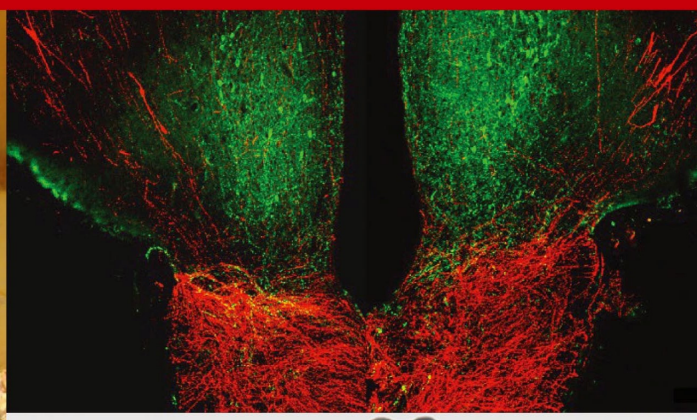
Knobil and Neill's

PHYSIOLOGY OF REPRODUCTION

FOURTH EDITION

VOLUME 1



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Pair bonded prairie vole family from Chapter 48 (J. Balthazart and L. Young). Photo by Todd H. Ahern

Relationship between gonadotropin releasing hormone GnRH (red) and kisspeptin neurons (green) in a coronal section of the monkey hypothalamus from Chapter 28 (A.J. Zeleznik and T.M. Plant).

Mouse uterus showing vascular permeability at the sites of implantation detected after an intravenous injection of a blue dye on day 8 of pregnancy. From Chapter 38 (S.K. Dey). Per Dey, acknowledgement/credit not needed.

Top back. Scanning electron micrograph of a bull sperm interacting with ciliated epithelium in the sperm storage reservoir of the oviduct (Chapter 5, Lefebvre et al., 1995).

Middle back from David Albertini. Confocal micrograph of a horse oocyte based on collaboration between Elaine Carnevale, Colorado State University and David Albertini and John Bromfield of the Kansas University Medical Center.

Lower back from Chapter 28 (A.J. Zeleznik and T.M. Plant). Pulsatile LH release from the pituitary and multi unit electrical activity in the mediobasal hypothalamus recorded from an intact (left panel) and ovariectomized (right panel) rhesus monkey.

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Introduction

This, the fourth edition of "Knobil and Neill's Physiology of Reproduction," is a major departure from its first three editions by having Editors in Chief not previously involved in managing the production of this work. Before his death in the year 2000, Ernst Knobil and I had made plans for the transition to an Editor in Chief who had been trained at the postdoctoral level in one of our laboratories. Dr Tony M. Plant was chosen from among several excellent candidates who had the requisite characteristics needed to continue production of a work that would have the same high standards we hoped to have maintained in the earlier editions. I first met Tony in the early 1970s while I was a young faculty member at Emory University in Atlanta, Georgia, and he was employed at the Georgia Mental Health Institute also located in Atlanta. I received a letter from Ernst Knobil inquiring about my impression of Tony, who had applied for a postdoctoral fellowship in Ernst's laboratory where I had trained as a fellow but had recently relocated to Emory. My reply was a strong endorsement of Tony, which was borne out in subsequent years. Tony Plant chose Dr Tony Zeleznik as a Co-Editor in Chief to share with him the demanding tasks of editorship requiring a broad base of information in the field and a high level of academic rigor. My perusal of the list of authors and their chapter titles suggests that the information presented in this edition will rival in quality that presented in the first three editions, i.e., the work is a modern synthesis of reproductive physiology of mammals at the molecular, cellular, and organismic levels. This is not surprising given the excellent past records of the two Editors in Chief.

Ernst Knobil and I believed strongly that any work claiming to be comprehensive and hewing to the highest principles of scholarship must include a summary of the founding and the founders of the field. Because Dr Roy O. Greep was one of the leading founders of the field, we requested that he provide that information together with an overview of the future of the field in his Foreword for the first edition in 1988. Thus, the original Foreword had the great strength of being written by one of the last surviving founders in the field, and thus avoided the greatest weakness of all historical analysis, namely that history is defined as what professional historians define it to be.

A new Foreword has been prepared by Dr M. Susan Smith to address, in part, Greep's predictions of progress that might occur in the field in the succeeding years after his Foreword first appeared. Susan is a prominent leader in reproductive physiology due, for example, to her Presidency of the Endocrine Society, Directorship of the Oregon National Primate Research Center, and maintaining an National Institutes of Health (NIH)-funded laboratory running for several decades. I am pleased that her fellowship training occurred in my laboratory at Emory University during which we published the most highly cited paper of my career, of which she was the senior author.

Jimmy D. Neill

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Preface

Having had the fortune of being mentored by Ernst Knobil in the early stages of our careers and serving as assistant professors in his faculty in the Department of Physiology at the University of Pittsburgh School of Medicine, we were greatly honored by the invitation to serve as Editors in Chief for the 4th edition of this book that has come to be recognized by many as the "Bible of Reproduction." At the same time, we undertook the task with considerable trepidation, being fully aware of how difficult it would be to fill the shoes of Professors Knobil and Neill, who, with their utmost attention to scientific rigor, had, together or individually, guided the previous three editions.

Given the foregoing challenge, we began by evaluating whether the structure and content of the previous edition needed to be refocused. It quickly became obvious that this was indeed the case because of the rapidly growing use of "omics" approaches in most, if not all, areas of reproductive biology. In this regard, it is noteworthy that the first edition of this text was published in 1988, a year before the first use of the "gene knockout" approach in mice was described. Over the course of the 25-year history of this text, the ability to delete or modify individual genes has increasingly and significantly added to the classical armamentarium of organ ablation/hormone replacement paradigms previously available to investigators studying reproduction. In addition, the application of genetics/genomics to elucidate the etiology of human disease has increased in parallel to the use of transgenesis in experimental animals. Collectively, these powerful approaches have provided new, and often serendipitous, information on the regulation of reproduction. For example, our view of the neuroendocrine control of the hypothalamic–pituitary–gonadal axis is now completely dominated by kisspeptin. This hypothalamic peptide, which, in the context of reproduction, was unheard of before 2003, surfaced because mutations of its receptor, then known as GPR54, were identified in individuals with delayed puberty.

Accordingly, our authors were confronted with the need to integrate information gleaned from "classical" whole animal studies with results generated by contemporary "omics" approaches in order to provide a holistic overview of reproductive processes. In keeping with this approach, we encouraged authors to discuss the historical underpinnings of their respective fields and to identify the burning questions that remain to be addressed.

In this 4th edition, Volume 1 focuses on basic processes of male and female reproduction while the thrust of Volume 2 is on the physiological control systems that govern reproductive processes, including those regulating sexual behavior. A chapter on meiosis has been included for the first time, and discussions of epigenetic mechanisms that are emerging as important regulators of reproduction, as well as fetal origins of adult diseases, have been expanded. New chapters on spermatogonial stem cells and hormone signaling in the testis that provide contemporary views of the control of testicular function replace the more classical approach to the control of spermatogenesis that was presented in prior editions of the text. We have also expanded the discussion of the regulation of reproductive behavior, which, having moved far away from its origins in ethology, is now dominated by genomic, epigenetic, cellular, and molecular approaches to the understanding of sexual motivation, partner preference, and parental behavior. For those chapters that have been updated, we are confident that these new versions present the most current discussion of the subject material.

Such restructuring of this book would not have been possible without the enthusiasm and commitment of our section editors, to whom we are greatly indebted. A feature of the 3rd edition that has been maintained is the inclusion of the Foreword by Professor Roy O. Greep that was written for the 1st edition, published in 1988. As recognized by Professor Neill, the Editor in Chief of the previous edition, Greep's account of the future of research on the physiology of reproduction continues to remain remarkably relevant, now 26 years after it was written. We are pleased to include a second Foreword that was written by Dr M. Susan Smith, who worked with Jimmy Neill as a postdoctoral scholar and was also a member of Ernst Knobil's Department of Physiology at the University of Pittsburgh School of Medicine. Dr Smith's broad understanding of both whole animal and molecular approaches used in the study of reproductive processes makes her uniquely qualified to comment on how the study of reproduction has progressed since Greep's original Foreword in 1988, as well as to identify the challenges to be met in future years.

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This 4th edition of *Knobil and Neill's Physiology of Reproduction* will be the last available in a print version. While our purpose here is not to debate the loss of hard copy, electronic publishing will enable, on the one hand, the addition of new chapters covering important emerging fields and, on the other, the replacement or revision of outdated chapters, both with relative ease. In this way, we anticipate that the *Physiology of Reproduction* will continue to be highly contemporary and comprehensive and therefore remain, for the future, the authoritative text of the field.

It is our expectation that this 4th edition of *Knobil and Neill's Physiology of Reproduction* will continue to kindle the importance of physiology in the understanding of reproductive processes and, in the words of Neill and Knobil in their preface to the first edition, be "useful to all serious students of reproductive physiology be they scientists, teachers or physicians."

Tony M. Plant
Anthony J. Zeleznik

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Foreword* by Roy O. Greep

I am pleased and honored to have been asked to prepare the Foreword to this volume of work depicting the progress in research on the physiology of reproduction as well as the resulting gains in understanding made over the past few years. The expertise that is represented by the numerous contributors to this work is so impressive that I am humbled even to contemplate adding anything of note. It is only by virtue of having personally witnessed a very large segment of twentieth century research on reproduction that I am emboldened to reflect on the byways and the trailblazings that have brought this field to its present proud state of enlightenment with regard to the long sought-after means of controlling the procreative process in humankind. Clearly, there are many important and knotty problems yet to be resolved, but the pace of progress over the past several years has quickened to the extent that one is left in expectant wonderment as to where and when the next revolutionizing development will occur.

The experimental method of studying reproduction was initiated in 1849 with Berthold's discovery of a blood-borne activity that came from the testis and stimulated growth of distant organs such as the comb and wattles. In so doing he utilized one of the most fundamental means of demonstrating the function of an endocrine organ, namely, surgical removal to determine what deficiencies follow, coupled with implantation or transplantation to ascertain whether the deficiencies were repaired. At that time it was not possible to take the next step, namely, preparation of an active extract of the testes, because nothing was known about the nature of the bioactivity. Forty years later, Brown-Séquard claimed to have prepared an active extract of dog testes; however, as is well known, his enthusiastic claims for restoration of his own sexual activity at an advanced age were not substantiated. Actually, these simple means of studying reproductive physiology persisted well into the twentieth century, including the studies of such pioneering stalwarts as Marshall, Heape, Prenant, Bouin, Ancel, Loeb, Cushing, and Aschner. Observations otherwise were limited to cyclic and seasonal changes in sexual behavior among common laboratory and small domestic animals. This type of eyeball research remained in vogue through the early 1920s and overlapped the extension of visualization to the microscopic level. The latter revealed, for the first time, the precise timing of events in the ovarian cycle through microscopically observable cellular changes in the vaginal fluid. My point in mentioning these early studies is to emphasize that although the tools and techniques were inordinately primitive by present standards, the results established a firm base of knowledge on which to build.

The study of cyclic changes in the vaginal smear in rats and the findings of estrogenic activity in follicular fluid during the early 1920s led to an explosion of interest in the study of reproduction. The field was fortunate in attracting to its ranks a small band of exceedingly able biologists and biochemists who, in 1932, were to become authors of the classic first edition compendium, Sex and Internal Secretions, a volume overwhelmingly devoted to reproductive endocrinology. It was this landmark of progress that finally gave propriety to the study of reproduction and put it on a par with the study of other major bodily systems. Incredible as it may seem, it was only a decade earlier that a distinguished panel of the National Research Council had declared that sex research was not a fitting topic for scientific study.

Lest our pride in today's spectacular pace of progress unduly bedazzle the mind, it should not be overlooked that the developments recorded in the 10-year span from 1926 to 1936 may never be equaled. Among those monumental achievements, all of the native sex steroid hormones were brought to light, their structures were determined, their functions were defined, and they were made available in pure form for research and therapy. Similarly, all of the pituitary, placental, and urinary tropic hormones were identified, and their functions were defined. Like today's competition for priority rights, publicity, and potential financial gain, these earlier periods also were times for intense rivalries, but rarely with prospects for financial rewards. It would be difficult to overstate the boost that was given to basic and clinical research in reproduction as a result of the availability of estradiol-17β, testosterone, and progesterone in pure form and of known potency. The replacement of

*This Foreword by Roy O. Greep is reprinted from the 1988 (first) 1994 (second) and 2006 (third) editions of this work (E. Knobil and J. D. Neill, The Physiology of Reproduction. Raven Press, New York. Copyright Elsevier). This now-deceased author wrote a remarkably prescient account of the future of research in the field that remains as relevant today as it did in the first three editions.

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homemade extracts and such elastic entities as rat units, mouse units, capon units, and so forth, with micrograms of pure hormone was revolutionizing and allowed the study of reproduction on a quantitative basis.

Prior to World War II the thrust of research on reproduction dealt predominantly with the steroid hormones. This was the heyday of steroid biochemistry. After World War II the emphasis shifted to the protein and peptide hormones, where it still remains strong. This prolonged and difficult effort yielded many biochemical triumphs. Most notable among these were the isolation of the pituitary, placental, and urinary gonadotropins, as well as the determination of their primary structure as glycoproteins comprised of two dissimilar and covalently bonded subunits, the isolation and synthesis of the gonadotropin-releasing hormone (GnRH) of hypothalamic origin, and the isolation and structural characterization of relaxin.

The availability of pure protein and polypeptide hormones made possible the production of hormone-specific antibodies as well as the application of immunological techniques to the study of reproduction. An outcome of great consequence was the development of radioimmunoassay as the new means of measuring all of the hormones relating to reproduction. The sensitivity of this new technique was so great that it made possible, for the first time, the measurement of all these hormones in the body fluids. It had the further distinct advantage of requiring such a small amount of fluid that the monitoring of blood levels of the hormones of reproduction could be done throughout an estrous or menstrual cycle by close serial sampling. This revealed still another and most unexpected finding, the pulsatile pattern of secretion.

Identifying the homeostatic mechanism(s) responsible for maintaining a steady state in various physiologic systems of the body has been fraught with many challenging problems, but these pale in comparison with the difficulties encountered in trying to elucidate the mechanisms maintaining a constantly changing system, a characteristic of the reproductive system of female mammals. The earliest piece of evidence suggested the existence of a "push–pull" mechanism that later came to be known as negative feedback. It was based on the demonstration that an estrogenic extract administered to immature rats would maintain the ovaries in an infantile state. This was quickly followed by conclusive evidence that estrogen acted to inhibit pituitary follicle-stimulating hormone (FSH) stimulation of follicular growth and maturation; however, the effect on luteinizing hormone (LH), ovulation, and luteinization remained unsettled. Gaps continued to exist in all proposed explanations of reproductive cycles. None of these explanations took into account the influence of photoperiodicity on seasonal breeders, nor did they account for the role of the stimulus of mating in nonspontaneous ovulators. Following the discovery of the hypothalamic control of pituitary function, estrogen was shown to exert its action on both the pituitary and the hypothalamus; however, the problem of accounting for cyclicity remained. Adding to the complexity, radioimmunoassay revealed an unexpectedly high level of blood estrogen just prior to ovulation, an event not in keeping with the negative feedback concept.

Finally, after many years of searching for a way out of this frustrating situation, a glimmer of light appeared at the end of this long dark tunnel—light that soon turned to brilliance. In 1969, Goding and associates found that the administration of large doses of estrogen to ewes at the time of estrus did not block, but instead entrained, ovulation. Shortly thereafter, in more elaborate examination of the relationship of blood estrogen levels and ovulation in rhesus monkeys in Knobil's laboratory, it was revealed that elevated estrogen levels preceded and appeared to trigger ovulation. On further examination, Knobil and colleagues found that when blood estrogen reached a critical level, the feedback mechanism switched from a negative to a positive, or stimulative, action. This utterly new finding greatly advanced our understanding of the endocrine mechanism governing reproductive cycles. There still remain, however, some uncertainties: Why does the switch in feedback action occur; to what extent and at what stage of the cycle does estrogen act at the level of the pituitary or the hypothalamus, or both; and lastly, what role, if any, do the ovarian peptides, especially inhibin, play in controlling reproductive cycles?

The progress of research on reproduction has been chronicled in numerous review articles by individual authors. Many have appeared in Recent Progress in Hormone Research, Volumes 1–42. Other major sources include the multiple editions of such titles as: Marshall's Physiology of Reproduction, Fourth Edition (1990); Sex and Internal Secretions, whose third and last edition was issued in 1961; two volumes on the Female Reproductive System (1973), and one on the Male Reproductive System (1975) in Section 7 of the Handbook of Physiology, published by the American Physiological Society; and four serial volumes on reproductive physiology in the International Review of Physiology, the last one being issued in 1983. The present volume will provide comprehensive coverage and meet the current needs of the field of reproductive physiology, a field that is rapidly gathering momentum from the application of new and highly sophisticated tools and techniques.

In viewing the vast literature dealing with research on the male and female reproductive systems and considering the rate at which it is accumulating, one might ask whether this staggering proliferation of books and articles is essential to progress; the answer is an emphatic "Yes!" The yardstick by which progress is measured

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in this or any other field is not in the number of articles published or the amount of financial support but in improved understanding. Such gains are generally marked by sharp peaks at indeterminate intervals, separated by avalanches of incremental gains, as recorded in an ever-growing list of journals. The point to remember is that without this persistent chipping away at a major problem there would be no solutions and no quantum leaps forward. In research very little comes from out of the blue. Part of the driving force in research is its adventuresome nature and ever-present possibility that one's efforts will pay off in an important manner. It may not be entirely fair, but in research (as in most human activities), the spoils go to the victor in the form of kudos, prizes, awards, public attention, and, increasingly in the present technological age, monetary gains—sometimes of great magnitude. What effect this latter may have, if any, on the long-cherished sanctity of science has not been determined, but it has become a matter of concern.

This volume bears the title The Physiology of Reproduction. Physiology, by traditional consensus, is that branch of science, which studies the functions of a living organism or any of its parts and includes the basic underlying processes. It will be understood that most of the studies reviewed here will be based more on holistic research than on research at the submicroscopic or molecular level. It is unfortunate that the excitement generated by recent fantastic advances in molecular biology and development has tended to downgrade the value of whole-animal research, and physiology in particular is sometimes looked upon as passé. Actually, the two categories of research are complementary, and both are essential for maximum advancement of knowledge. Whole-animal research cannot become outdated because it is the quintessence of biological relevance and the means by which molecular findings must ultimately be evaluated.

In the same vein, no one immersed in reproductive endocrinology can be unaware of the current tendency to regard research at the molecular level as representative of exceptional scientific talent. This is a common consequence of the opening of a new arena of investigation. I recall an incident that happened at a scientific meeting back in the 1930s. The first three papers in a session chaired by an eminent embryologist were on endocrine topics—mine was the third. That being ended, the chairman took pains to assure the audience that the meeting could now turn to considerations of more fundamental nature. One of the other three papers was given by Herbert M. Evans, who bristled noticeably but held his fire. There was also an earlier period when one either worked on steroid biochemistry or something of lesser appeal like biology. Anyone who remembers the 1950s will recall a flash in the pan ignited by cybernetics, a study of automatic control systems both neural and physical. The gurus of cybernetics captured the attention of the press and of audiences throughout the land, but eventually this obsession suffered the fate of other passing preoccupations. My own observation is that the closer one approaches the molecular level of research, the more one becomes dependent on highly sophisticated instrumentation to make the observations and to read out results that are often quite free of extraneous variables. Toward the obverse situation, one's dependence on an extensive background of experience and physiological increases as does the unavoidable complex of in vivo variables that must be taken into account. In either case we have today the availability of far more diverse approaches to a given problem in any field of biomedical research than has ever existed before. In Berthold's day there was only one experimental method available; today's number is untold but is probably in the hundreds, perhaps thousands. This is an exceedingly promising situation and one to which investigators of all persuasions must adjust. Open minds will experience exhilaration over substantive achievements at any point on this observational spectrum.

One of the major factors influencing research on reproduction has been the availability of funds or lack thereof. Prior to the institution of federal funding (i.e., prior to the middle of the twentieth century), reproductive research was sparsely supported by university departmental funds, industry, small grants from the Committee for Research in Problems of Sex within the National Research Council, and some aid from the Rockefeller Foundation. The National Institutes of Health were slow in providing significant support of research on reproduction because of restrictions on the support of work related in any way to birth control. This occurred despite the simultaneous postwar baby boom. What kept research afloat during this critical period was major support by the Ford Foundation plus lesser contributions by other major foundations. It was not until the establishment in 1968 of the Center for Population Research in the NICHD that major governmental funding in this area became available, but the boost was short-lived. As a result of the imposition of fiscal restraints in the early 1970s, federal support dwindled and has remained at a minimal level ever since. Support from all sources is woefully incommensurate with the distressing expansion of the human population and the need for safe, effective, economical, and readily available means of limiting human fertility.

The physiology of reproduction is predominantly under hormonal control. The first essential step in studying reproduction was identification of the hormones involved and the functions they serve. This having been accomplished, efforts turned to a detailed analysis as to how hormones act within the body. During the 1980s there has been a rising tide of interest

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in the binding of steroid, protein, or peptide hormones to receptors on specific target cells. Much effort is currently being directed toward the isolation and chemical characterization of these receptors. They are known to be composed of a protein or proteins, and some information has already been gained as to their partial or provisional structure. This, however, is only a preliminary step in the complex process whereby hormone action results in an end response such as growth, secretion of a target cell hormone, or altered behavior. The curtain has already been raised on the climatic and final chapter of the story on how hormones act. This involves linkage of the hormone–receptor complex with the nuclear genetic apparatus leading through a now well-defined series of processes to the manifestation of a physiological response in the living organism. Genes that bring about the expression of certain hormonal signals are being isolated, modified, transferred between species, and also inserted into bacteria where they direct the biosynthesis of specific hormones in large quantity. Thus genes are being manipulated in ways that raise the potential of altering the reproductive process. It is largely as a result of developments in endocrinology at the molecular level that bewildering possibilities loom on the horizons of reproductive research—they are within reach; they are science, not fiction; and they stagger the imagination.

It being granted that nothing succeeds like success, then the new edition of this highly successful two-volume compendium on The Physiology of Reproduction is destined for an illustrious fate. This second edition will maintain the same high standards of the first and again fulfill an existing need in a field that is experiencing rapid growth and exhilarating progress. Like the first edition, this one will provide a critical assessment of the state of the art in every aspect of research on the physiology of reproduction by eminent authorities.

In the years intervening between this edition and the last, notable changes have taken place in the study of reproduction. These stem largely from major advances in technology. Remarkable new instruments, techniques, and methods have enabled investigators to probe ever deeper into the interaction between hormones and genes, thereby eliciting in vivo responses. New parameters are being added to the target tissues of the classical reproductive hormones as revealed by the presence of receptor sites in tissues, the physiologic significance of which often remains tantalizingly obscure. Similarly, newly identified substances of endocrine or paracrine nature are being added to this domain of research with persisting frequency. Some of these substances—the endothelins, interleukins, activins, inhibins, and prorenin, to name a few—also exhibit a puzzling array of effects on extraneous tissues. Their study is being aided by the fact that their structure is known and, though rare, they are available.

Great strides are also being made in many other aspects of research on reproduction. Much work is being done on the structure of receptors and the loci of binding sites on segments of the folded gonadotropic molecules. A full-scale effort is underway seeking an elucidation of the neural mechanism underlying pulsatile secretion. Neuroendocrinologists are closing in on an elusive pulse generator located in the central nervous system. This looms as another landmark discovery in reproductive biology.

Research on reproduction is flourishing and the future appears bright. The taboos are gone. All aspects of the reproductive process are an open book. One area that has taken a quantum leap forward is the clinical application of an important body of relevant new knowledge gained in both basic and clinical spheres. Expanded opportunities have been opened by greatly improved diagnostic procedures, more effective treatment of disorders, and new methods of controlling fertility. Contributing greatly to this explosive development is the dissemination of information on reproductive matters to the lay public by the mass media. Concerned individuals have been made aware of the existing new means of manipulating the male and female reproductive systems for enhancement or inhibition of fertility. The joys and comforts that accrue respectively to these opposing modes of fertility control have enriched the lives of a grateful public. To that end I may note that it was by virtue of these frontier reproductive measures that my own progeny includes a new grandson and namesake.

Roy O. Greep

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Foreword by M. Susan Smith

I am truly honored to write the Foreword for the 4th edition of this book; it is a humbling task to follow in the footsteps of Professor Roy O. Greep, whose Foreword is herein reprinted from the 1st, 2nd, and 3rd editions of this work. His remarkable account of the field of the physiology of reproduction was written 26 years ago, yet as you read it, you will develop an overwhelming appreciation for how fortunate we were to have Professor Greep as one of the foremost leaders in our field. It is especially gratifying for me to be associated with this great work, since my scientific lineage is part of the Greep/Knobil/Neill 'family', having trained with Professor Neill and served as a faculty member in Professor Knobil's Department of Physiology at the University of Pittsburgh.

Since the publication of the 3rd edition of this book in 2006, there continues to be remarkable progress in the physiology of reproduction, reflecting the use of advanced technologies, such as those spurred by contemporary genetic and genomic approaches. However, the challenge today is to understand how this genetic and molecular information is integrated into the manifestation of a physiological response. Greep's thoughts on this are still prescient today, "Actually, the two categories of research are complimentary…." "Whole animal research cannot become outdated because it is the quintessence of biological relevance and the means by which molecular findings must ultimately be evaluated."

Professor Greep issued a challenge in his Foreword: "Developments recorded in the ten-year span from 1926 to 1936 may never be equaled." I posit that the 10-year span from 2004 to 2014 was, at the very least, equally important, as we have made a paradigm shift in how we conduct our science. In the more traditional way of science, experiments focused on understanding control systems; this then led to the discovery of new molecules. Today, in contrast, the use of massively parallel sequencing (exome sequencing, ChiP-seq, and RNA-seq) allows us to identify all the players, even though we may have no idea of their function. We then "reverse engineer" results from these studies to discover where a particular molecule is produced, how its production is regulated, and what its function is. These sequencing techniques were used in the groundbreaking studies that signaled the important role of kisspeptin and neurokinin B in regulating the pituitary–gonadal axis; subsequent studies located their sites of production in the hypothalamus and identified their critical function in controlling GnRH neuronal activity. All of these sequencing techniques generate massive amounts of data and necessitate application of the extensive bioinformatics infrastructure that goes with managing large databases. Greep was right when he stated, "the closer one approaches the molecular level of research, the more one becomes dependent on highly sophisticated instrumentation to make observations and to read out results that are often quite free of extraneous variables." As an example of the power of these databases that are available to the scientific community, there is one that identifies all the transcribed genes in the ovary of the nonhuman primate and maps changes in their individual activities throughout the duration of a menstrual cycle and into early pregnancy. Not too long ago it would have been impossible to imagine being able to follow the activity of one gene through such a time course. Importantly, these databases can be used for comparative analyses among species and should provide enlightenment about the evolution of different approaches to reproduction.

The material in the chapters of this 4th edition reflects advances that have been made at all levels of the hypothalamic–pituitary–gonadal axis and the reproductive tract; many of these advances have been made possible by the new tools available to scientists. The most notable is the discovery of kisspeptin in the hypothalamus that fundamentally changed our concept of the control of GnRH neurons and has expanded our knowledge of the neural networks that govern reproductive function. Significant advances have also been made in our understanding of how G-protein coupled receptors function; such as the insight gained by the discovery of GnRH receptor misfolding that results in a loss of trafficking of the receptor to the plasma membrane and, thus, functionality, a deficit that can be overcome by artificial chaperones. New areas of research have come forth, such as oncofertility, a term coined to signify the restoration or maintenance of fertility in cancer patients whose gonadal function is diminished or lost due to the side effects of their radiation treatment or chemotherapy. Studies using new techniques, such as 3-dimensional culture of follicles in an extracellular matrix and differentiation of gamete stem cells, have provided new insights into the processes controlling folliculogenesis and gametogenesis, with translational

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opportunities for promoting or controlling fertility. In addition, there is an explosion of interest in stem cell biology, a field that was rooted in reproductive research and the practice of in vitro fertilization. There is also a new appreciation for the role of the environment in fetal development and this has led to the birth of a new area of medicine known as DOHAD (Developmental Origins of Health and Disease). Maternal under- and overnutrition both lead to epigenetic changes in the fetus that can have long-lasting consequences in the adult. Similarly, exciting new research shows environmental influences on epigenetic modifications of DNA in sperm that may result in paternal transmission of disease risk.

When considering all of this new research, some caveats need to be kept in mind. There appear to be significant species differences in a number of reproductive processes, including puberty, ovarian cyclity, and parturition, yet current research focuses primarily on the transgenic mouse model because of the ability to manipulate the expression of specific genes. Therefore, many of our current ideas about the control systems regulating reproductive processes are "mouse-centric" and may not be directly applicable to other species. This may create problems as we look to translate this information into new therapeutics for use in human and veterinary medicine.

What new advances can we look forward to in the near future? New technologies, such as optogenetic/pharmacogenetic tools, designer receptors, and advanced imaging, will likely contribute to the making of another "best decade" from 2014 to 2024. But it will be important to develop better methods, with increased specificity and sensitivity, for measuring hormones and other substances in the blood. There are also growing concerns over the use of sex steroids in clinical therapies. As a result, there is considerable interest in developing "nonhormonal", i.e., nonsteroidal, selective therapies that act at the local or intracellular levels.

Future studies must also focus on the still many critical unanswered questions in the physiology of reproduction. Just in the area of neural regulation of reproduction alone, there are numerous examples. Many external signals modulate reproductive function, such as stress, endocrine disrupters, diet, photoperiod, and pheromones. Yet we still do not know how these external signals are transmitted to directly alter kisspeptin or GnRH neuronal activity and thus, reproductive function. Perhaps, Greep's assertion that "The physiology of reproduction is predominantly under hormonal control," needs to be expanded to include nonhormonal factors that are important modulators. Another mystery is what heralds the onset of puberty. Detailed information is known about the regulation of kisspeptin gene expression and its upregulation at the time of puberty, but the specific signals that bring about these epigenetic changes are still completely unknown. Similarly, although neuroendocrinologists are closing in on the GnRH pulse generator located in the central nervous system, the actual mechanisms involved in pulse generation remain elusive. Greep recognized that "This looms as another landmark discovery in reproductive biology." The processes involved in the positive feedback effects of estrogen on gonadotropin secretion also remain a mystery. With the current recognition that there are likely species differences in these processes, negative and positive feedback of estrogen might be achieved through two different populations of kisspeptin neurons (rodent model) or two different populations of GnRH neurons (nonhuman primate model).

While the future for research in reproduction appears bright, with more landmark discoveries in the making, there is also cause for concern. Funding for basic research in reproduction at the federal level is declining, as the emphasis shifts to translational research relevant to human health and disease. In the bench-to-bedside continuum of research, it is critical to keep "bench" in the equation. There are still many areas in reproduction where we do not understand the basic underlying controlling mechanisms, making it difficult to devise therapeutics. Studies of various species will also be harder to support. This is regrettable, since comparative studies have revealed differences in how reproduction is regulated, and this collective information may be critical to solving long sought after questions, such as, what constitutes the GnRH pulse generator, how is puberty initiated, how is parturition initiated, and what is the impact of environmental factors on germ cells. A good example of the value of studying various species is the seasonal regulation of reproduction that led to a focus on photoperiod and how time-of-day signals are transmitted to GnRH neurons. These studies were instrumental in advancing the field of chronobiology to the recognition today that all cells appear to have clock genes that regulate their function. It is also regrettable that there are still constraints on contraception research even though the link between the ability to control fertility and the economic development of a country is well established. Finally, in this age of "big data," it is well to remember that analysis of large databases can detect correlations, but it cannot determine whether the correlations are meaningful or provide information about causality or mechanisms. This is the province of basic research: to discover the critical information about control systems that then provides the underpinnings for translational research and the development of new human therapeutics.

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KNOBIL AND NEILL'S PHYSIOLOGY OF REPRODUCTION

FOURTH EDITION

VOLUME 1

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CHAPTER

1

Mammalian Meiosis

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INTRODUCTION

Basic Overview of Meiosis and Comparison with Mitosis

Mitosis and meiosis are the two types of cellular division that are common amongst eukaryotic organisms. While mitosis involves the faithful replication of cellular contents, including DNA, to produce two daughter cells of identical genetic and cellular composition, meiosis is confined only to those cells that participate in sexual reproduction: the germ cells (or gametes) in mammals. The goal of meiosis, in contrast to mitosis, is to produce gametes that have half the content of DNA within them so that, upon fertilization, the new zygote will contain the full complement of genetic information. This is achieved by virtue of the fact that each cell in a diploid organism contains two copies of every chromosome, each chromosome being referred to as "n" and a diploid organism therefore being referred to as a "2n"-containing organism. The two copies (or homologs), one of every chromosome, are provided by two parents: one maternal copy of every chromosome (chromosome 1, chromosome 2, chromosome 3, etc., up to chromosome 22, plus one X chromosome in humans) and one paternal copy of every chromosome (chromosomes 1 to 22, plus either an X chromosome or a Y chromosome in humans). Figure 1.1 provides a cartoon graphic depicting an imaginary meiotic species containing just four chromosomes: two paternal and two maternal. In this example, the long chromosomes (black and gray) are chromosome 1 homologs and the shorter chromosomes (white and off-white) are chromosome 2 homologs. Meiosis in germ cells begins with one round of premeiotic DNA replication to achieve "4c" DNA content, with each chromosome (each maternal "n" and each paternal "n" chromosome) now consisting of a pair of sister chromatids (each termed "c") that are tethered together through the process of cohesion. In Figure 1.1, therefore, the premeiotic replication results in the same four chromosomes, but now each chromosome consists of two chromatids, making a total of four chromatids for chromosome 1 and four chromatids for chromosome 2 (hence, two black chromosome 1 chromatids, two gray chromosome 1 chromatids, etc.).

Following DNA replication, cells enter meiosis, which consists of two consecutive rounds of division producing haploid gametes; these two rounds encompass meiosis I and meiosis II, respectively. In meiosis I, homologous chromosomes pair during prophase I (Figure 1.1: black and gray chromosomes pair, and white and off-white chromosomes pair) and then separate at metaphase I, resulting in the formation of two daughter cells each containing just one of the homologs for each chromosome (Figure 1.1: black or gray, and white or off-white). The meiosis I division is referred to as "reductional" because it results in daughter cells that contain only half the number of chromosomes that existed in the originating parent cell (although the daughter cells' chromosomes each now consist of paired chromatids). As will be discussed in detail in this chapter, some portions of these homologous chromosomes are exchanged in a process known as homologous recombination, and this is largely responsible for keeping the homologs together until metaphase I. Once the meiosis I daughter cells are formed, these can then undergo meiosis II, in which the paired sister chromatids now separate. This division is called "equational" because it results in the same overall number of chromosomes in the daughter cells as in the parental cell at the end of meiosis I. As can be seen in Figure 1.1, there are distinct differences in the number of products that arise following meiosis I and meiosis II in male and female germ cells, and these differences will be discussed in detail here.

The unique and defining features of meiosis occur during meiosis I, which consists of prophase I, metaphase I,

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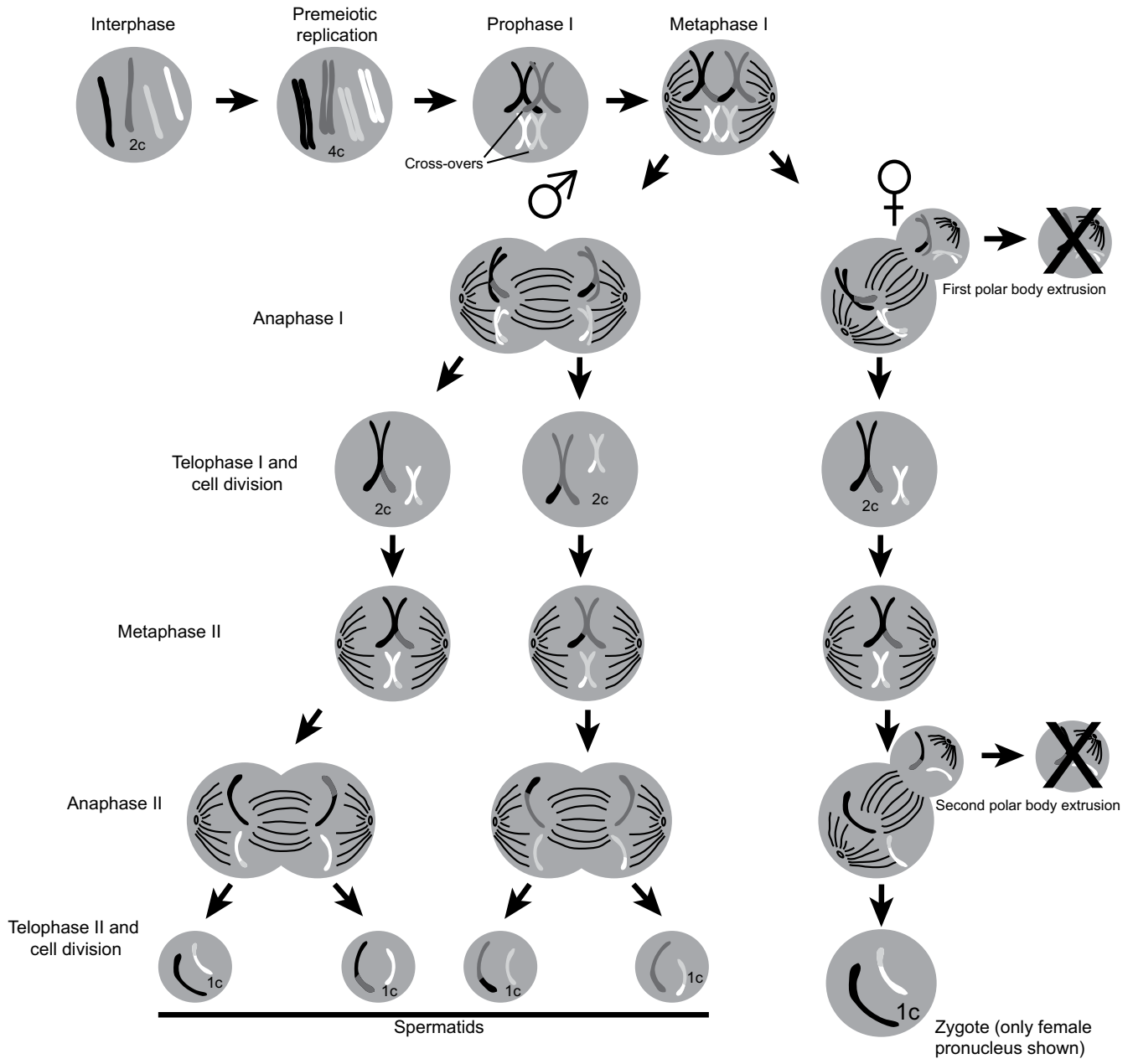

FIGURE 1.1 **Stages of meiosis.** Prior to the start of meiosis, the chromosomes are copied in a stage of premeiotic replication, taking the DNA compliment from 2c to 4c. In other words, there are still two copies of each chromosome (2n), but each of them has two sister chromatids (2c each, for a total of 4c). Upon entry into meiotic prophase I, the homologous chromosome pairs undergo two important events, synapsis and recombination. They then enter metaphase I, where the homologs line up on the metaphase plate and are attached to the meiotic spindle. Homologous chromosomes are separated during anaphase I, and the separation of the nuclei is completed during telophase I. Cell division (cytokinesis) then follows to produce two daughter cells with a chromosome compliment of 1n each, but with each chromosome still containing two sister chromatids (2c). In males, this process divides the cellular components equally between both daughter cells, while in females, half of the genetic complement is retained in the oocyte, and half becomes encapsulated into a smaller structure known as the first polar body. In this case, the asymmetrical division results in the majority of the cytoplasm and cell organelles dividing into the oocyte, with very little getting passed to the polar body. The second meiotic division is much more like a mitotic division, where the two sister chromatids are separated during metaphase II, anaphase II, and telophase II, resulting in haploid (1n and 1c) daughter gametes. Again, in the females, one of the products of this division is the second polar body, and it contains the second half of the genetic complement of the second division.

anaphase I, and telophase I (Figure 1.1), during which maternal and paternal homologous chromosomes come together and pair in a process known as synapsis, and then exchange genetic information through crossing over via the process of recombination. As will be described in this chapter, synapsis and recombination serve a single purpose: to keep homologous chromosomes tightly associated with each other so that they may then be

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segregated equally to daughter cells. These daughter cells, each containing just one of the originating homologous chromosomes, then enter meiosis II. Meiosis II, consisting of prophase II, metaphase II, anaphase II, and telophase II, resembles more of a mitotic (reductional) division. Mitosis is the process by which cells replicate their DNA from 2c to 4c (but still 2n), and then divide the nucleus in half, separating their sister chromatids (and, thus, their DNA) equally into two daughter (diploid but still 2n) nuclei. Thus, mitosis is "cyclical" in nature, and can truly be called a cell cycle, because the resulting 2n daughter cells can be used as starting material for future cycles of DNA replication and cellular division, while meiosis is less of a cell cycle and more unidirectional considering that, once produced, gametes need to undergo fertilization in order to achieve their full diploid status and become capable of cellular proliferation through repeated mitotic cycles.

History and Discovery of Meiosis

As with so many developmental processes, meiosis was first observed and described cytologically in sea urchin eggs by the German biologist Oscar Hertwig (1849–1922) and further characterized at the chromosome level by the Belgian zoologist Edouard Van Beneden (1846–1910) in eggs from the parasitic roundworm *Ascaris*. This new nematode model, with its larger sperm size (some 10-fold larger than that of sea urchins) and abundance of synchronously developing oocytes, facilitated more precise cytological observations of chromosome movements,[1] allowing Van Beneden to observe the halving of chromosome content even before the significance of chromosomes for heredity was fully realized. It was not until the studies of August Weismann (1834–1914), however, that the significance of the two earlier studies became clear. Weismann's *germ plasm* theory held that inheritance in a multicellular organism is the product of the germ cell, or *gamete*, while the normal proliferative activity leading to the growth and development of the individual is undertaken in the somatic cell lineages. Germ cells can give rise to soma or to germ cells themselves, whereas soma can only give rise to somatic cells, a concept referred to as the *Weismann barrier* and now arguably dispelled in the era of stem cells and reprogramming. Weismann went on to demonstrate that sea urchin eggs undergo both a reductional division and an equational one, and that both were essential to provide haploid gametes that could then collectively derive a diploid daughter cell following fertilization. His work preceded the rediscovery of Mendel's work by Hugo de Vries and others in the 1900s, and these events culminated in 1911 with the discovery by Thomas Hunt Morgan of crossing over, which is the exchange of genetic information between homologous chromosomes as a result of recombination, and the raison d'être of meiosis. Hunt Morgan's landmark studies in *Drosophila melanogaster* females earned him the Nobel Prize in 1933. The term *meiosis* was coined by Farmer and Moore in 1905 to explain the process of division of germ cells, but it was the accumulation of all of the studies described above that led to full realization of the crucial role of meiosis for gamete formation.

Conservation of Meiosis across Eukaryotes

Comparative evidence seems to indicate that meiosis appeared early in eukaryotic cell history, most likely arising only once, given its high degree of conservation among many species. Even in prokaryotes, similar machineries exist for repairing recombination intermediates to those found in highly evolved species. For example, the prokaryotic single-stranded binding protein RecA, which is essential for DNA repair, has evolved to form several RecA homologs in all eukaryotes, some of which appear to be specific to meiosis, while others are shared between different forms of recombination repair. Many other meiotic proteins, such as Spo11, MutS homologs 4 and 5 (Msh4 and Msh5, respectively), and MutL homologs 1 and 3 (Mlh1 and Mlh3, respectively), all of which are described in detail in this chapter, are also functionally conserved from yeast to human.

Meiotic processes involving the establishment and maintenance of cohesion between sister chromatids, and the physical attachment of homologous chromosomes through synapsis, recombination, and crossing over, are highly conserved, if not always achieved in the same manner in all species. For example, the hallmark structural component of prophase I, the synaptonemal complex, exists in the majority of eukaryotes but may consist of different proteins (examples of synaptonemal complexes from different organisms are provided in Figure 1.2). Meiotic recombination occurs in the majority of species studied, but does not occur in male *D. melanogaster*, only in the female flies. Msh4–Msh5 proteins are major components of the meiotic crossover (CO) pathway in eukaryotes (described in detail in this chapter) but do not exist in *Saccharomyces pombe*. Similarly, MLH1 and MLH3 proteins are the major determinants of COs in many eukaryotes, but *Caenorhabditis elegans* lacks these proteins. Interestingly, however, *C. elegans* does possess MSH4 and MSH5, the binding partners for MLH1–MLH3, raising the question of whether other functionally similar proteins are present in worms to substitute for MLH1–MLH3. Therefore, in these model organisms, meiosis itself is highly conserved, but the major players may be interchangeable or the processes slightly adapted to suit each organism.

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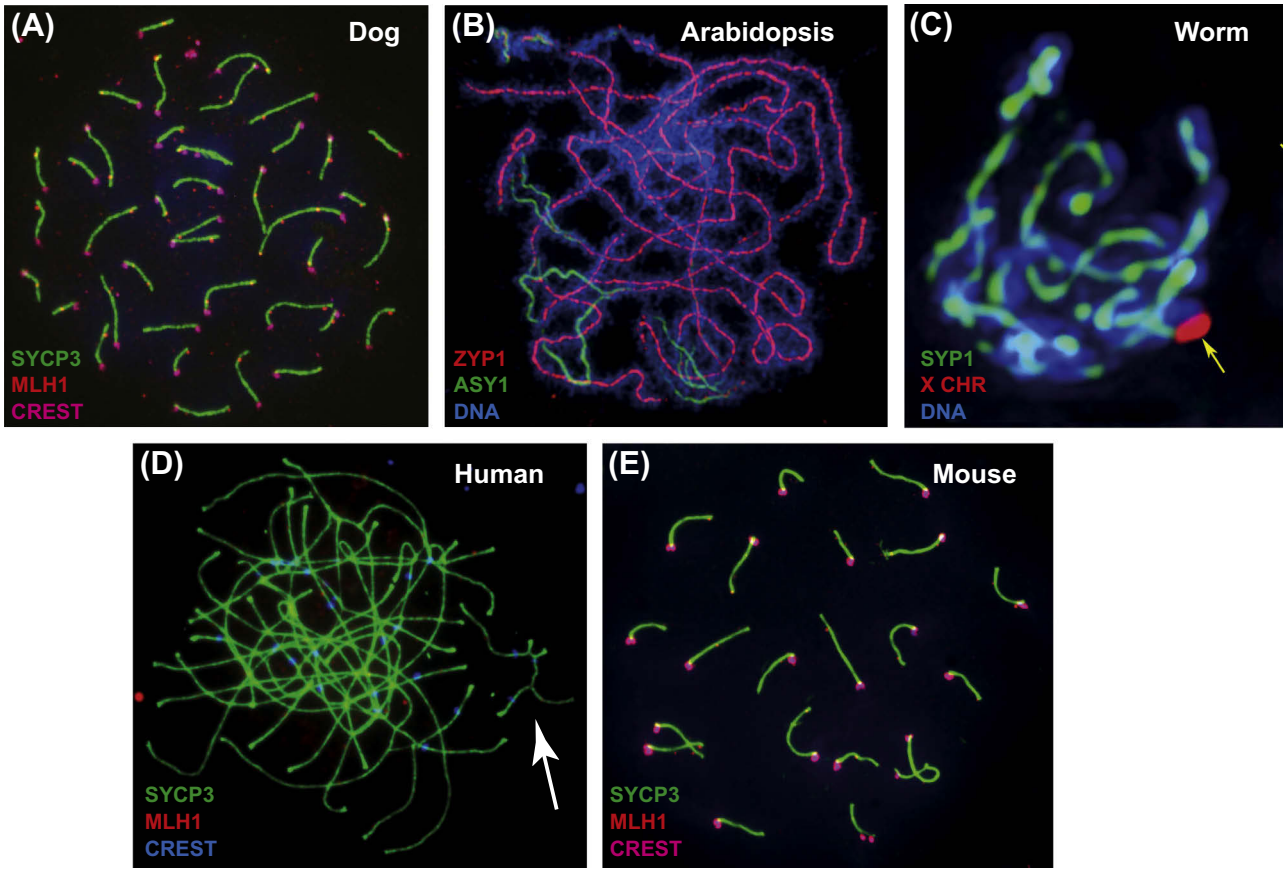

FIGURE 1.2 **Comparative meiosis across eukaryotic species.** This figure is reproduced in color in the color plate section. (A) Pachytene cell from an adult dog testis stained with antibodies against SYCP3 (green), MLH1 (red), and CREST (centromeres, pink). (B) Immunolocalization of the *Arabidopsis* synaptonemal complex transverses filament protein ZYP1 (red), polymerizing between the homologous chromosome axes marked by ASY1 (green) and the DNA loops (blue) in a late-zygotene nucleus. The ASY1 signal (green) is reduced in synapsed regions (red), and an interlock is being resolved. (C) A *C. elegans* pachytene nucleus immunostained to show that the synaptonemal complex (green) is normal and that the pairing center ends of the X chromosome are fully paired (red); *(Source: Adapted from Saito et al., PLoS Genet, 2009.)* (D) A trisomic human embryonic oocyte at the pachytene stage stained with antibodies against SYCP3 (green), MLH1 (red), and CREST (centromeres, blue). The three copies of chromosome 21 can be seen attempting to pair (arrow). (E) Mouse pachytene cell stained immunostained with SYCP3 (green), MLH1 (red), and cREST (centromere, pink).

Sexual Dimorphism and Timing of Meiosis

In mammals, one of the defining features of meiosis, and of gametogenesis in general, is that there are distinct differences in the mechanism, timing, and success of meiotic events between the sexes (reviewed by Ref. 2). The most fundamental difference lies in the number of products of meiosis, with four haploid spermatozoa being produced during meiosis in males, and only one oocyte in females, with the remaining chromosomal contents being extruded into two polar bodies, following a distinctly asymmetric division (Figure 1.1). In females, meiosis is not completed before fertilization occurs, and it is the process of sperm penetration of the oocyte cytoplasm that elicits the cascade of events that culminate in completion of the second meiotic division and extrusion of the second polar body (Figure 1.1, and see Chapter 4 for further details).

Meiosis in females begins in midgestational fetal life, with all oogonial precursors entering meiosis in a semisynchronous fashion. Oocytes undergo two periods of arrest; the first lasts weeks in mice and years in humans, and involves the entire oocyte pool. Following birth, each estrous or menstrual cycle is marked by the growth and development of a cohort of follicles, only a subset of which go on to be ovulated (one or two in humans), while the others undergo atresia. Only those oocytes that are ovulated will resume meiosis. In primates at least, the resulting diminution of the oocyte pool leads to reproductive senescence. In males, on the other hand, meiosis is initiated at or around puberty (or just after birth in rodents), and spermatogonial precursors enter meiosis in waves along the seminiferous epithelium of the testis, which reoccurs throughout the life of the individual.

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For humans, perhaps the most fundamentally important difference between males and females lies in the distinct difference in meiotic error rates observed in females compared to males. This is possibly most startling in humans, in whom meiotic error rates approach 40–60% in women but remain under 3% in men (Source: U.S. Centers for Disease Control). Studies of mouse mutants have pointed to the fact that female meiosis appears to be less stringent in its regulation, providing at least one possible explanation for the differences in error rates. In this chapter, many points of divergence between male and female mammals will be discussed, some or all of which may contribute to the differences in success rates for male versus female meiosis.

Scope of the Chapter

This chapter aims to describe the main features of the meiotic process, from the earliest stages of induction during embryogenesis in females, to the last stages of meiotic cell division. Complex mechanisms are described that control meiotic induction, cohesin assembly and disassembly, prophase I events such as synapsis, silencing and meiotic recombination, and meiotic segregation. For each of these, we provide an introductory overview at the beginning of each section before delving further into the often complex molecular mechanisms involved. Environmental and aging effects on meiotic mechanisms are discussed in detail, and diseases arising from meiotic errors also feature. Overall, the chapter focuses on mammalian meiosis, with an emphasis on those stages and events that differ from mitosis, but it draws evidence from non-mammalian meiotic species for the purposes of comparison and further illustration of key regulatory processes.

INITIATION OF MEIOSIS IN MAMMALS

One of the key features that distinguishes gametogenesis in male and female mammals is the time during development in which the oogonia or spermatogonia cease their proliferative activity and enter meiosis. Early in embryonic development, the primordial germ cells (PGCs) arrive in the bipotential or indifferent gonad and begin a rapid and rampant phase of proliferation, during which time they increase in number exponentially over several days to weeks. In the mouse, for example, approximately 3000 PGCs take up residence in the bipotential gonad at around 10.5 days postcoitum (dpc) at a time when the gonadal structure has barely arisen from the intermediate mesoderm. The PGCs continue to proliferate, reaching a number of some 30,000 by 13.5 dpc,[3,4] at which point the first sign of sexual dimorphism becomes evident. The PGCs of the female stop proliferating and enter prophase of the first meiotic division, while PGCs of the male arrest in G0/G1 of the mitotic cycle and then only resume proliferation and enter meiosis after birth[5] and, most usually (although not always), at puberty.

Retinoic Acid (RA) is the Key Trigger for Meiosis in Males and Females

It has been well established that male germ cells fail to enter meiosis during fetal life as a result of inhibitory factors secreted by the developing testis, while they can be induced to undergo meiosis when co-cultured with embryonic ovaries[6] or when cultured with conditioned medium from adult testes.[7–10] Such observations gave rise to the suggestion that meiotic entry is a cell-nonautonomous event, being regulated locally, either within the gonad or by tissues in the vicinity of the developing gonad, but not by the germ cells themselves. The mesonephros, which is closely apposed to the developing gonad, was suggested as the site of "meiosis induction" when it was demonstrated that co-culture of mesonephros with fetal bipotential gonads could induce feminization, or meiotic entry.[11–13] These studies were confounded, however, by early observations showing that ectopically located germ cells (that migrate aberrantly to other regions of the embryo) spontaneously initiate meiosis without close proximity to the mesonephros,[14,15] raising the possibility that gonad-intrinsic factors may also induce meiotic entry.

Evidence for the role of vitamin A–retinol metabolite, retinoic acid (RA), as the trigger for meiotic initiation in both sexes arose out of studies demonstrating that the entry into meiosis was dependent on expression stimulated by retinoic acid gene 8 (*Stra8*) in both sexes.[16–20] As its name suggests, *Stra8* expression is dependent on RA,[21] and both *Stra8* expression and meiotic entry by female germ cells during embryogenesis can be blocked by RA antagonists.[16–18] These observations were preceded by studies demonstrating the existence of RA receptors (RA receptor (RAR) and retinoid X receptor (RXR)) in embryonic gonads[22,23] and the finding that exogenous RA could induce premature meiosis in fetal ovary cultures.[22,24] The source of RA in vivo has been somewhat contentious, but it is thought to be produced in the neighboring mesonephros via a two-step process that converts retinol into retinaldehyde and then into RA (see Box 1.1). Accordingly, the mesonephros maintains high expression of the genes encoding RA synthetic enzymes, including retinol dehydrogenase 10 (*Rdh10*) and retinaldehyde dehydrogenase 2 (*Raldh2*, also called *Aldh1a2*),[25] along with *Raldh3*/*Aldh1a3*, the latter at lower levels.[26–28] Importantly, however, while the mesonephroi of both male and female embryos can synthesize large quantities of RA,[25] local levels of RA are maintained at 80% lower levels in the developing testis than in the ovaries as a result of high expression of cytochrome

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BOX 1.1

RETINOIC ACID SYNTHESIS AND ACTION

Retinoic acid (RA) is a lipid-soluble derivative of vitamin A (retinol) and is essential for many aspects of embryonic development (reviewed by Refs 32,33). RA regulates the expression of genes involved in body patterning and morphogenesis, while disruptions in RA signaling result in a wide range of congenital abnormalities, including skeletal and central nervous defects, cardiac malformation, and craniofacial abnormalities.

Retinol is bound to retinol-binding protein (RPB) for transport through the circulation. Upon reaching its target tissue, defined by the presence of STRA6 receptors on the cell surface, retinol is released from RPB and binds instead to cellular retinol-binding protein (CRBP). Both retinol and CRBP serve as the principal substrate for RA synthesis, through oxidation to retinaldehyde by retinol dehydrogenase 10 (RDH10), but free retinol can also undergo oxidation by more nonspecific alcohol dehydrogenases. This first step of RA synthesis is considered to be rate limiting and is reversible.[34] In light of the higher substrate affinity of RDH10, CRBP provides a substrate specificity mechanism by which specific RDH activity is promoted by the retinol–CRBP complex at the expense of free retinol oxidation through alcohol dehydrogenase.

The second step of RA synthesis is also enhanced through retinaldehyde binding to CRBP, and involves the irreversible oxidation of retinaldehyde to RA by the retinaldehyde dehydrodgenases (RALDHs), of which at least three have been described (RALDH1, RALDH2, and RALDH3; also known as ALDH1A1–3, respectively), encoded in the mouse by the genes *Aldh1a1*, *Aldh1a2*, and *Aldh1a3*, respectively. Each of these is expressed in unique patterns across developing mouse embryos, and each results in the production and secretion of RA from the cell to act in a paracrine fashion on neighboring cells and/or tissues.[27,35–38]

Uptake of RA into responding cells is achieved through binding to the cellular retinoic acid–binding protein (CRABP), which prevents the nonspecific degradation of RA in the target cell. Alternatively, RA may bind, and be degraded by, the P450 hydroxylase CYP26 enzymes, which include CYP26A1, B1, C1, and D1, and which may act in a partially redundant fashion to hydroxylate RA into less bioactive 4-oxo-RA. CYP26A1 is itself regulated by RA in a feedback regulatory loop.[32,33]

RA is transported into the nucleus, where it binds to one of a class of three retinoic acid receptors (RARα, RARβ, and RARγ), which are transcription factors of the retinoid receptor family. The retinoid X receptors (RXRα, RXRβ, and RXRγ) also belong to this group and, upon ligand binding, they dimerize with RAR. The RXR–RAR heterodimeric transcription factor, in association with co-activators and histone acetyl-transferase, then associates with RA response elements (RAREs) upstream of transcriptional targets across the genome, thereby initiating gene transcription.

P450 26B1(*Cyp26b1*), a gene that encodes a P450 enzyme that is responsible for degradation of RA[18,29–31] (see Box 1.1), specifically within the supporting somatic cells (Sertoli cells) of the male gonad. Thus, in females, meiosis is brought about by the expression of *Stra8* in PGCs, which is in turn stimulated by the production of RA, presumably in the mesonephros (Figure 1.3(A) and discussed further here). In males, on the other hand, fetal RA production by the mesonephros is counteracted by the expression of *Cyp26b1* by the developing testis, which acts to degrade RA and lower local RA concentrations (Figure 1.3(A)). Expression of *Cyp26b1* begins at 11.5 dpc in males, concurrent with the onset of *Raldh2* expression in the mesonephros of both sexes.[25] (See Refs 27,32–38.)

In male mice, RA production resumes postnatally, at around 3 days postpartum, as the PGC population (now known as *gonocytes*) differentiates into A1 spermatogonia. In both prepubertal and adult testis, *Raldh2/Aldh1a2* expression predominates in the Sertoli cell, although *Raldh1/Aldh1a1* is also expressed to appreciable levels.[39,40] Importantly, *Raldh2/Aldh1a2* is also expressed in premeiotic germ cells of the mouse testis, as well as in pachytene spermatocytes,[40] indicating perhaps some potential for RA synthesis by germ cells themselves. Moreover, studies exploring the relative contributions of different retinoid receptors have indicated that RA may act directly on the Sertoli cells through RARα/RXRβ receptors, as well as through RARγ receptors on spermatogonia[39,41] (Figure 1.3(B)). Thus, it is likely that RA may act in both autocrine and paracrine fashion to facilitate meiotic entry in males. This possibility is supported by recent studies involving cell-specific ablation of all three *Raldh/Aldh1a* genes.[40] Loss of all three *Raldh* genes specifically from Sertoli cells results in a failure of spermatogonial differentiation from A-type to A_1-type, which represents an irreversible first step toward meiotic initiation.[40] This effect is mediated specifically via the RARα receptor on Sertoli cells, indicating an important autocrine effect of RA in this context. Conversely, RA produced by preleptotene spermatocytes (those that are just about to enter prophase I of meiosis) may act in a cell-autonomous fashion to induce meiotic entry

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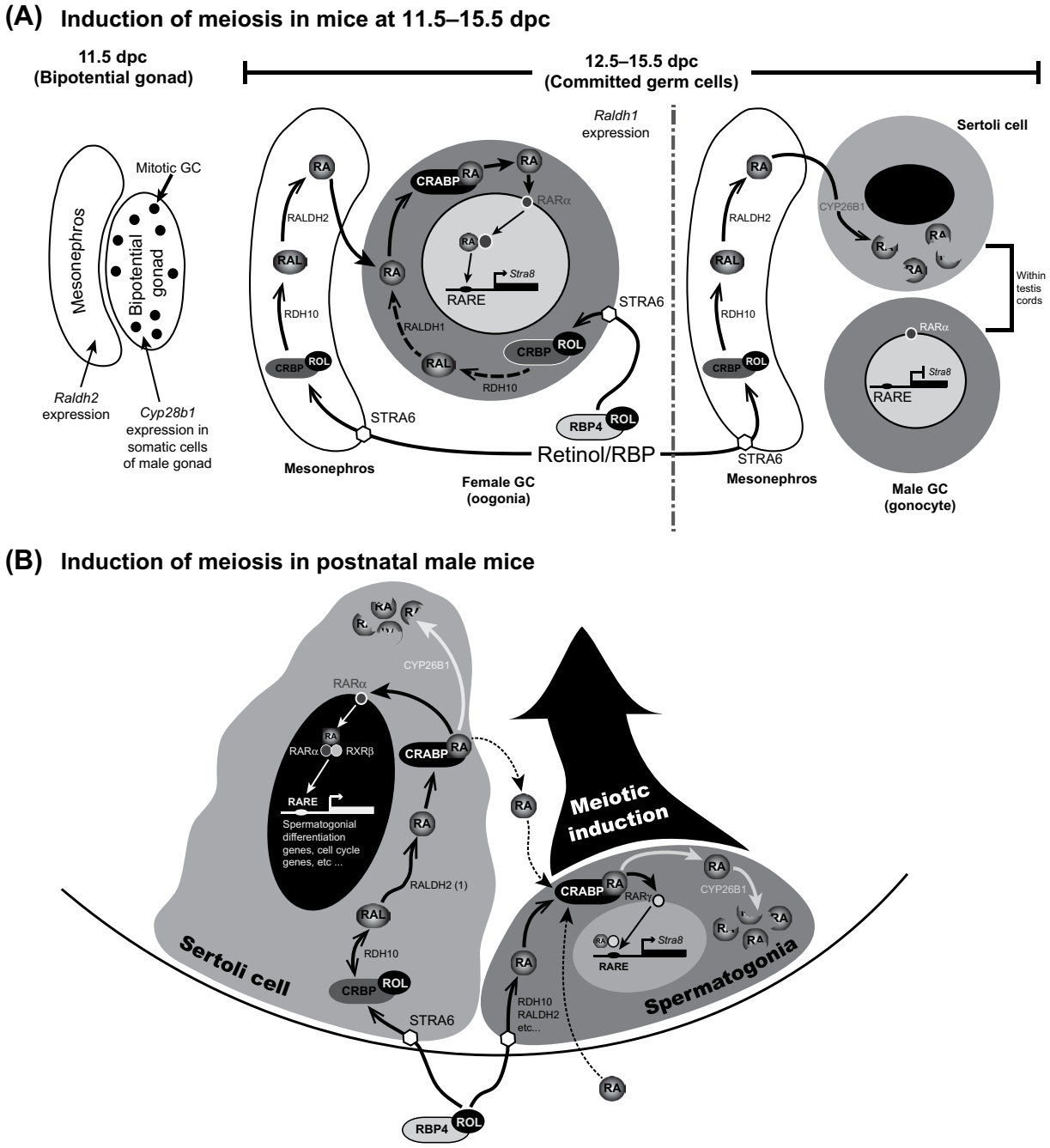

FIGURE 1.3 **Induction of meiosis in (A) female and (B) male mice.** (A) Females initiate meiosis during embryonic development, starting at around 12.5 dpc. Retinol and retinol-binding protein (RBP) are transported to the mesonephros and taken up via STRA6 receptors. Retinoic acid (RA) is produced through sequential steps, through retinaldehyde, and the RA is then released into the adjoining gonad. Alternatively, RA may be produced directly in germ cells, although this is somewhat contentious at the current time. Once in the oocyte, RA is taken up into the nucleus by binding to RARα, and the complex binds to retinoic acid response elements (RAREs) upstream of RA-responsive genes. In the cell, retinol and its metabolites are bound to appropriate binding proteins (e.g., CRBP and CRABP). In males, RA is degraded in the Sertoli cells of the developing testis cords by CYP26B1, preventing its activity on adjoining male germ cells. (B) In postnatal male mice, retinol is transported to Sertoli cells in which resides all of the appropriate machinery for RA synthesis. CYP26B1 is downregulated in the Sertoli cell, resulting in elevated RA levels. This RA is directed toward adjoining spermatogonia, and can induce expression of RA response genes through RA–RARγ uptake into the nucleus. Once again, CYP26B1-mediated degradation of RA is prevented by downregulating its expression in male germ cells. See the accompanying text for further details.

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via RARγ-mediated induction of *Stra8* expression[17,40,41] (Figure 1.3(B)). Importantly, however, the requirement for autocrine signaling of RA within the Sertoli cell to induce initial spermatogonial differentiation appears necessary only for the first wave of spermatogenic initiation, and is dependent on RARα, while RARγ is not required,[41] presumably because it resides within the germ cells. Conversely, Sertoli cell RA signaling through RARα does not appear to be required for subsequent waves, the induction of RA being now relegated presumably to the germ cells.[30] All of this information notwithstanding, it seems that the question of where RA is synthesized in the postnatal testis is not yet fully resolved, and more studies are required to confirm the spermatogonia and spermatocyte populations as sources of autocrine RA.

This potential role of germ cells in inducing meiotic initiation helps to clarify an important controversy regarding the role of RA in meiotic entry. Kumar et al.[42] utilized female mice bearing mutations in the *Raldh2/Aldh1a2* gene, or in both the *Raldh2/Aldh1a2* and *Raldh3/Aldh1a3* genes specifically in the mesonephros during embryogenesis, and showed that *Stra8* expression was still observed in fetal oocytes.[42] They also demonstrated that inhibition of CYP26B1 activity with ketokonazole allows for RA-independent induction of *Stra8* expression in the wild type or in the mutant embryos, but only if the mesonephros remains intact, while RA activity is not detected in wild-type fetal testes following ketoconazole treatment.[42] Taken together, these findings would suggest (1) that RA does not act directly on the gonad to induce meiotic entry during fetal life, and (2) that CYP26B1 acts to inhibit entry into meiosis but not at the level of gonadal RA activity. The authors concluded from these observations that RA was not acting to promote meiotic entry since they could find no evidence of another (gonadal) source of RA that could be responsible for meiotic initiation in females. Furthermore, they concluded that the target of CYP26B1 was a non-RA molecule within the mesonephros.[42] However, these data are in line with those described for the male here, and lead to a model in which RA produced by the developing ovary, and more specifically by the oogonia, is responsible for inducing meiotic progression either in a cell-autonomous manner to promote *Stra8* expression and/or indirectly by acting in a paracrine fashion to induce RA-responsive genes in the mesonephros (Figure 1.3(A)). Thus, ketoconazole inhibition of CYP26B1 could result in the loss of RA signaling specifically in the mesonephros, but with the RA originating from the fetal gonad. In support of this, a recent report suggests that RA can be produced from retinol by PGCs in the fetal ovary, with the RALDH1/ALDH1A1 protein being localized specifically in germ cells from 12.5 to 15.5 dpc[43] (Figure 1.3(A)). Such an observation is supported by those early studies of ectopic germ cell entry into meiosis, which is well clear of any influence of the mesonephros.[14] Taken together, these recent studies lend support to the idea that the germ cells themselves, rather than supporting cells and tissues, are the source of the RA that triggers meiotic progression, but that the effect of RA may require these supporting cells for paracrine interactions that lead to the onset of meiotic events.

Stra8 and the Induction of Meiosis

The major target of RA activity in mouse germ cells is to induce the expression of *Stra8*,[18,25] a gene initially described and characterized in P19 embryonal carcinoma cells.[21,44] In the mouse embryo, *Stra8* expression is observed from 12.5 dpc in females, approximately one day prior to the onset of meiosis. Prior to this time, male and female germ cells are indistinguishable, expressing similar genes such as Deleted in azoospermia-like (*Dazl*; Figure 1.4(A) and (B)). Interestingly, the entry into meiosis is not synchronous, but instead occurs in an anterior-to-posterior wave through the ovary.[45,46] Thus, germ cells in the anterior region of the ovary are exposed to RA, express *Stra8*, and enter meiosis from 12.5 dpc, while those of the posterior end enter somewhat later, with RA activity being evident from 13.5 dpc and *Stra8* expression extending to 16.5 dpc.[8,45] This *Stra8* expression occurs under a backdrop of high RA levels, coincident with downregulation of *Cyp26b1* expression (Figure 1.4(A), (C)–(F)), and leads within hours to the downregulation of pluripotency markers, such as POU-domain class 5 transcription factor 1 (*Pou5f1*; also known as octamer-binding transcription factor 4 (*Oct4*)), and the increased expression of meiotic markers such as Synaptonemal complex protein 3 (*Sycp3*), *Spo11*, and the meiosis-specific RecA homolog, Disrupted meiotic cDNA 1 (*Dmc1*).[9,25,47] *Stra8* expression is not required for proliferative activity of the female germ cells, but appears instead to be essential for the premeiotic S phase and meiotic entry.[16] The evidence that similar mechanisms exist in humans is provided by the observation that RA can induce *STRA8* expression in human fetal gonad explant cultures.[48,49]

In males, as in females, RA leads to induction of *Stra8*, but this occurs postnatally and usually around the time of puberty in most mammals. In mice, however, *Stra8* induction occurs co-incident with downregulation of *Cyp26b1* during early postnatal life, from around day 8 postpartum (pp), immediately prior to the onset of meiotic prophase I (Figure 1.4(A), (D), and (E)).[50] Loss of *Stra8* in males appears to result in a failure to transition from preleptotene stages into prophase I, but this appears to be a background-specific phenomenon, since the failure to enter meiosis

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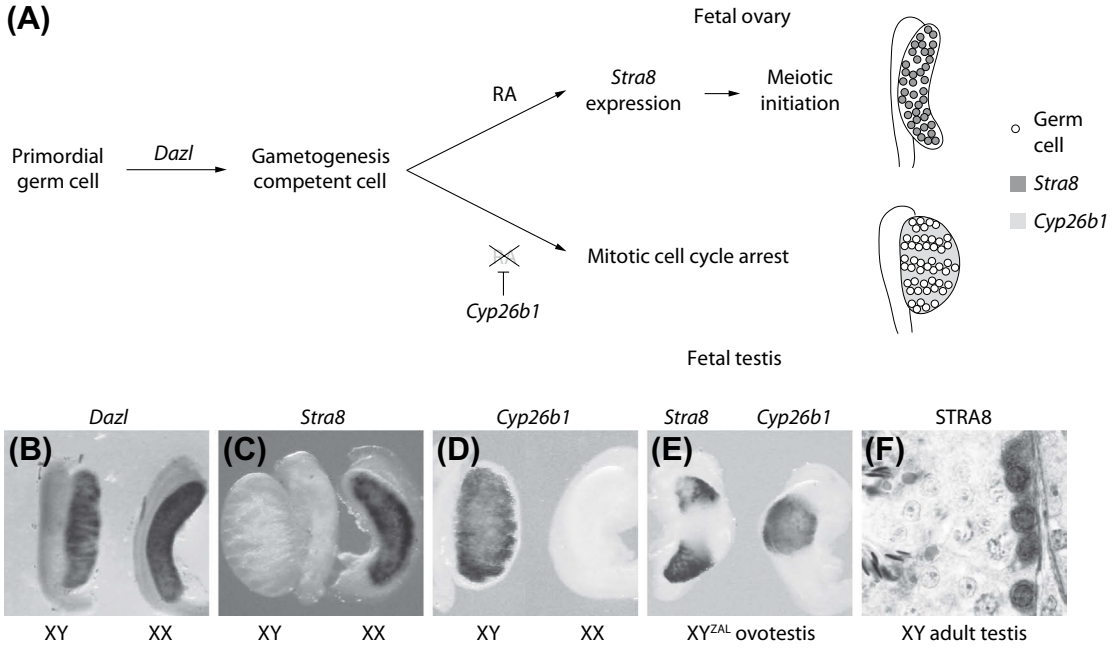

FIGURE 1.4 Regulation of meiotic initiation in mice. (A) Ovarian germ cells initiate meiosis during embryogenesis, whereas testicular germ cells undergo G0/G1 mitotic cell cycle arrest and do not initiate meiosis until after birth. Primordial germ cells in the fetal gonad express *Dazl*, thus acquiring competence for gametogenesis and gaining the ability to interpret RA as a meiosis-inducing signal. In the fetal ovary, signaling by retinoic acid (RA) induces *Stra8* expression and thereby meiotic initiation in germ cells. In the fetal testis, cytochrome p450 (*Cyp26b1*), an RA-metabolizing enzyme, is expressed in somatic cells surrounding the germ cells and prevents premature *Stra8* expression. (B–D) Whole-mount in situ hybridization on fetal testes and ovaries (B) at embryonic day 13.5 (E13.5) for *Dazl*; and at E14.5 for (C) *Stra8* and (D) *Cyp26b1*. (E) In E14.5 XY^{ZAL} ovotestes, *Stra8* is expressed in the polar regions exhibiting ovarian histology, and *Cyp26b1* is expressed in the central region exhibiting testicular histology. XY^{ZAL} embryos carrying a Zalende-derived Y chromosome on a C57BL/6 genetic background are often partially sex-reversed and have ovotestes. (F) Immunohistochemistry for STRA8 in adult testis. STRA8 is expressed in preleptotene germ cells in stage VII/VIII tubules. *Source: Figure kindly provided by Dr David Page and Shirleen Soh, Whitehead Institute, MIT, with additional images provided by Yanfeng Lin (panel B) and Tsutomu Endo (panel F). (A, C, D, E) Adapted from Refs 16,18,45,492,493.*

is strain specific.[16,17,51] In one *Stra8* mutant mouse line, meiotic entry was not impeded by the loss of *Stra8*, but instead meiotic progression was disrupted as a result of premature chromosome condensation.[51] As a result, Hogarth et al. concluded that genes other than *Stra8* must also be required to promote meiotic entry, and they went on to identify three potential candidates, at least one of which was RA responsive. Indeed, the RA-responsive gene, Establishment of cohesion 1 homolog 2 (*Esco2*), is expressed in embryonic ovaries at around 14.5 dpc, and in postnatal testis at day 10 pp, the protein appears to reside within the nucleus and is an essential component of the sister chromatid cohesion complex.[50,52] The roles of *Esco2* as well as of other genes whose profiles correlate with *Stra8* expression, such as *Setdb2* and *Uba6*,[50] remain unclear at the current time.

Recent studies have identified the mammalian Doublesex-related transcription factor 1 (DMRT1) as a transcriptional repressor of *Stra8* in both male and female germ cells.[53,54,55] However, it appears that the effect of DMRT1 on meiotic initiation is cell context specific and perhaps relies on differential posttranslational modification of the protein, resulting in sexually dimorphic regulation of meiotic entry. In adult males, expression of *Dmrt1* prevents the transition from spermatogonial to spermatocyte stages by limiting RA-induced transcription of *Stra8*, and at the same time promotes spermatogonial development by induction of genes required for differentiation from A- to B-type spermatogonia, including the Spermatogenesis and oogenesis-specific helix–loop–helix 1 transcription factor (*Sohlh1*).[54] DMRT1 was shown to bind to the *Stra8* promoter close to the RA response elements, presumably preventing binding of RA and thus limiting meiotic entry.[54] Interestingly, DMRT1 also alters expression of *Cyp26b1* and some of RA's synthetic enzymes, suggesting a direct action of DMRT1 on RA production and stability.[54] Alternatively, given recent evidence that pachytene spermatocytes can synthesize RA,[40] the reduced expression of these RA enzymes in the absence of DMRT1 may result from a loss of cells that are themselves a major source of RA. In either case, it appears that DMRT1 may modulate RA responsiveness

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by spermatogonia, thereby providing a modulating influence for meiotic entry in the premeiotic cell population. Taken together, these new observations present a mechanism by which meiotic entry is controlled by the exquisite interplay between opposing pathways to both limit and regulate how and when cells can initiate prophase I.

Unlike the situation in postnatal males, DMRT1 does not appear to play a role in preventing meiotic entry in fetal mouse testes.[55] *Dmrt1* expression arises prior to sex determination in the bipotential gonad at 11.5 dpc,[53,56] but its expression in the ovary is transient, disappearing from somatic cells by 13.5 dpc and then from germ cells by 15.5 dpc.[53,56] Despite this transient expression, loss of *Dmrt1* in *Dmrt*$^{-/-}$ female embryos results in significantly reduced expression of *Stra8* and a consequent failure to form the full complement of primordial follicles.[53] The level of expression of *Stra8* is sufficient to allow some cells to enter meiosis, albeit aberrantly, but these cells subsequently fail to proceed normally through prophase I. This is exactly the opposite situation to that seen in postnatal male germ cells, in which DMRT1 is required to suppress meiotic entry through downregulation of *Stra8* expression.[54]

Sry Induction of the Male Germ Cell Program during Fetal Development

While the destruction of RA by CYP26B1 is a major mechanism by which male germ cells avoid meiotic entry, a recent study has suggested that secretions from cells other than the germ cells may also function to restrict meiotic entry by male germ cells in utero. This was demonstrated by the observation that XY germ cells in cultured mouse fetal testes can occasionally enter meiosis when secretory activity is prevented by the compound Brefeldin A.[57] One such secretory protein appears to be fibroblast growth factor 9 (FGF9), which is essential for male germ cell survival during fetal life, and deletion of which is known to result in male-to-female sex reversal.[58,59] *Fgf9* expression arises in the somatic cells of the bipotential gonad prior to gonadal sex determination, but by 11.5 dpc becomes restricted to the developing testis,[60,61] having been suppressed in females by the female factor gene, wingless-related MMTV integration site-4 (*Wnt4*) (reviewed by Ref. 62). FGF9 treatment of cultured mouse gonads restricts meiotic entry, resulting in reduced *Stra8*, *Dmc1*, and *Sycp3* expression,[61,63] but at the same time it promotes the expression of pluripotency factors such as *Oct4* and Sex determining region Y (Sry)-box 2 (*Sox2*), along with markers of male fate, including Nanos homolog 2 (*Nanos2*) and DNA methyltransferase 3-like (*Dnmt3L*).[61,63] The action of FGF9 occurs directly on germ cells, most likely via the FGF receptor FGFR2, which is present on the surface of germ cells at 12.5 dpc. It has been postulated that the action of FGF9 on germ cells results in upregulation of the RNA-binding protein NANOS2 in primordial germ cells of both sexes and in premeiotic spermatogonia, leading to suppression of meiotic entry.[63] Studies using in vitro culture of fetal tissues indicate that FGF9 most likely acts by reducing the responsiveness of germ cells to RA, possibly via *Nanos2* regulation, reducing the ability of RA to induce *Stra8* expression,[64] while at the same time suppressing expression of *Wnt4*.[65] Thus, loss of *Fgf9* induces upregulation of *Wnt4*, which induces germ cells to become feminized, while loss of *Wnt4*, in turn, induces a male fate via upregulation of *Sox9* (reviewed by Ref. 66). *Sox9* is a direct target of the sex determination gene *Sry*, whose expression in males initiates a feedforward loop between *Sox9* and *Fgf9* (Figure 1.5), leading to upregulation of the latter.[65] Taken together, these observations suggest that suppression of meiotic entry in male gonads during embryogenesis is brought about by SRY-induced *Sox9* expression that, in turn, results in the expression of *Fgf9* (Figure 1.5(A)). The action of FGF9 is to suppress *Wnt4* expression and to impair the ability of male germ cells to respond to RA, through mechanisms that remain largely undefined. In addition, expression of *Cyp26b1* is enhanced both by SOX9 and by the transcription factor steroidogenic factor 1 (SF1) to promote RA degradation (Figure 1.5(A)).[67]

In the absence of SRY in females, germ cell sex determination is marked by increased expression of *Wnt4*, whose expression is regulated by R-spondin1 (RSPO1), leading to suppression of *Sox9* and *Fgf9* expression.[62]

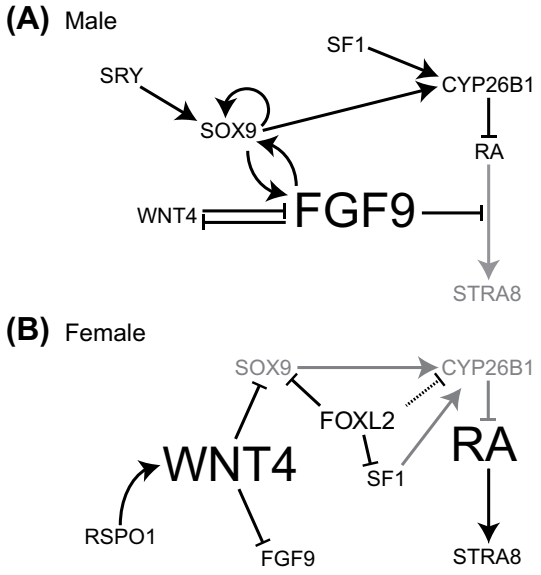

FIGURE 1.5 Summary of key sex determination signals that inhibit retinoic acid (RA) induction in male germ cells (A), and those that induce RA in female germ cells during embryonic development (B). See the main text for further details.

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At the same time, elevated expression of forkhead transcription factor 2 (*Foxl2*) acts to suppress production of CYP26B1 through several mechanisms: by further suppressing *Sox9* expression, by repressing *Sf1* expression,[67] and possibly also by other mechanisms. Thus, in females, the drivers of sex determination consist of WNT4 and FOXL2, which help to promote meiotic entry via suppression of *Cyp26b1*, thereby stabilizing RA levels in the gonad (Figure 1.5(B)). Whether WNT4 and FOXL2, or indeed any other factors, have a direct effect on RA metabolism and action is uncertain at the current time.

Early Indications for a Role for Small RNAs in Meiotic Entry

Recent studies have indicated a role for small RNA species in meiotic initiation. Members of the microRNA family *Mirlet7*, for example, are expressed in premeiotic and meiotic germ cells in response to RA signaling.[68] The induction of *Mirlet7* expression by RA signaling has been shown to occur via repression of the RNA-binding protein LIN28.[68] Furthermore, in mice lacking the Argonaute 4 (*Ago4*) gene, which encodes a member of the AGO family of proteins that are defining components of the small RNA–processing machinery, early postnatal spermatogonia enter meiosis approximately 4–6 days early.[69] This precocious entry into meiosis is associated with early upregulation of genes such as *Stra8*, *Esco2*, and *Sycp3*, and also altered expression of genes encoding the RA synthetic enzymes and *Cyp26b1*, suggesting that small RNAs that associate with, and function alongside, AGO4 may modulate meiotic entry through feedback mechanisms that regulate RA metabolism. These studies are at a very early stage, but they raise the possibility that meiotic induction and RA metabolism in germ cells are intricately linked to small RNA biogenesis and action.

PROPHASE I

Overview of Prophase I

Events during the first meiotic stage (meiosis I) are designed expressly to ensure that homologous chromosomes (one maternal and one paternal in origin) segregate equally at the first meiotic division (Figure 1.1). By contrast, during the second meiotic division, as in mitosis, it is the sister chromatids that must segregate equally. For meiosis II and mitosis, this is relatively easy, since the sister chromatids are already tethered together via the process of cohesion (discussed further in this chapter). It stands to reason, therefore, that the homologous chromosomes of meiosis I must first find a similar pairing mechanism that will enable them to segregate in a similarly efficient manner. Thus, the necessity of homologous chromosome pairing adds a level of complexity to meiosis I that is unique and defining for this stage. These events all take place during prophase I, and pairing is ensured by two distinct mechanisms, synapsis and recombination, the former through protein–protein interactions and the latter through DNA–DNA interactions. Both events require that homologous chromosomes must first locate each other, recognize their homologs, and then interact physically, and both are specifically designed to allow the tethering of homologs until the first meiotic division, much as sister chromatids are physically connected through cohesion.

As a result of the added complexity, meiotic prophase I is extended compared with its mitotic counterpart, and can take several days to complete in mammalian species. Separated into five distinct substages (Figures 1.6 and 1.7(A)–(E)) based on the cytological appearance of the chromosomes, prophase I is the most complex and long-lived stage of meiosis. Figure 1.6 summarizes the events of prophase I in temporal order from left to right, including the synapsis events (described in the upper portion of the figure) and recombination events (described in the lower portion of the figure). The status of the sister chromatids and homologous chromosomes is depicted in cartoon form in the middle of Figure 1.6.

The first substage of prophase I is known as leptonema (from the Greek *leptos*, meaning "thin"). It is the stage immediately following premeiotic DNA replication where the chromosomes are still fairly long, decondensed, and threadlike. The main event during leptonema involves the initial assembly of a proteinaceous structure, termed the synaptonemal complex, which begins to form a scaffold along each homologous chromosome between the sister chromatids. These scaffolds are termed axial elements, and they first assemble as short stretches along each homologous chromosome, the stretches coalescing to become longer filaments until they extend down the entire length of the chromosome in late leptonema and into the zygotene stage. Concurrent with synaptonemal complex assembly, meiotic cohesins are also assembled onto the homologous chromosomes, and subsequently disassembled, a process that is discussed in detail in this chapter.

During the second substage of prophase I, termed zygonema (from the Greek *zygon*, meaning "adjoining"), homologous partner chromosomes begin to pair, and then to synapse, the latter resulting in the physical tethering between homologs. Simultaneously, the DNA begins to condense, making the chromosomes more visible cytologically. This process of synapsis requires that the axial elements along each

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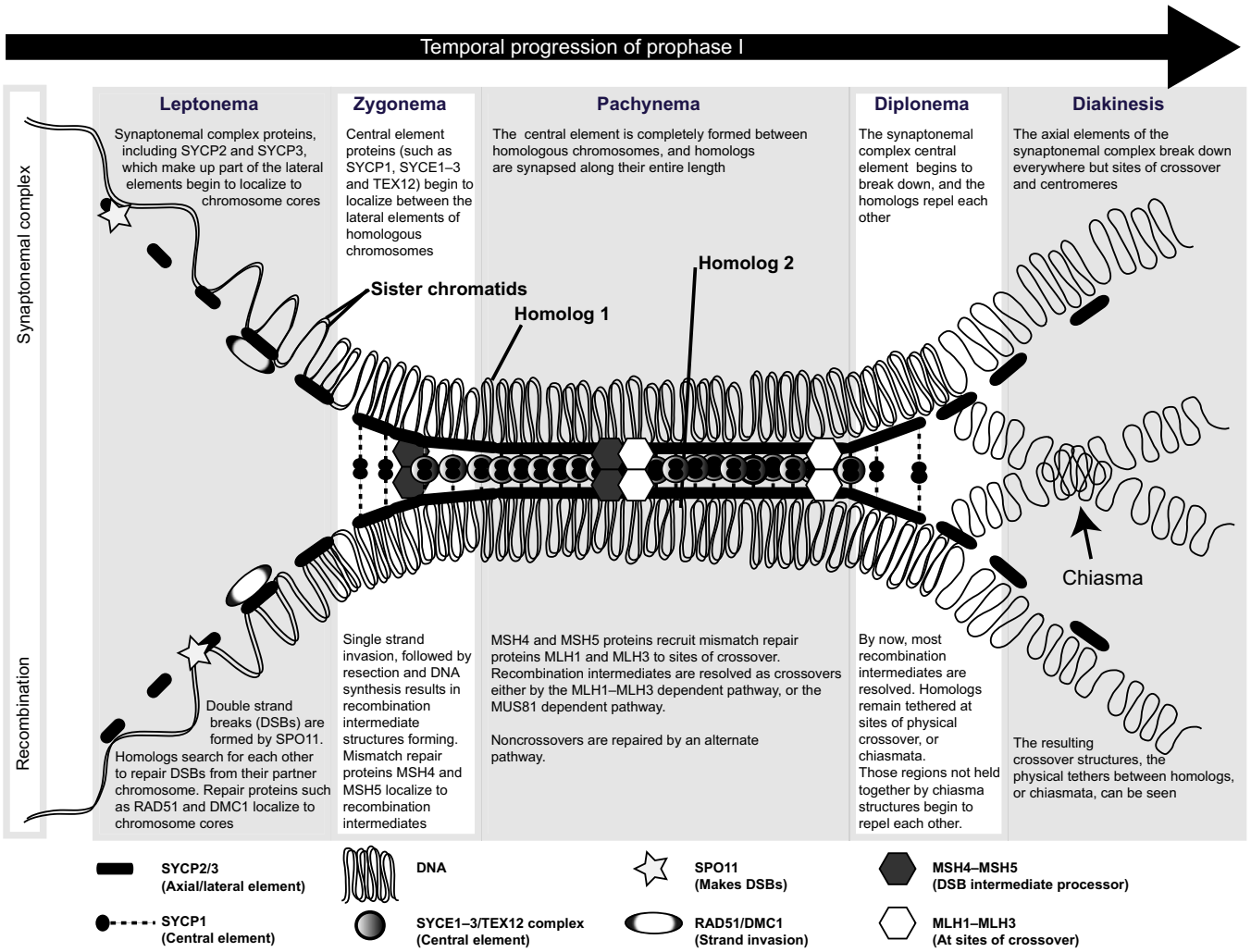

FIGURE 1.6 Dynamics of recombination and synapsis in prophase I. Events occurring during the five substages of prophase I (leptonema, zygonema, pachynema, diplonema, and diakinesis) are depicted schematically, according to their involvement in either synapsis events (along the top) or recombination events (along the bottom).

homolog are stabilized and joined together in a zippering process that involves multiple protein complexes, collectively termed the central element. Once the homologs have been joined together by the central element, they are said to have synapsed, and, once this occurs, the axial elements are reclassified as lateral elements. Upon complete synapsis along the entire length of the homologous axes, the cell enters the third substage of prophase I, known as pachynema (after the Greek *pachy*, meaning "thick", given that the chromosomes are now fully condensed and have a short, fat cytological appearance). Pachynema is the longest of the substages and can last up to 10 days in some rodent species, and even longer in humans. Once pachynema is complete, the central element of the synaptonemal complex begins to break down, and the chromosomes begin to repel each other. This fourth substage is known as diplonema (from the Greek *diplo*, meaning "double", given that two homologs become clearly visible again). Despite the central element breaking down and the synaptonemal complex disassembling, the homologs remain joined at the physical sites of meiotic COs, called chiasmata. These COs are generated by the process of repairing meiotic double-strand breaks (DSBs), discussed further in this chapter, in a process that is initiated in preleptonema and leptonema and that progresses concurrently with synaptonemal complex assembly. The fifth and final substage of prophase I, diakinesis, is named for the Greek *dia* (meaning "through") and *kinesis* (meaning "movement"), so in essence it means "moving through", indicating the final stage before proceeding to the next phase of meiosis, metaphase I. At this point, the homologs are connected only by the remnants of the synaptonemal complex found at the centromeres and at the CO sites.

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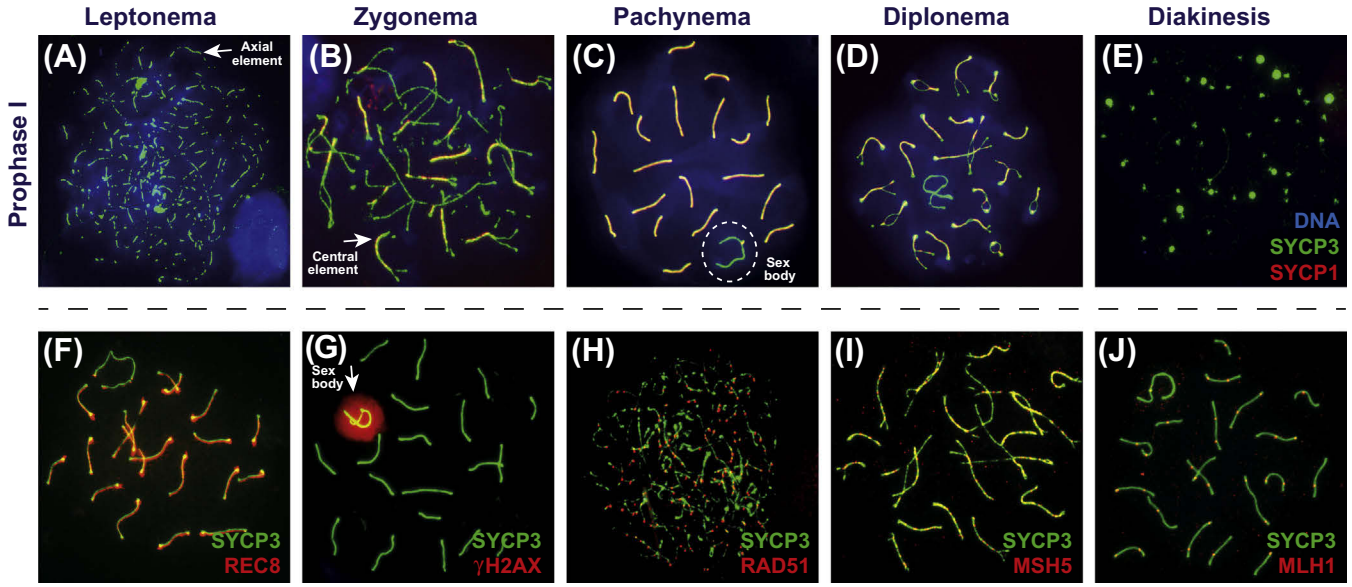

FIGURE 1.7 Immunofluorescent analysis of meiotic prophase I events in mouse spermatocytes. This figure is reproduced in color in the color plate section. (A–E) Synaptic events in the five substages of prophase I, visualized by staining with antibodies against axial–lateral element protein SYCP3 (green) and central element protein SYCP1 (red), where the overlay between the two appears in yellow. (F–J) Immunolocalization of SYCP3 (green) with key meiotic markers during prophase I; (F) REC8, (G) λH2AX, (H) RAD51, (I) MSH5, and (J) MLH1 all in red, and SYCP3 in green.

Chromosome Dynamics and Pairing in Early Prophase I

Chromosome Movements, Telomere Clustering, and Telomere Bouquet Formation

One of the fascinating aspects of meiotic prophase I is the delicate, yet dramatic, orchestrated "dance" that occurs between homologous chromosomes. The reorganization of the nuclear contents, and their situation with respect to the nuclear envelope, are, quite understandably, critical to the success of homolog alignment and, as a consequence, are essential for prophase I progression. It is notable that although there exists a large body of data concerning the molecular events that occur following synapsis of chromosomes, far less is known about how homologs recognize and pair with one another. Almost certainly, numerous mechanisms exist to promote homolog recognition and pairing, and these may be utilized to differing degrees in different organisms. For example, in many organisms, while pairing may occur at numerous sites along chromosomes, there are often defined locations where initial pairing interactions take place. The so-called pairing centers of *C. elegans* are a strong example of this.[70] The congregation of pairing centers coincides with the generation of highly conserved rapid chromosome movements, which have been demonstrated for many species, including mammals and both budding and fission yeast.[71,72] Driven by the Sad1p, UNC-84 (SUN)–Klarsicht, ANC1, syne homology (KASH) family of nuclear membrane–bound proteins, rapid chromosome movements are propagated by telomere connections to cytoplasmic motors that drive oscillatory movements.

Another important feature of chromosome interactions during early prophase I is the "telomere bouquet". The bouquet describes the clustering of telomeres at one location across the nuclear envelope, and is noted in many meiotic species.[73,74] However, the relative importance of rapid chromosome movements and bouquet formation appears to be species specific, since, for example, in *Sordaria macrospora*, the bouquet stage follows homolog pairing[75] and also follows early synapsis in female mice,[76] while in *Saccharomyces cerevisiae*, rapid chromosome movements appear to be more critical than bouquet formation for pairing.[77]

The oscillatory rapid chromosome movements of the fission yeast, *S. pombe*, are particularly noteworthy due to their long-lived activity and their dramatic motion through the nuclear space. During this so-called horsetail motion,[78] the telomeres gather in a tight bouquet adjacent to the spindle pole body (the microtubule organizing center that is itself outside the nuclear membrane on the cytoplasmic side) early in prophase I. The spindle pole body then proceeds to drag the entire nucleus backward and forward, powered by a meiosis-specific dynein motor while meiotic recombination is taking place.[79] Mutants in which telomeres coalesce normally but that are incapable of spindle pole body–driven nuclear movements show more severely impaired

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meiotic recombination than mutants with impaired bouquet formation, indicating that the horsetail movements of the nucleus are more critical for recombination events in *S. pombe*.[73] In contrast, mutants that exhibit altered bouquet formation have more defective asci (spore-bearing cells), suggesting that the bouquet may play additional roles in later or additional meiotic events.

Telomere clustering also plays a prominent role in meiotic prophase I in budding yeast (*C. elegans*) and in mammals (reviewed in Refs 80,81,82). However, the relative importance of rapid chromosome movements and bouquet conformations for homolog pairing and synapsis in mammals is relatively less well understood. In early prophase I in mouse meiocytes, rapid chromosome movements are readily observed and can be visualized in real time.[83] In addition, telomeres attach to the nuclear envelope via a conical thickening of the axial element.[81,82] These telomeres coalesce, as they do in other organisms, to form a defined bouquet structure. Moreover, in male mice, the bouquet is short-lived and often hard to find, but occurs concomitantly with homolog alignment and pairing, calling into question whether pairing is dependent on these telomere events in mammals. Loss of synaptonemal complex protein 3 (SYCP3) does not affect bouquet formation, despite the loss of axial elements,[84] whereas loss of the cohesion component (structural maintenance of chromosomes 1 beta (SMC1β)) results in failed nuclear envelope attachment.[85] Telomeric repeats also appear important for the clustering of telomeres in mouse, yeast, and worm (reviewed in Ref. 82).

Involvement of SUN–KASH Proteins in Telomere Tethering to the Nuclear Envelope

At the molecular level, the pathways that regulate telomere movements and bouquet formation are only just being elucidated, most of the prominent players having been identified in fission yeast. In *S. pombe*, Bqt1 and Bqt2 proteins connect telomeres to the spindle pole body via their interactions with Rap1, and, together, these three proteins form a bridge from the telomere to Sad1, a spindle pole body protein associated with the inner nuclear membrane.[86,87] Rap1, in turn, requires Taz1 for its association with the telomere in *S. pombe*.[88] Sad1 is the founding member of the SUN domain proteins, whose orthologs are present across the spectrum of meiotic species.[89] For example, in budding yeast, the telomere-associated protein Ndj1 is critical for telomere movements and bouquet formation,[82,90,91] and its function is dependent on interactions with the SUN domain protein, Mps3, present on the inner nuclear membrane.[91,92]

SUN proteins function together with KASH domain proteins, located on the outer nuclear membrane, forming a bridge that links telomeric DNA ends with specific elements of the cytoskeleton.[93] KASH domain proteins include Kms1 in *S. pombe* and ZYG12 in *C. elegans*, and they have also been reported for mammals (reviewed by Refs 89,94,95). In budding yeast, in contrast, Csm4 is required for telomere clustering and for homologous recombination[91,96,97] and, while not strictly a KASH domain-containing protein, appears to interact with Mps3 and Ndj1.[89]

In mammals, at least six SUN proteins have been described, only some of which have been associated with a specific KASH protein (reviewed by Refs 94,95). The two major SUN proteins involved in mammalian meiosis are SUN1 and SUN2, both of which utilize a common meiosis-specific KASH protein, KASH5, for tethering to cytoplasmic microtubules.[95,98,99] Localization of both SUN1 and KASH5 is restricted to meiotic prophase I, and more specifically to the telomeric regions of chromosomes during leptotene of prophase I onward.[98,100] Loss of *Sun1* in mice results in loss of telomere attachment to the nuclear envelope and synapsis failure during prophase I, resulting in male and female sterility.[100] Similarly, mutation of the *C. elegans* ortholog of *Sun1*, Matefin, disrupts its interaction with the KASH protein, ZYG12, and prevents synapsis and homologous recombination.[101]

What is clear from all these comparative studies of chromosome dynamics is that chromosomal connection to the cytoskeletal machinery, via the nuclear envelope, is a conserved feature of early prophase I progression and is essential for initial chromosome interactions and/or recombination. Conserved protein families mediate these interactions, including proteins of the SUN and KASH family (Figure 1.8). Most commonly, the attachments at the telomere may involve meiosis-specific proteins, such as Ndj1 in budding yeast and Bqt1 and Bqt2 in fission yeast, although no such linker protein has yet been identified in mammals. Rap1, which is essential for such interactions in fission yeast, appears to play no role in telomere attachment during meiosis in mice.[102] The SUN and KASH components involved in telomere clustering and homolog pairing in the plant kingdom remain to be elucidated, but are almost certainly conserved like their animal kingdom counterparts.

Pairing Centers and Noncanonical Mechanisms to Induce Homolog Pairing

It is interesting to note that in organisms that do not display a defined bouquet, nuclear envelope interactions remain critical for prophase I progression. *Caenorhabditis elegans* is an example of this in which pairing is mediated by pairing centers,[70] which themselves associate with the nuclear envelope during early stages of prophase I.[103] Pairing centers on each chromosome are bound by a single member of a family of zinc finger proteins, HIM8, and the ZIM proteins, ZIM1, ZIM2, and ZIM3.[103] These proteins mediate

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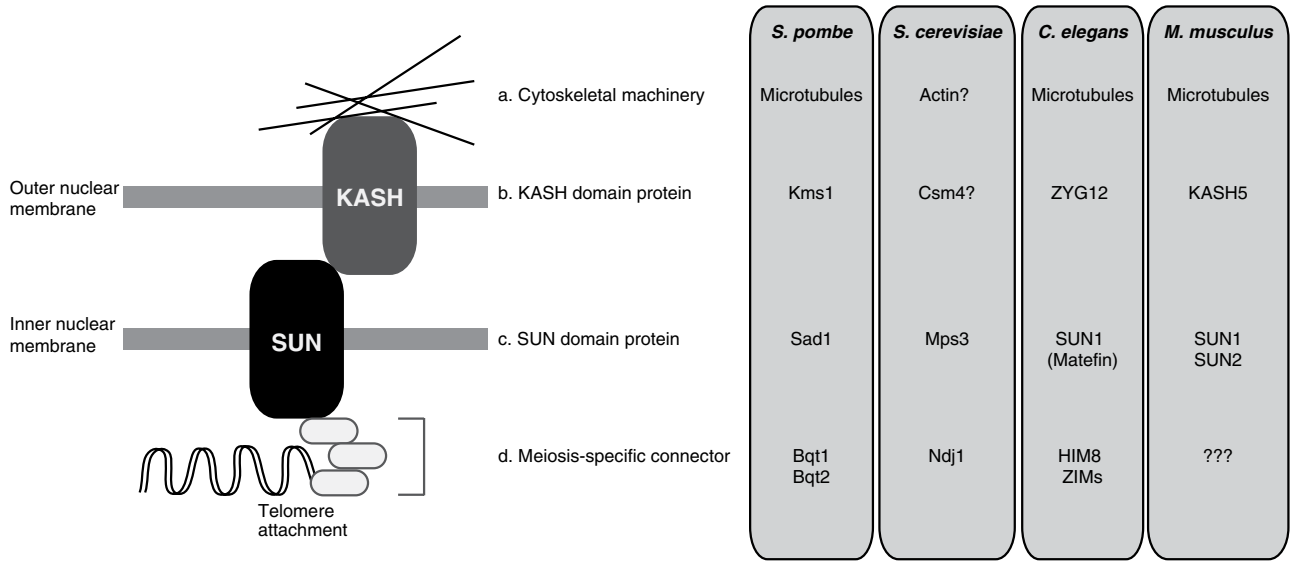

FIGURE 1.8 Chromosome movement and association with the nuclear envelope are coordinated through the highly conserved SUN–KASH family of nuclear membrane proteins. The left panel represents a simplified cartoon of SUN–KASH interactions with meiotic chromosomes and with the cytoskeletal machinery. On the right, known mediators of these events are shown for fission yeast, budding yeast, worms, and mice.

binding of the pairing center to the nuclear envelope in a bouquet-like fashion. Thus, while no specific bouquet is formed, the nucleus of early prophase I meiocytes in *C. elegans* displays a distinctly polarized appearance, and the chromosomes are clearly asymmetrically distributed.[104] This nuclear association of chromosomes is dependent on the SUN protein Matefin (SUN1) and the KASH protein ZYG12.[101,105] Thus, nuclear attachment is mediated at pairing centers rather than at telomeres, but is also mediated through SUN–KASH interactions with the ZIM proteins.

A recent report from studies in *S. pombe* indicates that a novel RNA-induced homolog-pairing process may exist in this organism.[106] Ding et al. demonstrate that the *SME2* locus is among the first to pair during the horsetail stage. This locus encodes a meiosis-specific noncoding RNA that appears to mediate homolog recognition. In *S. pombe*, meiotic initiation is regulated by Mei2, an RNA-binding protein. Mei2 forms a distinct focus at the *SME2* locus, and it sequesters Mmi1, another RNA-binding protein that is required for elimination of meiosis-specific mRNA transcripts in mitotic cells. Thus, the Mei2-induced removal of Mmi1 allows meiotic progression. At the same time, the binding of Mei2 and Mmi1 to *SME2* transcripts is sufficient to induce pairing at this locus, as well as between ectopic loci on nonhomologous chromosomes. It appears, however, that while *Sme2* transcripts can act as *cis*-acting pairing factors, there may be other, similar noncoding RNA loci that play similar roles in pairing and meiotic initiation across the *S. pombe* genome.

The Synaptonemal Complex and Synapsis

The synaptonemal complex was first visualized by Moses and Fawcett in 1956.[107,108] As a complete tripartite protein structure, it consists of two lateral elements that align each homolog and the central element. In addition, transverse filaments have one end anchored in the lateral element and the other in the central element, and function to "zipper" the two homologs together during early prophase I, in the process known collectively as synapsis (Figure 1.6). In the mouse, a significant amount of chromosome pairing occurs premeiotically, prior to the initiation of the DSBs at leptonema, and is dependent on the SPO11 and SUN1 proteins.[109]

Upon entry into meiosis, in the leptotene stage, the synaptonemal complex begins to form short stretches of axial element along the sister chromatids of each homologous chromosome, with the chromatin extending perpendicularly out from the development axial element to form loops (Figure 1.7(A)). The two major components of the axial elements are SYCP2 and SYCP3, first identified in the rat but later identified in many other mammalian species.[110,111,112,113] SYCP3 consists of two proteins in mice and rats (30 and 33 kDa in size) that are transcribed from the same gene, with the shorter isoform very highly conserved among vertebrate species,[114] whereas SYCP2 is a much larger protein at 173 kDa.[113] SYCP2 and SYCP3 form heterodimers that are dependent upon the conserved coiled-coil domain in SYCP2. Both proteins begin to localize to chromosomes to form axial elements during leptonema.[115,116] Mutations in either *Sycp2* or *Sycp3* in the mouse result in a

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similar phenotype, namely, the absence of functional axial elements and proper synapsis, along with impaired specificity of chromatin loop attachment, resulting in male sterility and female subfertility.[84,115,117,118] Intriguingly, mutant SYCP2 protein still localizes to the axial element in certain *Sycp2* mutants, but it cannot recruit SYCP3, demonstrating that functional SYCP2 is required for SYCP3 localization.[115] In addition, SYCP2, but not SYCP3, is known to be a physical link between the transverse filament and the axial element.[119,120]

During zygonema, homologous chromosomes are joined together in a "zipper"-like fashion by the transverse filament and central element (Figure 1.7(B)). This tripartite structure is highly evolutionarily conserved among species, from yeast to worm, mouse, and human. The C-terminal of SYCP1 is known to embed within the lateral element of the synaptonemal complex, while the N-terminal region is known to associate with the central element (Figure 1.6, pachynema). Additionally, the N-terminal of SYCP1 interacts strongly with itself, suggesting that SYCP1 subunits lie head-to-head within the space between the lateral elements of homologous chromosomes, and physically associate with the central element, effectively joining the two elements together.[119] More recent evidence shows that upon its localization, SYCP1 recruits other proteins to the central element, such as SYCE1, SYCE2, SYCE3, and TEX12.[121,122,123] SYCE1 and SYCE2 bind to one another and to SYCP1, while TEX12 forms a complex with SYCE2, thus only forming an indirect interaction with SYCP1.[122,124] Localization patterns of SYCP1, SYCE1, and SYCE3 are similar, forming a continuous staining pattern along the synaptonemal complexes of synapsed chromosomes,[121,123] whereas SYCE2 and TEX12 display a more punctate staining pattern and do not necessarily co-localize with SYCP1.[122,123] The complex formed by SYCE2 and TEX12 is thought to have an architectural purpose within the central element of the synaptonemal complex, as it consists of a hetero-octamer made up of one SYCE2 tetramer and two TEX12 dimers bound together.[124] It is postulated that this complex, within the confines of a "zipper" analogy for the synaptonemal complex, would be the slider that pulls the SYCP1–SYCE1–SYCE3 "teeth" together to zip up the synaptonemal complex. Other protein components are thought to be members of the central element scaffold, such as FKBP6, although very little is known about their role in synaptonemal complex assembly.[125] Recent studies suggest that FKBP6 may be a player within the RNA interference (RNAi) pathway during meiotic prophase I, with a role specifically in the biogenesis of P-element-induced wimpy testis (PIWI)-interacting RNAs (piRNAs) in premeiotic mouse testes, and in suppressing LINE1 retrotransposon integration into, and thus disruption of, the genome.[126] Given the long history of associations between LINE elements and the synaptonemal complex, and their possible role in facilitating and directing synaptonemal complex assembly, this is an exciting area of research that needs to be more fully explored.[127]

Mutations in synaptonemal complex protein genes have severe implications for meiotic progression in murine systems. Mutations in SYCP2 and SYCP3 show a very similar phenotype, with a failure to form axial element structures and defective synapsis in spermatocytes, resulting in a block of prophase I around zygonema and male infertility.[84,115,117] Female *Sycp2* and *Sycp3* mutants, however, are both subfertile, having severely reduced litter sizes and a reduced reproductive life span.[115,117] SYCP3 protein is required for correct CO formation, and its absence increases nondisjunction in these mutant female oocytes.[117] *Sycp3* mutant females show increased aneuploidy, in which the abnormal karyotype is inherited by the offspring, often resulting in fetal demise, a situation that increases with advancing maternal age. *Sycp1* null males also exhibit a fertility defect as a consequence of large-scale asynapsis in prophase I.[128] In *Sycp1* null females, there is a complete absence of follicles in adolescent female ovaries; however, this can be partially alleviated by crossing to an *Sycp3* null background. This, along with other evidence, suggests that SYCP3 may play a role in meiotic checkpoint mechanisms in mice.[129,130] However, *Sycp1–Sycp3* double-null mice remain male and female infertile due to a lack of synapsis and a block of the DSB repair pathway (discussed further in this chapter) prior to CO resolution. Interestingly, these mice are still able to load cohesins onto the chromosome cores (also discussed further), even in the complete absence of a functional synaptonemal complex.[129]

In central element–defective mice, such as those with homozygous mutations in *Syce1*, *Syce2*, and *Syce3*, severe defects in synapsis and DSB repair (discussed further here) are evident, despite an apparently "normal" loading of SYCP1, resulting in complete infertility in both sexes. This suggests that although SYCP1 can load onto the axial element, assembly of the central element is impaired and thus synapsis cannot be achieved.[123,131,132] Interestingly, SYCE2 interacts with the recombination repair protein RAD51 (also discussed further here), indicating that sites of RAD51 localization might be candidates for synapsis initiation sites in the mouse.[132]

In addition to appropriate synaptonemal complex formation, there is an additional requirement to monitor the regulation of homolog interactions in the mouse. HORMA (Hop1p, Rev7p, and MAD2) domain-containing proteins, HORMAD1 and HORMAD2, localize selectively along unsynapsed chromosomes in wild-type mice, and dissociate upon accumulation of central element proteins. This reciprocal distribution of HORMAD and central element proteins is not dependent upon DSB repair components, but is absolutely dependent upon the presence of a functional synaptonemal complex, in addition to the AAA

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(adenosinetriphosphatases (ATPases) associated with diverse cellular activities)–ATPase, thyroid hormone receptor interactor 13 (TRIP13).[133,134] HORMAD2 is also required for the accumulation of checkpoint kinase ATR (ataxia telangiectasia and Rad3-related protein) along unsynapsed cores in prophase I, as *Hormad2*$^{-/-}$ mice show defective ATR accumulation along the synaptonemal complex, suggesting that the HORMAD proteins play a major role in the meiotic prophase I asynapsis surveillance mechanism in mice (discussed further here).[135]

Very little is known about the synaptonemal complex disassembly process in mammals, but the beginning of the transition from prophase I to metaphase I involves both synaptonemal complex disassembly and chromatin compaction. During diplonema, the central element begins to disassemble, SYCP1 is lost from the chromosome cores, and the homologs repel each other[136] (Figure 1.7(D)). HORMADs relocalize to the cores upon desynapsis, while SYCP3, although remaining associated with the cores, begins to redistribute to the centromeres. Thus, the chromosomes are still held together by the synaptonemal complex at the centromeres and CO sites, and by the physical COs themselves, continuing into diakinesis[133,137,138] (Figure 1.7(E)). It is thought that central element disassembly begins with phosphorylation of SYCP1 and TEX12 by polo-like kinase 1 (PLK1), which facilitates their removal from the synaptonemal complex.[139] Subsequently, SYCP3 removal and lateral element reconfiguration processes are regulated by both the cyclin-dependent kinases (CDKs) and the aurora kinases (AURKs).[140,141] Further evidence to support the role of phosphatases and kinases in the transition process is that the phosphatase inhibitor okadaic acid can speed the G2–M transition in meiotic prophase cells.[142] SYCP1 remains localized to the centromeres until such time as meiotic kinetochores localize to the centromeres, consistent with a role for SYCP1 in assembly of these complexes in meiosis.[138] SYCP3 remains bound to the centromeres into diakinesis, where it promotes bi-orientation of homologous centromeres to help ensure proper segregation.[138]

Proper synaptonemal complex assembly and disassembly are critical steps in achieving appropriate DSB repair and subsequent correct meiotic segregation of chromosomes. This is true of humans as well as laboratory mouse models, and cases of mutation in synaptonemal complex component genes, such as *SYCE1* and *SYCP3*, have been implicated in women with premature ovarian failure and recurrent pregnancy loss.[143,144,145] Ectopic expression of synaptonemal complex genes has also been implicated in some rare incidences of cancer, demonstrating that meiosis-specific expression of these genes is crucial.[146]

Cohesins: Establishment and Assembly

As in mitosis, to ensure proper meiotic progression and correct chromosome segregation, sister chromatid cohesion needs to be established early in meiosis, and must be maintained until such time as the homologs and the sister chromatids need to separate at anaphase I and II, respectively. Thus, while cohesion is not meiosis specific per se, there are features of cohesion during meiosis that are very different from those of mitotic cells. The cohesion complex is similar in both cell cycles, consisting of two SMC proteins that enclose the DNA in a hinge, and two non-SMC proteins, the kleisin and stromalin subunits[147] (Figure 1.9(A)). In mitosis in higher organisms, cohesin loads onto chromosomes during replication and remains tightly bound until prophase, when the bulk of this cohesin dissociates from the chromosomes. By anaphase, all the cohesin has been removed, and the sister chromatids separate.[148,149] The timely removal of this cohesin is crucial for the proper segregation of sister chromatids. Cohesin binds along the entire length of the chromosome arms, but is intensely bound at the centromere, the region that is responsible for tightly binding sister chromatids.[150,151] During meiosis, however, the pattern of cohesin binding is very different; cohesion is established by cohesins binding to the chromosome arms, resulting in cohesion of paired homologous chromosomes, whereas once the first meiotic division has taken place, the sister chromatids remain joined by centromeric cohesion only.[152,153] Once the cohesin at the centromeres is disassembled in meiosis II, the sister chromatids may separate, similar to mitotic division.

Cohesion during cell division is maintained by specific cohesin complexes, the somatic cohesins SMC1 and SMC2, joined by kleisin RAD21 bound to stromalin SA1–SA2 (Figure 1.9(B)). In addition, most organisms contain meiosis-specific cohesins (Figure 1.9(C)), including SMC1β, REC8, RAD21L, and STAG3, which can combine with the canonical cohesins SMC1α, SMC3, and RAD21 to form varying complexes specific to meiosis. These proteins can be immunoprecipitated together as distinct complexes,[154] suggesting the presence of these varied subunit complexes in meiosis.

The timing of assembly and disassembly of meiotic cohesins through prophase I is complicated, and little is known about it in mammals; however, evidence from other eukaryotes suggests that cohesin assembly is a multistep process.[155] Cohesin assembly in mouse meiocytes begins with the localization of meiotic kleisin REC8 to chromosomes slightly before the premeiotic S-phase, followed by loading of the SMC proteins, SMC1β and SMC3, and synaptonemal complex axial element proteins, SYCP2 and SYCP3.[156] REC8 then remains localized to the chromosome arms longer than either SMC1β or SMC3, or the axial element proteins SYCP2 and SYCP3

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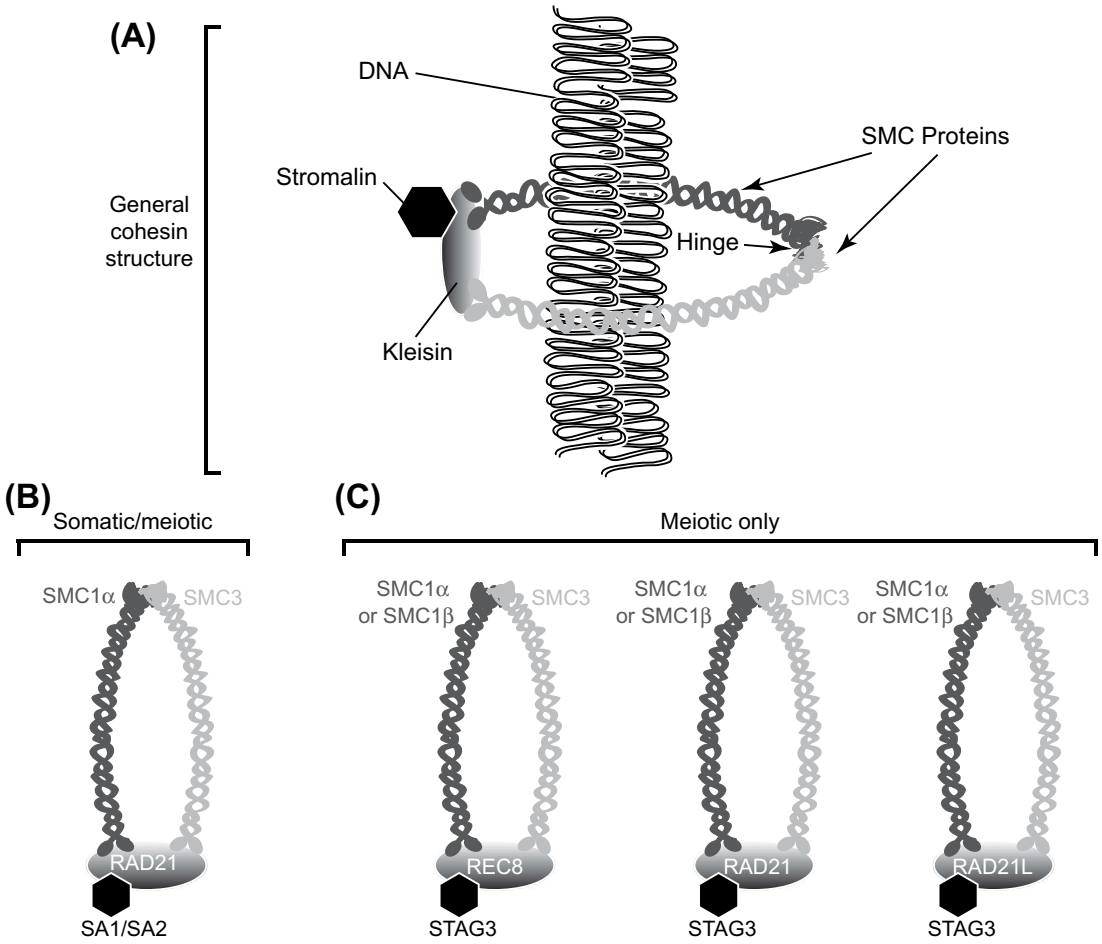

FIGURE 1.9 Cohesin structure in meiosis. (A) General cohesion structure consists of two SMC proteins (gray), which form a hinge around the DNA and are joined by a kleisin and stromalin subunit. (B) Somatic and meiotic cohesin. (C) Meiosis-specific cohesins.

(all of which dissociate from the arms by metaphase I, but remain localized at the centromeres) (Figure 1.7(F)). By anaphase I, REC8 is disassembled from the arms by separase cleavage, but is retained strongly at the centromeres. During meiosis II, all these proteins are disassembled from the centromeres simultaneously at anaphase II.[152,156,157] *Rec8* null mice are male and female infertile, with a complete arrest of spermatogenesis at meiotic prophase I, resulting from a failure to properly synapse. *Rec8* mutants do establish sister chromatid cohesion and accumulate synaptonemal complex proteins such as SYCP3 and SYCP1; however, the "synapsis" that they undergo is usually between sister chromatids, and thus they tend to suffer from precocious separation of sister chromatids, resulting in arrest at pachynema.[158,159]

A second kleisin subunit, RAD21L, also loads onto meiotic chromosomes early in leptonema, co-localizing with axial element proteins,[154,160,161,162] and it remains localized to the synaptonemal complex until pachynema or mid-diplonema, with strong staining on the axes of the X and Y chromosomes in males. Co-staining with REC8 in meiotic cells reveals mutually exclusive staining of these two kleisin complexes, suggesting that REC8 and RAD21L might form distinct cohesin-enriched domains along the axial elements, which consequently would form a unique pattern along the axial elements of chromosomes, resulting in a "barcode" that has been hypothesized to aid in homolog recognition.[154]

The third meiotic kleisin, RAD21, does not follow a localization pattern that is similar to those of REC8 and RAD21L. Instead, it tends to disappear during leptonema and zygonema, before reappearing in pachynema as RAD21L is dissociating from the cores, suggesting that it may be replacing RAD21L (and to some extent REC8, although the timing of REC8 removal from chromosome cores is not certain from data collected in several studies[154,156,157]). By metaphase I, RAD21 has also disassembled from the arms and remains only at the centromeres, similar to other cohesin localization patterns at this stage.[162,163,164] Given this differing localization pattern, it is thought that RAD21 may have a role in stabilizing sister chromatid cohesion at the time of DSB repair (during pachynema[165,166,167]), whereas REC8 and RAD21L may

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have a more canonical role in sister chromatid cohesion during early prophase I. It appears that REC8 may be the basis for the loading of cohesin complexes at this time, given that REC8 loads onto chromosomes before any other cohesin or synaptonemal complex proteins,[156] and that REC8 remains bound to chromosome arms longer than the other cohesins, therefore being the only means by which cohesion is maintained until anaphase I. *Rad21l* mutant mice exhibit total male sterility, resulting from a lack of synapsis in prophase I and subsequent meiotic arrest at zygonema, reminiscent of the situation in *Rec8* mutants, whereas females exhibit age-related problems with fertility, most likely due to premature depletion of the oocyte pool, as is common with other female meiotic cohesin mutants.[162,168,169]

In mammals, the meiosis-specific stromalin subunit of cohesin, stromalin antigen 3 (STAG3), and the meiotic SMC proteins, SMC1β and SMC3, all localize in early leptonema of prophase I to the axial element in mammalian oocytes,[164,170] until late in prophase I, when they disassemble from the chromosome arms. The disassembly of these cohesins from chromosome arms appears to be dependent on kinases, such as NEK1.[171] STAG3 is removed from the chromosome arms at or around metaphase I,[172] as are SMC3 and SMC1β.[173,174] SMC1β remains localized to centromeres until metaphase II, presumably to retain sister chromatid cohesion until separation of the sister chromatids at meiosis II.[173] *Stag3* mutant rats exhibit sterility, presumably as a result of the pachytene arrest due to loss of STAG3 protein; however, this mutation has not been fully characterized, nor has *Stag3* been knocked out in a mouse model to date.[175] *Smc1β* mutant males exhibit pachytene arrest of spermatocytes due to incomplete synapsis, whereas in the mutant females, oocytes progress to metaphase II. Axial elements in the mutants are shorter, and the chromatin extends further from the core, suggesting that SMC1β is involved in chromatin loop formation and organization.[85,176,177] In addition, in these mutants, CO structures and meiosis-specific telomere adjustments are absent. This, along with other evidence, indicates that SMC1β plays an important role, both in maintenance of telomeres during meiosis and as a chiasma-binding factor that stabilizes CO structures until anaphase I.[85,178] This is important evidence toward providing a link between axial element length and chromatin loop size with cohesin components. STAG2 (similar to mitotic stromalin (stromalin 2, or SA2)) has also been implicated in meiosis, although it is unclear in which complex it functions; it might be functionally associated with RAD21, as the localization patterns remain the same, and may cooperate with STAG3 complexes, although very little is known about its function in meiosis.[179]

Phylogenetic analyses have identified two further proteins belonging to the SMC family,[180] known as SMC5 and SMC6. In mammals, these proteins are highly expressed in the testes, and are associated with the XY chromosomes during prophase I of meiosis.[181] Very little is known about the role of these SMCs in mammalian meiosis; however, they have been implicated in repair processes in human somatic cells[182] and joint molecule resolution in yeast, not all of which are formed as a result of SPO11-dependent DNA breaks (discussed further in this chapter).[183,184] They do not appear to function in the cohesion-like processes for which other family members are best known. Other cohesin proteins have been implicated in meiotic repair pathways in many eukaryotic organisms, such as mouse, *C. elegans*, *D. melanogaster*, and *Arabidopsis thaliana* (mouse,[178] *C. elegans*,[185] *Drosophila*,[186] and *Arabidopsis*[187]), and in somatic repair.[165,188,189]

Meiotic Recombination

Overview of Meiotic Recombination

As described in this chapter, one of the ways in which homologous chromosomes can remain physically paired until the first meiotic division is via DNA:DNA interactions between maternal and paternal genomes. To do this, homologous chromosomes must break and interhomolog DNA connections must form. This process is known as crossing over, or reciprocal recombination, since there is a reciprocal exchange of DNA between maternal and paternal chromosomes (because exactly the same length of DNA must be swapped). Therefore, crossing over is the second fundamental process of prophase I and involves the exchange (or recombination) of genetic information between homologous (maternal and paternal) chromosomes.

In almost all species examined to date, crossing over is initiated by the formation of a DSB in one sister chromatid of one homologous chromosome during leptonema. Thus, while most cells of an organism must actively avoid DNA breaks, meiotic cells undergo genetically programmed breaks that must then be repaired in a specific way that is extremely tightly controlled both temporally and spatially. The formation of a DSB in leptonema and its subsequent repair through prophase I are called homologous recombination, which serves three crucial functions during prophase I. First, the exchange of genetic material between homologs generates diversity within gametes by shuffling the DNA and introducing variation; second, the physical events of recombination allow for homology searching between chromosomes (explained further here); and, third, and arguably most importantly, it creates the physical manifestations of crossing over, the chiasmata, that hold homologous chromosomes together at the metaphase plate during meiosis I, thus reducing the risk of

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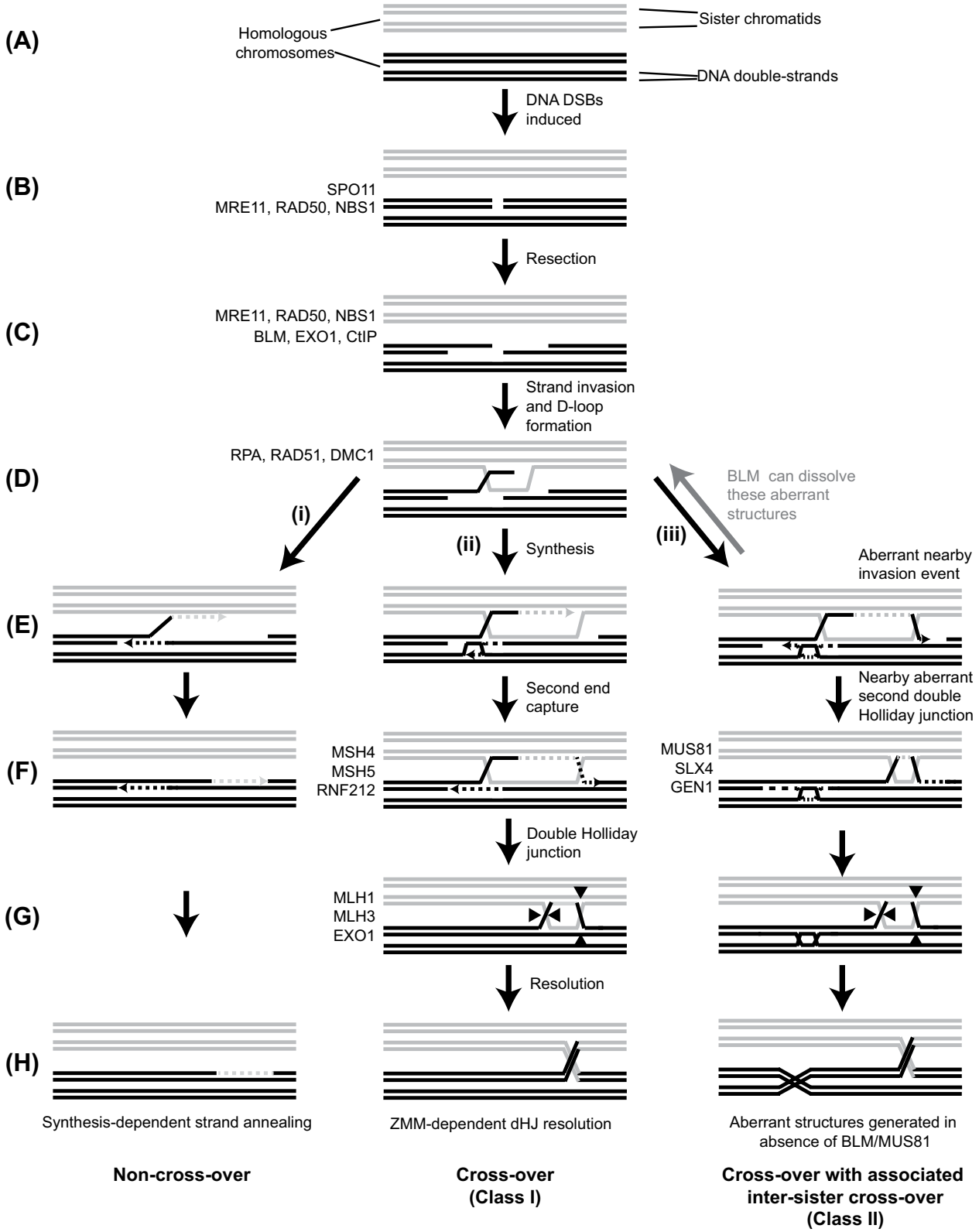

FIGURE 1.10 **Double-strand break repair model in eukaryotes.** A schematic representation of double-strand break repair (DSBR) in eukaryotic meiosis. (A) Homologous chromosomes (gray and black) undergo DSBR during meiotic prophase I. (B) SPO11 and its accessory factors form a DSB in the DNA of one homolog (in this case, the black one), which can then be repaired by one of several pathways: either as a non-crossover (left panel), as a class I crossover (middle panel), or as a class II crossover (right panel). (C) The cut DNA ends are resected back by exonucleases to form free 3′ overhanging strands. (D) These 3′ overhangs (black) may "invade" the opposing homologous partner (gray) to form a D-loop structure. (E) Depending on the method of repair, the D-loop can form several intermediate structures, and the DSB can be repaired in a number of ways. (F–H) Non-crossovers are thought to be repaired by a process known as synthesis-dependent strand annealing (SDSA, left panel, pathway i), during which no classic double

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aneuploidy in future gametes. This third requirement also demands that CO are placed at defined points throughout the genome, with at least one CO exchange event occurring on each chromosome, and with the placement of one CO limiting the placement of nearby CO events. Thus, as described here, the repair of DSBs is extremely tightly regulated at all stages of recombination.

DSB repair is extremely complex, and it is described in detail in this chapter. Briefly, however, the chromatid that is broken must be processed to allow one end of the break to become invasive. This invading end can then insert into other DNA strands to "test" them for homology (by simple base pairing) and to initiate homolog interactions. Once the appropriate homolog is located, these DSBs must then be repaired, either by forming hybrid DNA molecules with the opposing homologous sequence or by reannealing to the other end of DSB. Multiple DNA repair mechanisms exist for these purposes, many of which are similar between meiotic and mitotic cells, but which have been selectively "repurposed" to allow for the formation of distinct CO structures between homologous chromosomes. It is important to note, therefore, that DSB repair is not confined to meiosis, but also occurs in response to spontaneous (unprogrammed) DSBs that can arise in somatic cells. The molecular events of DSB repair in gametes are reminiscent of those in somatic cells, with a few major differences, as detailed in this chapter. Due to the problematic nature of studying these processes in detail in mammals, most of what is known about the mechanisms of meiotic DSB repair comes from research in yeast; however, due to the highly conserved nature of most meiotic processes, much of this data has been extrapolated to mammals. In fact, many of the fundamental steps of DSB repair involve proteins that are highly conserved across species, from yeast to humans.

Physical Events in Meiotic Recombination

The term "recombination" encompasses a number of DNA modification events that occur during prophase I, all of which are initiated by the induction of a DSB into the DNA, creating a blunt end nick in one sister chromatid of a recently replicated sister chromatid pair. The DSB is processed initially by resection of the two 5′ ends (Figure 1.10(C)), which results in 3′ single-stranded overhangs that are then capable of invading an opposing homolog and, in doing so, displace a stretch of DNA from the opposing homolog, known as the D-loop (Figure 1.10(D)). The D-loop may be repaired by several mechanisms, depending on the nature of the initial strand invasion event, the placement along the chromosome, and other factors. In the case of most CO events, the D-loop becomes further processed to form a structure known as a double Holliday junction (dHJ): (1) DNA synthesis from the end of the invading overhang (having lost a portion of DNA sequence due to the original resection event), using the opposing homolog as a template, stabilizes a "single end invasion" intermediate[190]; (2) subsequent religation of the invading strand with its original 3′ end results in what is termed "second end capture"; and (3) this occurs in tandem with DNA synthesis from the noninvading 3′ overhang, using the displaced D-loop as a template, followed by religation, all resulting in the formation of two stretches of heteroduplex DNA within what now is known as a dHJ (Figure 1.10(E), pathway (ii)).[191] Repair of this structure can give rise to COs, in which a flanking DNA is reciprocally exchanged, or non-crossovers (NCOs). However, for reasons that are not entirely apparent, dHJ repair in meiosis is heavily biased toward the formation of COs (discussed further here).

COs may also form from structures other than dHJs in yeast, but these have yet to be observed in mammals. In addition, it is possible for nearby DSB repair events and/or events that involve multiple-strand invasions from a single DSB to result in recombination intermediate structures involving all four chromatids (Figure 1.10(E)–(H), pathways (iii)). These structures, termed "multichromatid joint molecules" in *S. cerevisiae*, can be repaired in a number of ways, but appear to be rare events that are processed through specialized machinery.

As will be discussed here, the majority of DSBs do not become COs and instead are processed through NCO pathways, giving rise to repair events that do not involve exchange of parental genetic information. Given the bias of dHJ repair in meiosis toward COs, it appears that NCOs are achieved by temporally distinct mechanisms that arise during strand invasion and/or D-loop formation. For example, in *S. cerevisiae*, NCOs are obtained from DSB repair through a process called the synthesis-dependent strand annealing (SDSA) pathway, in which the invading strand is ejected from the D-loop and religates to its own 5′ end with the assistance of DNA synthesis using either the sister chromatid or the opposing homolog as the template, the latter resulting in a gene conversion event (Figure 1.10(E)–(H), pathways (i)).

◀ Holliday junction (dHJ) is formed (F, left panel). Conversely, class I crossovers (middle panel, pathway ii) are thought to be formed by the formation and repair of a dHJ (F, middle panel). Class II crossovers are seemingly more complex (right panel, pathway iii), perhaps being constructed as a result of repairing aberrant joint molecule structures (F,G, right panel) that arise too close to class I crossovers, or as a result of multiple invasion events during DSB repair, resulting in closely spaced crossover products (right panel, H). (F–H) Non-crossovers are thought to be repaired by a process known as synthesis-dependent strand annealing (SDSA; see left panel, pathway (i)), during which no classic double Holliday junction (dHJ) is formed (F, left panel). Conversely, class I crossovers (middle panel, pathway (ii)) are thought to be formed by the formation and repair of a dHJ (F, middle panel). Class II crossovers are seemingly more complex (right panel, pathway (iii)), perhaps being constructed as a result of repairing aberrant joint molecule structures (F, G, right panel) that arise too close to class I crossovers, or as a result of multiple invasion events during DSBR repair, resulting in closely spaced crossover products (right panel, H).

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An intriguing aspect of meiotic recombination is the fact that many more DSBs are formed than are actually processed into COs. This has prompted the conclusion that recombination serves multiple purposes beyond those that involve reciprocal recombination. In other words, physical tethering of homologous chromosomes through crossing over represents just one function of recombination. Indeed, in mammals, approximately 10-fold more DSBs are created than are processed into COs, with the remaining DSBs being repaired either through intersister chromatid interactions or through NCOs,[192] neither of which contribute to homolog segregation because they do not involve the formation of heteroduplex DNA between the homologous chromosomes. They may, however, contribute to the processes of homology searching and recognition since their repair progression includes initial strand invasion.

Initiation of DSBs

DSBs are introduced in high numbers in leptonema by the topoisomerase SPO11, an enzyme that introduces an asymmetric break in the DNA strand (Figure 1.10(B)). *Spo11* is expressed predominantly in the testes and ovaries of mice, although real-time polymerase chain reaction studies have found expression in certain somatic tissues in humans.[193,194,195,196] SPO11 protein is found in early prophase I, during leptonema, and mice lacking a functional *Spo11* gene show severe defects in prophase I progression. These defects include a complete absence of synapsis and a lack of DSBs, resulting in complete infertility of both male and female mice.[193,194,197,198,199] DSB induction and at least partial prophase I progression can be restored by cisplatin treatment, which induces DNA lesions that are similar to those created by SPO11.[199] Loss of *Spo11* in these mice also affects the downstream expression of several other genes involved in DSB initiation and processing, such as *Hop2*, *Brca2*, *Mnd1*, and *FancG*.[200] Importantly, the synapsis defects in *Spo11* mutant mice, and indeed in many mutants that disrupt DSB repair, demonstrate the requirement for recombination events to ensure homolog recognition and the ensuing pairing events.

In wild-type mice, SPO11 protein is expressed as two separate isoforms as a result of alternate splicing of exon 2, producing an exon 2-skipped isoform, SPO11α, and an exon 2-containing isoform, SPO11β. *Spo11β* is expressed from the onset of prophase I in juvenile male mice, whereas *Spo11α* is expressed from pachynema and is responsible for the unique, temporally distinct COs that form on the XY chromosomes (discussed further in this chapter). Both isoforms are present in female meiosis, although female mice expressing the *Spo11β*-only isoform are fertile, while their male counterparts are sterile, indicating this α isoform does not play a major role in female prophase I progression, which is expected given that its role in male meiosis is restricted to the X and Y chromosomes.[195,196,201,202]

Spo11 does not act alone; in yeast, it is known to require at least nine accessory proteins, both to localize Spo11 appropriately and to facilitate DSB formation (Figure 1.10(B); reviewed in Ref. 203). The catalytic activity of Spo11 comprises a nucleolytic attack of the DNA, resulting in a 5′ phosphotyrosyl bond and a 3′ hydroxyl, and Spo11 protein remains bound to the DNA ends until it is actively removed. It is these Spo11-bound DNA ends, or oligos, that are the basis for recent deep sequencing approaches that have identified Spo11-binding sites in the yeast genome.[204] This cleavage activity requires the MRX complex (MRX: Mre11, Rad50, and Xrs2), which is also known as the MRN complex in mammals (MRE11, RAD50, and NBS1).[205,206,207] The MRX complex works in conjunction with its partner Sae2–Ctp1 (reviewed in Refs 208,209), as well as several other factors such as Mei4, Rec102, Rec104, Rec114, Mer2, and Ski8, a subset of which have been identified in mammals as MEI4 and REC114[210] (and are reviewed in Ref. 203). MEI4 and REC114 have been shown to functionally interact in the mouse, and localize to meiotic chromosomes in early prophase I.[210] *Mei4*$^{-/-}$ mice show defective DSB formation, leading to infertility, indicating a role for these proteins similar to that of their yeast homologs, and of mammalian SPO11, in facilitating DSB formation.[210] A separate gene, *Mei1*, which is not conserved in yeast, has been identified by forward genetic screen to be crucial for DSB formation in mice.[211]

Previously, it was assumed that SPO11-induced DSBs were generated randomly throughout the genome and, while the presence of CO homeostatic mechanisms such as interference (discussed further in this chapter) dispelled this myth, only recently has it been possible to demonstrate the lack of randomness at the nucleotide level through next-generation sequencing methodologies. Recent advances in the mapping of DSB sites, along with techniques such as SPO11-associated oligo mapping (described here) in yeast and mouse, have allowed for a new understanding of how DSBs are targeted to specific genomic locations. Indeed, DSBs appear to be preferentially induced at very specific locations, termed DSB initiation hotspots, where recombination occurs at rates that are many thousandfold higher than the genome average.[196,212,213,214] Both the genomic map gleaned from the positions of single-stranded ends processed after DSB processing, and the more specific map extracted from sequencing Spo11 oligos, align very well (reviewed in Ref. 215). In agreement with many previous studies showing the preference for hotspot locations, the vast majority of Spo11 oligos map to nonrepetitive elements, mainly gene promoters, and GC-rich regions of the genome, both features associated

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with a more accessible chromatin structure, and where Spo11 can presumably more easily act on the DNA. SPO11-associated oligos have been extracted from mice also, although mapping of these has not yet been performed in detail.[196]

Initial DSB Processing

Following DSB formation, DNA resection takes place to leave single-stranded DNA overhangs. In budding yeast, resection initiation depends upon Sae2 protein and is severely hindered in the absence of Mre11 and Exo1.[216,217,218] Recent studies in yeast have determined that this resection is also bidirectional, with Mre11 first nicking the DNA several hundred bases away from the 5′ terminus of the DSB, and then resection occurring by Mre11 in a 3′ to 5′ direction toward the DSB, while Exo1 mediates 5′ to 3′ resection away from the DSB (Figure 1.10(C)).[217] In mammals, orthologs of these genes, *Mre11*, *CtIP* (the *Sae2* ortholog), and *Exo1*, are all required for meiotic prophase I progression. *Mre11* hypomorphic mice showed high levels of asynapsis of homologous chromosomes, with a different effect on male (increased) and female (decreased) frequency of MLH1 foci (indicative of nascent COs), whereas *Exo1* null mice progress apparently normally until diakinesis, where they lack sufficient chiasmata to progress further. This later phenotype of *Exo1* null mice might be due to a distinct role for EXO1 in promoting CO formation, entirely independent of its putative early prophase I role as an exonuclease, similar to that seen in yeast and other organisms[219,220,221,222] (discussed further here). Other factors, such as yeast Dna2, are required for mitotic DNA resection at sites of repair, but as yet, very little is known about their function in mammalian meiosis.[223]

Following resection, the single-stranded DNA (ssDNA) becomes coated in replication protein A (RPA), which prevents the ssDNA from looping back on itself and forming aberrant secondary structures (reviewed in Ref. 224). In mice, RPA localizes to meiotic chromosome cores, coincident with DSB sites.[225,226] ATM colocalizes with RPA along meiotic chromosomes, indicating a possible functional interaction between the two proteins.[225] The ssDNA then loads the RecA homologs, RAD51 and the meiosis-specific DMC1, both of which physically interact with RPA in human cells and may be loaded onto chromosome cores by the TEX15 protein.[226,227,228,229,230] Together, RAD51 and DMC1 facilitate invasion of an opposing homolog by the ssDNA end, which displaces one strand of the recipient homolog to form the D-loop, while in mitotic cells, RAD51 performs a similar role without the aid of DMC1. Both RAD51 and DMC1 localize at meiotic DSB sites in mammals, at a frequency of around 250 per nucleus in the mouse, beginning by associating with single cores but later lying between homologous cores as synapsis proceeds, a process that may be facilitated by the RAD51 associated protein 1 (RAD51AP1; Figure 1.7(H)). RAD51AP1 enhances the ability of DMC1 to capture the duplex DNA homologous partner and may mediate interactions between DMC1 and RAD51 in meiosis.[227,231,232] *Dmc1* mouse mutant spermatocytes fail to proceed past pachynema and both males and females are completely sterile (Figure 1.11(G)), indicating that DMC1 protein is absolutely required for proper meiotic DSB repair.[233]

Recent data suggest that D-loops coated with human RAD51 in vitro are more easily dissociated by factors such as RAD54 than those coated with DMC1 protein, and thus are more susceptible to repair as NCOs by SDSA (Figure 1.10, pathway (i)), suggesting that DMC1 might localize to D-loops destined to be repaired as COs, while sites designated as NCOs accumulate RAD51.[234] This is supported by recent evidence showing that the joint molecule (JM) function of Rad51 is completely dispensable for CO formation in yeast, while CO formation is entirely dependent on the JM function of Dmc1.[235] It is also known from yeast studies that Dmc1 and Rad51 are loaded using separate protein complexes and pathways, although these may differ from organism to organism, but are loaded in a side-by-side pattern on meiotic chromosomes.[236,237,238] Further evidence from *A. thaliana* suggests that DMC1 and RAD51 have functionally and spatially distinct roles, and each localizes to the opposite side of a DSB.[239] Additionally, Rad51 filaments are thought to be dissociated from D-loops in yeast by Srs2 protein, and indeed, *SRS2* mutants display an increase in COs, suggesting that due to RAD51 nonremoval from the D-loops, more COs are formed in the absence of Srs2.[240,241] In the mouse, RAD51 and DMC1 localize to the nucleus during leptonema, usually in large numbers of 250–300 foci per cell (Figure 1.7(H)),[242] but little is known regarding the preferential binding of these proteins for NCO and CO events, and it has been assumed until recently that the two proteins function in concert.

The serine–threonine kinases, ATM and ATR, master regulators of the ubiquitous DNA damage response, have been shown to play a major role in meiotic prophase in mammals: by localization studies showing they are present on meiotic cores, by mutational analysis showing that ATM is directly required for meiotic progression in mice, and by recent data showing that ATM is responsible for regulating the number of SPO11-induced DSBs in meiosis via a negative-feedback loop, in which DSB formation induces ATM expression and/or activation and ATM, in turn, downregulates SPO11 activity.[243,244,245] Thus, spermatocytes lacking ATM show a 10-fold increase in SPO11-linked oligonucleotides, indicating that ATM regulates the frequency of DSBs. Conversely, mice bearing one mutant allele of *Spo11* (*Spo11*$^{+/-}$) and two mutant alleles of

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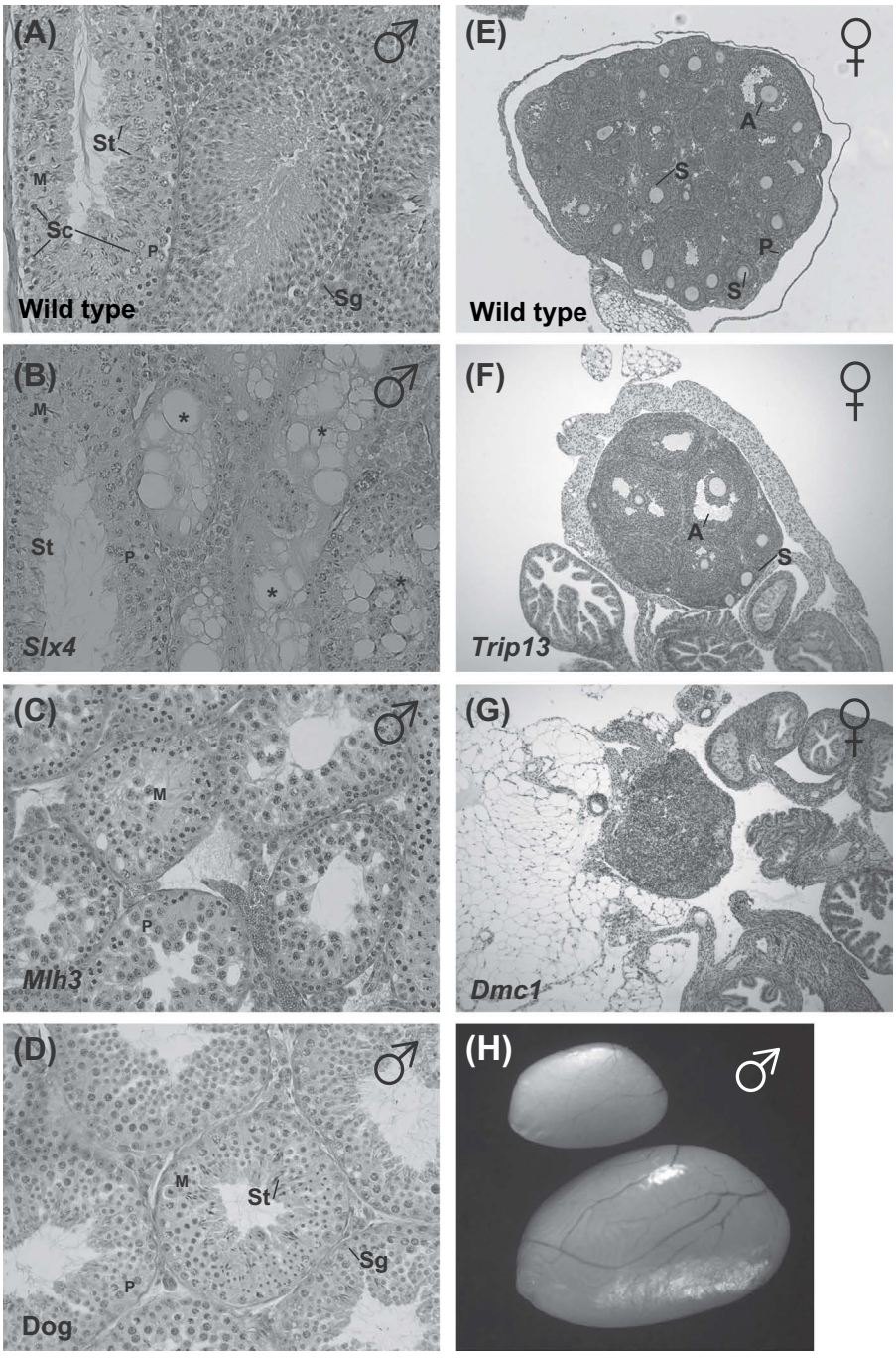

FIGURE 1.11 Phenotypic variation in meiotic mutants. (A–C) Testis sections from (A) wild type, (B) *Slx4* mutants, and (C) *Mlh3* mutants. Wild-type sections show a range of cell types from spermatogonia (Sg), to spermatocytes (Sc) in pachytene (P) and metaphase I (M) stages, to spermatids (St). *Slx4* mutants show these cell types in some tubules, but have other tubules completely devoid of meiotic cells (asterisk), indicative of a premeiotic stage error. *Mlh3* mutants show germ cells in every tubule, but no cells at a later stage than spermatocyte, indicating an arrest at the end of prophase I, due to meiotic errors. (D) Dog testis section shows a very similar morphology to that of the wild-type mice. (E–G) Ovaries from female mice that are (E) wild type, (F) *Trip13* mutant, and (G) *Dmc1* mutant. Wild-type ovaries show a mix of follicle stages, including primary (P), secondary (S), and antral (A). *Trip13* mutants show different stages of follicles, but vastly fewer in number in the whole ovary than wild type, and no primary follicles, indicating a premature loss of the oocyte pool. *Dmc1* mutants show no follicles at all, indicating they never made it past the very initial stages of meiosis before dying. (H) Size difference between testis of a wild-type male mouse and a meiotic mutant littermate (*Nek1* mutant).

Atm (*Atm1*$^{-/-}$) exhibit a less severe meiotic defect than do *Atm*$^{-/-}$ mice, suggesting a complex interdependent feedback loop between the two proteins. It is possible that ATM serves either to directly phosphorylate SPO11, or one of its accessory proteins, at DSB sites to prevent subsequent DSBs from forming in the same vicinity, or to restrict excessive DSB formation across the genome. ATM is functionally active at the sites of DSBs, given that one of its major targets, the histone H2AX, is phosphorylated at DSB sites in many organisms.[197,245,246] ATM–ATR homologs are also responsible for other important phosphorylation events during DNA repair, including phosphorylation of Sae2 in yeast meiotic DSB resection and phosphorylation of Rpa in mitotic yeast cells, and although these interactions might be true also in mammalian systems, little is known about their roles in meiosis.[247,248]

The Bloom's syndrome mutated (BLM) helicase also plays an important role in DSB repair in both meiotic and mitotic cells, and it has a critical role in meiotic progression in mice (as does its yeast homolog, Sgs1). BLM/Sgs1 is thought to act at sites of DSBs, where it localizes to chromosome cores in early prophase I, and also in the latter

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stages, during which it is thought to play a similar role to that in yeast, that of: untangling aberrant recombination structures, along with MUS81–EME1 (discussed further in this chapter).[249,250,251,252,253,254] Mice with a conditional knockout of BLM helicase in their spermatocytes display errors in meiotic synapsis, as well as in the proper repair of DSB intermediates, resulting in higher levels of unrepaired DSB intermediates at diakinesis and an increase in chiasmata-like structures at metaphase I.[254] BLM has been implicated as a major component of the Class II CO pathway in mice (also discussed further here).

CO versus NCO Fates: The Role of the ZMM Proteins

The decision to become a CO or an NCO following the initial common intermediate steps of DSB repair is regulated by several processes, not least of which is the fact that each and every chromosome must receive at least one CO, and that these COs must be appropriately spaced to prevent aberrant recombination events and/or to reduce the risk of genome instability. Following D-loop formation, DSB repair intermediates may be processed through one of several pathways, resulting in the formation of NCOs or COs (Figure 1.10). Studies in *S. cerevisiae* have revealed that NCO events are resolved much earlier in prophase I than COs, pointing to different resolution pathways for the two recombinant products. Instead, a temporally distinct NCO pathway has been proposed for yeast: the SDSA pathway (Figure 1.10, pathway (i)). In this scenario, failure to capture the second end of the DSB (discussed further here) leads to displacement of the invading strand in the D-loop, and instead the two strands can anneal, resulting in an NCO with minimal gene conversion (Figure 1.10, pathway (i)).[255,256]

Originally, it was thought that processing of DSBs through the dHJ intermediate and the subsequent cutting and resolution of that dHJ via one of two possible orientations could result equally in either COs or NCOs.[257] However, through mechanisms that are not clearly understood, dHJ resolution is extremely biased toward the formation of COs.[258] Members of the so-called ZMM family of proteins are thought to mediate this bias, including in yeast the synaptonemal complex protein Zip3 (along with Zip1, Zip2, and Zip4), the DNA mismatch repair proteins MutS homolog 4 (MSH4) and MSH5, and the adenosine triphosphate (ATP)-dependent helicase Mer3. Mammals and other organisms possess orthologs of the ZMM proteins, including worm ZHP3 and mouse and human RNF212 (orthologs of Zip3); MSH4/5 in mouse, human, and worm; and mouse HFM1 (ortholog of Mer3).[259] The cyclin B interacting protein, HEI10 (or CCNB1IP1), present in mouse and *Arabidopsis*, has recently been added to the list of ZMM proteins.[260,261] These proteins function collectively to ensure that dHJ processing results only in COs, as demonstrated by the fact that COs are significantly reduced or entirely absent in mutants for each of the ZMM genes across eukaryotes (reviewed by Ref. 192).

The DNA Mismatch Repair Pathway Regulates the Major Pathway for Crossing Over

Second end capture results in the formation of a dHJ, which is stabilized by the meiosis-specific MutS homolog heterodimer of MSH4 and MSH5, known as MutSγ. Hydrolysis of ATP through the MutSγ ATPase domains allows the MSH4–MSH5 heterodimers to form a sliding clamp that may displace the complex from the immediate vicinity of the dHJ, facilitating further clamp loading.[262,263,264] In mice, MSH4 and MSH5 proteins localize in distinct foci along chromosome axes during zygonema of prophase I, usually in much higher numbers than the eventual number of COs in the nucleus, at around 150 per nucleus (Figure 1.7(I)). MSH4–MSH5 numbers gradually decline into pachynema, until by the mid-pachytene stage they number approximately 30 per nucleus.[226,265,266] Recent data have suggested that certain MSH4–MSH5 foci are designated from the entire pool of foci as future CO sites, and these sites are stabilized in the mouse spermatocyte by the Zip3 homolog RNF212.[267] In addition, data from *C. elegans* suggest that preselected CO sites are processed by the cyclin-related protein COSA1, which is recruited only to sites of CO. Both MSH4 and MSH5 are critical for proper meiotic progression in the mouse, and the loss of either protein results in the absence of COs and complete sterility in males and females.[265,266] In addition, they load with similar temporal and spatial frequency on synaptonemal complexes from human oocytes, indicating similar functions.[268]

The MutSγ heterodimer recruits a second MMR heterodimer, this time composed of the MutL homologs, MLH1 and MLH3 (MutLγ).[269,270,271,272] Surprisingly, the complex is recruited sequentially in pachynema, MLH3 appearing on prophase I chromosome cores slightly prior to MLH1 in mouse, and localizing to cores even in *Mlh1*$^{-/-}$ mutants, indicating that MLH3 can bind to DNA without MLH1 present.[273] MLH1 then proceeds to co-localize with MLH3 at all but a small subset of foci in mouse spermatocytes, thus marking the position of the Class I COs (Figure 1.7(J)).[273] This Class I pathway is well characterized in lower eukaryotes, such as *S. cerevisiae* and *A. thaliana*, but very little is known about the control mechanisms in mammals.[274,275] Studies of a well-characterized recombination hotspot in the mouse, *Psmb9*, as well as cytogenetic studies in mutants for *Mlh1* and *Mlh3* have indicated that 90–95% of COs depend upon the Class I, MLH1–MLH3-dependent, pathway, while the remaining COs are processed through a Class II pathway that is regulated by the MUS81–EME1 endonuclease (discussed further in this chapter).[273,276,277,278] Additional proteins localize to sites of Class I COs in the

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mouse, and are crucial for proper progression of meiosis in the mouse. These include the cyclin-dependent kinase CDK2, which co-localizes with MLH1 in spermatocytes, and in which a mutation can cause infertility in the mouse; RAD51C, which localizes to sites of CO in late prophase I, is critical for meiotic progression in mice, and is required for HJ processing in somatic cells; and EXO1, which has been shown to have a crucial late role in CO processing following MLH1–MLH3 accumulation in the mouse, with *Exo1*$^{-/-}$ mice accumulating MLH1–MLH3 normally, but then having very few COs, leading to sterility.[221,279,280,281,282] Class I-processed dHJs are resolved into COs late in prophase I, by as yet unidentified resolvases. Many proteins have been implicated in this role in both mammals and lower eukaryotes, including MUS81, MLH3, EXO1, YEN1, and SLX4; however, no definitive single resolvase has yet been discovered.[283,284]

A Second Class of COs that are Independent of MutLγ

A second class of CO is thought to generate about 10% of COs in mammalian gametogenesis, given that *Mlh1* or *Mlh3* mutant mice have two to three residual chiasmata per cell and retain about 10% of total COs at hotspots.[270,272,276,277] These COs are not regulated by the ZMM proteins, and are instead thought to be dependent on the structure specific endonuclease, MUS81, because in *Mus81* mutant mouse spermatocytes the number of MLH1 foci increases in pachynema; however, the total number of chiasmata at diakinesis remains the same, indicating that the MLH1–MLH3 Class I CO pathway somehow compensates for the loss of MUS81.[278] In yeast, it is thought that Mus81 acts in concert with Sgs1 (the BLM helicase yeast homolog) to disassemble aberrant joint molecule structures that occur as a result of improper stand invasion events during DSB repair.[250,251,252,253] This may be true in mammals also, as mice displaying a testis-specific conditional knockout of *Blm* show an increase in chiasmata-like structures at diakinesis.[254] It might be the case that perhaps many resolvases are present to generate both classes of CO in meiosis, and that they have overlapping and compensatory roles in processing intermediates, which certainly appears to be the case in yeast, where several of these components can be removed and COs still processed.[222,285]

Multistage Regulation of CO Placement and Frequency through Prophase I

It is clear that the frequency and location of DSB induction in mice are tightly controlled. One example of their regulation was presented here, namely, the feedback control on Spo11 activity in both yeast and mouse by the ATM kinase.[245] Recent evidence has shown that DSB position is also tightly controlled, being preferentially initiated at regions that correspond to testis-specific trimethylation of lysine 4 on histone H3.[286] This methylation mark is a result of binding and methyltransferase activity by the zinc finger protein, PRDM9.[287,288,289] PRDM9 is thought to trimethylate histones upon binding to specific recognition sequences of the genome, which in turn attracts SPO11 to these hotspot sites.[286,290] DSBs occurring in *Prdm9* mutant mouse spermatocytes are not preferentially biased toward hotspots, but instead arise at alternative regions of the genome that have H3K4 trimethylation marks, such as gene promoters.[291] The formation of these DSBs is also kinetically slower than that in wild-type mice. PRDM9 is highly specific in its recognition of DNA sequence, and slight changes in the highly variable zinc finger motif render it incapable of binding certain alleles and more likely to bind elsewhere. Indeed, in humans, individuals with differing PRDM9 alleles can use entirely different sets of CO hotspots, raising a key issue known as the hotspot paradox.[292,293] This paradox is borne out of the fact that any PRDM9 allele that is active receives a DSB preferentially, while a nonactive allele does not. Due to the nature of dHJ formation, which by necessity includes repair of heteroduplex DNA, the intact homolog, which is inactive for hotspot activity, is used as a template for repair, effectively inactivating the recombination hotspot at this location through the process of gene conversion. This process, it is hypothesized, could eventually lead to the extinction of this initiating allele from a population, unless mechanisms (yet to be determined) exist to preserve the hotspot activity.[294,295]

Studies in a wide range of mammals have confirmed that PRDM9 is a global regulator of meiotic recombination in mammalian species. Intriguingly, though, PRDM9 is completely absent from dogs and their related species, with all canids showing a significantly different recombination landscape across the genome from that seen in humans and mice. These data indicate that there is an alternative pathway available for generating the recombination landscape across the mammalian genome.[291,296,297]

In yeast and mice, an interesting phenomenon occurs, by which CO frequency is maintained, even in the presence of fewer initiating DSB events, and is termed CO homeostasis.[298] This process ensures that, even in the case of fewer Spo11-induced DSBs, the final tally of COs is maintained, ensuring one CO per chromosome, or obligate CO. CO homeostasis is demonstrated in mice by the fact that number of DSBs induced during early prophase I far exceeds the number of eventual COs that result from repair of these breaks and that, although DSB numbers can change, final CO is exceptionally tightly controlled.[299] For example, an early marker for DSB repair is RAD51. The number of RAD51 foci in early zygonema is 100–500 per nucleus, with an average of around 219 foci per zygotene nucleus. A somewhat later (zygotene–pachytene) marker, MSH4, shows lower

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focus numbers per nucleus in the mouse, around 100–200, with the difference being accounted for by NCO mechanisms. Finally, the late pachytene marker MLH1 shows many fewer foci, in the region of 20–25,[270,300] again the balance being made up of DSB repair events that are destined toward NCO pathways. Not only does the number of foci for each of these recombination markers decline as prophase I progresses, but the regulation of marker frequency becomes increasingly stringent also, with lower variability in focus frequency, and steadily decreasing coefficients of variation in focus numbers as we proceed from zygonema to pachynema. The final tally of MLH1 foci in late pachynema is extremely conserved, with very, very low variability between nuclei. Even if DSB frequency is varied by changing the dosage of SPO11 protein in mice, the eventual number of MLH1 foci remains surprisingly unchanged, with the variable frequency being that of NCOs, in favor of COs.[300]

Given that this CO assurance ensures a minimum effective DSB rate, what mechanism prevents excessive DSBs from forming? Once DSBs are formed in early prophase I, a third level of regulation is implemented, which is entirely dependent upon ATM kinase. SPO11–oligo complex levels are highly increased in *Atm* mutant mice, and *Atm* mutant cells are very sensitive to variations in SPO11 level, indicating that ATM acts upon DSB initiation to prevent further DSBs from forming, in a negative-feedback loop.[197,245,301]

CO products marked specifically by MLH1–MLH3 are also tightly regulated, in both their frequency and their distribution along chromosomes, in a process known as CO interference.[302] The phenomenon ensures that, once a CO occurs at one point along a chromosome pair, no other COs will take place nearby. If two or more COs occur along homologous chromosomes, they will be spaced far apart, to ensure that the stability of the bivalents is maintained during segregation. The precursors to Class I COs, MSH4–MSH5 foci, are also controlled by interference, indicating this is a step-wise process in mammals.[303] COs that are designated as Class II COs, mediated by MUS81–EME1, are not subject to interference, such that in species that only have the Class II system (e.g., *S. pombe*), interference does not play a role in CO placement.[304] In mice, the loss of *Mus81* results in a compensatory increase in the number of MLH1–MLH3 foci at pachynema, resulting in normal CO rates by diakinesis. Interestingly, these additional MLH1–MLH3 foci reduce the overall level of interference between foci, indicating that the additional foci may not follow interference rules.[278]

COs occurring on the pseudo-autosomal region of the XY chromosomes appear to have a different level of regulation from that of autosomal COs. DSBs occurring on the PAR are formed later than those on the autosomes, specifically by the alpha isoform of SPO11, and the chromatin of the pseudo-autosomal region appears to be packaged differently from the autosomal chromatin, indicating that the initiating DSBs may be under separate control.[201] Further evidence for this comes from the *Prdm9* mutant mouse, in which the DSBs are redistributed from recombination hotspots to promoter regions on the autosomes, but are formed normally on the PAR, indicating a PRDM9-independent mechanism for DSB formation in this region.[291]

Role of Small RNA Pathways in Mammalian Meiosis

A conserved feature of small RNAs is that they must be bound to an Argonaute protein for stability and/or function, with different classes of small RNA binding to distinct classes of Argonautes. The Argonaute protein family is divided into two clades. Those that exhibit greater homology to *Arabidopsis thaliana* AGO1 are the AGO-like proteins and include AGO1–4 in mouse and human (also known as EIF2C1–4), AGO1 and AGO2 in *D. melanogaster*, and Ago1 in *S. pombe*.[305] AGO-like proteins bind both small interfering RNA (siRNA) and micro-RNA (miRNA) species. The second clade of Argonaute proteins constitutes the PIWI-like clade, including proteins related to *D. melanogaster* PIWI, including *D. melanogaster* Aubergine (Aub) and AGO3, and mouse and human MILI, MIWI, and MIWI2.[305] *S. pombe* has no known proteins in the PIWI-like clade. PIWI-like proteins bind another class of small RNAs, known as piRNAs in mammalian systems.

AGO-like Proteins, siRNAs, and miRNAs in Gametogenesis

The AGO-like proteins rose to prominence due to their involvement in RNAi, an evolutionarily conserved mechanism of gene silencing whereby small RNAs are able to target mRNAs for degradation or translational inhibition. RNAi relies on the small RNA-binding capabilities and nuclease activity of an AGO-like protein within the RNA-induced silencing complex (RISC) to "slice" target mRNA. Slicing is achieved via the PIWI domain; however, only a subset of AGO-like proteins has this catalytic activity, despite the presence of the PIWI domain in all AGO-like proteins. The single *S. pombe* AGO1 protein has slicing activity,[306] along with two of the 10 *A. thaliana* AGO-like proteins, AGO1 and AGO4.[307,308,309] Of the four mouse–human AGO-like proteins, only AGO2 has slicing activity, despite the fact that all four mouse AGOs are able to bind small RNAs.[310,311] Recent studies have implicated mammalian AGO3 and AGO4 in regulating meiotic entry and in transcriptional silencing of the sex chromosomes during meiotic prophase I in males (discussed further in this chapter), while *Ago1* has nearly undetectable expression in germ cells.[69]

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The biogenesis of miRNAs has been extensively reviewed[312,313] and involves transcription by RNA polymerase II to yield primary miRNAs, which are then cropped by the DROSHA–DGCR8 complex to yield shorter precursor miRNAs (pre-miRNAs). Pre-miRNAs consist of 60–70 nucleotide hairpin structures that are exported from the nucleus by exportin 5. Once in the cytoplasm, pre-miRNAs are processed by the RNase III nuclease DICER to remove the hairpin and to leave approximately 22-nucleotide miRNA–miRNA* duplexes. It is thought that the miRNA becomes loaded onto, and thus stabilized by, one of the four AGO proteins, while the miRNA* (or passenger) strand becomes degraded. Endogenous siRNAs (or endo-siRNAs) are produced in a similar fashion, except that they arise in situations where transcripts are able to form double-stranded RNA (dsRNA) or long stem–loop structures. These dsRNA transcripts are exported to the cytoplasm, where they, too, become the target for DICER and are then loaded onto AGO2.[313] Thus, a key distinction between miRNA and siRNA biosynthesis is the dependence (in the former) on DROSHA–DGCR8.

The relative roles of the siRNA and miRNA classes of small RNAs in germ cells have been discerned mostly through loss-of-function and conditional ablation of genes required for the generation and/or stability of these small RNA classes. For example, loss of *Dicer* by conditional deletion in the germline using Cre recombinase-mediated *LoxP* recombination has resulted in different phenotypes, depending on the *Cre* deletion mouse used and the lab from which the data were reported.[314] Since DICER is upstream of both the miRNA and siRNA pathways, it is not surprising that the most severe phenotype reported is that of complete testicular degeneration within days of birth in males, as a result of defects as early as the spermatogonial (premeiotic) stages when DICER is deleted from Sertoli cells.[315,316] *Dicer* deletion specifically in germ cells of male mice, in contrast, results in a variety of phenotypes, depending on the timing of CRE-mediated gene ablation. For example, loss of *Dicer* using a Neurogenin 3–*Cre* (*Ngn3–Cre*) mouse, which deletes around day 5 postpartum, results in slightly increased spermatocyte apoptosis but apparently normal meiotic progression for the majority of spermatocytes.[317,318] By contrast, deletion of *Dicer* during embryogenesis, using a *Ddx4–Cre* mouse, results in a more severe loss of spermatogenic cells and in infertility, resulting from delayed transition into, and through, early prophase I.[318] Two other groups utilized a *Stra8–Cre* strategy to delete *Dicer* prior to meiotic entry, and showed variable effects on meiotic progression, suggesting at least some role for DICER-driven events during prophase I in males.[319,320] Similarly, loss of *Dicer* in female germ cells results in meiosis I arrest and infertility.[321]

Loss of *Dgcr8* or *Drosha*, whose protein products play roles specifically in the miRNA pathway, also results in spermatogenesis defects from the spermatocyte stages onward,[322] while females remain fertile.[323] Loss of *Ago2*, which ablates siRNA function but not miRNA function, has no effect on germ cell proliferation or meiotic progression.[324] Taken together, these studies, while somewhat conflicting, point to a role for at least miRNA populations in meiotic progression. As will be discussed in this chapter, these functions may include regulating meiotic onset and/or transcriptional silencing.

Role of PIWI-Interacting RNAs in Mammalian Meiosis

The piRNAs were named for their exclusive association with Argonaute proteins of the PIWI clade, as opposed to the AGO clade that associates specifically with miRNA and siRNA species. PIWI clade proteins have been identified in a number of species, including *D. melanogaster*, for whose Piwi protein the clade was named. In mammals, three PIWI proteins exist—MILI, MIWI, and MIWI2—and the expression of these genes is restricted to germ cell lineages. Of the three major classes of small RNA found in mammals, piRNAs were discovered most recently and are highly enriched in germ cells. Importantly, while they were originally thought to be specific to germ cells, it now appears that they are found in other cells, including embryonic stem cells.[325] At 26–29 nucleotides in length, the piRNAs are larger in size than other small RNAs, and their biogenesis appears to be very different from that of other classes of noncoding RNA. Their synthesis is not dependent on DICER, and they arise from large genomic piRNA clusters that can exceed 100 kb in length and that encode many piRNAs of overlapping sequence.[326,327] Instead, their biogenesis appears to initiate by the formation of long single-stranded precursors transcribed from the piRNA loci, which are then processed by unknown mechanisms to form pre-piRNAs. These pre-piRNAs then load onto PIWI protein complexes for further processing and, in the case of pre-pachytene piRNAs, may be amplified through a *ping-pong* amplification mechanism, described in detail in Refs 313,327,328,329,330.

In mammals, piRNAs are most abundant during prophase I, but are also observed at high levels in prospermatogonia and, to a lesser extent, in oocytes.[328,331] Indeed, in male germ cells, two distinct populations have been identified: the pre-pachytene piRNA pool, which interacts with MIWI2 and MILI, and which is enriched in repeat-derived sequences,[328] and the pachytene piRNAs, which derive mostly from intergenic sequences and which interact with MILI and MIWI in pachytene spermatocytes and round spermatids.[332,333] Mouse knockouts for *Mili* and *Miwi2* result in spermatogonial stem cell loss, consistent with their interaction

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with pre-pachytene piRNA populations. Conversely, deletion of *Miwi* in the mouse results in meiotic disruption, but spermatogenic failure does not occur until the round spermatid stage.[332,334,335,336,337,338]

The major function of pre-pachytene piRNAs, and their associated PIWI proteins, is to silence retrotransposons, through both transcriptional (epigenetic) and posttranscriptional (mRNA) activities.[335,339] This activity is distinct from the DNA methylation mechanism in which de novo methylation of retrotransposons occurs via the action of DNMT3L during fetal life.[340] Prior to meiotic entry, MILI and MIWI2 act to facilitate this de novo methylation such that the phenotype of *Mili* and *Miwi2* mutants mimics that of *Dnmt3l* loss, and MIWI2 appears to play a direct role in facilitating this methylation event.[327,337] Prior to prophase I, MILI may also act to cleave transposon mRNAs through its slicer activity.[327] MIWI, in contrast, appears to function exclusively during prophase I, associating with regulatory proteins that include the RNA-binding protein, mouse vasa homolog (MVH–DDX4), the ortholog of *Drosophila* Maelstrom (MAEL), and various Tudor domain-containing proteins, TDRD1, TDRD6, and TDRD7.[341,342,343,344] All of these proteins reside in the perinuclear diffuse structure known as the *Nuage* (meanining "cloud"), which is rich in RNAs and associated proteins. The mechanisms by which MIWI acts during pachynema remain unclear, but it appears to affect both mRNA stability and translational activity.[327] Part of the difficulty in understanding the role of MIWI during prophase I has been due to the fact that loss of *Miwi* (or any of the nuage proteins) does not deplete all the pachytene piRNAs in mouse spermatocytes, since MILI-associated piRNAs persist. Instead, conditional deletion of *Mov10l1*, a putative RNA helicase, during prophase I appears to selectively remove all pachytene piRNAs, and these mice exhibit massive DNA damage in postmeiotic round spermatids. These data indicate that pachytene piRNAs are required for maintaining genomic integrity postmeiotically, but that this function is not associated with any effects on retrotransposon repression.[345]

Meiotic Silencing

Meiotic silencing is the process by which unsynapsed chromatin is transcriptionally repressed during prophase I, and has been described in many organisms, including mouse, nematodes, fungi, and birds.[346,347,348] In the animal kingdom, meiotic silencing most commonly occurs in the heterogametic sex as a means of silencing the largely asynapsed sex chromosomes. This process has been termed meiotic sex chromosome inactivation (MSCI), and it is absolutely crucial for normal prophase I, since mouse mutants that fail to silence their sex chromosomes display meiotic arrest.[348,349,350,351] MSCI is just one example of a larger silencing mechanism called *meiotic silencing of unpaired chromatin* (MSUC), which can be directed at any region of the genome that fails to undergo synapsis (asynapsed). Thus, MSUC itself can be considered a pathological response to aberrant asynapsis, and usually results in apoptotic cell death, whereas MSCI is a normal feature of meiotic prophase I in male mammals and meiosis cannot proceed without it.

The purpose of meiotic silencing is not entirely proven, but it is thought to represent an ancient mechanism for suppressing heterologous chromatin as a means to protect the host genome from exogenous DNA, such as retrotransposons.[352,353] Alternatively, or in addition, meiotic silencing may serve to limit transcription from regions of damaged DNA,[354] or may prevent the expression of sex-linked genes that would otherwise be lethal to the process of prophase I (discussed further here). The existence of non-sex-chromosome-associated silencing mechanisms would argue against this latter option, however.

Meiotic Sex Chromosome Inactivation

During pachynema in mammalian spermatocytes, the X and Y chromosomes become sequestered into a defined heterochromatin-rich subdomain of the nucleus called the *sex body*. This domain is enriched in specialized histones,[246,355] proteins that mediate DNA damage responses through phosphorylation cascades,[348,356] and RNA.[69,357] Together, these mediate both sex body formation and silencing itself through mechanisms that are only slowly becoming elucidated. Histone marks associated with MSCI include H3K9me2,[358] ubiquitylated H2A,[352] and the phosphorylated histone variant of H2AX, γH2AX.[359] The importance of this latter modification is highlighted by the fact that mice lacking the critical phosphorylation site on H2AX fail to establish a sex body and cannot undergo prophase I, leading to infertility.[246,360] Interestingly, XO female mice, who possess only a single univalent X chromosome, also show sex body formation in a proportion of oocytes as a result of the failure to pair their single X.[351,352] As a result, these cells die because of silencing of essential X-linked genes without the presence of autosomal "backups". A proportion of cells exhibit self-synapsis of the X chromosome, which loops back on itself in a hairpin configuration, thereby protecting itself from the MSCI machinery and allowing the cells to proceed through meiosis.[348,351]

BRCA1 is an early marker of the sex body,[361] and this protein recruits ataxia telangiectasia and Rad-3 related (ATR) protein and its activator, TOPBP1.[197,356,362,363] These proteins assemble on the unsynapsed sex chromosomes in early pachynema, and they spread throughout the sex chromatin as pachynema progresses. This process of spreading is co-incident with ATR-mediated phosphorylation of H2AX, to become γH2AX, and

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this mark becomes a defining feature of the sex body at pachynema.[246] Furthermore, the spreading of the BRCA1, ATR, and TOPBP1 signal throughout the chromatin may be mediated by MDC1, a binding partner of γH2AX, although their initial loading is not dependent on MDC1.[364] Importantly, the genesis of γH2AX at the sex body is temporally and functionally distinct from that of γH2AX found at sites of DSBs from leptonema to pachynema. Instead, it is likely that other kinases, perhaps ATM or DNA–PK,[365] are responsible for phosphorylation of H2AX and sites of SPO11-induced breaks.

The outcome of γH2AX appearance in the sex body is the condensation of the sex chromatin, and the exclusion of RNA polymerase II from the sex body, leading to transcriptional repression. The loss of expression from the X and Y is compensated for, in certain essential cases, by the expression of autosomal paralogs that are specifically active during male meiosis.[346] However, several sex-linked genes are known to be "toxic" during prophase I, and thus their expression must be repressed. These include the *Zfy1* and *Zfy2* paralogs, whose ectopic expression on the nonsilenced autosomes is sufficient to induce mid-pachytene apoptosis in transgenic animals.[366,367]

Analyses of meiotic mutants for genes involved in synapsis and/or DSB repair have indicated that the loss of these pathways also affects sex body formation and MSCI. Thus, spermatocytes with defective synaptonemal complexes or those that fail to properly repair their DSBs exhibit aberrant sex body formation, including those from homozygous mutant males for *Msh5*, *H2ax*, and *Sycp1*.[128,368] The loss of spermatocytes in these mutant animals occurs at mid-pachynema, when sex body silencing should be fully engaged, and is associated with increased expression of genes from the X and Y chromosomes.[368] However, it was unclear until recently whether loss of cells from these mutants was the consequence of checkpoint activation, due to persistent unrepaired breaks, or due to impaired MSCI. Royo et al. showed that in XYY males, in which single YY bivalents prevent transcriptional repression of Y-linked genes without any effects on DSB repair, the mid-pachytene loss was similar to that seen in DSB repair and synapsis mutants. Thus, it is likely that the loss of spermatocytes from many DSB repair mutant mice reflects improper Y-linked gene expression, and may account for the increased severity of the male meiotic phenotype compared to that of the female mutants for these genes.[350,368]

Meiotic Silencing of Unpaired Chromatin

As stated in this chapter, autosomes can also display meiotic silencing under certain conditions, and this is most clearly displayed in some of the mouse mutants for DSB repair and synapsis (some of which are listed here). This has led researchers to think more carefully about the links between synapsis, recombination, and the various silencing mechanisms. As described here, some of these meiotic mutants display synapsis defects that lead to normal MSUC, an example of this being *Spo11* homozygous mutant animals.[368] However, these mice exhibit disrupted MSCI.[198] Other mouse mutants, however, show aberrant MSUC as well as disrupted MSCI, and these include mice lacking *Msh5*, *Dmc1*, and *Sycp1*.[368] The interesting observation came with *Dmnt3l* homozygous mutant males, however, in which spermatocytes display increasing levels of asynapsis, and this is associated with a progressive decline in the efficacy of MSUC.[368] Importantly, the levels of MSUC-inducing BRCA1 associated with autosomal regions of asynapsis appear to be restricted such that it did not increase with the increasing severity of asynapsis in the *Dmnt3l* mutant spermatocytes, thus providing an explanation for failed MSUC. In addition, this BRCA1 relocation to the autosomes also results in reduced sex body-associated BRCA1, resulting in failed MSCI. Since BRCA1 initially localizes to DSB sites, this restricted pool of BRCA1 might also be sequestered at persistent DSBs in the face of impaired DSB repair pathways, perhaps providing an explanation for the impaired MSUC in DSB repair mutants (*Msh5*, *Dmc1*, etc.) but not in DSB-devoid mutants (*Spo11*).

Taken together, these different mouse mutants displaying extensive asynapsis with or without persistent unrepaired DSBs show differing MSUC responses, but all fail to silence their sex chromosomes.[368] This would suggest that the presence of asynapsed autosomes reduces the overall load of BRCA1 in the vicinity of the sex body, in turn reducing the recruitment of ATR. The amount of BRCA1 in females also appears to be somewhat limiting, since mouse oocytes bearing more than two or three asynapsed chromosomes fail to recruit enough BRCA1 to activate MSUC, whereas those with three or fewer asynapsed chromosomes possess enough BRCA1 to initiate silencing at each chromosome.[369]

One caveat to this model is the fact that some spermatocytes from *Brca1* mutant mice can evade meiotic arrest and might suggest an alternative mechanism for recruiting ATR. The obvious candidate for this alternative pathway is the 9-1-1 complex of RAD9, HUS1, and RAD1, which has been shown to accumulate in the sex body at pachynema,[354,370,491] and which is upstream of ATR in the somatic cell DNA damage repair pathway. Whether ATR is recruited to DSBs and the sex body by the same mediators is also the subject of debate at the current time.

Small RNA Participation in Meiotic Silencing

The involvement of small RNAs in meiotic silencing was first alluded to in *Neurospora crassa*, where the associated process of MSUD was first shown to involve Sad1, an RNA-directed RNA polymerase.[371] In

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mammals, recent studies have demonstrated that the small RNA–associated Argonaute protein AGO4 is localized to the sex body during pachynema and to regions of induced asynapsis (e.g., in translocation mutant mice) during prophase I.[69,372] This is an exciting finding because mammalian Argonautes commonly perform their small RNA functions exclusively in the cytoplasm and are not known to reside in the nucleus of somatic cells, unlike the situation in worms and other organisms where nuclear Argonaute complexes have been functionally characterized.[312,373] In mouse spermatocytes, however, AGO4 and, to a lesser extent, AGO3 both reside in the sex body, and are associated with many miRNAs that originate from both the sex chromosomes and the autosomes.[69] Loss of *Ago4* results in altered sex body morphology and failure to silence many genes on the X and Y chromosomes, including *Zfy1* and *Zfy2*, whose expression is toxic to the cells. However, AGO3 can partially compensate for loss of AGO4 such that *Ago4* mutant mice are only subfertile. Importantly, loss of *Ago4* results in dramatic downregulation of miRNAs from pachytene spermatocytes, over half of which originate from the sex chromosomes.[69] This is counterintuitive to the finding that RNA polymerase II access to the sex body is upregulated in these animals, but fits well with other studies demonstrating that specific sex-linked small RNAs evade the normal silencing mechanism that is characteristic of the sex chromatin during pachynema.[374] Moreover, these data fit with those from *Dicer* conditional knockout mice showing that deletion of *Dicer* (which is required for miRNA and siRNA pathways upstream of AGOs) results in failed MSCI.[320] Taken together, these observations suggest that small RNAs, and their associated AGO proteins, play essential roles in mediating the silencing of unpaired chromatin during meiotic prophase I in mammals in a mechanism that may resemble the process of RNA-induced transcriptional silencing found in *S. pombe*.[375,376] How these RNA-driven processes interface with the known molecular pathway that induces meiotic silencing is unclear at the present time, and is the subject of intense investigation.

Checkpoint Control of Synapsis and Recombination

There are many processes active during meiosis that ensure that the correct number of gametes arises and that defective gametes are eliminated from the population. Without these safeguards in place, defective gametes may well be utilized for reproduction, resulting in the inheritance of mutations in the subsequent generation. In prophase I, there are two important surveillance mechanisms, or checkpoints, that monitor the developing gamete for any abnormalities, namely, the synapsis checkpoint and the recombination checkpoint. These two checkpoints monitor nuclear events, ensuring that complete synapsis between homologs and effective repair of DSB intermediates, respectively, have been completed before the cell can progress beyond prophase I. So, for example, spermatocytes lacking *Spo11*, which fail to undergo complete synapsis of their chromosomes but which have no DSBs and no recombination defects, succumb to the synapsis checkpoint,[198,199] while mutation of mouse *Trip13*, the ortholog of yeast pachytene checkpoint 2 (PCH2), results in loss of prophase I cells at pachynema without affecting synapsis but instead resulting in recombination failure at a subset of DSB events.[377] The apoptosis of cells at the pachytene checkpoint in *Trip13* mutants is thus intact, despite normal synapsis (the opposite situation to that seen for *Spo11* mutant mice), providing evidence that failed recombination alone can trigger this checkpoint.

Other mutants that affect DSB repair (e.g., *Msh4*, *Msh5*, and *Dmc1* mutants) also succumb to the recombination checkpoint.[349,378] However, given that defects in DSB repair often result in synapsis failure too, it is often difficult to separate the two checkpoints from each other, and thus, they are collectively termed the pachytene checkpoint. Importantly, most of these mutants also exhibit failures in MSCI, while failure to induce MSCI itself results in activation of the pachytene checkpoint, leading to the proposal that failed MSCI may comprise a major mechanism for checkpoint induction in mammalian meiosis.[349]

In males, this checkpoint is extremely stringent and serves to eliminate defective cells at pachynema via the process of apoptosis. Indeed, even in wild-type male mice, there is an abundance of apoptotic cells present in the testes, indicating that the checkpoint is highly efficient in wild-type male mice.[379] In females, the checkpoint appears to be much less rigid, such that gametes bearing synapsis and recombination defects may progress beyond pachynema. Oocytes with more minor abnormalities can also progress to the end of meiosis, perhaps maintaining these errors from prophase I in the form of aneuploidies in the resulting gamete.[380] This sexual dimorphism is largely poorly understood in mammals; however, the general thinking is that because males make millions of sperm throughout their lifetime, whereas females only make oocytes once prior to birth, the amount of effort invested by the female organism in generating the gamete is such that they simply "make do" and repair their defects as best they can, whereas males eliminate these defective cells entirely.

It is important to note that defects later in prophase I, after synapsis and recombination are largely completed, may also result in apoptosis, but that this checkpoint may not occur until metaphase I in males, and even later in females. One example of this is *Mlh1*-deficient mice. *Mlh1* mutant males exhibit almost complete loss of chiasmata in diakinesis, with cells undergoing apoptosis

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around the time of metaphase.[270] *Mlh1* mutant oocytes progress somewhat further, however, with a proportion of them extruding a first polar body, albeit at low rates and with highly likelihood of nondisjunction.[381,382] Similar results are observed in *Mlh3* mutants,[272] and in both cases are associated with poor bipolar attachment on the first meiotic spindle and subsequent defects in spindle morphology. These data suggest that later prophase I defects evade the pachytene checkpoint, but instead are identified by a second checkpoint upon attachment to the meiotic spindle.

CHROMOSOME SEGREGATION AT METAPHASE I (MI) AND MEIOTIC PROGRESSION

A fundamental and defining feature of the mitotic and meiotic divisions is the ability to sequentially and equally segregate genetic material to opposite poles and thus to produce daughter cells that contain the same amount of genetic material through two rounds of division, one equational and one reductional (Figure 1.1). During mitosis or during the second meiotic division, this segregation involves sister chromatids that are, by necessity, held together by cohesion (discussed further here) until they become captured by microtubules emanating from each mitotic spindle pole (Figure 1.12). Sister chromatid cohesion is an essential prerequisite for their attachment to microtubules emanating from opposite poles, a configuration known as amphitelic attachment (or bi-orientation; Figure 1.13), which, in turn, ensures the equal segregation of sister chromatids at anaphase. By contrast, during the first meiotic division, segregation separates the homologous chromosomes, each of which consists of paired sister chromatids (Figure 1.1). The role of prophase I events is to maintain homologous chromosome interactions up until the time of segregation. Thus, by the end of prophase I and into metaphase I, chiasmata-intact chromosomes align along the midplate of the cell and initiate the first meiotic division. This process is tightly regulated to ensure that homologous chromosome pairs align appropriately along the midplate in the correct bi-orientation to facilitate segregation of homologs to opposite poles of the cell, while sister chromatids remain mono-oriented (unlike during mitosis), or in syntelic attachment, to ensure that they move toward the same pole (Figure 1.13). Thus, a fundamental distinction between the two meiotic divisions is the syntelic versus amphitelic attachment behavior of the sister chromatids.

In order for these events to occur normally, chromosomes must engage a functional bipolar spindle and must also stabilize the spindle to ensure appropriate tension with which to facilitate equal segregation. Destabilization of the spindle can result in loss of tension, which will increase the potential for one or more chromosomes to fail in their attempts to move to the appropriate pole. One of the key processes that prevents catastrophe at this critical time is the spindle assembly checkpoint (SAC), a system that detects imbalances in the meiotic spindle (caused by loss of tension and/or unstable chromosome connections) and induces metaphase arrest until the problem is resolved. Many of the components of the SAC are preserved between mitosis and meiosis; however, by necessity, their functions extend in meiosis to ensure that homologous chromosomes, rather than sister chromosomes, become stably bi-oriented and attach to the spindle.

Our ability to study meiotic spindle assembly and checkpoint control has been limited largely to studies in oocytes, since disruption of spindle structures in male gametes using microtubule-destabilizing drugs results in early prophase I defects and arrest, precluding analysis of metaphase I and beyond.[383,384] Thus, much of what will be discussed in this section will focus on studies performed in lower eukaryotes and in females, with extrapolation where possible to analogous events in males. The relative difficulties in monitoring spindle assembly and progression in males are just the tip of the iceberg when it comes to differences in meiotic progression between the sexes. Indeed, female meiosis imposes several complexities on the process of spindle assembly and stability, not least of which is the long length of time during which the chromosome chiasmata must persist from their establishment in utero during prophase I to their critical role on the meiosis I spindle following meiotic resumption in the adult. Indeed, the entire spindle stability rests on the integrity and robustness of the chiasmata, the *raison d'être* for prophase I events in the first place. The importance of proper chromosome segregation during meiosis cannot be overstated, since chromosome mis-segregation during the first meiotic division can result in the generation of gametes containing the wrong number of chromosomes, a condition known as aneuploidy. Aneuploidy most commonly arises due to mis-segregation of homologous chromosomes, referred to as nondisjunction, but also can arise during the second meiotic division. As will be discussed in this section, nondisjunction is particularly prevalent in humans and, more specifically, in females.

The Spindle Assembly Checkpoint

The SAC is conserved between mitosis and meiosis, and is essential for monitoring the accurate segregation of genetic material, thereby signaling to the cell cycle machinery to continue the cell cycle or to arrest the cell in metaphase. This system acts at the level of the spindle to ensure that all chromosomes are attached to microtubules emanating from the appropriate pole, and to

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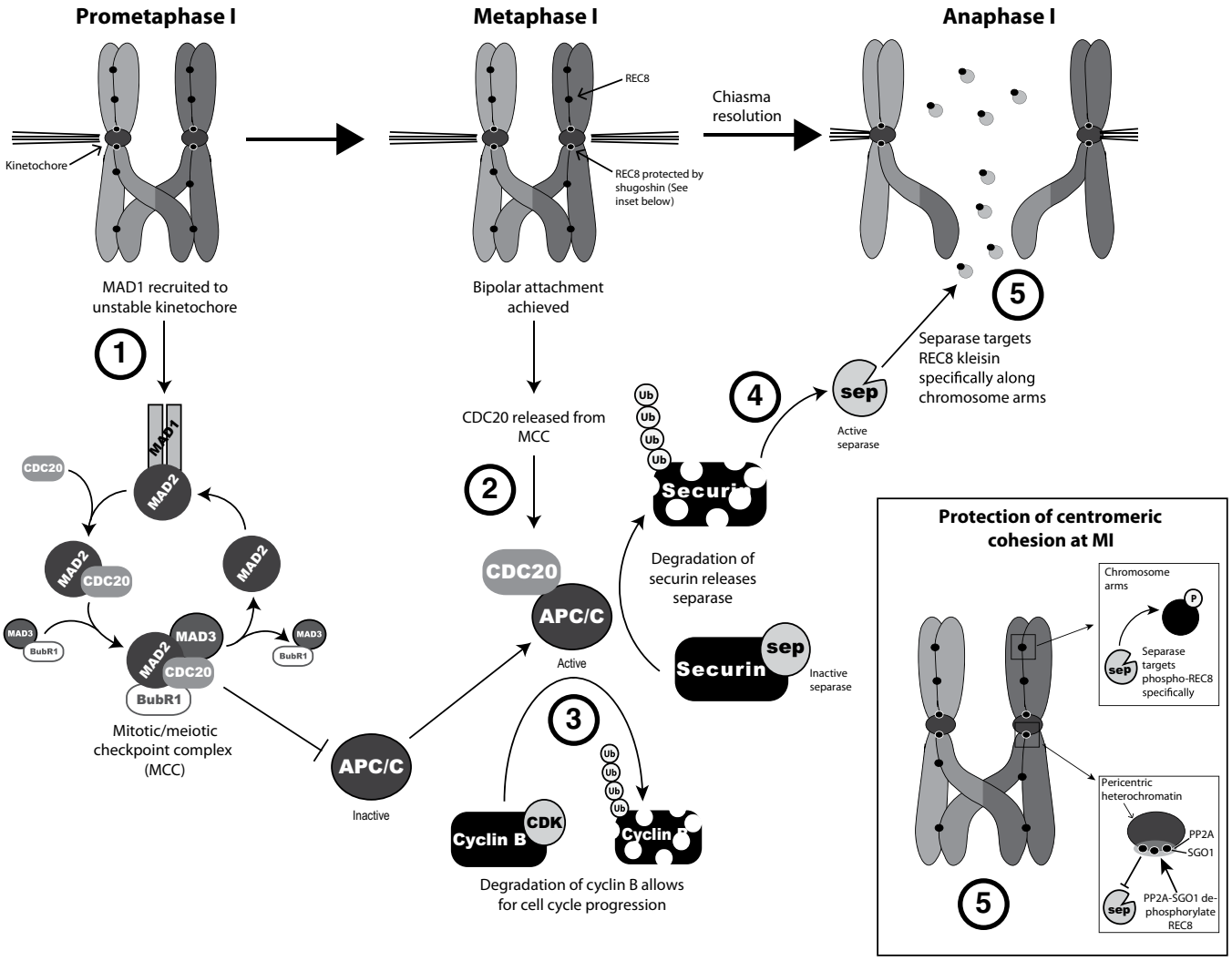

FIGURE 1.12 **Summary of events at the metaphase-to-anaphase transition in mammalian meiosis.** Step 1: Spindle assembly checkpoint (SAC) components are recruited to unstable kinetochores through the initial association of MAD1 and the subsequent recruitment of the mitotic–meiotic checkpoint complex (MCC). This inhibits APC/C activation. Step 2: If and when bipolar attachment is achieved, the MCC is destabilized, releasing CDC20, which can then bind to, and activate, APC/C. Step 3: APC/C can then ubiquitylate cyclin B–CDK complexes, resulting in their destruction, which facilitates cell cycle progression. Step 4: At the same time, APC/C ubiquitylates securin, releasing separase, which can then mediate phospho-REC8 destruction along chromosome arms, a process that is essential for disjunction of homologous chromosomes. Step 5 (inset panel): Importantly, SGO1 together with PP2A dephosphorylate REC8 at the centromere, which prevents separase-mediated destruction of REC8 at this location and thus preserves sister chromatid cohesion.

monitor tension within the spindle. Once all of these processes are in place, the SAC is silenced, and the anaphase-promoting complex/centrosome (APC/C) is activated, resulting in chromosome or chromatid movement to opposite poles and anaphase entry.

The SAC was discovered through a series of genetic screens in budding yeast that led to the identification of the key components of the checkpoint: the mitotic arrest deficient proteins, including Mad1, Mad2, and Mad3 (BUBR1 in vertebrates); and the budding uninhibited by benzimidazole proteins, Bub1 and Bub3, along with Mps1.[385,386,387,388,389,390,391] Classic laser ablation studies demonstrated that unattached kinetochores generate an inhibitory signal that delays anaphase onset, while destruction of the centromere (including both associated sister kinetochores) results in release of the checkpoint and anaphase progression. These studies indicated that activities in the vicinity of the kinetochore are responsible for monitoring mono-orientation and kinetochore attachment and for facilitating metaphase-to-anaphase progression.[392,393] Accordingly, most SAC components were later localized to the kinetochore,[387] and were found to be highly conserved in vertebrates.[394,395,396,397,398,399]

BUB1 is a protein kinase that localizes to kinetochores, senses microtubule attachment and stability, and may be involved in regulation of cyclin B (Figure 1.12, step 1). Similarly, MAD1 is recruited specifically to unattached, mono-oriented

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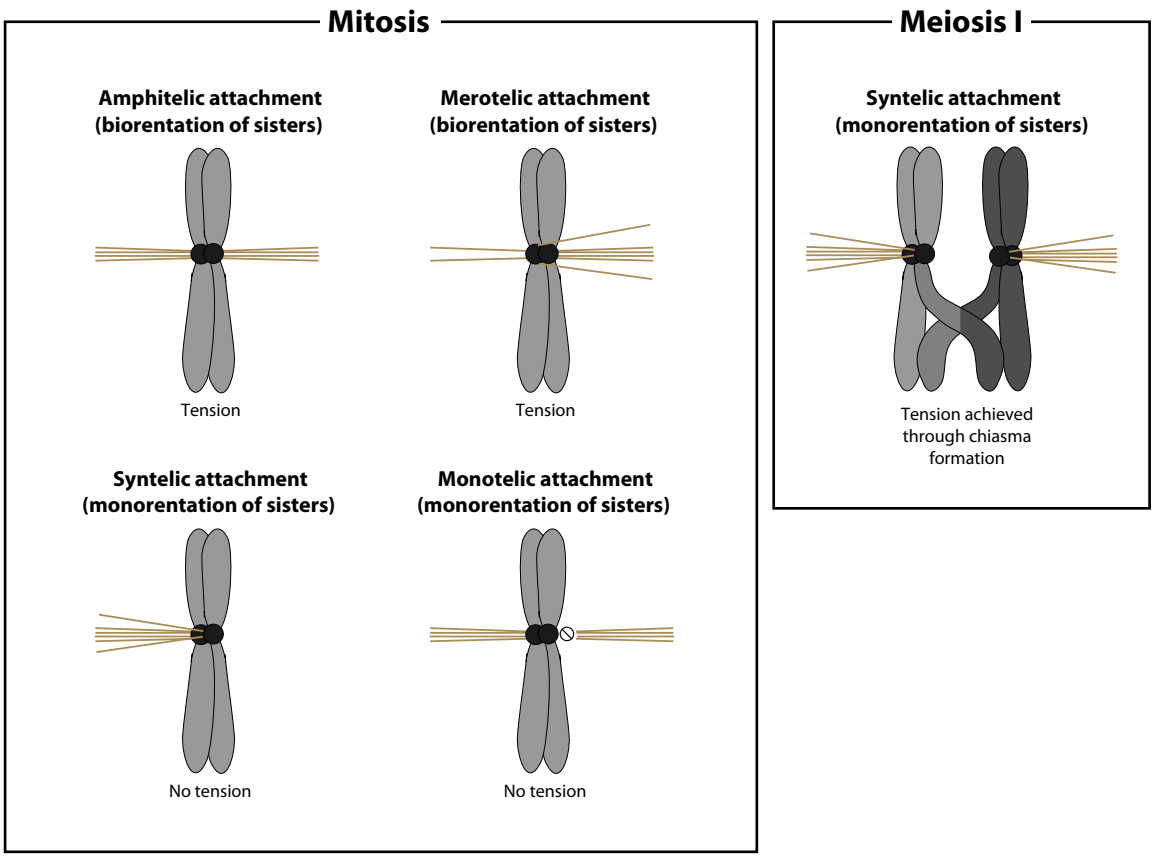

FIGURE 1.13 Sister chromatid interactions with spindle microtubules during mitosis and meiosis. During mitosis, sister chromatid kinetochores must attach to microtubules in bi-orientation to ensure tension across the spindle and, thus, equal segregation at metaphase. This attachment is referred to as amphitelic. However, bi-orientation can be achieved through merotelic attachment, although these interactions can be somewhat less stable. Mono-oriented attachment (such as those shown in the left-hand box) result in loss of spindle tension. During meiosis I (right-hand box), sister kinetochores must be mono-oriented toward the same pole, resulting in syntelic attachment. Tension across the metaphase I spindle is achieved through stable chiasmata formation.

kinetochores, and recruits with it MAD2. The localization of MAD1–MAD2 complexes at centromeres is at least indirectly dependent on MPS1.[400,401,402,403] MAD2 is capable of heterodimerizing and thereby recruits free MAD2 from the cytosol.[387] Upon spindle damage in mitotic cells or in the presence of unattached kinetochores, BUB1 phosphorylates MAD1, leading to the dissociation of MAD1–MAD2 complexes from the kinetochore. This dissociation allows MAD2 to sequester CDC20, an activator of APC/C. The formation of complexes of MAD2 and CDC20, together with BUBR1 and BUB3 (collectively known as the mitotic–meiotic checkpoint complex, or MCC; Figure 1.12, step 1),[387] prevents activation of APC/C and thus prevents the ability of the latter to invoke anaphase progression. Loss of this SAC mechanism in mammalian cells causes premature sister chromatid disjunction, leading to mis-segregation of chromosomes and cell death.[393,404,405]

During meiosis, the SAC acts at both metaphase I and II, and the functional components of the SAC appear to be conserved through both divisions.[403] During meiosis I, however, bi-orientation of sister kinetochores is prevented and instead the tension necessary to ensure stable microtubule attachment is provided by the chiasmata, which maintain homolog interactions (Figure 1.13). Moreover, during meiosis in the mouse, the preferred activator of the APC/C is CDH1, rather than CDC20, which is utilized during mitosis (Figure 1.12, step 2).[406] Studies of XO mice have demonstrated that, despite the absence of a pairing partner for the single X chromosome, failure to pair a single chromosome in oocytes does not result in meiotic failure, although the females exhibit somewhat reduced fertility. This has led to suggestions that the SAC is absent in vertebrate oocytes.[407,408] This does not appear to be the case, however, since overexpression of dominant negative forms of *Bub1* in mouse oocytes results in accelerated completion of MI and polar body extrusion (PBE).[409] Moreover, conditional mutant mice that do not express *Bub1* specifically in their oocytes display massive chromosome mis-segregation at meiosis I, precocious loss of cohesion between sister chromatids, and premature resolution of chiasmata and PBE.[410] These accelerated events

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are the result of early activation of APC/C.[410] BUB1 appears to have additional roles within the context of the meiosis I metaphase-to-anaphase transition, namely, it is required for congression of bivalents onto the metaphase I spindle, and to prevent premature destruction of sister–centromere cohesion (discussed further here). Interestingly, loss of one allele of *Bub1* in oocytes causes similar meiotic defects to homozygous deletion at this locus, whereas loss of a single copy of *Bub1* in spermatocytes has no such effect,[411] perhaps due to the existence of alternative testis-specific *Bub1* transcripts.[412]

In line with the differences in sister chromatid orientation during the first meiotic division, MAD2 localization during meiosis I in mouse spermatocytes differs from that seen during mitosis. MAD2 is present at most, if not all, kinetochores from prometaphase of the first meiotic division, and remains associated with the kinetochore throughout MI. It does not dissociate during metaphase and anaphase, as is the case for mitosis. Thus, progression to anaphase at the first meiotic division is not dependent on loss of the SAC complexes from kinetochores, at least in male mammals. In contrast, MAD2 localization during MII mimics that seen in mitotic cells, initially localizing to most kinetochores at the start of the second meiotic division, and then being lost as chromosomes arrive at the spindle equator.[413] Thus, loss of MAD2 from MII kinetochores during male meiosis occurs once stable spindle connections have been established and, presumably, facilitates the release of MAD2-mediated APC/C inhibition.

The aforementioned SAC proteins all function at the kinetochore, with the exception of MAD1 in mouse oocytes, which assembles at the spindle pole and from there is recruited to the kinetochore when unstable microtubular connections are formed.[414] By contrast, MAD1 in *Xenopus* oocytes remains kinetochore associated and is responsible for recruitment of MAD2 and other proteins to unattached kinetochores.[398] Regardless of its position on the spindle, MAD1 is thought to act as a sensor of spindle tension, whereby loss of tension induces and reinforces MAD1 localization at the kinetochore, which, in turn, inhibits APC/C activity.[403] This localization is dependent on preloading of MPS1, whose activity also appears to be required for meiosis in a number of organisms, including yeast, flies, mice, and zebrafish,[415,416,417] and this involvement extends to both meiotic divisions.[402] MPS1 activity may also be required for cytostatic factor (CSF) arrest at metaphase II (as discussed further in this chapter; and see Ref. 418).

The First Meiotic Division: Kinetochore Orientation and Cohesion Function

Given their similar progression and molecular regulators, the processes of mitosis and meiosis are often compared, particularly with respect to spindle assembly and chromosome segregation. The most obvious differences, however, are observed at the first meiotic division when, by the end of meiotic prophase I, when the synaptonemal complex breaks down, bivalent homologous chromosomes are seen to be connected by their chiasmata. The homologs each consist of paired sister chromatids, held together through cohesion, while cohesion also serves to maintain homolog interactions distal to the chiasmata sites. This arm cohesion must be lost during the first meiotic division, while maintaining cohesion at the centromeres to ensure that sister chromatid pairing remains intact through meiosis II (Figure 1.12, step 5). Meiosis-specific centromeric cohesion is also important as it serves to facilitate differences in the way that the centromeres attach to microtubules during the first meiotic division (Figure 1.12, step 5). In stark contrast to the events in mitosis, and in order to ensure spindle tension, the homologous sister chromatid pairs (i.e., two chromatids) must attach to microtubules emanating from the same pole such that their microtubule attachment points at the centromere (large protein complexes known as kinetochores) are mono-oriented toward one pole, while the opposing homologous sister chromatid pair is mono-oriented in the opposite direction (Figure 1.13). Indeed, in yeast, amphitelic attachment of sister chromatids is actively prevented by the monopolin proteins.[419] To this end, sister kinetochores are fused or closely aligned so as to ensure their syntelic attachment to microtubules from a single spindle pole, while the fused kinetochores from the opposing homolog (or bivalent) are captured in syntelic fashion by microtubules from the opposing spindle pole, to achieve bipolar tension and stability of the overall spindle.

Metaphase-to-Anaphase Transition during the First Meiotic Division

The downstream target of the SAC is the anaphase-promoting complex, or cyclosome (APC/C), a multimeric ubiquitin E3 ligase whose activity targets several proteins for degradation.[420] APC/C is activated by CDC20 or by CDH1, and the association of these activators with APC/C is downregulated by sequestration of CDC20–CDH1 by MAD2 when the SAC is activated.[406] Thus, when chromosomes align correctly and spindle stability is ensured, the release of CDC20 or CDH1 from the MAD2 complex allows for activation of APC/C (Figure 1.12, step 2). APC/C then targets a number of key proteins for degradation via ubiquitylation, and these include securin and cyclin B (Figure 1.12, steps 3 and 4). Securin is an inhibitory chaperone of the protease separase. During mitosis, separase cleaves the α-kleisin RAD21, resulting in the uniform loss of cohesion across the paired sister chromatids, and leading to chromatid movement to opposite spindle poles.[406]

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Studies in yeast, flies, and vertebrates have indicated that the APC/C–CDC20–CHD1 system is also responsible for metaphase–anaphase progression during meiosis and that securin is the major target of APC/C.[421,422] Events during meiotic prophase I are somewhat different with respect to cohesion, with the meiosis-specific α-kleisin, REC8, being used in place of RAD21. At the end of prophase I, with the synaptonemal complex degraded, cohesins distal to the chiasmata maintain cohesion along the chromosome arms and provide the tension and pulling force necessary for correct bivalent alignment on the metaphase I spindle. Once stable chromosome alignment is achieved, APC/C is activated, resulting in the degradation of securin and cyclin B, and this leads to cleavage of REC8 by active separase, thus inducing anaphase I onset in a manner analogous to that seen in mitosis. Importantly, this cleavage event occurs in concert with resolution of the chiasmata and only across the chromosome arms, allowing homologs to move to opposite poles, while retaining sister chromatid attachments' pericentromeric region (Figure 1.12, step 5).[423] The mechanism by which this differential protection of cohesion is achieved was uncovered in three distinct yeast screens for mutants affecting Rec8-mediated cohesion.[424,425,426] In the first, Kitajima et al. demonstrated that replacement of Rad21 by Rec8 during mitosis in fission yeast was not sufficient to replicate the stepwise loss of cohesion at anaphase, indicating that it was not Rec8 itself that promoted this centromeric protection phenomenon.[424] Furthermore, ectopic expression of a cleavage-resistant *Rec8* prevented sister chromatid separation, suggesting that, unlike in meiosis, mitotic separase is capable of cleaving Rec8 in a timely fashion. Thus, the existence of a meiotic-specific "centromeric protector of Rec8" was postulated, and a suitable candidate emerged in one screen for mutants that are toxic when expressed mitotically in the presence of Rec8, as well as in other screens for mutants that exhibit abnormal chromosome segregation during meiosis.[424,425,426] One gene was identified and named *Shugoshin*, a Japanese word meaning "guardian spirit", the budding yeast homolog for which was named *Sgo1*. Further homology searches revealed a second paralog in fission yeast, *Sgo2*, as well as orthologs in many species, including *Drosophila MeiS322*, which had been previously described as a gene essential for centromeric cohesion.[423,427,428]

As expected, both Sgo1 and its paralog, Sgo2, are meiosis specific, and both localize to centromeres in a Bub1-dependent fashion.[423,424,429] Importantly, their distribution alters radically in meiosis II, with SGO2 redistribution throughout the centromere facilitating separase-induced cohesin cleavage at this site.[430] During meiosis I, however, Shugoshin proteins prevent removal of cohesins from the centromere, at least in part by recruiting protein phosphatase PP2A to the pericentromeric region. PP2A is a serine–threonine phosphatase that, together with Shugoshin, can dephosphorylate the usually heavily phosphorylated Rec8 (Figure 1.12, step 5, inset).[431,432] In mice and in yeast, localization of PP2A to the centromere is dependent on Shugoshin,[433] while loss of PP2A in yeast results in premature loss of centromeric cohesion.[431,434]

While loss of Rec8 phosphorylation is now established as an important feature of maintenance of centromeric cohesion, the kinases involved in initial hyperphosphorylation remain the subject of debate. Clearly, Rec8 is heavily phosphorylated under normal conditions, and this phosphorylation is essential for cleavage by separase. In yeast, three kinases have been implicated in Rec8 phosphorylation, namely, polo-like kinase (Cdc5), casein kinase 1δ/ε (CK1), and Dbf4-dependent Cdc7 kinase (DDK)[423,435] and a number of reports present conflicting data with regard to the relative importance of each of these kinases. On the one hand, loss of Cdc5 in budding yeast during meiosis results in hypophosphorylated Rec8 and a failure to cleave cohesin,[436] while during mitosis in yeast, cohesion cleavage is promoted through phosphorylation of the cohesin subunit, Scc1, by Cdc5.[437] On the other hand, mutation of the Cdc5 target sites on Rec8 has surprisingly little effect on cohesin cleavage in meiosis I,[438] while, under certain mutant conditions, Rec8 is cleaved even when *Cdc5* is not expressed.[439,440] Thus, it is more likely that Cdc5 may act further upstream of Rec8 to modulate cleavage, although not directly on Rec8 itself. Instead, it appears that CK1 and DDK may instead be the kinases directly responsible for Rec8 phosphorylation since combined deletion of these kinases results in a block in Rec8 cleavage.[435] It is also possible that other kinases may participate in these events, since deletion of CK1, for example, does not cause as severe a block to Rec8 cleavage as does mutation on the CK1 phosphorylation sites on Rec8.[441]

Resumption of Meiosis in Females following Dictyate Arrest: The Roles of MOS, MPF, and CSF

A key feature of meiotic progression in female vertebrates is the punctuated progression of meiotic events. In mammals, prophase I progresses through fetal life up until the point that chromosomes are fully synapsed and COs are evident. This is the stage of diplonema, and the synaptonemal complex has, by this time, broken down. The process of prophase I arrests at this point, in a stage known as dictyotene or dictyate arrest. In many animals, this occurs at around the time of birth, and may be semisynchronous across all the oocytes in an individual.

At this stage, the oocytes are in a structure known as a cyst in which many common cytoplasmic components

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are shared. The role of the cyst is unknown, but one common belief is that the cyst structure allows for one or a few oocytes to gain nutritional and energy advantage, while the others die off, perhaps explaining the huge wave of oocyte death, or atresia, at birth. During dictyate arrest, the oocyte grows in size somewhat, and the pre-granulosa cells form primordial follicles, thus dissolving the cyst structure. The follicles wait in this state until the correct endocrine cues to resume division, or activation.

Meiosis does not resume in mammals until after puberty, at which time circulating gonadotrophins induce ovulation and meiotic resumption and completion of the first meiotic division. The major factor that induces meiotic resumption is the maturation promoting factor (MPF), so named in 1971 by Masui and Markert.[442,443] While its effects in oocytes had been known for several decades, it was not until the late 1980s that the constituents of MPF became known: cyclin B and CDK1.[444,445]

MPF activation is dependent on at least three activities of luteinizing hormone (LH; Figure 1.14(A)). Prior to the surge in LH at estrus, and in order to maintain dictyate arrest, MPF activation is prevented by high levels of cyclic adenosine monophosphate (cAMP) in the oocyte. cAMP levels are kept high by both intrinsic and extrinsic factors. In the case of the latter, cAMP is provided by the surrounding granulosa cells via gap junctions that pervade the zona pellucida, the glycoprotein coat surrounding the oocyte. These gap junctions also supply cyclic guanosine monophosphate (cGMP), which suppresses oocyte phosphodiesterase 3A (PDE3A), which would otherwise degrade cAMP (reviewed by Ref. 446). Elevated cAMP results in increased protein kinase A (PKA) levels that phosphorylate key kinases, WEE1 and MYT1. These kinases, in turn, phosphorylate MPF. Phosphorylated MPF is inactive and cannot induce meiotic resumption. PKA also phosphorylates (and inactivates) CDC25B, the major phosphatase required to activate MPF.

Upon LH stimulation, cAMP levels decline due to an effect of LH on the granulosa: First, LH induces a mitogen-activated protein kinase (MAPK) cascade that results in reduced cGMP levels, which facilitates increased PDE3A hydrolyzing activity to degrade cAMP in the oocyte. Next LH reduces cAMP in the oocyte by directly upregulating PDE3A hydrolysis of cAMP. Third, LH induces mitochondrial sequestration of PKA by A-kinase anchoring proteins (AKAP) and facilitates the dephosphorylation of CDC25B, which can then remove the phosphate group from CDK1 and thus activate MPF.

A key step in the activation of MPF, at least in females, involves MOS, the product of the Maloney murine sarcoma virus *c-Mos* proto-oncogene. MOS is a protein kinase that functions upstream of the MAPK cascade that ultimately results in the phosphorylation and activation of $p90^{RSK}$. MOS and $p90^{RSK}$ downregulate MYT1–WEE1, thus promoting further the activation of MPF and progression through to anaphase I.[447]

Induction of metaphase I results in completion of the first meiotic division, which involves condensation of the chromosomes, assembly onto the meiotic spindle, and nuclear envelope breakdown, also called germinal vesicle breakdown (GVBD). Instead of cleaving into two daughter cells, however, the excess half complement of chromosomes are shunted into a rudimentary structure called the first polar body (PB1 in Figure 1.14) in a clearly asymmetric division.

There is no intervening S-phase before entry into meiosis II, and oocytes progress to metaphase II rapidly, only to arrest again under the influence of cytostatic factor (CSF; Figure 1.14(B)). High CSF activity induces stabilization and reaccumulation of MPF again by inhibiting the action of the anaphase-promoting complex, APC/C, which would otherwise target MPF for proteosomal degregradation via polyubiquitination of cyclin B[406] (Figure 1.14(C)). MOS is also important for maintenance of high levels of MPF,[448] possibly upstream of CSF, in turn resulting in the second meiotic arrest at metaphase II (MII). It is only upon fertilization that this block is removed, via the influx of calcium ions that is triggered by sperm penetration, allowing MII progression once the sperm enters the egg.[449] Thus, if eggs remain unfertilized, the MII arrest persists. In contrast, *c-Mos* knockout female mice exhibit parthenogenetic activation of unfertilized oocytes, indicating that loss of *c-Mos* allows oocytes to proceed to the second meiotic division without the need for fertilization.[450,451] Importantly, MOS does not appear to be required for meiotic progression in male mice.

Despite its initial characterization in the 1970s, along with MPF, the molecular nature of CSF has never been completely resolved. It is plausible, given the relationship between MPF and APC/C activity, that CSF is an inhibitor of the latter and/or may constitute one or more components of the MCC (Figure 1.13). However, loss of one or more components of the MCC in oocytes does not affect APC/C function, nor does it affect calcium-induced loss of CSF activity,[452] suggesting that SAC proteins do not constitute the elusive CSF. Instead, it has been suggested that MOS–MAPK constitutes the true CSF activity, since introduction of *Mos* into a blastomere of a two-cell embryo results in CSF-like arrest, while both MOS protein and mRNA are rapidly degraded upon fertilization.[452] However, the mechanism by which the downstream target of MOS, $p90^{RSK}$, acts to suppress APC/C activity remained elusive for many years. Recent studies have pointed to EMI2 as a mediator of

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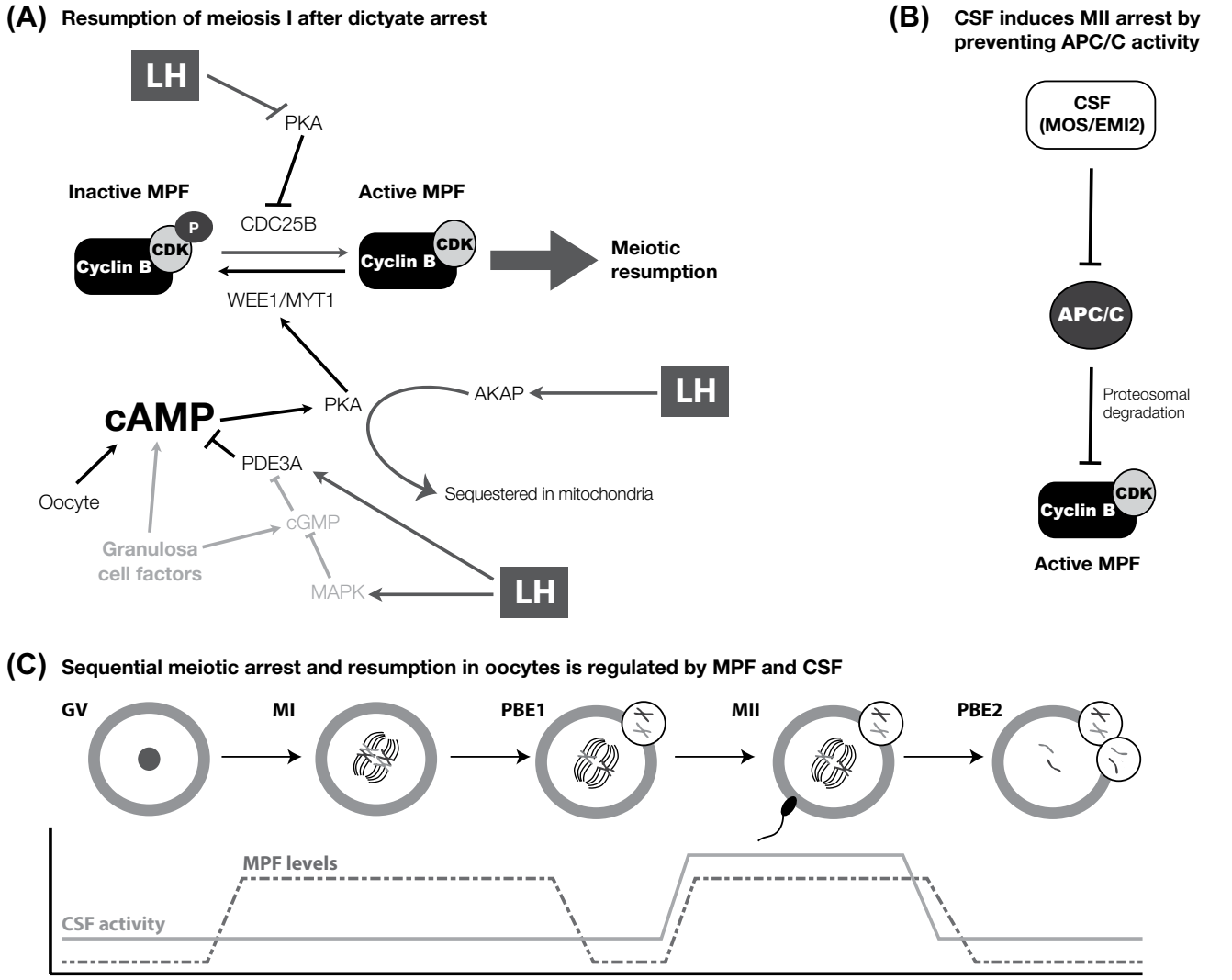

FIGURE 1.14 Resumption of meiosis I after dictyate arrest in females is mediated by MPF and CSF activity. (A) Dictyate arrest is achieved through the inactivation of MPF (cyclin B–CDK) via phosphorylation of CDK. This phosphorylation is brought about by maintaining high levels of cAMP within the oocyte. cAMP activates protein kinase A (PKA) that phosphorylates WEE1–MYT1 to maintain the phosphorylation status of CDK. PKA also inhibits CDC25B, the phosphatase that would ordinarily dephosphorylate cyclin B–CDK. Upon stimulation by luteinizing hormone (LH), PKA is inhibited through several routes, first by increasing PDE3A-mediated hydrolysis of cAMP through several routes. LH induces the MAPK cascade within the supporting granulosa cells that possess LH receptors, and this, in turn, suppresses cGMP release. In the dictyate-arrested oocyte, cGMP usually passes through gap junctions into the oocyte, where it suppresses hydrolysis of cAMP by PDE3A, and thus suppression of cGMP in the granulosa cells overcomes this suppression. In addition, LH induces closure of the gap junctions, further restricting movement of cGMP into the oocytes. Finally, LH stimulation results in upregulation of AKAP, which sequesters PKA within the mitochondria, preventing its access to the WEE1–MYT1 substrate. LH-mediated alleviation of MPF inactivity results in dephosphorylation and completion of the first meiotic division. (B) Following progression through the second meiotic division, oocytes arrest again in the presence of high CSF activity. CSF is thought to be composed of c-MOS and EMI2 and, together, they ensure suppression of APC/C activity, thus preventing inactivation of the steadily accumulating MPF. Fertilization of the oocyte by sperm results in an influx of calcium ions that, in turn, alleviates this inhibition, resulting in activation of APC/C and subsequent degradation of MPF. (C) Meiotic arrest and resumption are exquisitely timed events, all centering on the activation and inactivation of MPF and CSF activities. This graphical representation highlights key events in the bioactivity of these factors.

this APC/C inhibition, since depletion of EMI2 from egg extracts results in CSF release, while CaMKII, which is upregulated at fertilization, promotes EMI2 degradation, thereby relieving APC/C inhibition and promoting MII exit.[453,454] Importantly, EMI2 is a downstream target of $p90^{RSK}$,[455,456] providing the link between MOS activation and CSF activity.

DISORDERS OF MEIOSIS

Chromosome aneuploidy is a major cause of pregnancy loss and abnormal births in humans. Aneuploidy in fetuses tends to result in spontaneous miscarriage, as the vast majority of these render the fetus nonviable. Even those that survive to birth will have congenital

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abnormalities associated with an incorrect chromosome complement. The incidence of genetic aneuploidy in live births is around 0.3%; however, the true incidence of abnormal chromosome number during pregnancy is more likely to be in the region of 35–50%, taking into account those that are stillborn or spontaneously abort.[268,457] Upward of 70% of these errors are thought to arise due to maternal meiosis, with a vast proportion of these as a consequence of defective crossing over during prophase I in humans.[268] In contrast, mouse aneuploidies occur in <2% of meioses, which is surprising given the conservation of meiotic processes in mammalian systems. One reason for this massive discrepancy is likely to be the control of CO frequency and placement, which differs vastly from mouse to human. In mouse oocytes, MLH1 focus numbers are stringently controlled, numbering around 27.5 (±3.7) foci per nucleus.[268,458,459] In humans, in contrast, the number of MLH1 foci in fetal oocytes during pachynema is much more varied, ranging between 10 and 107 per nucleus, and representing a 10-fold difference in frequency from cell to cell, which is evident on the chromosomal level by chromosome arms missing MLH1 foci entirely or being overloaded with MLH1 foci.[268] This variation also differs between individuals, being more severe in some than others. Clearly, this vastly incompetent loading of MLH1 onto female oocytes in the human fetus would have a profound effect on the chromosomes during segregation. Given that these events occur in utero, they are distinct from any etiological causes in postnatal females or in adult women. As such, it is likely that genetic variation and/or changes in the molecular regulation of prophase I events are causes for such alterations in recombination frequency. In addition, it is possible that environmental factors may play a role in inducing this MLH1 variability, leading to the postulation that grandmaternal effects, such as smoking or drug use, may adversely affect meiotic events in female embryos.

Another fundamental cause of the high rate of maternal aneuploidy is the premature breakdown of cohesins associated with meiotic chromosomes in dictyate-arrested oocytes. Given the timing of human female meiosis, in which cohesins are sequestered on the chromosomes during fetal life and must remain there for several years until puberty, it is unsurprising that errors occur. There is evidence from mouse oocytes that once cohesins are recruited to the chromosomes prior to prophase I, they are not regenerated, and thus must maintain cohesion for a considerable period of time, possibly leading to nondisjunction in meiosis I.[460] This cohesin defect is also thought to be a major determinant of female age-related increases in aneuploidy (see below). Less than 10–20% of meiotic errors occur at meiosis II, and these are generally as a result of a precocious loss of centromeric cohesin.[461] Other meiotic genes also might be responsible for high levels of aneuploidy in mammals; a recent study found that mutations in *SYCP3* in females might increase the risks of pregnancy loss due to aneuploidy,[145] and *RNF212* haplotype variation has been implicated as a controlling factor in determining recombination rate in both males and females.[462] Recent evidence has suggested that different patterns of error origin are dependent upon the chromosome in question; for example, errors in separation of chromosomes 16 and 22 more often occur at meiosis II, whereas those in chromosomes 13, 18, and 21 occur at meiosis I.[463]

Maternal Age Effect

As mentioned above, cohesins in mammalian meiosis are thought to play a major role in age-related female aneuploidy, given the comparatively long time between the initial localization of cohesins on chromosomes during prophase I in the female in utero, and the time at which the oocyte emerges from dictyate arrest at the onset of ovulation. For example, *Smc1β* mutant mouse oocytes decrease in fitness with advancing age, with those from one-month-old females showing almost normal meiosis, while those from two-month-old females have vastly increased nondisjunction and meiotic errors.[178] A conditional knockout of *Smc1β*, which prevents SMC1β protein from being synthesized past dictyate arrest around birth, was, however, entirely fertile and showed no age-related loss in fertility, indicating that SMC1β production during prophase I was sufficient to ensure proper chromosome segregation.[464] However, several lines of evidence suggest that this is not the case with other cohesins. REC8 has been implicated as an important regulator of age-related meiotic errors, as it dissociates from chromosome arms and centromeres upon aging, and is not regenerated, leading to destabilized chiasmata and resulting aneuploidy in aged mice. Centromeric cohesin protector SGO2 is also required for proper segregation; however, its localization declines with age in mice.[460,465,466]

CO frequency and distribution are also thought to affect the rate of age-related aneuploidy. In divergent mouse strain crosses (divergence about 1%), aneuploidies in young mice increased relative to controls by around 10-fold, indicating that the sequence divergence was having a profound effect on recombination. In aged mice from the divergent crosses, aneuploidy rose by a further twofold, indicating that the problem was exacerbated in older mice.[467] Studies from human trisomies present a blurred picture as to the extent of influence that CO location has on aneuploidy in humans. One study showed that trisomies from young mothers contained COs more distal or proximal on chromosome 21 than those arising from older mothers; however, another

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study claimed that a single pericentromeric exchange correlated with increasing maternal age.[468,469,470] More recent high-resolution mapping of COs in human pedigrees reveals a significant decrease in recombination rate in older women, which is localized around the midsection of the chromosome arms and subtelomeric region.[471] It may be that COs in a certain position render the chromosome susceptible to age-dependent deterioration.

The SAC in human oocytes also may be a cause of age-related increases in aneuploidy. Decreased expression of critical SAC components, such as *Mad21l* and *Bub1*, has been observed in aged oocytes in humans and mice.[472,473,474,475] However, more recent data from mice reveal no apparent role for SAC in preventing aneuploidy in aged mice, given that MI lasts the same length of time in old and young mice, and they both have an equally robust checkpoint response.[465,476]

Human Diseases Caused by Meiotic Error

Many genetic diseases are caused by meiotic errors, the majority of these resulting from incorrect segregation of chromosomes in the oocytes and sperm. This mis-segregation can result in the offspring carrying too many (trisomy) or too few (monosomy) chromosomes. Typically, full chromosome autosomal monosomies are not found in the human population, but partial chromosome or mosaic monosomies do occur. The most prevalent monosomy in humans involves the deletion of one of the X chromosomes, known as Turner syndrome, which is viable in humans only because of the phenomenon of X inactivation, meaning that many of the cells will still possess a full complement of genes expressed. Diseases caused by chromosomal trisomy are more common in the human population, with the most common of these being trisomy 21, or Down syndrome. Down syndrome sufferers display developmental defects, along with severe mental retardation and a decreased life span. Down syndrome is the most common trisomy disorder seen in humans due to the fact that chromosome 21 is a small autosome, and therefore the trisomic cells can endure this extra chromosomal complement more so than a larger chromosome. Other trisomic diseases have been reported, including Edwards syndrome (trisomy 18) and Patau syndrome (trisomy 13), which have varying degrees of disease severity and life expectancy for the sufferer.[477,478] Trisomic disorders involving the sex chromosomes are more common than those involving autosomes, and include XXX, XYY, and XXY (Klinefelter syndrome). In the case of the latter, more than half of documented cases arise from paternal nondisjunction, rather than maternal, which is more common in humans.[479] All patient cohorts of sex chromosome trisomies exhibit some learning and behavioral difficulties as well as fertility problems, and XYY patients often have anger control issues.[480,481]

Environmental Effects on Aneuploidy

There is growing evidence supporting the fact that environmental factors have a large effect on the quality of gametes produced in mammalian species, particularly in oocytes. Diet has long been known to affect oocyte growth and maturation; however, a plethora of recent evidence has indicated the extent to which this is true. High lipid concentrations and maternal obesity have been demonstrated to have a negative correlation with oocyte health, inhibiting germinal vesicle breakdown and preventing the oocyte from proceeding beyond the prophase I stage[482,483]; whereas, conversely, animals maintained on a calorie-restricted diet do not display the usual aneuploidies associated with advancing maternal age.[484]

Smoking is also known to decrease fertility in both men and women, and for women the effect of smoking harms not only their own fertility but also that of their unborn female children.[485,486] Other toxic environmental factors include exposure to the estrogenic chemical bisphenol A (BPA), a component of many plastics and everyday items to which humans are exposed. Experimental evidence from mouse models and rhesus macaques has shown that BPA disrupts meiotic prophase sufficiently to cause synaptic defects, recombinational changes, and nondisjunction in the resulting chromosomes.[487,488] More recently, it has also been shown to have an effect on mouse germline stem cell differentiation, increasing levels of ovarian markers while decreasing levels of testicular markers.[489] So disastrous are these effects that BPA has subsequently been removed from much of the plastic sold in the United States. Temperature changes have also been shown to have an effect on oocyte growth in vitro, whereby the meiotic spindle of human oocytes was disrupted by transient cooling during culture.[490]

CONCLUSION

Our knowledge of the events of mammalian meiosis has evolved rapidly over the past 30 years, driven by impressive advances in our understanding of comparative cytogenetics and genomics, and our ability to extrapolate from similar processes that regulate events such as somatic cell mitosis and DNA repair through gene network analysis. The conservation of events between these processes and across species is remarkable and yet, at the same time, there are features that are unique to mammals, even differing between males and females, which continue to amaze and surprise us.

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These differences and similarities across species and sexes encourage and urge us to use comparative approaches at all times to elucidate the mechanisms of meiotic regulation. The next frontier in our understanding will come from continued understanding of the molecular pathways involved in recombination and checkpoint control, and how the epigenetic and RNA regulatory events integrate with those pathways. For example, our understanding of how DSB events are regulated at the chromatin level through *Prdm9* action has radically changed the way we think about DSB placement and CO assurance. More surprising has been the evidence from canids showing that, in the absence of a functional *Prdm9* allele, DSBs are directed to GC-rich regions and are evolutionarily more stable than recombination hotspots in other organisms.[296,297] This has led to speculation that recombination in the dog may be controlled by an ancestral mechanism that is found in other mammalian species but is outcompeted for in terms of DSB repair resources by the PRDM9-driven mechanism. What has also become clear in recent years is that CO placement is controlled by three distinct mechanisms: not only by interference, as had been previously thought, but by chromatin structure and CO homeostasis as well. The future in this area of research is particularly exciting. With exciting technologies to study recombination mechanisms, using methods such as ChIP-seq and SPO11-associated oligo mapping, already emerging, it seems the high-throughput approach to studying recombination is the direction that future studies will take. In addition, with the prospect of fast and inexpensive whole genome sequencing on the horizon, recombination events could be mapped over entire genomes in individual organisms or cells in the not-too-distant future.

A unique feature of mammalian, and specifically male, meiosis is the mechanism of meiotic silencing, particularly of the sex chromosomes. What has emerged in recent years is a picture whereby MSCI is inextricably linked to recombination and synapsis through the availability of specific mediators of each process, and these mediators (i.e., BRCA1 and ATR, but there may be others) act through heterochromatin formation and potentially utilize small RNA-driven mechanisms. This paradigm establishes an exciting interrelationship between gene-coding and noncoding mechanisms acting at the level of the DNA to regulate meiotic processes, and the elucidation of these RNA-based interactions over the coming decades is likely to change the meiotic landscape considerably.

Increasingly, studies specifically into meiotic processes in mice have helped advance similar studies in humans, and have led to major changes in the way we view human health. Advances in aneuploidy research and the massive effects that environmental factors can have on both male and, particularly, female meiotic health have led to changes in the way people choose to live their lives. The "grandmaternal effect" of exposing our unborn daughters to factors that may influence their reproductive health in years to come has changed international government policies on using these toxins in our plasticware. Research into female meiosis and the possible existence of oogonial stem cells isolated from both aged mice and humans has challenged the central dogma in reproductive biology stating that only a finite pool of oocytes exists at birth, and that this pool is never added to in later life. This area of reproductive biology remains controversial, but the broader questions of how germline stem cells become competent to undergo meiosis and, more importantly, how they do so with the exceptionally high fidelity needed to ensure the success of sexual reproduction represent an exciting area of continued and future research. Elucidating the molecular basis for meiotic entry and accurate progression has huge implications for our understanding of why human females exhibit such high rates of meiotic errors. Indeed this disparity between species in meiotic success rates remains one of the most poorly understood disease-related phenomena in human fertility despite the strict conservation of meiotic processes across eukaryotes.

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CHAPTER

2

The Mammalian Oocyte

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INTRODUCTION

The oocyte is distinct from all other metazoan cells by its ability to become a totipotent zygote. This fundamental property of female germ cells is not only retained in mammals but has required adjustments in design and function to meet the demands of the wide variety of reproductive strategies exhibited in mammals. While diversity between mammalian species exists with respect to the time required for oocyte development, there is remarkable conservation in basic processes governing the differentiation of a cell capable of effecting fertilization and embryogenesis. As stated above, among the most unique properties of the mammalian oocytes is the ability to generate totipotency. While subtle differences exist between mammalian species, the ability of the oocyte to reprogram the reconstituted diploid genome of the zygote, or that of a differentiated somatic cell nucleus, into a complete organism has been the culmination of researches encompassing more than two centuries.

Many historical accounts of the mammalian oocyte have been published, and for reaching into the deep past in this area, the reader is encouraged to examine "The Ovary of Eve".[1] That the ovum of vertebrates constitutes the generative cell was first suggested by William Harvey by the oft-cited statement, "*ex ovo de vivum*". It has taken many years to fully appreciate the ovum's totipotent character, requiring in retrospect the realization that the oocyte is contained within the ovarian follicle and undergoes a process of "maturation" as it is released from the ovary at ovulation, and finally accepting that fusion of egg with sperm launched a transformation of the ovum into the zygote, from which the embryo would develop.[2] These initial milestones are recorded in great detail in E. B. Wilson's treatise "The Cell in Development and Heredity," where the first glimpses of the process of oogenesis were described for the many studies on invertebrate animal models readily accessible in quantity and quality to analysis at the microscopic level.[3,4] Most important, these classical comparative studies on the oocyte formed the foundation upon which the basic principle that oogenesis presupposes embryogenesis was firmly established.

Appreciation that the oocytes of vertebrates more broadly applied to the biology of early development awaited the observational studies of the mid-twentieth century, where both the commonalities and distinctions of the oocytes of mammals were contributed by the likes of Austin, Beams, and Short. Among the principles reinforced from this era of research were: (1) the realization that the initial energy reserves for the embryo were deposited in the oocyte; (2) the appearance of extracellular coats for protection of the embryo and establishing the block to polyspermy; (3) the recognition that meiosis proceeds from diplotene to metaphase two in a highly developmental sequence under hormonal control; and (4) the interrelationship of oogenesis and folliculogenesis. This latter point was debated with vigor as the work of Sir Solly Zuckerman proposed that female mammals were born with a finite supply of oocytes that gradually diminished with advancing maternal age. The next significant phase of discovery took place in the latter half of the twentieth century, as has been summarized by Biggers.[4,5] Insights into the ultrastructure of the mammalian oocyte and the regulation of the meiotic cell cycle were obtained during this period of research.

Electron microscopic investigations defined in great detail the variety of organelles that constitute the "mother lode" of information that will carry the embryo to and through early development.[6,7] As shown in Figure 2.1, these descriptive studies revealed that the continuity of organelles through the female germ line to the embryo embodied mitochondria, the Golgi complex, endoplasmic reticulum, ribosomes, cortical granules,[8] and more specialized structures such as annulate lamellae (not

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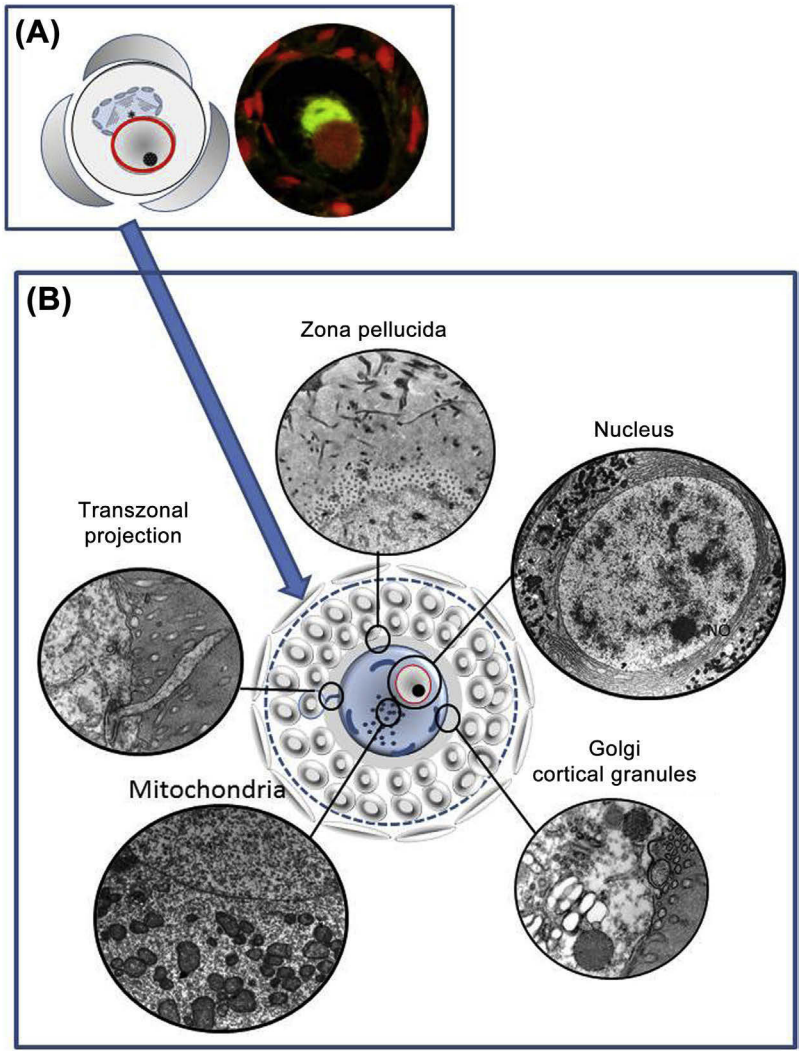

FIGURE 2.1 Structure of the mammalian oocyte. This figure is reproduced in color in the color plate section. (A) depicts the general organization of organelles in a primordial follicle; schematic (left) denotes centrally positioned nucleus and aggregation of organelles known as Balbiani's body (asterisk). Right is an immunofluorescence micrograph illustrating the concentration of the germ cell–specific marker VASA within Balbiani's body (green) located next to the oocyte nucleus (red). *(Image courtesy of Professor Alfredo Vitullo)*. Upon activation, oocytes enter the growth phase of oogenesis (B). (B) summarizes the major ultrastructural features of a growing oocyte within a preantral follicle noting the assembly of the extracellular coat or zona pellucida, the enlarged nucleus or germinal vesicle with prominent nucleoli (NO), subcortical Golgi complexes with associated cortical granules, abundant perinuclear mitochondria, and the elaboration of microvilli on the oocyte plasma membrane interacting with somatic cell projections known as transzonal processes.

shown), and cytoplasmic inclusions of various kinds that often were unique to certain mammalian species.[9] Moreover, ultrastructural studies reinforced and extended mechanistic thinking as it pertained to interactions of somatic cells with the oocyte and alterations in the structure and function of the oocyte plasma membrane known as the oolemma.

Concurrent with ultrastructural investigations, technologies were being honed that would permit the isolation and culture of oocytes, ova, and embryos, opening up an era of hypothesis-driven research making possible studies coupling structure with function. The foundations for this field were made by Gregory Pincus and colleagues at the Worcester Foundation for Experimental Biology during the 1930 and 1940s.[10] Although rarely acknowledged, Pincus' pioneering work established in vitro oocyte maturation (IVM) technology.[11] Some 20 years later, Bob Edwards established the utility of IVM for many mammalian species that conceptually laid the framework for much of what we know about the meiotic cell cycle today and led to the practice of human in vitro fertilization as we know it today.[12,13] Finally, experimental strategies for the ex vivo manipulation and analysis of virtually all stages of oogenesis in the mouse were established.[5,14] It was at this point that research flourished using the mouse model, as evidenced by the appearance of a number of papers that established the foundation for biochemical and molecular investigations of mammalian oogenesis.[15–18]

Important discoveries soon followed. For example, direct evidence documenting the dependence of the mouse oocyte on cumulus cell metabolism[19] was obtained. Work from Bob Moor's group validated the use of in vitro techniques in sheep oocytes, allowing for a detailed analysis of protein synthesis changes and meiotic cell cycle progression kinetics during IVM.[20–22] Importantly, these studies established the impact of culture on oocyte quality and developmental competence with respect to hormonal factors that remain relevant to

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TABLE 2.1 Competencies Acquired by Mammalian Oocytes during Oogenesis

Competency	Stage Acquired	Function
Meiotic	Late G	Resumption and completion of meiosis
Imprinting	Late G	Methylation of imprinted genes
Cortical granule fusion	M	Block to polyspermy
Calcium oscillations	M	Egg activation
Male pronucleus	M	Protamine S–S reduction; histone exchange
Cleavage	PM/M	Ability to sustain blastomere divisions
Compaction	PM/M	Ability to undergo cell polarization and lineage allocation
Embryonic stem cell derivation	G/PM/M	Ability to propagate stem cells from inner cell mass
Term birth	G/PM/M	Ability to sustain term gestation

Derived primarily from studies in the mouse based on phases of oogenesis described in Figure 2.2. Abbreviations: G, growth phase; PM, prematuration; M, maturation phase.

human assisted reproductive technologies (ARTs) to this day. Patterns of RNA synthesis were documented,[22] and the first studies describing an inhibitory role for adenosine 3′,5′-cyclic monophosphate (cAMP) in maintaining meiotic arrest appeared[23] (see Chapter 1). This discovery, the first to probe the mechanisms of cell cycle control during mammalian oogenesis, has become a centerpiece for much of the work exploring the relationship between cumulus cells and the oocyte over the past 40 years. Moreover, experiments of this kind paved the way for asking important clinical questions as to why some oocytes are able to develop after fertilization and why some fail. Whether mammalian oocytes are able to enact the events leading to successful embryonic development has been operationally defined as competence. Throughout this chapter, reference will be made to the oocyte competencies acquired at specific stages of oogenesis, as shown in Table 2.1.

The background literature up to this point sets the stage for much of the content of this chapter. While any attempt to cover the topic mammalian oocytes would fall far short of recognizing the many important contributions made prior to the "molecular genetic" era, there are several central tenets in the biology of the oocyte that have withstood the test of time and form recurrent themes of great importance to reproductive physiology.

The first theme embodies the notion that embryogenesis begins with oogenesis.[24,25] Maternal effect genes, products of oogenesis required for embryonic development, have been identified as have a variety of non-coding RNAs that are likely to play a role following fertilization.[26,27] Many of the proteins, and their posttranslational modifications, exerting control of the cell cycle from mitosis to meiosis and back to embryonic mitoses have been characterized (see Chapter 1). Together, the cytoplasm of the mammalian oocyte has been more critically defined in molecular terms, and to no surprise, the changing requirements at the protein level have taken on an aura of complexity that typifies the changing landscape within the oocyte as it proceeds through oogenesis. Figure 2.2 provides a framework for this chapter.

As Figure 2.2 illustrates, the process of oogenesis begins in the fetal ovary with the assembly of primordial follicles after the transition of primordial germ cells into meiotic prophase (see Chapter 1). With the formation of primordial follicles, oocytes have entered the diplotene stage of prophase one and will remain arrested in the cell cycle until luteinizing hormone (LH) induces ovulation and the resumption of meiosis. For the purposes of this chapter, oogenesis is divided into the growth phase, the pre-maturation phase, and finally, the maturation stage as shown in Figure 2.2. It is important to first appreciate that as the oocyte progresses through oogenesis, it maintains a temporal relationship within the ovarian follicle ensuring that the two processes, oogenesis and folliculogenesis, are integrated (see Chapter 21). When the primordial follicle is activated, oocyte growth proceeds in tandem with follicle growth in a gonadotropin-independent fashion. Once the follicle becomes sensitive to the action of gonadotropins, oocytes make the transition from the growth to pre-maturation phases, signaling the onset of the gonadotropin-dependent phase of folliculogenesis. As will be discussed below, the major factors that regulate development during the pre-antral growth phase involve paracrine and gap–junction mediated signaling mechanisms. In antral follicles, follicle-stimulating hormone (FSH) promotes growth and differentiation of somatic cells, ensuring that they eventually acquire sensitivity to LH, the primary trigger for ovulation and oocyte maturation. Each of these phases has been identified as error-prone in terms of nuclear (genetic, epigenetic) or cytoplasmic quality and have been implicated as determinants of embryonic developmental competence.[24,25,28–30]

Thus, the second major theme implies that oogenesis and folliculogenesis are inseparable from one another and any deviation from their coordination and integration spells failure to the reproductive fitness of a given species (see Chapters 21 and 22). From the vantage point of the oocyte, the growth phase of oogenesis relies heavily on direct forms of paracrine signaling between the oocyte and

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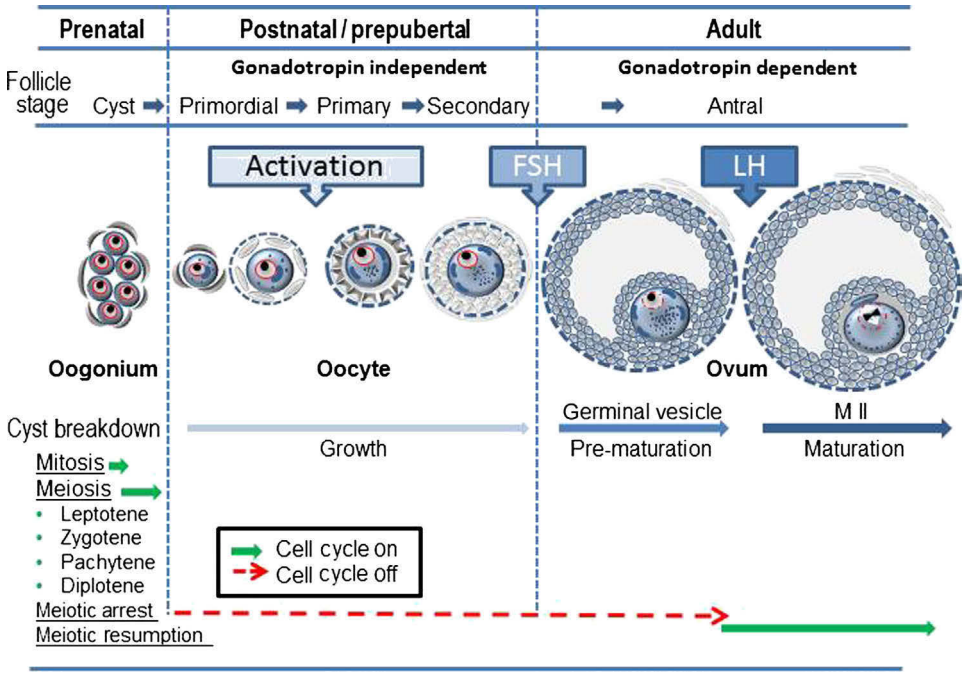

FIGURE 2.2 **Stages of oogenesis defined on the basis of the oocyte cell cycle state as it occurs in the mouse.** This figure is reproduced in color in the color plate section. Mitotic proliferation of oogonia occurs in the prenatal gonad and is accompanied by entry into meiotic prophase as follicle formation and cyst breakdown take place. The cell cycle is then turned off for the duration of the growth phase of oogenesis that occurs during the preantral stages of follicle development. While FSH is required to advance follicle development, the oocytes remain in meiotic arrest with a germinal vesicle in the prematuration stage. In response to the LH surge, meiotic arrest is released and the oocyte proceeds through maturation being ovulated at the metaphase two stage of meiosis, which is maintained until the fertilization signal elicits completion of meiosis two. Note that in general the growth phase of oogenesis occurs independent of gonadotropin stimulation, whereas the prematuration and maturation phases require gonadotropin stimulation.

surrounding somatic cells without direct involvement of gonadotropins (Figure 2.2). For basic and clinical scientists alike, the notion that the oocyte maintains communication with the soma throughout the various stages of follicle development has great significance.[31] The communication pathways at the interface of cumulus cells and the oocyte are now understood to involve many different forms of intercellular signaling that impact the development of both cell types.[32] Moreover, such communication pathways integrate germline and somatic cells to the overall benefit of embryo quality and the establishment of pregnancy.[33] There is good evidence to support the idea that the impact on the embryo is realized by epigenetic regulation. Importantly, epigenetics plays a role in separating genetic regulation of oocyte differentiation from that of the process of meiosis.[34] And during the growth phase of oogenesis, the expression of totipotency factors such as *Oct4* is modulated by gonadotropins through specific histone alterations.[35]

The third and final major theme for this chapter regards the problem of ovarian aging and the associated loss of oocyte quality. Humans are unique among mammals in the propensity with which their oocytes obtain an aneuploid condition, especially with advancing maternal age. Why our species is uniquely susceptible to genetic instability is not understood, but current opinion advocates faulty control of the meiotic and mitotic cell cycle.[36] Moreover, recent evidence suggests that other forms of genetic instability involving translocations, inversions, and DNA damage repair mechanisms are at play and that the oocyte itself may have a limited corrective potential for rectifying defects inherited paternally or maternally. Potential links between lifestyle alterations, environmental contaminants and reproductive failure have been proposed to find causality in these forms of genetic instability, clinically manifest in the high incidence of mosaicism observed in human embryos.[37]

Thus, in providing scope for this chapter, the link between oogenesis and embryogenesis, and the integration of oogenesis and folliculogenesis bear directly on the problem of ovarian aging and the clinical ramifications now being confronted in the context of human health and disease. This raises the interesting question as to the meaning of commonalities and differences in mammalian oocytes when compared between species. As shown in Figures 2.1 and 2.3, many structural properties exhibited by mammalian ova are conserved, such as the large size of the nucleus, prominent nucleoli, the presence of cortical granules, and the zona pellucida. Many of these properties are shared with the oocytes of lower vertebrates and invertebrates.[38] Where possibly of importance, attention

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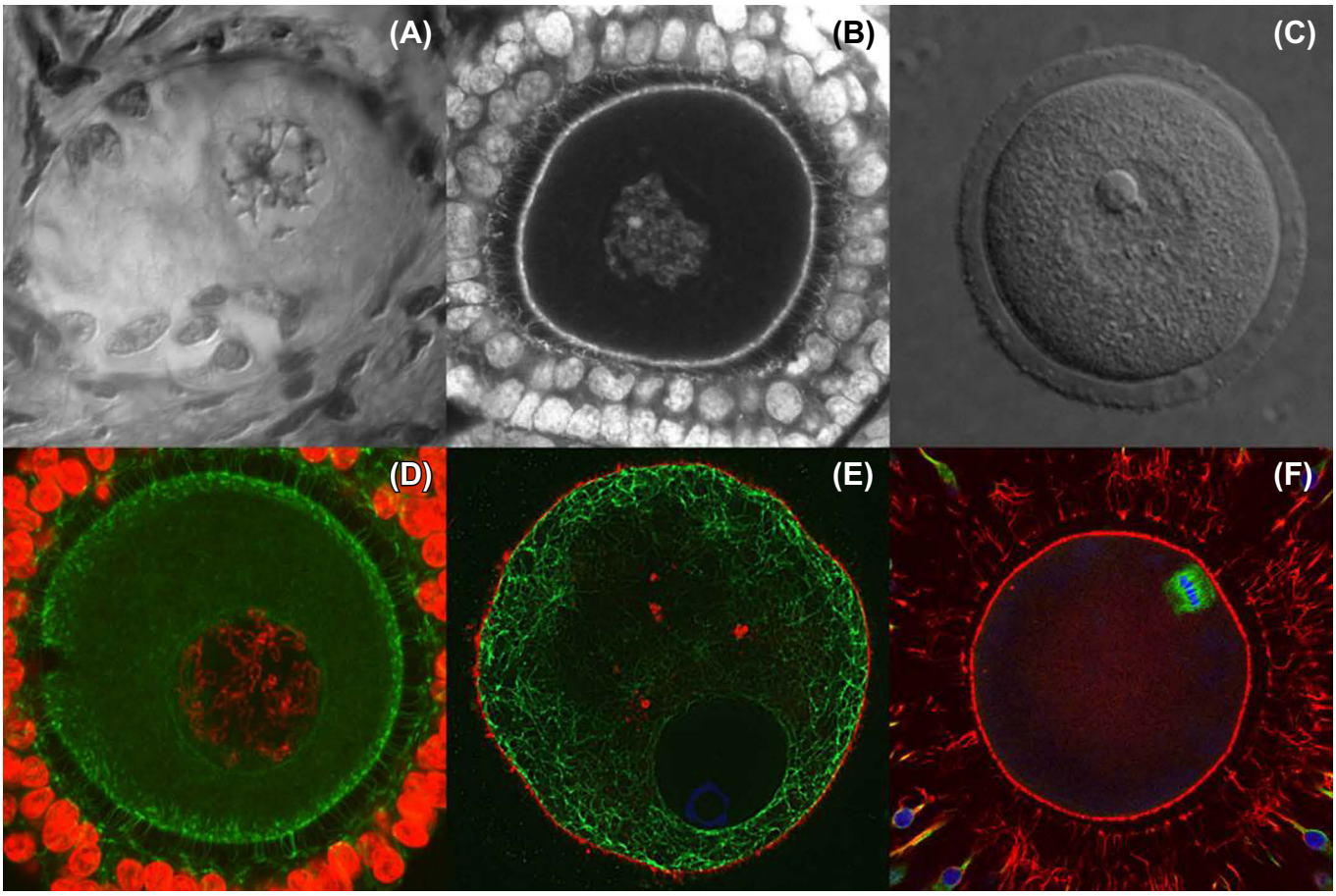

FIGURE 2.3 Prominent morphological features of the mammalian ovum at various stages of oogenesis in various species. This figure is reproduced in color in the color plate section. (A) depicts a resting primordial oocyte within the bovine ovary in which the germinal vesicle chromatin assumes a distinctly fibrillar pattern of organization. (B) represents a primary growing oocyte from the mouse containing a centrally positioned germinal vesicle and illustrating the prominent actin-rich cortex and numerous transzonal projections emanating from surrounding granulosa cells. (C) demonstrates the appearance of a full-grown immature mouse oocyte following isolation from the ovary; note the prominent eccentrically positioned nucleolus within the GV and the cell free zona pellucida. (D) depicts a full-grown immature bovine oocyte isolated from a Graafian follicle; note the clear organization of bivalents in the GV and the presence of numerous foci at the oocyte cell surface, which represent terminal connections with corona TZPs (preparation labels f-actin using rhodamine phalloidin). (E) illustrates an immature GV-stage human oocyte that has been labeled for chromatin and microtubules (fibrillar structures); note that the chromatin is aggregated around the nucleolus of the GV and that a dense network of cytoplasmic microtubules is present in the outer regions of ooplasm. (F) illustrates a mature metaphase two arrested oocyte from a horse following controlled ovarian hyperstimulation; this preparation has been dual-labeled for tubulin (green) that highlights the cortical anchored meiotic spindle and f-actin (red) that labels the prominent corona cell projections or TZPs that appear detached from the oolemma.

will be drawn to variations in mammalian oocytes that aid in understanding their physiology.[39]

CELL BIOLOGY OF THE OOCYTE

By far, one of the most striking features of the mammalian oocyte is its size. The transition from a quiescent non-growing state within the primordial follicle to that of active growth is one of the most dramatic examples of cellular hypertrophy known. Expansion in size occurs as a consequence to robust gene activation, selective protein synthesis, and hyperplasia of organelles. Of the new proteins and RNAs synthesized during the growth phase are those required for housekeeping and sustaining hypertrophy, those involved with regulation of the meiotic cell cycle, and those whose function will not be realized until later stages of development, so-called "maternal effect genes". Thus, the oocyte, arrested in diplotene of prophase in meiosis 1 (Chapter 1), exhibits a burst of transcriptional activity upon primordial follicle activation that persists until the oocyte has completed growth, as was first demonstrated in the mouse.[40] The unique challenge posed in the oocyte is how to selectively translate or store maternal RNAs of various classes, ensuring the readout of transcripts temporally restricted to the growth phase, cell cycle transitions, or after fertilization. Compounding this phase of development are emerging

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epigenetic requirements, exemplified by the dimethyl transferase variants used for maintenance methylation found in oocytes or embryos.[41] The importance of epigenetic regulation during the growth phase has been recognized in the now classical studies of Kono and colleagues, reinforcing the notion that events at early stages of oogenesis are critical for later development.[42] Thus, the conspicuous hypertrophy common to all mammalian oocytes is the first obvious sign of the investment the germ cell lineage will make toward the developmental potential of the embryo. And while inheritance of organelles like mitochondria has long been recognized, it is becoming increasingly clear that many other subcellular components will also contribute to embryogenesis.[42] Figure 2.1 illustrates the main features of the mammalian oocyte.

Traditional descriptions of growing oocytes relied heavily on the use of electron microscopy to validate and extend histological observations confirming the accumulation of mitochondria, Golgi complexes, and cortical granules, the specialized organelles that participate in the process of fertilization. Studies on the human oocyte identified constituents such as annulate lamellae and the characteristic perinuclear aggregate of organelles known as Balbiani's body[43] (Figure 2.1), a structure that will disperse upon follicle activation. Comparable constituents were described in bovine oocytes,[44] and Sathanathan has documented how these organelles are rearranged in human oocytes as they proceed from the growth stage through to the mature state.[45] In the oocytes of most mammals, it is generally appreciated that organelle amplification and migration to the oocyte cortex are a common feature of growing oocytes. Some exceptions do exist. For example, cortical granules are produced during oocyte growth in rodents[7] but formation is restricted to the maturation phase of oogenesis in sheep.[46] With interest shifting to the mechanisms that control entry into the growth phase of oogenesis,[47] more emphasis has been placed on the identification of specific molecules and organelles using contemporary imaging strategies.

The Germinal Vesicle—A Specialized Nucleus

One of the most prominent features of the mammalian oocyte is the large nucleus or germinal vesicle (GV) (Figures 2.3 and 2.4). The GV is located in a central or eccentric location, contains prominent nucleoli that can vary in number, and displays chromatin as condensed heterochromatin or diffuse euchromatin. Transitions between heterochromatin and euchromatin are generally believed to represent varying levels of transcription during oocyte growth, and there is wide variation between species with respect to whether the diplotene chromosomes remain chromosomal and fibrillar or are diffuse through the nucleoplasm (Figure 2.4). The states of chromatin organization are thought to reflect the level of ongoing transcriptional activity, as is discussed below. The presence of one or more nucleoli active in the generation of ribosomal RNAs is a common feature, with portions of the chromosomes bearing nucleolar organizing genes maintaining a stable pattern of heterochromatin at their surface (Figure 2.4). The nuclear envelope of the GV is rich in nuclear pores that mediate the robust level of RNA trafficking that occurs during the growth phase (Figure 2.4(F)).

Focus in recent years on the GV of mammalian ova has been placed on the nucleolus and interactions with surrounding chromatin (Figures 2.4 and 2.5). First described by Parfenov and colleagues, the compaction of chromatin around the nucleolus of human oocytes was named the karyosphere[48] (Figure 2.4(B,D,E)). It was proposed that this modification in chromatin reflected transcriptional repression near the end of the growth phase of oogenesis. Similar results were obtained in the rat[49] but the exact developmental timing and functional implications of this change in nuclear organization remained unclear. Studies in the mouse then showed that the gradual encapsulation of the nucleolus with heterochromatin (compare Figures 2.5(A,B,C)) occurred at the time of antrum formation, consistent with transcriptional repression known to occur at the end of the growth phase.[50] These and related studies then revealed that encapsulation of the nucleolus with heterochromatin coincided with a G2 to M phase cell cycle transition.[51] This implies coordination of chromatin remodeling with transcriptional and cell cycle control, a relationship reinforced in studies in rodents[52,53] and other mammals,[54] including the human.[55]

Specific reference to the transformation of chromatin from a diffuse (non-surrounded nucleolus (ND)) (Figure 2.4(A)) to perinucleolar and heterochromatized state (surrounded nucleolus (SN)) (Figure 2.4(B and C)) was proposed by Zuccotti,[52] Debey,[56] and others,[57–59] and general agreement now exists that this event also signifies a major change in cell cycle status that is associated with the acquisition of meiotic and developmental competence (Table 2.1). The experimental demonstration of meiotic competence refers to the ability of oocytes to reinitiate meiosis and proceed to metaphase of meiosis two when oocytes are removed from follicles and subjected to maturation in vitro.

Support for this broad generalization linking chromatin reorganization with competencies required for the completion of meiosis (meiotic) or embryogenesis (developmental) has been reported for the human,[60,61] bovine,[62] and primate[63] oocyte. Together, these findings demonstrate the importance of coordinating alterations in nuclear organization with key developmental transitions between the growth and pre-maturative phases of oogenesis. However, upon close examination, it has been reported that the basic distinctions for NSN and

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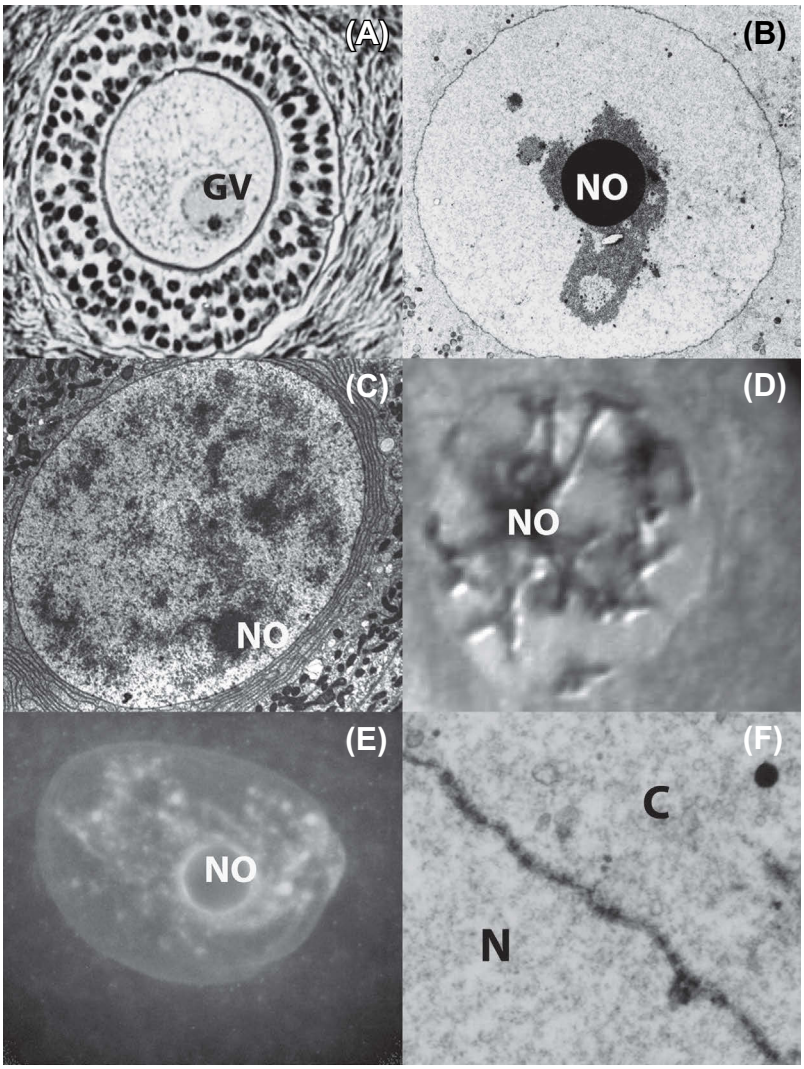

FIGURE 2.4 The immature mammalian oocyte nucleus or germinal vesicle (GV). (A) illustrates the general appearance of the GV using conventional histology; in this section from the rhesus monkey ovary, the GV assumes an eccentric position and exhibits a prominent nucleolus (NO). (B) is an electron micrograph of a human oocyte GV demonstrating the circular boundaries of the nucleus and the presence of heterochromatin surrounding the nucleolus (NO). In many mammals, fibrillar chromatin can be observed as shown here for the macaque (C) and the bovine (D); note that in both cases the bivalents are associated with the nucleolus centrally (NO) and at the inner aspect of the nuclear envelope. (E) illustrates the nuclear lamina in a mouse oocyte GV that has been labeled with antibodies to B-type lamins; the chromatin in this fully grown oocyte is superimposed on the lamin signal. In (F), a high-magnification electron micrograph of a human oocyte shows the high density of nuclear pores in the nuclear envelope that separates the nucleoplasm (N) from the cytoplasm (C).

SN used to describe the mouse oocyte do not hold when oocytes from other mammals are examined. Rather, the equivalent NSN configuration in ungulates and primates is more typical of condensed chromatin bearing the signature bivalent structure with crossover points well-defined (Figure 2.5(D–F)). And in human oocytes, the SN configuration is represented by the complete contraction of GV chromatin (Figure 2.5(G and H)) that is distinct from diakinesis (Figure 2.5(I)). Caution should be exercised in the use of such generalized descriptors given species variance in the organization of GV chromatin. Some of the factors that regulate chromatin reorganization in the GV have been identified through the use of transgenic mice.

For example, the oocyte specific gene for growth and differentiation factor 9 (*Gdf9*) produces a growth factor that is responsible for development of the follicle past the primary stage. In mice lacking *Gdf9*, the oocytes undergo pathological hypertrophy, exhibit impaired organelle proliferation, and show precocious transcriptional repression as evidenced by SN GV configurations.[64] In contrast, in mice bearing deletions in either the *FSH beta*[65] or *Gja37*[66] genes, follicles are arrested at the preantral stage, and contain oocytes exhibiting NSN configurations consistent with transcriptional activity. When animals are treated with FSH, follicle development is restored and oocytes proceed to an SN configuration showing the importance of gonadotropin stimulation in the regulation of GV organization and transcriptional status.[65] Many other factors have been identified that influence chromatin organization in mouse oocytes, including specific genes,[67] cell contact,[68] gonadotropins,[35] and the nuclear chaperone *Npm2*.[69]

It should be pointed out that some controversy remains as to the exact relationship between chromatin remodeling and transcriptional silencing in the mouse oocyte.[69,70] In other species, such as the cat, chromatin remodeling is not associated with the acquisition of meiotic competence.[70] The interested reader is referred to recent reviews on this topic.[57,71]

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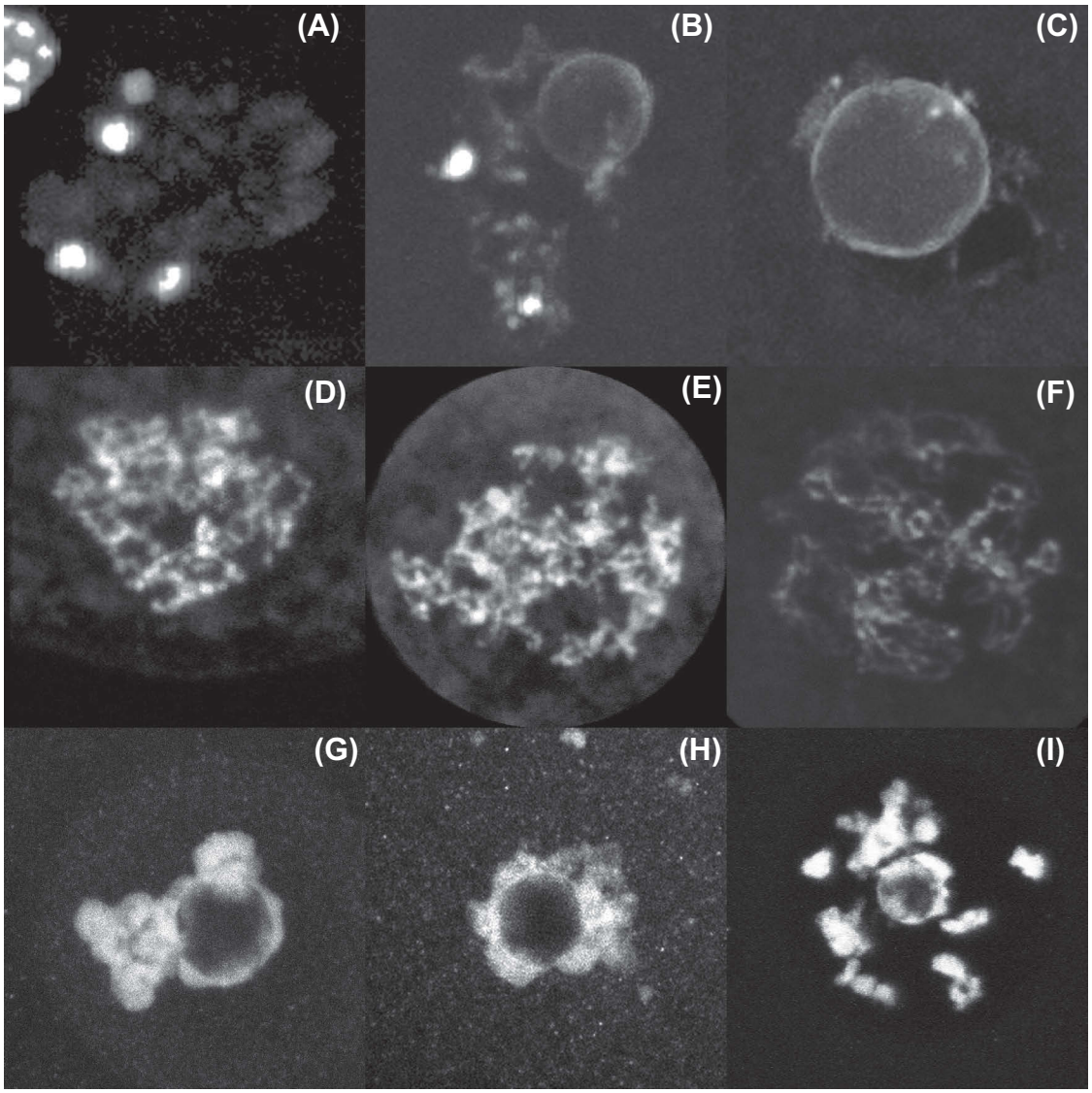

FIGURE 2.5 High-resolution fluorescence microscopy of patterns of chromatin organization in the GVs of immature oocytes from the mouse (A–C), Bovine (D–F), and human (G–I). It should be noted that all images were collected from fixed oocytes and chromatin is labeled with Hoechst dyes that detect only adenosine-thymidine–rich domains of chromatin; where apparent, unstained spherical structures represent the nucleolus. In rodents, growing oocytes exhibit a diffuse pattern of chromatin (A) that progressively heterochromatizes (B) and envelops the surface of the nucleolus (C). The GVs of most domesticated species tend to display fibrillar patterns of chromatin (D and E) that at the end of the growth phase assume a characteristic bivalent form typical of diplotene (F). Human oocytes that have been retrieved from ART cycles, and are presumed to have completed their growth, to exhibit condensed patterns of chromatin that encircle the nucleolus (G and H, karyosphere), or to exhibit properties of having entered diakinesis (I).

Cytoplasmic Composition of Mammalian Oocytes

Most initial characterizations of the composition of oocyte cytoplasm, known as ooplasm, drew upon the resolving power of the electron microscope and the use of histochemistry to gain insight into the enzymatic activities that distinguished oocytes from surrounding granulosa cells[72,73]. Many studies of this kind focused on the changes in distribution and structure of a particular organelle through the program of oogenesis and into early embryogenesis.[6,7] Studies of this kind laid a general foundation by identifying major organelles such as mitochondria, and documenting extensive hyperplasia of this organelle with changing functionality along the course of oogenesis. Besides documentation of the expected accumulation of ribosomes, endoplasmic reticulum, Golgi, and lysosomes, both new organelles such as annulate lamellae[74] and old favorites like Balbiani's body[43] were brought into the realm of oocyte biology (Figure 2.1). In keeping with the notion that oocyte ultrastructure

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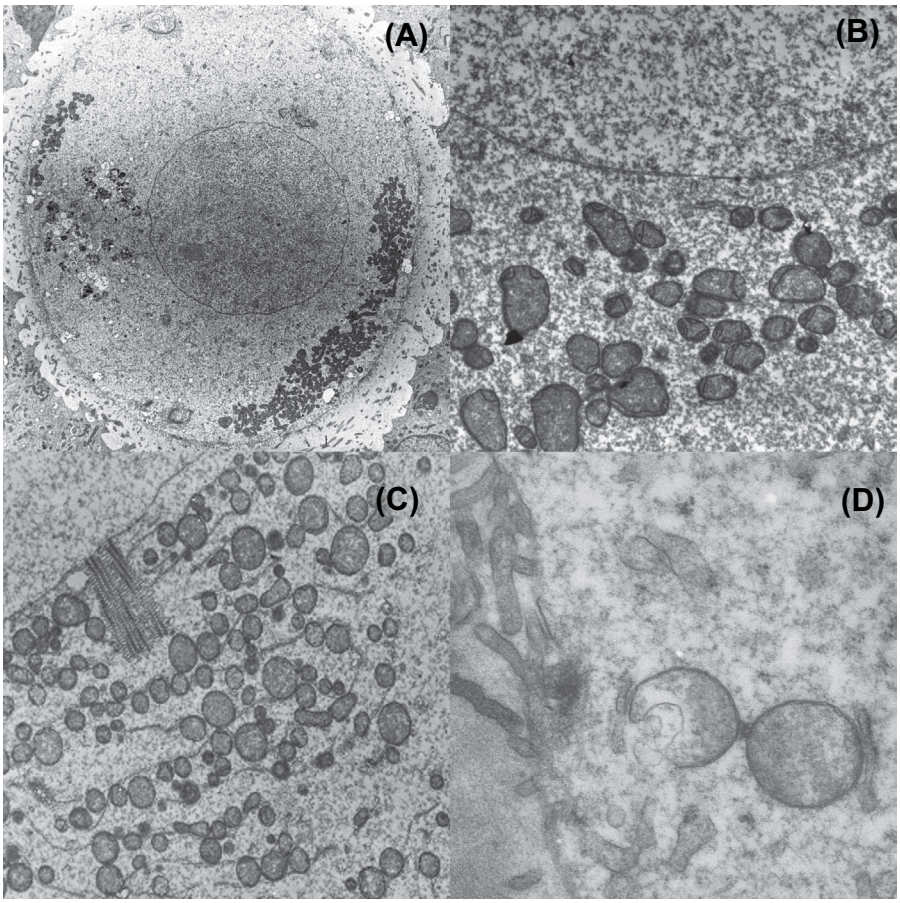

FIGURE 2.6 Ultrastructural appearance of mitochondria in resting primordial (A and C) or growing oocytes (B and D) from the primate (A–C) or mouse (D). In resting oocytes, the mitochondria tend to be collected at one pole and are either associated with the perinuclear Balbiani body or in a subcortical location (A). Independent of their position, there is great variation in the number and structure of mitochondria, but in general oocyte mitochondria are known to have few or no cristae, tend to be found in clusters of varying sizes, and lack electron-dense granules in the matrix (B). Not infrequently, oocyte mitochondria are associated with cisterns of smooth endoplasmic reticulum (C), and as the oocyte nears completion, many assume a cortical position with dumbbell configurations that may represent fission or fusion (D).

continues to play a role in our understanding of oogenesis, an overview of organelles is provided below and is followed by a discussion of the other major part of cytoplasm, that of the oocyte cytoskeleton formerly referred to as ground substance.

Without question, oocyte mitochondria continue to be a well-studied but poorly understood organelle (Figure 2.6). As noted above, what makes the oocyte mitochondria truly unique among organelles in general is their direct contribution to the immediate metabolic demands of the embryo and as providers of the mitochondrial genome to the next generation. From a structural point of view, the starting point for maternal inheritance begins with the formation of the primordial oocyte and the population of founder mitochondria from the primordial germ cells. In primordial oocytes, a mitochondrial cloud, or aggregate is typically situated in a perinuclear location in association with Balbiani's body (Figure 2.1). Oocyte mitochondria in arrested or growing oocytes are unusual in bearing relatively few cristae, usually thought to indicate a low level of metabolic activity. Once oocyte growth commences, expansion of the mitochondrial pool steadily occurs with amplified populations assuming a random (Figure 2.6 (B and D)) or highly polarized (Figure 2.6(A and C)) distribution that varies between species. Independent of their overall disposition, examples of mitochondria undergoing fission, or possibly fusion, are numerous (Figure 2.6(D)). How variations in number and location influence oocyte quality and development will be addressed in greater detail in a later section.

The themes of expansion and relocation noted above for mitochondria are similarly valid for the topic of the Golgi lysosome system (Figure 2.7). Being the control center for the biosynthesis of proteins, endosomes, cortical granules, and lysosomes, the Golgi complex of the oocyte has understandably been the subject of much attention given the proliferation and cortical dispersion observed during oogenesis. The primary functions of the Golgi that commence upon follicle activation are best characterized as secretory. The major secretory products at this stage include the glycoproteins that will comprise the zona pellucida, the membrane proteins designated for insertion into an expanding plasma membrane, and the proteins that must be synthesized, processed, and incorporated into lysosomes. With the availability of antibody reagents to identify these products, it has become apparent that a diverse distribution of Golgi-associated precursor molecules are evident in growing oocytes including components of the zona pellucida

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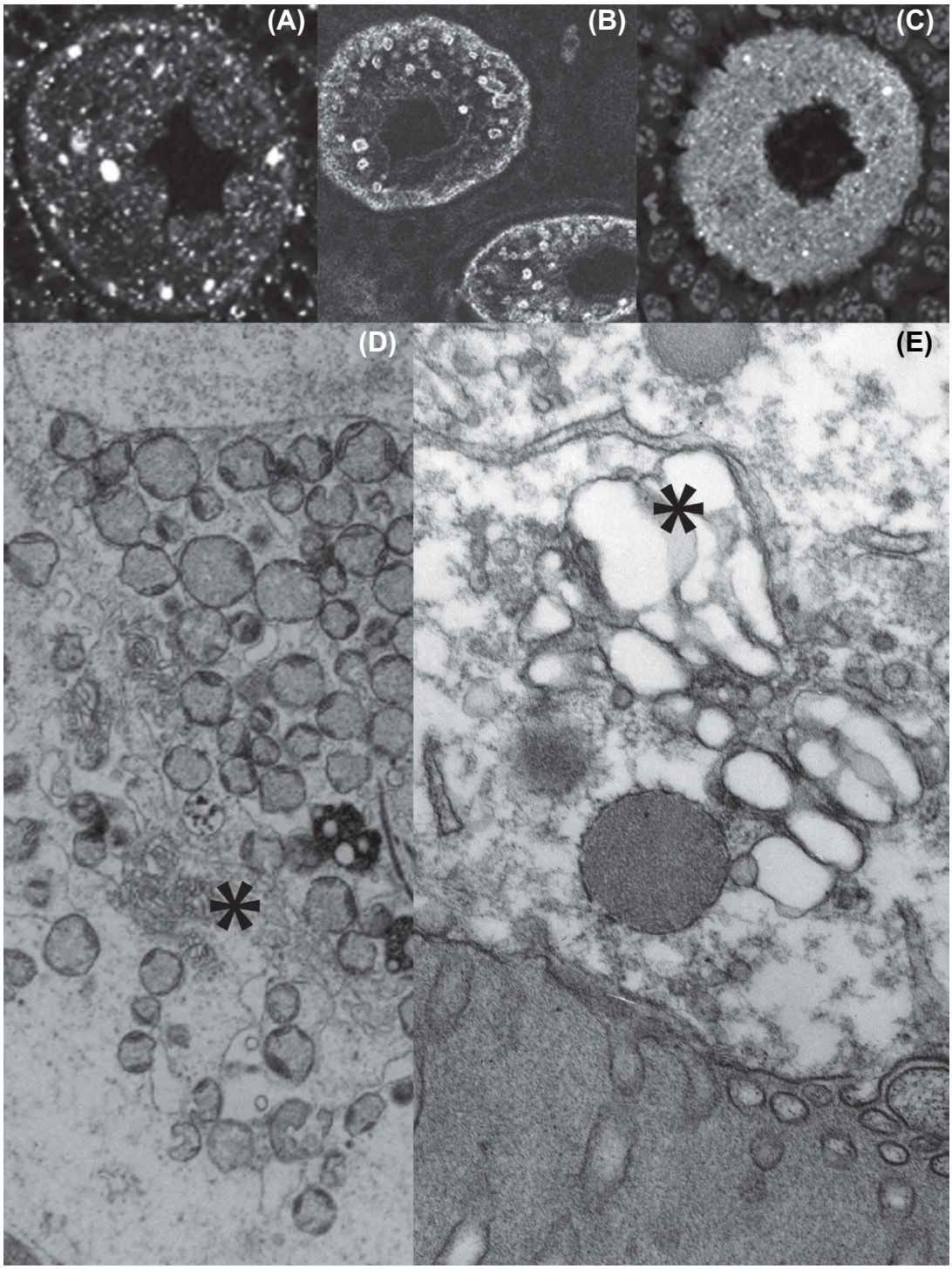

FIGURE 2.7 Disposition and structure of the Golgi lysosome system in mammalian oocytes of the mouse (A–C), human (D), and macaque (E). (A) illustrates the lysosome pattern revealed after staining with the lysosome membrane–specific antibody LAMP-2; small particles represent primary lysosomes, whereas large clumps are indicative of the multiple Golgi complexes found during the growth phase of oogenesis. A clear indication of the multiple Golgi complexes is shown in (B), where immunofluorescence microscopy has been used to label the zona pellucida protein ZP-1; in (C), a similar labeling strategy was used to detect the oocyte-specific factor BMP-15.

(Figure 2.7(A and B)), and lysosomes (Figure 2.7(C)). The spectrum of these patterns of distribution is consistent with earlier findings reporting the cytoplasmic and subcortical Golgi complexes that would be expected to be processing zona proteins.[9] Examples of this repositioning are illustrated in Figure 2.7. The Golgi disperses into multiple complexes that come to lie just beneath the oolemma (Figure 2.7(E)). The subcortical Golgi

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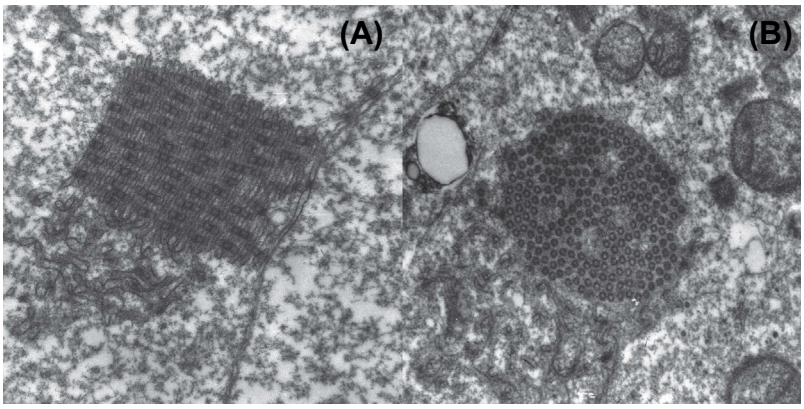

FIGURE 2.8 Ultrastructural appearance of annulate lamellae in a chimpanzee primary oocyte seen in cross section (A) and tangential (B) views. (A) shows the continuity of nuclear pores from the nuclear envelope into a stack of annulate lamellae. In (B), a tangential section through the surface of a stack of annulate lamellae revealing the closely packed organization of nuclear pore complexes.

complexes are actively engaged in the production of cortical granules, as first demonstrated by Nicosia.[8] The mechanisms underlying the dispersion and repositioning of the Golgi complex during oocyte growth remain poorly understood, but this dynamic and widely conserved feature of the mammalian oocyte emphasizes again the importance of positional information while a variety of products are being assembled whose functions will be realized in distinct ooplasmic regions. How such a division of labor is achieved will await further study using live cell imaging techniques.

An additional ultrastructural feature of mammalian oocytes that appears to be common to most mammals excluding rodents is that of annulate lamellae (Figure 2.8.) The distinct appearance of this historically recognized and elaborately designed organelle prompted earlier investigators to suggest that it provided stores of nuclear pore complexes that would be utilized after fertilization for the assembly of blastomere nuclear envelopes.[5] A detailed analysis of primordial oocytes by Hertig in the 1960s provided some support for this idea by documenting the physical contiguity of nuclear pores between the GV nuclear envelope and the adjoining annulate lamellae.[43] Given the subsequent ultrastructural analyses of human oocytes and embryos and the plausible role for annulate lamellae in embryonic development, it is surprising that this oft-neglected and yet prominent component of ooplasm has not received discriminating attention.

Largely ignored in the earlier descriptive ultrastructural work was the topic of cytoplasmic ground substance, classically defined as the highly cross-linked protein matrix within which organelles reside. The main reason for this omission of what has become an intensely studied aspect of oocyte biology was the failure to preserve components of the cytoskeleton using electron microscopy protocols that invariably damage all but the most stable of these structures, such as spindle microtubules, axonemes, and cytoplasmic lattices, a subject that will be dealt with in much greater detail below. Nevertheless, several salient and provocative features of the oocyte cytoskeleton have now come under scrutiny using more contemporary technologies, and are reviewed here.

Of central importance to the theme of maternal inheritance is the topic of the centrosome, a subject engrained in the study of oocyte biology that has fascinated developmental and cell biologists since the early twentieth century.[3,5] Centrosomes are classically recognized at the major microtubule organizing center (MTOC) in all cells displaying the prominent and telltale feature of asters due to a radiating pattern of microtubules. It was in the 1980s that Maro and colleagues first noticed these MTOCs within the cytoplasm of mouse oocytes and proposed that besides their involvement in the formation of the meiotic spindle, perhaps they represented a maternal supply of MTOCs that would be used to support the mitotic cell divisions in the early embryo.[75] As in other cell types, it was subsequently shown that rodent oocyte MTOCs contained the signature protein components gamma tubulin (TUBG1, TUBG2)[76] and pericentrin[77,78] and undergo cell cycle–specific alterations during meiotic progression from diplotene GV stage to metaphase two.[79] The biochemical characterization and essential role played by these structures in mouse oocyte and embryo cell cycle control have been thoroughly reviewed and reinforce the original suggestion that oocyte MTOCs are indeed a major maternal determinant of embryo quality.[80,81] But this more recent history of maternal MTOCs raised several questions in light of the seminal contributions of Szollosi and colleagues published in 1972.[82] This pioneering electron microscopic study documented a major change in MTOC composition that took place as mitotic oogonia transitioned into meiotic prophase in the mouse. Whereas classical centrosomes in somatic cells bear true centrioles within the pericentriolar matrix, Szollosi and coworkers documented that while oogonial centrosomes were indeed centriole-containing, the centrosomes within diplotene-arrested primordial and primary oocytes lack detectable centrioles. These findings, confirmed by others, raised the interesting question of how and when are centriolar centrosomes regenerated in the early embryo? While much debate has ensued

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over the origin and continuity of centrioles and centrosomes during oogenesis and embryogenesis, Szollosi, in collaboration with Ozil, went on to show that centriolar centrosomes could be generated de novo within rabbit embryos produced by parthenogenetic activation.[83] An excellent review on this topic has been authored by Sathananthan and colleagues[84] that describes the history of this problem[85] and the implications it poses for the treatment of human infertility as originally uncovered by Schatten and colleagues.[86] Together with more recent advances documenting the mechanisms by which mitochondrial inheritance impacts human oocyte quality and embryo development,[87,88] continuing efforts are indicated to explore the inheritance of centrosomes and other oocyte components whose direct contributions to the embryo impact the health and well-being of children produced by human ARTs.

Besides the microtubule cytoskeleton, two other classes of cytoskeleton proteins play important and somewhat distinct roles in the biology of the mammalian oocyte. It is worthwhile at this juncture to keep in mind that the transcriptional, translational, and post-transcriptional control of cytoskeletal proteins during oogenesis,[15,16] if compromised, could impose risks on the embryo where priorities are assigned to supporting key cell cycle and morphogenetic events necessary at later times. These classes of filaments include the contractile protein actin, and its associated motility-producing proteins such as myosin, and the proteins that comprise intermediate filaments which are thought to primarily confer rigidity within and between cells. The subject of actin filaments has been mostly studied in the context of meiotic maturation when dramatic remodeling of the actin cytoskeleton mediates meiotic spindle positioning, polarity, and polar body extrusion, and will be discussed in the section Clinical Parameters of Oocyte Biology.

Intermediate filaments in hamster oocytes have been characterized with respect to their assembly during oogenesis and utilization in the embryo with regard to establishment of junctional complexes required for compaction and blastocyst formation.[89] Subsequent to this work on the hamster, and following the earlier studies in the mouse,[7] the Capco[90–92] and Coonrod[93–96] laboratories have made major contributions to our understanding of the role of intermediate filaments in these rodent species.

This work has buttressed the notion that in order for the growing oocyte to maintain molecular order and physical stability, the deposition and arrangement of protein scaffolds composed of intermediate filaments is a necessary prerequisite that mediates a variety of biochemical processes. Among these are organelle positioning, the ordering of signal transduction systems that participate in the maintenance of meiotic arrest and the resumption of meiosis during maturation, and the provision of a stable matrix upon which meiotic spindle assembly can take place.[93] A slightly different but equally important tack has been taken in studies on the mouse oocyte with respect to the crystalline lattices that populate the cytoplasm of rodent species.

Here the emphasis and implications regarding the lattices have delved into the prospects that these stable components of the cytoskeleton undergo compositional variations that mediate processing of macromolecular complexes and/or positioning and protection of molecules destined for use in the early embryo. For example, PADI6 has been characterized as a lattice component that mediates the allocation of ribosomal constituents and ensures their availability for timely control of translation in the embryo.[94] In elaborating upon the role of PADI6, further studies have implicated the lattices in positioning of MATER, a maternal effect gene, as well as the control of microtubule organization and organelle positioning.[93–96] Taken together, this line of work further establishes the role of cytoskeletal stability in maintaining and regulating spatial organization required for the successful completion of oogenesis and the enactment of embryogenesis.

Membrane Specializations and Extracellular Coats

In achieving some semblance of order in such a large cell that is undergoing volume expansion, it comes as no surprise that physical forces generated from within and without the oocyte would assume pivotal roles in the design and shaping of the mammalian oocyte. As noted above, and as will be further discussed, the cytoskeleton provides an internal scaffold upon which to maintain shape, effect positioning of organelles, and stabilize the inner surface of the oolemma. It would be predicted that without balancing forces generated internally with those outside the oocyte, growth to extremely large proportions would result in the absence of externally imposed constraints. With this fundamental principle in mind, as evidenced by the invariant size and volume properties displayed by mammalian oocytes in each species examined, the hallmarks of the extracellular matrix and attendant modifications in the oolemma are next taken into consideration (Figure 2.9).

The most obvious external manifestation common to the oocytes of mammals is the zona pellucida (Figures 2.1, 2.3, and 2.9). The zona pellucida (ZP) is a specialized extracellular matrix serving a number of functions. Constructed from glycoproteins synthesized, processed and secreted from the oocyte (ZP1, 2, 3), this structure has long been recognized to serve as a protective barrier to the oocyte while within the follicle and following release at ovulation. Embedded within the substance of the ZP are two kinds of cellular projections originating from the granulosa cells externally and from the oolemma internally (Figure 2.9(A)).

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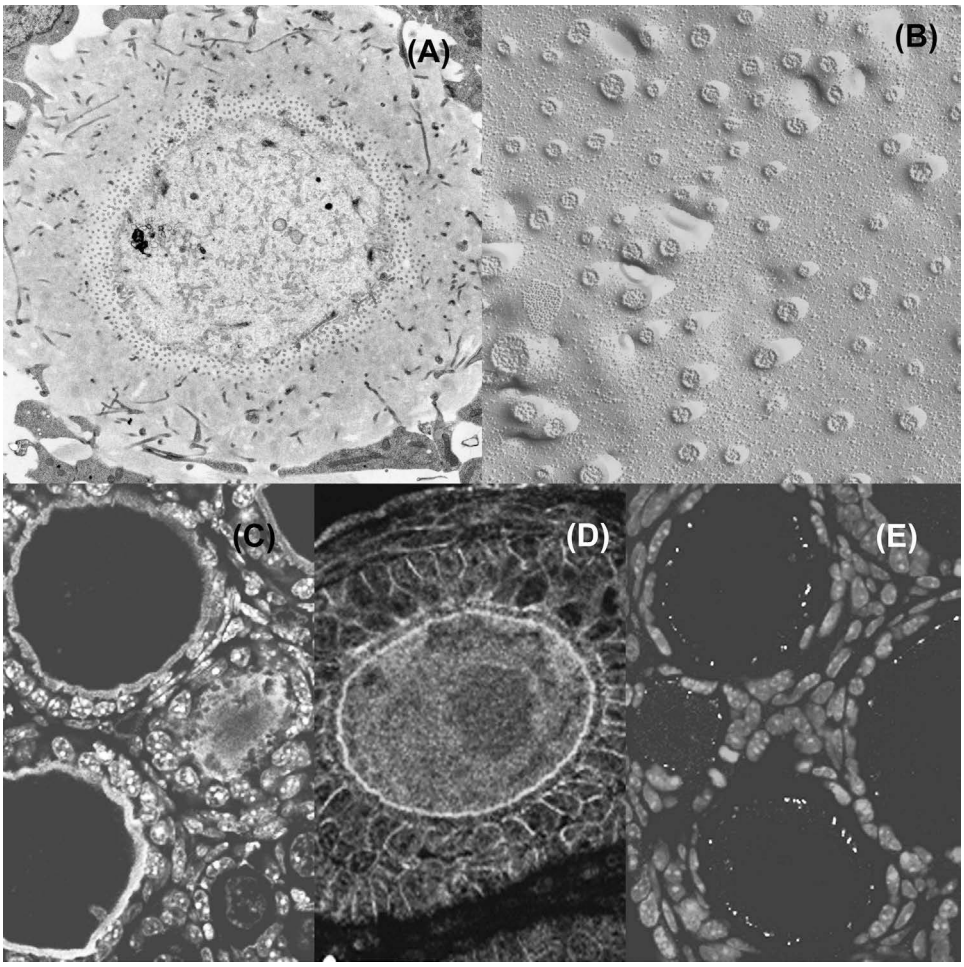

FIGURE 2.9 Membrane and extracellular coats in mammalian ova. (A) is an electron micrograph of a tangential section through the cortex of a mouse oocyte; note the extent of the zona pellucida and the penetration of numerous TZPs. (B) is a freeze-fracture electron micrograph through the oolemma of a rabbit oocyte showing the cleaved bases of microvilli and a solitary gap junction. C, D, and E illustrate disposition of ZP3 (C), beta-catenin (D), and CXN 37 (E) detected immunocytochemically on sections of adult mouse ovary.

The somatic cell projections are known as transzonal processes (TZPs) and will be dealt with in greater detail below. As the oocyte develops, the oolemma acquires a microvillus character that remains homogeneous until the time of oocyte maturation when the entire cortex becomes remodeled. As shown in Figure 2.9(B), a freeze-fracture image through the oolemma, the lawn of microvilli extend into and are anchored to the ZP, affording structural stability to the oocyte cortex. Additional membrane specializations reinforce the oocyte cortex as the process of growth comes to an end. These include the localization of the cell adhesion protein beta-catenin (Figure 2.9(D)) and the appearance of secretory granules containing zona proteins (Figure 2.9(C)) that gradually accumulate in the cortex as the oocyte enlarges. The most well-studied of the secretory vesicles is the cortical granule,[8] which along with the role of the ZP in mediating sperm–egg interactions[97] (see Chapter 4), demonstrate that the maturation of the oocyte cortex is designed, and the specializations contained therein, to ensure formation of the conceptus.

Cortical granules have long been understood to establish the block to polyspermy due to their secretion upon fertilization. But the pathway to assuring exocytosis is regulated in discrete steps, at least as shown in studies on the mouse oocyte. For example, Ducibella and colleagues showed that cortical granules and calcium sequestering vesicles are accumulated in full-grown oocytes and confer fusion competence to cortical granules that is independent of the distance between cortical granules and the oolemma.[98] Physical approximation of cortical granules and fusion competence were attained during the course of meiotic maturation, implying a major restructuring process that demarcates the boundary between the prematurative and maturative stages of oogenesis.[99] But the degree of cortical remodeling is significant for other reasons besides fertilization preparedness. As the meiotic spindle assumes its cortical location, a cortical granule free domain appears over the spindle, evidencing the first obvious signs of cell polarity. In the mouse, the mechanism underlying clearance of cortical granules from this region has been shown to be driven by chromatin.[100]

Several additional membrane specializations have been recognized as central features of the mammalian oocyte that further illustrate the mosaic nature of the oolemma. These include cholesterol-rich lipid rafts that are inherited by the embryo[101] and participate in

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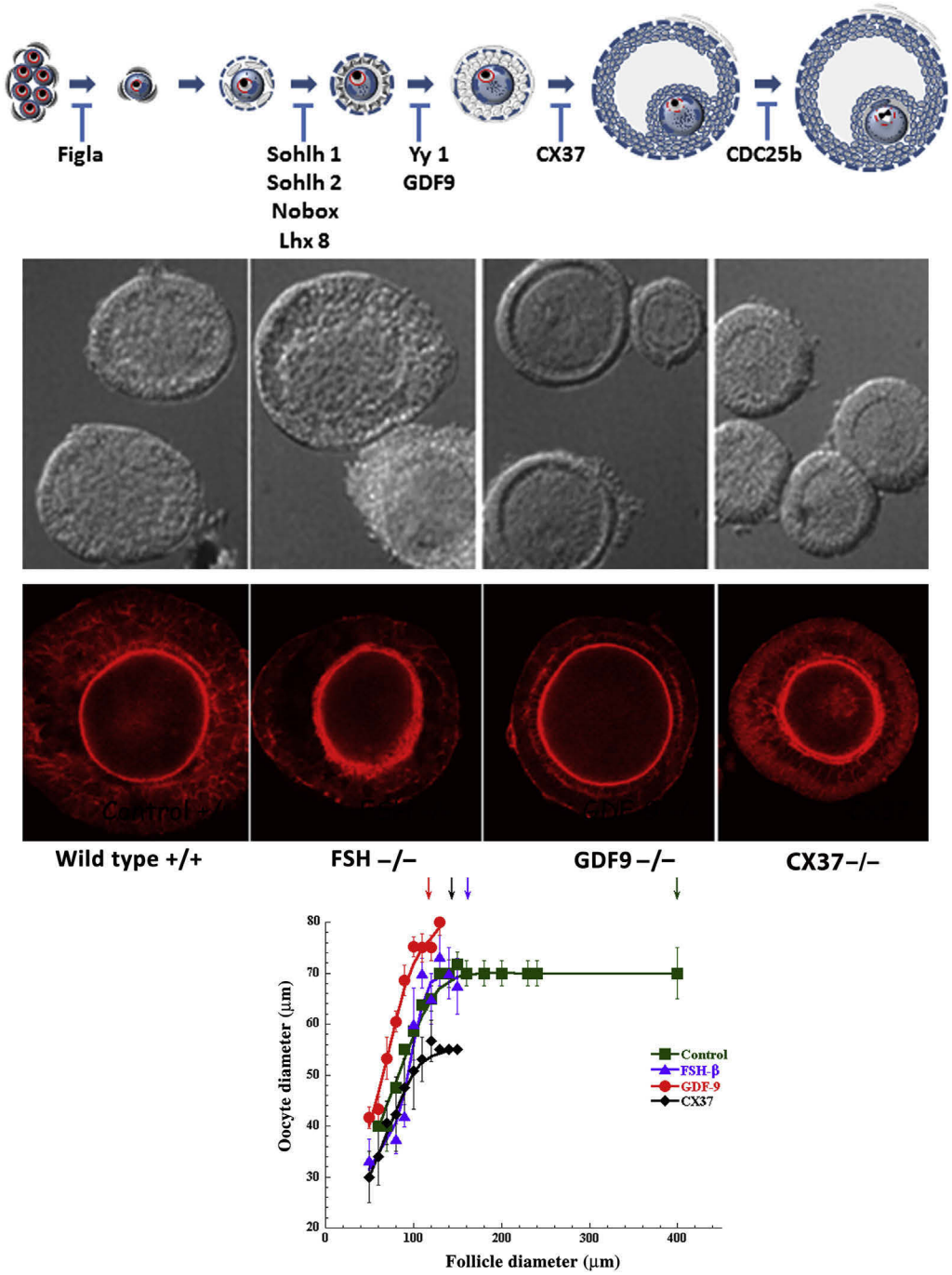

FIGURE 2.10 **Mouse knockout phenotypes with distinct defects during the growth phase of oogenesis.** This figure is reproduced in color in the color plate section. Schematic at top shows effects of targeted gene deletion on stage of follicle development. Images show phenotype of wild type (left), GDF9, FSH beta, and CXN 37 in sequence that in the top panel show living follicles, and in the lower panel, the organization of granulosa cell–oocyte interface. Graph (bottom) plots the relationship between follicle and oocyte growth for the same four groups.

the process of fertilization in the mouse.[102] Moreover, a variety of junctional specializations have been identified in the oolemma. It is thought that connexin-based gap junctions and adhesion junctions play a central role in communication and signaling between the oocyte and surrounding granulosa cells.[103] Of these, GJA37 has been shown to be the predominant connexin in mouse oocytes, but there is growing evidence that multiple connexin types exist in various mammals[104] with full classification awaiting the use of oolemmal proteomics.[105]

In conclusion, the compartmentalization and cellular specializations first suggested by ultrastructural

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studies of mammalian oocytes have been reinforced and extended in recent years to provide a more complete picture owing to the adoption of new technologies. The landscape that emerges is one that emphasizes the role of stage-specific modifications in oocyte gene expression that will serve the full-grown oocyte as it engages in and proceeds through meiotic maturation, fertilization, and the cleavage divisions of embryogenesis (Figure 2.10). These advances pale by comparison to the new knowledge accumulated over the past 20 years regarding the molecular biology of the mammalian oocyte during the growth phase of oogenesis.

THE GROWTH PHASE OF OOGENESIS

Patterns of Gene Expression

If one major theme fairly summarizes what has been nearly two decades of research on gene expression in mammalian oocytes, it is that feedback loops precisely regulate the pattern of gene expression toward the end of integrating the oocyte with the follicle, ensuring competencies that the oocyte must acquire, and depositing a genetic framework in time and space that is essential for the initiation and progress of embryonic development (Figure 2.10). While gleaned mostly from studies on the mouse,[28] these thematic issues find practical application in deepening our understanding of human oocyte biology at a level materially greater than what could have been imagined before the introduction of human ARTs. The pioneering efforts of Wassarman necessarily emphasized expression of abundant genes that were accessible with the technology available at that time.[106] Fast forward to the era of genomics and the exploitation of the mouse genome with transgenic approaches and, as illustrated in Figure 2.10 (and discussed in Chapter 21), we have witnessed the revelation of genetic networks driving the earliest stages of oogenesis as entry into meiosis and the growth phase are identified and characterized at the level of specific genes with demonstrable functions.[107,108]

Probing the upstream control of the birth of mammalian oocytes has uncovered first principles of gene expression in mammalian oocytes. Most significant among these was the discovery of the *Nobox* gene as a central regulator of follicle assembly and prime mover for genes with exclusive expression in the female germ line.[109] *Nobox* emerged as a transcription factor regulating the promoters of *Gdf9* and *Pouf51* based on this work, and set the stage for establishing a launching pad required for both the survival and eventual differentiation of the oocyte. Key players in this definitive transcriptional network include the transcription factors *Sohlh1*,[110,111] *Sohlh2*,[112] and *Lhx8*.[110] Excellent reviews on this subject have appeared[107,108] placing into context the linkage of these genes with subsequent patterns of transcription that had already been associated with advancing stages of oogenesis.

Among the downstream genes generally again bearing an oocyte-specific character of expression are *Bmp15*[113] and *Gdf9*.[114] Whereas *Gdf9* knockout mice exhibit a characteristic early block at the primary follicle stage with signs of aberrant oocyte growth, the phenotype of the *Bmp15* knockout mouse exhibits a temporally distinct later defect in oogenesis with more complex gene dosage requirements. The basic helix–loop–helix transcription factor *Figla* is another oocyte specific gene identified by the Dean laboratory that acts uniquely during the initial stages of oogenesis to promote oocyte gene expression but suppress gene expression in the male germline.[115,116] Thus, transgenic manipulations in the mouse have collectively provided a roadmap of interactions between oocyte-specific genes essential for the birth, survival, and earliest stages of oocyte differentiation (Figure 2.10). The more complete listing of oocyte-specific genes and their expression profiles have also been obtained through the use of transcriptomic approaches.

Using the strategy of single cell RT-PCR, individual mouse oocytes recovered follicles ranging from primordial to antral stages and transcript abundance was assessed semi-quantitatively with respect to the oocyte-specific genes *Nobox, Oct4, Bmp15, Gdf9, Oogenesin1,* and *Oogenesin*.[117] While transcript abundance for all of these genes exhibited a progressive increase during what would be equivalent to the growth phase of oogenesis in untreated mice, some subtle changes in expression were noted if oocytes were collected from animals receiving exogenous stimulation. Not surprisingly, upon completion of the growth phase, transcript numbers decreased progressively up to the metaphase two stage, with values very similar to those obtained for the primordial oocytes in both analyzed conditions. The transition from growth to pre-maturation was accompanied by enhanced expression of *Nobox* and *Oogenesin2* genes, whereas *Oogenesin1* was diminished. It was concluded that coordinate regulation of the *N*obox and *Oct4* genes distinguished oocytes with high developmental potential from those of lesser competence; the validity of these observations was strengthened by the corresponding demonstration of protein expression that is typically absent from transcriptomics studies of this kind. It is relevant to point out that associations between patterns of gene expression and developmental competence often invoke the use of the NSN and SN chromatin configurations as competence biomarkers at the time of transcriptional silencing. But the validity of this association remains questionable, as noted above, even though potential candidates for regulating gene

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switching, such as the TATA-binding protein have been implicated at specific stages of oogenesis and embryogenesis in the mouse.[118]

Central to any discussion that attempts to link differential patterns of gene expression in the oocyte to the growth, pre-maturation, and maturation stages of oogenesis and the later events of fertilization and embryogenesis is the state of knowledge regarding the specific products of transcription and their impending translational utilization and/or degradation. Some oocyte gene products are assigned functionality during oocyte differentiation whereas others are clearly reserved for use in the embryo. The latter, as mentioned previously, comprise the family of so-called maternal effect genes, of which *Spindlin* is one of the first to be identified.[119] But for the vast majority of transcripts made in the oocyte that define the female germ line in its "pre-totipotent" state, abundance and selectivity in processing and stability remain the *raison d'etre* for their existence.

One of the first in-depth transcriptomic studies took advantage of the ability to not only separate specific stages of mouse oocyte development from one another but also cultured oocytes isolated at one particular stage of development through to a later stage.[120] These and subsequent studies from the Eppig and Schultz laboratories revealed somewhat surprising trends in the overall complexity of gene expression in addition to more predictable features consistent with the eventual transcriptional silencing known to occur once the growth phase of oogenesis had been completed. Among these were the abundance and variety of transcripts evident in the primordial and primary oocyte and timely appearance of competency genes associated with the cell cycle and cytoskeleton alterations effected at later stages of oogenesis. When superimposed with results obtained from the same group of investigators for patterns of transcript degradation,[121] a complex scenario emerges for the regulation of gene expression that begs the overarching question: How is such a delicate balance between transcription, translation, and degradation of mRNAs accomplished with temporal and spatial precision in mammalian oocytes destined for life after fertilization? And a necessary corollary with clinical implications: To what extent are artificial conditions imposed by exogenous gonadotropin stimulation or in vitro manipulations directly or indirectly perturbing gene expression so as to compromise developmental potential prior to or following fertilization?

Evidence cited above for the mouse[117,120] has been confirmed and extended in other mammalian species,[122] with the resounding conclusion reached that indeed manipulations of the ovarian environment in which oogenesis must proceed do have a long and lasting influence on oocyte and embryo quality parameters that likely underscore some of the observed deficiencies apparent in offspring generated by ARTs.[25,41,42] One of the principal targets for explaining the etiology of such conditions has revolved around the discovery and subsequent investigation of genomic imprinting within the germline.

Classically recognized by the allele and gender-specific hypermethylation of promoter elements of certain genes, the critical role subserved by the family of dimethyl transferases expressed during oogenesis on embryonic development first came to light in the studies of Howell and colleagues in 2001.[41] With this formative finding, and the studies that followed, the general patterns of demethylation and remethylation have been well-established in the mouse, as summarized in Figure 2.11.

At the heart of how genomic imprinting effects oogenesis and later embryogenesis is the demonstration that the housekeeping methyltransferases responsible for de novo cytosine methylation in the oocyte genome (*Dnmt3a, Dnmt3b,* and *Dnmt3L*) are expressed in a temporally specific pattern during the growth phase of oogenesis, and the translation product for one of these, known as *Dnmt1o*, is stored in the cortex of the mouse oocyte until the eight-cell stage of development when it is deployed for the remethylation of zygotic genes.[42,123,124] This remarkable case of spatial and temporal control of gene expression during the course of an early stage of oogenesis exemplifies the role of maternal effect genes on development

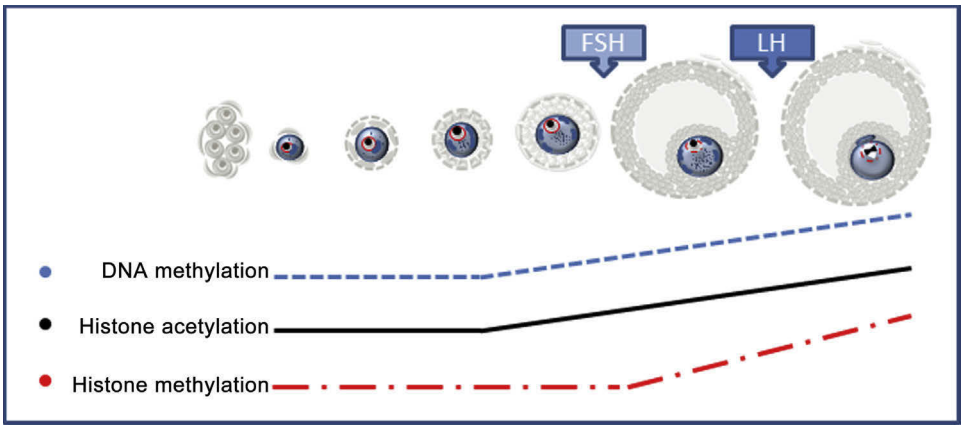

FIGURE 2.11 Epigenetic modifications of chromatin during the course of oogenesis. This figure is reproduced in color in the color plate section. Genes subject to imprinting show progressive signs of DNA methylation beginning during the growth phase of oogenesis and ending at some point in the preovulatory follicle. Histone acetylation in developing oocytes follows a similar time course as DNA methylation, whereas histone methylation is reported to occur following the growth phase. Data are based primarily on studies in the mouse.

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of the embryo. It places special significance on the link between oogenesis and embryogenesis in demonstrating a separation of function between the expression and utilization of *Dnmt* genes designated either for gametogenesis or development and proffers a plausible mechanism for how the maternal effect genes play out their accorded functions following fertilization. The list of maternal effect genes continues to grow (Table 2.2) and includes gene products likely to play similar roles in genetic or epigenetic processes conferring embryo quality.[27] In this category, *Tubg1*,[125] *Stella*,[126] *BRG1*[128], and *Basonuclin*[129] have been investigated and the recent studies on *Ring 1* and *Rnf2* set the novel precedent for components of the Polycomb-repressive complex 1 (PRC1) regulating chromatin remodeling during both oogenesis and embryogenesis.[127]

TABLE 2.2 Maternal Effects Genes Identified in Mammalian Oocytes

Name	Protein Function	Embryonic Defect	References
Mater (NLRP5)	Transcription enhancer	Two-cell block	26
Tle6	Transcription enhancer	Two-cell block	27
Zar1	Unknown	One-cell block	27
Padi6	Peptidyl arginine deiminase	Two-cell block	93,96
Filia	Unknown	Cell cycle delay	27
Floped (0oep)	Unknown	Cell cycle delay, abnormal cleavage	27
Tubg1	Microtubule nucleation	Blastocyst block	125
Dnmt1o	DNA methyltransferase one	Compaction block	41,124
Stella (Dppa3)	Methylation maintenance	Compaction block	126
Npm2	Nucleoplasmin two chromatin remodeling	Two-cell block	69
Ring 1, Rnf2	Transcriptional repression	Two-cell block	127
Spindlin	Unknown	ZGA	119
BRG1	Unknown	ZGA	128
Basonuclin	Nucleolar organizer	Two-cell block	129
TBP-2	TATA binding protein	Unknown	118

Abbreviations: MATER, maternal antigen that embryos require; DNMT1O, oocyte specific isoform of dimethyl transferase; ZGA, zygotic gene activation.

From a functional perspective, there is some indication that the products of some maternal effect genes may contribute to the loss of genetic stability that is associated with maternal aging in mouse models.[130]

And finally, for the sake of thoroughness, it should be emphasized that the relatively recent discoveries of small interfering (siRNAs), micro (miRNAs), and long-noncoding (lncRNAs) RNAs (see Chapter 52) will add a level of complexity to our understanding of gene expression in the mammalian oocyte that is only beginning to be unraveled.[131]

Regulation of Protein Synthesis

Just as we have seen for the expression and utilization of RNAs, so, too, the products of translation follow a temporal pattern that coincides with the major transitional milestones of oogenesis in mammals. These have been referred to as translational cascades extending well beyond the formal purview of oogenesis, given the inescapable contributions the oocyte makes to the embryo, a trait that is widely conserved among metazoans but adjusted to meet the demands of each species depending on the reproductive strategies displayed.[132] One of the most dramatic modifications, splendidly illustrated in the case of the oocyte, is the divergence in the processing of mRNAs for purposes of translating constitutive proteins, like those comprising the cytoskeleton or macromolecular entities such as organelles, from those proteins whose function is restricted to specific stages of oogenesis.[15–18] What has made it possible to distinguish between protein synthesis of the constitutive variety compared to that of a more regulative kind is the introduction of siRNA technology in conjunction with antibody neutralization and microinjection, and the attendant identification of oocyte proteins with limited lifespans.

The most tractable category of proteins studied in this way is those involved with cell cycle regulation. As mentioned earlier, developing oocytes acquire a series of competencies, the manifestation of which is generally realized by removing the oocyte from its in vivo context and assaying a change in behavior under in vitro conditions (Table 2.1). This experimental paradigm has been used to establish meiotic,[51] cortical granule fusion,[98] and embryonic competencies.[128] The expression of meiotic competence, manifest by the breakdown of the GV and progression to metaphase of meiosis one, is a clear example of a G2 to M cell cycle transition. Among one of the first proteins in mammalian oocytes whose mechanism of translational regulation was well-studied is cyclin B, the regulatory subunit that drives cell cycle progression from G2 into M phase. The timely translation of cyclin B mRNA is now known to require modifications at the 3′ untranslated region that control interaction of the

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cytoplasmic polyadenylation binding element (CPEB) necessary to initiate readout.[133] Coordinating CPEB phosphorylation, a requirement for translation initiation, is itself modulated by cell cycle status, thus ensuring synthesis of a burst of cyclin B to sustain M-phase progression through all of meiosis one and up to metaphase two arrest, itself a cell cycle arrest condition dictated by the c-mos proto-oncogene. In fact, many of the key cell cycle transitions exhibited in mouse oocytes appear to be mediated by selective translation of cell cycle regulatory proteins whose mRNAs are synthesized and stored at times preceding their actual utilization.[134] This is not likely to be the case in other mammals.

That protein synthesis is required for the G2 to M transition in nonmurine oocytes was first recognized in several species of primates,[12,135] along with the demonstration by Osborn and Moor[136] that de novo gene expression was an absolute requirement for germinal vesicle breakdown (GVBD), the morphological indication that cell cycle re-entry has indeed taken place. Thus, unlike the spontaneous progression from G2 to metaphase of meiosis one observed in the mouse, most other mammals require de novo protein synthesis and active transcription to reinitiate the meiotic cell cycle. That there is much more behind the various means of translational regulation in mammalian oocytes besides interspecies variations is also evidenced by differences in protein phosphorylation that discriminate between nuclear and cytoplasmic maturation[137] and the discovery of a new class of RNA granules seemingly acting as cyclin B mRNA storage sites that must be disassembled as a prerequisite for translation.[138]

As deeper databases of the transcriptome and proteome[139] become available, new levels of complexity for the control of translation in mammalian oocytes will become evident and redirect the current focus of attention. In the meantime, caution continues to be warranted in this area of oocyte biology. Eichenlaub-Ritter and Peschke have called attention to variations in translational efficiency in in vivo– and in vitro–produced oocytes that may underlie the loss of oocyte quality observed under culture conditions as well as implicated translational control in the etiology of oocyte aging and aneuploidy.[140,141]

Protein Posttranslational Modifications

A rapidly moving, but still immature aspect of mammalian oocyte biology is that of protein posttranslational modifications (PTMs). Here, too, there has been a focus on the alterations in protein function that mediate the maturative stages of oogenesis involved with key cell cycle transitions,[137] but the recent work remains descriptive. The kinds of studies performed to date have relied upon the use of PTM-specific antibodies to localize changes in candidate proteins at a microscopic or biochemical level[142] and suffer from a number of limitations. Among these are the lack of reagent specificity, the paucity of material available, and the quantitative limitations imposed by nuclear magnetic resonance mass spectroscopy approaches that have become the approach of choice in studies of other cell types. Because the work on oocyte PTMs has more direct bearing on the subject of meiotic maturation, the key advances in this area will be covered in the section Maturation Phase of Oogenesis: The Final Act.

Epigenetic Modulation of the Genome

The broad definition gaining acceptance for the field of epigenetics is one that engages the genome as a modifiable construct within which changes in gene expression are attributed not to alterations in nucleotide sequence but to modifications in the structure of chromatin that not only regulate patterns of gene expression but effect long-term performance of the genome at times distal to the original modification (see Chapter 52). With this principle in mind, we have already introduced the notion of genomic imprinting, and in so doing emphasized how the expression of enzymes responsible for promoter methylation impact de novo methylation during oocyte growth and methylation of imprinted genes later in development.[42,123,124] Here, we turn our attention to the more specific matter of when these epigenetic modifications occur during the course of oogenesis and why these alterations in the oocyte genome are relevant to the zygote (Figure 2.11). As a prelude to this section, it should be noted that understanding the epigenetics of oogenesis ultimately led to the production of the first completely parthenogenetic offspring in a mammal.

In an elegant series of nuclear transplantation experiments using mouse oocytes, Kono and colleagues first established that the epigenetic marks conferring developmental competence were acquired during the growth phase of oogenesis[143] (Table 2.1). They then went on to demonstrate that the completion of oocyte growth was required for maternal imprinting[144] and direct manipulation of gender specific methylation marks overcame the restricted development previously observed in digynic parthenotes.[145] Following this accomplishment, the birth of the first viable and reproductively competent mice was reported[146] and the insights gained from these experiments on the expression of imprinted genes has since been exploited to improve the overall efficiency for producing bimaternal offspring.[147] Not to be constrained with their work on the mouse, this group is now applying their experimental stratagem to the bovine oocyte with hopes of further dissecting the pattern of genomic imprinting in other mammalian species.[148]

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In this regard, several outstanding questions remain. Are there similar temporal properties for executing methylation in the oocytes of other mammals? When one considers the relative brevity of oogenesis in the mouse (c. 20 days) compared to that of the human (c. 100 days), is the expression pattern for *DNMT*s and the storage of *DNMT1o* in the oocyte cortex a rodent attribute or one generally shared with the oocytes of other mammals? And in addition to DNA methylation, a number of histone modifications collaborate with *DNMT*s to effect the chromatin remodeling needed for successful imprinting that have yet to be fully studied. One report has appeared implicating histone methylation in the expression of meiotic and developmental in cat oocytes.[149]

Clearly, epigenetics will continue to be an important area of enquiry in the field of oocyte biology and recent work is already expanding the molecular mechanisms at play beyond those of the traditional methyltransferases given the discovery of Tet enzymes in the female germline that hydroxylate 5-methyl cytosine residues, as recently reported by the Zhang laboratory.[150]

Coordination of Oogenesis with Folliculogenesis

Research over the past decade has contributed to a deeper understanding of how the oocyte is tightly integrated with the follicle (Figure 2.2). The emphasis placed on this topic, and the compelling body of evidence supporting it, make clear that the processes of oogenesis and folliculogenesis are not dissociable from one another. The physiological manifestation that arises from the integration of the somatic and germ cells within the follicle is manifold with each cell type contributing to the stepwise differentiation of its companion cell. As shown in Figure 2.12, integration appears to be accomplished through the deployment of distinct signaling pathways that operate at sequential stages of oogenesis that range from direct communication via gap junctions to the feedback control exercised by the array of growth factors implicated at various steps beginning with follicle activation. Thus, timely differentiation of the oocyte or granulosa cell necessitates deployment of diverse signaling pathways, the consequences of which are alterations in gene expression, cell cycle status, survival. and differentiation commencing with the initiation of follicle development,[152] as discussed in Chapter 21.

Momentum for this concept first arrived with the demonstration of the potent array of influences that the oocyte can impose on the surrounding granulosa cells (GC). Thus, oocytes were shown to promote GC proliferation,[153] enhance FSH-induced expansion of the cumulus,[154] suppress the expression of the LH receptor gene (*Lhr*),[155] regulate synthesis and secretion of kit ligand,[156] maintain metabolic cooperativity,[157] and modulate cumulus cell mitogen-activated protein kinase (ERK1/2).[158] While this work was being carried out in the mouse, the broader implications of oocyte influences in the follicle were made apparent with the realization that the oocytes' developmental potential was a byproduct of enforcing this bidirectional interaction[159,160] beginning with the elaboration of the hyaluronate-rich matrix at ovulation which ensures ovum capture[161,162] (see Chapter 22). As noted in Figure 2.1, the GCs of the follicle are of two types. Those immediately in contact with the oocyte are known as

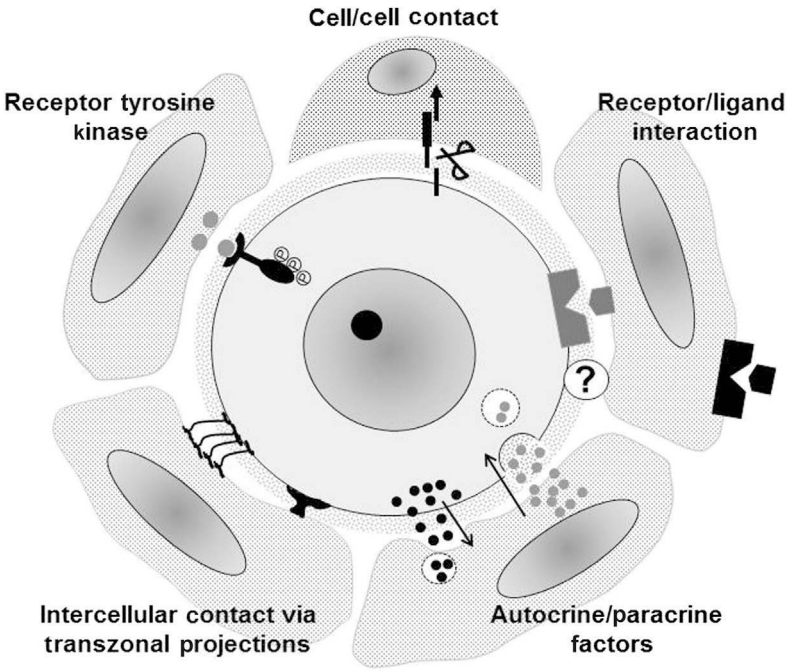

FIGURE 2.12 **Diagram summarizing types of signaling interactions known to exist between oocyte or granulosa cells that impact the survival, growth, and differentiation of the oocyte.** Cell–cell contacts involving proteolytically cleavage-based receptor activation shown at top and counterclockwise examples include receptor tyrosine kinases such as c-kit ligand, direct gap junction–mediated communication via transzonal projections, paracrine modes of communication involving secretion and/or uptake of ligands, and classical receptor–ligand interactions expressed on the granulosa cell surface but not confirmed for the oocyte cell surface (?). *Source: Image is redrawn from McGinnis et al. (2012) where specific examples are cited[151] and content is based primarily on results from targeted gene deletion experiments in the mouse.*

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cumulus GCs, whereas those lining the wall of the follicle are known as mural GCs. The latter are the precursors of the luteal cells that will produce progesterone after ovulation, while cumulus GCs mediate metabolic support of the oocyte during growth and maturation.

The documentation of metabolic integration between oocytes and GCs is one of the most significant outcomes from this line of investigation[163] (Table 2.3). Specific ion transporters in the oocyte require cumulus GCs for their regulation during maturation,[170] and modulation of calcium impacts oocyte survival via caspase-2.[168] The broad spectrum of events occurring during the various stages of oogenesis which are reliant on somatic cells is but one manifestation of how the oocyte influences follicle development, currently envisioned to be mediated by oocyte-secreted factors like GDF9 and BMP15.[159] But the reverse is also true; the granulosa cells direct specific developmental transitions in the oocyte.

One of the best examples of this pertains to the process of meiotic competence acquisition (Table 2.1). In the mouse, meiotic competence acquisition is strictly correlated with the size of the oocyte. Whether oocytes initiate but fail to complete maturation in vitro distinguishes those oocytes that have acquired only partial rather than complete competence.[51,171,172] As noted previously, the gradient reflected in meiotic competence according to a growing or fully grown state is also linked to the acquisition of genomic imprinting[42] and the accumulation of cell cycle factors whose posttranslational modifications indicate the earliest signs of a G2 to M cell cycle transition.[172,173] Follicle culture experiments have further established that meiotic competence acquisition can occur ex vivo, but also emphasize that excess gonadotropin can delay or inhibit the acquisition of full competence as a result of deficiencies in metabolic cooperation.[174–177] These studies and others implicate failure to maintain oocyte chromatin in an open NSN state as one consequence resulting in competence acquisition impairment in rodents and primates.[174,178] It is interesting to note that among the genes whose continued expression seems necessary for continued growth and competence acquisition in rodents is the transcription factor *Oct4*.[179]

TABLE 2.3 Metabolic Substrate Sharing between Granulosa Cells and Oocytes

Substrate	Contact-Dependent	Documentation	References
Pyruvate	Yes	Tracer-based	19,164
Alanine	Yes	Tracer-based, Transcriptome Monitoring (TM)	165
Cholesterol	Yes	Tracer-based, Transcriptome Monitoring (TM)	157
Glutathione	Yes	Direct measurement of oxidative metabolism (REDOX) potential	163,166,167
Calcium	Yes	Ratiometric measurement	168
Cyclic guanosine monophosphate	Yes	Tracer-based	169
Bicarbonate/chloride exchange	Yes	Tracer-based	170

Contact-dependent refers to the requirement for cell coupling between oocyte and granulosa. Documentation refers to radioactive tracer usage (tracer-based) or transcriptome profiling to validate expression of enzymes in a given metabolic pathway as present in granulosa and absent in oocytes.

In summary, the initiation of oocyte growth within the follicle is critically dependent on the maintenance of an integrated metabolism between oocytes and GCs (Figure 2.12). This dialogue forms the basis for modulating the progressive differentiation of oocyte and GC and appears to be a conserved feature for mammalian oocytes that will eventually ensure full developmental competence[165] as specific genes are identified and their interactive networks documented.[180] This has been achieved in the mouse, where a series of transgenic lines have been used to map out the network of genes responsible for maintaining quiescence or activating primordial oocytes, a topic recently reviewed[181] (see Chapter 21). What has remained more elusive, however, is the definition of pathways for communication that would mediate the sequential episodes of signaling during and after the growth phase.

One of the first modalities for signaling identified was that of the gap junction, a well-established plasma membrane specialization known to mediate metabolic cooperation between cells (Figures 2.1, 2.12, and 2.13). First described by Zamboni,[182] freeze fracture electron microscopy verified the existence of gap junctions between granulosa cells and oocytes in several mammalian species and implicated gap junctions in the exchange of molecules that would maintain meiotic arrest.[23,183,184] Dekel and Beers then provided direct evidence for their role of oocyte-granulosa cell contact in the transport of cAMP.[185,186] Eppig and colleagues have since summarized these and other findings to elaborate a tractable scheme for the role that gap junctions play in conjunction with paracrine modalities of communication to effectively syncitialize the oocyte and its companion GCs[62] throughout the course of oogenesis,[31,187] explaining in part their earlier observations implicating GC support in the acquisition of developmental competence.[188] One of the more elegant examples of how paracrine signaling and heterocellular contact ensure metabolic synergy between oocytes and granulosa cells derives from the demonstration of cholesterol synthesis in the cumulus cell supplying the oocyte with stores of this substrate, since

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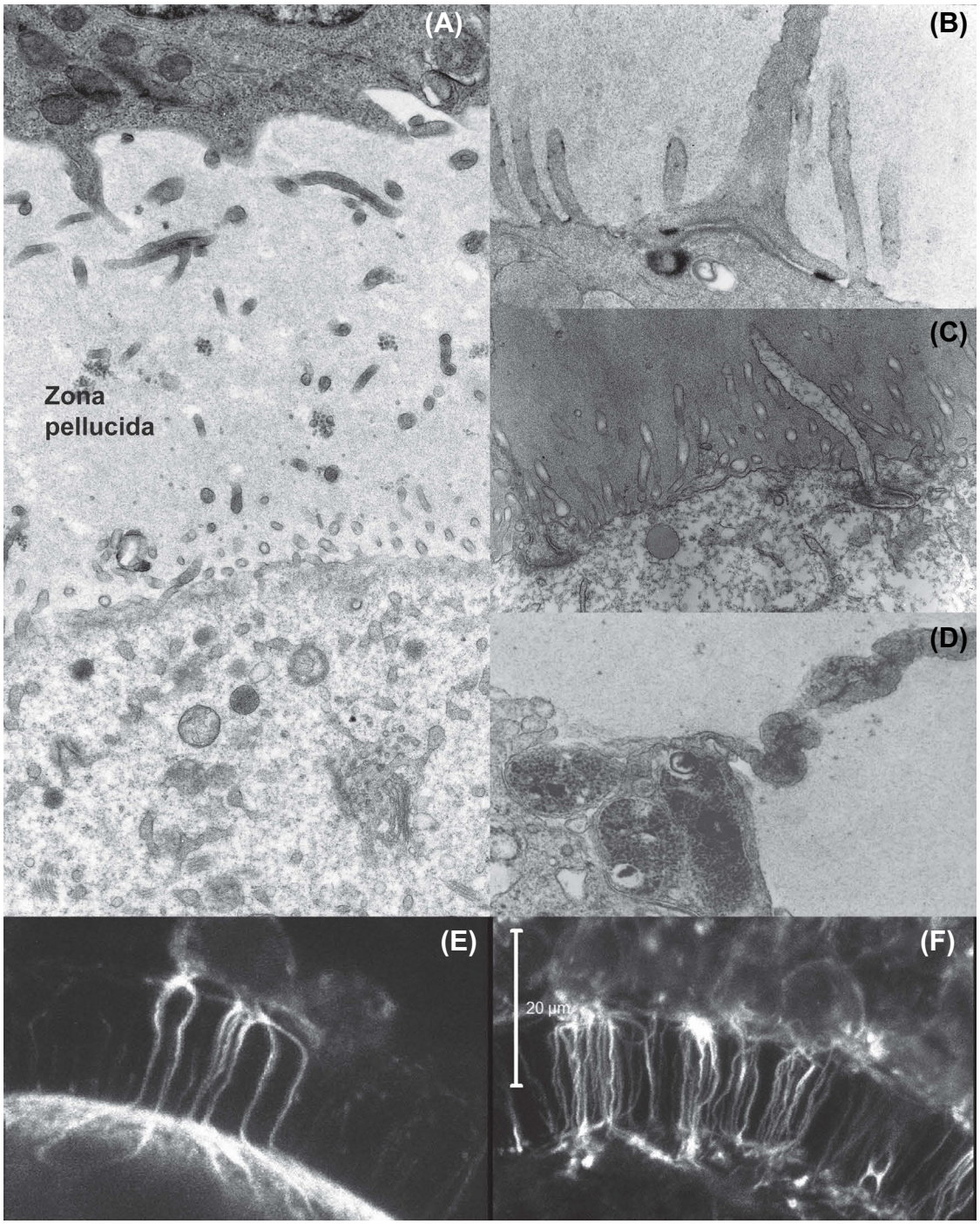

FIGURE 2.13 Appearance and structural organization of transzonal projections (TZPs) between somatic granulosa cells and oocyte during oogenesis and in different mammalian species. The electron micrograph in (A) is from a full-grown mouse oocyte within an antral follicle; portions of a cumulus cell are at the top, and in descending order, TZP roots are apparent at the outer margin of the zona pellucida traversing through the zona and attaching to the microvillus-rich oolemma (bottom). (B) and (C) are electron micrographs of rhesus macaque oocytes in which the planar attachment of the TZP is situated between oocyte microvilli; the dark reaction product in (B) highlights the location of gap junctions at the terminus of the TZP. (D) The TZPs after oocyte maturation are less dense and take on a highly tortuous course terminating as large dilations filled with various organelles (in vitro matured porcine oocyte, courtesy of Virginia Rider). (E) and (F) are three-dimensional projected views from a confocal stack of images which illustrate the arborization of TZPs from immature bovine oocytes retrieved from Graafian follicles; the preparation was labeled with rhodamine phalloidin and highlights the density of actin filaments within the TZPs and at attachment sites with the oolemma.

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the oocyte lacks and therefore must rely upon somatic cell–derived resources for its survival and development.[164]

In what may be referred to as the final act of oogenesis, the concept of metabolic regulation has been invoked to advance our understanding of the mechanisms involved at ovulation that foster release of meiotic arrest and the resumption of meiosis (Chapters 1 and 22). This field has been advanced using transgenic murine models and molecular physiology to unravel what has lingered as a controversial and yet pivotal event in the course of oogenesis in mammals (Figure 2.14). The cascade of signaling events elicited by the LH surge at ovulation is now believed to be initiated by de novo production of epidermal growth factor (EGF)-like molecules from the granulosa causing selective closure of gap junctions as a result of MAP kinase-dependent phosphorylation of connexin 43 gap junctions.[169,189] Modulating cyclic guanosine monophosphate[190] and EGF signaling pathways conspire to initiate and sustain meiotic progression[191] in a manner reminiscent of the interplay between gonadotropin and growth factor receptors in the regulation of follicular steroidogenesis.[192] Coupled with the genetic dissection of tumor growth factor-beta in other mammalian species,[193] the weight of the evidence supports the presence of a feedback loop of counteracting molecular signaling machines that ultimately serve to coordinate the processes of oogenesis and folliculogenesis to the benefit of yielding developmentally competent ova (Chapter 21).

The structural substrate for achieving integration of function within the follicle resides at the interface of the oocyte and granulosa cells. Transzonal projections (TZPs) were introduced above as specialized extensions from granulosa cells that penetrate the zona pellucida and attach to the oolemma.[182–184] Without TZPs, intercellular junctions could not exist. As noted above, gap junctions form at the TZP–oolemmal interface and in rodents are known to require interaction of connexin 37 subunits of oocyte origin with connexin 43 subunits originating from granulosa cell.[194] The changing architecture of the cumulus–oocyte complex during ovulation first implicated TZPs as regulators of meiotic maturation based on the persistence of gap junctions with the oocyte, but also raised the intriguing prospect for transport and delivery of molecules and organelles toward the oocyte as maturation proceeds.[195] In fact, the generalized notion that gap junction uncoupling engages cell cycle reentry in rodents does not appear to be operative in the oocytes of other mammals.[167] Studies on several lines of mice in which gene ablation has been imposed leading to fertility defects, following deletion of genes for GDF9, connexin 37, and the beta subunit of FSH (Figure 2.10), collectively establish the role of TZPs in the regulation of oocyte growth and meiotic competence acquisition and demonstrate a direct role for FSH in modulating their formation and stability.[64–66] It follows, then, that defects in TZP maintenance, and hence gap junction functionality, could lead to impairments in genomic imprinting that would compromise subsequent embryonic or fetal development, as has been recently demonstrated.[196] The possibility that must then be seriously entertained is that the original studies implicating TZP withdrawal as a signal for meiotic resumption[197] fail to identify the need for prolonging gap junction communication well into the maturative phase of oogenesis (Figure 2.2). In fact, it has been recognized for many years that the bovine oocyte acquires and accumulates reduced glutathione based on the metabolic provisions received by oocytes from the cumulus cells with which they maintain contact.[166] Reduced glutathione, required for remodeling of the male genome after fertilization, may then qualify as one of many metabolic substrates of somatic cell origin that serve to fuel the maturing oocyte as it acquires the final properties that will ensure developmental competence.

MATURATION PHASE OF OOGENESIS: THE FINAL ACT

Background: The Importance of Spatial Patterning

Certain generalizations can be gleaned from the foregoing discussion that set the stage for a comprehensive treatment of oocyte maturation. First, the cell cycle of

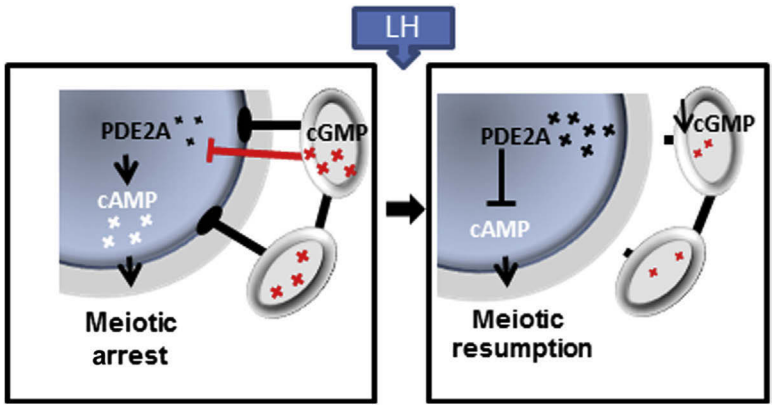

FIGURE 2.14 Modifications in cell communication proposed to explain the resumption of meiosis following the LH surge. Panel on left depicts the role of cyclic guanosine monophosphate as a meiosis arresting agent requiring an intact system of gap junctions. Following the LH surge (right panel), junctional uncoupling results in enhanced activation of oocyte PDE2A permitting the oocyte cell cycle to proceed from G2 to M-phase.

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the mammalian oocyte must acquire the molecular machinery needed to formally enact a G2 to M transition in order to complete meiosis (Chapter 1). Second, transcriptional competence following a profound period of gene expression becomes repressed, and will remain so until the zygotic genome reactivates after fertilization. Third, the fully grown but immature GV oocyte resting in a cell cycle arrested in diplotene of meiosis one awaits reception of somatic cell signals that will elicit meiotic progression to meiotic metaphase two, the state in which it will be ovulated (Figure 2.2). By inference, then, the maturative phase of oogenesis, encompassing a time course of hours in rodents to days in humans, must reliably support in a temporally and spatially regulated manner the final stage of oogenesis that will predate the onset of embryogenesis. Most of what we have learned about oocyte maturation over the past decade derives from studies in the mouse that address two questions: How is the cell cycle regulated? What role does the cytoskeleton play in preparing the oocyte for fertilization?

Insights into the molecular underpinnings of oocyte maturation have been reviewed in the context of the requirements that must be fulfilled to effect oocyte activation at fertilization and ensure embryonic development up to the point of zygotic gene activation[198,199] (Chapter 6). But the first principles drawn upon to explain switching the cell cycle from an off–on–off–on motif common to all mammalian oocytes have led to the realization that much of the cell cycle controls displayed are best understood through a consideration of spatial organization achieved primarily by the remodeling of the cytoskeleton. A striking example of this was first alluded to by Kubiak and colleagues, who showed that the critical switch governing the transition from meiosis to embryonic mitosis required the intact metaphase two spindle to elicit the prompt and complete destruction of the cyclin B.[200] Cytoskeletal control of oocyte maturation was also suggested by protein localization and gene expression studies confirming the importance of spatial patterning and compartmentalization of cell cycle molecules.[201–204] Bypassing inhibitory control elements[205] added further evidence for a role for microtubule stability in initiating GVBD,[66] as did new evidence identifying the involvement of protein kinase B.[206] Together, the landscape for the execution of meiotic competence takes on a level of complexity invoking control features of temporal and spatial dimensions.

Over a similar time period, characterization of the metabolic determinants for the maturative phase of oogenesis was taking place largely as a result of the continuing efforts of Downs and colleagues.[207] In fact, their original studies brought to surface the notion of EGF and FSH as players in the resumption of the oocyte meiotic cell cycle,[208,209] a subject that has received thorough coverage in a highly recommended recent review.[210] With the adoption of fluorescence imaging techniques affording greater temporal and spatial resolution, oocyte maturation has come under closer scrutiny. In this vein, Carroll and colleagues have now provided compelling evidence in support of the somatic cell influences for ATP provisions that guide and support the energy-consuming demands of the oocyte cell cycle.[26] It is important to emphasize the impact of cell culture on the study of meiotic maturation. The obvious benefits obtained from experiments conducted in vitro include the ability to control environmental conditions, carefully monitor and assay time-dependent processes, establish a paradigm conducive to live cell imaging, and afford the opportunity for subsequent in vitro fertilization and embryo culture permitting the evaluation of developmental competencies (Table 2.1). But adverse consequences from in vitro maturation have been noted in several mammalian species.[199]

The Meiotic Spindle: A Microtubule-Based Scaffold

The meiotic spindle is the major structure involved with the segregation of chromosomes at anaphase of meiosis one and meiosis 2 (Figure 2.15). Invariably, the spindle is anchored to the oocyte cortex and deploys centrosome proteins at the spindle poles. In mice, meiotic spindle organization is strikingly altered when in vivo matured oocytes are compared to oocytes matured in vitro.[211,212] Moreover, differences in culture conditions impact both spindle morphogenesis and the expression of developmental competence despite apparently normal rates of meiotic progression and efficiency.[213] In fact, modifications in culture media composition such as growth factor supplementation can have profound effects on embryonic developmental competence.[214] Importantly, with the move from using oocyte in vitro maturation as a basic science tool to one with clinical indications, there is a mounting practical imperative to explore and define conditions that will most likely recapitulate this phase of oogenesis in a fashion that limits disruptive influences on oocyte quality pertaining especially to defects in spindle organization that have been associated with aneuploidy.

Toward this end, the use of chemical agents that inhibit the spontaneous G2 to M meiotic cell cycle transition elicited when oocytes are removed from the follicle is an experimentally acceptable and widely employed manipulation that permits the synchronization of meiotic cell cycle progression. A number of studies have employed phosphodiesterase type–specific drugs to maintain G2 meiotic arrest in mouse[215] and human[216] oocytes, with generally favorable results with respect to the completion of maturation and demonstration of developmental competence. Comparative

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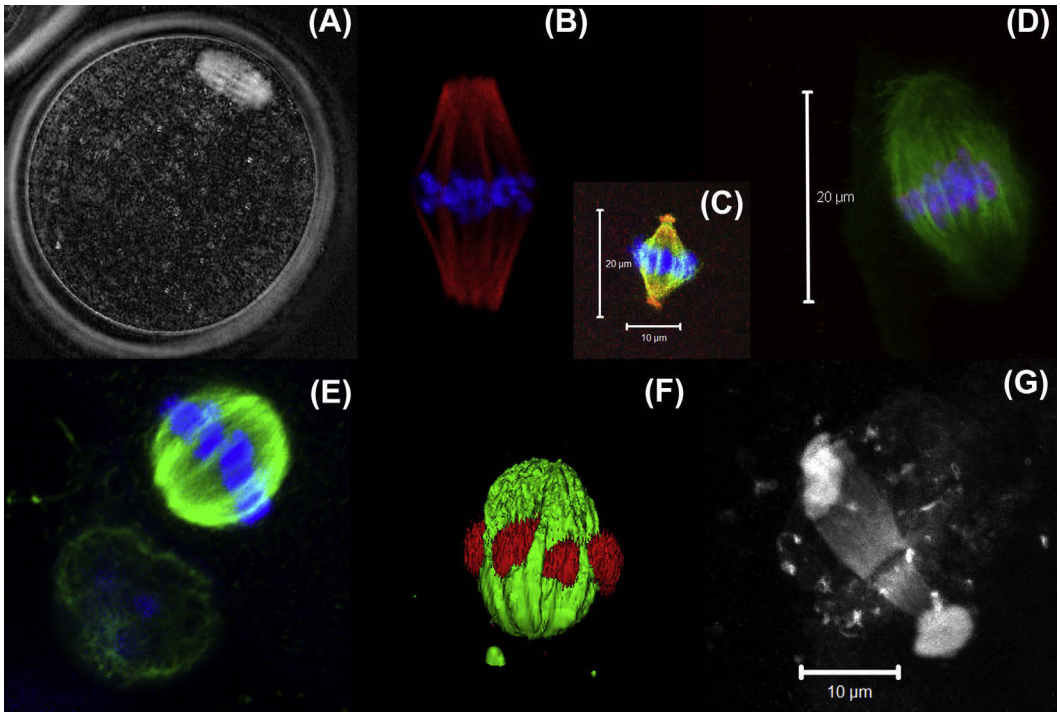

FIGURE 2.15 Meiotic spindles of mammalian oocytes. This figure is reproduced in color in the color plate section. (A), (B), and (C) depict metaphase two–stage mouse oocytes under polyscope in a living oocyte (A), and after confocal immunofluorescence of tubulin (B, red; C, green); chromosomes aligned on the metaphase plate are rendered blue, and in (C), the poles of the spindle from a naturally ovulated oocyte show gamma-tubulin in red. (D) illustrates kinetochore microtubules as green stripes in a metaphase one horse oocyte that has been stained for chromosomes (blue) and phosphohistone H3 (red). (E), (F), and (G) are human oocytes at metaphase two (E and F, with the latter rendered as a three-dimensional projection); and (G) illustrates a projection of telophase one at the time of polar body extrusion.

gene expression profiling in human oocytes matured in vivo or in vitro has revealed differences in transcript abundance and type suggesting that in vitro conditions may compromise the stability of certain mRNA species whose significance awaits further research.[217] While exercising caution in the use of IVM for clinical purposes remains an important perspective for the treatment of infertility, the benefits cited above have been of enormous value in contributing to our understanding of the relationships between the cell cycle and cytoskeleton in animal models.

For in-depth coverage of the oocyte cell cycle, the reader is encouraged to see the review of Jones, which provides a complete description of the biochemical components that mediate the various starts and stops typically displayed by mammalian oocytes.[218] This foundational biochemical approach has complemented a series of more recent live cell imaging studies that have focused directly on processes such as spindle dynamics and chromosome segregation. Among the most telling of these are studies coming from the Ellenberg laboratory.[219] By introducing reporter constructs for the cytoskeleton, and tracking individual mouse oocytes over the course of meiotic maturation, novel insights have been obtained that provide a workable hypothesis for how, in the absence of classical centrosomes, MTOCs orchestrate the morphogenesis of the meiotic spindle through self-assembly intervals coinciding with important cell cycle transitions. The principle of "self-organization," especially as it applies to maturation-associated remodeling of the oocyte cytoskeleton, is gaining traction as a driving force that oversees the successful separation of chromosomes during meiosis and embryonic mitoses, a subject reviewed by Dumont and Brunet.[220] The biological relevance of studies of this kind bear directly on the problem of age-related aneuploidy and the mechanistic origins of chromosome imbalance propagated during meiotic maturation (see Chapters 1 and 37)

One likely mechanism contributing to female meiotic aneuploidy concerns the functionality of cell cycle checkpoints that in somatic cells eliminate imbalanced products of cell division. It appears as though the checkpoints engaged in mouse oocytes differ from those deployed in somatic cells in fundamental ways such that surveillance of the spindle is dissociable from the checkpoints that monitor chromosome position[221] (see Chapter 1). The mechanisms behind checkpoint control in mouse oocytes seem based on unique ways to ensure stability of cyclin B and in effecting activation of the anaphase-promoting complex/cyclosome (APC/C).[221–223]

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Whether comparable mechanisms are at play in human oocytes remains unknown.

The Oocyte Cortex: An Actin-Based Scaffold

One of the most distinguishing and universal features of mammalian oocytes is the asymmetric nature of cytokinesis that ensures the extrusion of polar bodies. Polar body extrusion serves two vital roles for the maturing oocyte. First, the spatial restriction of the contractile forces required to form both polar bodies guarantees that the byproducts of anaphase-1 and anaphase-2 chromosome segregation result in retention of one chromosome set in the ooplasm and one being discarded. Second, by limiting the size of the polar bodies, the oocyte is able to retain most of the molecular and organellar constituents destined for utilization and consumption in the zygote. This was first elucidated by Longo and Chen, who defined the mosaic patterning of the actin cytoskeleton and cortical granules at the ultrastructural level.[224] With the documentation of spatial reorganization for many organelles, and the suggestions of cytoskeletal dependence for inward or outward displacements,[98,225,226] only a fragmentary picture of cytoplasmic dynamics during oocyte maturation could be assembled with the exception of the more obvious events associated with meiotic spindle assembly and function noted above. Progress in this field was again limited due to the lack of experimental reagents and the compounding issue resulting from the effects of culture conditions on organellar redistribution.[227] In the end, tracking organelles in ooplasm fails to identify the operative motility systems in biochemical terms; and with rare exception, few pathways invoked in meiotic cell cycle progression appear linked to cytoplasmic reorganization. One possible link may be through the action of tyrosine protein kinases, based on recent studies in the mouse oocyte.[228]

FYN kinase exhibits activation latency with respect to the consequences of inhibition on later stages of meiosis involving spindle location and cytokinesis.[229] This kinase targets a spatially distinct cortical set of protein substrates when compared to the protein substrates targeted by the CDK1 kinase that drives the cell cycle.[230] In fact, recent studies on protein tyrosine kinase signaling suggest involvement in motility-based processes more in line with known actin cytoskeleton targets in somatic cells and influencing events in the zygote that follow the completion of maturation, a subject that has been recently reviewed.[231] This general theme of spatial organization and the possible role that the actin cytoskeleton plays was first raised by studies showing that the actin linker protein, beta catenin, was deployed in the oocyte cortex and used at later times in embryogenesis, confirming its identity as one of the first maternal gene products recognized in mammalian oocytes.[232] Since then, and as alluded to above, the role of calcium,[233] a potent modulator of the actin cytoskeleton, and the reorganization of calcium sequestering endoplasmic reticulum[234] among other organelles, raise the likelihood that fundamental remodeling events during maturation have both a short- and long-term impact on oocyte quality, possibly as a result of the selective activation of SRC family kinase members.[235] From a regulatory point of view, the historical focus on calcium metabolism, and the recent discovery of the role of zinc ions as a potent mediator of meiotic cell cycle progression,[236,237] imply the existence of a far more complex array of ionic factors that are likely to impact the oocyte cytoskeleton.

As noted above, the mammalian oocyte exhibits a distinct polarity in the mature metaphase two state and the actin cytoskeleton provides a driving force for the generation of polarity. Overlying the meiotic spindle, an actin-rich and microvillus free cap appears that is rich in myosin two and lacks cortical granules.[99,238,239] Chromosomes provide the signal for the localized reorganization within actin caps,[100] which have also been shown to contain markers of cell polarity such as usually encountered at the apical surface of epithelial cells.[240] The mechanism by which chromosomes exert their influence on actin reprogramization has been thoroughly studied and involves MAP kinase,[241] myosin light chain kinase,[242] and RAN GTPase.[243] As summarized in a recent review, the actin cytoskeleton in mammalian oocytes is a dynamic system that directs the establishment of cell polarity by a cytoplasmic streaming activity that positions and anchors the meiotic spindle in the cortex.[244] Significant biophysical alterations have been documented to occur as a result of the combined actions of the myosin two motor molecule and members of the Ezrin/Radixin/Moesin (ERM) family of actin-binding proteins.[245] In summary, remodeling of the cortex in mouse oocytes is a major feature of meiotic maturation that is essential for spindle migration and attachment, and stabilization of the microvillus portions of the plasma membrane. It seems likely that the positioning and localization of maternal effect gene products, as mentioned previously for MATER, which assumes a similar asymmetric disposition, is also dependent on the forces deployed to achieve cell polarity.[94] In the mouse oocyte, one additional cortical specialization deserves mention in the context of polarity as it has practical bearing on the topic of cloning.

Microtubule organizing centers were discussed earlier with reference to their role in assembly of the meiotic spindle. These structures are silenced with respect to their microtubule nucleation capacity at specific cell transitions such as metaphase of meiosis one or meiosis two, but upon anaphase onset they nucleate a cortical network of asters excluded from the actin cap.[78] In enucleated oocytes prepared for somatic cell nuclear transfer or cloning, MTOCs are retained ensuring the inheritance of maternal centrosomes in reconstructed embryos.[246,247] This situation implies that the introduction of a somatic cell into an enucleated oocyte will result in an admixture of

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centrosomes of somatic and maternal origin.[248] Cell cycle progress and chromosome segregation involve MTOCs due to their AURORA kinase-A activity, a situation that has been proposed to complicate cell cycle regulation in the embryo, especially after somatic cell nuclear transfer (SCNT).[249,250] The general theme that emerges from this discussion is that scaffold functions served by both actin-based and tubulin-based components of the cytoskeleton drive the maturative phase of oogenesis, and interactions between these components appear to be bridged by the family of proteins known as formins.[251,252] As shown in Figure 2.16, the remodeling of the cytoskeleton as finally enacted during the maturation phase of oogenesis also drives the positioning of organelles involved with developmental competence. Thus, CGs assume a subcortical location and maternal effect gene products become localized in anticipation of their recruitment for events such as compaction in the embryo (see Chapter 6).

Because most of our knowledge to date comes from studies on the mouse, it is difficult to predict the importance of cytoskeletal remodeling as manipulations of mammalian oocytes are attempted for clinical purposes. With some assurance, however, it can be stated that the requirements for egg activation imposed by SCNT have been met through a deeper understanding of the role of calcium transients[253] and their application to the production of cloned embryos in other mammalian species.[254] Moreover, when cytoskeletal dynamics as described above are manipulated in the context of the cell cycle state, the retention of egg quality contributes to an improved efficiency for procedures like SCNT[255,256] or embryo splitting.[257]

CLINICAL PARAMETERS OF OOCYTE BIOLOGY

The human oocyte is now well within the purview of contemporary research, given the enormous growth in the field of human ARTs. Because of the large-scale use of human oocytes for the treatment of infertility, and emerging strategies for fertility preservation including oocyte cryopreservation and in vitro follicle development, exploration of human oocyte biology has been realized. Thus, for the first time, modern investigative approaches are providing a knowledge base exceeded in content only by that ascribed to rodent animal models. What makes the human oocyte unique relative to those of other mammals is now gaining clarity and will soon aid in the diagnosis and treatment of reproductive disorders for which there has been little information with which to proceed. This section will introduce topics of clinical relevance in hopes of providing a basis of comparison between the human oocyte and those of other mammals. It is designed to guide and inform future work for purposes of therapeutic advancement.

Regulating Genetic Integrity

Among the distinguishing features of the human oocyte, relative to those of other mammals, is the high degree of chromosome imbalance that arises during oocyte maturation and early cleavage divisions of the embryo. What makes the human ovum so predisposed to genetic instability and what is the underlying basis for the age-related increase in aneuploidy? These questions prompt consideration of this problem on two levels that have been reviewed above. First, and at the level of the organism, advances in our general understanding of genetic stability have been contextualized for human oocytes at the mechanistic level with respect to the management of DNA damage. Second, data are now in hand that allow for a detailed examination of the origins of chromosome imbalance with respect to the relationship between the cell cycle and cytoskeleton.

Metazoans have evolved mechanisms for the detection and repair of DNA lesions that can be incurred by any variety of factors such as oxidative stress, chemotherapy, radiation exposure, or lifestyle conditions.

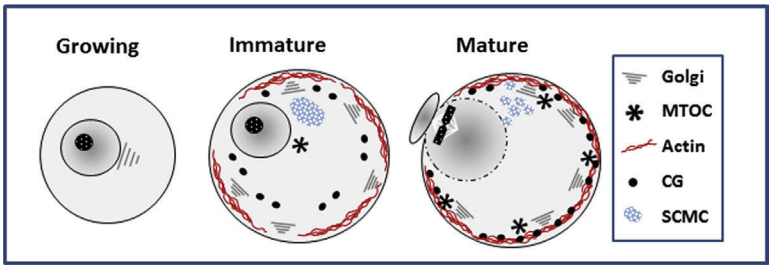

FIGURE 2.16 Illustration depicting the patterning of organelles and the cytoskeleton in mouse oocytes during growth phase (left), as a fully grown oocyte (center), and following meiotic maturation to the metaphase two stage (right). Legend shows summary of principal organelles that undergo remodeling, including the Golgi complex, cortical granules (CG), and the subcortical maternal complex (SCMC). Note dispersion of perinuclear Golgi into multiple subcortical complexes, elaboration of multiple microtubule organizing centers (asterisk, MTOC), and the assembly of a prominent network of f-actin that subtends the oolemma at all sites except where the spindle is located.

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Better known as the DNA damage and repair response (DDR), lesions in DNA elicit a cascade of enzymatic reactions localized to single- or double-stranded breaks that are repaired through high-fidelity (homologous recombination) or error-prone (non-homologous recombination) mechanisms as occurs during meiotic prophase (see Chapter 1). Transcriptome analyses of human oocytes and embryos have confirmed the expression of proteins for both of these repair pathways, and only recently has evidence been provided that the DDR is alive and well in human oocytes.[258] As the DDR serves as a vital checkpoint for genetic stability in somatic cells, failure to effectively repair DNA damage typically results in the elimination of damaged cells through a p53-mediated programmed cell death pathway. Given the hallmark attrition of oocytes accompanying aging in female mammals, analysis of this pathway in mouse models has been a point of departure for probing mechanisms of genetic stability in the female germ line.

As shown in Figure 2.17, the expression of DDR in the mouse follows a developmental course that utilizes a surveillance system that is distinct from both the male germ line and somatic cells. Rather than drawing upon p53 for execution of damaged cells, as occurs in somatic cells and sperm, the surveillance system in oocytes uses the p63 variant. This has been demonstrated using mice bearing a null mutation for TAp63 in which radiation-induced DNA damage results in oocyte loss due to apoptosis unless p63 is genetically ablated.[259] Earlier work had identified the protective role of TAp63 in eliminating damaged oocytes as they remain arrested in diplotene[260] and prompted the suggestion that the accumulation of DNA damage, as would occur during maternal aging or after inducing double-strand breaks, if carried through the course of oogenesis might explain why oocytes show an aneuploid tendency after ovulation. It remains unclear as to whether the TAp63-mediated checkpoint evident at diplotene is linked to age-related aneuploidy or whether distinct checkpoints operate at sequential stages of oogenesis to ensure genetic stability of fully matured mouse oocytes. This topic has been the subject of recent reviews.[261,262] One of the reasons for the lack of clarity in this field is due to the fact that both DNA damage–dependent and DNA damage–independent pathways have been shown to mediate oocyte loss.[263] In the case of DNA damage–dependent oocyte loss, recent mechanistic studies have identified mediators of oocyte elimination as described below.

A reliable marker for DNA double-strand breaks is an antibody that recognizes the gamma-phosphorylation of histone H2AX. Since the phosphorylation of this histone two variant represents the activation of the DDR, both the resident capacity for eliciting a repair response and the loss of signal signifying completion of repair can be monitored in sections of ovarian tissue. Using gamma irradiation to induce the DDR in neonatal mouse ovaries, Kerr, Hutt, and colleagues demonstrated that TAp63 elicits the expression of Puma gene as an effector of apoptosis.[264] Interestingly, a recent report implies that failure to complete the DDR impacts chromosome segregation. This study reports that the persistence of H2AX double-strand breaks is correlated with a loss of the apoptosis

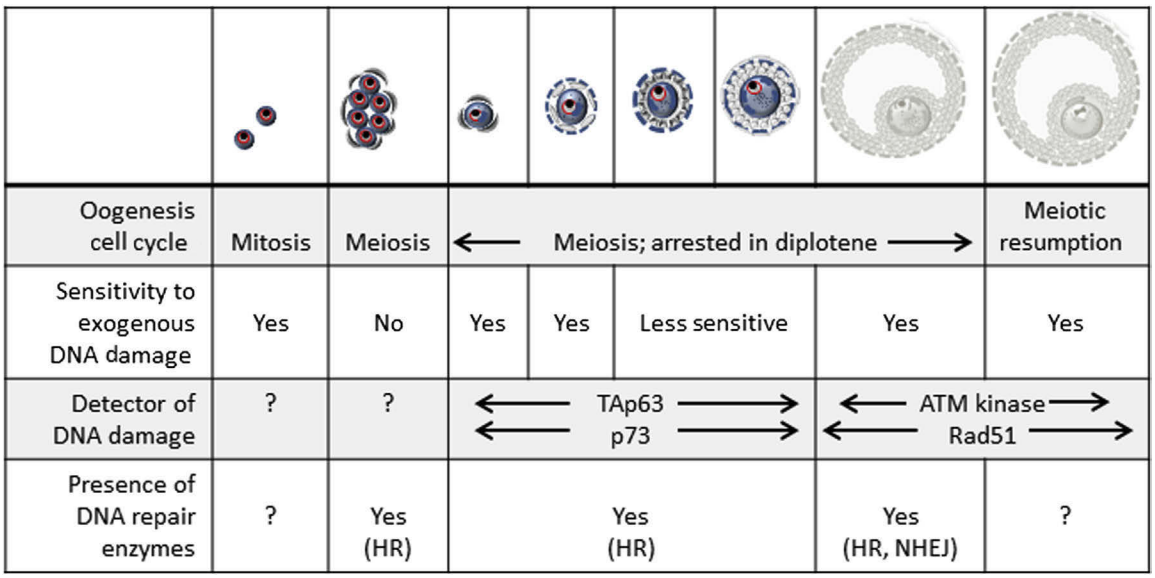

FIGURE 2.17 Schematic demonstrating stages of oogenesis that are most sensitive to DNA damage. Oogonia to full-grown oocytes are shown at the top as a function of the type of cell cycle engaged and current estimates of relative sensitivity to genotoxic agents; sensitivity may also vary depending on the damaging agent as gamma-irradiation or chemotherapeutic compounds. Line four shows checkpoint targets at later stages, and the bottom line summarizes types of DNA damage and repair pathways thought to be operative.

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effector PARP-1,and oocytes presenting these properties are likely to undergo aberrant chromosome segregation resulting in aneuploidy.[265] They further show that PARP-1 mediates the recruitment of the checkpoint protein BUB3 to centromeric regions of meiotic chromosomes, providing a potential mechanism that would link the DDR of diplotene oocytes with ploidy balance at the end of oogenesis. While these studies are derived from the mouse oocyte, as will be shown below, there is mounting evidence that comparable mechanisms may be at play in the human oocyte that could account for the age-related loss of genetic stability.

Reproductive Aging and the Human Oocyte

Many factors have been implicated in the well-documented loss of oocyte quality that occurs upon advancing maternal age (see Chapters 1 and 37). Keefe and co-workers have long proposed that oocytes resident in the primordial follicle for protracted periods of time would be subjected to repeated episodes of oxidative stress, which if exacerbated by the accumulation of DNA mutations in the mitochondrial genome, would contribute to a burden of lesions in the nuclear and cytoplasmic genomes.[266] One contributory factor widely recognized to be central to the loss of oocyte quality in humans is aneuploidy,[267] and the incidence of this form of genetic instability has been confirmed repeatedly from clinical studies. As noted above, mitochondrial function is suspected as being a causative factor in oocyte aging due to a decline in efficiency of free radical management with advancing maternal age.[268] The theme of declining metabolic or organellar functionality upon loss of oocyte quality resonates through many of the studies attempting to provide a molecular understanding of the aging effect in mammalian oocytes. By far, the best studied, as might be expected, is that of cell cycle checkpoint control, a topic of mounting interest that has been recently reviewed[269] (see Chapter 1).

Because the events involved with the generation of aneuploidy occur at the level of the interaction between the meiotic spindle and chromosomes, concerns about the stability of cyclin B and that of the spindle have remained an important target for investigation in animal models.[221] Similarly, the role of cohesins, molecules that maintain the interaction of homologous chromosomes and sister chromatid pairs, has been implicated in studies on mouse oocytes, and there is some indication that as a result of advancing maternal age, the cohesin-mediated linkages between chromosomes are weakened through an as-yet ill-defined process.[270] It is generally assumed in these and related studies that either mammalian oocytes have robust checkpoints for spindle assembly and/or chromosome alignment whose efficiency fails upon advancing maternal age, or these and related checkpoints are intrinsically leaky relative to somatic cells or male germ cells and therefore predispose female germ cells to genetic imbalances that may have a long-term adaptive benefit for reproduction.[271]

Whatever the case may be, studies using mouse models have been informative in guiding research on human oocytes and will likely continue to be so. Studies in animal models have also raised concerns regarding current ART practices and how they may compromise oocyte or embryo quality. Examples of this again have focused on mitochondrial metabolism and the impact of in vitro maturation or controlled ovarian hyperstimulation,[272,273] a subject critically reviewed with respect to clinical considerations.[274] In this regard, it is interesting that work on cohesins in human oocytes obtained from women of different ages supports the findings in rodents cited above validating some aspects of oocyte aging worthy of further pursuit in humans and other species.[275]

The Oocyte in Human Assisted Reproductive Technologies

In a clinical context, many factors have been evaluated in an attempt to explain why of the many human oocytes collected for embryo production, so few give rise to embryos of high developmental competence and even fewer give rise to live offspring. This has raised a number of questions regarding the practice of human ARTs and how the procedures currently in use may alter egg quality either directly or as a result of hormonal manipulations involved in controlled ovarian hyperstimulation (COH). While current evidence suggests that offspring health is not adversely affected by human ARTs, the causes of inefficiency remain important to address and may provide insights into the unquiet biology of the human oocyte.[276]

Lifestyle Factors and Oocyte Quality

Although the impact of lifestyle has been rigorously studied in animal models, focus on the periconceptional period—that is, immediately prior to and following fertilization—has been receiving greater attention. Interest has centered on the role of diet during the preimplantation period of mammalian development and whether dietary changes or conditions such as diabetes would impact the metabolism of offspring and contribute to early-onset adult disease.[277] Obesity has been identified as a contributory factor to many forms of human infertility, sometimes linked to polycystic ovary syndrome, and the fact that altered metabolism during oogenesis influences oocyte quality brings to the table the increasing likelihood that preconceptional health may be as important as postconceptional.[278] Obviously, given the focus of this chapter, the manner in which lifestyle influences oocyte quality is of great importance to human

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reproduction. But how close are we to identifying a link between maternal health and oocyte quality?

Several laboratories have made progress in this arena. It appears that a diabetic state influences the process of oogenesis through impairments in mitochondrial biogenesis and function, likely the result of altered ovarian metabolism.[279] Secondary to metabolic perturbations are defects in the assembly and function of the meiotic spindle that predispose mouse oocytes to genetic instability. In the cow, hyperlipidemia during oocyte maturation leads to altered metabolism in resulting embryos.[280] And in one study, the adverse consequences of obesity on oocyte quality were found to be partially reversible after treatment with rosiglitazone.[281] Taken together, there is mounting evidence to suggest that the protracted period of ovarian oogenesis is subject to lifestyle interventions.

Controlled Ovarian Hyperstimulation—More Oocytes but are They Better?

Ovarian stimulation with gonadotropins has been used in experimental and clinical settings as a means to rescue follicles from atresia and thereby gain access to a greater number of oocytes than might be obtained from an unstimulated ovary (see Chapters 28 and 29). The widespread use of COH in the practice of human ARTs has increased oocyte retrieval efficiency, but for patients classified as poor responders due to low oocyte yields, multiple rounds of COH are often conducted in an effort to increase the number of embryos available for transfer. Concerns over the long-term effects of repeated COH have been raised with respect to increased cancer risks and premature follicle depletion, but the practice continues.[282] Because repeated COH accelerates the rate of follicle development while sensitivity to exogenous gonadotropins is retained, the potential negative consequences on egg quality have been the subject of many investigations in animal models.[283] In one study using mice subjected to four rounds of stimulation, a progressive decrease in oocyte ATP levels was noted in relation to the number of rounds of stimulation. It was further shown that while when oocytes were fertilized in vivo or in vitro, only embryos maintained in culture exhibited fetal defects after transfer to pseudopregnant animals, indicating that metabolic deficiencies affect in vitro developmental competence. Embryos allowed to develop in vivo were indistinguishable from one another despite multiple rounds of COH.[284] Studies of this kind suggest that altering the pace of follicle development with exogenous gonadotropins does not exert long-term effects on developmental competence unless additional challenges are imposed on embryos by maintaining them in culture. As mentioned earlier, one of the major determinants for successful completion of meiosis and egg activation is the ability to sustain calcium transients after fertilization. In fact, it is well established that if this competence for calcium oscillations is instilled in the oocyte during maturation, then many of the sequelae that launch early development will proceed on schedule.[285–288] Thus, as a biomarker of developmental competence, calcium oscillations emerge as a major property for oocyte quality.

This has been borne out further by studies linking the fidelity of metabolic changes during egg activation to adult body weight.[289] As the spermatozoon's contribution of phospholipase C zeta (PLCz) is known to trigger the onset of calcium oscillations (see Chapter 4), it is of special interest that in cases of male infertility, where intracytoplasmic sperm injection is unable to launch the program of developmental competence, replacement with purified PLCz can rescue human oocytes and sustain early development.[290] Manipulations such as these may in the future be used for the treatment of infertility where specific deficiencies are identified that compromise gametic quality.

In Vitro Maturation of Human Oocytes

In vitro maturation is likely to be used clinically as conditions for optimizing cytoplasmic and nuclear maturation are refined in the future. Already, some of the differences between in vivo and in vitro matured mouse oocytes have led to the identification of specific competency deficits that can be rectified using inhibitors or activators of signaling pathways.[291] As mentioned earlier, inhibitors of PDEs can improve the maturation and developmental competence of IVM human oocytes,[216] but concerns remain given that studies in primates suggest a delay or blockage in zygotic gene activation as a consequence of IVM.[292] Even though there is now solid evidence that recapitulation of all phases of oogenesis under in vitro conditions results in the birth of mice exhibiting normal adult health and lifespan, caution should be exercised when applying such approaches to human material for purposes of reproduction.[293] Thus, while live births have occurred after human oocyte IVM, careful follow-up of offspring health has yet to be evaluated.[294]

Human Oocyte Cryopreservation

Cryopreservation of human oocytes is becoming widely adopted in human ARTs when indicated for purposes of achieving or preserving fertility. In recent years, comparisons have been made between mature metaphase two oocytes cryopreserved by either vitrification[295] (rapid freezing) or slow-freeze methods where the effects of either approach have been evaluated based on oocyte survival after thawing and the ability to produce embryos.[296] There has been general agreement that vitrification is less damaging to mature human oocytes, and coupled with the convenience and expense associated with it, it has become the method of choice clinically.[297]

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Oocyte Biology in the Context of Fertility Preservation

The increased survival of female cancer patients observed over the past 20 years has motivated scientists and clinicians alike to pursue avenues of research that might enhance prospects for preserving fertility. As noted above, the mechanisms by which mammalian oocytes die of natural causes or as a result of iatrogenic therapies indicated for cancer patients are gradually being understood to a level where it may be possible to preserve and/or protect the livelihood of oocytes.[298] It has been recognized that both chemotherapy and radiotherapy, alone or in combination, cause extensive damage to the oocyte and follicle pool in the human ovary.[299] To offer patients the prospect of preserving fertility, a number of strategies are under development to achieve this end, and these have been summarized by Woodruff[300] and are illustrated in Figure 2.18.

It has become possible to cryopreserve ovarian cortical strips from patients using either slow-freeze or vitrification technology and subsequently perform autologous grafting to those patients once they have fully recovered from their original malignancy. To date, 16 children have been born by transplantation of previously frozen ovarian cortical strips. Because the risk of reintroducing cancer cells is high, an alternative to this approach has been initiated that involves culturing ovarian strips to allow for the activation of primordial follicles and the attendant initiation of the growth phase of oogenesis (Figure 2.1). An ex vivo procedure has been developed by Telfer and colleagues recently.[301] This three-step procedure is designed to mimic the growth phase by culturing intact strips of ovarian cortex, followed by a prematurative phase when somatic cell contact is maintained outside the follicle, and finally subjecting oocytes to IVM. Thus far, small numbers of oocytes have been shown to initiate and complete meiotic maturation (Figure 2.18).

Oocyte Biology and Regenerative Medicine

Achieving totipotency is the *raison d'etre* for the mammalian oocyte. Thus, understanding how this cell acquires and sustains the ability to reprogram the genome brings it to center stage in the field of regenerative medicine. The most significant advance in the field of regenerative medicine has recently been achieved in the demonstration that human oocytes subjected to SCNT are capable of producing high-quality blastocysts with the competence to generate embryonic stem cells (ESCs).[302] On a more fundamental level, the possibility that stem cells could be coaxed back into the female germ line has been proffered as an experimental strategy for defining the molecular blueprints that guide and inform the process of oogenesis. The recent demonstration that ESCs or induced pluripotent stem cells (iPSCs) can be manipulated to form primordial-like germ cells, that if placed in the correct ovarian context proceed to acquire and express all of the competencies resulting in live offspring, establishes the feasibility of directing stem cells down the pathway of gametogenesis.[303] Other strategies have been proposed to recapitulate oogenesis de novo.

There has also been great interest in the field of oocyte biology with regard to prospects of identifying oogonial stem cells from which mature oocytes might be propagated, as originally suggested from the studies on mice by Johnson and colleagues.[304] While this work has elicited much debate and attention, more recent studies showing that more complete methodological approaches can be used to purify oogonial stem cells from mouse or human ovarian tissues offer some promise that the existence of such cells can be investigated in a broader context.[305] As

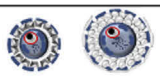
	Follicle stage			
	Germ cell cyst	Primordial	Primary & secondary	Antral
Technique				
Cryopreservation	NA	Cortical strips	NA	Oocytes; GV or MII
Culture	NA	Cortical strips	Intact follicles	In vitro maturation
Transplants	NA	Yes	No	NA

FIGURE 2.18 Summary of in vitro methods being used to support oocyte development in the field of fertility preservation. Figure illustrates application of techniques at specific stages of oogenesis represented by cortical strips containing primordial follicles, isolated follicles, or oocytes subjected to IVM. NA, not applicable. Cryostorage of cortical strips of ovary containing primordial follicles has been shown to result in live births following culture and autologous grafting in humans; and while oocyte cryopreservation is widely used, successful attempts for embryo production have yet to be accomplished cryopreserving follicles. For growing oocytes, methods have been used to culture primary or secondary follicles, but as yet, complete development and maturation in all mammals have not been achieved except in the mouse.[301]

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there is little direct evidence to suggest that such cells recapitulate the entire process of oogenesis in vivo or in vitro, earlier questions regarding the validity of this provocative idea remain.[306]

As advances in stem cell and germ cell plasticity continue to be made using mouse models, the prospects of achieving in vitro oogenesis remain a formidable challenge for the oocytes of other mammals.[307] Given that recent technological advances are showing some signs of progress that some aspects of oogenesis may indeed proceed ex vivo (Figure 2.18), a deepening understanding of the complexities of oogenesis looms as the next chapter in the study of mammalian oocytes.[301]

CONCLUSION

The above discussion of the mammalian oocyte summarizes the present state of knowledge on this remarkable cell. As noted in the introduction, the fundamental property of totipotency that allows the fertilized egg to develop into the conceptus and adult organism has spawned intense interest in the areas of reproductive physiology, stem cell biology, and human regenerative medicine. Many questions remain regarding the biological underpinnings of the oocyte whose resolution will be required to advance research at basic science and clinical levels. In the end, understanding how an organism draws upon the ovarian follicle reserve to support ovulation of a developmentally competent mature oocyte is the overarching question awaiting resolution.

Two outstanding questions prevail. First, how will the key determinants of oocyte quality be best defined using present and future technological innovations? The introduction of "omics" approaches has certainly opened new avenues for study, and the increased availability of databases on mammalian oocytes suggests that "deep" technologies and bioinformatics will foster mining of genomes, metabolomes, and transcriptomes in relevant animal models where functional outcome studies are feasible. Such efforts are likely to reveal the molecular and physiological determinants of oocyte competencies and for the first time permit the linking of each phase of oogenesis to specific events in early embryogenesis and beyond. Discovery of the array of maternal effect genes, in this regard, will provide a detailed biochemical roadmap with which to generate new hypotheses pertinent to reproductive health and well-being of females and their offspring.

Second, what is the difference between mammalian oocytes of good or bad quality? Human ARTs reinforce long held beliefs from studies on other mammals that ovulated oocytes are not equal in their ability to yield live young. To date, our perspective for defining oocyte quality has been largely restricted to understanding properties emerging at the time of ovulation when the oocyte is proceeding through the penultimate stages of oogenesis. But as discussed above, many of the properties essential for sustaining later development of the conceptus in fact are conferred at much earlier times in oogenesis, typically three or more ovarian cycles prior to ovulation. So, while the technology is in hand for monitoring the various competencies the oocyte must acquire during the protracted course of oogenesis, our understanding of how and when these events take place remains fragmentary.

The bulk of accumulated knowledge in this area has come from studies on the mouse and the introduction of technologies such as transgenesis. Hence, attributing functional phenotypes to oocyte-specific genes is now possible using conditional gene deletion strategies as well as siRNA knockdowns along the pathway of oogenesis. However, oocyte-specific Cre mice are limited in availability for most stages of oogenesis, and as noted above, must also be generated to tease apart the interdependence of oogenesis and folliculogenesis (see Chapter 21). Nonetheless, the mouse, and likely other species like the rat, afford animal models with great potential owing to their genetic tractability and development of novel transgenic technologies. While these technological advantages are unlikely to be realized for species more related to the human, both nonhuman primate and various domesticated species have served well in the domain of oocyte biology in the past, and all indications are that appropriate models will be exploited with the "omics" approaches required to systematically solve important questions such as: (1) how are maternal mRNAs differentially processed to direct the overlapping events of cell cycle control and cytoplasmic maturation in response to physiological signals propagated by the ovulatory surge of LH versus ex vivo maturation? (2) What are the immediate and delayed consequences of oocyte cryopreservation on mitochondrial function after fertilization? (3) What competing interests at the level of RNA or protein allocation are served by maternal versus zygotic gene products? and (4) How will the range of newly discovered RNA species play into the growth and maturative stages of oogenesis as genetic or epigenetic modifiers?

Several limitations of our current knowledge are in evidence. First, while much is known about the biology of the mouse oocyte, we are only now gaining insight into the ova of other mammalian forms. Second, almost all studies of oocytes, especially in rodents, involve analyses of somatic cell free systems that, as has been shown, greatly impact the maturation and postmaturative stages of oogenesis. Of course, this problem is the basis for the experimental intractability in this system due to the highly integrative nature of the cumulus oocyte complex. And third, the use of COH to obtain as many oocytes as possible, while serving a practical experimental need, is

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now recognized to severely compromise oocyte quality from bench studies with rodents to human ARTs. In fact, and as discussed earlier, gonadotropin stimulation of follicle development appears to disrupt the delicate balance of signaling pathways within the follicle, and what appears, at least on the surface, to be good for the follicle seems not to be so for the oocyte.

In the vein of clinical importance, the study of high-quality oocytes remain enigmatic and a source of frustration to patients and clinicians alike. Fewer than 5% of all oocytes retrieved for infertility treatments ever result in a live birth. Coupled with the severity of the aging problem in humans, and the desire to maintain fecundity in cancer patients, the sources of genetic and epigenetic instability remain poorly understood.[307] Are there intrinsic surveillance mechanisms that can detect and repair damaged DNA as a result of prolonged oxidative stress during aging, exposure to environmental toxins or endocrine disruptors, or manipulations from human ARTs (cryopreservation, culture). If molecular alterations can be identified that distinguish high- from low-quality oocytes, what measures can be devised that might restore and/or ameliorate the compromised performance of these oocytes? Will metabolomics approaches define these differences and will they be rectifiable by medium supplements? These and other questions in the realm of clinical practice will await the acquisition of basic knowledge obtained from studies in animal models.

The final prospect of oocyte biology that leaves many questions is that concerning the capability to manipulate the process of oogenesis in vivo or in vitro. Here, the power and potential of stem cell biology have already gained inroads in demonstrating proof of principle for the ability of ESCs and iPSCs to reenter the female germline and propagate oocytes after transplantation in murine models.[303] Such in vivo approaches emphasize the importance of the ovarian niche for directing appropriately primed stem cells to reenter meiosis and recapitulate oogenesis in a surrogate ovary, reinforcing the notion that somatic cell interactions with oocytes are essential for folliculogenesis to proceed.[31] But this approach is of low efficiency and long-term consequences on offspring health have yet to be fully evaluated. Will the generation of oocytes and the completion of oogenesis ever be possible in an ex vivo environment? Some steps have been recapitulated in in vitro experiments using cow and human materials, but only with the original support of the oocyte–follicle complex. Any attempts to recapitulate oogenesis from stem cells will require close attention to the detailed structural and functional requirements that establish and maintain the elaborate communication system between the oocyte and granulosa cells; the connectome, as it were, is emerging at the heart of many efforts to obtain some degree of ex vivo functionality for various organs. Herein lies a future for the biology of the mammalian oocyte that will likely contribute to the advancement of our basic understanding and at the same time continue to impact the fields of reproductive and regenerative medicine.

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CHAPTER

3

The Spermatozoon

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INTRODUCTION

The term "sperm" is derived from the Greek word "sperma," meaning seed. The Dutch merchant Antonie van Leeuwenhoek is credited with using his handcrafted lenses and microscopes to be the first to observe live spermatozoa and to report in letters to the British Royal Society in 1637 that sperm have a head and a motile tail. Although initially controversial, the observations by van Leeuwenhoek and others led to the development of the cell theory: that all living organisms are made of cells, new cells are created by division of old cells, and cells are the basic building block of tissues, organs, and organisms. Another Dutch microscopist, Nicolaas Hartsoeker, who learned lens making from van Leeuwenhoek, is credited with observing in 1694 tiny men inside human sperm and calling them homunculi or animalcules. This led to the development of the spermists' theory explaining conception, that the sperm was a little man that grew into a child in the womb.

The German zoologist, Oscar Hertwig, is credited with discovering fertilization by observing in 1876 the penetration of a sperm into an egg cell in sea urchins. The many variations in the size and shape of sperm of different organisms were studied by biologists while the light microscope was the main research tool, but it was not until the development of the electron microscope that the remarkable complexity of spermatozoa was appreciated.

Studies beginning in the early 1960s by Don Fawcett and others, and continuing to the present, have led to a rich accumulation of knowledge regarding the nature and organization of the structural components of spermatozoa. Around this same time, biochemical studies began to identify enzymes responsible for the energy production in sperm needed by the flagellum to maintain motility and the major proteins of the acrosome, nucleus, and flagellum. It became possible to pinpoint the location of some of the proteins when antibodies to known proteins became available. The subsequent development of methods for generating monoclonal antibodies led to the identification and localization of previously unknown sperm components and to the definition of the restricted surface domains of spermatozoa. However, it has only been during the last 20 years that gene targeting methods have allowed studies to determine experimentally the roles of specific proteins in the structure and function of spermatozoa. Most of these studies have been carried out in the mouse for technical reasons, but have led to the identification of genes essential for fertility in the human male. One of the surprising findings of the genomic era has been that many of the proteins present in sperm are encoded by genes or variant transcripts that are not expressed in other cells of the body. Although there is a great deal to be learned about the regulation of expression of the genes and proteins that form the sperm and how they assemble and function, there is now a wealth of knowledge about the structure and physiology of sperm, as will be described in this chapter.

The spermatozoon is the end product of spermatogenesis proceeding through successive processes of mitotic, meiotic, and postmeiotic phases within the seminiferous tubules in the testis. During the mitotic phase, the germline stem cells undergo a series of divisions that expands the spermatogonial population. The DNA of male germ cells is reprogrammed in this phase. The first meiotic

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phase begins with the last cell cycle S phase and culminates in the second meiotic phase that rapidly occurs without DNA replication to produce haploid spermatids. Spermatozoa are formed during the postmeiotic phase characterized by extensive remodeling of spermatids into spermatozoa by formation of the acrosome, nuclear condensation, flagellar development, and shedding of the cytoplasm. These events result in production of a sperm cell highly differentiated in structure and function. It is developmentally totipotent and capable of combining with an egg to initiate the next generation. The spermatozoa have a small amount of cytoplasm and lack most organelles common to somatic cells.

The sperm head region is elaborately organized and efficiently leads to gamete fusion with the aid of flagellar motility. During the transit from the male to the oocyte, sperm DNA packaged very tightly in the nucleus is protected from damage by external conditions. Spermatozoa released from the testis are immature and in a transcriptionally and translationally silent state. They become mature to gain fertilizability and motility during passage through the epididymis and female reproductive tract. This maturation occurs in the absence of gene transcription and de novo protein synthesis, but depends upon posttranslational modifications and remodeling of constituent molecules under the influence of products of the epithelial cells lining the reproductive tract.

The main components of a sperm are the *head* and *flagellum* (tail), joined by the *connecting piece* or the neck (Figure 3.1). The head contains the nucleus, acrosome, and a small amount of cytoplasm, in which cytoskeletal structures are elaborately organized. The nucleus contains highly condensed chromatin and is capped anteriorly by the *acrosome*, a membrane-enclosed cytoplasmic vesicle containing hydrolytic enzymes and novel molecules required for fertilization. Starting from the connecting piece (neck), the flagellum is divided successively into the midpiece, principal piece, and endpiece regions. The connecting piece contains centriolar derivatives and the segmented columns. The flagellum contains a central complex of microtubules forming the *axoneme*, surrounded by *outer dense fibers* extending from the neck into the principal piece. The midpiece contains the *mitochondrial sheath*, a tightly wrapped helix of mitochondria surrounding the outer dense fibers and axoneme. Most of the length of the flagellum is the principal piece, defined by the presence of a *fibrous sheath* surrounding the outer dense fibers and axoneme. The outer dense fibers and the fibrous sheath are cytoskeletal structures novel to the sperm flagellum in higher vertebrates.[1] The endpiece is the final segment and contains only axonemal microtubules. The head and flagellum are tightly enclosed by the plasma membrane and contain a sparse amount of cytoplasm. There are substantial species-specific differences in the size and shape of the head, and in the length and relative amount of the different components of the flagellum. Spermatozoa from invertebrates usually have a head with an acrosome and a flagellum containing an axoneme and mitochondria, but lacking accessory cytoskeletal structures.[2]

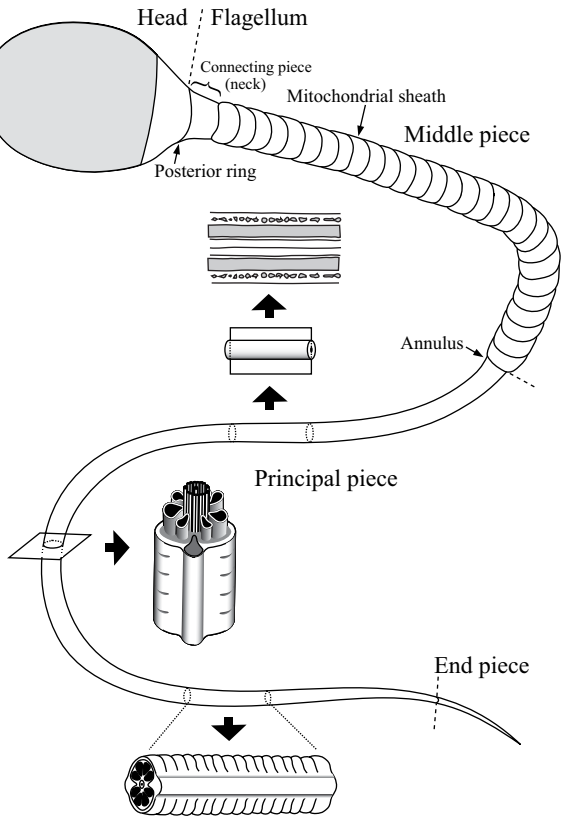

FIGURE 3.1 General features of the mammalian spermatozoon. The head of the sperm is attached to the flagellum by the connecting piece at a border termed the posterior ring. The regions of the flagellum are the middle piece, the principal piece, and the endpiece. The annulus forms a border between the middle piece and the principal piece. The middle piece contains the mitochondrial sheath, while the principal piece contains the fibrous sheath. Longitudinal and cross-sectional views of the principal piece and a segment of fibrous sheath are indicated by arrows. The internal components of the flagellum are identified in Figures 3.8–3.12.

The specialized structural features of mammalian spermatozoa reflect the unique functions required for fertilization. The acrosome contains specific enzymes and matrix molecules required for penetrating the egg investments and achieving competency to achieve fertilization, while the flagellum contains the energy sources and machinery to generate the motility necessary for the sperm to reach the egg. These functions are essential for delivery of the paternal genome to the ooplasm, where the haploid male and female pronuclei combine to produce the zygote and initiate development for the next generation. In most mammals, the sex chromosome carried in the haploid sperm nucleus determines the sex of the resulting animal. Both a paternal and a maternal

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genome are required for normal development to proceed to term, usually due to differential imprinting of genes during gametogenesis in males and females.[3]

This chapter focuses on the characteristics of mammalian spermatozoa,[4] with particular attention to the molecules currently known to contribute to their structures and functions. The major topics considered are the organization, composition, and function of the plasma membrane, the head, and the flagellum. Other chapters and the previous edition[5] should be consulted for fundamental and additional information, necessary for understanding the role of the structural and functional features of spermatozoa in reproductive processes.

THE SPERM PLASMA MEMBRANE

The Mosaic Sperm Surface

Spermatozoa have a characteristic polarized morphology and their surface is highly differentiated and subdivided into regional domains that differ in composition and function. Sperm surface differentiation and segregation are established during spermatogenesis. This segregation is presumably important for preventing the premature initiation of events such as the acquisition of motility, hypermotility, capacitation (acquisition of the ability to undergo the acrosome reaction and achieve hyperactivated motility), the acrosome reaction, and the sperm-egg interaction that should occur in the most appropriate timing during fertilization. However, even if once established, some molecules that are sequestrated in these domains can be redistributed within and between defined domains after release of mature spermatids into the seminiferous tubule lumen (spermiation). These changes cause selective mixing and shuffling of proteins between domains, which eventually induces coordinated protein interactions and specific signaling pathways that culminate in functional changes. Thus, the sperm functional domains are dynamic during the life of the cells.[6,7]

The major regions of the plasma membrane on the sperm heads of most mammals are the *acrosomal region* (anterior head) and the *postacrosomal region* (posterior head) (Figure 3.2). The plasma membrane of the acrosomal region can usually be subdivided into the *marginal segment* domain (apical segment, peripheral rim) over the anterior margin of the acrosome, *principal segment* domain (anterior acrosomal domain) over the major portion of the acrosome, and *equatorial segment* domain (posterior acrosomal domain) over the posterior part of the acrosome (Figure 3.3). The size and shape of these domains vary between species[5] (Figure 3.2). The marginal and anterior acrosomal domains together are referred to as the acrosomal cap. A central crescent

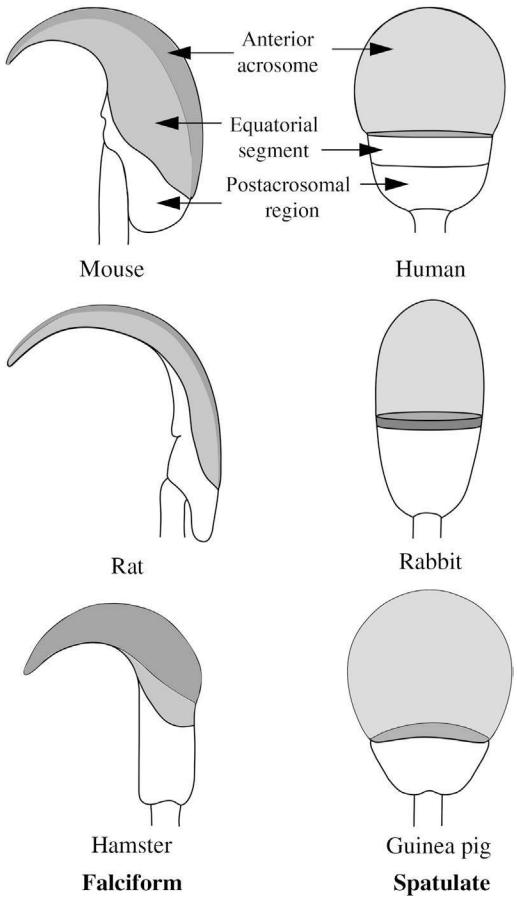

FIGURE 3.2 General features of the heads of falciform-shaped (mouse, rat, and hamster) and spatulate-shaped (human, rabbit, and guinea pig) spermatozoa. Lateral view. The size and shape of the spermatozoon head is specific to each species and varies considerably between species. The major regions of the sperm head are the acrosomal region or anterior head (anterior acrosome and equatorial segment (posterior acrosome)) and the postacrosomal region or posterior head. The acrosomal region of the falciform-shaped mouse sperm head has a relatively small anterior acrosome compared to the spatulate-shaped human sperm. The postacrosomal region is that portion of the sperm head not covered by the acrosome.

domain that separates the marginal and principal segment is found in the guinea pig.

The plasma membrane of the postacrosomal region (posterior head, postacrosomal segment) covers the region between the posterior margin of the acrosome and the connecting piece. The margin between the acrosomal and postacrosomal regions is delimited in some species by the serrated band (subacrosomal ring), which girdles the sperm head at the posterior margin of the equatorial segment.

The posterior ring (nuclear ring, striated ring) is located at the junction between head and connecting piece and believed to form a tight seal (barrier) between the cytoplasmic compartments of the head and flagellum (Figures 3.1, 3.3 and 3.4). The plasma membrane of the flagellum is divided into the middle piece (midpiece)

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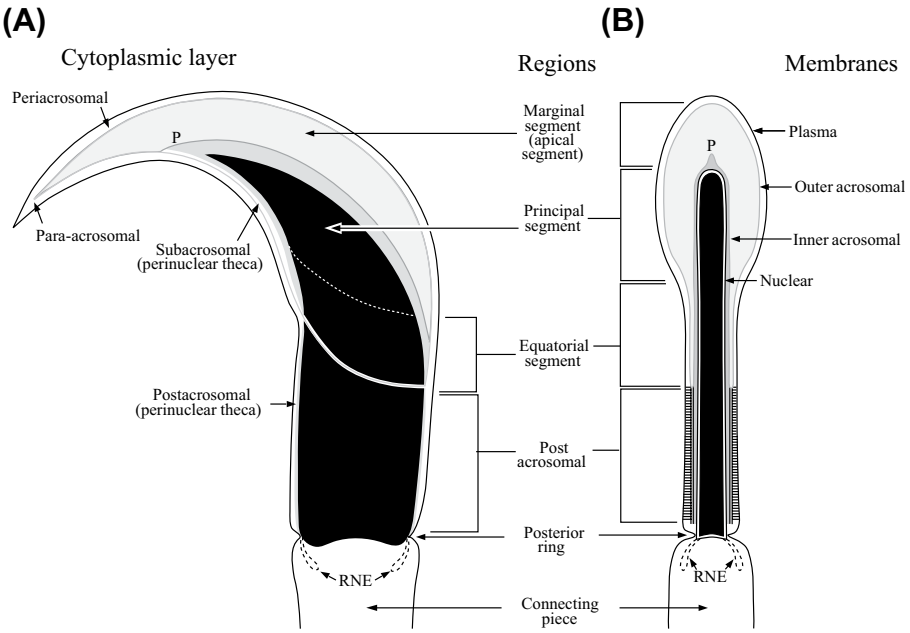

FIGURE 3.3 The distribution of the cytoplasmic layer, regions, and membranes on a falciform-shaped hamster sperm head is shown schematically. (A) Sagittal section. (B) Frontal section. The cytoplasmic layer includes the para-acrosomal layer, the subacrosomal layer, and the postacrosomal layer. The regions include the marginal segment (apical segment) and principal segment of the anterior acrosome, the equatorial segment or the posterior acrosome, and the postacrosomal region between the posterior margin of the acrosome and the posterior ring. The outermost plasma membrane encloses the entire spermatozoon, while the outer acrosomal and inner acrosomal membranes form a continuous enclosure of the acrosomal contents. The nuclear envelope is a double membrane enclosing the nucleus. The subacrosomal cytoskeleton (perforatorium) is located between the inner acrosomal membrane and the nuclear envelope. The postacrosomal cytoskeleton is located between the plasma membrane and the nuclear envelope. Together these are referred to as the perinuclear theca. *Source: Modified from Ref. 6.*

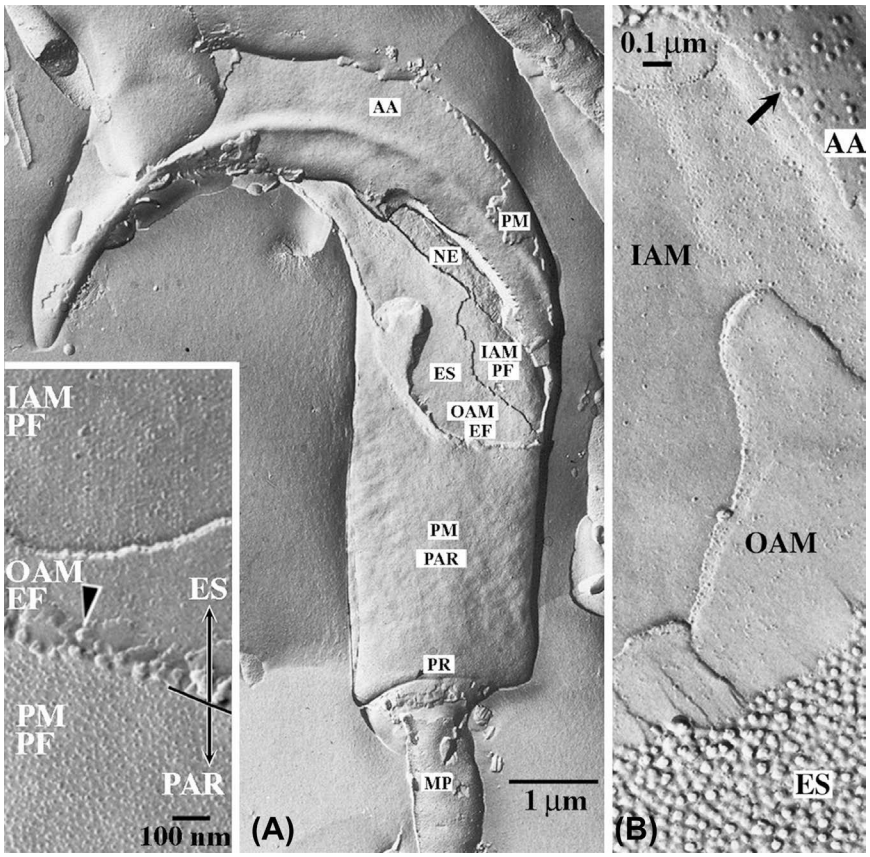

FIGURE 3.4 A freeze-fracture image of golden hamster sperm generated with a membrane cholesterol probe, filipin. (A) Lateral view. This image corresponds to that shown in Figure 3.3(A). Inset; freeze-fracture images of the plasma membrane, and outer and inner acrosomal membranes at the posterior acrosome (ES) and the postacrosomal region (PAR). (B) Freeze-fracture images of the plasma membrane, and outer and inner acrosomal membranes at the anterior acrosome (AA) and the equatorial segment (ES). The membrane features and compositions of the plasma membrane, outer and inner acrosomal membrane, and nuclear envelope appear to be different in the domains. Cholesterol is abundant in the equatorial segment (B), but low in amount in the postacrosomal region (A, inset). Arrowhead in the inset indicates the complex of filipin–cholesterol. An arrow in B indicates the border of the marginal domain and the principal domain of the anterior acrosome region. EF, E-face of the fractured membrane; IAM, inner acrosomal membrane; MP, middle piece; NE, nuclear envelope; OAM, outer acrosomal membrane; PF, P-face of the fractured membrane; PM, plasma membrane; PR, posterior ring. *Source: Modified from Toshimori K, et al.* Cell Tissue Res *1987;**250**(3):673–80.*

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region, principal piece region, and endpiece region. The middle piece and principal piece regions are separated by the annulus (Jensen's ring), a fibrous ring that is a cytoskeletal component adhered to the inner surface of the plasma membrane (Figure 3.1). Most sperm-surface domains are established during spermiogenesis as spermatids are remodeled into spermatozoa. However, spermatozoa undergo additional shape and surface changes during epididymal maturation, and some domains acquire their final form and composition. The middle piece is modified as sperm transit the epididymis by the cytoplasmic droplet migrating from the anterior to the posterior end and being shed. The cytoplasmic droplet does not move beyond the annulus. The shape and composition of the sperm head can change, the acrosome can undergo a reduction in size, and proteins on the surface of the sperm head can migrate to other domains.[6,7]

The surface of the sperm tail (also of the head) binds proteins secreted from the epithelial lining of the epididymal duct. Secretions from the epididymis, which contain a decapacitation factor and a variety of other components, coat the sperm surface. It is hypothesized that the temporal regulation of surface expression of surface components directs them to their correct surface domains, with newly synthesized proteins being inserted into sperm domains. Proteins might not diffuse into more posterior domains because they either are anchored in the anterior domain, or barriers exist to prevent diffusion between domains. Although this model suggests that localization of surface components to specific domains might not require a specific sorting mechanism, signals might exist that identify them for removal from inappropriate domains.

Identification of Domains

Surface Charge, Lectins, and Freeze-Fracture

Sperm domains have been identified using a variety of labeling methods.[5] These include surface charge labeling (e.g., net negative charge on the tail), lectin staining, freeze-fracture/freeze-etch/fracture-labeling, surface replication, and atomic force microscopy (AFM). In addition, specific membrane-intercalating agents such as a polyene that complexes β-hydroxysterols with sterol, filipin, and a fluorescent lipid analog, 1,1′-dihexadecyl-3,3′-tetramethyl-indocarbocyanine perchlorate (C_{16}diI),[5] have been used. For example, AFM revealed that the equatorial subsegment has a species-specific size and shape and that its topography is distinguishable from the plasma membrane overlying the anterior acrosome, equatorial segment, and postacrosome in *Artiodactyla* spermatozoa.[8] Filipin develops filipin-sterol complexes between plaques of intramembranous particles and clearly identifies the difference of sperm membrane domains (Figure 3.4). Domains are also observed using C_{16}diI, which intercalates into the outer leaflet of the plasma membrane. The acrosomal region of ram sperm is labeled more intensely than the postacrosomal region with this agent.[9] The difference in affinity of the probe for these regions apparently results from its interactions with lipids and proteins heterogeneously distributed within the plane of the membrane.

Antibodies and Green Fluorescent Protein

Antibodies can be conjugated directly with various labels and detected directly or indirectly with secondary antibodies for light or electron microscopy. Many antisera have been produced, but the multiple and variable specificities of these polyclonal antisera to whole cells or mixtures of antigens often has limited their usefulness for dissecting the distribution of specific sperm-surface components, for defining the biochemical characteristics and functional roles of such components, and for identifying the molecules recognized. Some of these problems were circumvented using monoclonal antibodies to identify molecules localized to subdomains of the sperm surface. Their use confirmed that the surface of sperm from many different species consists of well-defined domains and demonstrated that different proteins and glycoproteins may be either confined to individual domains or shared by multiple domains. However, attempts to use immunoprecipitation with monoclonal antibodies to identify specific proteins can also result in the isolation of multiple proteins that might either be subunits of a molecular complex or functionally unrelated molecules that share a common epitope. For example, a glycosphingolipid, GalNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glcβ1-ceramide that is recognized by MC121 monoclonal antibody is present on different molecules, including the 54-kDa sialoglycoprotein in mouse cauda epididymal spermatozoa and lipids of human red blood cells.[10] Specific protein-tagged probes such as green fluorescent protein (GFP) used for GFP-transgenic mice have proven useful for determining the localization and behavior of specific molecules of plasma membrane domains under in vivo conditions.[11]

Localized Sperm Membrane Components

The sperm surface has a mosaic surface and reflects the localized distribution of different proteins. Ca^{2+} channel proteins localized to specific regions of the sperm plasma membrane are involved in regulating Ca^{2+} homeostasis required for various sperm functions. The CATSPER (cation channels, sperm associated) family contains four sperm-specific alkalinization-activated, voltage-gated Ca^{2+} channel proteins (CATSPER1, 2, 3, 4) and is highly conserved in humans and mice. CATSPER1 is a six transmembrane spanning, voltage-gated Ca^{2+}-channel protein necessary for regulation of Ca^{2+} entry,

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and is present in the plasma membrane of the principal piece region of the mouse sperm flagellum.[12] CATSPER2 is also located in the plasma membrane of the flagellum. Ca^{2+} ions entering the channels in the principal piece trigger intracellular Ca^{2+} concentration increases in the midpiece and head through unknown mechanisms.[13] Both CATSPER1- and CATSPER2-null male mouse are infertile due to failure of hyperactivation of spermatozoa, and the null sperm cannot fertilize zona-intact eggs in vivo.[12,14] CATSPER protein complex contains pore-forming proteins and three additional proteins, CATSPERB, CATSPERG, and CATSPERD. The trafficking and/or assembly of CATSPERB and CATSPERG depend on CATSPER1.[15,16] CATSPERD was identified as the product of the *Tmem146* gene.[17] In humans, at least two mutations in the *CATSPER1* gene are found to cause *CATSPER1*-related nonsyndromic male infertility.[18] Disruption of the gene for a subunit (alpha 1E) of $Ca_v2.3$ (*Cacna1e* gene) resulted in increased straight-line velocity and linearity parameters of sperm motility as determined by computer-assisted sperm analysis.[19] Another group of voltage-gated Ca^{2+}-channel proteins is present in the plasma membrane of the acrosome ($Ca_v1.2$ and $Ca_v2.2$), the postacrosomal region ($Ca_v2.3$), and the principal piece ($Ca_v1.2$, $Ca_v2.2$, and $Ca_v2.3$) of mouse sperm.[12] $Ca_v1.1$–$Ca_v1.4$, $Ca_v2.1$–Ca_v3 and $Ca_v3.1$–$Ca_v3.3$ are recently identified.[20]

Plasma membrane Ca^{2+}-transporting ATPase 4 (PMCA4, ATP2B4) is a widely expressed housekeeping enzyme that is a member of a family of proteins (PMCA1-4) that move Ca^{2+} from the cytosol across the plasma membrane, and is localized primarily to the principal piece and to a lesser extent to the acrosome of mouse spermatozoa. PMCA4 can be activated by PDC-109, a secretory protein of bovine seminal vesicles.[21] PDC-109 provokes a cholesterol and phospholipid efflux from membranes by binding to sperm surface choline lipids.[22] Deletion of the *Pcma4* gene resulted in a substantial increase in sperm intracellular Ca^{2+} concentrations and in male infertility due to a failure of hyperactivated sperm motility.[23–25] In the mouse, the major splice variants are PMCA4a and PMCA4b. PMCA4a differs from PMCA4b in the mechanism of calmodulin binding and activation, showing a much higher basal activity and being more effective than PMCA4b in returning Ca^{2+} to resting levels.[26] In the bull, PMCA4b is the major splice variant in testis, caput, and corpus epididymidis, but PMCA4a is the major variant in the cauda epididymidis for transferring Ca^{2+} across sperm membranes, suggesting the importance of isoform switching for facilitation of a higher Ca^{2+} turnover in epididymal spermatozoa.[26]

Other proteins are identified in restricted regions of the sperm surface. GLUT8 (*Slc2a8* gene) is a class three sugar transport facilitator predominantly expressed in testis and brain. Targeted disruption of *Slc2a8* reduces ATP levels, motility, and mitochondrial potential of spermatozoa.[27] Mouse GLUT9 protein (*Slc2a9* gene) has multiple splice variants. GLUT9a comprises a long form having 12 transmembrane segments, and GLUT9b comprises two short forms each having a deletion resulting in the loss of two transmembrane-spanning domains.[28] GLUT9a is present in the midpiece, while GLUT9b is present in the acrosome, midpiece, and principal piece.[28] Basigin (BSG, EMMPRIN, CE9, HT7) is involved in cell–cell interactions in the testis and is essential for the completion of spermatogenesis in mice, since deletion of the *Bsg* gene caused meiotic arrest.[29] BSG is expressed during spermatogenesis beginning in spermatocytes and associates primarily with the principal piece in caput epididymal spermatozoa and the middle piece in cauda epididymal spermatozoa.[30] BSG interacts with H^+-monocarboxylate co-transporters (MCT), MCT1 and MCT2, to locate them properly in the membrane of spermatogenic cells.[31] This suggests that BSG may enable sperm to utilize lactate as an energy substrate, contributing to sperm survival.

Some sperm proteins undergo chemical processing and modification of their location while sperm traverse the epididymis. In addition, proteins are attached to the sperm surface during epididymal maturation. They include chaperones calmegin (CLGN) and calreticulin (CALR, calsperin); a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif 10 (ADAMTS10); proprotein convertase subtilisin/kexin type 4 (PCSK4, PC4); cysteine-rich serine protein 1 (CRISP1); defensins; phospholipase C zeta 1 (PLCZ1); and the antigens SMA4 and T21.

Chaperones are cell stress-response or heat-shock proteins. Because molecular chaperones participate in normal cellular functions such as metabolism, growth, differentiation, and apoptosis,[32] they were initially thought to be restricted to intracellular organelles. However, molecular chaperones exist on the sperm surface and play secondary roles during functional transformation in the epididymis and female reproductive tract.[33–35] For example, testis-specific molecular chaperones, CLGN and CALR, are necessary for a disintegrin and metallopeptidase domain 1a (ADAM1A)—ADAM2 dimerization, subsequent maturation of ADAM3, and/or presentation on the sperm surface.[33,36] CLGN and CALR-null male mice are infertile with impaired sperm transport in the female reproductive tract in vivo and the loss of sperm–zona binding ability.[33,36] These phenotypes are the result of misexpression, misprocessing, and/or misfolding of fertilization-related proteins due to impairment of chaperone functions. A similar phenotype was documented by targeted deletion of *Adam1a* and *Adam3* genes.[37] These data suggest the link between ADAM protein expression and fertilization.

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ADAMTS10 is a novel sperm protein of the metalloprotease superfamily. ADAM family metalloproteases are known to mediate diverse cell and extracellular matrix adhesion events across a variety of cell types.[38] In the mouse, ADAMTS10 is initially expressed during the late stages of spermatogenesis and incorporated into the acrosomal domain of developing spermatids. This protein is chemically processed during sperm maturation and then expressed on the surface of the periacrosomal region.[35] ADAMTS10 is a candidate molecule for the functional redundancy involved in the mediation of sperm–oocyte interactions such as binding to zona pellucida.

PCSK4 originates in the testis and is integrated into the sperm surface. PCSK4 belongs to a nine-member family of Ca^{2+}-dependent serine endoproteinases involved in the proteolytic activation of secretory precursor proteins into their bioactive forms and is conserved in mammals. Deletion of the *Pcsk4* gene did not impair spermatogenesis but caused severe male subfertility, rendering sperm incapable of fertilizing oocytes.[39] In mouse sperm, PCSK4 is localized on the plasma membrane overlying the acrosome.[40] ADAM2 (fertilin beta) is a substrate for PCSK4.[41]

CRISP1 is secreted by the epididymal epithelium, and attached to the sperm surface. It is redistributed to the equatorial segment after capacitation in the rat.[42] CRISP1-null mouse sperm are fertile, but they showed lower levels of tyrosine phosphorylation than controls, suggesting a regulatory role during sperm capacitation.[43] CRISP4 is secreted predominantly in the mouse and rat epididymis as a soluble form associated with small membranous vesicles secreted into the epididymal lumen, called epididymosomes in the luminal fluid, and becomes bound to the sperm surface.[44] The CRISP domain of CRISP4 inhibits the transient receptor potential ion channel transient receptor potential cation channel, subfamily M, member 8 (TRPM8).[45] TRPM8 is a major cold receptor in the body and is found in the testis and on the tail and head of mouse and human sperm. CRISP4-null mouse spermatozoa are compromised in their ability to undergo the acrosome reaction in response to the physiological agonist progesterone, suggesting that CRISP4 is an endogenous regulator of TRPM8 in normal sperm function.[45] Defensin beta 126 (DEFB126) in macaques and defensin beta 22 (DEFB22) in mice are glycosylated peptides derived from the epididymis adsorbed onto the entire sperm surface.[46,47] DEFB126 has a conserved defensin beta core and a unique long glycosylated peptide tail. The carbohydrates of this domain contribute substantially to the surface glycoproteins that contribute to the sperm glycocalyx. DEFB126 loss is required for sperm recognition of the zona pellucida.[48] DEFB126 is critical for efficient transport of sperm in the female reproductive tract, preventing their recognition by the female immune system, and might facilitate the delivery of capacitated sperm to the site of fertilization.[47] Mouse DEFB22 is a 130-kDa protein, but migrates at 54–57 kDa under reducing conditions.[49] Although the presence of a cell coat is a common feature of many cell types, the carbohydrate structure of such coats and their contribution to the negative cell surface charge are yet to be clarified.

The T21 antigen, a 54-kDa glycoprotein recognized by T21 monoclonal antibody, is secreted from the epididymal epithelium and attaches to the mouse sperm tail.[50] It is heavily sialylated and the epitope (carbohydrate region) for T21 antibody is effectively masked.[50] The nature of the 54-kDa sialoglycoprotein is similar to DEFB22, but it is unclear whether the 54-kDa sialoglycoprotein is the same as DEFB22. The carbohydrate typically associated with a negative charge is sialic acid. The sialic acid is most often found as the terminal sugar in the oligosaccharide, thereby creating a negative shell around the molecule and extending its life.

Formation and Maintenance of Domains

The separation of different membrane proteins into specific domains allows the compartmentalization of responses by spermatozoa to external conditions and signals. In some cases, barriers to diffusion of plasma membrane proteins develop during spermiogenesis at the same time as membrane domains.[51] Most surface domains overlie distinct cytoplasmic organelles or features. In spermatozoa, transmembrane proteins stabilized by linkage through membrane skeleton proteins to cytoskeletal structures may be important for defining the boundaries and contents of different sperm-surface domains.

Actin and Associated Proteins

Cytoskeletal proteins in spermatozoa participate in the rearrangement and shaping of nuclear and cytoplasmic components, rearrangement of domains, and have a role in the acrosome reaction. The acrosomal region contains the testis-specific proteins, actin-related protein T2 (ACTRT2),[52] activity regulated cytoskeletal-associated protein (ARC),[53] and fascin homolog 3, actin-bundling protein, testicular (FSCN3).[54] Actin filaments were found in the subacrosomal space in rat sperm,[55] acrosome in guinea pigs,[56] the postacrosomal region of rat, rabbit, bull, and boar sperm, and in the connecting piece of hamster, rabbit, bull, and human sperm.[57] They also were detected in the posterior region of the head and in the connecting piece, middle piece, and principal piece of human sperm,[58,59] and along the concave margin of the hamster sperm head.[58] The testis-specific profilin 3 (PFN3)-actin related protein T3 (ACTRT3) complex was implicated in conformational changes in the organization of the sperm-specific nucleus.[60] In addition, myosin

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is a component of the nuclear plate that connects the acrosome with the nuclear envelope.[61] Also, the perinuclear theca contains actin-related proteins Arp-T1 and Arp-T2,[62] the actin-binding protein calicin (CCIN),[63] and the capping protein (actin filament) muscle Z-line, alpha 3 (CAPZA3, CPα3) and capping protein (actin filament) muscle Z-line, beta 3 (CAPZB3, CPβ3) subunits of a testis-specific actin-capping protein complex.[64] Furthermore, human spermatozoa contain spectrin in the anterior acrosome and principal piece regions[65] and rabbit sperm contain spectrin in the anterior acrosome region and postacrosomal region of the head and the principal piece of the tail.[66] Finally, Ca^{2+}-dependent protease, calpain-1 (CALPN1), originally located in the sperm cytoplasm, relocates from the cytoplasm to the plasma membrane during capacitation in guinea pigs, possibly serving to cleave spectrin and facilitating the acrosome reaction.[67]

Maintenance of Surface Domains

The maintenance of sperm-surface domains and the segregation of specific proteins into domains probably involves several processes: restriction of mobility of surface molecules in their final domain, existence of barriers to movement of surface components at the domain boundary, and thermodynamic partitioning of molecules into specific regions.[68] For example, ADAM2 is evenly distributed over the entire head of testicular sperm but becomes restricted to the posterior head as sperm transit the epididymis in the guinea pig probably due to proteolytic processing of the β fertilin subunit in the epididymis.

Associations between the plasma membrane and internal structures are believed to form barriers between domains and serve to restrict the movement of some proteins. The plasma membrane is closely associated with the posterior ring at the junction between the head and neck (connecting piece), and with the annulus at the junction of the middle piece and principal piece. Two antigens (2B1, 2D6) present over the whole surface of the flagellum of rat sperm showed antibody-induced patching, but did not migrate onto the head,[69] perhaps due to the posterior ring. However, BSG apparently was not restricted from redistribution by the posterior ring and annulus.[30] The PT-1 antigen exhibited free diffusion within the plasma membrane of the distal tail domain, but was prevented from migrating into the midpiece, possibly by the annulus.[70]

Other structures that might serve to constrain or anchor plasma membrane components are the groups of intramembranous particles seen in freeze-fracture studies. These include zipper-like array of particles associated with the ribs of the fibrous sheath and oblique strands of intramembranous particles associated with the underlying mitochondria.

Sperm Plasma Membrane Lipids

Lipid Composition

Sperm plasma membranes contain relatively high amounts of ether phospholipids known as plasmalogens (20–40%), other ether-linked phospholipids,[71] and lipids with long, polyunsaturated aliphatic chains.[72] Phospholipids make up about 70% of the total plasma membrane lipid in boar sperm,[73] and choline phospholipids account for almost two-thirds of phospholipids in the plasma membrane of the anterior head of ram sperm. Sterols are the next most abundant lipid, resulting in a cholesterol/phospholipid molar ratio of about 0.12.[73] The total cholesterol/total phospholipid ratio, the choline/ethanolamine glycerophospholipid ratio, and the proportion of plasmalogens within these two classes, were much larger in the head than in the tail in the rat.[74] It was suggested that the high cholesterol to phospholipid ratio accounts for the lack of thermotropic phase transitions in the plasma membrane of live human sperm detected by Laurdan fluorescence spectroscopy.[75] The amount of sterol is much higher in the anterior acrosome than in the postacrosomal region in mammalian sperm (Figure 3.4). The postacrosomal region contains few sterols or anionic lipids. Also, cholesterol sulfate makes up only a small fraction of the total sterol content, but is a major component of the plasma membrane over the acrosome of human sperm.[76]

Free fatty acids comprise a relatively small amount of the lipid in boar spermatozoa, whereas diacylglycerols are present in about the same amounts as glycolipids.[73] The phospholipid/protein ratio is approximately 0.68 on a weight basis in plasma membranes isolated from boar spermatozoa,[73] suggesting that the amounts of total lipid and protein are about the same. However, this is for the total plasma membrane, and the amounts and types of lipids and the lipid/protein ratios probably vary between different domains.

Glycolipids may have a significant role in lateral domain organization.[73,77] The major glycolipid in sperm, sulfogalactosylglycerolipid, is present in both the head and tail fractions of spermatozoa.[78]

Diffusion of Lipids

Fluorescence recovery after photobleaching (FRAP) measurements indicate that a large fraction of the plasma membrane lipids of mammalian sperm is not free to diffuse laterally.[9,79] The nondiffusing lipid pool increases to more than 50% of the lipid during epididymal maturation.[9] FRAP studies showed that the lipid diffusion rates were significantly higher over the acrosome and postacrosomal region than on the midpiece and principal piece in live bull, boar, ram, and mouse spermatozoa.[80] FRAP studies also observed a free exchange of a lipid analog by lateral diffusion in the plasma membrane between

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the head and midpiece regions of ram and boar sperm[81] and between the midpiece and distal tail of ram sperm.[9] However, studies using the method of fluorescence loss in photobleaching detected a diffusion barrier to ~200-nm particles between the equatorial segment and postacrosomal domains, but not between the principal and equatorial segments.[82]

When spermatozoa were subjected to hypo-osmotic shock to produce surface blebs, the diffusion rate for lipids were comparable on blebs and on untreated spermatozoa, suggesting that interactions of plasma membrane lipids with underlying cytoskeletal elements do not affect diffusion.[77] In addition, when artificial bilayers were formed from sperm plasma membrane lipid extracts, the fraction of nondiffusing lipid was about the same as that on intact spermatozoa, suggesting that lipid–lipid interactions are responsible for restricting lateral lipid movement.[77] Differential scanning calorimetry studies indicated that significant amounts of gel-phase lipids are present in the anterior region of the head of ram spermatozoa, which allows sperm plasma membrane lipids to segregate by lateral phase separation into coexisting fluid and gel domains at physiological temperatures.[71] The gel domains may contain the nondiffusing lipid fraction present in the sperm plasma membrane.

Lipid Rafts

Lipid rafts (also known as lipid microdomains) are discrete lipid domains present in the external leaflet of the plasma membrane. The rafts are enriched in cholesterol, glycosphingolipids, and glycosyl-phosphatidylinositol GPI-anchored proteins, and are insoluble in low concentrations of nonionic detergents. Lipid rafts are present throughout the surface of mammalian spermatozoa and not restricted to particular domains. Lipid raft-related proteins detected in spermatozoa include caveolin1 (CAV1) on the anterior acrosome and principal piece.[83] Transient receptor-1 homolog (TRPC1) was co-immunolocalized with CAV1.[84] Other lipid raft markers, CD59, GM1 ganglioside, and flotillin2 (FLOT2), were found on the posterior head, and FLOT2 was localized mainly on the posterior head and middle piece in human spermatozoa.[85] Serine protease 21 (PRSS21) was localized in the head, cytoplasmic droplet, and midpiece.[86] Sperm lipid raft dynamics are reviewed elsewhere in relation to sperm–zona binding and the zona pellucida-induced acrosome reaction.[87]

Changes in Lipids with Maturation and Capacitation

Changes in the lipid content and distribution in the spermatozoon plasma membrane occur during epididymal maturation and capacitation, and these may have substantial effects on the composition and function of the membrane in different domains. The total lipid and cholesterol content decreases during epididymal maturation in most species. In addition, the cholesterol/phospholipid ratio and concentration of phosphatidylserine, phosphatidylethanolamine, cardiolipin, and ethanolamine plasmalogen decreases. However, sulfoconjugated sterols and unsaturated fatty acids increased in hamster and human.[88] There was a decrease in the level of fatty acids and an increase in diacylglycerol, but no change in the degree of saturation of fatty acids. Plasma membrane from the anterior head region of ram sperm is particularly rich in ethanolamine and choline phosphoglycerides, and the amount of desmosterol and ethanolamine in this region of the plasma membrane decreases while the cholesterol to phospholipid ratio increases during epididymal maturation. The decrease of plasma membrane fluidity is regionally different, as evidenced by FRAP.[9] However, the diffusion rate of a fluorescent lipid analog increased in all regions of the sperm except the midpiece during maturation.

Cholesterol efflux from the sperm plasma membrane occurs during capacitation. A sulphogalactolipid analog incorporated into the marginal segment of ram sperm migrated to the equatorial segment with capacitation.[89] Capacitation also resulted in the redistribution of CRISP1 on rat spermatozoa,[42] ADAM2 on guinea pig spermatozoa,[90] and BSG on mouse spermatozoa.[30] Freeze-fracture studies of filipin-treated sperm showed a patch-like loss of cholesterol during capacitation from the plasma membrane overlying the acrosome in mouse spermatozoa. This cholesterol change alters the membrane fluidity and the lateral mobility of associated proteins and initiates the protein kinase A signal transduction pathway that promotes capacitation.[91] This membrane condition is presumed to lead to facilitating fusion between the plasma membrane and acrosomal membrane during the acrosome reaction, and between the plasma membrane overlying the equatorial segment and microvilli during gamete membrane fusion. ATP-binding cassette transporter G2 (ABCG2) is an efflux pump that belongs to the ABCG subfamily of the ATP-binding cassette (ABC) transporters and can extrude a wide variety of compounds, including cholesterol, from the cell using the energy produced from ATP hydrolysis. ABCG2 is present in the plasma membrane overlying the acrosomal membrane from bovine testis and epididymis.[92] This location of ABCG2 suggests its possible involvement in the translocation of cholesterol sulfate and its role in epididymal sperm maturation.

THE SPERM HEAD

The mammalian sperm head contains the nucleus and acrosome surrounded by moderate amounts of cytoskeletal components and a small amount of cytoplasm.

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The acrosome caps the anterior end of the nucleus and cytoskeletal components lie in the narrow space between the acrosome and the nucleus and between the acrosome and the plasma membrane. Spermatozoa of most mammalian species have a spatulate head (Figure 3.2), with the nucleus and acrosome flattened in the plane of the anterior–posterior axis of the spermatozoa. The acrosome and nucleus are symmetrical structures. In contrast, the sperm of some rodents have a falciform-shaped head (Figure 3.2), with the acrosome overlying the convex margin of the nucleus. Although spermatozoa are uniform in size and shape within most species, a third or more of human spermatozoa are abnormal in size and shape and frequently have various sizes of nuclear vacuoles or lacunae.

The Sperm Head Cytoplasm (Layer)

The head of the spermatozoon contains a thin layer of cytoplasm. The space between the anterior tip and convex surface of the acrosome and the plasma membrane of falciform sperm is the para-acrosomal space (Figure 3.3). The space between the outer acrosomal membrane and nuclear envelope is the subacrosomal space (perinuclear space) that contains perinuclear theca components. The subacrosomal space can be divided into two subspaces. One is the subacrosomal space of the anterior part of the head and the other is the postacrosomal space where the postacrosomal sheath is present. The cytoplasm or space between the plasma membrane and outer acrosomal membrane is the periacrosomal space. This space is narrow and not clearly visible in mature spermatozoa, but it may be important in maintaining acrosome shape and in early events of capacitation and the acrosome reaction or in sperm-egg membrane fusion.

The Sperm Nucleus

Sperm deliver the paternal genome to the embryo in a novel configuration. The chromatin is highly condensed to 1/13th that of the oocyte,[93] and the nuclear volume is considerably less than 5% of a somatic cell. Sperm chromatin is resistant to much greater mechanical disruption than somatic cells.

Nuclear Proteins

Mammalian sperm have two major nuclear proteins, protamines and histones. Protamines are the predominant nuclear proteins, composed of 27–65 amino acids, and are enriched in the highly basic amino acids arginine and cysteine. The mRNAs encoding mouse protamines are transcribed from the haploid genome in round spermatids and translation is delayed until spermatids are undergoing elongation.[94] The remaining histones are highly acetylated. Most mammals have only one active protamine gene, but mice and humans have two. Both protamines are essential for fertility; in mice heterozygous for a mutation in either gene, the amount of the protein was decreased and sperm DNA stability was disrupted.[95,96] Male mice null for testis-specific serine kinase 6 (TSSK6, SSTK) are sterile due to profound impairment in motility and morphology of spermatozoa resulting from a defect in DNA condensation.[97] Nuclear disruption occurs in elongating spermatids at a step in spermiogenesis coincident with chromatin displacement of histones by transition proteins.

It was suggested that the histones in human spermatozoa might be part of a sequence-specific component of the genome that is programmed for expression in early development. TSSK6 phosphorylated histones H1, H2A, H2AX, and H3, but not H2B or H4 or transition protein one in vitro.[97] The chromobox 1 protein (CBX1, M31) was detected in mouse total sperm proteins on western blots and presumably was associated with centromeres, but histone H1 was not detected.[98] Hamster sperm nuclei appear to contain a DNAse,[99] and enzymes involved in de novo pyrimidine biosynthesis were detected in the nucleus of human spermatozoa.[100]

There are two general models for the association of protamines with DNA. The more widely accepted model suggests that protamines are present in an extended configuration and lie in the major groove of the DNA helix, causing the DNA to become packaged into toroidal loops.[101] Most of the mammalian sperm chromatin is compacted into toroids that contain roughly 50 kb of DNA. Each protamine-DNA toroid forms a loop domain, and these are stacked side to side. The toroid configuration is thought to protect chromatin from oxidizing and from the effects of other harmful molecules within the reproductive tract.[102,103]

Although the large majority of DNA is packaged by protamines, a smaller amount (2–15%) retains histones and the DNA is attached to the proteinaceous nuclear matrix at roughly 50-kb intervals.[101,104] Histones are nonrandomly distributed in the sperm genome and are associated with specific genes.[105,106] Histone-bound segments of DNA are scattered throughout the paternal genome, largely at gene promoter regions.[105] This can allow spermatozoa to transmit histone-based chromatin structural features and histones with specific modifications in the sperm to newly fertilized oocytes,[107] including both histone-bound chromatin and matrix attachment regions in the newly formed paternal pronucleus. It is suggested that the nuclear matrix organization is required for DNA replication in the one-cell embryo, with the histone-bound chromatin identifying paternal genes important for embryonic development.[101,103] Chromatin is stabilized by the formation of covalent disulfide linkages between protamines

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on adjacent DNA strands. Defects in sperm DNA integrity are linked with DNA fragmentation and apoptosis.[108]

Epigenetics

Epigenesis is the heritable transmission of non-DNA-based information. Epigenetic modifications occur in the methylation recycling stages in primordial germ cells, gametogenesis (reprogramming), and postfertilization or embryogenesis.[109] Mammalian sperm carry the paternal genome to the oocyte, and each sperm contains one copy of each gene that bears epigenetic modifications. There are two types of heritable epigenetic changes, DNA methylation and histone modification.

DNA methylation is the status of methylation of cytosine nucleotides of DNA (examples are genomic imprinting and X-chromosome inactivation), leading to monoallelic expression. This occurs on cytosine residues within CpG dinucleotides that exist at differentially methylated regions, and is involved in regulation of gene expression. For example, the *H19* gene is normally methylated and silent in males (paternally methylated), and hypomethylated or unmethylated in females and expressed in the offsprings (maternally demethylated). However, the occurrence of abnormal methylation (e.g., incomplete methylation acquisition) at the *H19* locus was significantly increased in oligozoospermic patients compared with normozoospermic patients.[110] Similarly, unusual hypomethylation of the *H19* gene and hypermethylation of the *MEST* gene (paternally expressed gene) were observed at a higher frequency in oligozoospermic men than in normozoospermic controls.[111]

Histone modification occurs by acetylation, phosphorylation, methylation, sumoylation, and ubiquitylation. During spermatogenesis, canonical histones (H2A, H2B, H3, H4) are replaced with protamine, resulting in chromatin compaction. However, 5%–15% of the sperm genome in fertile men remains bound to histones rather than undergoing protamination.[109,112] During protamine replacement, histones and nucleosomes were retained on promoters of genes for transcription factors and signaling pathway components associated with early embryo development, and these regions also showed significant DNA hypomethylation in the sperm genome.[106,109] A study employing chromatin immunoprecipitation of DNA bound to histone H3 acetylated at lysine 12 (H3K12ac) showed that in human sperm H3K12ac was primarily localized in the subacrosomal region, and that in mouse sperm H3K12ac was more strongly observed in the male pronucleus than the female pronucleus.[113] A positive relationship was found between H3K12ac enrichment at specific gene promoters and an elevated level of mRNA transcription of those genes. Histone H4K12ac was localized in the nucleus at the postacrosomal region, while protamine-1 is present throughout the nucleus.[113] Because histones are retained for potential epigenetic regulatory control of the sperm genome postfertilization, aberrant histone acetylation (e.g., H4K12 deacetylation) that is found within developmentally important gene promoters in infertile men could reflect insufficient sperm chromatin compaction and result in inappropriate transfer of epigenetic information to the oocyte.[113]

Thus, the status of the sperm epigenome implies not only a poising of the paternal genome for embryogenesis and a possible role in the establishment of totipotency of the embryo, but also an association with different types of male infertility.[114]

RNA

Mature ejaculated mouse and human spermatozoa carry messenger RNA, antisense RNAs, and microRNAs.[115,116] For example, ropporin 1 (ROPN1) RNA is localized to the nuclear matrix, suggesting that paternal RNA could contribute to early embryonic development.[117]

Nuclear Envelope

The sperm nucleus is enclosed by an unusual nuclear envelope, which is established during spermiogenesis. Over most of the nucleus, nuclear pores are absent. Caudal to the posterior ring, in the redundant nuclear envelope, sperm nuclear pores are abundant (Figure 3.5).

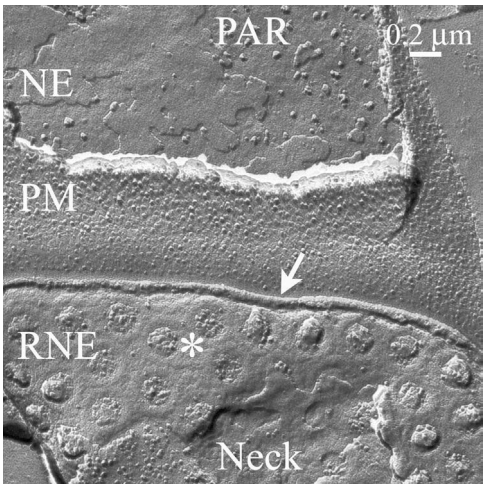

FIGURE 3.5 Freeze-fracture image of the membranes at the neck region. The neck region is connected to the postacrosomal region by the posterior ring. The nuclear pores (*) of the redundant nuclear envelope (RNE) are seen in the neck region. The plasma membrane covering the neck region is not seen because of detachment of the head from the neck region during sample preparation. NE, nuclear envelope; PAR, postacrosomal region; PM, plasma membrane.

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Nuclear Lamina

The nuclear lamina is a protein meshwork lining the inner surface of the nuclear envelope and forms part of the nuclear skeletal network (karyoskeleton) anchoring the chromatin. It consists primarily of lamins, members of the intermediate filament protein family. This network contains lamins A and B (LMNA and LNMB), and splice variants of LMNA and LMNB, lamina-associated polypeptide 2 (LAP2), and the lamin B receptor (LBR). These proteins are detected during spermatogenesis, but except for lamin B1, usually are not detected in spermatozoa. Deletion of the *Lmna* gene resulted in a high level of apoptosis in pachytene spermatocytes and the disruption of spermatogenesis.[118] Deletion of the germ cell-less homolog 1 (*Gmcl1*) gene caused abnormally shaped spermatozoa, suggesting involvement for head shaping via the association between the nuclear lamin and perinuclear theca substance in mice.[119] The dpy-19-like-2 (DPL19l2) protein is located in the inner nuclear membrane. This new member of a family of structural transmembrane proteins was reported to anchor the acrosome to the nucleus.[120] In DPY19l2-null male mice, the acrosome and the manchette (a transient microtubular structure surrounding the elongating spermatid nucleus) failed to link to the nucleus. This led to the formation of round-headed sperm (globozoospermia) apparently caused by disruption of vesicular trafficking and destabilization of the nuclear dense lamina and the junction between the acroplaxome and the nuclear envelope.

Nuclear Pocket

During chromatin condensation in spermiogenesis, smaller areas of the nucleus (0.1–0.2 μm) remain uncondensed and manifest a fine granulofilamentous substructure. While this process takes place, pores move caudally and concentrate in the redundant nuclear envelope that demarcates the clear nuclear pocket around the sperm neck.[121] Polyubiquitinated proteins are found in the nucleus of rat spermatids and human spermatozoa, and proteasomes are present in the small uncondensed chromatin regions and nuclear pocket area.[122] In most cells, proteasomes are proteolytic macromolecular complexes that digest polyubiquitinated proteins.

Nuclear Lacunae or Vacuoles

When observed with the electron microscope, nuclear lacunae or vacuoles appear as large (1–3 μm) defects of irregular shapes. They are found in human sperm, but not in sperm of other primates and mammalian species. The vacuoles are sharply delimited from normally compacted chromatin.[121] The nuclear vacuoles sometimes contain cytoplasmic or acrosomal components.[123,124] Because surplus cytoplasm usually is phagocytosed by Sertoli cells and discarded during normal spermiogenesis, the nuclear vacuoles that contain surplus cytoplasm could have deleterious effects on the function of the paternal genome in the fertilizing sperm.[124]

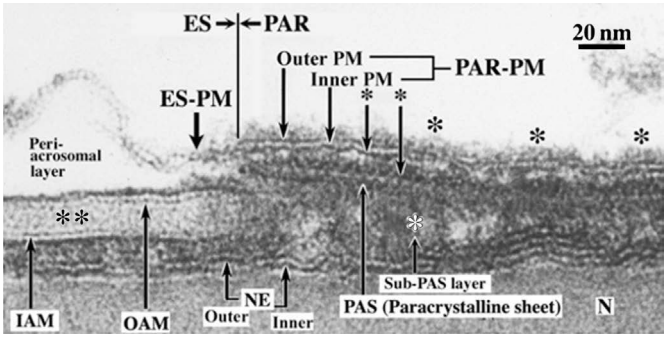

FIGURE 3.6 Transmission electron micrograph of the border between the equatorial segment (ES) and the postacrosomal region (PAR) in mouse sperm. In the ES, the periacrosomal layer is expanded and appears to be electron-lucent. The acrosomal lumen between the outer acrosomal membrane (OAM) and inner acrosomal membrane (IAM) at the equatorial segment is filled with the acrosomal matrix (**). The PAR has complex features. Glycocalyx-like substance (*) is seen over the postacrosomal plasma membrane (PAR-PM). The postacrosomal sheath (PAS) or the paracrystalline sheet is connected by an electron-dense periodic substructure (asterisks with arrows) to the plasma membrane. The sub-PAS layer is filled with electron-dense material (white asterisk), which is a component of the perinuclear theca surrounding the enitre nucleus (see Figure 3.3). N, nucleus; NE, nuclear envelope. *Source: Modified from Toshimori K, et al.* Mol Reprod Dev *1991;**29**(3):289–93.*

The Sperm Head Cytoskeleton

Cytoskeletal structures are associated with the different regions (domains) of the mammalian sperm head (Figure 3.3), including the *subacrosomal cytoskeleton* located in the subacrosomal space, the *postacrosomal cytoskeleton* in the postacrosomal space, and the *para-acrosomal cytoskeleton* in the para-acrosomal space in falciform sperm (Figures 3.6 and 3.7). The periacrosomal space is narrow, but molecules in this region could function in sperm–egg interactions.

Structural Aspect

The subacrosomal cytoskeleton, referred to as the *perforatorium* and postacrosomal cytoskeleton, is composed of amorphous, electron-dense material. These structures can be isolated together and are referred to as the *perinuclear theca* (Figure 3.3). The perforatorium is a prominent feature of falciform-shaped heads of rodent spermatozoa, but is a minor component of the spatula-shaped heads of spermatozoa in most mammalian species. The perforatorium becomes more resistant to solubilization during epididymal transit due to extensive disulfide bond formation, and may have a mechanical role in egg penetration.[125]

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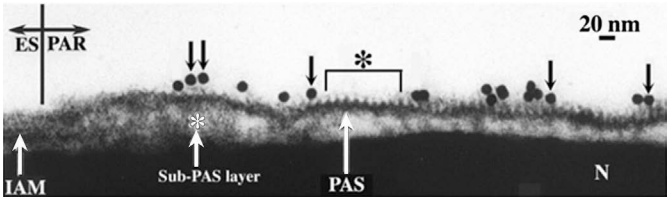

FIGURE 3.7 The cytoskeletal structure at the postacrosomal region. Immuno-electron microscopy with anti-MN13 monoclonal antibody. MN13 epitope regions (arrows) are present on the electron-dense periodic substructures (typically found on the part indicated by asterisk) that emerge from the postacrosomal sheath (PAS). The plasma membrane was removed with 0.1% Triton X-100 treatment before antibody application. The sub-PAS layer (white asterisk) is filled with electron-dense material. ES, equatorial segment; IAM, inner acrosomal membrane; N, nucleus; PAR, postacrosomal region. *Source: Modified from Toshimori K, et al.* Mol Reprod Dev *1991;**29**(3): 289–93.*

The perinuclear theca covers the external surface of the nuclear envelope and is divided into two components, the subacrosomal perinuclear theca and the postacrosomal sheath perinuclear theca. The postacrosomal sheath perinuclear theca is continuous with the posterior end of the subacrosomal perinuclear theca and extends caudally to the posterior ring. The ring region is a zone of close adhesion between the plasma membrane, the postacrosomal cytoskeleton, and the nuclear envelope (Figures 3.3–3.5). The isolated perinuclear theca retains the shape of the sperm nucleus in the absence of DNA and the acrosome.[126] In human spermatozoa, the perinuclear theca is composed of six proteins of variable molecular mass (16, 22, 29, 34, 50, and 68 kDa) similar to those previously described in other mammals.[63,127–131] In addition, the MN13 molecule is localized in the perinuclear theca just beneath the plasma membrane overlying the postacrosomal sheath (Figure 3.7). It is involved in maintaining the shape of the posterior head and is absent in round-headed spermatozoa (globozoospermia) in both human and in mouse spermatozoa deficient in the golgi-associated PDZ- and coiled-coil motif-containing protein GOPC.[132]

Composition

The evidence that the sperm-head cytoskeleton shows resistance to chemical treatments suggests that it has structural roles in defining the shape of the sperm head and functional roles in helping sperm penetration into the egg investment at fertilization, and presumably regulates other functional molecules required for the acrosome reaction.[125] The proteins of the sperm-head cytoskeleton are listed in Table 3.1.[133–146] In addition to these molecules, other proteins associated with the perinuclear theca include actin, spectrin, and calmodulin,[147] thecins,[148] DP71F isoform of dystrophin, β-dystrobrevins,[149] antigen AJ-p90,[150] and PLCZ1.[151] Of these, PLCZ1 is best-characterized and is a candidate for the sperm-borne oocyte activation factor. PLCZ1 is the smallest among the five or more phopholipase C (PLC) isoforms and lacks an N-terminal pleckstrin homology domain that is present in other PLC isoforms.[151] The reported location of PLCZ1 is variable from species to species, possibly due to the different antibodies used. Although PLCZ1 is initially thought to be a constituent of the detergent-resistant perinuclear theca, it is localized in the acrosomal region, equatorial segment, and postacrosomal region in human.[152] In humans and mice, PLCZ1 is incorporated into the acrosome and not detected in testicular sperm during spermatogenesis. However, a recent study showed that PLCZ1 is secreted by the epididymal epithelial cells, binds to the sperm surface during epididymal maturation, and disappears after the acrosome reaction.[153] In addition, PLCZ1 is not found in the detergent-resistant perinuclear theca fraction, which may not support a role for it as a sperm-borne oocyte activation factor. The postacrosomal sheath's other proteins include the WBP2 N-termina like protein (WBP2NL) localized to the postacrosomal sheath in the rat[154] and the MN13 antigen. An inhibition assay with the MN13 antibody blocked egg activation in the mouse.[155]

Function

Sperm-head cytoskeletal components have a structural role in defining the shape of the sperm head and have a functional role in aiding sperm penetration of the egg investments at fertilization.[156] In early spermiogenesis before the perforatorium forms, the para-acrosomal theca materials support acrosome biogenesis and apparently shield and shape the nucleus. At the end of spermiogenesis, the perforatorium and the postacrosomal sheath develop as a distinct structure after elongation and flattening of the spermatid head, and these postacrosomal sheath components provide structural reinforcement to maintain the acrosome and nuclear shapes.[157]

Because the anterior acrosome is involved in the acrosome reaction, the molecules including periacrosomal or para-acrosomal cytoskeletons are important in relation to the initiation of the acrosome reaction. The para-acrosomal cytoskeleton (filamentous cytoskeletal complex) is present in hamster sperm.[158] Complexins I and II (CPLX1, CPLX2) proteins are reported to bind tightly to the soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) complex and regulate exocytosis. These proteins are found in the peri-acrosomal region of spermatozoa.[159] CPLX1-deficient sperm performed the acrosome reaction in response to a Ca^{2+} ionophore, but they did not respond to egg zona proteins and showed reduced fertilizing ability in vitro.[159] SNARE regulatory proteins, including *N*-ethylmaleimide sensitive fusion protein attachment protein alpha (NAPA, a-SNAP), *N*-ethylmaleimide sensitive fusion protein (NSF), ras oncogene family

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TABLE 3.1 Sperm Head Domains and Associated Cytoskeletal Proteins

Domain	Protein	Gene	Features	References
Whole head	T-actin 2	*Actl7a*	Actin-like protein, postmeiotic, testis-specific	52
Acrosomal	Spectrin		Human, also in principal piece	65
			Rabbit, also in postacrosomal region, principal piece	66
	ARC	*Arc*	Activity regulated cytoskeleton-associated protein, postmeiotic, highly expressed in testis, also in neck and principal piece	53
	CASK	*Cask*	Ca^{2+}/calmodulin-dependent serine protein kinase, postmeiotic, widely expressed	133
	Testis fascin	*Fscn3*	Fascin homolog 3, postmeiotic, testis-specific	54
Periacrosomal	Calpastatin	*Cast*	Between plasma membrane and outer acrosomal membrane in macaque monkey	134
	Calpain1	*Capn1*	Translocates from acrosomal cytoplasm to plasma membrane	67
	Calpain11	*Capn-11*	Contains motif for Ca^{2+}-binding and protease activity	135
	Complexin1	*Cplx1*	KO; subfertile due to defect in zona penetration	136
Perinuclear theca *subacrosomal region*	PERF 15	*Fabp9*	Fatty acid binding protein 9, meiotic and postmeiotic, testis-specific	129
	SubH2Bv	*Subh2bv*	H2B variant PT15, postmeiotic, testis-specific	131
	Myosin V	*Myo5*	Component of acroplaxome, testis-specific	61
	ODF2	*Odf2*	Component of acroplaxome, cenexin/ ODF2 gene	137
Perinuclear theca *postacrosomal region*	Cylicin I	*Cylc1*	Basic protein of sperm head cytoskeleton 1, postmeiotic, testis-specific	138
	Cylicin II	*Cylc2*	Basic protein of sperm head cytoskeleton 2, postmeiotic, testis-specific	139,140
	Calicin	*Ccin*	Actin-binding protein, postmeiotic, testis-specific	141,142
	CPα3	*Capza3*	Actin-capping protein alpha subunit 3, postmeiotic, testis-specific	142,143
	CPβ3	*Cabzb*	Actin-capping protein beta subunit, postmeiotic, tissue-specific isoform (splice variant), high in testis and lower in brain	64
	Arp-T1	*Actrt1*	Actin-related protein T1, postmeiotic, testis-specific	62
	Arp-T2	*Arpm2*	Actin-related protein M2, postmeiotic, testis-specific	62
	CYPT1	*Cypt1*	Cysteine-rich basic protein, postmeiotic, testis-specific, at least 10 members, Cypt1-10	144,145
	STAT4	*Stat4*	Signal transducer and activator of transcription, postmeiotic, expressed in testis and a few other tissues	146
Nucleus	Arp-M1	*Arpm1*	Actin-related protein M1, postmeiotic, testis-specific specific complex with profilin III	60

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member 3a (RAB3A), and synaptotagmins (SYT), are detected around the acrosomal region, suggesting involvement of the SNARE complex in regulation of acrosomal exocytosis.[160–162]

The perinuclear theca components presumably associate with the other matrix proteins and play roles in assembling the acrosome and shaping the nucleus (head) during spermatogenesis and in oocyte activation during fertilization.[132,163–165] If acrosome biogenesis and perinuclear theca formation are impaired, developing sperm become round (globozoospermia) and lack the ability to activate mouse or human oocytes.[165] In addition, the perinuclear theca appears to link the nuclear envelope to the postacrosomal plasma membrane. In round-headed sperm of GOPC-null males, the association between the nuclear envelope and the postacrosomal plasma membrane is weak due to the lack of a postacrosomal sheath and posterior ring, allowing the mitochondrial sheath to shift proximally to coil around the nucleus.[166]

The Acrosome

The acrosome is a unique sperm organelle that originates from the Golgi complex and contains enzymes necessary for the sperm to penetrate through the cumulus cells and zona pellucida of the egg to achieve fertilization. It is a membrane-enclosed vesicle sitting as a cap over the nucleus in the anterior part of the sperm head. Most of the acrosomal components are produced in haploid spermatids during spermiogenesis, but a few begin to be synthesized in late pachytene spermatocytes.

Structure

The shape of the acrosome is characteristic of the species (Figure 3.2). The inner acrosomal membrane overlies the anterior part of the outer membrane of the nuclear envelope and is continuous with the outer acrosomal membrane at the posterior end of the acrosome (Figure 3.3**)**. The outer acrosomal membrane lies close to the inner surface of the plasma membrane of the anterior sperm head. The acrosome consists of two segments, the acrosomal cap (anterior acrosome) and the equatorial segment (posterior acrosome), which corresponds in distribution to the plasma membrane domains with the same names. During the acrosome reaction, the outer acrosomal membrane and the overlying plasma membrane fuse and vesiculate, and most of the acrosomal contents are discharged. The inner acrosomal membrane and the contents at the equatorial segment persist until sperm–egg fusion in most species. Acrosome shape and size varies widely between species (Figure 3.2), and the distribution and relative prominence of these two segments differ accordingly.[167]

The portion of the acrosomal cap that extends beyond the anterior margin of the nucleus is referred to as the marginal segment (apical segment, peripheral rim), and the portion overlying the nucleus is referred to as the principal segment (acrosomal segment). In the human, monkey, bull, boar, rabbit, and bat the acrosome is relatively small, with no appreciable extension beyond the nucleus, whereas in the guinea pig, chinchilla, and ground squirrel the acrosome has a large apical segment.[167] The acrosome, particularly the marginal segment, often has a more complex shape than is obvious when examining sperm smeared onto a slide. The equatorial segment forms a band that approximately overlies the equator of spatulate head spermatozoa, while in falciform head sperm, the equatorial segment may cover much of the lateral surfaces of the head (Figure 3.2).

The anterior acrosome is subdivided into two or three subdomains as evidenced by immunocytochemical and electron microscopy methods. In mouse spermatozoa, the cortex domain faces the outer acrosomal membrane, while the medulla domain is deep and faces the inner acrosomal membrane. The MC101 molecules appear to locate at the border of these subdomains.[168] In guinea pig sperm, three subdomains (M1, M2, M3) are present.[169] Acrosomal molecules are present in these subdomains and integrated into the acrosomal membranes.

The final shape of the acrosome is influenced by extrinsic forces generated by cytoskeletal elements in the spermatid and/or Sertoli-cell cytoplasm or by forces intrinsic to the nucleus. Generally, mammalian acrosomes and their constituents continue to undergo differentiation structurally and chemically after spermatogenesis, and the definitive morphology is not achieved until sperm reach the distal portion of the epididymis.[170] Genetic factors influence acrosome formation and shape, as evidenced by disruption of this process in many genetically modified mice and in mutant mice.[171]

The membrane of the acrosome, particularly the equatorial segment, contains particles that form a crystalline array, giving the membrane a highly regular, granular appearance (Figure 3.4). These may be an indication of ordered structure in the underlying acrosomal components. The outer acrosomal membrane appears to be fragile and easily displaced at the time of the acrosome reaction. However, it has electron-dense coating substances on the inner (luminal) surface.[172] This inner surface coat of the outer acrosomal membrane is composed of glycoproteins, since lectin wheat germ agglutinin binds to the inner surface of the membrane. These glycosylated molecules at this site might help to stabilize the membrane or play a functional role in the membrane fusion events of the acrosome reaction.[173] Some of the membrane components could be phosphorylated in a cyclic adenosine monophosphate (cAMP)-independent manner and include calmodulin-binding proteins.[173] This coating may also function as protection, because the outer acrosomal membrane is greatly deformed

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to contact the plasma membrane, accompanying acrosomal swelling prior to the acrosome reaction in human sperm.[174] Zonadhesin (ZAN) is restricted to the outer acrosomal membrane of the apical and principal segments and to the contents of the posterior acrosome in mammals including humans.[175] ZAN is dissociated from the outer acrosomal membrane and becomes incorporated into the forming acrosomal matrix during sperm maturation in the epididymis.[176] ZAN is exposed to the sperm surface during capacitation to be involved in binding to the zona pellucida independently of the acrosome reaction by a rapid transient fusion ("kiss and run") mechanism.[175]

Development of the inner acrosomal membrane begins in early spermatids when the membrane of the proacrosomal granule abuts and then flattens against the nuclear envelope. The granule spreads over the apical end of the nucleus during acrosomal development and nuclear elongation.[177] The inner acrosomal membrane contains glycoproteins and is generally quite resistant to chemical and physical disruption. However, the inner acrosomal membrane appears to be fluid because antigens recognized by monoclonal antibody PH-20 (SPAM1) migrate from the plasma membrane of the postacrosomal region of the guinea pig sperm head to the inner acrosomal membrane following the acrosome reaction.[178] Acrosomal proteins equatorin (EQTN) and izumo (IZUMO1) are anchored on both the inner and outer acrosomal membranes and are translocated to the overlying equatorial plasma membrane.[179–181] IZUMO1 also moves to the postacrosomal plasma membrane.[180] In contrast, the equatorial segment is stabilized by the extensive scaffolding network that connects the outer acrosomal membrane to the inner acrosomeal membrane, but this cytoskeletal network is not seen before sperm entry into the oocyte, and becomes apparent upon fertilization, presumably due to the release of acrosomal contents.[125,155]

Contents

The acrosome contains multiple enzymes that serve a critical role in the process of fertilization in mammals (Table 3.2).[182–221] During the acrosome reaction, acrosomal contents are released by exocytosis in response to Ca^{2+} signals. Following the release and activation of acrosomal enzymes, spermatozoa penetrate the zona pellucida surrounding the oocyte, a process that can be blocked by protease inhibitors. Some of the acrosomal enzymes appear to associate with nuclear matrix structural proteins.

Acrosin (ACR) is a member of the serine protease superfamily and present only in spermatogenic cells (Table 3.2). It is synthesized as a precursor (proacrosin) predominantly in round spermatids, but proacrosin mRNA or protein are detected in pachytene spermatocytes of mouse, guinea pig, and human. Proacrosin is present mainly in the anterior acrosome in human, boar, bull, and rabbit spermatozoa and is localized to the inner acrosomal membrane.[186] ACR-null male sperm are fertile,[185] indicating that other serine proteases participate in this process. ACR appears to contribute to zona penetration because acrosin-null male mouse sperm are moderately slower to fertilize than wild-type sperm.[222]

Acrosin-binding protein (ACRBP, sp32) binds proacrosin, but not mature ACR, and is tyrosine phosphorylated during capacitation.[206] In PCSK4-null male mice, ACRBP, which normally undergoes processing from a 58.5-kDa precursor to a 27.5-kDa mature form, is not proteolytically processed, suggesting that ACRBP is a substrate for PCSK4.[175] A recent report showed that ACR and matrix metalloprotein 2 (MMP2) are bound to the inner acrosomal membrane.[186] ACR inhibitors are present both within the acrosome[223] and in seminal plasma.[224] Sperm-associated inhibitors in the boar resembling Kazal-type inhibitors are present in seminal plasma.[224] Another serine protease inhibitor closely related to the plasma protein C inhibitor is present in the acrosome of human spermatozoa.[225] Thus, both ACR and ACR inhibitors are present in the acrosome.

Sperm hyaluronidase is different from the common lysosomal form.[226] Although neither of the two sperm hyaluronidases, sperm adhesion molecule 1 (SPAM1, PH20) and hyaluronoglucosaminidase 5 (HYAL5), are essential for fertilization in the mouse, SPAM1/ACR-deficient and SPAM1/serine protease 21 (PRSS21 testisin, TESP5) deficient sperm have reduced ability to traverse the cumulus matrix compared to sperm lacking SPAM1. This suggests that ACR, PRSS21, or both may function cooperatively with SPAM1 in sperm/cumulus penetration.[227]

Other acrosomal enzymes that have been reported include β-galactosidase, β-*N*-acetylglucosaminidase, arylsulfatase, collagenase-like peptidase, a cathepsin D-like protease, cathepsin H, trypsin-like proteases distinct from acrosin, dipeptidyl peptidase II, calpain II, neuraminidase, nonspecific esterases, arylamidase, aspartylamidase, arylsulfatase A, acid phosphatase, β-*N*-acetylglucosaminidase, a phospholipase C, and phospholipase A_2.[5] Although it is generally believed that these enzymes are soluble components that are released when the acrosome reaction occurs, the genes encoding most of these enzymes have not been identified and they remain to be characterized at the molecular level. Testis-specific serine proteases 39 and 40 (PRSS39 and PRSS40)[210] and a ubiquitously expressed homolog of pancreatic trypsin, serine protease 2 (PRSS2),[211] are also present. PRSS21-deficient sperm are fertile, but are reduced in fertilizing ability in vitro.[212] Sperm acrosome associated 3 (SPACA3, SLLP1) is an acrosomal protein

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TABLE 3.2 Acrosome Components

Protein	Gene	Expression	Features	References
N-acetylglucosaminidase	*Naglu*	Ubiquitous	Soluble compartment; putative testis-specific isoform	182
Acrogranin, granulin	*Grn*	Ubiquitous	Soluble compartment; cysteine-rich	183,184
Acrosin, proacrosin, preproacrosin	*Acr*	Testis-specific, mRNA and protein low in spermatocytes, high in spermatids, in mature sperm	Soluble compartment; serine protease	185
			KO; fertile	185
			IAM associated	186
AM50 (p50, apexin)	N.D.	Testis-specific	Soluble compartment; similar to neuronal pentaxin II, Ca^{2+}-dependent binding	187
CRISP2 (TPX1)	*Crisp2*, *CRISP2*	Testis-specific, protein in spermatocytes	Soluble compartment; cysteine-rich protein	188
			Interacts with GGN1 and SHTAP	189,190
β-galactosidase	*Bgal*	Ubiquitous	Soluble compartment; putative testis-specific isoform, differs in size and enzymatic properties from lysosomal form	182,191,192
Hyaluronidase SPAM1 (PH-20)	*Spam1*, *SPAM1*	Testis-specific, protein	Glyosylphopshatidylinopsitol-anchored plasma membrane, inner acrosomal membrane retained on acrosome-reacted sperm	193
			KO; fertile	194
HYAL5	*Hyal5*, *HYAL5*	Testis-specific, protein	Glyosylphopshatidylinopsitol-anchored plasma membrane, released during acrosome reaction	195
			KO; fertile	196
MC41	N.D.	Testis-specific epitope	Matrix of marginal segment; putative ZP2-binding protein	197
SPACA1 (SAMP32)	*Spaca1*, *SPACA1*	Testis-specific	Matrix; inner membrane in principal and equatorial segments	198
			KO; infertile (globozoospermia)	199
SPACA3 (SLLP1)	*Spaca3*, *SPACA3*	Abundant in testis, low in pancreas and Raji cells	Matrix, principal and equatorial segments; C lysozyme-like protein	200
			Novel binding partner for oocyte SAS1B	201
SPACA4 (SAMP14, Izumo4)	*Spaca4*, *SPACA4*	Testis-specific	Matrix; inner and outer membranes; similar to Ly6/urokinase plasminogen activator receptor superfamily protein	202
SPACA7	*Spaca7*, *SPACA7*	Testis-specific	Acrosomal protein; novel protein	203
SPESP1 (ESP)	*Spesp1*, *SPESP1*	Testis-specific	Matrix; equatorial segment	204
			KO; fertile, equatorin increased	205
sp32	*Acrbp*, *ACRBP*	Testis-specific	Matrix; proacrosin-binding protein	206
sp56 (AM67)	*Zp3r*, *SELENBP1*	Testis-specific, protein in late pachytene spermatocytes	Matrix; marginal segment; putative zona binding protein	207
			Zp3r KO; fertile	208
Sp-10	*ACRV1*, *Acrv1*	Testis-specific	Matrix, principal segment and posterior equatorial segment; putative zona binding protein	209

(Continued)

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TABLE 3.2 Acrosome Components—cont'd

Protein	Gene	Expression	Features	References
Synaptotagmin VI	*Syt6*, *SYT9*	Ubiquitous	Outer acrosomal membrane; Ca^{2+}-binding protein	160
TESP1	*Press39*, *TESP1*	Testis-specific	Matrix, principal segment; serine protease, putative role in zona penetration	210
TESP2	*Press40*, *TESP2*	Testis-specific	Matrix, principal segment; serine protease, putative role in zona penetration	210
TESP4	*Prss2(Try2)*	Ubiquitous	Matrix, principal segment; pancreatic trypsin-like serine protease, putative role in zona penetration	211
TESP5 (testisin, esp-1)	*Prss21*	Testis-specific	Matrix; glyosylphopshatidylinopsitol-anchored serine protease, localize in head, cytoplasmic droplet, midpiece	86
			KO; fertile, but reduced fertility in vitro	212
TMEM190	*Tmem190*, *TMEM190*	Testis-specific	Matrix; inner acrosomal membranes, co-localize with Izumo1. Present in human pharynx and ovary KO; fertile	213
Zonadhesin	*Zan*, *ZAN*	Testis-specific	Matrix; inner and outer membranes in spermatids, matrix in sperm; zona binding protein, acrosomal matrix	176
			Zan KO; fertile	175
ADAM3 (cyritestin)	*ADAM3* (pseudogene in human)	Testis-specific	Matrix; inner and outer acrosomal membrane sperm migration into the uterus KO infertile	37
Izumo1 (OBF13)	*Izumo1*, *IZUMO1*	Testis-specific (from Japanese word for shrine for uniting) in sperm	Matrix; soluble compartment, inner and outer acrosomal membrane associated at apical segment	180,181
			KO; infertile	180
Equatorin (MN9, afaf)	*Eqtn*, *EQTN*	Testis-specific	Matrix; inner and outer crosome membrane	179
			N-*O*-sialoglycoprotein, acrosome reaction	214
			Acrosome formation-associated factor	215
CD9	*Cd9*, *CD9*	Ubiquitous	Matrix; inner acrosomal membrane	216
			KO; male fertile, female infertile	217
CD46 (MCP)	*Cd46*, *CD46*		Matrix; inner acrosomal membrane	
			KO; fertile	218
MC101	N.D.	Testis-specific epitope	Matrix; marginal segment border between cortex and medulla	168
MMP2	*Mmp2*, *MMP2*	Ubiquitous	Matrix; inner acrosomal membrane associated with protainase	186
ZPBP1 (SP38, IAM38)	*Zpbp1*, *ZPBP1*	Testis-specific?	Matrix; inner acrosomal membrane inner and outer acrosome membrane after acrosome reaction	219
			KO; infertile (astheno-globozoospermia)	220
			Mutation; abnormal head in infertile men	221

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exposed and retained on the inner acrosomal membrane in 90% of acrosome-reacted sperm.[201] In astacin-like metalloendopeptidase (M12 family) (ASTL, SAS1B)-null mice, oocytes showed a 34% reduction in fertility, suggesting that sperm ASTL functions as a binding partner for oocyte ASTL during fertilization.[228]

Some other partially characterized acrosomal proteins that might function during the acrosome reaction include inhibitory guanine nucleotide-binding regulatory proteins (G_i proteins) that function as signal transducing elements mediating this exocytotic event, acrogranin in guinea pig sperm, proenkephalin in rats, gastrin in humans, and the related cholecystokinin peptide in mouse, rat, and monkey.[5] The functional roles of these peptides during spermatogenesis or fertilization have not been determined. Soluble components that are released when the acrosome reaction occurs include AM50 (p50, apexin) in the guinea pig, which is similar to neuronal pentraxin II,[187] and the cystein-rich protein CRISP2.[188]

The majority of the non-enzymatic acrosomal proteins serve as components of the acrosomal matrix. The acrosomal matrix is relatively insoluble in nonionic detergents and largely remains associated with the sperm after the acrosome reaction. Proteins associated with the acrosomal membranes include sperm acrosome associated 1 (SPACA1, SAMP32),[198] synaptotagmin IV (STY4),[160] and ZAN.[229] Some matrix components that might be involved in zona binding include the MC41 antigen,[197] zona pellucida 3 receptor (ZP3R, SP56),[207] acrosomal vesicle protein 1 (ACVR1, SP-10),[209] and zona pellucida binding protein (ZPBP, Sp38, IAM38).[220] ZAN-deficient ($Zan^{-/-}$) male mice are fertile, although $Zan^{-/-}$ sperm have reduced species specificity for zona pellucida adhesion.[175] ZP3R is not essential in sperm–zona pellucida binding and/or penetration of zona pellucida, because ZP3R-deficient male mice were fertile.[208] Other matrix proteins that have been identified include ACRBP[230] and sperm acrosome associated 4 (SPACA4, SAMBP14), which has regions of sequence similarities to the plasminogen activators.[202] Equatorin (EQTN, Mn9),[179,215] IZUMO1, POU domain, class 2, and associating factor 1 (POU2AF1, OBF1)[180] are released during the acrosome reaction, and become relocated and retained on the overlying plasma membrane. IZUMO1 is a type one immunoglobulin superfamily protein localized in the anterior acrosome and becomes translocated onto the overlying plasma membrane of the whole sperm surface after the acrosome reaction.[180,181,199] IZUMO1-null sperm are infertile.[180] TSSK6-null sperm failed to relocate IZUMO1 during the acrosome reaction due to a failure of actin polymerization.[231] Sperm equatorial segment protein 1 (SPESP1) is localized to the equatorial segment of ejaculated human spermatozoa.[204] SPESP1 persists in the equatorial segment in the acrosome-reacted spermatozoa and to spermatozoa tightly bound to the oolemma of hamster eggs.[204,232] SPESP1 deficient male mice were fertile, although $Spesp1^{+/-}$ and $Spesp1^{-/-}$ sperm had a reduced fusing ability.[205] In SPESP1-null sperm, although various sperm proteins were aberrantly distributed, EQTN was increased in amount, suggesting a redundancy of EQTN. EQTN is expressed in spermatids, integrated into spermatids during spermatogenesis, and becomes a type 1 *N*,*O*-sialoglycoprotein anchored to the outer and inner acrosomal membrane in mammals, including human.[179,214] Anti-EQTN antibody MN9 inhibited the sperm–oocyte fusion or egg activation in vitro[179] and in vivo.[233] Transmembrane protein 190 (TMEM190) is an inner-acrosomal membrane protein in cauda epididymal sperm. TMEM190 also partly relocated onto the surface of the equatorial segment during the acrosome reaction. TMEM190 and IZUMO1 are co-localized in mouse sperm both before and after the acrosome reaction, but immunoprecipitates of TMEM190 did not include IZUMO1.[213]

Abnormal Sperm Heads

Teratozoospermia is a condition in which human spermatozoa have abnormal morphology, including abnormally shaped heads.[234] A widely accepted notion is that a well-conserved organization of sperm transcripts in the healthy male originates from a well-orchestrated process of spermatogenesis, and failures in that process result in abnormal spermatozoa without heads, called acephalic or decapitated sperm. Decapitated tails are detected in up to 10%–20% of spermatozoa in subfertile patients, but are rare in the semen of fertile men.[235,236] Round heads are formed by altered early acrosome biogenesis in mouse and human globozoospermia.[132,237] This alteration is related to the failure of perinuclear theca proteins to properly associate with the nuclear surface and significant deficiencies in specific perinuclear theca components that are necessary for proper acrosome formation, implantation, and expansion over the spermatid nucleus.[237] Round head formation is also related to disorganization of tail components. Mutations associated with the occurrence of round-headed sperm (globozoospermia) in humans have been identified.[238]

THE FLAGELLUM

The flagellum is connected to the head (nucleus) at the basal plate between the connecting piece and base of the nucleus. The indentation of the base of the sperm head containing the basal plate is known as the implantation fossa. The flagellum of the mammalian sperm consists of four distinct segments: the connecting piece (neck), the middle piece (midpiece), the principal piece,

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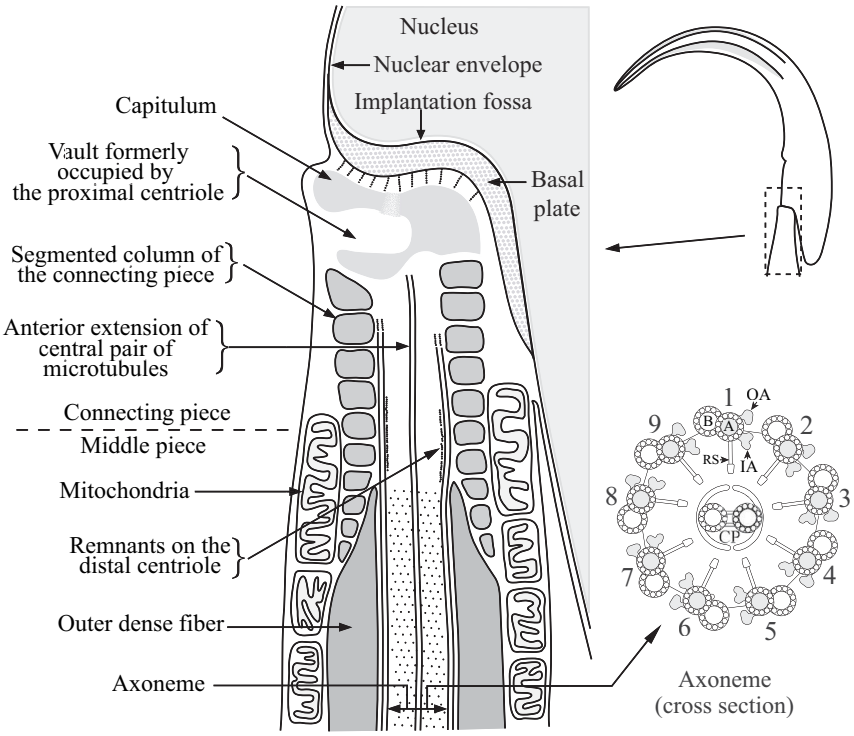

FIGURE 3.8 Connecting and proximal middle piece regions of sperm flagellum. Left schematic shows the internal details of the boxed area at the top of the right side of the diagram of a rat sperm head. The basal plate is adherent to the nuclear envelope, defining the implantation fossa and forming the site of attachment of the flagellum to the sperm head. The connecting piece is topped by the capitulum, with the segmented columns extending from it caudally to fuse with the outer dense fibers. The axoneme extends from the connecting piece to the distal tip of the flagellum. *(Source: This schematic drawing is modified from Ref. 239.)* The details of the axoneme are shown in bottom right diagram. The axoneme is composed of a nine-plus-two (9+2) complex of microtubules; 9 outer doublet microtubules (from 1 to 9) and a central pair of singlet microtubules (CP). When the axoneme is viewed from the sperm head towards to tail, the peripheral doublets are numbered 1 through 9. Number 1 is the doublet situated on a plane perpendicular to that bisecting the microtubules of the central pair, and the next clockwise doublet is number 2. Outer dynein arms (OA) and inner dynein arms (IA) extend from the A microtubule (A) towards the B microtubule (B) of the adjacent doublet.

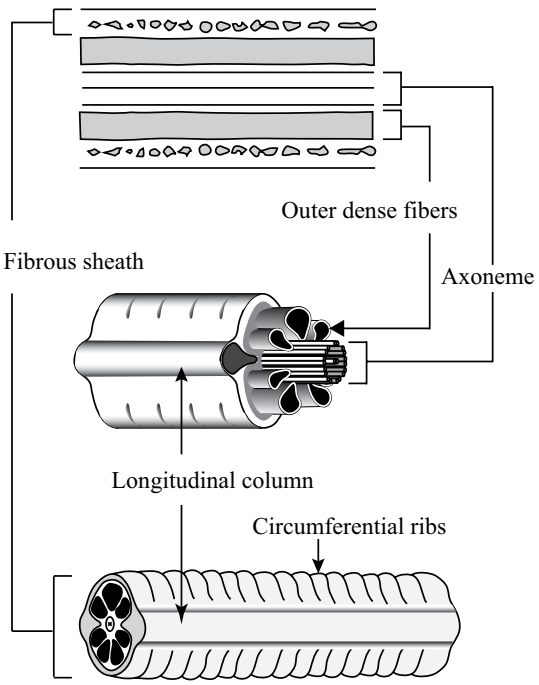

FIGURE 3.9 The cytoskeletal components in the principal piece region of the sperm flagellum. The outer dense fibers lie between the fibrous sheath and axoneme in the principal piece of the flagellum. The fibrous sheath is composed of two longitudinal columns connected by circumferential ribs and surrounds the axoneme and outer dense fibers in the principal piece region.

and the endpiece (Figure 3.1). The main structural components within the flagellum are the axoneme, the mitochondrial sheath, the outer dense fibers, and the fibrous sheath (Figures 3.8 and 3.9). The axoneme is composed of a nine-plus-two (9+2) complex of microtubules that extends from the connnecting piece through the full length of the flagellum. Early during spermiogenesis, sperm axonemal microtubules are recruited to the distal centriole as the proximal centriole migrates towards the spermatid nucleus. In mammalian sperm flagella, two accessory structures, the outer dense fiber and the fibrous sheath, surround the axoneme. Most flagellated sperm of lower organisms, such as sea urchins, do not contain either outer dense fiber or the fibrous sheath. The outer dense fibers are adjacent to the axoneme, and extend from the connecting piece to the posterior portion of the principal piece. The fibrous sheath begins anteriorly at the annulus and is a characteristic feature of the principal piece segment. The mitochondrial sheath defines the extent of the midpiece. It immediately surrounds the outer dense fibers, whereas the fibrous sheath surrounds the outer dense fibers. The endpiece lacks fibrous sheath or outer dense fiber components and contains only axonemal components. The components of these domains have unique functional roles.

The human sperm is about 60 μm long, and the flagellum is greater than 1 μm in diameter, 55 μm in length, and tapers towards the distal tip (Figure 3.1). However, sperm vary considerably in length between

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species: for example, 120 μm in the mouse, 190 μm in the rat, 250 μm in the Chinese hamster, 46 μm in the rabbit, and 50 μm in sea urchins.[5]

Connecting Piece

The connecting piece (the neck or head–tail coupling apparatus) comprises the capitulum (the dense fibrous plate-like structure) and the segmented (striated) columns (Figure 3.8). The capitulum conforms to the shape of the implantation fossa and connects to the top of the segmented column. The implantation fossa conforms to the basal plate, an electron-dense structure that is adherent to the outer membrane of the nuclear envelope. The connecting piece functions as a structural anchorage or articulation between the head and tail, retaining some centriolar proteins, to stabilize the proximal part of the tail. The elastic compliance of the connecting piece is thought to provide initiation of the sperm beat cycle and bend propagation in guinea pig sperm, suggesting the importance of the pale band region of the connecting piece.[240]

A recent electron cryotomography study revealed the three-dimensional structure of the connecting piece in bovine spermatozoa.[241] The connecting piece has asymmetrical architecture, with the segmented columns held together by filamentous linkages. At the proximal end, the segmented columns fuse with each other into structures that form the centriolar vault, where the structure reminiscent of the proximal centriole is localized, while at the distal end, the segmented columns fuse with the outer dense fibers to form the distal centriolar vault, where a novel bar structure associates with outer dense fibers 3 and 8. The architecture of these two outer dense fibers extending into the principal piece as part of the fibrous sheath suggests an intrinsic structural constraint on the waveform of sperm movement.

The segmented columns extend posteriorly from the capitulum, 1 to 2 μm in length, and connect to the outer dense fibers at their distal end (Figure 3.8). However, the segmented columns and outer dense fibers are different in their origin. During spermiogenesis, as centrioles initiate docking to the plasma membrane in an early stage of axonemal development, segmented structures form around the centrioles, presumably by the aid of a novel component of the connecting piece, speriolin (SPATC1).[242] The connecting piece region is strengthened by these molecules. Centrioles are involved in development of the connecting piece and the axoneme, but not required for sperm motility.[4,239] In humans and bovines, but not in rodents, the microtubule organizing center found in the fertilized egg arises from the sperm aster derived from the fertilizing sperm centrioles. Proteins that mediate the binding of the connecting piece to the sperm head are not known at present.

Centrosome

The centrosome is an amorphous cytoplasmic region, approximately 0.5 μm in length and 0.2 μm in diameter. The centrosome functions as the primary microtubule organizing center in animal cells and regulates the nucleation and spatial organization of microtubules. The centrosome is formed by the perpendicularly oriented proximal and distal centrioles and by the surrounding matrix of pericentriolar material. The proximal centriole retains its structure, but the distal centriole is disorganized in late spermatids in most species.[239] The pericentriolar material is a protein complex containing gamma 1 tubulin (TUBG1) and is involved in microtubule nucleation. Although information about the proteins found in the sperm centriolar region is limited, molecules found in the microtubule organizing center include TUBG1, nuclear mitotic apparatus protein 1 (NUMA1), and pericentrin (PCNT).[243] TUBG1 is found in germ cells in the mouse, rat, hamster, guinea pig, rabbit, bull, rhesus monkey, and human. It is shed during late spermatid differentiation in most species, but can be detected in human and bull sperm. Centrin (CETN1) is contained in the pericentriolar material associated with the proximal and distal centrioles in spermatids in the mouse, rabbit, pig, rhesus monkey, and human.

There are other proteins localized in the connecting piece region. SPATC1 is thought to provide architectural strength to the connecting piece region of mouse and human sperm.[242] SPATC1 is associated with the seventh tryptophan–aspartic acid (WD) protein structural motif of the CDC20 spindle checkpoint protein that activates the anaphase-promoting complex/cyclosome. SPATC1 surrounds the intact proximal centriole in human sperm and is localized at the periphery of the distal centriole in mouse sperm. Although the paternal centrioles enter the oocyte with the fertilizing sperm and form the sperm aster in humans and most other mammals, they are degenerated and not inherited by the oocyte in mice. SPATC1 is the first sperm centrosomal protein identified in the connecting piece region that is transmitted to the mouse zygote.[242]

The testis-specific serine kinase (TSSK) protein family is composed of six members (TSSK1-6). TSSK1, TSSK2, TSSK4, and TSSK6 (SSTK), but not TSSK3, are present in mouse testicular germ cells and in epididymal spermatozoa. TSSK1, TSSK2, and TSSK6, but not TSSK3, are detected in human spermatozoa.[244] Testis-specific serine kinase substrate (TSKS) is the substrate of TSSK1 and has two coiled-coil regions. The coiled-coil region is related to its centriolar localization, and through its binding activity with TSKS, TSSK2 is recruited to the spermatid centrioles to fulfill its essential function.[245] TSKS is localized during spermiogenesis to the centrioles, associating with TUBG1 of postmeiotic spermatids, and persists in

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ejaculated human spermatozoa, while centriolar TSKS diminishes in mouse spermatozoa.[245] Mouse TSKS and the TSSK2 persist and localize to the tail and acrosomal regions of epididymal spermatozoa, while human TSSK2 is found in the equatorial segment, neck, and midpiece. TSSK2 kinase is enzymatically active in both testis and mature spermatozoa. Male chimeras with a deletion of one allele of both of the closely located *Tsks* and *Tssk2* genes were unable to transmit the mutations to the next generation due to haploinsufficiency.[245] Testes of chimeras exhibited arrested spermatogenesis, failure to form elongated spermatids, increased apoptosis, and a greater number of round spermatogenic cells in the epididymides.[245] The TSSK6-null sperm exhibited impaired postmeiotic chromatin condensation and showed abnormal spermiogenesis.[97] Another group reported that the TSSK1/2 double knockout males are infertile, with the mitochondrial sheath severely disarranged, presumably due to impaired annulus migration, a process that is required for maturation of sperm mitochondria during late spermiogenesis.[246] This phenotype was somewhat similar to the feature of SEPT4 deficient mice.[247,248]

Ornithine decarboxylase antizymes (OAZs) control the intracellular concentration of polyamines. The rigid connection of the sperm head and tail are affected by the actions of OAZs.[249] OAZ-t/OAZ3 is expressed specifically in germline cells during spermiogenesis, while OAZ1 and OAZ2 are expressed ubiquitously. OAZ3 appears to play a role in haploid germ cell differentiation by binding to ornithine decarboxylase and inactivating it to regulate the local concentration of polyamines. OAZ3-deficient male mice are infertile, and the sperm heads and tails are easily separated.[249] Most of the headless tails contain all the components except the basal plate, indicating that the head-tail separation occurs at the plane between the basal plate and the capitulum. A similar separation is induced by trypsin treatment, but not by sonication, which causes cleavage at any place of the tail. The decapitated head that contains the nucleus and the basal plate fails to acrosome-react, but is capable of fertilizing eggs via intracytoplasmic sperm injection. OAZ3 may have a function other than as an antizyme that inactivates decarboxylase. However, a 12-kDa protein variant encoded by *Oaz3* lacks the antizyme domain that interacts with ornithine decarboxylase.[250] It is localized to the connecting piece, outer dense fibers, and fibrous sheath. In *Oaz3*-null male mice, the p12 variant does not affect ornithine decarboxylase levels and the testis does not display altered polyamine metabolism.[250] A major p12-interacting protein is myosin phosphatase targeting subunit 3 (MYPT3), a regulator of the protein phosphatase 1, catalytic subunit, beta isoform (PPP1CB). MYPT3 is present in sperm tails and its ankyrin repeat binds p12.

Centrobin (CNTROB, Nip2, Nek2-interacting protein 2, NIPS) was initially identified as a centrosome protein that is critical for centrosome duplication and spindle assembly. CNTROB is associated with daughter centrioles and implicated in microtubule stabilization. In the rat hypodactylous (*hd*) mutation males, the sperm head is detached from the tail. The mutation is caused by an insertion of an endogenous retrovirus into intron 10 of the *Cntrob* gene. In the *hd* mutant, spermatids fail to form an acroplaxome marginal ring and unusually separate the centrosome from the normal attachment site, which leads to spermatid decapitation during the final step of spermiogenesis.[251] Such *hd*/*hd* mutant spermatozoa resemble a type of (oligo)teratoasthenospermia in humans, with marked sperm tail fragility and decapitated sperm heads.

Axoneme

Microtubules

The organization of the axoneme (axial filament complex) is the same in the mammalian sperm tail and in the cilia and flagella of most plants and animals. Cytoskeletal tubulin and motor protein dynein are the major components. The typical axoneme nine-plus-two (9 + 2) axonemal structure is composed of 9 outer doublet microtubules (A and B microtubules) and 2 central singlet microtubules. Each doublet consists of a complete A microtubule, and a "C-shaped" B microtubule attaches onto the side of the A microtubule. Two dynein arms extend from the A microtubule toward the B microtubule of the anterior doublet. When the axoneme is viewed from the sperm head (proximal) towards the tail (distal), the arms project clockwise, and the doublets are numbered one through nine. Number one is the doublet situated on a plane perpendicular to that bisecting the microtubules of the central pair, and the next clockwise doublet is number two (Figure 3.8). In most mammals, the central pair of microtubules appears to extend into the connecting piece to the implantation fossa or capitulum, whereas the other microtubules appear to end on the remnants of the base of the distal centriole. Radial spokes are structures that extend from the base of A-tubules to the central pair apparatus.[252]

The microtubules are composed of dimers of β-tubulin (tubulin, alpha 1a, TUBA1A) (56 kDa) and α-tubulin (tubulin, alpha 1b, TUBA1B) (54 kDa). Multiple tubulin genes are expressed during spermiogenesis, including testis-specific isoforms, and the diversity of these proteins are further expanded by posttranslational modifications.[253] One of the first postmeiotically expressed genes identified was for a spermatid α-tubulin with a unique sequence.[254] This led to the prescient suggestion that the gene encoded a novel α-tubulin involved in determining special properties of the sperm axoneme.[254]

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Radial spokes are composed of at least 23 polypeptides (8–210 kDa) in *Chlamydomonas* flagella. These proteins include several components involved in Ca^{2+}/calmodulin (CALM) or (cAMP)-dependent intracellular signaling, such as CALM, a calmodulin-binding protein, a protein with an A-kinase anchor protein (AKAP) domain RSP3 (RS protein 3), a protein with a nucleoside diphosphate kinase domain and an isoleucine–glutamine motif, and a protein with a regulatory subunit (RII) alpha motif.[255]

The central pair apparatus contains at least 17 proteins, including CALM, CaM-binding protein primary ciliary dyskinesia protein 1, FAP221, AKAP-binding protein AAT-1 (FAP91), CPC1, a protein with an adenylate kinase domain, a CPC1-binding protein (enolase), WD-repeat protein paralyzed flagella PF20, Ala/Pro-rich protein PF6, a kinesin-like protein 1, the hydrocephalus-inducing gene product hydin, and armadillo-repeat containing protein PF16. The binding between spoke CaM and central apparatus CaM-binding protein and between the protein with an AKAP domain RSP3 and central apparatus AKAP-binding protein AAT-1 might mediate radial spoke/central pair apparatus interaction.[252]

Dyneins

The dyneins are a large family of motor proteins associated with microtubules, including the outer doublet microtubules in axonemes of cilia and flagella. Their ATPase activity generates force unidirectionally towards the minus (−) end of microtubules to generate sliding forces between adjacent doublets of microtubules during flagellar movement. Dyneins consist of heavy, intermediate, light intermediate, and light chains. The motor activity is associated with heavy chains and axonemal dyneins are present as heterotrimers, heterodimers, and monomers of heavy chains. Most of what is known about the function of axonemal dyneins comes from studies on ciliated unicellular organisms and sea urchin sperm and is presumed to apply to mammalian sperm. The targeted disruption of genes for axoneme components in mice that result in drastically altered sperm motility and male infertility are reviewed elsewhere.[252]

The flagellar movement that results from axonemal bending is generated by the active sliding of axonemal doublet microtubules by the action of two molecular motor dyneins, the outer and inner arm dyneins. They play different roles in the production and regulation of flagellar motility. Dynein itself contributes to the formation and propagation of flagellar bending, while the other axonemal structures, such as the radial spoke/central pair apparatus, also have essential roles in the regulation of flagellar bending.[252] Also, outer or inner arm dyneins are presumed to be involved in the elevation of microtubule sliding velocity (increasing beat frequency) and formation and propagation of flagellar bending, respectively.[256]

Tektins

Tektins, first identified in sea urchin sperm flagella, are a family of cytoskeletal proteins associated with microtubules and are expressed in spermatids. Tektins are coiled-coil proteins that form filaments in the walls of ciliary and flagellar microtubules.[257] At least five tektin proteins (TEKT1-5) have been identified in mammals. TEKT1 is localized to the centrosome in round spermatids and to the caudal end of elongating spermatids, but disappears as spermiogenesis proceeds. TEKT1 may be involved in the nucleation of the flagellar axoneme and is required for assembly of the basal body.[258] TEKT2 is localized to the axoneme. TEKT2-null male mice are infertile and TEKT2-null sperm display flagellar bending and reduced motility[259] due to a disruption in the dynein inner arm. TEKT3-deficient males are fertile and hyperactivation is intact, but TEKT3-null sperm have reduced motility and forward progression and increased flagellar structural bending defects.[260] TEKT4 is located on outer dense fibers and interacts with the outer dense fiber-associated protein, spermatogenesis associated 18 (SPATA18, SPETEX1), in rat sperm flagella,[261] but is not associated with the axonemal tubulins of flagella in mice.[260] TEKT4-null male mice are subfertile and TEKT4-null sperm have drastically reduced ATP levels and forward progressive velocity and uncoordinated waveform propagation along the flagellum.[262] In addition, the peri-outer dense fiber space is extended, the submitochondrial reticulum (Figure 3.10) is diminished,

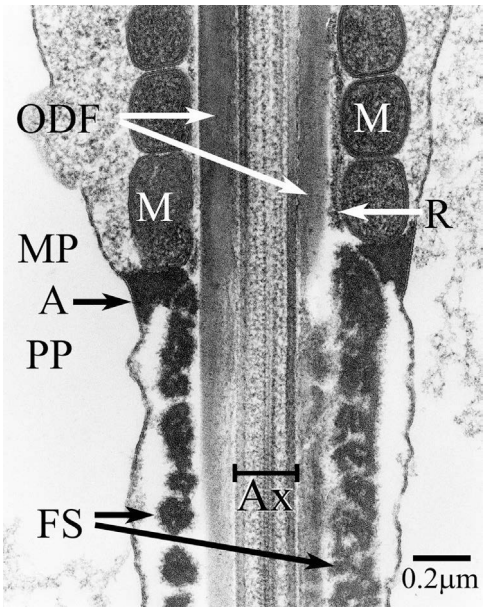

FIGURE 3.10 Transmission electron micrograph showing the annulus region. The mitochondrial sheath (M) is found in the middle piece (MP), and the fibrous sheath (FS) is found in the principal piece (PP). Outer dense fibers (ODF) and sub-mitochondrial reticulum (R) are found longitudinally between the mitochondrial sheath and the axoneme (Ax). A, annulus.

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and the circumferential ribs of the fibrous sheath frequently break, suggesting that the lack of TEKT4 causes a decrease in the tensile strength of flagella. Thus, TEKT4 likely functions in the transmission of the bending force from the axoneme through the outer dense fiber to the connecting piece.[262] TEKT5 is localized to the inner surface of the mitochondrial sheath in the rat sperm flagellar midpiece.[263]

Mitochondrial Sheath

The middle pieces differ considerably in length, and the organization of mitochondria is quite variable between species. The midpiece length is related to the length of the sperm, and in most mammals is between 5 and 12 μm (8.5 μm in rabbits, 9 μm in guinea pigs, 10 μm in rhesus monkeys, 12 μm in bulls, and 5.5 μm in humans), but is 64 μm in rats and 100 μm in Chinese hamsters.[5]

Sperm mitochondria are elongated, flattened, and arranged circumferentially in an end-to-end fashion to form a helically coiled sheath around the outer dense fibers (Figures 3.10 and 3.11). The number of helices, the number of spiral turns, and the length of the middle piece vary between species; for example, in the mouse, there are two parallel helices, with an average of 87 windings.[5] The morphogenesis of this mitochondrial arrangement occurs in the spermatid by (1) the migration of the annulus from the connecting piece down the flagellum to the beginning of the fibrous sheath, (2) the formation of a dextral helix of elongated mitochondria around the flagellum, (3) the division of these mitochondria into spherical mitochondria, and finally (4) the elongation and end-to-end apposition of these mitochondria into two tight sinistral helices.[264] In the mouse, the mitochondria are usually of variable length and abut end-to-end at random along the helix. The organization of the mitochondrial sheath is highly uniform in most species, but the precise numbers of mitochondria differ between species.[5]

The mitochondrial sheath adheres to an underlying complex of filaments named the sub-mitochondrial reticulum[265] (Figures 3.10 and 3.11). The sub-mitochondrial reticulum complex is a network of ribbons of filamentous material that are laterally interconnected and terminate to fuse with the annulus at the junction between the midpiece and the principal piece.

A structural protein, sperm mitochondria-associated cysteine-rich protein (SMCP), localizes to the capsule associated with the mitochondrial outer membranes and is thought to function in the organization and stabilization of the helical structure of the sperm mitochondrial sheath. SMCP is synthesized in elongating spermatids in the rat, but the levels of SMCP mRNA in humans are much lower than in other mammals. SMCP-null male mice on the 129/Sv background are

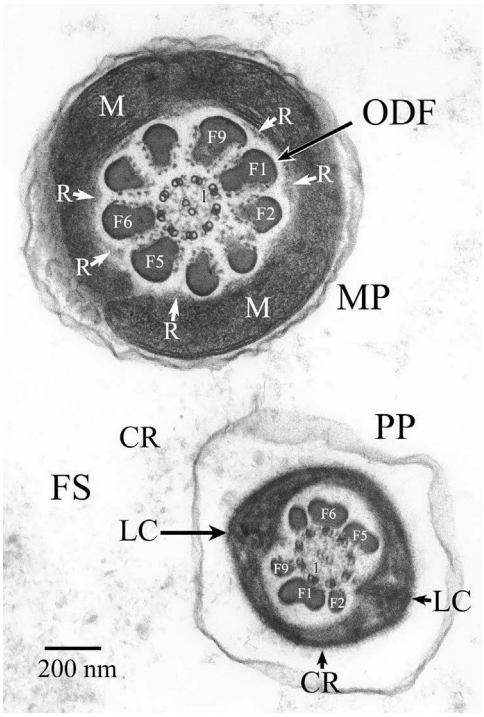

FIGURE 3.11 Cross-section of the middle piece and the principal piece. When the outer dense fibers (ODF) are viewed from the sperm head towards to tail, the outer dense fibers are numbered 1 (F1) through 9 (F9). Outer dense fiber number 1 (F1) is adjacent to microtubule doublet number 1 of the axoneme. It is situated on a plane perpendicular to that bisecting the microtubules of the central pair. The next clockwise outer dense fiber is number 2 (F2). The sub-mitochondrial reticulum (R) is located in the gap between the adjacent outer dense fibers and the indentation on the abaxial surface of outer dense fibers. The fibrous sheath (FS) is formed by two longitudinal columns (LC) connected by circumferential ribs (CR). M, mitochondria; MP, middle piece; ODF, outer dense fiber; PP, principal piece.

infertile due to asthenozoospermia (reduced sperm motility), and the decreased capability of sperm to penetrate oocytes.[266] Five triple gene knockout lines, *Acr/H1.1/Smcp*, *Acr/Tnp2/Smcp*, *Tnp2/H1.1/Smcp*, *Acr/H1t/Smcp*, and *Tnp2/H1t/Smcp*, displayed decreased fertility on mixed genetic backgrounds.[267] The decreased fertility is due to defects in sperm motility in vitro correlated with reduced migration in the female reproductive tract, and decreased fertilization due to defects in adhesion of sperm to the zona pellucida. SPATA19 (SPERGIN1) is localized to the midpiece and associated with the mitochondrial sheath as an adhesive molecule in the mouse and human.[268,269] Glutathione peroxidase 4 (GPX4, PHGPX) is a structural component of the mitochondrial capsule that serves to maintain normal sheath structure. Phospholipid hydroperoxide glutathione peroxidase (PHGPX) is an intracellular antioxidant enzyme that directly reduces peroxidized phospholipids produced in cell membranes.[270] GPX4, first described as an antioxidant enzyme, is the predominant selenoenzyme in testis and required for spermatogenesis. GPX4-deficient male

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mice are infertile and show multiple structural abnormalities such as bent tail, decaudated heads, dislocation of mitochondria along the midpiece, and extrusion of microtubules and outer dense fibers from the principal piece, features that likely would lead to infertility in humans.[271] In GOPC-null sperm, SPATA19 remains on the stratified mitochondria, while GPX4 is unevenly distributed on the mitochondrial sheath, suggesting that GPX4 is necessary to maintain normal sheath structure, and SPATA19 prevents dispersal of mitochondria.[166]

Outer Dense Fibers

General Features, Structure, and Formation

The outer dense fibers are cytoskeletal structures specifically found in the sperm tails of vertebrates but not in eukaryotic cilia and flagella.[4] The outer dense fibers are important for the stability and the elastic recoil of the sperm tail as well as for support of the flagellar beat, but they seem not to be involved in active motility.[272]

The outer dense fibers consist of nine fibers located between the mitochondrial sheath and the axoneme in the midpiece and surrounded by the outer dense fibers in the principal piece (Figures 3.10 and 3.11). The fiber shows a "9+2" complex numbered corresponding to the adjacent microtubule doublets. The fibers run longitudinally throughout the length of the middle piece and most of the principal piece of the flagellum of mammalian sperm, and are attached to doublet microtubules of the axoneme. Their cross-sectional profiles are not uniform at the ultrastructural level[4] (Figure 3.11). In general, the fibers 1, 5, 6, and 9 are larger than the others, and fibers are thickest in the proximal part of the middle piece and gradually taper in size toward the distal tip and terminate in the proximal half of the principal piece in human, macaque, and bat sperm, but extend to near the end of the principal piece in rat, hamster, guinea pig, and ground squirrel sperm.[4] Fibers 3 and 8 organize the distal centriolar vault, closely adhering to the connecting piece at the proximal end, whereas they terminate in the principal piece as the longitudinal columns of the fibrous sheath extend inwardly. The large fibers 1, 5, and 6 are usually the last to terminate.

The formation of outer dense fibers is best characterized in the rat, where it begins in spermatids; first as a small dense fiber immediately adjacent to microtubule doublets at the head–tail junction in step 8 spermatids, extends into the principal piece by step 14, and continues to be laid down in a proximal-to-distal direction during steps 15 to 19.[273] The precursor of the outer dense fibers apparently are synthesized in the cell body, and transported down the axoneme to the distal portion of the principal piece.

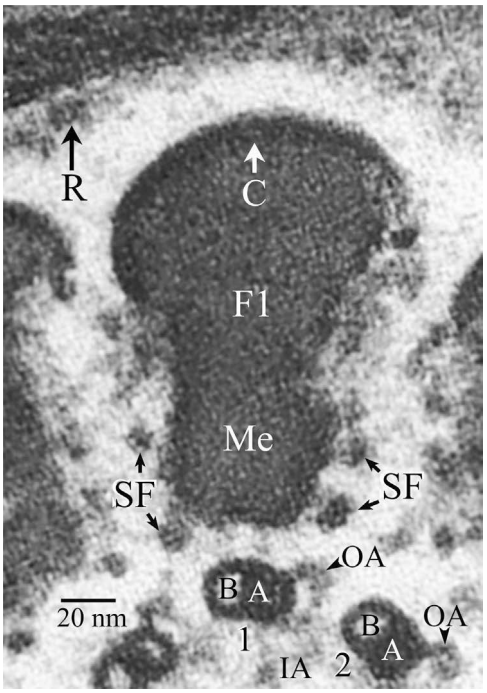

FIGURE 3.12 Higher magnification of outer dense fiber. Outer dense fiber 1 (F1), which is adjacent to peripheral microtubule doublet number 1, has cortex (C) and medulla (Me) regions. Satellite fibers (SF) are found associated with the medulla region at the adaxial surface of the outer dense fiber. A, A microtubule; B, B microtubule; IA, inner dynein arm; OA, outer dynein arm; R, sub-mitochondrial reticulum.

Each outer dense fiber is composed of cortex and medulla as determined by electron microscopy (Figure 3.12). The cortex is a thin layer stained more intensely than the medulla, and appears to be composed of a single lamina of globular particles 6–7 nm in diameter, whereas the medulla appears electron dense with no obvious substructure[274] (Figure 3.12). The cortex appears to be continuous with the satellite fibrils located at the adaxial surface of the outer dense fibers (Figure 3.12).

Composition

A number of the constituent proteins of the outer dense fibers and their genes have been identified and are listed in Table 3.3.[275–306] The outer dense fibers (ODF) are composed of two main ODF proteins, outer dense fiber of sperm tails 1 and 2 (ODF1 and ODF2).[288] The ODF1 protein interacts with ODF2, and both are located to the ODF, basal plate, capitulum, and segmented (striated) columns.[307] Linking of ODF to axonemal microtubules may be mediated via binding of ODF1 to sperm-associated antigens 4 and 5 (SPAG4, SPAG5) and of ODFs to mitochondria via kinase light chain 3 (KLC3) binding. ODF1 interacting proteins are SPAG4, KLC3, ODF1-interacting protein (OIP1), and SPAG5.[294] SPAG4 is located in the transient manchette and associated with the axoneme and manchette in elongating spermatids and epididymal sperm, and binds to ODF1 but not to

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TABLE 3.3 Outer Dense Fiber-Associated Proteins

Protein	Gene	Features	References
ODF1 (RT7, Odf27, rts5/1)	*Odf1*	Outer dense fiber of sperm tails 1, also as HSPB10 in human structural protein, present only in medulla of ODF postmeiotic, high in testis and low in a few other tissues	275–283
ODF1/HSPB10	*HSPB10*	Self-interacting protein, family of small heat-shock protein KO; sperm head-tail detachment, dysarrangement of ODF and mitochondrial sheath	284
ODF2 (Odf84)	*Odf2*	Outer dense fiber of sperm tails 2, structural protein, also present in acroplaxome, connecting piece of sperm, centrosomes of other tissues interacts with ODF1, meiotic and postmeiotic, partial homology with cenexin	285–290
ODF3 (SHIPPO1)	*Odf3*	Outer dense fiber of sperm tails 3, structural protein, postmeiotic, high expression in testis, low in epididymis and brain	291
ODF4 (OPPO 1, Tisp-62)	*Odf4*	Outer dense fiber of sperm tails 4, oppo (from Japanese word for tail) 1, structural protein, meiotic and postmeiotic, testis-specific	292,293
Rhophilin (ODF5)	*RHPN1*	Outer dense fiber of sperm tails 5, also as rhophilin	
		Rhophilin-associated protein 1, AKAP-binding sperm protein, postmeiotic, testis-specific	291
Roppoein (ODF6)	*Ropn*, *ROPN1*	Outer dense fiber of sperm tails 6, also as ropporin-1	
Spag4	*Spag4*, *SPAG4*	Sperm associated antigen 4, structural protein, interacts with ODF1, present in manchette and axoneme, not in ODF, postmeiotic, testis-specific	294,295
Spag5 (deepest, Astrin)	*Spag5*	Sperm associated antigen 5, structural protein, interacts with ODF1, meiotic and postmeiotic, testis-specific	296,297
		KO; fertile	297
OIP1	*Rnf38*	ODF1 interacting protein, ring finger protein 38, structural protein, interacts with ODF1, localizes to sperm tail, meiotic and postmeiotic, high expression in testis, present in several other tissues	292
CRISP2 (Tpx-1)	*Crisp2*	Testis-specific protein 1, cysteine-rich secretory protein 2, also present in acrosome and connecting piece and longitudinal columns of FS, meiotic and postmeiotic, testis-specific	189,190
		Interacts with GGN1 and SHTAP	298
SAK57	*Krt5*	Sperm associated keratin of mass 57 kDa, keratin 5, keratin complex 2 gene 5, structural protein, also found in manchette and acroplaxome, meiotic and postmeiotic, expressed in many tissues	299
VDAC2	*Vdac2*	Voltage dependent anion channel 2, ubiquitously expressed, bind to KLC3	287,300,301
VDAC3	*Vdac3*	Voltage dependent anion channel 3, ubiquitously expressed	300
KLC3	*Klc3*	Kinase light chain 3, motor protein, interacts with ODF1 and VDAC2 postmeiotic, expressed in multiple tissues	302–305
Cdk5/p35	*Cdk5r1*	Cyclin-dependent kinase 5 regulatory subunit (p35) 1, regulator of proline directed serine/threonine kinase, associated with developing ODF, ubiquitously expressed	306

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ODF2.[294] SPAG4 has a leucine zipper that interacts with a leucine zipper in ODF1.[294] SPAG4 interacts with two microtubule-containing structures in spermatids, the manchette and the axoneme, but not outer dense fibers in epididymal spermatozoa. SPAG4 is presumably involved in outer dense fiber assembly by forming a link between ODF1 and axonemal microtubules.[294] SPAG5 associates with ODF, but not with the axonemal microtubules.[308] OIP1 binds to the conserved C-X-P motif in the C-terminal end of ODF1,[309] and ODF1-OIP1 interaction is enhanced by cyclin-dependent kinase 5 (CDK5), p35-mediated ODF1 phosphorylation.[310] ODF1 and ODF2 also are located in the manchette.[311]

The genes for ODF2, ODF3, and ODF4 are not homologous to each other or to the gene for ODF1. Their names relate only to their original source. The *Odf1* gene encodes a cysteine-proline-rich 27-kDa protein that is the major protein of rat ODFs. The *Odf1* gene encodes a protein highly conserved in evolution.[288] ODF1-null male mice are infertile, and the sperm show detachment of the head from the tail.[284] In addition, the organization of the mitochondrial sheath and the outer dense fibers are disturbed.

A major 84-kDa outer dense fiber protein encoded by the *Odf2* gene interacts through leucine zipper motifs.[289] ODF2 is also found in rat, mouse, and human. ODF1 was detected only in the medulla, but ODF2 was detected in both the cortex and the medulla of the outer dense fibers.[307] Although ODF2 is abundant in spermatogenic cells, it is also a cell-cycle dependent scaffold component of centrosomes in somatic cells.[290] ODF2 is known as a protein marker of the mother centriole and is a coiled-coil protein containing two leucine zipper motifs that mediate interactions with itself, ODF1 and microtubules.[290] In the testis, ODF2 is found localized to the acroplaxome, tail, connecting piece, and the nucleoli of Sertoli cells, spermatogonia, and spermatocytes.[137] ODF2 and cenexin 1 and 2 are the products of differential splicing of the *Odf2* gene consisting of 24 exons. ODF2 and cenexin 1 and cenexin 2 differ in a short stretch of amino acids at their N-terminal regions encoded by exon 3b. A peptide of 42 amino acids specific for cenexin is necessary for the formation of ODF2/cenexin fibers that are associated with acetylated microtubules and targets ODF2/cenexin to the centrosome and the primary cilium.[312] Conditional knockout (KO) mice have been generated, in which exons 6 and 7 of *Odf2* are disrupted.[313] In these conditional KO mice, multiple phenotypes such as coughing and sneezing were induced due to impaired structure and function of the basal bodies of cilia, but the effects on the flagellum of the sperm tail are unknown.

ODF3, a highly conserved 28-kDa protein is expressed only during the postmeiotic phase of spermatogenesis.[291] ODF4, a 33-kDa protein, is expressed only in the testis.[292] The human *ODF4* gene encodes a 30-kDa protein and differs from the mouse gene in genomic structure and in exon/intron usage.[293]

Three other ODF1-interacting proteins, SPAG4, SPAG5, and OIP1, are identified in rat sperm. SPAG5 also interacts with ODF1 via leucine zippers.[295] However, ODF2 does not interact with either SPAG4 or SPAG5, indicating that their interactions with ODF1 involve more than leucine zipper binding. The *Spag5* gene product, a 200-kDa protein, is synthesized in pachytene spermatocytes and spermatids. SPAG5 has a 73% similarity with DEEPEST, a putative mitotic spindle protein of unknown function.[296] SPAG5-null male mice are fertile[297] and the role of the protein remains unknown.

OIP1, a RING-finger family protein, is present in the outer dense fibers of rat sperm. It is encoded by the *Rnf38* gene, which appears to have biphasic expression during spermatogenesis, with one peak in spermatogonia and another in round spermatids. *Rnf38* also is expressed at lower levels in epididymis, heart, and smooth muscle.[309] ODF1 phosphorylation by CDK5 enhances ODF1 interaction with OIP1.[310]

Other proteins found in outer dense fibers are CRISP2, keratin 5 (KRT5, SAK57), voltage dependent anion channels 2 and 3 (VDAC2 and VDAC3), kinesin light chain 3 (KLC3), and the CDK5/p35 complex. CRISP2 is a member of the CRISP family including CRISP1, CRISP2, CRISP3, and CRISP4. They share 40%–80% sequence similarity at the protein level, and all are found in sperm. CRISP2 is a component of outer dense fibers and is present in the developing acrosome in spermatids, and in the perinuclear theca, connecting piece, and longitudinal columns of the fibrous sheath in rat sperm.[189] CRISP2 has a potential role in fertilization through the regulation of ion signaling in the acrosome reaction and sperm motility. The ion channel regulatory region within the CRISP domain of CRISP2 interacts with gametogenetin 1 (GGN1), a novel CRISP2-binding protein in the mouse sperm tail expressed during spermatogenesis, suggesting a role for the GGN1–CRISP2 complex in sperm tail development and/or motility.[190] In addition, CRISP2 specifically binds to multiple ~20–87 kDa isoforms of the sperm head and tail associated protein (SHTAP), a novel binding partner in the mouse.[314] SHTAP protein is localized to the periacrosomal region of round spermatids, and the head and tail of the elongated spermatids and sperm tail, where it co-localizes with CRISP2.

KRT5, an acidic keratin, is temporarily stored in the manchette, and then became a component of ODFs and the longitudinal columns of the fibrous sheath after the dispersion of the manchette.[299]

VDAC2 and VDAC3 are identified in bovine sperm outer dense fibers.[300] VDAC2 is located in the acrosomal membrane or plasma membrane of human spermatozoa and plays putative roles in sperm functions through mediating Ca^{2+} transmembrane transport.[315] VDAC2

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is also localized on the acrosomal region and principal piece, while VDAC3 is localized on the acrosomal region and midpiece.[301]

KLC3 binds to the surface of outer dense fibers in spermatids, isolated outer dense fibers, and to ODF1 in a microtubule-independent fashion. In a recent study using a transgenic mouse model in which a KLC3ΔHR (heptad repeat) mutant protein is specifically expressed in spermatids, KLC3 mutant protein bound mitochondrial protein VDAC2 and caused aggregate formation. It did not bind outer dense fibers,[304] but it was found to be localized between outer dense fibers and mitochondria,[305] suggesting it plays a role during the formation of sperm tail midpiece and in sperm function.

CDK5 is a proline-directed serine/threonine kinase that pairs with an activator, either p35 or p39. Both CDK5 and p35 are present in the outer dense fibers and in the basal plate, capitulum, and striated collar of the connecting piece.[306] ODF2 interacts with CDK5 and p35 but not with the CDK5/p35 heterodimer.[310] In addition, an in vitro phosphorylation assay showed that although CDK5/p35 did not phosphorylate ODF2, it phosphorylated ODF1.[306] Such phosphorylation of ODF1 enhanced ODF1–OIP1 interaction, suggesting that CDK5 may be important in promoting ODF1 degradation.

The germ cell-less homolog 1 (*Drosophila*)-like (GMCL1) protein is predominantly expressed in rat elongating spermatids and is integrated into outer dense fibers.[316] It is localized at the abaxial (convex) surface of outer dense fibers. TEKTIN4, TEKTIN5, and GMCL1 interact either with each other or with some molecules in the sub-mitochondrial reticulum to tether the axoneme–outer dense fiber complex to the mitochondrial sheath.

Satellite Fibers

Satellite fibers are present in the flagellar matrix between the outer dense fibers (Figure 3.12). These fibers form in step 19 of spermiogenesis in the rat, just before spermatozoa are released from the seminiferous epithelium.[273] The satellite fibers appear to separate from the edge of the outer dense fibers. SPETEX1, a protein with two coiled-coil motifs, appears to be associated with the satellite fibers and involved in the structural stability of the middle piece in cooperation with TEKTIN4.[317] It is also present in the capitulum and connecting piece in *Suncus*.[318]

Function

The outer dense fibers function in improving the bending torque of the tail,[272] and/or protecting the sperm tail against shear forces encountered during epididymal transport and ejaculation. The fibers are also important for the stability and the elastic recoil of the sperm tail, as well as for support of the flagellar beat, but are not involved in active motility. Stabilization of outer dense fiber proteins by abundant disulfide cross-linking may give them significant passive elastic properties that serve to stiffen or provide elastic recoil for the sperm tail.[4] The presence of VDAC2 and VDAC3,[300] and a serine/threonine kinase CDK5, its activator p35, and Cdk5/p35 activity[306] suggest that the outer dense fibers serve as scaffolds for proteins involved in regulating sperm function.

Annulus (Jansen's Ring)

The annulus is located at the junction of the midpiece and the principal piece in mammalian mature spermatozoa (Figure 3.10). It is essential for the structural and mechanical integrity of the flagellum. Cytoplasmic droplets do not move to distal regions beyond the annulus. During spermatogenesis when the axoneme starts to extend, the annulus is already formed as an electron-dense structure in the round spermatid phase, associating with the chromatoid body at the base of the flagellum. As sperm tails elongate, the annulus and the ring-shaped chromatoid body move distally along the axoneme towards its final position. During its migration, the annulus is located at the leading edge of the cytoplasm that encompasses the midpiece area of the axoneme.

Some of the biochemical components of the annulus have been identified in the mouse, bovine, and human.[247] Septin (SEPT) proteins, originally named from being in the "septin ring" in yeast, are localized at the annulus. Septins are generally regarded as cytoskeletal proteins involved in various cellular functions through interaction with tubulin or actin. Septins are members of the GTPase superfamily (polymerizing GTP-binding proteins), and there are at least 14 septin genes. Several are shown to be associated with male infertility and other diseases in humans.[319] In SEPT4-null sperm, the annulus is replaced by a loose fibrous network, beyond which kinesin-mediated intraflagellar transport is impaired (stalls). Although the ability of ATP usage by the dynein motor is decreased, the axonemal 9+2 structure is unchanged.[247] In addition, in SEPT4-deficient sperm, domain confinement was lost, allowing unrestricted lateral diffusion of BSG on the whole tail.[320] BSG is normally confined to the principal piece domain on testicular and caput sperm and is relocated into the midpiece during epididymal transit.[30] Thus, SEPT4 functions as a membrane diffusion barrier at the midpiece–principal piece junction. Decreased expression (haploinsufficiency) of SEPT12 (*Sept12*$^{+/-}$) results in maturation arrest at the spermatid stage, broken acrosomes, bent tails, disorganized mitochondria, and nuclear DNA damage.[321] SEPT4, SEPT5, and SEPT6 deficiencies, however, do not show overt phenotypes. In infertile men, two missense SEPT12 mutations, c.266C>T/p.Thr89Met and

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c.589G>A/p.Asp197Asn, were found inside the GTPase domain, implying alteration of the protein structure.[322] The SEPT12^{D197N} patient spermatozoa showed annulus defects with bent tail and loss of SEPT12 from the annulus.[322]

DnaJ (Hsp40) related, subfamily B, member 13 (DNAJB13) and solute carrier family 26, member 8(SLC26A8, TAT1) appear to associate with the annulus.[323,324] DNAJB13 is a member of the HSP40 cochaperone family produced in the mouse testis and located in the radial spokes of the flagellar axoneme in mature sperm and similarly in the flagella of *Chlamydomonas reinhardtii*.[325] DNAJB13 is co-localized with the annulus in late spermiogenesis and migrates towards the midpiece–principal piece junction. When the annulus reaches its final position, DNAJB13 appears to gradually disappear from this structure and persists as a radial spoke protein.[324]

SLC26A8 is a member of the SLC26 family of anion transporters that is specifically expressed in male germ cells.[326] SLC26A8 is localized to the annulus region in normal mouse and human spermatozoa. The structural defects in SLC26A8-deficient sperm are similar to those in SEPT4-null sperm, but SLC26A8-deficient sperm lack or have an incomplete oval annulus that is detached from the plasma membrane and the fibrous sheath, strongly reducing ATP consumption.[323]

During spermatogenesis, the annulus guides the growth of the flagellum to coordinately align the mitochondria along the axoneme. Then, the annulus/septin ring serves as a cortical scaffold to organize the fibrous sheath immediately distal to the mitochondrial array, and also serves as a barrier between the midpiece and principal piece, forming a complex of septins 1, 4, 6, 7, and 12 in mammals. This complex is associated with the co-chaperone DNAJB13 and possibly with integral protein SLC26A8, which could play a critical role in anchoring the annulus to the plasma membrane.[327]

Fibrous Sheath

General Features, Structure, and Formation

Most of the genes encoding the known fibrous sheath components are expressed during the postmeiotic period of spermatogenesis and found only in spermatogenic cells. Constituent proteins, features, and the genes associating to the fibrous sheath are listed in Table 3.4.[328–359]

Structure

The fibrous sheath defines the extent of the principal piece (Figure 3.1), by far the longest segment of the flagellum.[167] It is a cytoskeletal structure that is probably unique to the flagellum of spermatozoa in mammals and some birds. It closely underlies the plasma membrane, is attached to the annulus (Figure 3.10), and is a tapering cylinder formed by two longitudinal columns connected by circumferential ribs (Figures 3.9 and 3.11). The columns are formed by longitudinally oriented, loosely packed filamentous structures 15–20 nm in diameter. The longitudinal columns run peripheral to microtubule doublets 3 and 8 of the axoneme and are attached to outer dense fibers 3 and 8 in the proximal part of the principal piece. Distal to the termination of these two dense fibers, the tapered central edges of the longitudinal columns attach to ridges projecting from microtubule doublets 3 and 8.[167] The columns lie approximately in a plane sagittal to both of the inner doublet microtubules of the axoneme. The dorsal longitudinal column is nearest outer doublet 3, while the ventral column is nearest outer doublet 8 in sperm with a falciform shape.[167] The ribs broaden toward their ends, where they merge with the longitudinal columns and with each other. The thickness of the ribs diminishes toward the distal end of the principal piece, and the ribs and outer dense columns end abruptly at the posterior margin of the principal piece.[4,167] In human spermatozoa, the ribs are 10–20 nm apart and about 50 nm wide.

Formation

Fibrous sheath assembly, most thoroughly studied in the rat, proceeds throughout much of spermiogenesis from distal to proximal, opposite to the direction of outer dense fiber development.[273] The longitudinal columns first appear as thin rods joined to microtubule doublets 3 and 8 near the distal end of the flagellum in step 2 of spermiogenesis and increase in length until step 10. The ribs appear in step 11 as evenly spaced pairs of fibrous bands lying against the plasma membrane in the posterior part of the flagellum. The rib precursor associates with the columns in a distal to proximal direction during steps 12–14. During steps 15 and 16, additional ribs are formed and the longitudinal columns thicken to complete fibrous sheath development. Fibrous sheath proteins continue to be synthesized during the entire 15-day period of fibrous sheath formation in the rat.[273] This assembly occurs similarly in the mouse.[273]

Composition

The fibrous sheath is highly resistant to acid solubilization and contains disulfide bonds that cross-link the proteins for stabilizing the fibrous sheath. There are presumably more than 20 proteins that are located in or are closely related to the fibrous sheath of mammalian spermatozoa (Table 3.4).

Six cAMP-dependent protein kinase A (PRKA or PKA) anchoring proteins (AKAPs) have been identified in sperm, including AKAP1, AKAP3, AKAP4, AKAP11, microtubule-associated protein 2 (MAP2), and WAS protein family, member 1 (WASF1, WAVE1).[360] Of these,

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TABLE 3.4 Fibrous Sheath-Associated Proteins

Protein	Gene	Features	References
AKAP4 (p82, FSC1, AKAP82, FS75)	*Akap4*	"PRKA (PKA) anchoring protein 4," major structural protein, two kinase activity, PKA anchoring sites, postmeiotic, testis-specific	328–332
		Transmembrane receptor serine/threonine kinase signaling pathway	333
		KO male; infertile due to lack of sperm motility	331
AKAP3 (AKAP110, FSP95)	*Akap3*	"PRKA (PKA) anchoring protein 3," structural protein, PKA anchoring site, postmeiotic, testis-specific	334,335
TAKAP-80 (AKAP14)	N.D.	"Rat testis-specific, developmentally regulated, RII-binding protein," postmeiotic, testis and other tissues	336
FSIP1	*Fsip1*	Fibrous sheath interacting protein 1, binds to AKAP4, postmeiotic, testis-specific	332
FSIP2	*Fsip2*	Fibrous sheath interacting protein 2, binds to AKAP4, meiotic and postmeiotic, testis-specific	332
ASP (AKAP-asp)	*Ropn1l*	"AKAP-associated sperm protein," ropporin 1-like protein, binds to PKA anchoring site on AKAP3, testis-specific	337
Ropporin (RPPN, ODF6)	*Ropn1*	Rhophilin-associated protein 1, AKAP-binding sperm protein, postmeiotic, testis-specific	337
Rhophilin (rhophilin 1 or odf5)	*Rhpn1*	Rho GTPase binding protein 1, meiotic and postmeiotic, high expression in testis and low in some other tissues	338–340
Sp17	*Spa17*	Sperm protein 17, meiotic and postmeiotic, mainly in principal piece, detectable in middle piece and dorsal acrosome, testis-specific	339,341
CABYR (fibroushethin 2)	*Cabyr*	Ca^{2+}-binding tyrosine phosphorylation regulated protein, mainly expressed in testis, bind to AKAP3 and ropporin	342,343
FS39 (DDC8)	*Ddc8*	39-kDa fibrous sheath protein, postmeiotic, testis-specific	344
Tsga10		Testis-specific 10, meiotic and postmeiotic, testis and other tissues	345
HK1-S	*Hk1*	Hexokinase 1-S, spermatogenic cell HK-1 splice variants, meiotic and postmeiotic, mainly in principal piece, testis-specific tethered to PFKMS and GSTM5 and the fibrous sheath	346–349
GAPD-S (GAPD-2)	*Gapds*	Glyceraldehyde 3-phosphate dehydrogenase-S, sperm-specific glycolytic enzyme, postmeiotic, testis-specific	350–353
		KO male; infertile	354
GSTM5	*Gstm5*	Glutathione S-transferase mu 5, meiotic and postmeiotic, high expression in testis and low in other tissues	355
Sptrx-1 (TXNDC1)	*Txndc2*	Sperm thioredoxin-1, associated with assembling longitudinal columns of fibrous sheath in spermatids, not detected in sperm, postmeiotic, testis-specific	356
Sptrx-2	*Txndc3*	Sperm thioredoxin-2, present in longitudinal columns and ribs in fibrous sheath in sperm, postmeiotic, testis-specific	357
36K-A	*Pdhb*	Pyruvate dehydrogenase E1 component β subunit, possible testis-specific splice variant, meiotic and postmeiotic, multiple tissues	358
PDHA2	*Pdha2*	Pyruvate dehydrogenase E1 component α subunit, possible testis-specific splice variant, meiotic and postmeiotic, multiple tissues present in fibrous sheath and co-localize with AKAP4	359

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three AKAPs are identified in the fibrous sheath. PRKA are defined by their ability to bind one or more of the regulatory subunits (PRKAR1A, PRKAR2A, PRKAR1B, and PRKAR2B, formerly RIa, RIIa, RIb, and RIIb) of PRKA, and these subunits interact with an amphipathic helix on the AKAP.[360] The catalytic subunits are PRKACA, PRKACB, and PRKACG (formely Cα, Cβ, and Cγ).[361]

AKAP4, a ~80-kDa protein, is the most abundant AKAP and a major structural component.[328,329] AKAP4 is present in both the longitudinal columns and the ribs of the fibrous sheath of mouse spermatozoa. AKAP4 has two regions of homology with PKA-anchoring domains characteristic of AKAP proteins, one domain with dual specificity for binding either the PRKAR1A or PRKAR2A subunit of PKA and one domain that binds only PRKAR1A.[330] AKAP4-null males are infertile, and have reduced motility (one-eighth the level of sperm motility from wild-type male). In the absence of AKAP4, the framework of the fibrous sheath formed, but the fibrous sheath remained incompletely developed, and the flagellum was shortened.[331] Also, AKAP4-null sperm showed disruption in subcellular distributions of PRKA, phosphatidylinositol 3-kinase, and sperm autoantigenic protein 17 (SPA17), as well as significant alteration in activity and phosphorylation of PPP1CC.[361]

AKAP3, a ~110-kDa protein, has a single PRKAR2A binding site. In the mouse, *Akap3* mRNA and AKAP3 protein were both detected in step 2 round spermatids, but AKAP3 protein was not detected in the flagellum until the beginning of step 8 spermatid elongation.[332] In human sperm, the AKAP3 protein was detected by immunoelectron microscopy only in the circumferential ribs.[335] AKAP3 is involved in formation of the framework of the fibrous sheath, and AKAP4 is deposited on this framework late in spermiogenesis.[331]

Other proteins associated with the fibrous sheath include AKAP14 (TAKAP-80), a 55-kDa protein found in the rat, which has a single PRKAR2A binding site.[336] In addition, fibrous sheath interacting proteins 1 and 2 (FSIP1 and FSIP2) have been identified by yeast two-hybrid screening. FSIP1 binds to the C-terminal portion of AKAP4, and AKAP4 binds to a region at the C-terminal end of FSIP1.[332] The FSIP2 binding site on AKAP4 encompasses the dual PRKAR1A/PRKAR2A-binding domain, suggesting that FSIP2 might have a role in regulating PKA anchoring to AKAP4.

ROPN1 and roppolin 1-like protein (ROPN1L, ASP) interact with the PKA-binding domain of AKAP3.[337] ROPN1L is 39% identical to ROPN1 and both proteins have N-terminal regions similar to the AKAP-binding domain of the PRKAR2A subunit. Due to the sequence similarity of ROPN1 and ROPN1L to the N-terminal dimerization and docking domain of PRKAR2A, these proteins are named R2D2 proteins.[362] The R2D2 domain is also contained in SPA17 and Ca^{2+}-binding tyrosine phosphorylation-regulated protein (CABYR, formerly FSII).[343] ROPN1 was identified previously as a rhophilin-binding protein and forms a heterodimer with SPA17.[339] Rhophilin (RHPN1) contains a binding motif for the ras homolog protein (RHO), a small GTPase that triggers reorganization of the cytoskeleton and regulates motility and cell-substrate adhesion.[340] Ultrastructurally, ROPN1 is mainly on the inner surface of the fibrous sheath, while RHPN1 is tightly bound to the outer surface of the fibrous sheath.[339]

SPA17 and CABYR contain regions similar to the N-terminus of the AKAP binding region of PRKAR2A.[337] SPA17 is found in the principal piece, middle piece, and equatorial region of the acrosome, co-localizes with AKAP3 in the fibrous sheath of mouse sperm and binds to AKAP3 in vitro, but SPA17 is expressed more widely. CABYR is expressed mainly in the testis.[343] Multiple variants of CABYR were identified in human testis, not all of which contained the RII-like domain or the Ca^{2+}-binding EF-hand domain. Some of the variants gained Ca^{2+}-binding capacity when phosphorylated during capacitation.[343] Recent yeast two-hybrid assays showed that CABYR variants form a complex with AKAP3 and ROPN1 in human sperm fibrous sheath.[244] This binding to AKAP3 may reflect a functional need for Ca^{2+} signaling transduction and it may play a role in motility at sites along the fibrous sheath.

Protein FS39 (gene unknown) is testis-specific in the mouse and has similarities to myosin and intermediate filament-related protein.[344] The testis-specific 10 protein (TSGA10) contains a myosin-like domain similar to the Ezrin/radixin/moesin family domain. The gene is expressed at high levels in mouse testis, but the transcript is also present in some fetal tissues and tumor cells.[345] Mouse TSGA10 is a 65-kDa protein synthesized in elongating spermatids and processed to a 27-kDa protein in the purified mature fibrous sheath.[345]

Testis-specific isozymes or proteins associated with enzymatic activity also are found in fibrous sheath. They include three glycolytic pathway enzymes, hexokinase type 1, spermatogenic cell variant (HK1S), glyceraldehyde 3-phosphated dehydrogenase, spermatogenic (GAPDHS), and pyruvate dehydrogenase subunits (PDHA E1α and E1β subunits; PDHB).[5] In addition, there are enzymes associated with free radical metaolism, including mu-class glutathione S-transferase (GSTM5) and two thioredoxins, thioredoxin-related transmembrane protein 1 (TMX1, TXNDC1, SPTRX1) and thioredoxin domain containing 2 (spermatozoa) (TXNDC2, SPTRX2).

HK1S and GAPDHS are associated with the principal piece. Alternate transcripts of HK1 (hexokinase type 1) are present in spermatogenic cells, and the N-terminal porin-binding domain usually present in HK1 is absent and replaced with a spermatogenic cell-specific region

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(SSR).[348] There are at least three alternatively spliced transcripts that encode the same protein. HK1S is present mainly as a monomer in motile sperm with an increase of hexokinase activity accompanied by dephosphorylation and reduction of the disulfide bond in HK1S.[349] A yeast two-hybrid assay using the SSR as bait determined that HK1S is tethered to a sperm variant isozyme of muscle-type phosphofructokinase (PFKMS), which in turn is bound tightly to GSTM5 in the fibrous sheath.[363] Thus, direct interactions occur between two glycolytic enzymes and between a glycolytic enzyme and a fibrous sheath protein. A hierarchy of binding affinities exists from low to high between HK1S and PFKMS, between PFKMS and GSTM5, and between GSTM5 and other fibrous sheath components.[363]

GAPDHS is as a testis-specific ortholog of GAPDH (glyceraldehyde 3-phosphate dehydrogenase) that is integrated into the fibrous sheath and is encoded by the *Gapdhs* gene.[353] GAPDHS-null male mice are infertile, and the motility of the null sperm was reduced to a weak, twitching motion.[354] Oxygen consumption of the GAPDHS-null sperm was unchanged, but ATP levels were greatly reduced. Thus, the energy required for sperm motility is derived primarily from ATP produced by glycolysis in the principal piece and not by oxidative phosphorylation in mitochondria.[354] A recent study showed that active metabolism of glucose, rather than mitochondrial ATP production, results in hyperactivated motility of spermatozoa, by affecting sperm redox pathways that could play a role in regulating sperm changes that occur during capacitation.[364]

GSTM5 is a member of the mu-class glutathione S-transferase family. The GST proteins are scavengers of cytotoxic substances, such as electrophilic compounds and peroxides. GSTM5 found in the fibrous sheath of mouse sperm[329] might serve to protect sperm from damage by such agents.

Thioredoxins are able to reduce disulfide bonds in the presence of thioredoxin reductase. Thioredoxin family SPTRX1 protein is associated primarily with the longitudinal columns of the fibrous sheath during flagellar formation in rat spermatids, but little SPTRX1 was detected in rat epididymal spermatozoa. Thus, SPTRX1 is suggested to be a transient component whose role is to participate in the formation of the longitudinal columns of the fibrous sheath, but the SPTRX2 protein is an integral component of the fibrous sheath in epididymal spermatozoa.[357]

PDHB was identified in associated with the fibrous sheath in hamster sperm.[358] PDHA has two active sites and requires thiamine pyrophosphate (TPP) and Mg^{++} as cofactors for its enzymatic activity. PDHA co-localized with AKAP4 in the fibrous sheath and exhibited tyrosine phosphorylation during hamster sperm capacitation.[359] Using a proteomics approach, eight peptide fragments were found to match regions covering 32% of the sequence of PDHB.[358] PDHA catalyzes the decarboxylation of pyruvate, thereby forming a tightly bound enzyme intermediate and liberating carbon dioxide. PDHA is a component of pyruvate dehydrogenase complex that exhibits tyrosine phosphorylation during hamster sperm capacitation.[365]

Function

The fibrous sheath serves as a mechanoelastic structure involved in shaping the flagellar waveform.[4] The fibrous sheath serves as a scaffold for both glycolytic enzymes and constituents of signaling cascades mediated by tyrosine phosphorylations, plays a role in the regulation of sperm motility, and also is involved in sperm maturation, capacitation, and hyperactivation.[331,366,367] Glycolytic enzymes associated with the fibrous sheath include GAPDHS, HK1S, aldolase a (ALDOA), lactate dehydrogenase A (LDHA), triose phosphate isomerase (TPI), pyruvate kinase (PK), lactate dehydrogenase-C (LDHC), sorbitol dehydrogenase (SORD), and ADP/ATP carrier protein 4 (SLC25A4).[5] Several signaling proteins localized in the fibrous sheath include SPA17, CABYR, AKAP3, AKAP4, AKAP14, ROPN1, and GSTM5.[336,339,343,367,368]

The attachment of axonemal doublets 3 and 8 to the longitudinal columns of the fibrous sheath seems to restrict their participation in microtubule sliding and axoneme binding during flagellar motion. The longitudinal columns themselves also limit bending of the flagellum in this same plane, whereas these features would not restrict flagellar bending perpendicular to this plane.[4] Although sperm appear to swim with a planar effective stroke and recovery stroke somewhat like cilia, they also rotate, and the propagated waves have a three-dimensional component in the distal portion of the tail when viewed in a microscope.[4]

The cAMP-signaling pathway participates in the regulation of processes associated with sperm maturation, motility, capacitation, hyperactivation, and the acrosome reaction.[125,369] PKA is the predominant target for cAMP, synthesized from ATP by adenylate cyclase in response to HCO_3^- and Ca^{2+} stimuli, and PKA in turn phosphorylates proteins on serine and threonine residues. The downstream targets for PKA in this cascade are tyrosine kinases that modify proteins in the flagellum associated with these processes.[369] The presence of AKAP4, AKAP3, and AKAP14 in the fibrous sheath strongly suggests that one of its main roles is to anchor the PKA involved in such processes.

Components of the Rho-GTPase signaling pathway also are associated with the fibrous sheath. RHOPN1 binds to Rho, a small GTPase that acts through effector molecules to modulate reorganization of cytoskeletal components. ROPN1 and ROPN1L bind to the RII-anchoring

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site on AKAP3 and contain an N-terminal domain similar to the AKAP-binding region of PKAR2A.[337] Other proteins associated with the fibrous sheath that also have similar domains are SPA17, and CABYR.[337] This led to the suggestion that AKAPs serve as scaffolding proteins for the Rho-GTPase pathway and that Rho activates kinases and phosphatases involved in regulating sperm motility.[332,337] The evidence that Rho GTPases (RhoA, RhoB, Rac1, and Cdc42), as well as the effectors RhoGDI, PI(4)P5K, and ROCK-1, were present in the sperm head in boar, human, rat, ram, bull, and elephant sperm suggests that Rho GTPases in spermatozoa are conserved throughout mammalian evolution and Rho GTPase is involved in regulating the acrosome reaction in the sperm of several mammalian species as well as in motility of bovine sperm.

Although glycolysis is a highly conserved process, several of the glycolytic enzymes in sperm have biochemical or enzymatic properties that differ from those of glycolytic enzymes in somatic cells.[370] Four glycolytic enzymes in sperm that have been examined closely to determine the basis for these differences are PGK2, GAPDHS, LDHC, and HK1S. Sperm require glucose for in vitro fertilization and are not able to use pyruvate or lactate as energy substrates. Since blocking mitochondrial oxidative phosphorylation with oligomycin does not inhibit fertilization, sperm do not depend on mitochondria for their energy. Indeed, targeted disruption of the *Gapdhs* gene drastically reduced ATP levels and sperm motility, leading to male infertility.[354]

Abnormal Flagella

Spermatozoa with abnormal flagella are not common in many mammals, but are relatively common in humans. Asthenozoospermia is found frequently in infertile men.

Two categories of flagellar defects have been defined.[371,372] One category includes nonspecific or nonsystematic defects, seen as heterogeneous combinations of alterations affecting variable percentages of sperm among different individuals. These are usually acquired secondary to pathological conditions that affect the testis or duct system. The second category includes systematic defects, often seen as a particular sperm phenotype that predominates in a sample from an individual and resembles the phenotype of sperm from other individuals. These are likely to have a genetic origin and tend to show family clustering.[372] These two categories are recently described as nonspecific flagellar anomalies (NSFAs) and various genetic disorders including primary ciliary diskinesia and the dysplasia of the fibrous sheath, and are reviewed in sperm pathologies affecting the head, neck, and tail.[121]

Heritable Effects on Human Sperm Flagellar Structure

One hereditary condition causing sperm flagellar defects is referred to as immotile-cilia syndrome (ICS),[373] Kartagener's syndrome, or primary ciliary dyskinesia (PCD).[374] It often results in male sterility, situs inversus (major visceral organs are reversed from their normal position), chronic respiratory problems, and sinositis.[373] These conditions are directly or indirectly a consequence of an autosomal recessive trait that makes cilia or flagella unable to perform normal and coordinated movements.[375] Sperm from men with the syndrome have defective axonemes with such alterations as a partial or complete lack of dynein arms, a lack of outer dynein arms, a lack of inner arms, or abnormally short spokes, and a lack of the central sheath.[375] Because of the different types of defects seen in sperm and cilia, mutations in multiple genes might contribute to the syndrome.[373] Over 200 axonemal proteins are present in human cilia[376] and many of these probably are present in the sperm flagellum. However, the relatively low incidence of ICS suggests that a smaller number of genes are involved.[377] Two strategies being applied to identify such genes include screening for mutation in candidate genes selected on the basis of their expected function in axonemes, and genetic linkage analysis in families with ICS to map chromosomal regions associated with the disease.[377] Such studies have led to the identification of two genes for components of the outer dynein arms that are associated with ICS and the elimination of other candidate genes.[377]

A different type of flagellar defect probably of genetic origin is known as dysplasia of the fibrous sheath (DFS). This syndrome is characterized by severe asthenozoospermia and by the sperm having hyperplastic and disorganized fibrous sheaths and short, rigid, thick, and immotile tails.[372] Other associated changes seen include distortion or severe disruption of the axoneme, extension of outer dense fibers 3 and 8 into the principal piece, and failure of the annulus to migrate caudally. The occurrence of this syndrome in brothers suggests that DFS can be caused by recessive mutations in autosomal genes involved directly or indirectly in the assembly and organization of the fibrous sheath. If DFS is the only symptom present, the mutations might occur in spermatogenic cell-specific genes expressed in spermatids. However, about 20% of DFS patients have respiratory symptoms from early childhood, suggesting that their sperm defects might be due to ICS,[371] that mutations in some genes might cause both ICS and DFS, or that these individuals have mutations in multiple genes associated with these syndromes.

An additional type of flagellar defect that occurs in sperm from infertile men is called flagellar dyskinesia.[378] It has been observed in brothers, suggesting that it can

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have a genetic cause.[379] The sperm have normal axonemes but altered placement of periaxonemal structures. These include abnormal extension of individual dense fibers along the axoneme, altered order of termination of these structures, and a modified number and location of longitudinal columns of the fibrous sheath.[378] The outer dense fibers have abnormal positions with respect to each other, being placed symmetrically with respect to a plane that passes through microtubule doublet 1 and between doublets 5 and 6. The most common defect of the longitudinal columns of the fibrous sheath is the presence of only one column adjacent to doublets 3 or 8.[378] One suggestion was that this might be due to a defect in the A microtubules of the outer doublets that affects the adjacent formation or assembly of the outer dense fibers and longitudinal columns of the fibrous sheath.[380]

Heritable Effects on Mouse Sperm Flagellar Structure

Several spontaneous mutations affecting formation of the flagellum have been identified in mice. The mutations usually are pleiotropic, causing defects in other tissues in addition to the seminiferous epithelium.[381] This is known because they were identified by other effects of the mutation and subsequently found to cause defects in sperm. Homozygous mice with the wobbler mutation, caused by a nonsense point mutation in the leucine rich repeat containing 32 (*Lrrc32*) gene and characterized by spinal muscular atrophy and defective spermiogenesis, apparently produce normal numbers of sperm, but few sperm are motile.[382] The sperm appear to have average-length tails and normal heads, but 70% of the sperm in the vas deferens have ultrastructural defects in the flagellum. These defects include the absence of one to four outer doublets and the corresponding dense fibers, most commonly those from positions 4 through 7. Other defects seen less frequently are supernumerary microtubules or the absence of central pair tubules in the axoneme.[383] Defective sperm are common in mice heterozygous for a mutation in the T-locus that causes a shortened tail and/or defects in sacral vertebrae,[384] with the most frequent flagellar defects seen in sterile mice being a lack of four outer dense fibers and doublets. The male-sterile mutation, hydrocephalic-polydactyl (*hpy*), results in the absence of a flagellum or in partially assembled axonemal structures and/or poorly organized aggregates of other tail components.[381] The axoneme usually is absent or abnormally formed and the outer dense fibers and fibrous sheath are morphologically atypical, when present. The axoneme dysgenesis might be due to failure to form stabile structures because of defective subunits.[381]

A defect often seen in an abnormal or degenerating flagellum is the absence of microtubule doublets 4 through 7 and the associated dense fibers. Doublets 3 through 8 and the associated dense fibers appear to remain firmly attached to the fibrous sheath, while doublets 4 through 7 are extruded from the sperm tail.[385] This suggests that sperm seen with this defect may be degenerating. In some situations, the flagellar axoneme appears to form normally and then to become progressively disorganized, as occurs during spermiogenesis in quaking[386] and in hpy/hpy mice.[381] However, in wobbler mice, the percentage of abnormal sperm increases as they transit the male tract. Only about 5% of sperm tails are abnormal in the testis, while nearly 70% of sperm from the vas deferens have abnormal flagella.[382]

Gene Knockouts Affecting Mouse Sperm Flagellar Structure and Function

Sperm flagellar defects and dysfunctions found in gene knockout mice models have been reviewed in detail elsewhere.[252,387] This accumulated evidence shows various mutants generate different phenotypes relating to the axoneme, fibrous sheath, outer dense fibers, motility, and other parameters, sometime affecting head components. More than 400 mutant mouse models with an altered reproductive phenotype have been generated.[388] Only about 10% of knockout mouse models that disrupt male fertility have spermatozoa with impaired motility and/or sperm flagellum structure anomalies, with the remainder being infertile due to disruption of developmental, spermatogenic, or endocrine functions.

Flagellar Motion

The Flagellar Wave

The evidence that the proximal part of the midpiece remains collinear with the axis of the head suggests that motion of the head is minimal with respect to the connecting piece or within the connecting piece itself.[241]

The flagellar wave is propagated in a plane perpendicular to the central pair of microtubules of the axoneme. It passes through doublet 1 and between doublets 5 and 6, with the active stroke being toward doublet 1.[389] The flagellum twists during bend propagation, with the plane of bending moving toward doublet 2.[390] The part of the axoneme containing doublets 1 through 5 is thought to be active during this phase of flagellar motion.[390] The outer dense fibers and fibrous sheath appear to play a passive role in flagellar motion. The tapering architecture of the dense fibers appears to relate to the progressive increase in the amplitude of curvature observed during flagellar wave propagation.

As sperm mature while passing through the epididymis, sperm gain a vigorous motion that results in rapid forward movement, referred to as progressive motility. With this change, sperm flagella appear to become more rigid due to the increased disulfide bond formation that

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occurs in outer dense fibers and the fibrous sheath and reduce the arc of curvature in swimming pattern. However, cauda sperm from different species show different patterns of motility, flagellar beat, and flagellar rigidity.

Regulation of Sperm Motility

Sperm undergo two types of motility: progressive motility and hyperactivated motility. The beating mode of sperm flagella can be categorized into two different types: symmetrical waves and asymmetrical waves. Progressive motility is generated by symmetrical waves propagating down the flagellum with net linear propulsion. Hyperactivation is associated with asymmetrical waves propagating down the flagellum with higher amplitude, resulting in circular trajectories.[391] Hyperactivation is characterized by a whip-like beating showing a vigorous figure-eight pattern of the flagellum. It occurs with the capacitation process leading to the acrosome reaction and is found in sperm during ascending of the female reproductive tract.[125,392] The details are still unclear concerning whether hyperactivation is a consequence of capacitation or whether hyperactivation and capacitation are separate processes that occur in parallel. However, hyperactivation mediated by Ca^{2+} signaling is regulated by similar yet distinct signaling events that distinguish it from capacitation that involves cross-talk between the cAMP/PKA and tyrosine kinase/phosphatase signaling pathways.[393]

ACTIVATION OF MOTILITY

Physiological factors such as Ca^{2+}, HCO_3^-, and cAMP are essential for the initiation and maintenance of progressive motility and hyperactivated motility. Ca^{2+} plays a major role in regulating hyperactivated motility by acting on the axoneme. Demembranated sperm produced by disruption of the plasma membrane and mitochondrial membranes with Triton-X100, leaving the axoneme and cytoskeletal support intact, show hyperactivation. However, it is poorly understood how Ca^{2+} reaches the axoneme in the flagellum to switch on hyperactivation. Sperm motility quiescence factors such as immobilin are present in rat epididymal fluid, suggesting that sperm motility is prevented in the epididymis.[394]

There is limited information about receptors on the sperm surface that might be involved in triggering progressive or hyperactivated motility. Olfactory receptors are a large family of membrane-associated G-protein-coupled receptors, some of which are found on the sperm surface. They are suggested to be involved in triggering progressive or hyperactivated motility as well as chemotaxis. Olfactory receptors are localized in the midpiece of mature sperm. Bourgeonal (a ligand for human olfactory receptor 1D2 (OR1D2)) causes an increase in intracellular Ca^{2+} as well as chemotaxis in human sperm.[395] Sperm from transgenic mice overexpressing the olfactory receptor 16 (OLFR16, MOR23) showed positive chemotactic swimming behavior.[396] In mammals, 29 olfactory receptors were identified in the testis by RNAse protection assays, northern blotting, RT-PCR, western blotting, in situ hybridization, DNA arrays, and bioinformatics.[397]

Gamma-aminobutyric acid (GABA) receptors are present in the sperm membrane, and exposure of human sperm to GABA causes changes in sperm motility parameters and hyperactivation.[398] Bicuculline and picrotoxin, antagonists of GABA, blocked the effects of both GABA and progesterone on the acceleration of rat sperm capacitation and hyperactivation, and sperm capacitation required both Cl^- and HCO_3^-.[399] This suggests that activation of a GABA receptor contributes to sperm capacitation and hyperactivation and that both HCl^- and HCO_3^- are essential for its function.

CALCIUM AND MOTILITY

Ca^{2+} has important roles in motility, especially hyperactivation. CATSPER1, CATSPER2, $Ca_v2.3$, and PMCA4 participate in the regulation of intracellular Ca^{2+} levels, and are involved in the initiation of sperm motility. CATSPER1 is a testis-specific voltage-gated Ca^{2+} entry channel localized to the sperm principal piece. CATSPER1-null male mice are infertile and have reduced progressive motility.[400] CATSPER2, another voltage-gated ion channel, is closely related to CATSPER1 and is present in the flagellum. CATSPER1- and CATSPER2-null mouse sperm fail to hyperactivate but exhibit normal protein tyrosine phosphorylation.[12,14]

Ca^{2+} channels have important roles in regulating sperm motility. Disruption of the gene for the Ca^{2+} channel, voltage-dependent, T type, alpha 1G subunit (CACNG1) resulted in increases in straight-line velocity and linearity in mutant sperm.[19] Disruption of the gene for PMCA4, a Ca^{2+}-dependent ATPase involved in Ca^{2+} efflux, also resulted in male infertility. One research group reported that there were no differences in motility of sperm with or without PMCA4 when incubated in a medium that did not induce capacitation, but sperm without PMCA4 became immotile when incubated in capacitation medium.[23] However, another group reported that the majority of sperm lacking PMCA4 were immotile.[24] In addition, the latter group indicated that the motile sperm present had decreased average velocity, progressive velocity, and track speed, and intracellular Ca^{2+} levels over two times higher than normal sperm.

Other regulators of ion movements across the plasma membrane also are known to be important in sperm motility. Disruption of the gene for a voltage-dependent anion channel (*Vdac3*) resulted in markedly reduced sperm motility, frequently causing alterations in the axoneme structure.[401] A sperm-specific sodium–hydrogen exchanger called solute carrier family 9, subfamily C (Na^+-transporting carboxylic acid decarboxylase),

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member 1 (SCLNC1) is also involved in regulating intracellular pH and is located in the principal piece. Disruption of its gene (*Sclnc1*, formerly *Slc9a10*) resulted in male infertility and markedly reduced sperm motility.[402] This immotility phenotype of SCLNC1-null spermatozoa appeared to result from an intimate association of SCLMC1 and the atypical spermatogenic cell adenylyl cyclase (ADCY10, sAC).[403] Furthermore, a unique Na,K-ATPase alpha4 isoform subunit (ATP1A4) found only in testis and located in the midpiece, is also required for sperm motility. It is responsible for transporting Na^+ out and K^+ into the cell. Specific inhibition of the alpha4 isoform with the cardiac glycoside ouabain substantially reduced the percentage of motile sperm over time.[404] In addition, an inositol 1,4,5-triphosphase (IP_3) receptor-gated release of Ca^{2+} from an intracellular store in the sperm neck region was suggested to regulate hyperactivated motility.[405]

cAMP AND MOTILITY

cAMP is a key second messenger to regulate sperm motility. cAMP levels increase when adenylyl cyclase is stimulated to convert ATP to cAMP. This results in activation of PKA to trigger a cascade of phosphorylation of serine and threonine residues on neighboring proteins. This occurs primarily in the flagellum and in turn results predominantly in phosphorylation of proteins on tyrosine residues.

Plasma membrane-bound adenylyl cyclases (ADCYs) are regulated by G proteins in response to external stimuli. There is evidence for G-protein activated ADCYs in mouse sperm that are involved in the acrosome reaction and in human sperm that are involved in olfactory receptor-mediated chemotaxis and hyperactivation.[406] However, sperm appear to depend primarily on the soluble adenylate cyclase ADCY10 that lacks a putative transmembrane domain and is not G-protein regulated.[407] Two alternatively spliced transcripts are expressed in rat testis, which encode a 187-kDa and a C-terminal truncated 52-kDa form.[407,408] The 187-kDa form is present predominantly in soluble fractions from rat testis.

HCO_3^- and Ca^{2+} are involved in the cAMP-regulated activation of sperm motility.[409] HCO_3^- directly stimulated ADCY10 of spermatozoa.[410] This elevates intracellular pH, increasing sperm intracellular cAMP, and activating protein kinase A, which in turn initiates a downstream signaling cascade that enhances tyrosine phosphorylation. Increased tyrosine phosphorylation is an indication that spermatozoa are undergoing capacitation.[410,411] HCO_3^- increased ADCY10 activity in vitro by increasing the rate of the enzyme reaction and by relieving substrate inhibition that occurs at high ATP-Mg^{2+} concentrations.[409] The evidence that HCO_3^- concentration in the epididymis is low suggests that sAC activity is limited in sperm stored in the epididymis.[409] Ca^{2+} acts directly on ADCY10 and acts synergistically with HCO_3^-.[409]

ADCY10-null male mice were infertile and their sperm showed only a trembling movement in the midpiece and no forward motility, and this motility was restored by loading the sperm with a cAMP analog.[412] ADCY10-null spermatozoa, adenylyl cyclase activity and cAMP content are greatly diminished, and HCO_3^- is unable to rapidly accelerate the flagellar beat or facilitate evoked Ca^{2+} entry into sperm.[413] Thus, ADCY10 and cAMP coordinate cellular energy balance in sperm, and ADCY10 plays a critical role in cAMP signaling in spermatozoa.

PKA AND MOTILITY

PKA phosphorylation of proteins on serine and threonine residues activates a signaling cascade leading to tyrosine phosphorylation of flagellar proteins. The presence of PKA anchoring sites (AKAP3, AKAP4, and AKAP14) in the fibrous sheath strongly suggests that one of the major roles of this structure is to anchor PKA in the principal piece region. The defining characteristic of an AKAP is the presence of an amphipathic helix region that binds PKA, usually in a subcellular location that places it close to its target proteins.[414] The PKA holoenzyme consists of two catalytic I subunits and two regulatoI(R) subunits. Genes have been identified for three C subunits (PRKACA, PRKACB, PRKACC) in humans and two C subunits (PRKACA, PRKACB) in mouse, and for four R subunits (PRKAR1A, PRAKR1B, PRKAR2A, and PRKAR2B) in humans and mice. There are at least three isoforms for PRKACB and two isoforms for PRKACA in mice, one of which (PRKACA2) lacks the N-terminal myristoylation signal and is present only in the testis.[415] The R subunits dimerize by their N-terminal domains and bind to AKAPs by this region. The R and C subunits have a cAMP-binding site and when cAMP binds to the R subunits, the C subunits are released and their catalytic activity is triggered by cAMP. PRKAR2A was present mainly in the axonemal region of the flagellum of bovine sperm and in the middle piece and distal portion of the principal piece of the flagellum of mouse sperm,[416] while PRKAR1A was seen in association with outer dense fibers and the fibrous sheath of boar sperm.

In PRKAR2A-null male mouse sperm, the majority of the PKA was not anchored in the flagellum, and sperm motility and fertility were not changed.[416] RI appears to be present in low amounts in the flagellum,[416] and bind to a PRKAR1A-specific domain and a dual PRKAR1A/PRKAR2A domain of AKAP4 in vitro.[330] Although significant amounts are present in specific locations within the flagellum, the PRKAR1A knockout is an embryonic lethal[417] and the role of PRKAR1A in sperm motility was unclear.

PRKAR2A is present in the midpiece and axoneme of ovine and mouse sperm.[418] $C\alpha_2$-null male mice generated

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from the targeted disruption of exon 1b of the Cα gene using a conditional Cre-loxP knockout system produced normal numbers of sperm, but were infertile.[419] The null sperm have progressive motility, but failed to hyperactivate or to accumulate tyrosine-phosphorylated proteins.[419] The level of PRKAR1A in sperm was reduced considerably, while the level of RIIα was reduced somewhat. The PRKAR2A remaining was most abundant in the principal piece, suggesting that it anchors to AKAPs in that region in the absence of C subunits.

PHOSPHORYLATION AND MOTILITY

Increased protein tyrosine phosphorylation is found in the flagellum and associated with capacitation, but not hyperactivation. Capacitation and protein tyrosine phosphorylation are regulated by cAMP at the level of PKA.

Studies defining the correlation between capacitation and the pattern of changes in tyrosine phosphorylation have greatly facilitated studies leading to a better understanding of the roles of bovine serum albumin (BSA), Ca^{2+}, HCO_3^- and other factors involved in the regulation of protein phosphorylation during capacitation.[420]

A number of changes need to occur in the sperm before the tyrosine phosphorylation begins, and the time course is relatively slow. Newly phosphorylated proteins are first seen in mouse sperm after 30–45 min in capacitation medium and the maximum level of phosphorylation is reached at about 90 min. Although some of the increases in phosphorylation might be involved in regulating hyperactivated motility, initiation of progressive motility occurs within a few seconds after exposure to medium containing HCO_3^-.[421] This suggests that rapid PKA-mediated serine/threonine phosphorylation events are involved in initiation of progressive motility.

A few sperm proteins have been identified that are serine/threonine phosphorylated in response to HCO_3^-. These include 96-, 64-, and 59-kDa bands recognizable on western blots of boar sperm extracts with an antibody against the consensus target sequence for PKA.[422] Proteomics approaches indicated that the 96- and 59-kDa bands were ODF2. It was suggested that PKA phosphorylation of target sites within a leucine zipper domain might result in changes in protein–protein interactions to alter mechanical properties of outer dense fibers.[422] In addition, AKAP4, AKAP3, and valosin-containing protein (VCP), the homolog of a SNARE-interacting protein, were found to be serine and tyrosine phosphorylated in human sperm.[423] Another sperm protein that is serine phosphorylated is glycogen synthase kinase 3 beta (GSK3B). Stimulation of bovine sperm motility with a general phosphodiesterase inhibitor (IBMX) or a serine/threonine-specific phosphatase inhibitor (calyculin A) resulted in a dramatic increase in GSK3B serine phosphorylation and a decrease in its catalytic activity.[424] In addition, the pyruvate dehydrogenase E1 component β (PDHB) associated with the fibrous sheath in hamster sperm is serine phosphorylated in a cAMP-dependent manner in association with the initiation of motility.[358] However, it is not obvious how serine/threonine phosphorylation of these proteins affects motility.

Several of the proteins that undergo changes in tyrosine phosphorylation during capacitation include the fibrous sheath proteins AKAP3,[423,425] AKAP4,[328,335,423] and CABYR.[343] Others reported are VCP[423,426] and NSF, another homolog of the SNARE-interacting protein.[423] Fifteen additional human sperm proteins that undergo tyrosine phosphorylation were identified by microsequencing.[423] These include proteins that are axonemal (α-tubulin) and fibrous sheath or outer dense fiber components (ODF1, VDAC2, GSTM3, PDHB) (Tables 3.3 and 3.4). Some of these also were identified (previous paragraph) as serine and threonine phosphorylated proteins.

Kinases responsible for tyrosine phosphorylation during capacitation must be present in sperm that are either direct targets of PKA or in the downstream signaling cascade. A 42-kDa tyrosine kinase (sp42) was isolated and purified from ejaculated boar sperm.[427] It is male germ cell-specific and localized to the midpiece. Another tyrosine kinase isolated from boar sperm (TK-32) was shown by an in-gel tyrosine kinase activity assay to require Ca^{2+} and HCO_3^- in the incubation medium. An additional tyrosine kinase identified is present in the connecting piece and principal piece of the boar sperm flagellum.[428] This 72-kDa protein is not extracted by NP-40, indicating it is bound to cytoskeletal components of the sperm. It is recognized by an anti-phospho-Sky antibody and is likely to be protein tyrosine kinase 3 (TYRO3, SKY), a Src family protein tyrosine kinase. It was shown to respond to cAMP-PKA signaling by tyrosine phosphorylating proteins mainly in the connecting and middle pieces. In addition, Yamaguchi sarcoma viral (v-yes) oncogene homolog 1 (YES1, c-Yes), another member of the Src family, has been identified in human sperm.[429] However, it was shown by immunohistochemistry to be present in the sperm head and unlikely to be involved directly in regulation of motility.

Although the primary signaling pathway for protein phosphorylation passes through PKA, other routes for the initiation of this process are present. Stimulation of sperm motility occurred when human sperm were treated with an inhibitor (LY294002) of phosphatidylinositol 3-kinase (ataxia telangiectasia mutated homolog, ATM). This resulted in an increase in cAMP production and tyrosine phosphorylation of AKAP3, leading to the suggestion that PI3-kinase is a negative regulator of sperm motility.[425] Treating sperm with the inhibitor also resulted in stimulation of binding of PKA to AKAP3 through PRKA2B, apparently due to tyrosine phosphorylation of AKAP3. This was interpreted

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to indicate that PI3-kinase negatively regulates sperm motility by interfering with binding of PKA to AKAP3. PI3-kinase also might be an upstream regulator of GSK3B, also a negative regulator of sperm motility. In somatic cells, PI3-kinase-generated phosphoinositol lipids activate pyruvate dehydrogenase kinase, isoenzyme 1 (PDK1), causing it to phosphorylate thymoma viral proto-oncogene 1 (AKT1), which in turn phosphorylates GSK3B. Treating bovine sperm with protein phosphatase inhibitors (calyculin A, IBMX) resulted in phosphorylation of serine 21 of GSK3Aα, reduced GSK3B kinase activity, and initiation and stimulation bovine sperm motility.[430] Thus, GSK3B also acts as a negative regulator of motility. GSK3B is located in the principal piece region (and postacrosomal region) of bovine sperm, where it could participate in the regulation of motility.

In addition, the proline-directed serine/threonine kinase CDK5 and its activator p35 are present in the outer dense fibers and in the basal plate, capitulum, and striated collar of the connecting piece in mouse sperm.[305] The substrates for CDK5 in the brain include actin, microtubule-associated proteins, and other cytoskeletal proteins, suggesting that CDK5 could have a significant role in modifying axonemal proteins in association with initiation of progressive or hyperactivated motility.

The inhibition of phosphatase activity results in the stimulation of motility, strongly suggesting that phosphatases have an important role in regulating this and other processes in sperm. While phosphatases are important components of signaling and regulatory pathways in other cell types, little is known about phosphatases in sperm. A sperm phosphatase isoform that has received some attention is protein phosphatase 1, catalytic subunit, gamma isoform (PPP1CC). It is a proline-directed serine/threonine phosphatase and thus could serve to buffer the serine/threonine kinase activity of PKA. It is a testis-specific isoform and the only PP1 present in spermatozoa of several mammalian species. There is an inverse relationship between PPP1CC activity and the motility of sperm from different regions of the epididymis, suggesting that PP1γ2 limits motility of sperm in the epididymis. PPP1CC is regulated by a homolog of the yeast protein phosphatase binding protein, protein phosphatase 1, regulatory (inhibitor) subunit 7 (PPP1R7, SDS22), and PPP1CC complexed with PPP1R7 is catalytically inactive.[431] A phosphorylated form of PPP1CC is present predominantly in the sperm head and may be involved in signaling events associated with fertilization.[432] The PPP1CC-null males were infertile due to spermatogenesis failure and only a few motile sperm were present in the epididymis, but the details are unknown.[433]

CONCLUSION

A rich knowledge of the structural and functional features of spermatozoa has been gained since 2006 (3rd edition) by a combination of studies using both modern molecular technologies and classical methods of light and electron microscopy. This information provides the foundation on which current studies are continuing to build an understanding of the molecular composition and functional mechanisms responsible for the unique properties of spermatozoa. The use of newer visualizing tools such as nanoscopy and live imaging techniques in addition to molecular tools have led to advances during the last decade in understanding the molecular components responsible for the novel structural and functional features of spermatozoa. Although earlier studies provide information valuable for interpreting the results of current investigation, they often are no longer readily accessible and therefore overlooked. This chapter has included information gained both in earlier and in recent studies that have led to the current understanding of the biology of the spermatozoon.

The role of the spermatozoon is of course to deliver the male haploid genome to the egg so that it can combine with the female haploid genome to initiate development of the next generation. The novel structural and functional features of spermatozoa are exquisitely well designed for this purpose. The major features of spermatozoa were divided here into three arbitrary categories: the plasma membrane, the head, and the flagellum. They can be considered analogous in some ways to a space ship: (1) the plasma membrane forms a barrier to a hostile environment; (2) the head contains the payload being delivered; and (3) the flagellum provides the propulsion required for delivery of the payload.

Since 2006, significant advances in knowledge about spermatozoa have come from the large expansion of the use of the tools of molecular biology and proteomics to identify the genes and proteins responsible for its composition, and of gene targeting methods for determining the role of specific proteins in sperm functions. Some of these advances concern (1) the basic information of structures and molecules during spermatogenesis that affect sperm morphology and function; (2) the identification of acrosomal proteins that have essential roles in fertilization; (3) dynamic redistribution of constituent proteins from a domain to different domains to play essential roles; (4) how the nuclear elememts are inherited by the next generation; (5) how surface molecules are posttranslationally modified in the male reproductive tract and in the female reproductive tract; (6) the identification of Ca^{2+} channels that have an essential role in sperm motility; (7) the further identification of a novel bicarbonate activated adenylyl cyclase; (8) the further findings that capacitation is accompanied by the

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tyrosine phosphorylation of a characteristic subset of flagellar proteins; (9) the expansion of the identification of genes encoding proteins that contribute to the structure and function of cytoskeletal elements in the head and flagellum; (10) the identification of heritable effects on the structure and function of the flagella of human spermatozoa and the genes responsible for similar effects on mouse spermatozoa; and (11) the demonstration that many of the proteins that comprise the features specific to spermatozoa are the products of novel genes or alternative transcripts that are expressed during the postmeiotic phase of spermatogenesis.

Notwithstanding a very active research period in the last three decades, the molecular basis controlling gamete adhesion and species specificity in mammals remains a conundrum. There also are large holes in our knowledge of how different molecular components are assembled into the complex structures of spermatozoa, the mechanisms responsible for sperm function, and how these mechanisms are regulated. Although great progress has been achieved particularly at the molecular and gene levels, the following questions still remain: (1) How are sperm-surface domains established and maintained? (2) What mechanisms are involved in delivery of surface components to specific domains? (3) What is the role of surface domain and lipid raft components in plasma membrane function? (4) What are the mechanisms responsible for producing and maintaining the species-specific sperm head shapes? (5) What are the roles of the sperm head cytoskeletal components or the other cytoplasmic substances? (6) Are the acrosomal enzymes identical to lysosomal enzymes, or are they products of novel genes or alternative transcripts? (7) How do acrosomal membrane and matrix proteins affect fertilization events? (8) How is assembly of the complex cytoskeletal structures of the flagellum regulated? (9) Are the components of the outer dense fibers and fibrous sheath found only in mammals, or are there homologs in nonmammalian vertebrates? (10) What mechanisms regulate the initiation of progressive motility and hyperactivated motility? (11) What are the targets of PKA, and what are the components of the downstream signal transduction pathways that lead to initiation of motility, capacitation, hyperactivation, and the acrosome reaction? Newly emerged questions are: (l) In human sperm, how do sperm nuclear changes such as epigenetic modifications and DNA fragmentation affect fertilization or early embryonal development? (2) What are the roles of carbohydrates and lipids on sperm proteins that are composed of a myriad of unknown combinations?

Although there have been considerable advances in knowledge, clearly there is a great deal more to be learned that is important to human male reproductive health. More knowledge is required to understand the long-term effects of assisted reproductive technologies, particularly intracytoplasmic sperm injection, on the integrity of the sperm genome and the risk of transmitting genes causing infertility in males to their male offspring. There is a need to identify the gene mutations detrimental to sperm function to allow identification of patients who will or will not benefit from further medical treatment. A better knowledge of the composition, organization, and function of spermatozoa is also needed to allow development of highly specific approaches to regulating sperm function in ways beneficial to family planning, either to improve sperm function and male fertility or to inhibit sperm function and disrupt male fertility. Furthermore, additional knowledge about mechanisms regulating function of spermatozoa is also essential for identifying the effects of environmental agents on male fertility and the genetic integrity of spermatozoa. There clearly are many important reasons for learning much more about the structure and function of the mammalian spermatozoon.

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CHAPTER

4

Fertilization in Mammals

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INTRODUCTION

Fertilization is the process by which haploid gametes, sperm and egg, unite to produce a genetically distinct individual. Interest in this process extends back more than two millennia,[1–3] but recently research in this area has exploded. Here are some numbers: a query of the PubMed database (htttp://www.ncbi.nlm.nih.gov/pubmed) lists more than 62,500 publications on *fertilization*; in 1967, for the first time there were more than a hundred new articles in a single year, in 1987 more than 1000, and since 2003 more than 2000 reports each year. A philosophical interest in the beginnings of development is ancient and longstanding, but several factors contribute to this more recent growth in the study of fertilization. An understanding of the mechanisms of fertilization may hold the key to both population control, where expansion past 7 billion has refocused efforts to develop effective contraceptive methods, and infertility, where there is an increased reliance on and concern about the therapeutic interventions that comprise assisted reproductive technologies.

In order for fertilization to occur, sperm must attain a fertile state through the process of capacitation (see below and Chapter 3). Sperm and egg must interact so that gamete fusion and the introduction of paternal information into the egg occurs, and the program of development must be activated. As we discuss these events, our focus will be narrow. First, we concentrate on these processes in eutherian mammals, although it is not possible to tell this story without reference to nonmammalian models. Second, we will not address the related areas of gametogenesis and meiotic regulation, sperm maturation within the epididymis, gamete transport, or preimplantation development after the first cell cycle. Those issues are considered in depth in other chapters of this volume (Chapters 1-3, 5,6 and 17). Finally, we will concentrate on developments that have occurred since the third edition of this series appeared in 2006.[4] Context for recent progress requires discussion of earlier results, but in those cases we will attribute only the initial observations and then point readers to relevant reviews. This is particularly the case for the unusually fertile period of approximately four decades beginning in the mid-to-late 1940s, during which many of the foundations of this field were established. It was during this time that the key events of mammalian fertilization, such as capacitation, the acrosome reaction (an exocytotic event in sperm that is required for fertilization), and the block to polyspermy, were discovered; methods for analysis of fertilization in vitro were developed; the first "test tube babies" were produced; and the oral contraceptive "Pill" was developed. The pioneering work of Chang, Austin, and numerous others who ended their research careers within that time frame will, of necessity, be referred to briefly herein. Yet their work remains highly relevant for the continued progress of this field. A more thorough discussion of that foundational period can be found in the magisterial reviews by Yanagimachi in 1988 and 1994 in the first two editions of this series.[5,6]

SPERM CAPACITATION

Definitions and General Comments

Mammalian sperm that await ejaculation in the cauda epididymis are not able to fertilize eggs (Chapter 17). The requirement for additional maturation was

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first reported in 1951 by Austin[7] and by Chang,[8] who observed that sperm must reside in the female reproductive tract in order to fertilize eggs. A similar conclusion was reported by Noyes and coworkers in 1953,[9] although Yanagimachi suggests that those experiments were completed in the late 1940s but not reported for several years.[6] The process by which sperm develop fertility under instruction from the female is referred to as "capacitation".[10] The importance of capacitation should not be understated. It was the last key step that needed to be understood in the effort to fertilize mammalian eggs in vitro, which began with the work of Schenk in 1878 but produced equivocal results for a variety of reasons.[11] Once the requirement for capacitation was recognized, it was a relatively brief 8 years until rabbit eggs were fertilized in vitro using sperm recovered from the uterus, and hence at least partially capacitated in vivo,[12] and 4 years further until the report by Yanagimachi and Chang in 1963 of the fertilization of ovulated hamster eggs in vitro using cauda epididymal sperm capacitated in vitro.[13] This set the stage for the analysis of sperm–egg interaction and fertilization under controlled conditions. In addition, capacitation is a factor in evolution through postcopulatory sexual selection[14,15] and a plausible target for contraceptive intervention.[16]

A characteristic feature of capacitation is a relatively long time course. This varies among mammalian species in vivo, from ~1 h in mice to >6 h in some primates, but in all cases sperm must reside for a prolonged period within the female reproductive tract.[6,17] In contrast, sperm of some well-studied nonmammalian model systems fertilize eggs soon after release from the male; for example, in echinoderms fertilization may occur in <1 min after spawning.[18–20] This difference may suggest that capacitation is restricted to mammals. Yet the general phenomenon of capacitation—the control of sperm function by factors from eggs or from the female reproductive tract—is not unique to mammals but instead similar to events in a wide range of animals and plants. In the hydroid *Campanularia flexuosa*, sperm fertility requires the secretion of factors from epithelial cells surrounding the egg.[21] A jelly coat, secreted by the oviduct, is required for fertilization of amphibian eggs,[22,23] and the intracellular signaling pathways that are activated in sperm by jelly are similar to those that participate in mammalian sperm capacitation in vitro.[24] In sea urchins the egg jelly coat contains chemokinetic and chemoattractant regulators of sperm motility, as well as stimulators of sperm metabolism,[19,25,26] and females of other vertebrate and invertebrate animals also produce egg-derived sperm chemoattractants.[27–29] Similar events occur in mammals and are regulated by capacitation (see below).

The notion that, as Austin states, "sperm must undergo some form of physiological change or capacitation before it is capable of penetrating the egg,"[10] and so linking capacitation to fertilizing potential, has been difficult to approach experimentally. Fertilization assays in vitro require sperm:egg ratios of 100–1000:1, and so one can only assess the capacitation state of the small fraction of the sperm population that enter eggs. Mammalian sperm are functionally heterogeneous,[6,30–33] and it is difficult to correlate the physiological status of the few fertilizing sperm with population-averaged biochemical or molecular properties. Related problems are presented by in vivo studies. Only a small fraction of the ejaculate reaches the oviduct ampulla and are available to fertilize eggs[34–36] (see Chapter 5 in this volume for additional details.). Those sperm that reach the ampulla exhibit hyperactivated motility[37] and are strongly capacitated, but they are difficult to study due to the low numbers involved and inaccessibility within the oviduct.

One effort to resolve these experimental difficulties has been to develop a set of markers that permit the identification, and possible purification, of a capacitated subpopulation. In this regard, many events during capacitation can be followed with fluorescence probes or by observing changes in sperm behavior. Monitoring such capacitation-associated changes, in conjunction with methods of fertilization in vitro using small numbers of sperm (sperm:egg ratios <10:1),[38,39] may provide some resolution. However, a comprehensive set of appropriate markers is not yet available.

Several general features of capacitation should be emphasized:

1. Capacitation is usually thought to reflect the functional reprogramming of sperm within the female reproductive tract. However, it may be that capacitation begins when sperm are exposed to seminal fluids during the ejaculation. Sperm are quiescent during storage in the cauda epididymis and activate progressive motility rapidly upon release.[6,40] This process, which is essential for fertility, is driven in part by HCO_3^- concentrations in seminal fluids through a cAMP/protein kinase A (PKA) pathway[41,42] and continues within the female reproductive tract.[6,43–45] The significance of seminal plasma-induced capacitation has not been fully explored and is likely to vary among mammalian species depending on the degree of sperm mixing with seminal fluids and the time of sperm exposure.[6,46,47] Capacitation is completed in vivo in the oviduct.[6,48]
2. As a consequence of capacitation, sperm express the ability to interact with eggs, exhibit unique patterns of flagellar motility, and display novel responses to external signals (see the section Consequences of Capacitation). Many of the biochemical and biophysical changes that accompany capacitation are likely to relate to these behavioral modifications.

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3. Capacitation is not a unitary process but rather the sum of several component reactions that proceed with separate time courses and regulation.[4,6,49,50] The notion of multiple distinct reactions is illustrated by the elegant chemical genetic study of Morgan et al., in which a genetically modified Cα2, the sperm-specific isoform of the catalytic subunit of protein kinase A,[51,52] was used to show that the cAMP/PKA pathway has separate early and late roles in capacitation.[53]
4. Sperm within a population capacitate in a highly asynchronous manner, such that the representation of strongly capacitated sperm in a population at any one time may be low both in vitro and in vivo.[30–32,54,55] This might plausibly extend the fertile lifespan of an ejaculate by continually replenishing a pool of capacitated sperm and so may be essential for efficient reproduction in species where ovulation and sexual receptivity of the female are not tightly coupled.[6,14,30] In any case, this heterogeneity complicates an analysis of the underlying mechanisms.
5. Capacitation in vitro and in vivo may differ in important regards. Studies in rodent models use cauda epididymal sperm that have not been mixed with seminal fluids. Those fluids contain: negative modulators of fertility (so-called "decapacitation factors");[6,56–58] positive modulators of fertility, including *c*ysteine-*ri*ch *s*ecretory *p*roteins (CRISP) family proteins; Fn-2 domain proteins; spermadhesins; other secreted proteins and peptides; as well as prostaglandins.[47,59–62] Also contained in these fluids are exosomes, shed by the epithelium in distal regions of the male reproductive tract after sperm release from the cauda epididymis, that regulate sperm function,[63,64] including some properties that may be involved in capacitation.[65] Even when ejaculated sperm are used, in vitro culture conditions represent a simplification of the environment within the female reproductive tract. For example, Yanagimachi noted that the ionic composition of the female reproductive tract fluids differs from the standard culture media used for capacitation, and this may influence sperm physiology.[6] In addition, female reproductive tract fluids contain an array of bioactive molecules that may affect sperm function but which are not present in simple culture medium. Stated differently, the development of culture conditions that support sperm fertility in vitro has provided valuable information on the intracellular pathways mediating capacitation, but does not necessarily inform us of the extracellular triggers that activate these pathways in vivo.

In the next sections, we consider the physiological process of capacitation and current thoughts regarding the underlying mechanisms. For additional details of the understanding of these events up through 1994, readers should consult Yanagimachi.[6] Recent progress in specific aspects of capacitation is discussed in a number of reviews.[29,66–70]

Consequences of Capacitation

Capacitation is associated with widespread changes in the biochemical and biophysical properties of sperm cells. These include alterations in the following: plasma membrane and cell surface properties through such processes as desorption of noncovalently attached (glyco) proteins,[71–73] redistribution of integral plasma membrane proteins, altered rates of membrane protein and lipid diffusion,[74–76] reorganization of detergent-resistant membrane domains,[77–79] alterations in the asymmetric distribution of plasma membrane phospholipids, and loss of membrane cholesterol;[76,80,81] altered cAMP metabolism;[82,83] increased phosphorylation state of a range of proteins through the regulation of both protein kinases and phosphatases;[84–86] changes in metabolism;[87,88] a strong hyperpolarization of membrane potential;[31,89] and an altered internal ionic environment, including changes in pH, intracellular calcium concentrations (Ca^{2+}_i) and other ion activities.[68,90] This extensive reprogramming takes place in both the sperm head and flagellum.[4,6,49,50]

As a consequence of capacitation, sperm exhibit at least three modifications in behavior that appear related to fertility: hyperactivation of flagellar motility; regulation of signal transduction pathways permitting responses to chemoattractants; and expression of the ability to acrosome react and to interact with eggs. These are considered separately as, at present, there is not a unified mechanism that accounts for the range of behavioral changes in sperm during capacitation.

Hyperactivated Motility

Sperm are immotile during storage in the cauda epididymis and initiate motility upon release either into culture medium in vitro or into seminal fluids. This initial pattern of motility (referred to as "progressive" or "activated" motility) consists of relatively symmetrical, low amplitude flagellar bends that produce linear, forwardly progressive sperm movement in aqueous media. In contrast, a different type of motility is observed in sperm that were incubated under capacitating conditions in vitro,[91–94] recovered from the oviduct,[95] or observed through transilluminated, explanted oviducts following mating or artificial insemination.[94,96,97] Sperm in the hyperactivated state display a number of behaviors, including (but not restricted to) vigorous, deep, asymmetric flagellar bending that produces nonprogressive cell movement and that alternates with periods of progressive motility during observations in culture media

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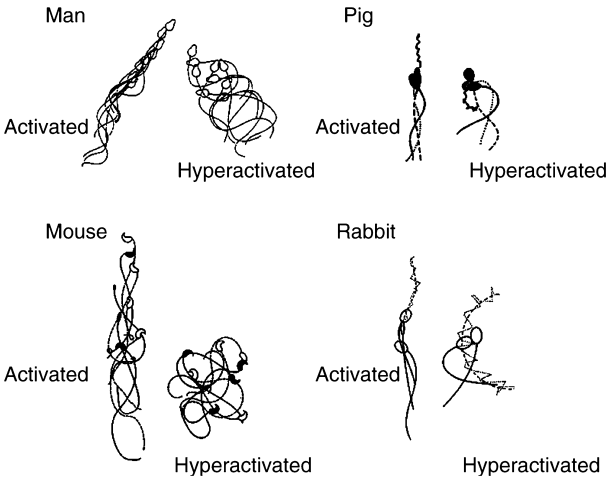

FIGURE 4.1 Progressive and hyperactivated motility of mammalian sperm. Motility patterns of mammalian sperm are displayed in time-lapse drawings. Activated (or progressive) motility is characteristic of uncapacitated sperm, while hyperactivated motility is observed in sperm incubated under capacitating conditions in vitro and in sperm recovered from the oviduct. Note that sperm in the activated state exhibit low amplitude and relatively symmetrical flagellar bends, and linearly progressive path of the sperm head. The hyperactivated mode consists of transitions to deeper and less symmetrical flagellar bending and results in a path of the sperm head that is less linear than that of activated state motility. *Reprinted from Ref. 4 with permission from Elsevier, Inc. Courtesy of Dr Ryuzo Yanagimachi.*

(Figure 4.1). However, there are species differences in the particular details of flagellar bending and in the frequency of nonprogressive/progressive transitions.[4,6,96,98–100]

Fertility depends on the timely development of hyperactivated motility. Hamster sperm that were incubated under noncapacitating conditions in vitro and did not display hyperactivated motility were able to penetrate into the oviduct from the uterus following artificial insemination, while sperm that had been subjected to preincubation in a capacitating medium, and that displayed high levels of hyperactivation, failed to enter the oviduct in appreciable numbers.[101] This suggests that the transit through the uterotubal junction into the oviduct may act as physiological filter and select sperm that have not completed capacitation prematurely (see Chapter 5). Hyperactivation increases the thrust and force generated by sperm[102] and has been suggested to facilitate release of sperm from oviduct storage sites in the lower isthmus.[103,104] Additionally, it permits sperm to move through viscous media more readily[105] and may be essential for progression along the surface of the female reproductive tract and for penetration of cervical mucus, the cumulus oophorus matrix, and zona pellucida, each of which is a formidable barrier to fertilization.[106–113] Finally, mode switching between hyperactivated and progressive motility is linked to sperm chemotaxis in vitro (see the section Sperm Chemotaxis).[114,115]

Hyperactivation is dependent on elevated Ca^{2+}_i,[100,116] and can be evoked by treatment of intact sperm with Ca^{2+} ionophore or of demembranated sperm with elevated Ca^{2+}.[100] CATSPER (cation channel, sperm associated), an evolutionarily conserved ion channel, is the major Ca^{2+} entry pathway controlling sperm hyperactivation.[68,108,117–121] Loss of function mutations of any of the channel subunits result in loss of male fertility in mouse models and have been linked to cases of human male infertility.[68,117,122,123] Sperm with defective CATSPER activity fail to ascend the oviduct to the site of fertilization in vivo[124] or penetrate the zona pellucida in vitro.[117]

The channel is constructed from pore-forming subunits that have the architecture of a classic voltage-sensitive ion channel,[68,117,118] with six transmembrane α-helices (S1–S6) consisting of a voltage sensor domain (S1–S4) with a set register of fixed positive charges in the S4 helix and an ion conductance domain (S5–S6).[125,126] CATSPER exhibits weak voltage sensitivity.[127] Sperm with a slightly acidic internal pH (pH_i), a characteristic of uncapacitated cauda epididymal sperm,[4,128,129] require depolarization to strongly positive potentials to activate CATSPER conductance.[127] There is no evidence that sperm generate such strongly positive membrane potentials.[4,31,67,89,130,131]

How then is CATSPER activated? A variety of factors have been shown to modulate CATSPER channels and facilitate conductance. First, sperm pH_i rises during capacitation[128,132] and will shift the conductance–voltage relationship such that CATSPER can be activated by physiological membrane potentials.[127] Several mechanisms may contribute to this internal alkalinization. These include: SLC9A10, a sperm-specific Na^+-H^+ exchanger regulated by membrane potential[133,134] and related to the voltage-sensitive Na^+-H^+ exchanger of sea urchin sperm;[135] HVCN1, a voltage-sensitive proton channel mediating acid efflux from human sperm[136] that participates in pH_i regulation; and HCO_3^- entry pathways, such as CFTR (*cystic fibrosis transmembrane conductance regulator*)[137–139] and other anion transporters;[129,140,141] as well as formation of HCO_3^- by sperm carbonic anhydrase[142] following diffusion of the CO_2 that is reported to be present in the oviduct during the periovulatory period at high levels.[44] Second, CATSPER is a ligand-gated channel that can be directly activated by progesterone and by prostaglandins, which are present in the oviduct,[143] and certain odorants and other small molecules.[144–146] This will be discussed in greater detail in the next section.

The emerging model is that CATSPER is essential for hyperactivation and is designed to be activated by the environment of the oviduct, where capacitation is completed.[6,48] However, the reader should be cautious—CATSPER may do more than simply control the switch to hyperactivated motility, as indicated by the sluggish

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motility of sperm from nullizygous mice[117] and some aspects of the behavior of human sperm,[147] and so all aspects of the phenotype of CATSPER nullizygous mice may not reflect a failure of hyperactivation. Alternatively, ligands that activate CATSPER may also have additional, CATSPER-independent mechanisms of action.

Sperm Chemoattraction

That male gametes orient motility in gradients of factors released by eggs or by the female reproductive tract is a widely conserved phenomenon. The earliest report in plants was in 1884 for the case of bracken fern sperm,[148] and in animals in 1912, for the cases of an echinoderm, *Arbacia punctulate*, and a polychaete worm, *Nereis limbata*.[149] However, the chemical nature of attractants in taxa examined to date varies and includes proteins, peptides, amino acids, olefins and other lipids, and gases; thus, it is likely that the chemoattraction process has evolved independently on multiple occasions. The focus here is on events in mammals and their relationship to capacitation. Events in other taxa and the cellular mechanisms by which sperm receive attractant signals and respond to those signals are detailed elsewhere.[27–29,54,150,151]

Chemotaxis of mammalian sperm was first reported in 1958 in a study using human sperm and (among other attractants) follicular fluid,[152] and was soon followed by a study of rabbit sperm.[153] Interest in this area was reignited following reports that human sperm reorient swimming direction in vitro toward sources of follicular fluid or media conditioned by oocyte–cumulus complexes in a manner that correlated with the ability of oocytes to be fertilized in vitro.[154,155] In addition, assay systems were developed that differentiate chemoattraction from other mechanisms of sperm accumulation.[54] Finally, an expanding understanding of the complex environment and the physiological roles of the oviduct has mitigated concerns of functional relevance.[36,156,157] Current models suggest that sperm are guided through the mammalian female reproductive tract by several mechanisms: processes such as rheotaxis[113] and thermotaxis[158,159] may operate over long distances, while chemotaxis operates when sperm are in the vicinity of the egg/cumulus complex.[54] Chemotaxis has been studied more extensively than other processes and will be discussed here.

Soluble sperm attractant activity is present in the follicular and oviductal fluids from a number of mammalian species,[154,155,160–162] as well as from the egg/cumulus complex.[163] Figure 4.2 shows that human sperm reorient motility toward a medium conditioned by human eggs and containing follicular fluid.[155] In human sperm preparations, which have been studied extensively in recent years, the proportion of responsive sperm was low (~10%), consistent with observations that a small fraction of human sperm populations reside in a capacitated

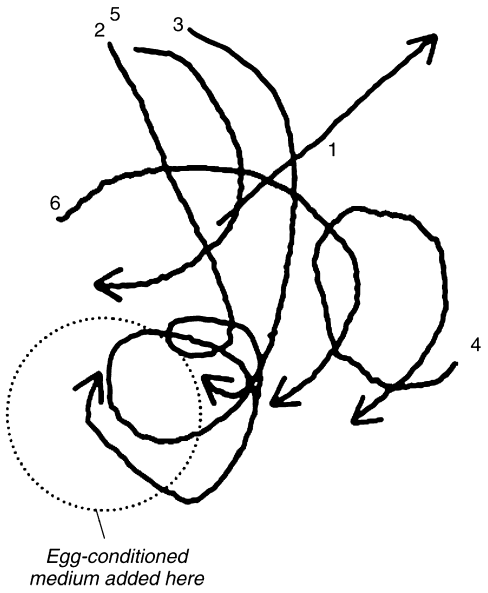

FIGURE 4.2 Diagram of mammalian sperm chemotaxis. Capacitation is associated with the ability of sperm to reorient motility toward chemoattractants. Culture medium was conditioned by incubation with human oocyte/cumulus complexes and also contained ~5% follicular fluid (which was recovered with oocytes during aspiration from follicles). Aliquots of conditioned medium were added to fields of human sperm (site of addition shown by dotted line). Human sperm motility was tracked using video microscopy. Paths of six sperm are shown, of which five reorient toward the conditioned medium. *Adapted from Ref. 155 with permission from Elsevier, Inc. and is provided through the courtesy of Dr Michael Eisenbach.*

state at any one time, and that only capacitated sperm execute taxis in response to chemoattractants.[30,54,164,165]

At least one of the active factors in follicular fluid and oviduct fluid appears to be progesterone,[166–169] although other factors may also be present.[54,163,170] Cumulus cells synthesize progesterone during the periovulatory period and apparently retain this activity following ovulation.[167,171–175] One model predicts that progesterone from cumulus cells at the site of fertilization (lower ampulla region of the oviduct) establishes a local gradient that guides sperm from the side channels formed by the highly arborized projections of the oviductal mucosa toward the egg.

The behavioral mechanism of taxis in response to chemoattractant gradients appears to be regulated by switching between progressive and hyperactivated motility modes, as demonstrated in gradients of the floral odorant, bourgeonal, and of progesterone.[114,115] Human sperm exhibit progressive motility during ascent of spatial gradients but initiate episodes of hyperactivation when they are moving down the concentration gradient (Figure 4.3). This permits a directional change in that sperm emerging from transient hyperactivation will enter a progressive run if pointing up the gradient but reinitiate a hyperactivated phase if moving to zones of lower attractant concentration. Armon and Eisenbach

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point out that, despite mechanistic differences, this mode switching serves the same purpose as bacterial tumbling in driving a directional reorientation in a spatial gradient.[115]

A plausible mechanism of mode switching is through modulation of CATSPER channel activity. Bourgeonal (a chemoattractant of human sperm in vitro that is unlikely to play a role in vivo) and progesterone are CATSPER agonists that act in a direct and membrane delimited fashion on the channel, either by interacting with one of the identified channel subunits or with another protein intimately associated with the channel.[144–146] Inhibition of hyperactivation occurs during sperm ascent of spatial gradients of progesterone.[115] While CATSPER mediates hyperactivation,[108,119] the channel may also exhibit agonist-induced desensitization in a spatial gradient, similar to that reported following repetitive agonist stimulations, and so continue to ascend the spatial gradient.[123]

A number of questions must be resolved in order to explain this sperm behavior. First, taxis toward chemoattractants has been demonstrated in stable gradients in vitro, but do stable gradients exist in vivo in the presence of the reported enhanced fluid flow following coitus[113] and the high beat rate of motile cilia on the epithelium lining the ampulla of the oviduct?[176] Second, in addition to progesterone, many candidate mammalian sperm chemoattractants have been identified, including *N*-formyl peptides,[177,178] chemokines,[179] atrial natriuretic factor,[180,181] and odorants.[114,182,183] The reader is directed toward focused reviews for additional detail.[54,170,184]

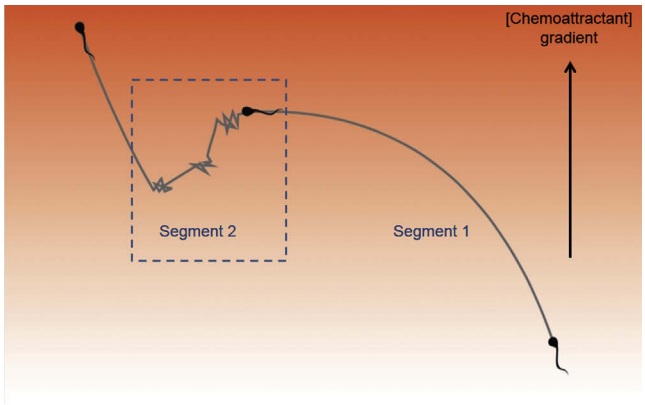

FIGURE 4.3 A model of sperm behavior in a spatial gradient of a chemoattractant. This model is based on mode switching between progressive and hyperactivated motility. The local chemoattractant concentration is indicated by the intensity of the background shading. *Segment 1*: Sperm that sense an increasing local chemoattractant concentration suppress hyperactivation turns, resulting in swimming along approximately a straight path. *Segment 2*: Sperm cease to sense an increasing concentration gradient of chemoattractant switch motility modes transiently from progressive to hyperactivated, resulting in turns. One or more episodes of hyperactivation reorient sperm movement so that they once again ascend the spatial gradient of chemoattractant. This behavior can be based on the activation of the CATSPER ion channel and suggests that this channel may adapt to increasing chemoattractant concentrations. *This figure is adapted from Ref. 115 through the open access license policy of the Public Library of Science and* PLoS One, *and is provided through the courtesy of Dr Michael Eisenbach.*

Are all of these bona fide chemoattractants, and, if so, how does CATSPER integrate such diverse signals? For example, we have discussed the case of bourgeonal, an odorant that can activate CATSPER directly. Yet sperm also appear to have G protein-coupled odorant receptors.[182,185–187] Are these functional and do they also transmit chemoattractant (or other) information?

Regulation of Sperm–Egg Interaction by Capacitation

Uncapacitated sperm cannot interact with eggs in vivo as their failure to hyperactivate motility precludes ascent to the site of fertilization. However, in vitro studies suggest additional layers of direct modulation of gamete interaction. Following capacitation, sperm develop the ability to penetrate the cumulus oophorus.[188] This reflects, at least in part, a role of hyperactivation in the penetration process. There is also an enhanced ability to adhere tenaciously to the zona pellucida[189,190] and to undergo acrosome reactions evoked by zona pellucida agonists.[191,192] This is discussed in greater detail elsewhere.[4,6]

Some Considerations of the Mechanisms of Capacitation

Our view is that capacitation is a cluster of processes running in parallel and that a single, unified mechanism is not apparent at present. That said, there are several well-established mediators of capacitation that are likely to provide an entrée to the underlying mechanisms.

Ion-mediated events. It has been suggested that altered activities of a number of different intracellular ions are essential for capacitation. It may be premature to design mechanistic pathways, but at least the key components may have been identified.

There is strong evidence for a role of Ca^{2+} entry in capacitation,[6,84] as underscored by the recent demonstration that elevation of Ca^{2+}_i activity is sufficient for fertilization in vitro.[193] CATSPER is the major influx pathway for this ion that can be observed in electrophysiological experiments[68] and plays an important role in hyperactivation and mechanisms of activation as noted above. In addition, data from gene disruption models and fluorescent probe experiments indicate that $Ca_v2.3$, a member of the family of high-voltage activated Ca^{2+} channels, is present and is regulated during capacitation.[194] Other Ca^{2+} entry pathways may be present,[67,195] although not evident in electrophysiological recordings due to channel

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density, restriction to small but functionally important membrane domains,[194] or to a lack of voltage sensitivity.

In addition, sperm membrane potential is hyperpolarized,[31,68,89] and pH_i shifts in an alkaline direction rise during capacitation.[68,128,129,195] These may be linked as membrane potential is set, at least in mouse sperm,[196] by KCNU1, a pH-dependent K^+ conductance channel.[197–199] A number of potential regulators of sperm pH have been discussed previously, including extracellular pH, the sperm-specific Na^+-H^+ exchanger SLC9A10, and the voltage-sensitive proton channel HVCN1, which conducts a proton current in sperm and may regulate pH_i.

A number of other ion activities and transport pathways are also implicated in capacitation.[67,68,195] In considering these events, the reader should view the ion milieu in any cell, including sperm, as a dynamic network in which changes in any one component drive the whole system to reset at a different equilibrium point. As such, treatments that focus on a single ion (for example, the effects of ionophore addition or depletion of a single extracellular ion) should be interpreted cautiously.

Sperm phosphoproteins. The phosphorylation state of an array of sperm proteins increases during capacitation, and both in vitro studies and gene disruption models suggest that these are important regulators of sperm functional state.[66,82,84,195,200] Efforts are focused on the identification of these phosphoproteins and their role in capacitation, as well as on the protein kinases and phosphatases that ultimately control phosphorylation state.

Sperm membrane changes. Membrane remodeling accompanies capacitation, including: cholesterol efflux and the associated alterations in membrane domains; phospholipase activation, with the potential generation of lipid regulators of ion channels and other membrane proteins.[49,76,201]

Final considerations. A wide range of factors control the capacitation state of sperm in vitro. Readers are directed to other issues in this series for earlier thoughts and References 4 and 6, and to more recent reviews for a more detailed examination of the role of protein phosphorylation, ion channels, and sperm membrane state in this process.[66,68,195,201] An increased understanding of the physiological environment for capacitation, as established in the periovulatory oviduct, should provide insight into the relevant mechanisms.

SPERM–EGG INTERACTION

Models of Gamete Interaction

The development of well-controlled methodologies for the study of fertilization in vivo and the application of new and powerful microscopic tools (phase contrast and transmission electron microscopy) lead to the identification of core principles for gamete transport in the female, capacitation and the acrosome reaction, and the cortical reaction and zona block to polyspermy. This initial phase led to the articulation of the first coherent model of mammalian fertilization in the early 1960s.[5,6]

The first version of this model (referred to here as Model 1; Figure 4.4) was based on the following observations: that ovulated eggs in the oviduct are surrounded by a cumulus mass;[202] that the cumulus mass can be dispersed by a sperm-derived enzyme[203,204] shown later to be hyaluronidase;[205,206] that mammalian sperm undergo an acrosome reaction (Figure 4.4(A)) before penetration of the zona pellucida;[207,208] and that hyaluronidase is within the acrosome and released during the acrosome reaction.[206,209] Based on these observations it was proposed that: (1) capacitated sperm initiate, and possibly complete, the acrosome reaction immediately before or during penetration of the cumulus mass; (2) the acrosome reaction releases hyaluronidase, which is essential for sperm penetration of the cumulus mass; and (3) sperm attached to the zona pellucida have at least initiated, and possibly completed, the acrosome reaction.[5,6,209]

This model was subsequently modified during two overlapping historical periods. First, the systematic study of the cellular events of fertilization began in earnest with the development of methods for the fertilization of eggs in vitro using sperm capacitated in vitro.[13] Controlled in vitro gamete interaction studies were carried out using then-novel tools (hybridoma reagents, ion selective fluorescent probes, and single-cell imaging). In particular, a structure/function analysis of the zona pellucida led, by the mid-1980s, to a reformulation of Model 1 of sperm–egg interaction (Model 2; Figure 4.4). The core elements in Model 2 include: (1) capacitated sperm with intact acrosomes are able to penetrate the cumulus mass, possibly by means of a cell surface hyaluronidase;[210] (2) sperm with intact acrosomes bind to the zona pellucida (ZP)[211] through the interaction of an unknown sperm adhesion site and an oligosaccharide domain of ZP3;[212–216] (3) ZP3 then triggers the sperm acrosome reaction;[217] (4) following the acrosome reaction, sperm penetrate the zona pellucida and fuse with the egg plasma membrane; and (5) the block to polyspermy includes the release of the ovastacin,[218] which catalyzes the proteolytic modification of ZP2[219] thought to prevent sperm penetration of the zona pellucida, as well as triggers an unknown modification of ZP3,[212,214] preventing additional sperm from binding to the matrix and acrosome reacting. Additional descriptions of the ZP proteins and their roles in fertilization are given later in this chapter.

Finally, two separate experimental threads are brought together in a competing model (Model 3; Figure 4.4), which states: (1) the fertilizing sperm begin, and possibly complete, acrosome reactions during transit through the cumulus;[220] (2) sperm that are completely[39]

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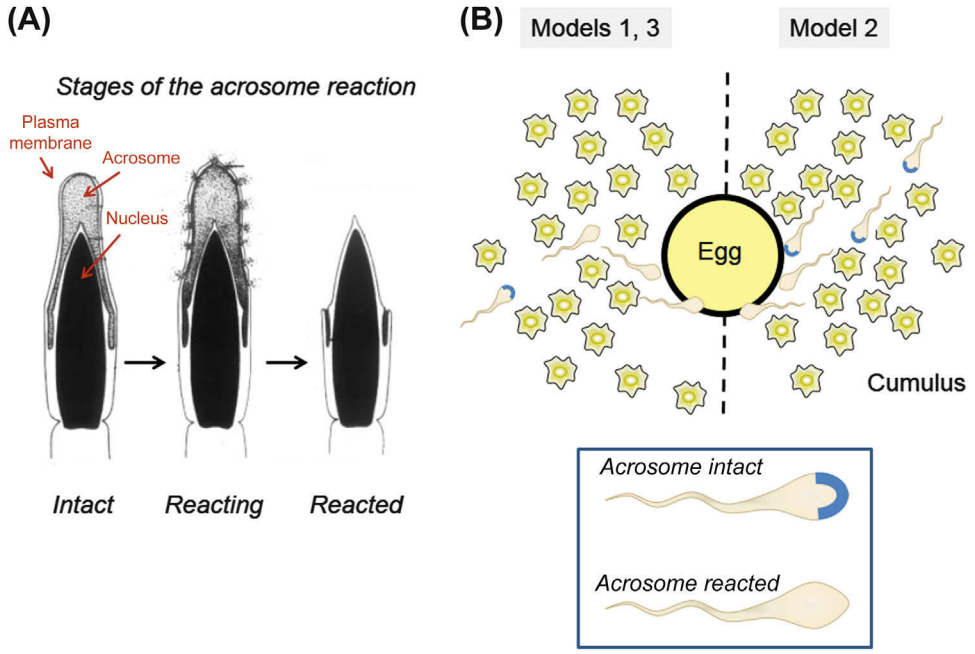

FIGURE 4.4 Models of sperm interaction with the zona pellucida. (A) Drawings of the sperm head are shown with the plasma membrane, acrosome, and nucleus indicated (*intact*). During early stages of the acrosome reaction, the plasma and acrosomal membranes fuse at multiple points, permitting release of acrosomal vesicle contents (*reacting*). The completion of the acrosome reaction is marked by complete release of vesicle contents, sloughing of acrosomal/plasma membrane hybrid vesicles, and the exposure of a new cell surface domain in the apical sperm head. Sperm must complete the acrosome reaction to penetrate the zona pellucida and fuse with the egg plasma membrane. (B) Models of the cellular events of sperm–zona pellucida interaction. Models 1 and 3 both propose that sperm acrosome react within the cumulus mass, that acrosome reaction-inducing agonists are found within the cumulus mass, and that acrosome reacted sperm bind to the zona pellucida. Model 2 proposes that sperm with intact acrosomes penetrate the cumulus mass, bind to the zona pellucida, and then initiate acrosome reactions on the surface of the zona pellucida. *Inset*: key for acrosome intact and acrosome-reacted sperm for Panel B.

or partially acrosome reacted adhere to the zona pellucida by the association of adhesion sites, either on the inner acrosomal membrane or within the acrosomal matrix,[221,222] with domains on ZP2;[223] (3) following adhesion sperm penetrate the zona pellucida and fuse with the egg plasma membrane; and (4) the block to polyspermy includes proteolytic cleavage of ZP2 by ovastacin.[218] Note that Model 3 is, in fact, Model 1 with additional molecular details.

The thumbnail description above identifies the salient elements of these models. More details, including additional data, are available in numerous reviews.[4,6,224–229] In the following discussion we focus on two questions raised by these models: (1) Do sperm acrosome react in the cumulus matrix (Model 3) or on the zona pellucida (Model 2)? (2) How do sperm establish initial interactions with the zona pellucida?

Sperm Interaction with the Cumulus Oophorus

Eggs of eutherian mammals are ovulated and enter the oviduct surrounded by several thousand cumulus cells.[6,202] Initially, a number of passive roles were suggested for the cumulus, including: enhancement of the efficiency of fertilization by increasing the target size (relative to a denuded egg) and by orienting sperm that enter the cumulus toward the egg; and functioning as a sperm barrier that may minimize polyspermy.[230] It is now understood that the cumulus is a more dynamic structure that, in in vitro experiments, may be both regulated by the presence of sperm and also secrete factors that regulate sperm function. Such factors include progesterone,[54,167–169,231] chemokines[232,233] and possibly other proteins,[234] prostaglandins,[233,235] extracellular matrix components such as hyaluronic acid fragments,[232,236] and other bioactive components.[237]

Do these or other factors trigger acrosome reactions during sperm transit through the cumulus in vivo? Acrosome-reacted sperm have been observed within the cumulus by phase contrast[208] and transmission electron microscopy,[238] but it was not certain whether these were trapped cells in the process of dying or fertilizing sperm captured en route to the egg.[6,228] Recently, Jin and coworkers followed living, acrosome reacted mouse sperm penetrating the cumulus and fertilizing eggs in vitro.[220] In addition, acrosome-reacted sperm recovered from the perivitelline space can penetrate the zona pellucida and fertilize new, zona-intact eggs.[39,239–241]

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These reinsemination experiments clearly indicate that acrosome-reacted sperm can penetrate the cumulus mass and fertilize eggs in vitro.

Several cumulus-derived factors, including hyaluronan fragments, progesterone, and chemokines, elevate sperm Ca^{2+}_i.[144–146,236] It is well known that Ca^{2+}_i elevations in the sperm head, whether driven by agonists or ionophores, can initiate acrosome reactions in vitro.[6] There is cytosolic continuity between the sperm head and flagellum,[127] and so it follows that elevations of Ca^{2+}_i arising anywhere in sperm, if sufficiently large to override the intrinsic Ca^{2+}_i clearance mechanisms,[242,243] may initiate acrosome reactions during in vitro experiments. Whether such acrosome reactions are physiologically relevant will depend on such factors as the kinetics of stimulation. This is illustrated by the case of progesterone: in low (pM–nM) concentration gradients it regulates motility and can be a chemoattractant, but does not evoke acrosome reactions,[167,244–247] whereas the stepwise presentation at higher doses (μM) triggers acrosome reactions.[248] Whether acrosome reactions are evoked in sperm transiting the cumulus by progesterone, or by other cumulus-derived factors, will depend on how the agonist is presented.

Thus, candidate agonists for induction of the acrosome reaction are present in the cumulus, but the physiological relevance of these molecules is unresolved. The question of how this impacts the models of gamete interaction will be discussed in a subsequent section.

Sperm Interaction with the Zona Pellucida

The zona pellucida is the extracellular coat of the mammalian oocyte. It acts as a selective filter before fertilization that can only be penetrated by sperm that have completed the acrosome reaction.[6,208] Sperm interaction with the zona pellucida has been examined extensively using two distinct strategies and two distinct models (mouse and a mouse/human hybrid). These models (Model 2 and Model 3, Figure 4.4) were discussed above (see the section Models of Gamete Interaction) and in greater detail in the references cited there. Here, we consider the strengths and assumptions of those strategies.

A reductionist approach, pioneered by Wassarman and his coworkers, starts with the assumptions that (1) the biological activities of the intact zona pellucida can be recapitulated from the properties of its component constituents; (2) purification of those zona pellucida glycoproteins from an insoluble matrix and subsequent examination of their actions on sperm in solution does not alter essential biological properties of those glycoproteins; and (3) the cumulus-denuded egg that is used to study interactions with the zona pellucida is an appropriate model for gamete interaction in vivo. This approach led to the core data supporting Model 2 (Figure 4.4). As noted previously, the core data led to proposals that sperm adhere to the zona pellucida by interacting with a glycan chain that (at least in mouse) is on ZP3, that ZP3 triggers acrosome reactions in bound sperm, and these bioactivities are lost following fertilization and contribute, along with a proteolytic modification of ZP2, to the block to polyspermy.[4,225,249,250]

A different proposal for sperm–zona pellucida interaction is presented in Model 3 (Figure 4.4). Dean and his colleagues studied the biological properties of the structurally intact zona pellucida. A knockout/knockin strategy was used to replace mouse ZP proteins with their human orthologs, and the interactions of human sperm with this modified matrix were then examined. The assumption in this case is that the hybrid zonae pellucidae, containing mouse and human glycoproteins, is a useful model for sperm interaction with native structures. Although conserved cysteine residues in the ZP domain indicate similar protein folding patterns,[251–255] the primary sequences of mouse and human ZP2 and ZP3 are only 56% and 67% identical, respectively. The effects of these sequence differences on the structure and behavior of hybrid zonae pellucidae are not known. Finally, glycosylation is specified by the host cell, and so human proteins synthesized in mouse oocytes will have mouse glycans.[256,257] The genetic manipulation approach that is the basis for Model 3 assumes that these factors can be accounted for and do not alter the properties of the hybrid zona pellucida. The core features of Model 3, as it pertains to sperm–zona pellucida interaction, are that human sperm bind to the zona pellucida by interactions with an N-terminal domain of ZP2,[223] that this adhesion depends inversely on the proteolytic cleavage state of ZP2 (as does Model 2),[258,259] and that this adhesion does not trigger the acrosome reaction.[260]

These models are internally complete and coherent, and agree on certain points (for example, that ZP2 proteolysis is associated with downregulation of sperm adhesion to the zona pellucida), but it may be instructive to focus on points where they disagree. First, different approaches to solid-phase assays have yielded different results; in one case, human sperm bind only to beads conjugated with fragments of human ZP2 synthesized in mouse oocytes,[223] consistent with Model 3, and in the other mouse sperm bind only to beads coated with native ZP3,[261] consistent with Model 2. Second, isolated glycans from human zonae pellucidae[216] and glycans or low molecular weight glycopeptides from mouse ZP3[213,214] bind directly to sperm and inhibit sperm binding to the zona pellucida, consistent with Model 2 and inconsistent with Model 3. Finally, Model 2 asserts that an acrosome reaction-inducing agonist is present in the zona pellucida, as has been reported in mouse,[217,262,263] bull,[192] and human.[263–267] However, experiments supporting Model 3 assert that sperm do not acrosome react

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on the zona pellucida surface.[260] An examination of the experimental points at which these models depart from each other may provide a means of resolving this apparent impasse.

Is There an Obligatory Sequence of the Early Events of Fertilization?

There is general agreement that sperm must acrosome react before penetrating the zona pellucida. However, disagreement exists regarding where the exocytotic reaction begins, on how it is triggered (in the cumulus or at the zona pellucida surface), and on the cellular and molecular mechanisms of sperm interaction with the zona pellucida (acrosome intact or reacted sperm binding to ZP2 or ZP3, and whether interaction is mediated by zona pellucida glycans or a protein domain). The underlying premise is that there is a single conserved sequence of events during sperm–egg interaction. However, reproduction is characterized by diversity,[268] and this may clarify the understanding of mammalian fertilization.

First, we have considered the question of whether acrosome reactions are initiated in the cumulus or at the surface of the zona pellucida. It should be recalled that eutherian eggs enter the oviduct within a cumulus, but this cellular investment disperses with time in the oviduct. In those species in which the female displays a restricted period of sexual interest and receptivity, and in which the period of interest is regulated such that capacitated sperm arrive in the ampulla either before or at approximately the same time as eggs, it is likely that sperm are confronted with a cumulus mass that has not started to disperse, and that may be an obstacle to sperm penetration. This is the situation in, for example, the mouse, but it is not the case for all eutherians. There are reports of the absence of adherent cumulus cells around the egg within the oviduct in cows, sheep, horse, goat, and other species.[6,269] In particular, this may be the case when there are long periods of sexual receptivity by the female, and where ovulation occurs early in that receptive period. There are additional examples (such as human) in which sexual receptivity is not restricted to the periovulatory period. It then seems likely that there will be many cases in eutheria in which sperm must be capable of fertilizing eggs irrespective of the presence of the cumulus. The presence of an acrosome-reaction evoking agonist in the zona pellucida is expected as acrosome-intact sperm cannot proceed past the zona pellucida surface. Potential agonists are also present in the cumulus, but their functional significance may vary across species and depend on reproductive behavior and other factors.

Similarly, sperm interactions with the zona pellucida may also be characterized by diversity. Four genes encode mammalian zona pellucida proteins (ZP1-4), but there are extensive differences in the composition of these egg coats. Eutherian mammals can be divided into three classes based on the composition of the zona pellucida: those assembled using four glycoproteins (human, chimpanzee, macaque, and rat); those built from ZP2, ZP3, and ZP4 (dog, cattle, and possibly pig, cat, and rabbit); and those using ZP1, ZP2, and ZP3 (mouse).[270] These categories may simplify as ZP1 is most closely related to ZP4,[270,271] yet the possibility of functional differences in eutherian zonae pellucidae cannot be discounted.

The suggestion here is that there may not be a canonical reaction sequence for mammalian fertilization. If there is extensive diversity in the process of sperm–egg interaction, then these questions should be examined in a wide range of species. This was the case to some extent during an early period that began approximately with the discovery of capacitation (1951) and ended during the 1980s. Since then the choice of model systems has narrowed to mouse (where genetic and other tools became available first), human, and a limited number of domestic species (where studies have high translational value). Generalized conclusions may be more evident when our knowledge base is more generalized.

SPERM–EGG PLASMA MEMBRANE INTERACTIONS

Plasma Membrane Domains and Factors that Affect Fusion

Only acrosome-reacted sperm undergo fusion, and the acquisition of a fusogenic capacity occurs within minutes of initiation of the acrosome reaction.[6] Fusion is preceded by adhesion, and experimental methods have been designed to evaluate and distinguish these processes, such as strength of sperm attachment to the oolemma and transfer of nuclear dyes between gametes.[6,272] These techniques and others have made possible the determination of whether adhesion or fusion has taken place, but they do not provide insight into the mechanisms or molecules that underlie these events. The availability of proteomic and genetic approaches is progressively filling this gap.[272,273]

Mere contact anywhere at the gamete surface is not sufficient to ensure fertilization. The sperm membrane is highly regionalized, and specific domains must come into intimate association with the egg surface. Initial contact and adhesion is followed by fusion in neighboring regions.[6] Evidence for the specialization of the membranes involved in gamete interaction also comes from antibody studies demonstrating specific localization of proteins and inhibition of fertilization.[274,275]

Indirect evidence for an initial binding step prior to fusion comes from studies in which bound, non-fused sperm on eggs can be identified, and the two processes

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(binding and fusion) can be dissociated. After fertilization in vitro of zona-free eggs, fusion failure of residual surface-bound sperm is inferred from the absence of the transfer of a DNA dye from the egg cytoplasm to the sperm nucleus.[276] Experimental conditions have been found in which gamete binding occurs but fusion does not. For example, despite the fact that media requirements for sperm-oolemma fusion are minimal compared to those required for capacitation, it was observed that the absence of glucose or presence of glycolytic inhibitors, or in an acidic external pH, sperm–egg binding occurs without fusion.[6,277] Reduced binding also occurs in the absence of extracellular Ca^{2+}, and restoration of Ca^{2+} or glucose or pH levels allows some bound sperm to fuse.

Identification of the membrane interactions and the structures involved in both the initial association of sperm and egg membranes, as well as in the subsequent steps that result in the incorporation of the sperm head, were revealed by electron microscopic examination of the acrosome reaction and fertilization.[278] These formative observations have been recently expanded upon by studies using time-lapse confocal microscopy of fluorophore-tagged proteins.[279,280] The sperm-acrosome reaction exposes the inner acrosomal membrane (IAM), where initial interactions with the oolemma occur,[281] but it is the sperm's equatorial segment and posterior head regions that become closely associated with the egg surface and undergo fusion with the oolemma (Figure 4.5(A)–(D)).[6] The IAM is subsequently

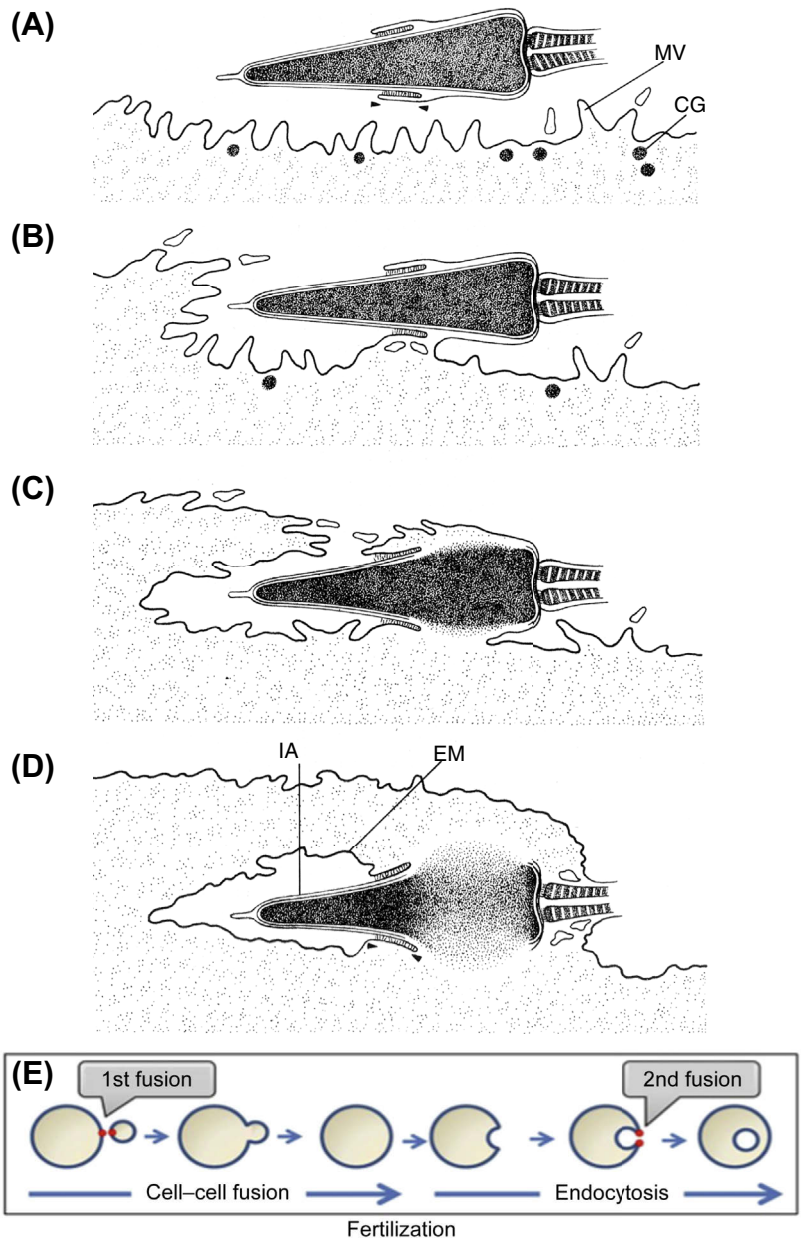

FIGURE 4.5 Diagram of the early stages of sperm interaction with the egg plasma membrane. (A–D). Although the inner acrosomal membrane (IA) of the sperm may make initial contact with egg microvilli, this region does not seem to undergo fusion. The equatorial segment of the head of an acrosome-reacted sperm undergoes the initial fusion with the egg plasma membrane (A–B). The amount of continuity between the sperm and egg cytoplasm increases in a posterior direction on the sperm head. Anteriorly, the inner acrosomal membrane and egg plasma membrane (EM) form a vesicle inside the egg cytoplasm (C, D), whose internalization requires a second round of fusion (E). The loss of cortical granules can be observed to occur during the initial process of sperm incorporation (A–C). *(Reprinted from Ref. 4 with permission from Elsevier, Inc. Courtesy of Dr Michael Bedford.)* (E) The two distinct fusion processes during fertilization are shown in a diagram. The first, cell–cell fusion, combines two independent membranes (sperm and egg) into one. Second, a second round of fusion is required to incorporate the vesicle. Dots indicate where fusion occurs. CG, cortical granule; MV, microvillus. *Reprinted from Ref. 280 with permission from The Company of Biologist. Courtesy of Dr Masaru Okabe.*

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internalized[6,282] through a second membrane fusion event (Figure 4.5(E)).[280] Sperm enter a fusion competent state immediately before interaction with the oolemma; in contrast, competence develops in oocytes well before ovulation.[283]

Mammalian sperm fuse with microvilli on the egg surface,[6] consistent with the notion that membrane fusion is facilitated by the regions of high curvature,[284,285] and conditions that alter the integrity of microvilli, including destabilization of actin microfilaments, prevent fusion.[286,287] The egg surface may be further regionalized. In rodents, a non-microvillous membrane domain is established over the metaphase II spindle during oocyte maturation[288] through a process that involves signaling through FYN kinase-dependent pathways.[289] This domain does not seem to participate in fusion.[6,290] However, such morphological and functional regionalization is not observed in all mammalian eggs, as human eggs display uniform distribution of microvilli.[291]

Candidate Sperm Proteins for Binding and Fusion

The observation that gamete plasma membranes exhibit specialized domains involved in adhesion and fusion provided a strategy for identifying specific molecules that mediate gamete interaction. These molecules were characterized and localized initially using anti-gamete monoclonal antibodies that inhibit fertilization in vitro,[292,293] and later by application of proteomic[290,294]

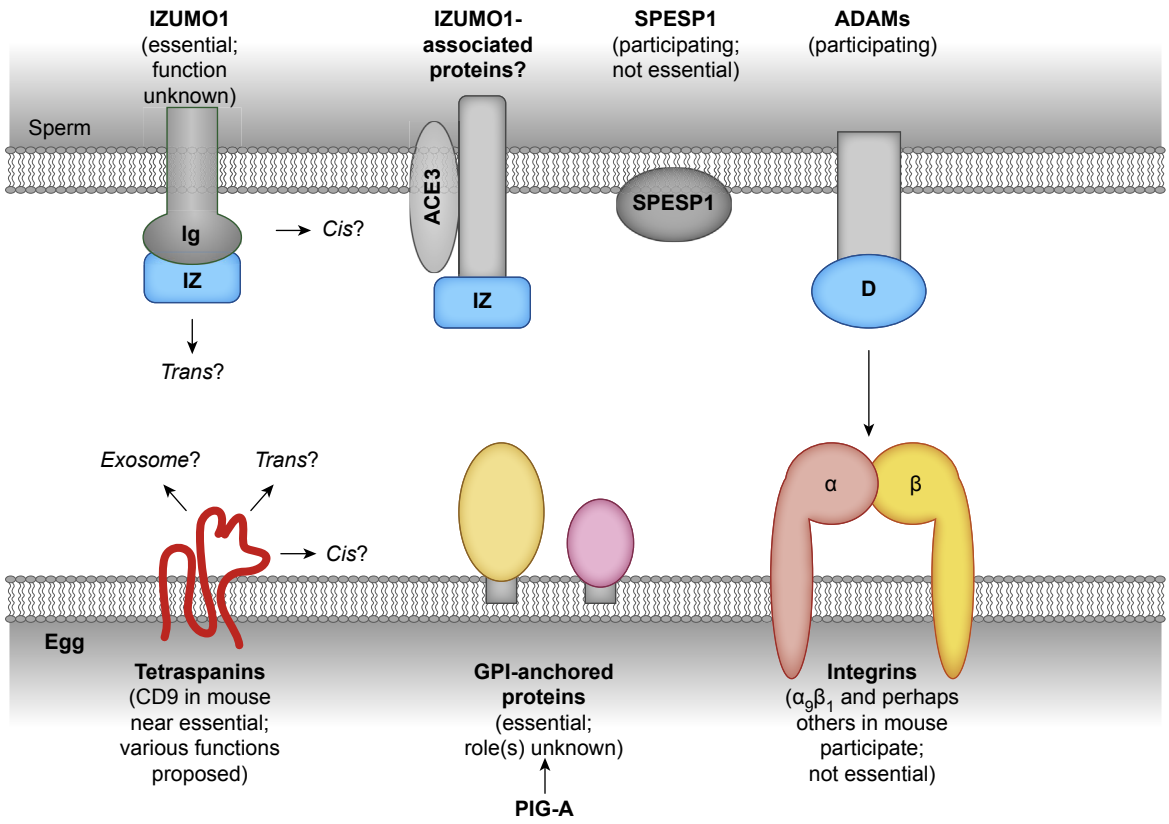

FIGURE 4.6 Sperm and egg molecules implicated in gamete membrane interactions. This diagram illustrates the molecules proposed to participate in sperm–egg membrane interactions. IZUMO1 is essential for sperm–oocyte fusion. ACE3 (angiotensin-converting enzyme 3) is one of IZUMO1-associated proteins recently identified. The position of the Izumo domain (IZ), which has been detected in other proteins, is noted in the diagram. The tetraspanin CD9 is the major player identified thus far on the mouse egg and is likely to function in conjunction with another tetraspanin, CD81. The two extracellular loops, a small and a large one, characteristic of this family are noted. Also noted are the different ways in which the CD9 may interact with putative partner proteins, cis, trans, and exosomes. Glycosyl phosphatidylinositol (GPI)-anchored proteins on the egg are also implicated on the basis of the phenotype of the oocyte-specific conditional knockout of phosphatidylinositol glycan anchor biosynthesis, class A (PIG-A). Other proteins both in sperm and eggs that for their location and/or antibody-blocking studies have been suggested to participate in sperm–egg fusion are also shown. For most of these proteins, knockout lines have not yet been done or if available have failed to confirm the need of the specific protein in the fusion process. Among those proteins are the sperm equatorial segment protein 1 (SPESP1) and several sperm ADAMs, where ADAM stands for a disintegrin and a metalloprotease domain and the D in the diagram depicts the disintegrin domain thought to mediate interactions with their putative partners, the integrins, which are membrane proteins composed of an α subunit and a β subunit. Refer to the text for specific citations and experimental data of the proteins presented in the diagram. *Figure adapted from Ref. 272 with permission from Annual Reviews. Courtesy of Dr Janice P. Evans. See Addendum.*

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and gene knockout technologies.[295] These efforts identified an array of sperm/male reproductive tract proteins thought to participate in adhesion and fusion, however, subsequent in vitro and in vivo studies ruled out most of these candidates. A comprehensive list of these early contenders is discussed in earlier editions of this series,[4,6] while more recent candidates are considered in specialized reviews[272,295] and discussed below.

Candidate fusion protein were identified in rodents by the inhibition of fertilization in vitro by function-blocking antibodies, although subsequent studies found in some cases that these proteins participated in preliminary events antecedent to fusion[272,295] (Figure 4.6). The first strong candidate sperm proteins were members of the ADAM family (*A D*isintegrin *A*nd *M*etalloprotease domain).[296,297] Fertilin, the founder member of this group, is a membrane-anchored heterodimer composed of fertilin-α (ADAM1B) and fertilin-β (ADAM2) subunits. Fertilin is distributed on the entire sperm head in testicular sperm, but during transport through the epididymis it relocalizes to the posterior head, where sperm bind to the oolemma, and both subunits undergo proteolytic processing exposing N-terminal disintegrin domains.[298] ADAM2 was suggested to participate in sperm–egg binding and fusion based on the following observations: (1) it contained a Gln/Thr-Asp-Glu peptide motif that could mediate interactions with integrin(s)[299] on the egg suface;[300] (2) it also contained a 20-residue amphipathic α-helix with a diprolyl motif that was similar to viral fusion peptides;[301] and (3) addition of peptides that interact with integrins, antibodies, native fertilin-β, or recombinant fertilin inhibited sperm binding and fusion with zona-free eggs.[302,303]

However, the assignment of a function to sperm ADAM2 is complicated by the pleiotropic effects of gene disruption. Nullizygous *Adam2* male mice lack fertilin and are infertile, but this infertility is associated with defects in sperm migration into the oviduct and in interaction with the zona pellucida, as well as reduced interaction with the oolemma.[304] More telling, disruption of *Adam1b* also resulted in a loss of sperm surface fertilin, but in this case there was no associated fertility phenotype. In addition, deletions of *Adam2* were associated with compensatory downregulation of other ADAM family members in sperm.[305] These results were consistent with the notion that fertilin was not required for sperm–oolemma fusion and that another, possibly ADAM-related protein, might underlie the fertility defects of *Adam2*-deficient mice. Further studies showed that disruption of two other ADAM family members, *Adam1a* and *Adam3*,[306,307] as well as other unrelated genes, phenocopied the fertility defect associated with *Adam2* deficiency and also resulted in a loss of sperm surface ADAM3.[295,308] Finally, eggs are fertilized in vivo when sperm from *Adam3*$^{-/-}$ mice were injected directly into oviducts at a point distal to the uterotubal junction, and in vitro.[309,310] The emerging picture is that fertilin and other ADAM family genes do not mediate gamete fusion, but that Adam3 or an associated protein plays an essential role in uterotubal transport.

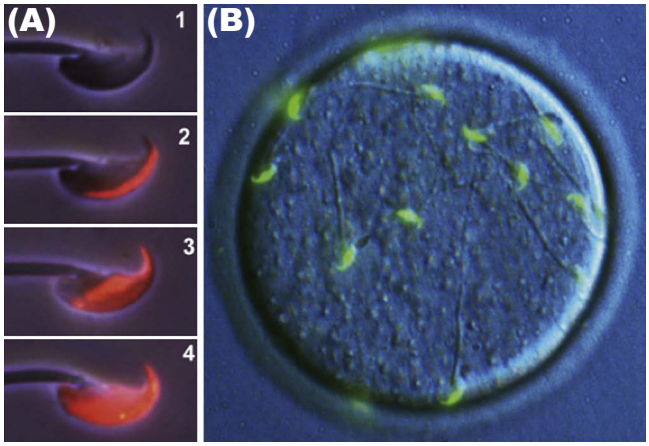

FIGURE 4.7 Gamete fusion-related factor IZUMO1 and localization in sperm. This figure is reproduced in color in the color plate section. (A) IZUMO1 is an acrosomal membrane protein that is not exposed before the completion of an acrosome reaction (1). Acrosome-reacted sperm can be classified into three major groups by their IZUMO1-staining pattern: acrosomal cap (2), equatorial (3), and whole-head (4). (B) Accumulation of many sperm in the perivitelline space of the eggs recovered from females mated with *Izumo1*$^{-/-}$ males. Sperm in the perivitelline space were labeled with acrosome-reacted sperm-specific monoclonal antibody MN9. *Figure reprinted from Ref. 273 with permission from the* Asian Journal of Andrology. *Courtesy of Dr Masaru Okabe.*

A stronger case can be made for a second candidate: IZUMO1. Antibodies against this protein block fertilization and *Izumo1* nullizygous male mice are infertile.[311,312] IZUMO1 remains the only sperm protein required for gamete fusion in the mouse. A role in other species has not been explored extensively; addition of antihuman IZUMO1 antibodies prevented fusion of human sperm to zona-free hamster eggs, but sperm binding to the oolemma was also disturbed.[312]

IZUMO1 is a testis-specific member of the immunoglobulin (Ig) superfamily of proteins, and there are three other closely related members of the family: IZUMO2–4. IZUMO1 is a type I membrane protein with a single extracellular Ig domain and shares a unique "Izumo domain" with other IZUMO proteins.[313] IZUMO1 is located in the acrosomal membrane before the acrosome reaction.[273,314] Following the acrosome reaction, IZUMO1 relocalizes in one of three patterns (Figure 4.7), and the functional consequences of this process are to relocate IZUMO1 from the anterior sperm head to the equatorial segment or to the complete sperm head, where sperm–egg fusion starts and progresses, respectively.[273,280] Sperm from *Izumo1* nullizygous mice display normal morphology, penetrate the zona pellucida, and attach to the oolemma in vitro,

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although are unable to fuse with the oolemma and therefore accumulate in the perivitelline space (Figure 4.7).[39] Importantly, sperm from *Izumo*-disrupted males used for intracytoplasmic sperm injection (ICSI) fertilize and promote development to term, which suggests that the function of IZUMO1 is exclusively associated with sperm–oolemma fusion.

It is unclear whether IZUMO1 is the fusogen, as it lacks the characteristic features of a viral fusion protein. However, it may be of interest that Ig domain proteins are implicated in osteoclast cell–cell fusion.[315,316] Alternatively, IZUMO1 may be more responsible for organizing the membrane and providing the appropriate framework for the sperm membrane fusion machinery to operate.[313] One approach to resolving the mechanism of IZUMO1 action has been to identify interacting partners. When *Izumo1* was reintroduced into an *Izumo1*$^{-/-}$ background, the protein interacted with angiotensin-converting enzyme-3 (ACE3), an acrosomal protein. The functional significance of this association is unknown, and generation of *Ace3*-deficient mice had no fertility defect.[308] Interestingly, disruption of the genes that encode SPESP1 (sperm equatorial segment protein-1) and TSSK6 (testis-specific serine kinase-6) altered the localization and/or redistribution of IZUMO1, compromised the ability of sperm to fuse with the oolemma, and impaired male fertility in mice.[317,318] Disruption of *Spesp1* altered the organization of the equatorial segment membrane, as assessed by scanning electron microscopy, and modified the localization of other equatorial segment resident proteins.[318] Disruption of *Tssk6* leads to defects in actin polymerization in the posterior sperm head, suggesting that redistribution of IZUMO1 during the acrosome reaction may be regulated by the actin cytoskeleton.[317] At present, IZUMO1 is believed to mediate gamete fusion either directly or through its association with a fusogenic partner.[318] (See Addendum.)

The equatorial segment protein equatorin is another candidate sperm protein to mediate gamete fusion. Addition of the MN9 antibody, which recognizes equatorin, prevents fertilization in vivo and in vitro by blocking fusion, but had no effect on sperm penetration of the zona pellucida.[319] Equatorin is an acrosome protein that redistributes to the equatorial region after the acrosome reaction. The N-terminal region of the protein, which contains the MN9 epitope and is *O*-glycosylated, is present on the acrosomal membrane facing the acrosome lumen.[320] Further studies of equatorin are required to establish its function during fertilization and fusion.

The CRISP family of sperm proteins are also candidate mediators of gamete fusion. CRISP proteins are synthesized in the epididymis and deposited on the sperm surface.[321] The evidence for their participation in fusion includes: (1) antibodies against these proteins reduced fertilization rates in vitro, largely by inhibiting sperm–egg fusion; and, (2) recombinant CRISP1 protein also inhibited gamete fusion. *Crisp1*$^{-/-}$ males had normal fertility, although their sperm showed reduced capacitation parameters and some reduction of fertilization in vitro.[322] Complete assessment of the role of CRISP family proteins is complicated by the presence of other family members that may compensate for *Crisp1* gene disruption, and therefore must await the generation of more comprehensive genetic models.

In summary, a single sperm protein, IZUMO1, has emerged as essential in the pathway leading to gamete fusion. It remains uncertain whether this acts directly as a fusion protein, or indirectly. Finally, it is worth noting that while dismissed as fusogen candidates, several members of the ADAM family play essential functions in fertilization. Therefore, besides identifying additional key effectors of sperm–oolemma fusion, future studies should ascertain the role and mechanisms of action of proteins such as ADAM3.

Candidate Egg Proteins for Binding and Fusion

Interest in identifying oolemmal molecule(s) that participate in gamete fusion has been hampered by the limited availability of oocytes. To date, only three families of proteins remain as possible candidates/effectors for gamete binding and fusion: integrins, glycosyl phosphatidylinositol (GPI)-anchored proteins, and tetraspanins; only the last two are known to be required for gamete fusion[272,273] (Figure 4.6). Interest in the role of integrins was sparked upon the discovery of the ADAM family proteins in sperm and the presence in ADAM2 of an integrin-binding domain.[301] Integrins are a large family of heterodimeric proteins mediating cell interaction with extracellular matrix and with other cells, as well as intracellular signaling.[323] Consistent with their proposed role in gamete interaction, mouse eggs express several α and β integrin subunits, making possible the presentation of multiple αβ pairs on the surface of eggs; integrins have been detected in other mammalian eggs as well.[272,324] Their functional role in gamete interaction was supported by the inhibition of sperm binding to and fusion with oocytes by integrin-targeted antibodies and by peptide studies.[300,303] In addition, integrin α6β1 was shown to be associated on the oolemma with CD9, one of the tetraspanins required for sperm–oolemma fusion (see below).[325] However, subsequent studies involving animals or eggs with disrupted expression of single integrins displayed normal fertility in vivo or in vitro, casting doubts on the importance of these proteins in fertilization.[326,327] Collectively, this work suggests that individual loss from the egg surface of most integrin family members expressed in eggs is dispensable for sperm–oolemma fusion and fertility, although ancillary roles for these proteins cannot be fully ruled out by genetic

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studies due to possible redundancy, incomplete knockdown, and/or compensatory mechanisms.[272]

A family of proteins involved in mediating sperm-oolema fusion in mouse eggs is the GPI-anchored proteins. Initial studies by Hirao and Yanagimachi noted that treatment of eggs with phosphatidylinositol-specific phospholipase C (PI-PLC), which cleaves the phosphatidylinositol link and releases proteins from the oolemma, prevented gamete fusion in hamsters.[328] While the authors themselves questioned the results, comparable experiments by Coonrod and colleagues with more purified versions of the enzyme produced similar results and narrowed down the fertilization phenotype to defects of fusion.[329] Proteomic analysis of exudates of PI-PLC-treated eggs revealed that one of the proteins released was CD55, although mice null for this protein are fertile.[330,331] Nevertheless, widespread removal of GPI-anchored proteins from the oolemma accomplished by conditional targeting of the *p*hosphat*i*dylinositol *g*lycan *a*nchor biosynthesis, class A gene (*Piga*), which participates in the initial steps of GPI synthesis, generated females that were sterile and displayed lack of sperm–oolemma fusion in vitro.[332] However, the precise GPI-anchored protein(s) responsible for the fusion defect was not uncovered. What also remains obscure is how the absence of GPI-anchored protein(s) affects fusion. GPI-anchored proteins usually associate with lipid rafts, where signaling molecules congregate. A possibility is that in the absence of these proteins, productive interactions between sperm and the oolemma are not possible and appropriate signaling pathways are not engaged. It is also possible that without these proteins, the oolemma becomes widely disorganized and prevents fusion, although this seems less likely, as acute removal of these proteins produces similar outcomes than those caused by persistent absence of these proteins, as in the case of the knockout model where oocyte membrane is organized in the absence of GPI-anchored proteins.[331] (See Addendum.)

CD9 is to date the only identified egg plasma membrane protein required for gamete fusion in mammals. Tetraspanins display four transmembrane domains: two extracellular loops, a small and a large one; and two intracellular tails, which can interact with cytoskeletal components and with molecules involved in signal transduction[333] (Figure 4.6). Tetraspanins interact with other tetraspanins as well as with other transmembrane and cytoplasmic proteins forming "tetraspanin webs", which are specialized membrane microdomains.[331] Tetraspanin proteins are widely expressed and participate in myriad cell functions, including cell proliferation, signaling, cell–cell interaction and fusion.[334] Regarding the latter, CD9 interacts in various cell types with several β1 integrins including α6β1,[333] which is also the case in mouse eggs.[335] A monoclonal antibody raised against the extracellular loop of CD9, JF9, proved highly potent at inhibiting sperm–oolemma adhesion and fusion.[335] Subsequent genetic studies showed that eggs from females with disrupted CD9 rarely fused with wild-type sperm, even in zona-free assays in vitro, and females were nearly infertile;[286,336,337] disruption of another tetraspanin, CD81, showed similar, albeit less severe, phenotype.[338] Importantly, the fusion defects observed in CD9 null eggs were partly overcome by injections of mRNAs encoding for CD9 or CD81,[339] and fertilization of these eggs by ICSI caused egg activation and supported development to term, demonstrating that CD9 null eggs are developmentally competent.[286] CD9 appears to play a role in gamete interaction in other mammalian species,[340] and CD151 appears to be one of the tetraspanins involved in human sperm–oolemma interaction and fusion.[341]

The mechanism by which CD9 modulates gamete fusion is not known.[272,331] One possibility is that, via its numerous interactions, one of its associated proteins effects fusion. Consistent with this prospect is the finding that preincubation of recombinant peptides representing the large extracellular loop of CD9 prevents fertilization when incubated with eggs but not with sperm.[342] In addition, proteomic studies support this notion, as one of the few known CD9-associated proteins in eggs, EWI-2 (also known as immunoglobulin superfamily member 8, or IgSF8), is absent from the surfaces of CD9-null eggs, and similar fates may befall other currently unknown CD9-partner proteins. Such protein losses may undermine the fusion competence of CD9-null eggs. This remains a possibility, but it should be noted that IgSF8 knockout mice did not show a fertility phenotype.[343] CD9 may also be critical in determining overall membrane topology and thereby enhancing the interaction between gametes. In support of this view, the shape and density of the microvilli is severely altered in CD9 null mice,[344] and a recent, more exhaustive assessment of sperm–oolemma interactions showed that while the number of adhesion opportunities between sperm and eggs was unaffected by the absence of CD9, the strongest adhesion interactions were decreased.[345] These results suggest that on the plasma membrane CD9 may favor the formation of sites responsible for stronger adhesion events that facilitate the subsequent fusion process. The underlying mechanism(s) responsible for the superior adhesion properties of CD9-containing membranes is unknown. CD9 is also reportedly released from oocytes in cell-derived vesicles (exosomes) into the perivitelline space, where it can be adsorbed onto surrounding sperm.[346] Adsorption may render sperm fusion competent,[279] although this has been disputed.[347] Lastly, CD9 may interact with sperm proteins, thereby facilitating adhesion and fusion between gametes. There is evidence for this type of association in other cell types, although such interactions have not been found in sperm. In summary, research over the

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last decade on both sperm and eggs has identified key molecules that participate in sperm–oolemma adhesion and fusion. Nevertheless, how these and other yet-to-be-discovered molecules function in mediating gamete adhesion and fusion remains a significant challenge for present and future investigators interested in this field.

Incorporation of the Fertilizing Sperm into the Egg and Fate of Sperm Components

One indication that gamete fusion is underway is the sudden reduction of the movements of the sperm tail,[348] a property of fertilizing sperm shared by mammals and invertebrates.[6] In mammals, fusion results in the entire sperm, including the tail, being incorporated into the ooplasm.[349] Sperm incorporation induces modifications of the oolemma and of the underlining egg cortex, causing the formation of an actin-rich fertilization cone[350] that progressively expands to engulf the sperm head (Figure 4.5(B) and (C)). The anterior portion of the fused membranes form a vesicle derived from the inner acrosomal and oolemmal membranes, which is endocytosed and requires a second round of membrane fusion, as already pointed out (Figure 4.5(D) and (E)).[280,351] Nevertheless, the role of actin in the process of sperm entry in mammals is not yet settled. Some reports show no inhibitory effects of actin inhibitors,[352] others find that the organization of oocyte actin is essential and required for sperm head entry, decondensation, and for incorporation of the axoneme;[353,354] the blunted $Ca^{2+}{}_i$ responses induced by fertilization of mouse eggs with disrupted microfilament organization confirm the pivotal role of actin in promoting complete and timely sperm incorporation in these species.[354] PYK2, a calcium-sensitive tyrosine kinase from the focal adhesion kinase (FAK) family, reportedly participates in the reorganization of actin at the site of sperm entry.[355]

Once in the ooplasm, the components of the sperm undergo different fates. Simultaneous with sperm head decondensation, which is brought about in part by the reducing environment of the ooplasm, the mitochondrial sheath is separated from the connective piece, making possible access of ooplasm material to the sperm centriole. Recruitment of pericentriolar material to this site and assembly of microtubules leads to formation of the sperm aster, which guides the apposition of the male and female pronucleus and brings both genomes in close proximity in preparation for the first mitotic cleavage.[6,356,357] This sequence of events occurs in most mammals, although in rodents, which lack a sperm centriole, centrosomes made up of egg pericentriolar material serve as the microtubule-organizing center.[358] Centrosomes organize meiotic spindles, and their function during the initial stages of development appears to be more important than centrioles, as parthenogenetically activated diploid embryos can commence embryo development and reach early fetal stages without sperm contribution.[359] Given that centrosomes and centrioles can form "de novo" from egg extracts and during early cleavage stages,[358] the significance of paternal centrioles and components of both maternal and paternal centrosomes to the early embryos remains to be determined. The displaced paternal mitochondria, which are ubiquitinated during spermatogenesis, are targeted for degradation in the ooplasm and do not persist more than 2 or 3 days, although it is possible that some may evade degradation and fuse with the egg's mitochondria;[356,357] their elimination seems determined by certain developmental events rather than by time postfertilization, and it appears to be species specific, as it is not seen in cases of interspecies crosses.[357] Other components of the sperm tail and principal piece also undergo degradation, with loss of the fibrous sheath preceding the disintegration of the outer fibers and axoneme microtubules.[356,360]

Other sperm components delivered during fertilization include plasma membrane and RNA.[4] Remnants of the sperm plasma membrane are detectable on the surface of marine and mammalian eggs on the site of entry,[361,362] from where they disperse into the oolemma.[363] The role of this exchange is unknown, although it may contribute to the membrane block to polyspermy, which in mammals is observed after fertilization but not after ICSI.[364,365] However, prophase-I stage oocytes do not mount such a block following fertilization with sperm, raising questions regarding the contribution of the donated sperm membrane.[366] In terms of RNAs, it is now widely accepted that sperm carry and deliver into the ooplasm diverse RNA populations, including rRNAs, mRNAs of genes actively transcribed during spermatogenesis, and miRNAs;[367] sperm also carry an endogenous reverse transcriptase, which reportedly may be active both prior and after fertilization.[368] While the implications of this type of genetic transmission to fertilization and early embryo development remain tantalizing,[369,370] additional studies are needed to demonstrate the scope of their functions as well as their possible application as a diagnostic method.

EVENTS FOLLOWING SPERM–EGG MEMBRANE FUSION

The onset of zygote development depends on completion of a series of so-called "events of egg activation" that are induced by fertilization and occur sequentially prior to the first cleavage. The early events include secretion of egg cortical granules (see loss of cortical granules from the egg cortex in the diagram of Figure 4.5(B) and (C)), the blocks to polyspermy (see below) and onset of paternal chromatin decondensation. In most mammals,

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these events are well underway within the first hour of fertilization. Later events include completion of maternal meiosis, maternal chromatin decondensation, male and female pronuclear formation, and recruitment of maternal mRNAs resulting in expression of new proteins. Before the first cleavage, the zygote also undergoes the onset of genomic reprogramming and limited zygotic genome activation. Egg activation is universally induced by an increase in the concentration of free Ca^{2+}_i, and all events of egg activation are directly stimulated by Ca^{2+}_i or rely on changes in the activity of specific kinases brought about by the release of Ca^{2+}.

Block(s) to Polyspermic Fertilization

Mammalian females employ several mechanisms to prevent incorporation of extra sperm into the ooplasm, "block(s)" to polyspermic fertilization,[4,371,372] as polyploidy caused by entrance of more than one sperm results in abnormal development. The blocks to polyspermy occur sequentially and arise from two sources: the oviduct and the egg itself. Despite these barriers, low rates of polyspermic fertilization are observed under in vivo conditions in all mammals, including humans.[373] Rates of polyspermy increase considerably when fertilization is performed in the absence of oviductal fluids and in the presence of excess numbers of sperm, which is the case when assisted reproductive technologies (ART) such as in vitro fertilization (IVF) are applied. For example, in the case of human IVF, reported rates of polyspermy are between 5% and 10%,[374] and similar rates are reported for most other mammals including bovines, sheep, rodents, and rabbits, although in the case of porcine eggs polyspermic fertilization following IVF can reach levels higher than 50%.[6,375]

The oviduct contributes to the polyspermy block in two manners (Figure 4.8). The best-characterized effect is the physical barrier imposed by transport through the uterotubal junction, which limits the number of sperm available for storage in the isthmus and posterior release to the ampulla.[6,376] This mechanism in several species reportedly achieves sperm/egg ratios in the ampulla of <10:1,[377] which considerably lowers the risk of multiple sperm penetration.[378]

A less-characterized oviduct-based mechanism is predominantly functional in ungulates and relies on molecular components of the oviductal fluid. This effect is evidenced in in vitro matured porcine and bovine oocytes that when incubated with tubal fluids for different periods of time prior to IVF show greatly reduced rates of polyspermy.[375,379] Exposure of eggs to tubal fluids in these species render the zona pellucida resistant to proteolysis, a change that is not induced by fertilization or by release of cortical granules,[375,380] as it occurs in rodents; human eggs seem insensitive to the effects of oviductal fluids.[381] This mechanism has therefore been called prefertilization zona hardening (see below for more details for postfertilization zona hardening), and it does not interfere with sperm penetration.[382] Remarkably, this process is reversible, restricted to the fluids of the periovulatory period[382,383] and thought to be mediated by the combined and sequential action of oviduct-specific glycoprotein 1 (OVGP1) and sulfated-glycosaminoglycans

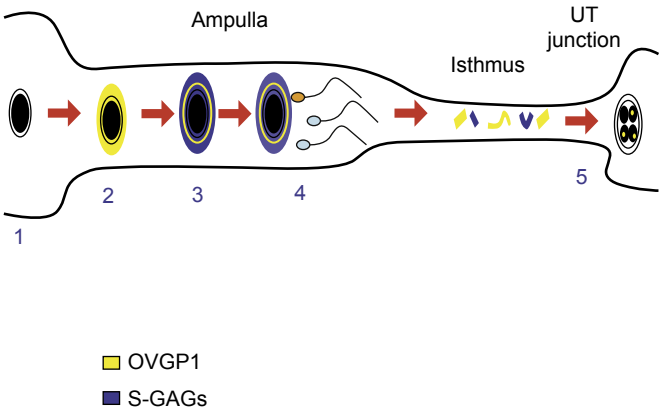

FIGURE 4.8 Depiction of the proposed role of oviductal contents in preventing polyspermy in ungulates. This figure is reproduced in color in the color plate section. After the egg is shed into the ampulla following ovulation (1), oviduct-specific glycoprotein (OVGP1) surrounds it in a "shell" that renders the zona pellucida (ZP) resistant to proteolysis (2). Other oviduct molecules such as heparin-like glycosaminoglycans (S-GAGs) are thought to aid and strengthen the binding of OVGP1 to the ZP (3), modifying interaction with spermatozoa (4). As the zygote advances to the uterus, the aforementioned factors are shed or absorbed into the egg (5). *Figure reprinted and legend adapted from Ref. 375 with permission from John Wiley & Sons. Courtesy of Dr Pilar Coy.*

TABLE 4.1 Classification of Animals Based on the Mechanisms of Polyspermy Block

	Group		
	I	**II**	**III**
Species	Golden hamster	Rat	Rabbit
	Dog	Mouse	Pika
	Sheep	Guinea pig	Mole
	Field vole	Cat	Bat
	Ferret		
	Human		
Presence of sperm in PVS	Very rare	Often 1 sperm	Often≥a few
Primary source of polyspermy block	ZR	ZR and PMB	PMB

PMB, plasma membrane block; PVS, perivitelline space; ZR, zona reaction.
Adapted from Table 4, Ref. 4.

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(S-GAGs) (Figure 4.8), although the expression of these proteins in specific regions of the oviduct and the structural modifications imposed on the ZP are unknown and remain to be characterized.[384]

The egg-based blocks to polyspermy have been thoroughly investigated in mammals and consist of the zona and plasma membrane blocks. The relative contributions of these two blocks vary in different species[4,6] (Table 4.1) with the plasma membrane block unfolding earlier than the zona pellucida block. The membrane block reportedly unfolds within a few minutes after sperm–egg fusion in eggs of mice[385] and hamsters,[386] although a more recent study suggests a slower block.[287] The goal of the plasma membrane block is to prevent binding/fusion of additional sperm, as demonstrated by in vitro fertilization of zona-free eggs that have large numbers of bound sperm but remain (in >50% of eggs) monospermic even after many hours of culture. Little is known about the biochemical basis of the membrane block,[6,287,366] which lacks the membrane depolarization component observed in marine eggs.[371] Nevertheless, the observation that this block is induced following sperm–egg fusion but not after ICSI[364,365] suggests contribution of a sperm membrane component, although participation of oocyte/egg factors is required, as it is only engaged in metaphase II stage eggs.[366] (See Addendum.)

The zona block is intended to prevent sperm penetration and/or binding;[229] inhibition of penetration unfolds rapidly after fusion,[6,385–387] while prevention of sperm binding occurs after several hours.[219,229] Several mechanisms have been proposed to account for the zona block, including those that might cause specific modifications in key sperm-binding residues,[388] or changes in the organization of the zona or of its components,[219,229,389] and/or by addition of new proteins following the release of cortical granules (see below).[390] Ultrastructural studies in human eggs and those from other mammals showed changes in zona filaments and structure after fertilization,[391–393] although unfertilized human eggs display significant variability in these parameters, raising questions about their contributions to the zona block.[391,394] Initial studies to elucidate the molecular basis of the zona block focused on modifications of presumed sperm binding residues such as *O*- and *N*-linked glycans on ZP3, as studies with purified ZP3 from unfertilized eggs but not from two-cell embryos demonstrated that it, but not ZP1 or ZP2, abrogated sperm binding to the zona, and it induced acrosome reaction.[212,217] Further, ZP3 glycopeptides and purified ZP3 *O*-glycans retained sperm-binding ability but not the capacity to induce the acrosome reaction.[213,214] The glycan model (Figure 4.9(A)), as this theory is known, envisions release of glycosidases following the cortical granule reaction that removes *O*-linked glycans from ZP3 thereby preventing sperm binding.[214,395] Nevertheless, the proposed glycan composition has thus far not been detected in native zonae pellucidae,[396] hindering further characterization of the model. In addition, demonstration that sperm binding to the zona continues unimpeded in transgenic mice where candidate *O*-glycans residues have been eliminated raises the prospect that other mechanism(s) may underlie the zona block.[397] The ZP2 cleavage model (Figure 4.9(A)) arises from observations in mice showing that after fertilization ZP2 undergoes cleavage, after which sperm are no longer able to bind the zona pellucida.[219] More specifically, this model proposes that after fertilization the N-terminal domain of ZP2, which is where sperm binding is thought to occur, undergoes a series of cleavages eliminating the binding site.[258,259] While ZP2 fragments remain bound by disulfide bonds and are undetectable during fertilization, they can be visualized by electrophoretic methods[219] even in human eggs;[398] it is speculated that these modifications change the higher-ordered structure of the zona pellucida. Studies using transgenic and genetic models where human zona pellucida proteins were used to replace their mouse counterparts provide support for this model, including: (1) human sperm only bind eggs that express human ZP2 (Figure 4.9(B)); and (2) human sperm bind to beads decorated with human ZP2 N-terminal peptides, and excess of these peptides prevents binding of human sperm to ZP2 humanized zonae pellucidae.[223] Therefore, these studies suggest that proteolytic cleavage of ZP2 prevents sperm binding and underlies the slow developing zona block in mouse and most likely in other mammals. While it is speculated that structural reorganization of the zona pellucida accounts for the downregulation of sperm binding, there is no available biophysical or biochemical evidence supporting such conformational shifts.

The zona pellucida block results from the action of proteins secreted by the egg upon fertilization. This evidence is well established in marine and amphibian eggs where the secreted proteins are known to contribute to the extracellular matrix-level block.[399] A major source of secretion is the exocytosis of thousands of egg cortical granules. These granules undergo reorganization during oocyte maturation, which includes migration to the cortex and acquisition of exocytosis competence.[4] In fact, during late stages of maturation, some granules undergo premature exocytosis and their contents are found in the perivitelline space; the role of this prefertilization release is not well established. In mammalian eggs, thousands of 0.1- to 1.0-μm-diameter, membrane-delimited cortical granules are found in the peripheral cortex underlying the plasma membrane.[400] The major wave of cortical granule exocytosis occurs within 15 min of sperm entry, as the egg initiates the "cortical reaction", and it is triggered by the Ca^{2+}_i rises induced by fertilization.[386,401] The cortical granule exudate diffuses across the perivitelline space, where it removes or masks sperm

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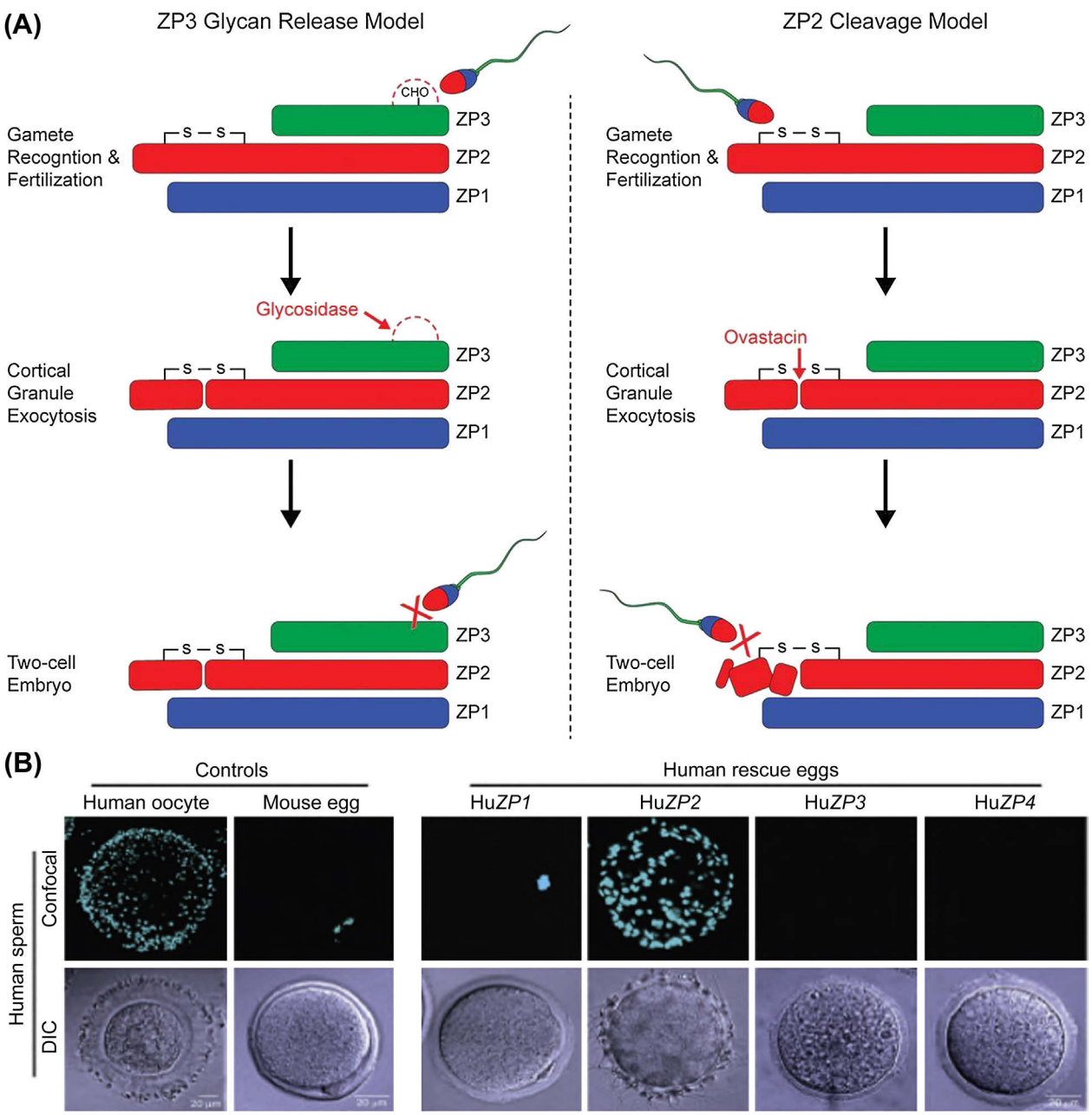

FIGURE 4.9 **Models of zona pellucida block to polyspermy.** This figure is reproduced in color in the color plate section. (A) A specific glycan-release model (left panel) proposes that *O*-glycans on ZP3 Ser^{332} and Ser^{334} or possible others act as ligands for a sperm surface receptor. Following fertilization, cortical granules would release a glycosidase that would remove the *O*-glycans and account for the inability of sperm to bind to two-cell embryos. The ZP2 cleavage model (right panel) proposes that sperm bind to an N-terminal domain of ZP2. Following fertilization, ovastacin, a cortical granule metalloendoprotease, is exocytosed and the proteolytic destruction of the ZP2 docking site prevents sperm from binding to two-cell embryos. (B) Human sperm bind to mouse eggs from transgenic strains expressing human ZP2, but not other human ZP proteins, in the ZP. Confocal and differential interference contrast images of capacitated human sperm binding to the ZP of mice expressing human ZP1, human ZP2, human ZP3, or human ZP4, in the absence of the corresponding mouse protein. Human oocytes and mouse eggs served as positive and negative controls, respectively. Specific references and experimental details can be found in the text. *Figure reprinted from Ref. 229 with permission from Oxford Journals. Courtesy of Dr Jurien Dean.*

receptors in the egg's extracellular investments, or otherwise modifies those investments.[390] Some of the cortical granule-derived macromolecules that remain in the perivitelline space have been suggested to form a "cortical granule envelope",[390,402] although the function and molecular composition of this envelope warrant further investigation.

Cortical granules in mammals are estimated to contain up to 14 different proteins.[400,403] A number of enzymatic activities are present, including trypsin-like and

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tissue-type plasminogen activator activities, an ovoperoxidase and an *N*-acetylglycosaminidase activity that is thought to use ZP3 as a substrate,[4,6] but these activities were not directly linked to the zona pellucida block. Recently, characterization of the ZP2 cleavage site led to a focus on an astacin family of metalloendoproteases,[259] and to the identification of ovastacin, an oocyte-specific astacin,[404] which localizes to mouse cortical granules and is released by exocytosis.[218] Transgenic expression of ZP2 with a mutated cleavage site or ablation of the *Astl* gene, which encodes ovastacin, left ZP2 intact, even after cortical granule release, and prevented the inhibition of sperm binding component of the zona pellucida block to polyspermy.[218,259]

Targeted deletion of the liver-derived plasma protein fetuin-B confirmed the role of ZP2 cleavage in the deployment of the zona block under in vivo conditions.

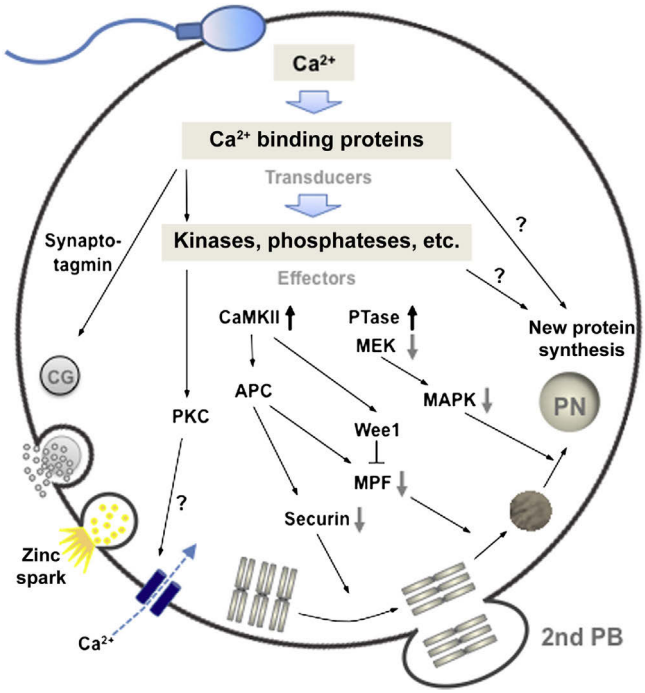

FIGURE 4.10 Signaling diagram of egg activation. The model includes the pathway downstream of multiple elevations of $Ca^{2+}{}_i$ and the events of egg activation initiated by these rises. Some Ca^{2+}-sensitive transducers (e.g., calmodulin, not shown) and effectors (CaMKII) have important roles (see text). Others, for example those promoting new protein expression, which is Ca^{2+} dependent, are yet to be identified. Small vertical arrows indicate whether the activity of an effector increases or decreases to promote the pathway with which it is associated. New protein synthesis results from the recruitment of maternal mRNAs. APC, anaphase promoting complex; Ptase, phosphatase; MAPK, MAP kinase; MEK, MAPK kinase; 2nd PB, second polar body; PN, pronucleus; CG, cortical granule; CaMKII, calmodulin-dependent protein kinase II; MPF, maturation promoting factor, PKC, protein kinase C. Synaptotagmin is a Ca^{2+}-sensitive protein that regulates secretion in other cells. *Modified from Ref. 410 with permission from Elsevier, Inc.*

The so-called zona pellucida hardening, a descriptive term for the zona block and so named for the diminished susceptibility of zona pellucida to proteolytic digestion, occurs naturally after fertilization, and prematurely when oocytes are matured in vitro in the absence of serum;[4,6,405] in both cases, it is associated with ZP2 cleavage.[219,404] Addition of serum, or fetuin prevents in vitro hardening of the zona including cleavage of ZP2,[406] although the molecular target was unknown. Fetuins are part of the cystatin superfamily, which are structurally related to protease inhibitors.[407] Consistent with this, in vitro studies showed that fetuin-B directly inhibits ovastacin, and eggs of *Fetub*$^{-/-}$ mice showed preovulatory ZP2 cleavage, which prevented sperm from penetrating the zona pellucida and caused female infertility.[408] This indicates that plasma fetuin-B restricts the activity of ovastacin in vivo, thereby rendering the zona pellucida permissive to sperm penetration. It is worth noting that ZP2 cleavage is observed in human eggs that fail to fertilize during clinical IVF procedures, but it is unknown if ZP2 changes were responsible for the lack of fertilization or arose due to the prolonged in vitro culture.[409]

Cell Cycle Progression and Completion of Meiosis

Sperm entry promotes cell cycle progression that entails the transition from meiosis to mitosis. In temporal order, these include the completion of meiosis and extrusion of the second polar body, formation of male and female pronuclei, DNA replication, pronuclear envelope breakdown, and the first mitotic metaphase prior to first cleavage. This review will focus more attention on the mechanisms responsible for early events in this series because they are more directly regulated by fertilization (Figure 4.10).

Fertilization of most (but not all) mammalian eggs occurs at metaphase II (MII) of meiosis. This arrest coordinates completion of meiosis with fertilization: ovulated mammalian eggs are arrested at MII by the elevated maturation-promoting factor (MPF) activity, whose two components are cyclin-dependent kinase 1 (CDK1) and cyclin B. MPF activation is necessary for promoting entry into the M-phase stage in all cells. In mitotic cell cycles, MPF levels rise transiently during metaphase and then decrease following cyclin B degradation with each cell cycle.[411] In oocytes (Figure 4.11), activation of MPF promotes the transition from the germinal vesicle stage to metaphase I. Complete activation of MPF requires association of CDK1 with cyclin B, site-specific phosphorylations/dephosphorylations of CDK1, and increasing concentrations of cyclin B.[4,411–414] Oocytes that reach MI transition to MII following decrease in MPF activity and cyclin B degradation, which are prematurely terminated by increasing levels of early mitotic inhibitor 2

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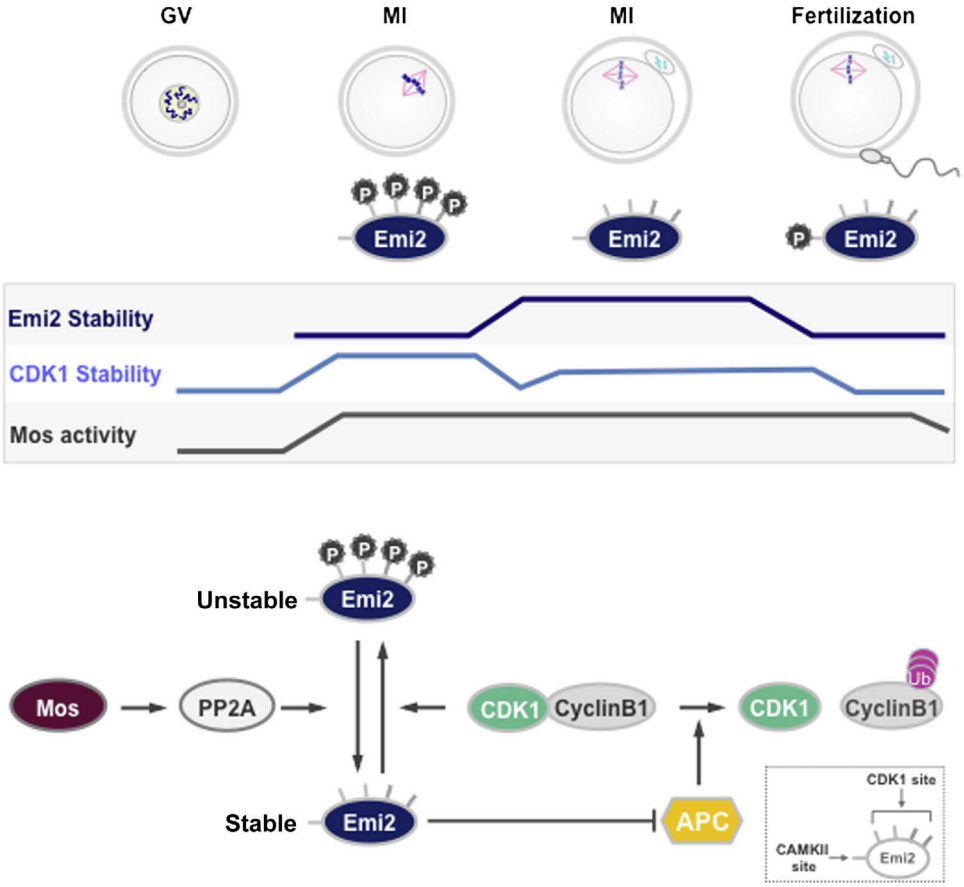

FIGURE 4.11 Molecular effectors that contribute to resumption of meiosis and MII arrest in vertebrate eggs. Stages of oocyte maturation are depicted in the top panel. Phosphorylation controls EMI2 stability during oocyte maturation. At MI, CDK1 phosphorylates amino-terminal sites on EMI2, at least in *Xenopus* oocytes, and this triggers EMI2 degradation, required for MI exit. At MI anaphase, cyclin B is degraded, leading to a drop in CDK1 activity. EMI2 is stabilized by dephosphorylation triggered by Mos signaling and mediated by PP2A type of phosphatase. EMI2 accumulates, resulting in APC inhibition, critical for S phase block and MII entry. CDK1 activity is low in MII relative to MI. EMI2 is stable in MII, as required for CSF-mediated arrest. At fertilization, EMI2 is quickly degraded through a CaMKII-mediated pathway, allowing for activation of the APC and exit from MII. At the onset of MI anaphase, APC-mediated cyclin B degradation results in decreased CDK1 activity. With the Mos-PP2A pathway predominant, dephosphorylated and stabilized EMI2 protein prevents complete ubiquitylation of cyclin B by the APC. This is essential for the inhibition of S phase between MI and MII. *Figure and legend adapted from Ref. 412 with permission from CSHLP. Courtesy of Dr Sally Kornbluth.*

(EMI2), an inhibitor of the anaphase-promoting complex cyclosome (APC/C)[415] that stalls cyclin B proteolysis, promoting instead its accumulation, increase in MPF activity and transition into MII with formation of a bipolar spindle.[416] Unlike mitosis, the activity of MPF at MII remains elevated for hours. The so-called "cytostatic factor" or CSF stabilizes MPF by restraining the proteolytic activity of APC/C.[417,418] EMI2, a component of CSF, is mostly responsible for inhibiting the APC/C, although the precise mechanism(s) of inhibition is not entirely resolved.[419,420] EMI2's activity and stability during vertebrate meiosis are regulated by activation of the c-MOS/MEK/MAPK/p90RSK pathway, which phosphorylates it on several key residues;[411] in mice, the function of p90RSK seems assumed by another substrate of MAPK, the stress-mediated mitogen-activated protein kinase.[421] A PP2-type of phosphatase binds phosphorylated EMI2 and modulates its inhibition of the APC/C by erasing destabilizing CDK1-mediated phosphorylations and in this manner maintains stable levels of MPF activity despite continuous synthesis of cyclin B (Figure 4.11, lower panel).[411] Downregulation of EMI2 or pharmacological inactivation of PP2A in mouse eggs causes the release of the CSF-dependent MII arrest and induces changes associated with fertilization, confirming the role of these molecules in mammalian eggs.[416,422] PP2A, at least in *Xenopus* eggs, seems to be negatively regulated by the action of the Greatwall kinase by means of the small peptide ARPP19 (cAMP-regulated phosphoprotein-19) and α-endosulfine[423] (Figure 4.12, right panel); the timely regulation of PP2A is required both for the arrest and exit of MII.[424]

The stability of MPF ceases after fertilization, which causes inactivation of CSF and release from the MII

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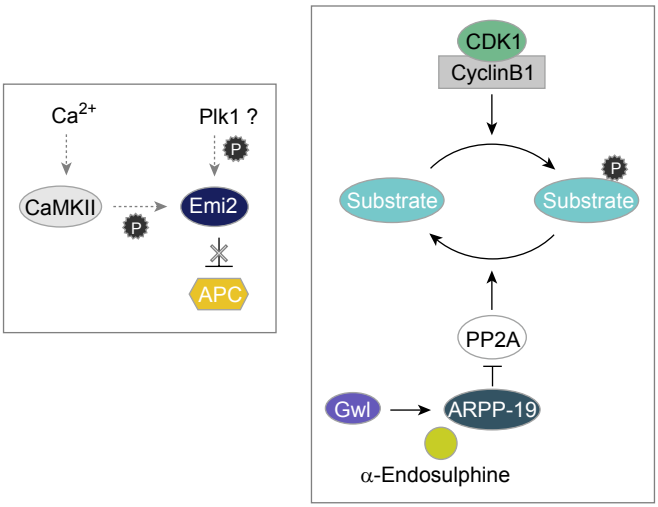

FIGURE 4.12 Molecular events induced by Ca^{2+} release promote MII exit and progression into interphase in vertebrate eggs. As a consequence of a $Ca^{2+}{}_i$ rise, CaMKII is activated, which induces phosphorylation at a conserved consensus site of EMI2. Phosphorylation of EMI2 promotes Plx1 binding and phosphorylation, which triggers SCFb-TrCP-dependent degradation of EMI2 and consequent reactivation of the APC in *Xenopus* eggs; in mouse eggs, a phosphodegron is present, although its position shifted, and therefore the molecular intermediaries from CaMKII to EMI2 degradation remain to be determined in these species (Plk?). Following inactivation of MPF, progression into interphase is facilitated by inactivation of the Greatwall kinase (Gwl), which via ARPP-19 and alpha-endosulphine negatively regulates PP2A, which dephosphorylates many of the M phase CDK1 substrates. *Figure modified from Ref. 412 with permission from CSHLP Dr Sally Kornbluth.*

arrest.[410,425] Sperm entry and the increase in the concentration of free $Ca^{2+}{}_i$ associated with it induce EMI2 degradation[426,427] (Figure 4.12, left panel). $Ca^{2+}{}_i$ increases are transduced by the calcium-responsive protein calmodulin (CaM), which activates several signaling cascades (Figure 4.10). One pathway involved is calcineurin/PP2B, which reverses previous phosphorylations by MPF, thereby facilitating resumption of meiosis and release of the second polar body;[428,429] the function of calcineurin is seemingly not required in mouse eggs.[430] CaM also activates CaM-dependent protein kinase II (CaMKII), which is responsible for proteolysis of EMI2, subsequent APC-mediated destruction of cyclin B, and loss of MPF activity (Figure 4.11; Figure 4.12, left panel).[426,427] CaMKII phosphorylates EMI2 promoting binding by polo-like kinase, whose phosphorylation of EMI2 renders it a substrate for SCFβ-TRCP-mediated ubiquitylation and complete destruction.[426,427,431] Consistent with this, expression in mouse eggs of cRNAs encoding constitutively active CaMKII induced MII exit and egg activation,[432] although in vitro phosphorylation studies by others failed to show phosphorylation of EMI2 by CaMKII.[430] Further, the polo-like kinase phosphodegron in mouse EMI2 is shifted to a different position than in *Xenopus*, therefore making it unclear how CaMKII targets EMI2 for degradation and what is the E3 ligase involved.[430] Nevertheless, the role of CaMKII on MII exit was confirmed by studies where eggs devoid of CaMKIIγ, the predominant isoform on the enzyme in mouse eggs, failed to exit MII following fertilization or activation by $SrCl_2$.[433,434] Another target of CaMKII is WEE1B, which phosphorylates and inhibits CDK1, inactivating MPF and facilitating exit from MII.[435] Once fully active, the APC/C, which targets proteins for destruction via ubiquitylation, also degrades securin, making possible the cleavage of cohesins, which are the proteins that hold sister chromatids together, allowing anaphase onset[436,437] (Figure 4.10 and Chapter 1). Therefore, the current Ca^{2+}-CaM-CaMKII model of EMI2 inactivation and MII release that has been comprehensively characterized in *Xenopus* eggs is largely conserved in mammals, although additional studies are needed to identify the molecular players and pathways that execute the program in the latter species.

Given the cortical location of the second meiotic spindle and small constriction required for extrusion, the second polar body forms relatively rapidly after fertilization. In preparation for polar body extrusion, the meiotic chromosomes cause remodeling of the cortex above the spindle, which includes the formation of the F-actin cap[288] and differential accumulation of several proteins such as myosin IIA and B, PAR proteins, and the small GTPase RAC.[4] A RAN-GTP gradient emanating from the meiotic spindle is responsible for recruiting actin filaments and the actin nucleating machinery to the cortex and for the organization the F-actin cap,[438] which is necessary to maintain the eccentric location of the spindle.[439] The RAN-GTP gradient also controls the polarized distribution of the RHO-GTPase CDC42,[438] which regulates the function of the actin nucleating machinery[439] and whose inactivation prevents extrusion of the second polar body.[440] Following the $Ca^{2+}{}_i$ response induced by fertilization and progression to anaphase but prior to the telophase stage, the cortical spindle must rotate to attain a perpendicular position relative to the surface of the egg. The myosin-light chain kinase (MYLK), which is another target of CaM and the upstream regulator of myosin II, a protein involved in cytokinesis, controls rotation of the spindle and constriction of the cytokinesis ring, as pharmacological inhibition of either protein prevents these events and release of the second polar body. The molecular mechanism(s) that regulates MYLK during these processes remains to be demonstrated.[441]

The Pronuclear Stage

In mammalian fertilization, the haploid genome of each gamete becomes surrounded by its own nuclear ("pronuclear") envelope, in which the first mitotic "S"

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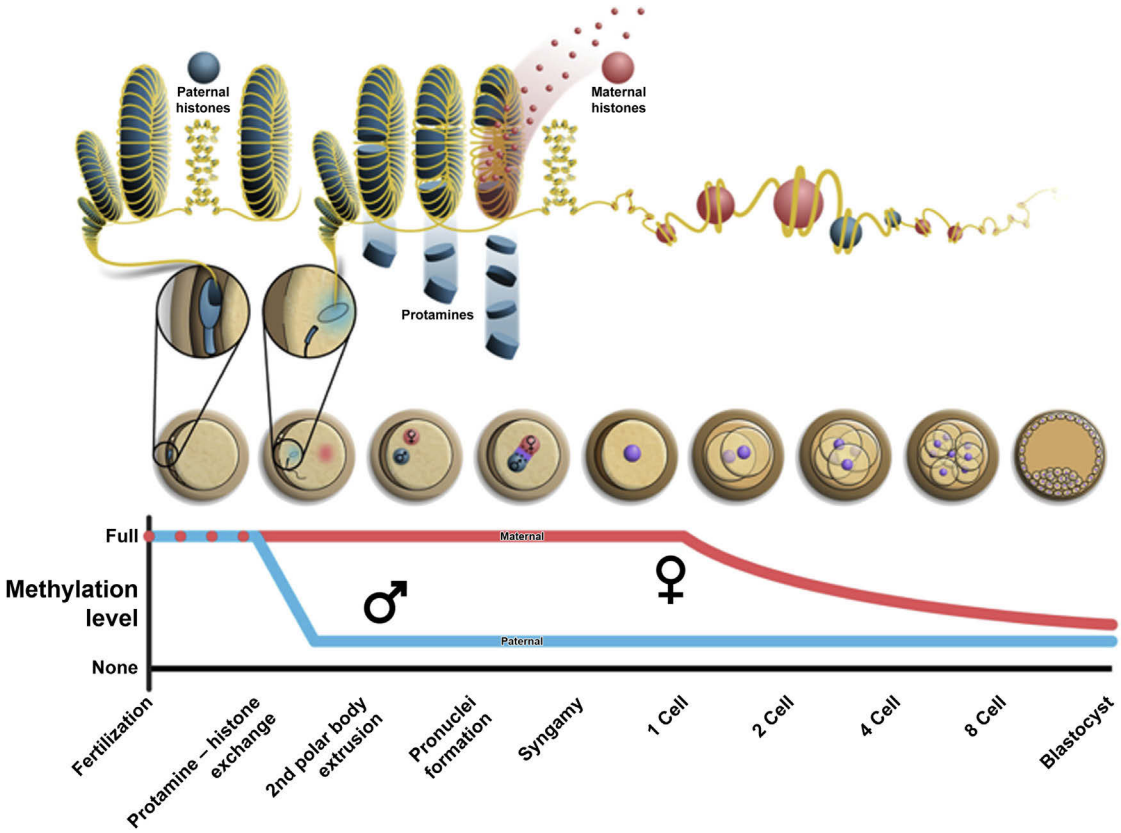

FIGURE 4.13 Reorganization of the male chromatin after fertilization and alterations to the epigenome postfertilization. This figure is reproduced in color in the color plate section. The top panel illustrates the chromatin structure of the mature sperm immediately following fertilization (highly protaminated with some retention of paternally derived histones). Soon after sperm entry, the protamine to histone transition takes place where maternally derived histones replace protamines resulting in the decondensation of the sperm head. The middle panel illustrates the various stages of early embryonic development. The bottom panel shows the methylation changes that occur over time in the maternal and paternal pronucleus, where the paternal pronucleus undergoes active demethylation and the maternal DNA is demethylated passively in a replication-dependent manner. The approximate chronology of major events in the early embryo is outlined along the bottom of the figure and correlates to the illustrations of embryos above. *Figure reprinted from Ref. 444 with permission from Frontiers. Courtesy of Dr Douglas Carrell.*

phase takes place to generate two copies of each chromosome. Thus, there are two distinct pronuclei. Examination of this pronuclear stage has been crucial for human IVF programs, because the large size of the pronucleus allows for rapid identification of fertilized eggs, and on occasion, if required, removal of abnormal polyspermic or triploid embryos (with >2 pronuclei). Following this transient pronuclear stage, the pronuclear envelope disassembles and chromosome condensation reoccurs to set up the first mitotic cleavage division. Despite assembling in a single metaphase plate, the two sets of chromosomes remain separated according to parental origin, and this organization is maintained up to the four-cell stage in mouse embryos. It is speculated that chromatin segregation may be required to accommodate the extensive and distinct reprogramming that occurs in both genomes early in embryogenesis.[442]

Pronuclear formation is dependent upon factors in the egg cytoplasm that accumulate during maturation and require protein synthesis.[443] The male chromatin is organized in a highly compact manner, toroid complex, and is associated with protamines, which are sperm-specific basic proteins that replace the majority of histones during late spermatogenesis (Figure 4.13).[444,445] Prior to becoming a male pronucleus, the sperm nucleus undergoes a multistep reprogramming, which consists of an initial phase of chromatin dispersal, followed by a brief recondensation, and finally a complete decondensation into the male pronucleus. Upon gaining access to the ooplasm, the sperm's nuclear envelope, which lacks a lamin lining and is poreless except on its posterior region, undergoes breakdown. Egg kinases, especially PKC and possibly M-phase associated kinases, are thought to regulate this process, although findings that paternal chromatin decondensation continues unimpeded in the presence of PKC inhibitors and the sperm nucleus can undergo decondensation during interphase suggest participation of additional molecular regulators.[4] The loss of nuclear envelope exposes the sperm head contents to the ooplasm, leading to limited sperm

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head decondensation, which coincides with reduction of the protamines' disulfide bonds. This relaxation of the toroid complex enables the egg's histones to associate with the sperm DNA, causing the formation of nucleosomes and inducing recondensation of the sperm nucleus. Finally, once the deposition of histones is complete, the paternal chromatin fully expands into the male pronucleus (Figure 4.13),[446] whose formation is also associated with incorporation of other proteins.[445]

The exchange of protamines and histones in the ooplasm is regulated by different mechanisms. The required reduction of disulfide bonds is primarily controlled by the egg's glutathione content, which increases during maturation, and abrogation of this increase prevents sperm chromatin decondensation.[4] The nucleoprotein exchange also involves phosphorylation events[447] as well participation of chaperone proteins. In *Xenopus*, an oocyte-specific acidic protein, nucleoplasmin (NPM), assists both with the removal of protamines and deposition of histones onto the paternal chromatin.[448] The mammalian homologues NPM1, NPM2, and NPM3 are expressed in oocytes, and recent in vitro studies showed that NPM1 and the complex NPM1-3 efficiently promoted sperm nuclear decondensation and nucleosome organization.[447] Earlier studies have shown that mouse oocytes with deletion of NPM2, which is the most abundant isoform, underwent fertilization and sperm nucleus decondensation, although females displayed fertility defects associated with chromatin organization during failed preimplantation development.[449] Thus, in mammals, NPM1 and 3 are likely additional sperm head decondensation factors. Other proteins are likely to contribute to nuclear reprogramming as well, such as the NAP/SET family of proteins, the histone chaperone HIRA[450] and the chromatin remodeling protein CHD1,[451] both of which associate with the histone H3.3 replacement variant in the paternal genome until DNA replication.[452] Given that several of these factors contribute to sperm nucleus remodeling, future studies should ascertain their relative contribution.

The formation of the female pronucleus also requires chromatin decondensation, although this process is simpler, as the DNA remains associated with maternal histones and without a pronuclear envelope. At the time of fertilization, the MII-stage chromatin is condensed from the high levels of CSF activity reigning in the ooplasm, and remains in this condensed state until the completion of meiosis and extrusion of the second polar body. Decondensation and entry into interphase requires a sustained decrease in MPF activity, which is triggered by the oscillations in $Ca^{2+}{}_i$ levels induced by fertilization.[453]

Despite the different pathways involved in chromatin remodeling, the formation of the male and female pronuclear envelopes occurs at approximately the same time. Pronuclear envelope assembly begins at the periphery of decondensing chromatin,[454] and in sea urchins forms by ATP-dependent recruitment of vesicles to chromatin and GTP-dependent processes.[455] The latter studies provide evidence that a lamin B receptor-like protein, an integral membrane protein of the interphase nuclear envelope, mediates targeting of the nuclear envelope precursor vesicles to chromatin.[456] Pronuclei appear to draw on a reserve of egg endoplasmic reticulum to generate the large surface area that each pronucleus requires, which is not surprising given the extensive endoplasmic reticulum that is present in oocytes and zygotes. Remnants of the sperm envelope persist in zygotes at the tip and on the base of the sperm head, and these structures are thought to be important for the assembly of the new male nuclear envelope. These nuclear remnants are rich in sterols, contain polyunsaturated phospholipids, and are enriched in polyphosphoinositides and other phospholipids.[457] In sea urchins, fusion of the nuclear envelope precursors seems to be promoted by vesicles containing PLCγ and polyphosphoinositides that produce diacylglycerol, promoting localized fusion and formation of the pronuclear envelope.[458] In zebrafish, fusion of individual chromatin masses, which are surrounded by nuclear envelope, into a large mononucleus relies on a newly discovered maternal-effect protein named Brambleberry.[459] In mouse zygotes, the ooplasm has strong propensity to form nuclear envelopes around individual chromosomes, resulting in the formation of micronuclei and multinucleated cells, a condition that negatively affects fertility.[460,461] This tendency is prevented by the chromokinesin Kid/kinesin10, a plus-end directed microtubule-based motor with both microtubule and DNA binding domains, which is responsible for compacting chromosomes at the anaphase stage, thereby favoring the formation of large pronuclei.[462] Pronuclear envelope assembly also occurs in the presence of decreasing MAPK activity,[463] and studies suggested that inactivation of MAPK was required for pronuclear formation.[463,464] MAPK inactivation follows the drop in MPF activity by several hours or more, depending on the species. The decrease in MAPK activity and formation of pronuclei are Ca^{2+} dependent and require a greater number of $Ca^{2+}{}_i$ rises than do the decrease in MPF activity and anaphase II onset.[453] In spite of these findings, recent studies performed on individual oocytes where inactivation of MAPK and pronuclear formation were evaluated simultaneously showed that pronuclear formation precedes inactivation of MAPK, and can even occur in the presence of high MAPK activity, suggesting that other factor(s), most likely Ser/Thr protein phosphatases, also play a role in pronuclear assembly.[465]

The pronuclei are far from merely a passive staging area for the two parental genomes prior to assembling the zygotic genome. Both sets of chromatin undergo asynchronous DNA synthesis at the pronuclear stage,

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generating the 4C amount of DNA required for the ensuing first mitosis.[466] In addition, they undergo changes in DNA methylation, with the paternal genome appearing to experience earlier and "active" demethylation (Figure 4.13 and Chapter 52) that occurs prior to and during DNA replication[467] and is mediated by oxidation of the 5-methylcytosine by the DNA dioxygenase ten-eleven translocation 3 (TET-3);[468,469] but see Ref. 470. In spite of this so-called global demethylation, specific paternal sequences such as those containing paternally imprinted genes, intracisternal A particle elements (IAPs), retrotransposons, and centromeric regions, avoid demethylation and preservation of these marks is important for embryo development.[471] The maternal genome also avoids demethylation at the zygotic stage, an effect that seems mediated by the specific binding of the maternal factor STELLA or DPPA3.[472] The maternal genome, however, experiences passive demethylation over the course of subsequent cleavages.[444,473] Other differences found in the paternal pronucleus include the presence of inherited sperm histones (Figure 4.13), which are significantly enriched at loci of developmental importance,[474,475] higher levels of acetylated histone (H)4,[476] and hypomethylation, which is specifically reflected by lower levels of methylation on H3 at lysine(K)-9 and H4K20me3.[477–479] Further, the histone variant H3.3 is predominantly associated with the paternal genome, and mutation of lysine 27 on H3.3 but not on H3.1 prevents embryo development.[467] Of note, the paternal genome retains binding of the heterochromatin protein 1β HP1β, which is also associated with the maternal genome.[479] Thus, these differences in methylation, acetylation, and histone composition may be the basis for the earlier initiation of transcriptional activity from the male than female pronucleus in the mouse.[478,480,481] The acquisition of transcriptional permissiveness and activation of the zygotic genome, which is first detected approximately at the mid S-phase in mouse zygotes[477,481] and coincides with massive degradation of maternal RNAs,[481] is made possible by multiple maternal factors,[482,483] and requires recruitment of maternal mRNAs[484] and protein synthesis.[485] Activation of the zygotic genome is vital for preimplantation development, as demonstrated by the early developmental arrest caused by the elimination of any of the genes encoding for maternal factors (for recent review, see References 483,486; also see table of maternal effect genes and products in Chapter 2). Other factors important for the regulation of zygotic/early embryonic gene expression are those that control chromatin remodeling and structure and DNA replication. Chromatin remodeling is important to ensure specific patterns of gene expression during early development, as demonstrated by the finding that mutation or elimination of remodeling or transcription factors such as BRG1, a homologue of the brahma protein of *Drosophila*, or transcriptional intermediary factor 1α, TIF1α, cause early cleavage arrest.[487,488] The role of DNA replication was elucidated in studies that showed that while inhibition of the first or second round of DNA replication did not prevent activation of the zygotic genome, it reduced expression of genes transiently expressed during the two-cell stage and reduced BrUTP incorporation (RNA production), respectively, suggesting that it is required to impose the repressive chromatin state during the few embryonic cleavages.[489,490]

While some of these molecular events are underway, the two pronuclei migrate toward each other to become apposed prior to lining up for the first mitosis. Actin microfilaments and microtubules are required for pronuclear apposition in sea urchin and mouse eggs,[352,491] although in humans and other large domestic animals, pronuclear migration and apposition relies exclusively on microtubules.[492] The molecular mechanism by which the cytoskeleton actually translocates these relatively large pronuclei, compared to other organelles, a distance of 50–100 μm from opposite directions, remains to be established. However, it is likely to involve cytoplasmic, microtubule-associated motor proteins found in eggs.[493]

The pronuclear stage ends when MPF levels rise again, resulting in nuclear envelope breakdown and chromosome condensation. While CDK1 is responsible for phosphorylation of the lamin A/C proteins that results in nuclear/pronuclear envelop breakdown, recent studies have shown that inhibition of poly(ADP-ribosylation), a process that involves transferring negatively charged poly ADP ribose polymers to proteins, causes zygote arrest at the pronuclear envelope breakdown stage, suggesting that poly(ADP-ribosylation) may contribute to the regulation of this process; the precise role of poly(ADP-ribosylation) in pronuclear envelope breakdown remains to be determined.[494] With meiosis completed, the duplicated chromosomes line up for the first mitotic cleavage. In contrast to the previous meiotic divisions, the spindle is centrally placed, resulting, after first cleavage, in two blastomeres of similar size. An F-actin cloud-like structure that surrounds the anastral spindle, and possibly the lack of MAP kinase activity at this stage, are responsible for the central location of the first mitotic spindle.[495]

Other Cytoplasmic Events

The recruitment of maternal mRNAs is another event of egg activation, and translated products are known to contribute to several zygote functions, including conferring transcriptional competence that underlies the activation of the zygotic genome.[483,484] Other proteins that accumulate in mouse zygotes are those associated with

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protein synthesis[496] and degradation,[497] especially components of the ubiquitination pathway and the proteasome, which may assist with the degradation of maternal proteins that occurs soon after fertilization.[496] The molecular mechanism(s) whereby maternal mRNAs are recruited for translation after fertilization remains unknown, although increases in $Ca^{2+}{}_i$ as well as cell cycle progression are necessary, unlike CaMKII activity that seems dispensable.[434,453] Despite recent progress in the recognition of proteins produced at this stage,[4,498] the identity of most translated zygotic proteins remain unknown, and their contribution to the activation of embryonic genome and preimplantation development needs to be ascertained.

The aforementioned degradation of mRNAs that occurs during maturation and post fertilization[499] is also vital for activation of the zygotic genome.[498] The decapping complex proteins DCP1A and 2, which are maternal products, assist in this process, and reduction of their levels reduces activation of the zygotic genome.[498] Micro RNAs are also associated with message degradation, but they are inactivated in oocytes and embryos.[500]

In many organisms, the polarity of the unfertilized egg, i.e., the orientation of the axis that runs through the animal pole, corresponding to the spindle region and the vegetal pole, the animal–vegetal (A–V) axis, provides positional cues for the organization of the body pattern of the early

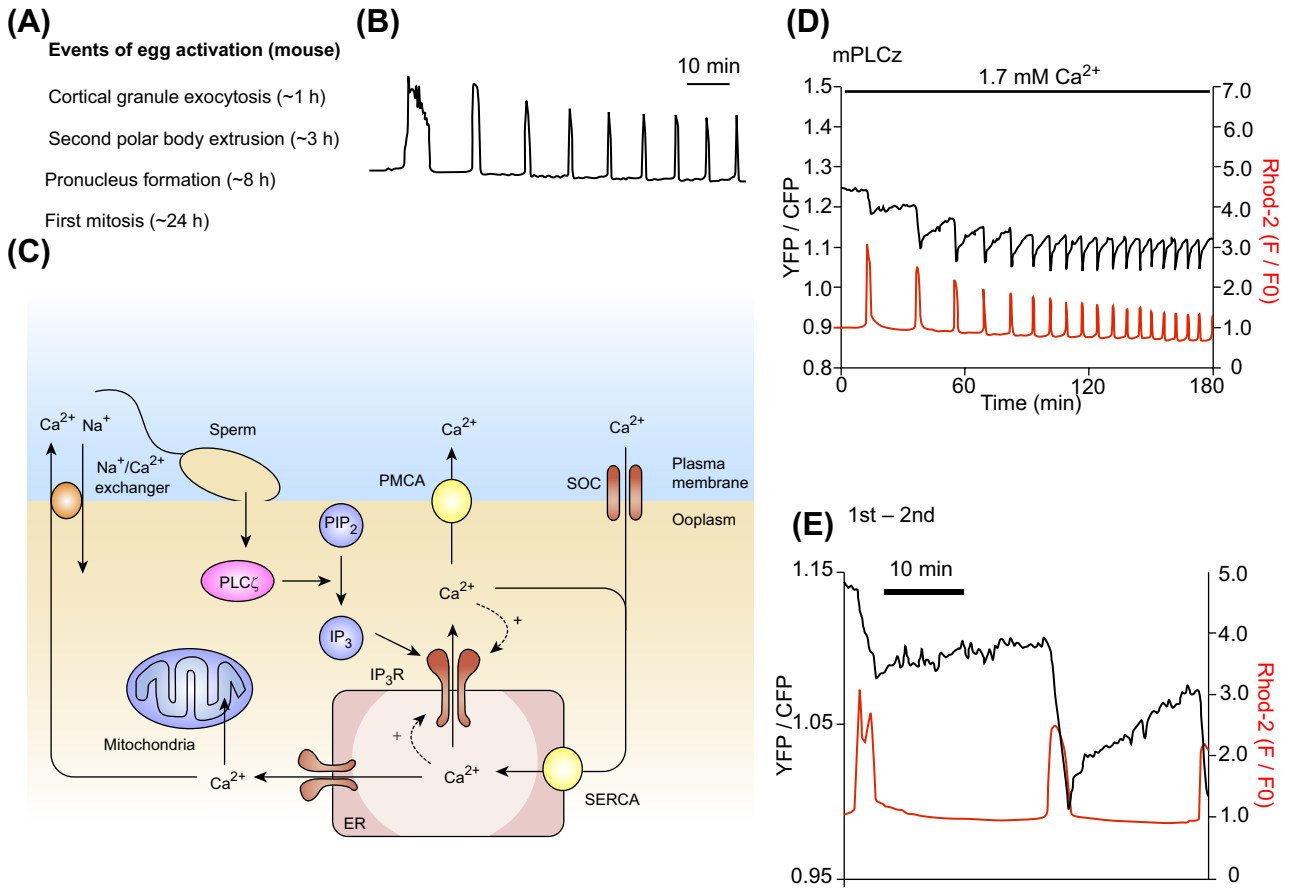

FIGURE 4.14 Temporal course of activation events in mouse eggs with a characteristic Ca^{2+} response and candidate molecules involved in the initiation oscillations and in Ca^{2+} homeostasis. This figure is reproduced in color in the color plate section. (A) Main cellular events of egg activation after sperm entry and approximate time in hours (h) required for their completion. (B) A typical pattern of $Ca^{2+}{}_i$ oscillations associated with fertilization or with injection of PLCζ cRNA. Note that recordings were terminated prematurely. (C) Upon fusion, the sperm is thought to deliver phospholipase C (PLC) ζ, which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol 1,4,5-trisphospahte (IP_3) and diacylglycerol (DAG). IP_3 binds its receptor, IP_3R1, causing Ca^{2+} release out of the endoplasmic reticulum (ER). Following Ca^{2+} release, basal $Ca^{2+}{}_i$ levels are regulated by the combined action of the sarcoendoplasmic reticulum Ca^{2+} ATPase (SERCA), plasma membrane Ca^{2+} pump (PMCA), Na/Ca^{2+} exchanger, and mitochondria. Store operated Ca^{2+} channels (SOC) or other Ca^{2+} channels are proposed to mediate Ca^{2+} influx, which is required to fill the ER and maintain oscillations. Broken lines suggest feedback action of Ca^{2+} on IP_3R1. *(Adapted from Ref. 507 with permission of CSHLP.)* (D) $Ca^{2+}{}_{ER}$ and $Ca^{2+}{}_i$ undergo simultaneous but opposite changes in concentration during oscillations in mouse MII eggs. $Ca^{2+}{}_i$ responses were induced by injection of 0.05 μg/μl mouse PLCζ cRNA into MII eggs. In vitro transcribed D1ER cRNA was injected into eggs 5h before the initiation of $Ca^{2+}{}_i$ measurements. The emission ratio of D1ER (YFP/CFP) was used to estimate relative changes in $[Ca^{2+}]_{ER}$ (left axis, black trace). $[Ca^{2+}]_i$ (right axis, red trace) was recorded using Rhod-2. (E) The first and second $Ca^{2+}{}_i$ rises do not elicit significant refilling of the ER, but subsequent rises, especially after ~ the sixth rise do (D). *Adapted from Ref. 508 with permission from The Company of Biologists.*

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embryo.[501] Other positional determinants have been suggested in mammals such as the point of sperm entry[502] or the plane of first cleavage, which is defined by the second polar body and the sperm point of entry.[503] Nevertheless, equally significant literature contradicts these findings.[504,505] Therefore, whereas bias in the pattern and lineage contribution in early cleavage stage mammalian embryos is observed after the two-cell stage,[506] the emerging notion is that mammalian embryos are developmentally flexible and that patterning driven by differential intrinsic concentration of molecules is not and does not need to be established by the egg or zygote.[504,506]

SIGNALING PATHWAYS RESPONSIBLE FOR THE EVENTS OF EGG ACTIVATION

Ca^{2+} is the Predominant Messenger of Activation

Over the last decades, steady progress has been made in elucidating the underlying pathways of egg activation, although mechanistic details remain to be worked out in all animal and plant systems. A universal step in the signaling cascade leading to egg activation is the increase in the intra-oocyte/egg levels of free Ca^{2+}_i, which initiates a multitude of cellular responses, including the previously described events of egg activation that render eggs into zygotes and meiotic cells into mitotic blastomeres (Figure 4.10; Figure 4.14 (A) and (B)).[509] Work by Loeb and colleagues at the end of the nineteenth century associated egg activation with changes in the intracellular concentration of several ions, as egg activation and initiation of development in sea urchin eggs was accomplished by varying the concentration and composition of the fertilizing medium.[510] Nevertheless, a few decades later concrete evidence started to emerge regarding the role of Ca^{2+} in fertilization. Ca^{2+} signaling is important for many cellular processes,[511,512] and many of the parameters that make it such a widespread signaling mechanism have been worked out in somatic cells. Significant for its function is the steep gradient between its intracellular and extracellular concentrations, 10,000 to 100,000-fold greater concentrations in the extracellular milieu, and the energy-dependent mechanisms that cells use to restore Ca^{2+}_i levels to ~100 nM, which is the basal level in most cells.[511] In eggs, the activating Ca^{2+} signal is generally associated with sperm–egg interaction/fusion, which occurs at different stages of meiosis depending on the species,[513] although in insects, where fertilization is dissociated from activation and where embryos can develop parthenogenetically, the presumed Ca^{2+}_i increase is thought to be induced by mechanical stimulation during ovulation/oviductal transport.[514,515] Therefore, regardless of the mode in which Ca^{2+}_i is increased in oocytes and eggs, Ca^{2+} signaling is thought to drive the majority of the events of egg activation with the possible exception of the initial decondensation of sperm chromatin. For an updated and comprehensive review of the signaling pathways associated with Ca^{2+} release during fertilization in a number of model species, please refer to a special issue of *Cell Calcium* "Ca^{2+} signaling and ionic homeostasis in gamete maturation and function".[516]

The role of Ca^{2+} ions as the pivotal change responsible for driving all events of egg activation was established by studies where the calcium ionophore A23187, a reagent capable of mobilizing Ca^{2+}, was used to activate sea urchin eggs as well as eggs from other invertebrates and vertebrates species.[517] These studies also showed that most of the Ca^{2+} required for egg activation was from intracellular stores, as activation was unaffected by the absence of extracellular Ca^{2+}.[518] Follow-up studies focused on determining the spatial and temporal characteristics of the activating Ca^{2+} signal. Using the then recently synthesized luminescent protein aequorin, explosive Ca^{2+}_i rises were detected following fertilization or addition of Ca^{2+} ionophore in medaka fish eggs.[519] Subsequent studies showed that the Ca^{2+}_i rise propagated as a wave starting from the point of the sperm entry,[520] an observation that was later extended to several other species, including mammals.[513,521]

Unlike the single Ca^{2+}_i increases detected in fertilized sea urchin and medaka eggs, measurements of Ca^{2+}_i changes in mammals revealed that their eggs displayed repeated Ca^{2+}_i responses, also known as oscillations.[522] Ca^{2+}_i oscillations were subsequently reported in non-mammalian species, although the duration in these species rarely exceeded 30 min, whereas in mammals they last for many hours.[513] These oscillations are required for egg activation, as their abrogation, which can be accomplished by a Ca^{2+} chelator such as BAPTA, prevents egg activation and thwarts the initiation of development in mouse zygotes.[523] In mammals, the first Ca^{2+}_i rise is of greater magnitude and duration than subsequent ones, which are mostly uniform, last approximately 1 min and occur at intervals between 10 and 40 min according to the species[507,521,524] (Figure 4.14(A) and (B)). Information is apparently encoded in the number of Ca^{2+}_i rises, as different egg activation events require different numbers of oscillations for their initiation, and, for most events, more rises are required for their completion than for their initiation.[453] Changing the Ca^{2+}_i oscillation parameters also alters the extent of parthenogenetic mammalian development.[359,525] These periodic Ca^{2+}_i increases generate nearly synchronous oscillations of activity of the kinase CaMKII,[526] which regulates the resumption of meiosis, as previously discussed (Figure 4.12, left panel). It is worth noting that the Ca^{2+}_i rises induced by fertilization cause coordinated cytoplasmic contractions that can be detected by advanced imaging technology,[521] providing a noninvasive method to assess in real time the presence and frequency of oscillations; analysis of

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these cytoplasmic contractions has been successfully used to predict the developmental competence of mouse zygotes.[527] Also associated with sperm-initiated Ca^{2+}_i oscillations, and especially with the first few rises, is extrusion of intracellular zinc into the extracellular milieu, which lowers the levels of zinc in the ooplasm. This extrusion can be visualized with fluorescent dyes and is generically termed as zinc sparks. Zinc sparks, which also occur around the time of cortical granule exocytosis (Figure 4.10), are thought to facilitate the resumption and completion of meiosis following fertilization.[528]

The universal and indispensable role of Ca^{2+} release for egg activation[513] as well as the diverse temporospatial configuration in which the responses are deployed, provoked interest in understanding the underlying mechanisms responsible for their initiation and regulation. Several hypotheses have been put forth, including sperm–egg contact or receptor-mediated activation and various fusion models, invoking the addition of sperm membrane ion channels, or the passage of Ca^{2+} from the sperm into the egg, or the release of a sperm-signaling protein (for reviews see References 4, 529, 530). Although initially it was thought that a single mechanism could underlie initiation of Ca^{2+} signaling in all species, it became apparent that this was not the case, and the more accepted view is that signaling mechanisms have been adapted according to the species' Ca^{2+}_i profile of egg activation. Therefore, in species that display single Ca^{2+}_i rises, i.e., *Xenopus* and sea urchin, signals emanating at the site of interaction between sperm and egg initiate the signaling cascade,[530,531] whereas in species that rely on protracted Ca^{2+}_i signaling such as mammals, the initiation depends on the release of a sperm-signaling molecule, most commonly known as the "sperm factor" into the ooplasm.[532] For more details and supporting data on these hypotheses, see specific reviews on the subject.[533–535]

IP_3, IP_3R, and Other Components of the Ca^{2+} Tool Kit

1,4,5-Inositol trisphosphate (IP_3)-mediated Ca^{2+} release from the endoplasmic reticulum (ER), the main cellular Ca^{2+} reservoir, is associated with Ca^{2+} release and cellular events in myriad cell types (reviewed in References 512, 536) (Figure 4.14(C)). It is also primarily responsible for the initial Ca^{2+}_i wave and oscillations during fertilization in most species studied to date.[522,537] Its cognate receptor, IP_3R, is the main intracellular Ca^{2+} release channel in nonmuscle cells and is appropriately located in the ER. The ER and IP_3Rs undergo reorganization during oocyte maturation,[538,539] and by the MII stage, IP_3Rs are organized in cortical clusters, near the entry point of the sperm, which is the site from where the first Ca^{2+}_i wave is initiated. Abnormal ER reorganization at the end of maturation affects Ca^{2+}_{ER} content and the ability to initiate persistent oscillations, as recently demonstrated in oocytes deficient in the maternal effect protein Mater.[540]

The suggestion that IP_3 may play a role during fertilization arose from studies in sea urchin and amphibian eggs where an increase in phosphoinositide metabolism was associated with fertilization,[541,542] an observation that was soon followed by the demonstration that injection of IP_3 triggered Ca^{2+}_i release.[541] IP_3 production requires phosphatidylinositol-4,5-bisphosphate (PIP2) hydrolysis by a PLC,[544] and injection of antibodies against PIP2 in *Xenopus* eggs inhibited the sperm-initiated Ca^{2+}_i wave, suggesting that this sequence of events is conserved in fertilization.[545] Measuring IP_3 changes during mammalian fertilization has proven difficult due to the small size of eggs and asynchrony of fertilization, although an increase in intrazygote concentrations of IP_3 has been observed during Ca^{2+}_i oscillations using fluorescence resonance energy transfer (FRET) technology.[546] In addition, injection of IP_3 and guanine nucleotides into mammalian eggs initiated Ca^{2+} release from intracellular stores,[547] albeit of short duration, but injection of a nonhydrolyzable analogue of IP_3 such as adenophostin A[548] or repeated release of caged IP_3 generated long-lasting Ca^{2+}_i responses and triggered all events of egg activation.[549] Another channel thought to participate in Ca^{2+} release during fertilization is the ryanodine receptor (RyR), the main Ca^{2+} channel of muscle cells, which is also expressed in oocytes and eggs of vertebrate and nonvertebrate species.[550,553] Despite studies reporting Ca^{2+}_i responses induced by RyR agonists in mammalian eggs, their role remains largely undefined and is likely minor, as inhibitors of these channels have no effect on egg activation.[550,554]

The overwhelming evidence for the phosphoinositide pathway in mammalian fertilization led to the search for expression of IP_3Rs in oocytes and eggs. The IP_3R is a large protein with distinct domains, which functions as a tetramer.[555] There are three IP_3R isoforms,[512] and mammalian oocytes and eggs and their surrounding cells express all isoforms,[556,557] although the type I IP_3R isoform is overwhelmingly the most commonly expressed version.[556,558,559] Besides IP_3, gating of IP_3R1 requires Ca^{2+}, although at high concentrations Ca^{2+} inhibits the channel's conductivity,[560,561] and this biphasic regulation of IP_3R1 makes it especially suited to mediate oscillations. The function of IP_3R1 is enhanced during oocyte maturation by its increase in concentration, its own phosphorylation, redistribution to the cortex, and increased levels of Ca^{2+} in ER, all of which optimize Ca^{2+} release at the time of fertilization.[507,562] As oscillations persist after sperm entry, IP_3R1 undergoes protracted degradation, which is solely induced by the production of IP_3, whose persistent production is required to underlie the

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oscillations.[559] Lastly, the essential role of IP_3R1 in mammalian fertilization was also demonstrated by injection of a functional blocking antibody, which blocked both initiation of Ca^{2+}_i oscillations[537] and egg activation.[563] Subsequent studies confirmed the role of IP_3R1 in fertilization in other species.[564–568]

For long-lasting oscillations to unfold, besides IP_3 production, Ca^{2+}_i levels must be returned to baseline after each Ca^{2+}_i rise and stores refilled (Figure 4.14(D)) as oscillations cease prematurely without external Ca^{2+}.[569,570] Cells use a variety of mechanisms to maintain basal Ca^{2+}_i levels, including sarcoendoplasmic reticulum Ca^{2+} ATPases (SERCAs), which uptake Ca^{2+} into the ER, plasma membrane (PM) Ca^{2+} ATPases (PMCAs), and Na^+/Ca^{2+} exchangers, which extrude/exchange Ca^{2+} out of the cell (Figure 4.14(C)).[512,571] These mechanisms are functional in mammalian eggs,[507] as revealed by pharmacological inhibition of SERCAs or PMCAs, which profoundly alter and/or terminate oscillations,[507,572,573] by the recent demonstration of SERCA1B protein expression in these cells,[507] and by the transient effects on Ca^{2+}_i responses caused by culturing eggs in Na^+-free conditions.[574] Because only a fraction of Ca^{2+} from each Ca^{2+}_i rise is returned to the ER during oscillations in mammalian eggs,[508] internal stores must be refilled to maintain oscillations. Somatic cells as well as oocytes and eggs use a variety of plasma membrane channels to influx Ca^{2+},[507,573] but which of these channels operates in mammalian eggs remains unknown. Unlike fertilization in sea urchins,[575] the changes in membrane potential during mammalian fertilization are negligible, which likely limits the role of voltage-gated channels during this process. More recently, mammalian oocytes and eggs have been shown to express components of the store-operated Ca^{2+} entry (SOCE) mechanism,[576] stromal interaction molecule 1 (STIM1)[577,578] and ORAI1.[579,580] SOCE is based on the notion that stores refill following their depletion, and this function relies on a Ca^{2+} sensor in the ER, STIM1, which when Ca^{2+} in the stores falls below a certain threshold, it engages ORAI1 in the plasma membrane, which mediates Ca^{2+} influx. Functional studies show that depletion of the Ca^{2+} stores occurs during sperm-initiated oscillations (Figure 4.14(D)) or after inhibition of SERCA by pharmacological methods, both of which induce Ca^{2+} influx.[508,581] Nevertheless, the role of SOCE in mammalian fertilization needs to be further investigated, as while its inactivation precluded oscillations in porcine oocytes,[582] it was without effect in mouse eggs.[573,583] Other channels that might contribute to Ca^{2+} influx in mammalian eggs are the transient receptor potential (TRP) ion channels, which are widely expressed, nonselective cation channels[584] for which transcripts have been found in mammalian oocytes.[585,586] Their contribution to Ca^{2+} homeostasis in mammalian oocytes and eggs, however, remains to be demonstrated, although a member of the vanilloid family of TRP channels, TRPV3, is expressed in mouse oocytes during the late stages of maturation.[587] Genetic deletion of TRPV3 did not modify sperm-initiated oscillations but abolished $SrCl_2$ influx and egg activation associated with exposure to this commonly used parthenogenetic agent;[587] a more formal role in sperm-induced oscillations cannot be discounted for other members of the TRP-family of ion channels. Elucidating the channels that underlie influx in oocytes and eggs will have broad implications, as this knowledge might be applied to parthenogenetic activation, enhancing developmental competence and contraception.

Egg Phospholipase C and SRC Family Kinases

The involvement of IP_3, IP_3R, and diacylglycerol (DAG) in the events of egg activation implied the participation of PLCs. Besides being involved in the production of IP_3 and DAG, PLCs are a large family of enzymes that are capable of responding to a variety of stimuli, as they can be activated by interacting with plasma membrane receptors, either guanine nucleotide-binding protein (G-protein)-coupled-receptors or tyrosine kinase receptors, or by other means.[544,588] Transcripts for nearly all members of each of the PLC families have been detected in mammalian eggs,[589] and protein expression has been demonstrated for a few isoforms.[533,590,591] Evidence for their functional presence in eggs was initially gathered from pharmacological studies, as injection of GTP-γS and activation of trimeric G proteins resulted in Ca^{2+}_i transients, whereas GDP-βS, a nonhydrolyzable GDP analogue that inhibits G-protein function, prevented sperm-induced Ca^{2+}_i oscillations.[547,592] Expression of exogenous m1 muscarinic receptor followed by addition of its ligand acetylcholine[593] led to oscillations and events of egg activation, confirming the presence of target PLC(s) and the G-protein-signaling machinery in eggs. Nevertheless, other studies do not support a role for G-protein-coupled receptors, including those using ADP-ribosyltransferase exotoxins such as cholera toxin, an activator of Gs function, and pertussis toxin, an antagonist of Gi, both of which failed to prevent egg activation.[547,593,594] The role of the Gq-protein family members, Gq, G11, G14, and G15,[595] which are insensitive to the mentioned exotoxins but are capable of activating PLCβ isoforms,[596,597] was also examined. Despite expression of Gq and G11 in mouse eggs, their inhibition failed to modify fertilization-induced Ca^{2+}_i oscillations.[598] Intriguingly, deletion of PLC-β1 and PLC-β3 genes[533,599] resulted in female infertility, although follow-up studies including oocyte-specific downregulation of PLC-β1 did not eliminate Ca^{2+}_i

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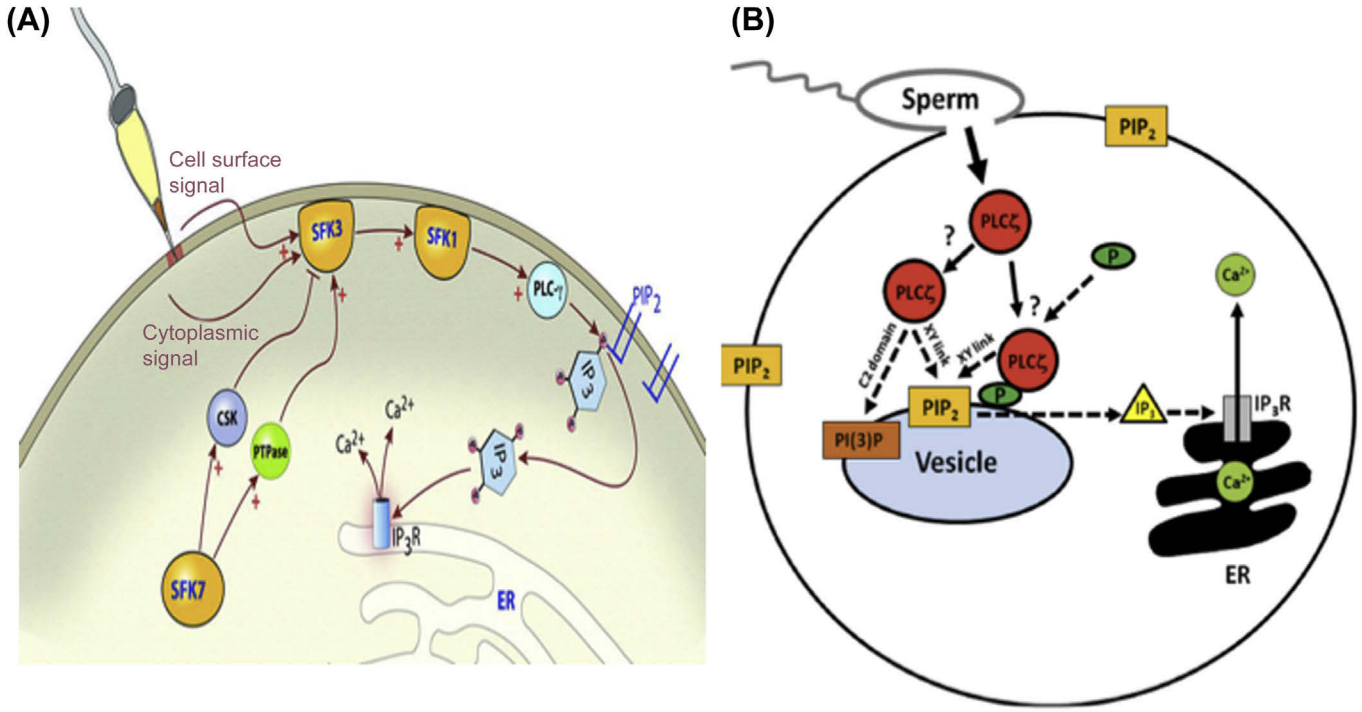

FIGURE 4.15 Model for initiation of Ca2+ signaling at fertilization in echinoderm and mammalian eggs. (A) In echinoderms, the initial signal leads to activation of a Src family kinase 3, SFK3, which binds and activates SFK1 that in turn activates PLCγ. This is followed by production of IP_3 and $Ca^{2+}{}_i$ release, which activates multiple aspects of oocyte function. New data suggest that this pathway could be regulated in several ways. SFK7 could affect calcium release either via phosphorylation of CSK, a negative regulator of SFK3, or via a hypothesized phosphatase that would activate SFK3. *(Adapted from Ref. 604 with permission of John Wiley & Sons. Courtesy of Dr William Kinsey.)* (B) Hypothetical mechanism of PLCζ domain regulation of function based on studies on sperm PLCζ. After sperm–egg fusion, PLCζ diffuses from the sperm head into the egg cytosol and targets a distinct intracellular vesicular PIP_2-containing membrane. The EF hand domains of PLCζ confer its high Ca^{2+} sensitivity, enabling the enzyme to be active at resting $Ca^{2+}{}_i$ levels (nM) in the egg. Association of PLCζ with its specific membrane target may be mediated by interaction of the C2 domain with either PI(3)P and/or an unidentified egg membrane-targeting protein (P). The positively charged amino acids in the XY-linker region (XY link) further assist the anchoring of PLCζ to the membrane, thus enhancing the local PIP_2 concentration adjacent to the catalytic domain via electrostatic interactions with the negatively charged PIP_2. Once PLCζ is associated with the membrane PIP_2 the catalytic X/Y barrel binds and hydrolyzes its substrate to produce IP_3 that in turn induces Ca^{2+} release from the ER. *(Adapted from Ref. 529 with permission of John Wiley & Sons. Courtesy of Dr Karl Swann.)*

oscillations,[589] which is consistent with results demonstrating that the infertility of PLCβ1 knockout female mice is mostly associated with implantation defects.[600] PLCβ isoforms can also be activated by monomeric small GTP-binding proteins such as RHO GTPases, which although present in mouse eggs, seem to participate in sperm incorporation rather than in the generation of oscillations.[354]

PLCγs are downstream of tyrosine kinase receptors and are expressed in mouse eggs.[590,601] A burst of protein tyrosine kinase activity accompanies fertilization in sea urchin and *Xenopus* eggs, and PLCγ is among the phosphorylated targets in these eggs[602,603] (Figure 4.15(A)). Consistent with these results, inhibition of PLCγ activity by expression of own SH2 domains delayed or prevented fertilization-induced Ca^{2+} release in sea urchin and ascidian eggs,[604] but not in *Xenopus* or mouse eggs.[566,601] Nevertheless, the role of PLCγ is indispensable for Ca^{2+} release and fertilization in *Xenopus*,[601] suggesting that in this species the enzyme might have a different mechanism of activation, which is consistent with the recently reported role of phosphatidic acid during *Xenopus* fertilization.[605] Conversely, inhibition of PLCγ in mouse eggs either by pharmacological[606] or dominant negative constructs against nonreceptor SRC family kinases (SFKs), the upstream activator of PLCγs, did not prevent oscillations,[607] suggesting that this pathway does not contribute to the initiation of the sperm-induced $Ca^{2+}{}_i$ responses in the mouse and possibly all mammals. It is noteworthy that mouse oocytes and eggs abundantly express several protein tyrosine kinases such as the SFKs FYN and YES, FAK kinases as well as FER,[604,607] and injection of recombinant c-FYN-induced mouse egg activation, although evidence that it does by causing Ca^{2+} release has not been shown.[608,609] Therefore, the expression and presence of PLCγs, SFKs, and FAK suggests important roles for these pathways in oocyte maturation and fertilization in mammals, although the effects in these species seem more a consequence of their role in actin and cortical

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organization than in the regulation of Ca^{2+} release and oscillations.[604,610]

Sperm Phospholipase C

The inability of egg PLCs to replicate sperm-initiated oscillations,[592,611] the evidence that sperm–oolemma interactions are brief,[6] and the demonstration that oscillations start seconds after fusion of the gametes[612,613] raised the prospect that an alternative mechanism(s) might underlie oscillations in mammalian eggs. Prompted by the studies of Dale and colleagues, one of the mechanisms examined was that a sperm-derived product, the sperm factor (SF), might cause oscillations. Toward this end, observations first in sea urchins and then in ascidian eggs reported that injection of sperm extracts caused plasma membrane currents similar to those observed in fertilization,[614,615] although more recent studies cast doubts on the role for a sperm factor in sea urchin fertilization.[616] More convincingly, injections of mammalian sperm extracts into mouse, rabbit, and hamster eggs induced oscillations highly similar to those of fertilization as well as caused egg activation.[617,618] Subsequent studies extended these results by demonstrating that sperm extracts initiated oscillations in eggs of other mammalian and nonmammalian species.[532,619,620] Additional evidence for a sperm-based factor came from clinical studies, as ICSI, which bypassed any interaction of the gametes' membranes, produced offspring in humans.[621] Later, it was shown that fertilization by ICSI initiated fertilization-like oscillations in eggs of numerous mammalian species.[622–624] This sperm activity is associated with demembranated sperm heads, as it was present after light sonication and it was lost after residence in the ooplasm.[625,626] Function-blocking antibodies to IP_3R1 inhibited sperm extract-induced oscillations, indicating that it acts upstream of IP_3R1.[627] These studies consolidated the concept that a sperm component participates in the initiation of oscillations, although the active principle remained unknown.

Several small molecules and proteins were proposed as the active candidate(s) in the sperm extracts,[533,628,629] although earlier studies have shown that the Ca^{2+}-inducing activity was dependent upon a protein component(s).[617,618] Injection of the candidate factors caused egg activation in some studies, but without exception they failed to initiate sperm-like $Ca^{2+}{}_i$ oscillations, a result that undermined their proposed role.[533,629] This was in contrast to the significant PLC activity displayed by mammalian sperm extracts in in vitro assays.[630,631] Importantly, this activity was highly sensitive to Ca^{2+},[632] which implied that the putative PLC(s) should be active at the reigning basal $Ca^{2+}{}_i$ levels of approximately 100 nM Ca^{2+}; this is in contrast to most other PLCs that require $Ca^{2+}{}_i$ levels of approximately 1 mM,[544,633] which are not compatible with MII arrest. Not surprisingly, injection of recombinant proteins representing most of the known isoforms expressed in sperm[631,634,635] failed to initiate oscillations in mouse eggs[635] or did so at nonphysiological concentrations,[636] which suggested that the PLC(s) in sperm extracts must contain especial properties.

A novel sperm-specific PLCζ[637] was identified in a homology screen of mouse testis – expressed sequence tags. Results from this and other subsequent studies point at this molecule as the sperm's Ca^{2+} active component (Figure 4.15(B)), as PLCζ is present almost exclusively in active fractions of sperm extracts,[637,638] its removal by specific antibodies abrogates their activity,[637,639] injection of PLCζ cRNAs induced sperm-like oscillations in mouse, rat, human, bovine, porcine, and equine eggs;[535] injection of recombinant PLCζs in mouse or human eggs induced similar responses.[529,640–642] Further, in vitro PLC assays with recombinant PLCζ confirmed the enzyme's high sensitivity to Ca^{2+},[641] and its accumulation in the postacrosomal area of mouse sperm[640] and in the equatorial area of bull and human sperm,[643,644] which at the time of gamete fusion are the regions to first come in contact with the ooplasm, respectively,[645] reinforced the hypothesis of PLCζ as the initiator of oscillations. In this context, lower PLCζ expression should compromise fertility. Although this association has not yet been examined using knockout models due to abrogation of spermatogenesis caused by deletion of PLCζ, a knockdown approach using transgenic RNAi produced males whose sperm displayed reduced $Ca^{2+}{}_i$ oscillatory activity and produced smaller litters.[646] More significantly, the sperm of human patients that repeatedly fail to fertilize after ICSI, besides being incapable of initiating $Ca^{2+}{}_i$ responses when injected into mouse eggs, also display severely reduced or absent expression of PLCζ,[643] and point mutations in the catalytic regions of the molecule that inactivate the enzyme have been found in other infertile males.[647] Patients with globozoospermia, an affliction characterized by round-headed, acrosomeless sperm that repeatedly fail ICSI and have low PLCζ expression, have their infertility overcome by artificial activation.[648,649] These results suggest that the impediment to fertility in these patients is most likely their inability to induce egg activation because of abnormal expression of PLCζ. Of note, recent studies have shown that these patients and a mouse model of the disease have deletions or lack expression of the Dpy19l2 gene, which encodes for a novel inner nuclear membrane protein whose presence is required for anchoring the acrosome to the nucleus and for the organization of the perinuclear theca, the site of storage of PLCζ in the sperm.[650,651]

The structure of PLCζ is similar to that of other PLCs in that it has a domain-based structure with two pairs of EF-hand domains, X and Y catalytic regions, which are the active sites of the enzyme, and a C2 domain.

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Prominently, it lacks a pleckstrin homology domain, which makes it soluble, and other regulatory domains present in other PLC family members, and therefore its regulation is presently not well known.[529,637] Nevertheless, PLCζ is more than 20 times more effective at inducing oscillations in eggs than PLCδ, its closest relative,[641] and this is possibly due to its high Ca^{2+} sensitivity, which is conferred by the first pair of EF-hand domain, as its deletion greatly decreases PLCζ EC50 for Ca^{2+}.[633,652] Another unique feature of PLCζ is its longer and more basic linker domain,[637] which is the region joining the X and Y domains. It is thought that this domain together with the C2 domain contribute to the localization of PLCζ in the egg, which is uniformly distributed in the ooplasm.[653,654] These domains via electrostatic and/or low affinity interaction with membrane phospholipids are thought to target PLCζ to internal sites and/or vesicles whose membranes are enriched in PiP2, the enzyme's substrate (Figure 4.15(B)). Therefore, several features of PLCζ makes it uniquely qualified to be the trigger of oscillations in mammalian eggs and possibly in other vertebrate species.[655] Nevertheless, future studies should establish the mechanism(s) that limit the function of this enzyme in sperm, and the mechanism(s) regulating its function and localization in the egg, as answers to these questions may have applications in both overcoming infertility and developing novel contraception methods in humans and animals alike.

CONCLUSION

This chapter highlights progress in our understanding of the complex process of mammalian fertilization. We have focused on the cellular and molecular events associated with gamete interaction, sperm–egg fusion, and the activation of development. Since the third edition of this series in 2006, some of these topics are better understood. The understanding that CATSPER is a ligand-gated channel and a target for oviduct factors, including progesterone, clarifies a number of issues and points to a potential mechanism for the control of sperm motility during capacitation. The discovery of ovastacin opens new avenues for the examination of the ZP block to polyspermy.

In contrast, a number of unresolved questions remain and are targets for future studies. A partial list of these unanswered questions is as follows. We do not understand how the oviduct capacitates, and possibly selects, sperm. In particular, the environment within the oviduct is not understood in detail, despite the essential role of that environment in supporting capacitation, fertilization, and the first days of embryonic development. The early events of sperm–egg interaction, from entry into the cumulus matrix to association with the ZP, have not been resolved completely. It is understood that sperm IZUMO and egg CD9 are essential for gamete fusion, but the specific roles of these proteins and their interacting partners have not yet been revealed. Ovastacin permits a detailed study of the molecular and biophysical events of the ZP block to polyspermy, but events of the plasma membrane-level block remain elusive. We understand that in mammalian eggs, as in the eggs of other taxa, Ca^{2+} responses are required for the activation of development, and key regulators such as PLCζ and IP3R1 have been identified, but we have an incomplete vision of how those Ca^{2+} oscillations are sculpted and interpreted. Important proteins, such as EMI2, have been identified, but questions remain about the regulation of its stability and of how it, and other components, that control the imposition and release of MII arrest. Finally, the events of sperm nuclear decondensation and the epigenetic remodeling of chromatin are key unresolved issues. We anticipate that these and other aspects of fertilization may be understood in greater detail when the fifth edition of this series appears.

ADDENDUM

Candidate Egg Proteins for Binding and Fusion

The sperm's IZUMO1 binding partner(s) in the egg plasma membrane and its mechanism(s) of action during mammalian fertilization remain unknown. A recent manuscript by Bianchi et al.[656] shows that the folate receptor 4 (FOLR4) is the required egg receptor for IZUMO1. FOLR4 does not bind folate and given its expression in eggs and role in fertility, it is proposed to be renamed JUNO, after the Roman goddess of fertility and marriage. JUNO's cellular distribution and participation in fertilization suggests that it is the long sought-after binding partner for IZUMO1. (1) JUNO is a GPI-anchored protein expressed on the surface of mouse eggs, which is consistent with the requirement for these types of proteins for gamete fusion during mammalian fertilization. (2) Anti-JUNO antibodies prevented fertilization in in vitro trials. (3) JUNO -/- females but not males are sterile. (4) JUNO is rapidly shed from the plasma membrane after fertilization, a finding that suggests participation on the membrane block to polyspermy. The molecular underpinning(s) of the membrane block are unknown, and the role of JUNO provides the first evidence towards its understanding. This is also consistent with the evidence that ICSI, which does not induce the membrane block, does not induce shedding of JUNO. (5) In vitro binding studies showed that among members of the Folr family only JUNO, which contains a single globular domain, binds IZUMO1 and not other IZUMO isoforms. (7) JUNO-IZUMO1 interaction,

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which is conserved among mammals, is proposed to mediate the initial adhesion step between gametes but not their fusion.

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CHAPTER

5

Gamete and Zygote Transport

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INTRODUCTION

Overview

How do mammalian sperm and oocytes reach each other? It might seem a simple matter of chance, because millions of sperm are usually deposited in the female by the male. Nevertheless, there is strong evidence for the existence of mechanisms that regulate the passage of sperm and oocytes through the female reproductive tract. These mechanisms not only promote successful fertilization but also tend to select morphologically normal and vigorously motile sperm for passage to the oocyte.

In mammals, oocytes are usually fertilized within hours of ovulation.[1] Sperm, however, may be stored within the female for days or even months before the arrival of the oocyte in the oviduct. In mice, fertilization takes place within 7 h of mating,[2,3] but in some bats, sperm are stored throughout winter hibernation.[4,5] Because sperm are terminally differentiated cells deprived of an active nucleus and a synthetic apparatus, they must survive the wait without benefit of transcription and translation.

Sperm are subjected to physical stresses during ejaculation and some phases of transport through the female tract, and they may sustain oxidative damage. Furthermore, because sperm are allogenic to the female, they must endure or avoid the defenses of the female immune system.[6] Thus, in order to succeed, sperm must somehow overcome these challenges. On the other hand, it is also advantageous to the female to promote the successful migration of sperm all the way to the site of fertilization, yet without promoting the invasion of disease organisms.

The major consideration for oocyte transport is to get the ovulating oocyte directly and quickly from the surface of the ovary into the ampulla of the oviduct. Speed is crucial, because oocytes of at least some mammalian species are known to age within hours of ovulation in vivo.[1]

Historical Aspects

It has been challenging to learn about gamete transport, because of the tremendous amount of variation among species with regard to gross and microscopic anatomy of the female reproductive tract, site of insemination, timing of reproductive cycles, mechanisms of ovulation induction, numbers of oocytes ovulated per cycle, level of intensity of sperm competition, and more. Information obtained from one species cannot be reliably translated to another species.

In the first half of the last century, attempts to understand the processes involved in transport of sperm and oocytes relied primarily on three approaches: (1) preparing histological sections of the female reproductive tract at various times after insemination to examine distribution of gametes; (2) removing the tract, dividing it into segments, and flushing each segment to assess distribution of gametes in the tract and the motility patterns of sperm in each segment; and (3) surgically ligating the tract at various points along its length and at various times after insemination in order to ascertain when sperm reach regions above the point of ligation in sufficient quantities to enable fertilization to occur. Although much information was obtained using these approaches, handling the tract can often cause redistribution of gametes, particularly by stimulating contraction of smooth muscles in the walls of the tract; therefore, results obtained were not always reliable.

In the 1950s, cinematography became a valuable tool for examining the process of transferring the oocyte–cumulus complex from the surface of the ovary to the ostium of the oviduct. Richard Blandau pioneered the use of cinematography to study transfer, particularly in the rabbit, where the process can be visually accessed in anesthetized animals via surgery.[7] In 1980, Katz and Yanagimachi adapted high speed cinemicrography to study the movement of sperm within the oviductal ampulla of the golden hamster.[8] This was possible

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because the oviduct could be removed from the hamster after mating, placed in a chamber on a warm microscope stage, and transilluminated to reveal the movement of sperm within the oviductal lumen. Sperm would continue to swim vigorously in the ampulla for several minutes. Later, sperm behavior throughout the entire oviduct of the mouse could be recorded in this fashion, enabling the study of the oviductal reservoir of sperm.[9]

In the first decade of this century, the development by Masaru Okabe's group of gene-modified mice that produce fluorescent sperm made it possible to use epifluorescence with highly sensitive digital video cameras in order to see sperm more clearly within the oviduct. Sperm with green fluorescent acrosomes made it possible to ascertain where acrosome reactions take place in the oviduct.[10,11] Other uses of the tools of gene manipulation, particularly gene disruption, have contributed to our understanding of various mechanisms driving and regulating gamete transport, including semen coagulation,[12] passage of sperm through the uterotubal junction,[13] and the role of the cumulus matrix in oocyte transport.[14,15] Selection of genes for manipulation has been aided by advances in microarray analysis and mass spectrometry.[16]

In order to understand fully the regulation of movement of gametes in the female reproductive tract, studies of biochemical factors must be complemented by studies of the physical aspects of interactions of gametes with the reproductive tract. The development of mathematical models to describe swimming of organisms the size of sperm (micron scale) began in the latter half of the 1800s with calculations of Reynolds numbers, which indicated that inertial forces acting on sperm are negligible compared with viscous forces.[17] Interest of biophysicists and mathematicians in understanding movement of sperm was stimulated in 1951 by Sir Geoffrey Taylor, who developed the analysis of an infinite sheet, which could be idealized as a swimmer (for example, a sperm flagellum) that was much longer than its radius.[18] In 1976, James Lighthill proposed a mathematical approach for analyzing the hydrodynamics of the movements of eukaryotic flagella.[19] Application of physics to sperm interactions with mucus in the female tract was pioneered by David Katz[20] in the late 1970s. In 1995 and beyond, mathematical modeling was used by Lisa Fauci to explain the interaction of sperm with boundaries, such as the walls of the female reproductive tract.[21] More recently, development of the technology to produce microfluidics devices enabled Denissenko et al. to design microchannels that could actually guide the movement of human sperm.[22] The current explosion in the advances of biophysics, microfluidics technology, and mathematical modeling should rapidly advance our understanding of how sperm move through the female reproductive tract and inspire development of new treatments for infertility and of more effective contraceptives.

In the second edition of this book, Michael J. K. Harper,[23] a leader in the study of gamete transport, provided more detailed information on the earlier work on gamete transport.

SPERM TRANSPORT

Sperm Competition and Cooperation

For the males of many species, the ability to reach the site of fertilization in the oviduct is not enough to ensure success, because their sperm may be forced to compete with the sperm of other males.[24] There is strong evidence in a variety of species that increased competition among males correlates with increased velocity of their sperm.[24] In some lineages, the increased competition appears to have selected for evolution of longer flagella that produce faster sperm; however, in other lineages, competition appears to have selected for increased production of sperm, suggesting that evolutionary tradeoffs occur between production of larger, faster sperm and production of greater numbers of sperm.[24]

In some species, sperm appear to cooperate by joining with each other to form aggregates that move with greater speed than individual sperm. In the wood mouse, *Apodemus sylvaticus*, sperm have small hooks on their heads that latch them onto each other, resulting in the formation of trains of several (Figure 5.1), or even thousands, of cells[25]. The trains swim faster than solitary sperm.[26] Furthermore, in the highly promiscuous deer mouse, *Peromyscus maniculatus*, sperm aggregate preferentially with sperm from the same male over sperm from other males; whereas, sperm from the monogamous species, *Peromyscus polionotus*, aggregate indiscriminately with sperm from other males.[25] The nature of the tendency of sperm to aggregate preferentially with others from the same male is unknown, but proposed to be due to a homophilic adhesion protein.

There are other examples of sperm joining with each other by their heads. Sperm of new world marsupials, such as the opossum species *Didelphis virginiana*[27] and *Monodelphis domestica*,[28] form pairs that break apart

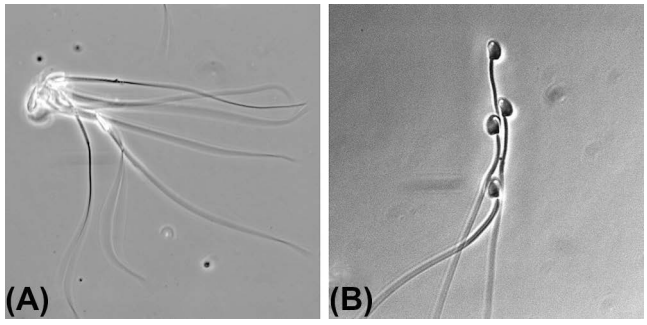

FIGURE 5.1 Phase contrast images of deer mouse (*Peromyscus maniculatus*) sperm (230×). (A) Sperm aggregates attached at heads. (B) Sperm attached head hook to midpiece. *Source: From Ref. 25.*

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shortly before fertilization. Pairing enhances passage of marsupial sperm through viscous media and thus may provide an advantage for sperm swimming in the viscous fluids of the female tract.[29] Guinea pig (*Cavia porcellus*) sperm heads stack together in rouleaux in the epididymis, and the rouleaux are maintained in the female tract, even into the oviduct.[30–32] Sperm of flying squirrels (*Glaucomys volans*)[33] and armadillos (*Cabassous unicinctus*)[34] also form rouleaux. Hamster sperm (*Mesocricetus auratus* and *Cricetulus griceus*) retrieved from the caudal epididymis agglutinate by their heads when incubated under capacitating conditions in vitro, then break apart as they become hyperactivated,[35–37] although it is not known whether this behavior occurs in vivo. Whether or not these last examples represent sperm cooperation, it is evident that cooperation has evolved in sperm of diverse species.

Timing of Insemination with Respect to Ovulation

Sperm may be required to survive in the female tract for a long time in species that spend several days in estrus or that delay ovulation during hibernation. Prolonged survival is promoted by adaptations of the female for storing sperm. Mares (*Equus caballus*) ovulate about five days after the onset of estrus and must therefore be able to store sperm for that period.[38] Many species of hibernating vesperilionid and rhinolophid bats mate before they enter hibernation in the autumn, and their sperm are stored until spring, when ovulation occurs shortly after arousal.[39,40]

One solution to shorten the length of time that sperm must survive in the female tract is coitus-induced ovulation. Female rabbits, for example, remain in estrus for long periods of time until they mate, which triggers ovulation about 12 h later.[41] In the Sumatran rhinoceros (*Dicerorhinus sumatrensis*), mating activity induces an LH surge within 1–2 h, followed by ovulation.[42] Other induced ovulators include domestic cats and other feline species,[43,44] camels,[45] the American black bear (*Ursus americanus*),[46] the grasscutter (*Thryonomys swinderianus*),[47] voles,[43] hedgehogs, moles, and shrews.[48]

Insemination

Vaginal Insemination

The site of semen deposition is not easy to establish, because it must be determined by examining the female immediately after coitus and by considering the anatomy of the penis, vagina, and cervix during coitus. This has been accomplished for humans, in which semen has been observed pooled in the anterior vagina near the cervical os shortly after coitus (Figure 5.2). Within minutes

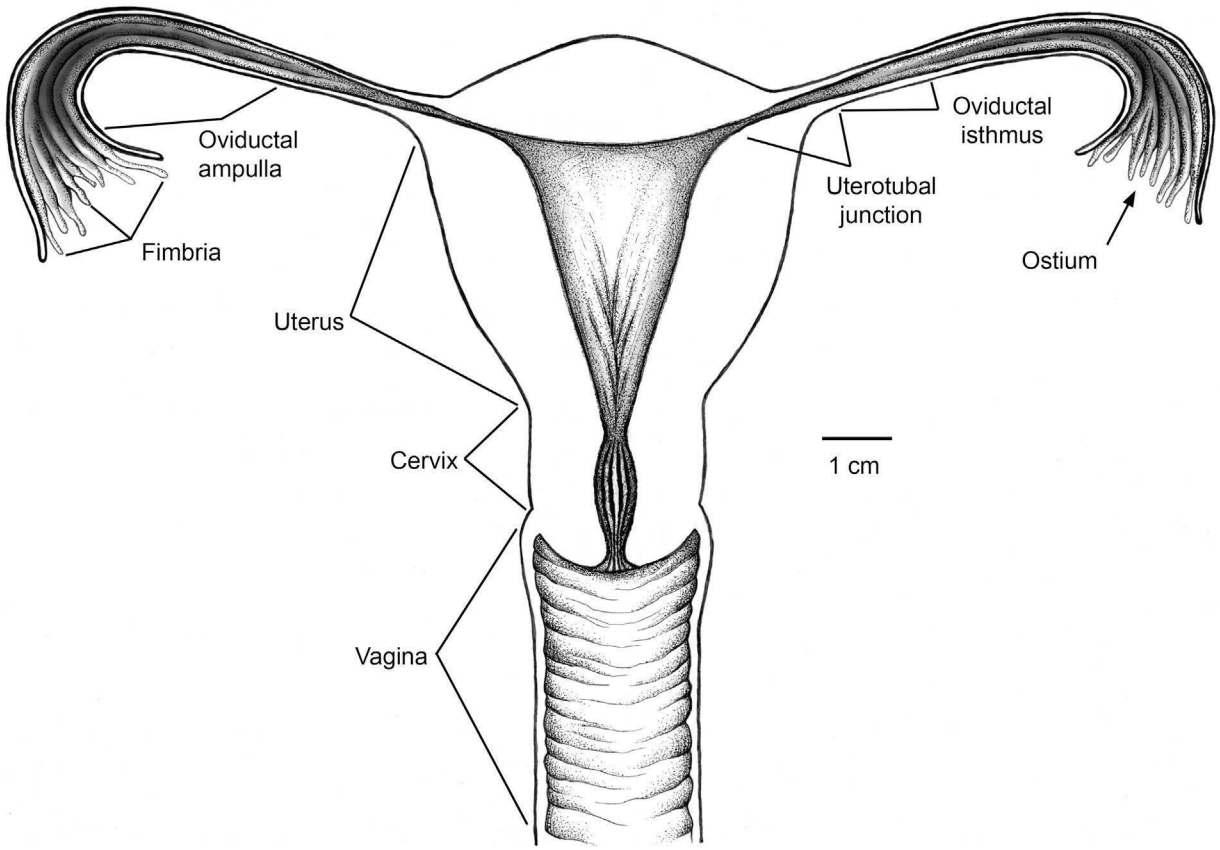

FIGURE 5.2 Human female reproductive tract. *Source: Adapted from Ref. 49.*

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of vaginal deposition, however, human sperm begin to leave the seminal pool and enter the cervix.[50] Semen of rabbits (*Oryctolagus cuniculus*) is also deposited in the anterior vagina, but a few million sperm have been recovered from the cervix 1 min after coitus.[51]

Rats (*Rattus norvegicus*) and hamsters (*M. auratus*) have been classified by some as uterine depositors of semen, because their sperm can be found in the uterine horns minutes after coitus. Nevertheless, results of a careful study by Bedford and Yanagimachi[52] revealed that the sperm are deposited in the vagina but transported through the cervix into the uterus within a few minutes. At the time of vaginal deposition, the sperm are immotile or only weakly motile[52]; therefore, it is assumed that they are swept through a relaxed cervix into the uterus by muscular contractions of the female tract. In support of this assumption, Carballada and Esponda[53] detected several seminal plasma proteins in rat uterine fluid. As in the rat, the mouse uterine lumen is distended only minutes after coitus by a dense mass containing sperm and bacteria,[3] indicating that mouse semen is rapidly swept through the cervix into the uterus as well.

Copulatory Plugs and Gels

Whereas most of the semen of murine rodents is rapidly transported into the uterine cavity, some remains in the vagina where it coagulates to form a copulatory plug. The plug forms a cervical cap that promotes sperm transport into the uterus.[7,53,54] The plugs formed by semen of guinea pigs and mice extend into the cervical canals and thus would seem to form a seal against retrograde sperm loss[7] (Figure 5.3). Ligation of the vesicular and coagulating glands of rats prevented the formation of plugs and the transport of sperm into the uterus.[55] The major protein components of the copulatory plugs of rodents are a family of seminal vesicle-secreted proteins (SVS) family.[56–58] A transglutaminase secreted by the mouse coagulating gland polymerizes SVS1-3 and is thought to be responsible for plug formation in vivo.[56,59]

Male mice deficient for the gene encoding the protease inhibitor known as protease nexin-1 (PN-1) show a marked impairment in fertility.[12] Vaginal plugs formed in females after mating with PN-1 null males were small, soft, and fibrous, and did not lodge tightly in the dual cervical canals. When proteins extracted from plugs produced by mutant and wild-type males were compared by SDS/PAGE analysis, significantly fewer protein bands were detected in the mutant extracts, suggesting that, in the mutant, many of the proteins normally forming the plug had been degraded into peptides too small to be retained by the gel. No sperm could be found in the uterus 15 min after mating with PN-1 null males, demonstrating the importance of the plug for promoting transport of mouse sperm into the uterus.[12]

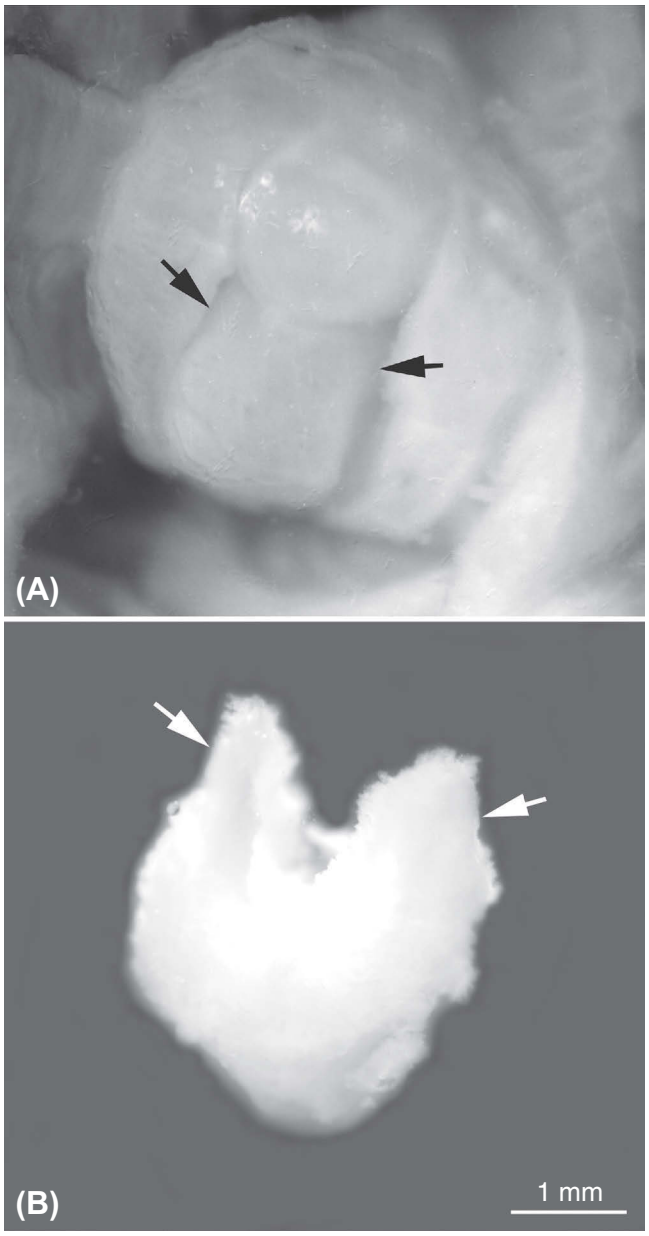

FIGURE 5.3 Copulatory plugs. (A) External os of mouse cervix. *Arrows* indicate openings. (B) Anterior portion of a mouse copulatory plug removed from the vagina. *Arrows* indicate projections that fit into cervical openings. *Source: Photomicrographs by S. S. Suarez.*

The semen of humans also coagulates, although it forms a loose gel rather than the compact fibrous plug seen in rodents. The coagulum forms within a minute of coitus and soon is enzymatically degraded[50] by prostate-specific antigen (PSA), a serine protease secreted by the prostate gland.[60] The predominant structural proteins of the gel are the 50 kDa semenogelin I (SEMG1) and the 63 kDa semenogelin II (SEMG2), as well as a glycosylated form of SEMG2, all of which are secreted primarily by the seminal vesicles.[60] The semenogelin genes, *SEMG1* and *SEMG2*, are considered homologous to the mouse genes that encode SVSI and SVSII.[60]

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SEMG1 binds to epididymal protease inhibitor (EPPIN) on the surface of sperm. Sperm acquire a coating of EPPIN in the testis, where it is produced by Sertoli cells, and in the efferent ductules and epididymis, where it is produced by the epithelial linings.[61]

It has been proposed that temporary entrapment of human sperm in the coagulum serves to keep the sperm at the cervical os and protects them from the acidic environment of the vagina. EPPIN has strong bacteriocidal activity, and may also serve to protect sperm from vaginal bacteria and help to prevent bacterial transport into the cervix.[61]

Like humans, some primates produce semen that forms a soft gel. However, in chimpanzees (*Pan troglodytes*), a species in which females mate with more than one male in a brief time, the semen coagulates into a compact plug resembling that of rodents.[62,63]

Some carnivores (e.g., domestic dogs, *Canis familiaris*) and some rat and mouse species of the family Cricetidae use the penis as a copulatory plug; i.e., the mating pair remains joined together for a period of time after coitus.[64] About a minute after a dog has mounted and locked with a bitch, he turns around, such that the two animals face opposite poles. They remain locked together in this position for 5–45 min. Close examination of the anatomy of the joined genitalia led Grandage[65] to propose that locking serves to "encourage uterine insemination".

Uterine Insemination

Some species bypass the vagina altogether and deposit semen directly into the uterine cavity, where sperm may quickly gain access to the oviduct. In the pig (*Sus scrofa*), the penis is shaped like a corkscrew and the cervix contains complementary furrows. During copulation, the penis fits into the cervix and passes a large volume of semen (about 250 ml) into the uterine cavity.[66–68]

Even those species that do not pass the penis through the cervix may use the penis to open the cervical canals. The glans of the rat penis contains projections that seem designed to push open flaps guarding the cervical canals.[55]

Artificial Insemination

In dairy cattle (*Bos taurus*), bypassing the vagina and cervix has proven advantageous for artificial insemination. Whereas a bull normally deposits several billion sperm into the vagina (Table 5.1), artificial inseminators deposit 5–20 million frozen/thawed sperm directly into the body of the uterus.[69–71]

Artificial insemination into the peritoneal cavity, near the ovary, can result in pregnancy and has been used to treat infertility in humans and repeat breeding syndrome in dairy cows.[72] Access to the peritoneal cavity is obtained through the abdominal wall or vaginal wall.[72] Evidently, inseminated sperm are swept into the ostium or picked up by the cilia on the surface of the oviductal fimbria and transported into the ampulla to fertilize oocytes. Oviductal pickup of sperm from the peritoneal cavity may also occur naturally. To demonstrate that sperm can exit an oviduct via the ostium of the ampulla, enter the peritoneal cavity, and then enter the contralateral oviduct through its ostium, Larsson[73] ligated and resected a 2–3 cm segment of isthmus of one oviduct of dairy heifers (virgin cows), then deposited semen into the uterus at estrus. Sperm were recovered only 2 h after insemination from the resected oviducts, cranial to the resected segment. Presumably, these sperm had reached the resected segment via the peritoneal cavity.

There are case reports of human tubal pregnancies that arose in spite of lack of access of sperm from the uterus into the oviduct on the side of ovulation.[74–76] The only route available to the sperm in these cases was through the peritoneal cavity.

TABLE 5.1 Semen Characteristics in Domestic Animals

	Bull	Stallion	Ram (Buck)	Boar	Dog	Cat
Volume (ml)	4[a] (1–15)[b]	70 (30–250)	1 (0.7–3.0)	250 (125–500)	10 (1.0–25.0)	0.04 (0.01–0.12)
Sperm concentration (millions/ml)	1200 (300–2500)	120 (30–600)	3000 (1000–6000)	150 (25–1000)	125 (20–540)	1730 (96–3740)
pH	6.8 (6.2–7.5)	7.4 (7.0–7.8)	6.8 (6.2–7.0)	7.4 (7.0–7.8)	6.7 (6.0–6.8)	7.4
Total sperm/ejaculum (billions—approximate)	4.8	8.4	3.0	37.5	1.25	0.057

[a] *Mean.*
[b] *Range.*
Ref. 68.

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Vaginal Defenses

The vagina is open to the exterior and therefore to infection, especially at the time of coitus; therefore, it is well equipped with antimicrobial defenses that can work against sperm as well as against pathogens. To enable fertilization to take place, both the female and the male have adopted mechanisms for protecting sperm from these defenses. As discussed before, some species bypass the vagina and deposit semen directly into the uterus, whereas, in all other species, vaginal deposition occurs right at the entrance to the cervix.

Vaginal pH

For vaginal inseminators, sperm must contend with the acidic pH of vaginal fluid. The vaginal pH of women is normally 5 or lower, which is microbicidal for many sexually transmitted disease pathogens, including those that cause bacterial vaginosis, yeast infections, urinary tract infections, and HIV/AIDS.[77] Evidence indicates that the acidity is maintained through lactic acid production by anaerobic lactobacilli that feed on glycogen present in sloughed vaginal epithelial cells.[78] Lowering pH with lactic acid has been demonstrated to immobilize bull sperm.[79,80] The pH of seminal plasma ranges from 6.7 to 7.4 in common domestic species (Table 5.1)[68] and has the potential to neutralize vaginal acid. Vaginal pH was measured by radiotelemetry in a fertile human couple during coitus. The pH rose from 4.3 to 7.2 within 8 s of the arrival of semen; whereas, no change was detected when the partner used a condom.[81]

The bacteria that inhabit the vagina of healthy women consist of a number of species. The composition of the vaginal microbial ecosystem was examined in samples from asymptomatic sexually active women using a high throughput method of pyrosequencing of barcoded 16S rRNA genes. The samples were acquired from 396 North American women, equally representing Asian, white, black, and Hispanic communities. A total of 282 taxa were detected among the microbiota of these samples. The majority of vaginal communities were dominated by *Lactobacillus* species. The remaining types were other anaerobic species of bacteria; however, all communities contained species that are known to produce lactic acid,[77] indicating that lactic acid plays an important role in cervical function, presumably for its toxicity against a number of aggressively infectious microbes.

Leukocytic Response in the Vagina

In addition to providing protective substances for sperm in seminal plasma, males may also overcome female immunological defenses by inseminating millions or even billions of sperm. This strategy would be particularly effective in overcoming a cellular immune response. In the rabbit, deposition of semen results in an invasion of neutrophils into the vagina. This invasion takes time, however, to build to an effective level. Numerous leukocytes, many containing ingested sperm, were observed in the vaginas of rabbits 3–24 h post coitus.[82,83] By that time, however, thousands of sperm had already reached the oviduct.[84]

Sperm Transport Through the Cervix

Cervical Mucus

After deposition in the cranial vagina, sperm of humans and dairy cows enter the cervical canal rapidly, where they encounter large volumes of cervical mucus produced when systemic levels of estrogen are high. Under the influence of estradiol, the cervix produces highly hydrated mucus, with a water content that often exceeds 96% in women.[85] The extent of hydration is correlated with penetrability to sperm.[86] Coitus on the day of maximal mucus hydration in women, which occurs within the six-day window prior to the day of ovulation, is more closely correlated with incidence of pregnancy than coitus timed with respect to ovulation detected using basal body temperature.[87]

The viscoelastic nature of cervical mucus is due to the presence of large, polymeric, glycosylated proteins in the mucin family that range from 2 to 50 MDa in mass.[88] RNAseq analysis of bovine endocervical linings indicated that transcription of some genes associated with mucus production was higher during estrogen dominance than during progesterone dominance, including *Muc5B*, *Muc1*, *Muc16*, and *Muc20. Muc5B* encodes secreted mucin, while the other three *Muc* genes encode transmembrane mucins. Some genes involved in glycosylation of mucins were upregulated at estrus, whereas others were downregulated, suggesting that the glycosylation patterns of mucins differ between the time that cervical mucus is watery and accessible to sperm and the time that it is inaccessible to sperm.[88] Staining of histological sections of bovine cervix with lectins indicated that the presence of some terminal sialic acids is maximal during estrus, while that of terminal fucose is lowest.[89] Because bovine sperm bind to fucosylated molecules,[90] the reduction in fucosylation of mucin molecules would facilitate sperm passage through the cervix.

Proteomic and glycomic analyses of samples of secreted human cervical mucus collected from 12 Caucasian women did not detect cyclical changes in mucin glycoproteins; however, glycosylation changes were detected and were associated with ovulation. MUC5B

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was the most abundantly secreted, gel-forming mucin, but MUC5AC and MUC6 were also detected, along with two transmembrane mucins, MUC16 and MUC1. Sialylated oligosaccharides were the predominant forms identified in mucins before and after the ovulatory period, but the dominant oligosaccharides detected during the ovulatory period were neutral oligosaccharides.[91]

Cervical mucus presents a greater barrier to abnormal sperm that cannot swim properly or that present a poor hydrodynamic profile than it does to morphologically normal, vigorously motile sperm, and is therefore thought to be one means of sperm selection.[85,92–94]

The greatest barrier to sperm penetration through cervical mucus is at its border, because here the structure is more compact.[95] Components of seminal plasma may assist sperm in penetrating the mucus border. More human sperm were found to enter cervical mucus in vitro when an inseminate was diluted 1:1 with whole seminal plasma than when it was diluted with Tyrode's medium, even though the sperm swam faster in the Tyrode's dilution. It was concluded that components of seminal plasma facilitate penetration of sperm into cervical mucus.[96]

A role for penetration of cervical mucus has been attributed by Tollner et al.[97] to the coating of sperm by specific members of the defensin family of proteins, namely DEFB126 in humans and rhesus macaques, and DEFB22 in mice and rats. Defensins are cationic peptides comprised of 29–42 amino acids. They contain six conserved cysteine residues that form three intramolecular disulfide bonds, which fold the peptide into a conformation that resists protease cleavage. Defensins have been detected in a wide array of animal and plant species, where they are known to play a role in innate immunity and to possess microbicidal activity against bacteria, fungi, and some viruses. DEFB126 and DEFB22 are large defensins (32–35 kDa) that have extended carboxyl tails with numerous glycosylation sites. DEFB126 is secreted in the epididymis and coats the entire surface of macaque sperm. Removal of the DEFB126 coating reduced the ease by which macaque sperm swam through watery estrous cervical mucus, but regained the ease when the supernatant, which contained DEFB126, was added back to sperm.[97,98] A similar reduction in mucus penetration occurred when the macaque sperm were treated by neuraminidase, which was proposed to remove terminal sialic acid groups from the DEFB126 coating.

Homozygosity for a sequence variant of the *DEFB126* gene in humans has been associated with reduced fertility. The variant consists of a deletion of two nucleotides that creates a nonstop mutation. Sperm of men who are homozygous for the delection variant do not penetrate hyaluronate gels as well as men who are heterozygous or homozygous for the wild-type allele. Hyaluronate gel is supposed to serve as a model for cervical mucus. The variant allele is common in Asian, European, and African populations, suggesting that the allele is old and has not been subjected to significant negative selection. Nevertheless, because the incidence of heterozygotes is more frequent than expected, heterozygosity may confer a selective advantage to the variant.[97]

Immune Responses in the Cervix

The cervix is immunologically competent. In rabbits and humans, vaginal insemination stimulates the migration of leukocytes, particularly neutrophils and macrophages, into the cervix as well as into the vagina.[99,100] Neutrophils migrate readily through midcycle human cervical mucus.[101] In rabbits, neutrophils heavily infiltrate the posterior region of the cervical canal near the external os within a half hour of mating or artificial insemination.[99] Interestingly, it was discovered that if female rabbits were mated to a second male during the neutrophilic infiltration induced by an earlier mating, sperm from the second male were still able to fertilize.[102] Thus, although the cervix is capable of mounting a leukocytic response, and neutrophils may migrate into cervical mucus, the leukocytes may not present a significant barrier to sperm, at least in the rabbit. It has been demonstrated that neutrophils will bind to human sperm and ingest them only if serum that contains both serological complement and complement-fixing antisperm antibodies is present.[103] This can happen if the female somehow becomes immunized against sperm antigens. In conclusion, the evidence indicates that leukocytic invasion serves to protect against microbes that accompany semen and does not normally present a barrier to normal motile sperm, at least not shortly after coitus.

Immunoglobulins IgG and IgA have been detected in human cervical mucus. Secretory IgA is produced locally by plasma cells in subepithelial connective tissue. The amount of IgG and IgA increases in the follicular phase and then decreases at about the time of ovulation.[104] The immunoglobulins provide greater protection against microbes at the time when the cervical mucus is highly hydrated and offers the least resistance to penetration. However, when there are antibodies present that recognize antigens on the surface of ejaculated sperm, infertility can result.[6]

TGF-β and other components of human seminal plasma induce cervical epithelium to activate cytokine and chemokine gene expression, including genes that encode GMC-SF and IL-6.[105] This results in recruitment of macrophages, dendritic cells, and memory T cells to the cervical epithelium and underlying connective tissue.[105] The recruited cells may serve to protect the female against infectious disease microbes that accompany semen; however, the infiltration process is too slow to affect the rapid migration of many thousands of sperm through the cervix into the uterus.

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Preferential Pathways Through the Cervix for Sperm

An elegant three-dimensional reconstruction of serial sections of the bovine cervix produced by Mullins and Saacke[106] led them to conclude that there are narrow channels in the cervix, not much wider than the heads of sperm, that lead to the uterine cavity (Figure 5.4(A–D)).

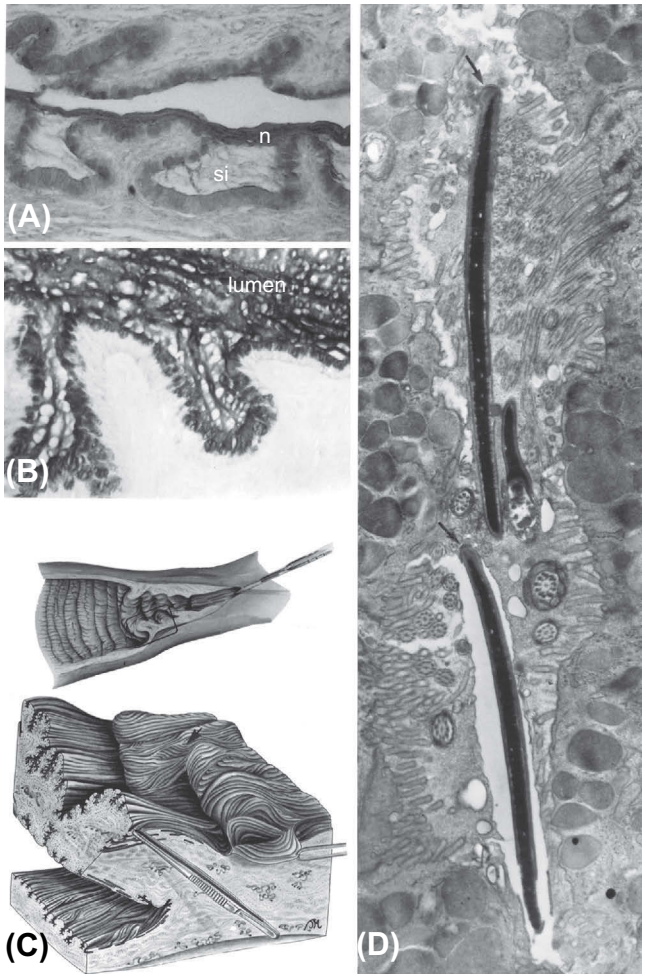

FIGURE 5.4 The bovine cervix (*Bos taurus*). (A) A section of a primary fold of cervical mucosa taken from a cow in the follicular phase of the estrous cycle. The tissue was fixed in Bouin's solution, dehydrated, embedded in paraffin, sectioned, and stained with Alcian blue (AB) and high-iron diamine (HID). AB-positive sialomucins (si) predominate in basal areas of minor grooves in the mucosa, underneath a layer of denser-staining HID-positive neutral (n) and sulfomucins. This staining pattern demonstrates that sperm encounter a different type of mucus in the base of grooves (magnification × 170). (B) In a similar section, taken from a cow in the luteal phase, the AB and HID staining reveal a loss in the layered organization seen in the estrous cow (magnification × 170). (C) An illustration by K. J. Mullins of the three-dimensional structure of the folds of cervical mucosa, derived from stereomicroscopical examination of tissue stained on its mucosal surface and from three-dimensional reconstruction of serial sections. (D) Transmission electron micrograph of cervical tissue showing sperm within grooves of cervical mucosa. *Arrows* indicate the rostral tips of the heads of sperm (magnification × 17,850). *Source: Adapted from Ref. 106.*

Furthermore, based on histochemical staining characteristics of the mucus in tissue sections of cervix, they concluded that mucus in the channels is different in composition and less dense than that in the cervical lumen during the follicular phase (Figure 5.4(A) and (B)). They proposed that sperm reach the uterine cavity by traveling through the narrow channels and avoiding the more viscous mucus in the center of the cervical lumen that serves to discharge uterine contents and perhaps also to prevent the ascent of pathogens. Mattner[107] found that when he flushed the cervices of goats and cows 19–24 h after mating at the onset of estrus he recovered approximately 90% of the mucus and more than 90% of the luminal leukocytes but only about half of the sperm. The remaining half of the sperm were found in the periphery of the cervical lumen, at the base of mucosal folds and within small channels. Recent histochemical staining of sections of bovine cervix with an array of lectins revealed differential distribution of glycans between the apical and basal regions of mucosal folds.[89]

Sperm may also be guided through the cervix by the microarchitecture of the cervical mucus itself. Mucins, the chief glycoproteins comprising cervical mucus, are long, flexible linear molecules. It is thought that these long molecules become aligned as they are secreted by the flow of secretions through mucosal channels and thus serve to guide sperm. Human[108] and bull[109] sperm have been demonstrated to orient themselves along the long axis of threads of bovine cervical mucus. Human sperm swimming through cervical mucus swim in a straighter path than they do in seminal plasma or medium (Figure 5.5(A) and (B)).[110]

Are Sperm Stored in the Cervix?

Little is known about how long sperm spend traversing the cervix or whether sperm are stored there. About one day after natural mating, a few million sperm were recovered from the cervices of cows and goats, and more than 60% of these sperm were motile.[107] Vigorously motile sperm have been recovered from the human cervix up to five days after insemination.[111] Nevertheless, it is not known whether sperm collected from cervices this long after coitus would reach the oviducts and succeed in fertilizing, nor could it be known whether these sperm had re-entered the cervix from the uterus. In vitro, human sperm swim through estrous cervical mucus at a rate of 2–3 mm/min.[110] At this rate, the time for traversing the cervical canal would be about 10–15 min.

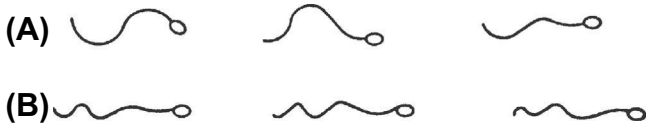

FIGURE 5.5 Flagellar beating patterns of human sperm. (A) Sperm swimming in seminal plasma or aqueous medium. (B) Sperm swimming in cervical mucus. *Source: Adapted from Ref. 110.*

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Sperm Transport Through the Uterus

Rate of Transport

In uterine semen depositors, some sperm leave the uterus rapidly to enter the uterotubal junction. In pigs, for example, ligation of the uterotubal junction at various times after mating revealed that sperm in numbers sufficient to achieve fertilization reach the oviduct within 30 min of coitus.[66] The large volume of porcine semen could ensure that sperm are washed up against the uterotubal junction shortly after coitus. In mice, rats, and hamsters, even though semen is deposited in the cranial vagina, enough sperm-filled seminal fluid is soon transported into the uterine horns to cause visible distension.[52,112] Mouse sperm move beyond the uterus to reach the oviduct in substantial numbers within 30 min of mating.[3]

At only a few centimeters in length, the human uterine cavity is relatively small and could be passed through in less than 10 min by sperm swimming at about 5 mm/min, which is the swimming speed of human sperm in aqueous medium.[113] The actual rate of passage of human sperm through the uterus is difficult to determine, due to experimental limitations. In studies of sperm distribution in reproductive tracts of women, variation of data was high among individuals.[114] In one set of experiments, fertile women were inseminated into the cranial vagina shortly before surgical excision of both fallopian tubes. Sperm were recovered from the fimbrial segment of the ampulla in two women whose tubes were removed 5 min after insemination. Sperm were recovered all along the tubes of two more women 10 min after insemination.[115] Unfortunately, the condition of these sperm was not assessed; therefore, it could not be determined whether the sperm were capable of fertilizing. In another study,[116] several motile sperm were recovered from fallopian tubes following hysterectomy 30 min after insemination in one patient and 1 h after insemination in three out of seven patients; however, these women underwent surgery for treatment of fibroids, polyps, or endometriosis and therefore sperm transport may have been abnormal.

In contrast to human sperm, bovine sperm must pass through a uterine body 2.5–4 cm long and uterine horns that are 20–40 cm long before reaching the uterotubal junction (Figure 5.6).[68] At an approximate swimming speed of 7 mm/min, bull sperm would require about an hour to reach the uterotubal junction if they swam directly from the internal os of the cervix to the uterotubal junction. Hunter and Wilmut[117–119] ligated the uterotubal junction at various times after mating and later flushed the oviduct to recover embryos. Their results indicate that about 8 h are required for bull sperm to reach the oviduct in sufficient numbers to achieve fertilization. A similar experiment in sheep also indicated that 8–10 h are required for sufficient numbers of sperm to reach the oviduct.[120]

Uterine Peristalsis

Transport of sperm through the uterus is likely aided in some species by pro-ovarian contractions of the myometrium. Ultrasonography of the human uterus has revealed cranially directed waves of uterine smooth muscle contractions that increase in intensity during the late follicular phase.[121,122] The uterine contractions occurring in women during the periovulatory period are limited to the layer of myometrium directly beneath the endometrium.[121,123] This is in contrast to contractions occurring during menses, which involve all layers of the myometrium. In cows and ewes, electromyography has indicated that strong contractile activity occurs during estrus, while contractions are weak and localized during the luteal phase.[124]

Kunz et al.[122] deposited 5–40 μm albumin microspheres radioactively tagged with technetium into the cranial vaginas of women to determine how such contractions might transport sperm. They found that spheres were rapidly and maximally transported into the uterine cavity and even into the oviductal isthmus during the late follicular phase. Interestingly, transport of the spheres was greater to the isthmus ipsilateral to the dominant follicle than to the contralateral isthmus. This preferential transport may result from signals passed via a vascular communication between the preovulatory follicle and the uterus and oviduct. An arterial anastomosis lies between the ovarian and uterine arteries (which also supply the oviduct) in the corneal region of the human uterus. Doppler flow sonography revealed increased perfusion of the anastomosing vessels on the side of the preovulatory follicle.[125] It is thought that these vessels carry hormones from the dominant follicle directly to the uterus and oviduct without first passing through the systemic circulation, because the ovarian artery associates closely with the ovarian vein. This association is thought to enable a countercurrent transfer of ovarian hormones from the venous drainage of the ovary to the ovarian artery and then to the arterial supply of the uterus and oviduct.[126,127] In addition, lymphatic drainage of the ovary might transfer hormones to the ovarian artery.[127] Although these anatomical relationships appear to be widespread in mammals, the data demonstrating transfer of hormones or other signals along this route are still preliminary.

Studies of uterine contractions during estrus should be interpreted with caution if the females were not mated. Video laparoscopic examination of mated and unmated rats revealed significant changes in contractile patterns of the uterine horns after mating. Unexpectedly, the change consisted of several-fold increases in both cranially and caudally propagating contractions.[128] Caudally directed peristalsis would be expected to carry sperm away from the uterotubal junction. In estrous domestic cats, both ascending and

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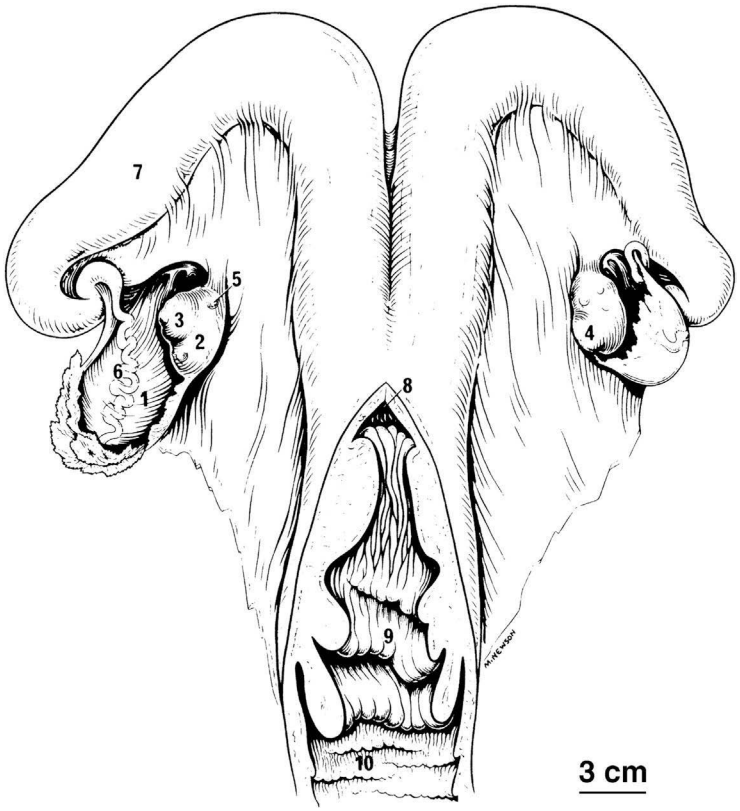

FIGURE 5.6 The dorsal aspect of the female reproductive tract of a cow (*Bos taurus*). (1) Ovarian bursa, (2) ovary, (3) corpus luteum, (4) follicle in ovary, (5) corpus albicans in ovary, (6) oviduct (uterine tube), (7) uterine horn, (8), uterine body, (9) cervix, (10) vagina. *Source: From Ref. 68.*

descending contractions were observed by fluoroscopy.[129] Perhaps the ebb and flow of contractions direct fresh waves of sperm to the junction.

Myometrial contractions may be stimulated by seminal components. When female rats were mated with vasectomized males, the incidence of strong uterine contractions was lower than when they were mated with intact males, indicating that sperm or testicular or epididymal secretions have stimulatory activity.[128] The secretions of male accessory sex glands may also play a role, because removal of the seminal vesicles significantly reduced the pregnancy rate in mice.[112] In boars, there is evidence that estrogens, which may reach 11.5 μg in an ejaculate, increase myometrial contraction frequency.[130] Since boar semen is deposited directly into the uterine cavity, the uterus is exposed to the full amount of estrogens in the semen. There is evidence that the estrogens enhance contraction by stimulating secretion of PGF-2α.[130]

Immunological Responses in the Uterus

Rapid transport of sperm through the uterus by myometrial contractions can enhance sperm survival by propelling them past the immunological defenses of the female. Particularly in species in which seminal plasma rapidly enters the uterus, coitus induces a leukocytic infiltration of the uterine cavity, which reaches a peak several hours after mating in mice.[3] The leukocytes are primarily neutrophils and have been observed phagocytizing uterine sperm in mice, rats, and rabbits.[3,131] This phagocytosis was observed several hours after insemination and therefore might be directed primarily against damaged sperm.

Sperm of rhesus monkeys retain the protective coating of the protein DEFB126 after migrating through the mucus of the cervix. This coating has been proposed to mask highly antigenic proteins of the sperm plasma membrane.[97] Because DEFB126 is highly conserved among mammals, this proposal likely applies to many other species. As time passes, the leukocytes in the uterine lumen begin to outnumber the sperm and phagocytosis of sperm occurs. Loss of the DEFB126 coating, which occurs during capacitation of sperm,[97] could accelerate the killing and phagocytosis of uterine sperm by leukocytes. (Note: capacitation is broadly defined as physiological changes in sperm that confer on them the ability to fertilize oocytes. The processes associated with capacitation are described in Chapter 4. Thus, it is imperative that sperm pass through the uterine cavity before significant numbers of leukocytes arrive.

In addition to phagocytosing uterine sperm, uterine neutrophils also trap sperm by extruding nuclear contents, particularly DNA, which form neutrophil extracellular traps (NETs).[132] NETs were initially discovered as part of the response of neutrophils to bacteria,[133] but neutrophils have also been demonstrated to respond

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similarly to sperm. Interestingly, DNase activity has been detected in equine seminal plasma and is capable of breaking down NETs.[132] Since equine seminal plasma enters the uterus during mating, the DNase activity could offer sperm some protection from NETS.

Sperm Transport Through the Uterotubal Junction

The Junction as a Regulated Entrance to the Oviduct

The uterotubal junction presents anatomical, physiological, and/or mucous barriers to sperm passage in most eutherian mammals. Anatomically, the lumen in species as distantly related as dairy cattle and mice is particularly tortuous and narrow.[134–138] The narrowness of the lumen is especially apparent in living tissue[9] and in frozen sections (Figure 5.7), in which tissue does not shrink as it does during standard preparation of paraffin-embedded sections.[138]

The entrance to the junction is fairly simple in humans; whereas, it is complicated by mucosal folds in cows, pigs, rabbits and many other species (Figure 5.8).[134–137] In mice and rats, the entrance forms a projection into the uterus called a colliculus tubarius (Figure 5.9).[3,9,140]

The mucosal folds that line the uterotubal junction vary in amplitude, architecture, and complexity among species. When the junction of the cow is slit open longitudinally and laid flat, the mucosal folds appear to form cul-de-sacs that would seem to block ascent of sperm[137,141] (Figure 5.10). Nevertheless, when the tube is intact, the cul-de-sacs might actually form funnels that direct sperm to the center of the channel for rapid passage.

In cattle and other species, a physiological valve may be created by a vascular plexus in the lamina propria/submucosal layer of the wall of the junction. When engorged, the plexus can compress the lumen.[137] The walls of the bovine junction and adjacent tubal isthmus also contain a thick muscular layer that could further constrict the lumen. The bovine uterotubal junction is sigmoidal in shape and supported by muscular ligaments that appear capable of increasing the flexure of the curve to compress the lumen.[134,135] Altogether, these structures could control the diameter of the lumenal channel and accessibility of the oviduct to sperm. The human junction traverses a thick muscular layer of uterine wall.[135] In the mouse, the junction (Figure 5.11) was reported to be patent shortly after coitus, but to close tightly about an hour later.[3,9]

In some species, the junctional lumen is filled with a viscous mucus that could impede the progress of sperm (Figures 5.7 and 5.12). Mucus has been found in the uterotubal junction in rabbits,[142,143] pigs,[144] cattle,[138,145]

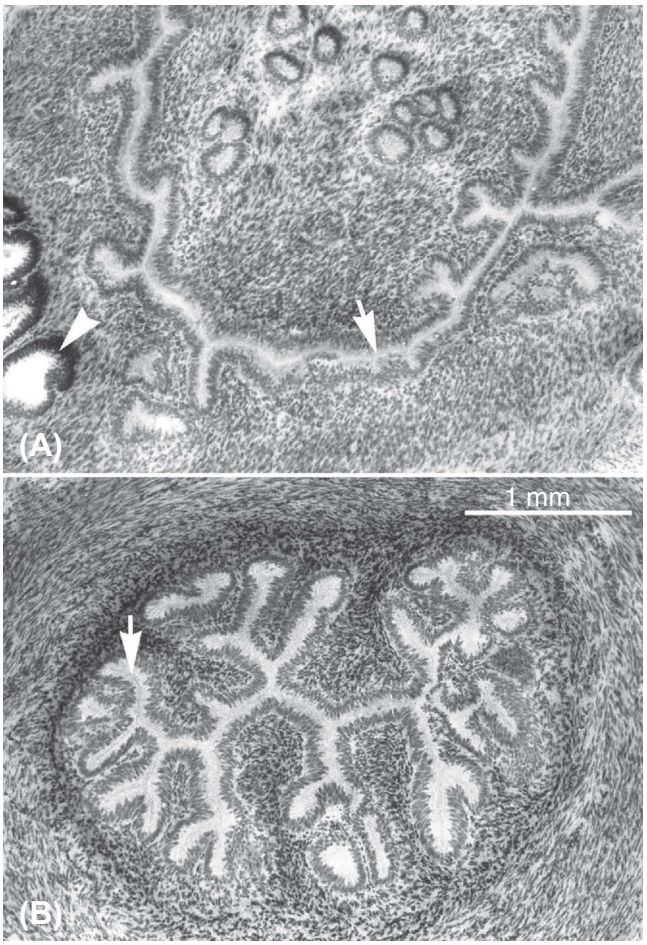

FIGURE 5.7 Frozen sections of the bovine uterotubal junction and caudal isthmus, stained with periodic-acid Schiff reagent and counterstained with hematoxylin. (A) A section of the uterotubal junction proximal to the uterus. *Arrow* indicates lumen of the uterotubal junction; *arrowhead* indicates a uterine gland. (B) A section of caudal isthmus, proximal to the uterotubal junction. The narrow, mucus-filled lumen (*arrow*) may be distinguished as a slightly darker region bounded by the lightly stained apical cytoplasm of the mucosal epithelial cells. *Source: Adapted from Ref. 139.*

and humans.[146] In pigs at estrus, the mucus becomes watery, similar in appearance to that of human midcycle cervical mucus (Suarez, personal observation).

The Junction as a Filter

For uterine semen depositors, the uterotubal junction, particularly when filled with mucus, can serve the filtration function served by the cervix in vaginal depositors. That is, it may filter out pathogens introduced with the semen, as well as morphologically abnormal sperm or sperm with poor motility. In pigs,[147] rats,[140] and hamsters[148] motile sperm pass through the uterotubal junction much more successfully than immotile sperm. Sperm demonstrating linear, progressive motility are more successful at passing through the uterotubal junction than are sperm swimming in nonlinear patterns,

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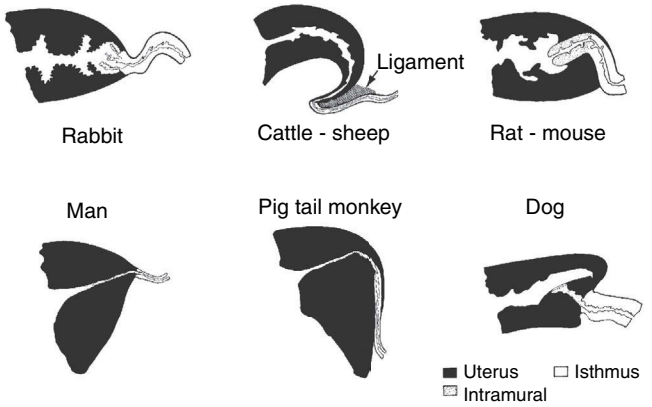

FIGURE 5.8 Diagrammatic illustration (not drawn to scale) to show species differences in the morphology of the uterotubal junction. Various anatomical relationships between the oviduct and uterus can be seen: the conspicuous folds in the rabbit, the flexure of the junction and isthmus in cows, the colliculus in the rat and mouse, the funnel shape in humans and other primates, and the moundlike papilla in the dog. *Source: From Ref. 135.*

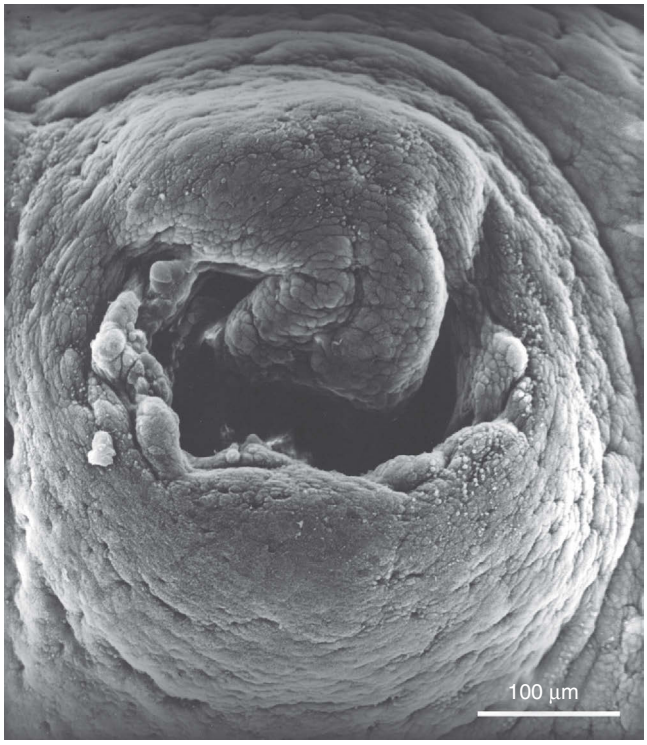

FIGURE 5.9 Scanning electron micrograph of the colliculus tubarius, the uterine entrance to the uterotubal junction in the rat. *Source: Provided by David M. Phillips.*

such as those circling due to an asymmetrical flagellar beating pattern.[140,149] In species in which whole semen is deposited in the uterus, the uterotubal junction may also serve to filter out seminal plasma. Seminal plasma components are left behind in the uterus and are not detected in the oviducts of rats.[150]

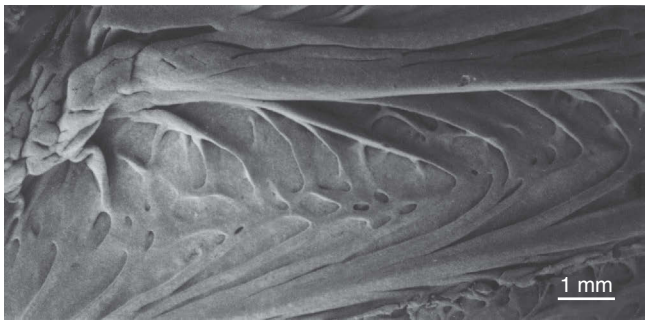

FIGURE 5.10 Scanning electron micrograph of the mucosal (inner) surface of the bovine uterotubal junction, opened longitudinally. Between terminal primary folds are secondary folds that form cul-de-sacs that open toward the uterus. *Source: From Ref. 141.*

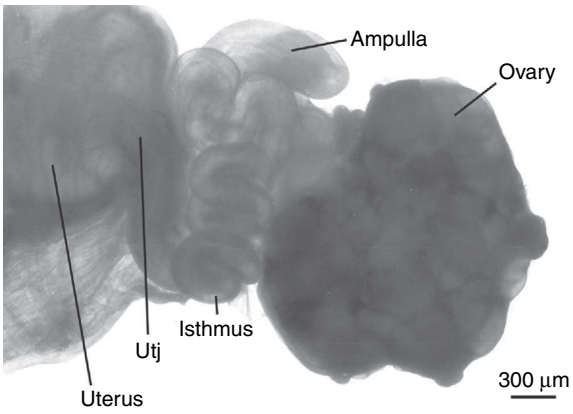

FIGURE 5.11 Photomicrograph of a freshly dissected oviduct of a mouse, illustrating the long, sigmoidal uterotubal junction (utj) and the highly coiled isthmus and ampulla. *Source: From Ref. 139.*

Proteins Required for Passage of Sperm Through the Junction

Male mice that are null mutants for the genes *Adam3*,[151] *Clgn*,[152,153] *Ace*,[154,155] *Adam2*,[156] *Adam1a*,[157] *Calr3*,[158] *Tpst2*,[159] or *Pdilt*[13] are infertile because their sperm cannot pass through the uterotubal junction. In these null mutants, both the motility and morphology of the sperm are normal when examined in vitro. The sperm are unable to bind to the zonae of cumulus-free oocytes; however, sperm from *Adam1a–/–*, *Adam3–/–*, and *Pdilt–/–* males have been reported to fertilize cumulus-intact oocytes in vitro. Therefore, the main cause of infertility in vivo is failure of sperm to pass through the uterotubal junction. All of the aforementioned gene products are proteins that are involved, directly or indirectly, in correct folding and disulfide bond formation of ADAM3 (a disintegrin and metalloprotease 3) in the endoplasmic reticulum of developing male germ cells in the testis, such that ADAM3 is placed in a proper configuration in the sperm plasma membrane.[16]

So far, the protein that is most indirectly involved in the proper placement of a properly folded ADAM3

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in sperm membranes is ACE (angiotensin I-converting enzyme). ACE is normally shed from mouse sperm when they enter the epididymis; therefore, it has been proposed that it has an enzymatic effect on the surface of maturing sperm that somehow enables them to pass through the uterotubal junction.[160] The testis-specific isoform of ACE has dipeptidase activity similar to that of somatic ACE, but it also has GPI-anchored protein-releasing activity (GPIase). Although ACE does not act directly on ADAM3, removal of the GPI-anchored protein TEX101 from developing male germ cells by ACE in the testis has been shown to be necessary for normal distribution of ADAM3 in sperm membranes and for passage of sperm through the uterotubal junction.[161]

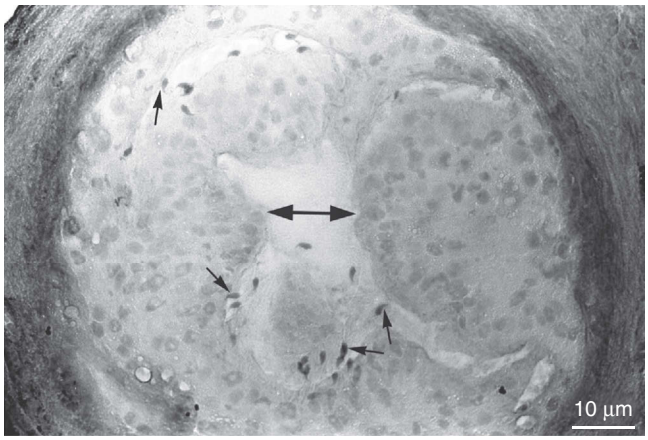

FIGURE 5.12 Frozen section of sperm in the extramural segment of the mouse uterotubal junction, close to the isthmus, stained with periodic acid Schiff reagent to show mucus and with hematoxylin as a nuclear counterstain. The *two-headed arrow* indicates the central part of the lumen, filled with mucus; *arrows* indicate heads of some of the sperm in the lumen. *Source: Provided by Robert P. DeMott.*

Although there is strong evidence that ADAM3 is required in sperm plasma membranes in order for sperm to pass through the uterotubal junction, little is known about how ADAM3 serves this purpose. Some indication of how ADAM3 functions was obtained using mice that are null mutants for *Clgn*. CLGN acts as a chaperone protein in testicular male germ cells and mediates the heterodimerization of ADAM1A with ADAM2. In turn, the ADAM1A/2 dimer is required for correct folding of ADAM3. The role of CLGN in enabling sperm to pass into the oviduct was examined more closely using chimeric males that produced a mixture of testicular germ cells with intact and disrupted *Clgn*. The question addressed was whether CLGN-chaperoned proteins are required by individual sperm in order to pass through the uterotubal junction, or would the presence of wild-type sperm enable the mutant sperm to pass? Such would be the case, for example, if the proteins on the sperm surface signaled the uterotubal junction to open. Chimeric males were created by fusing embryos from wild-type mice that had normal *Clgn* genes with those from a double transgenic line of mice that were homozygous null for *Clgn* and expressed enhanced green fluorescent protein in their acrosomes. The resulting chimeric XY/XY males produced a mixture of sperm, about half of which were derived from mutant germ cells, as identified by the presence of the fluorescent acrosomes. When these males were mated with wild-type females, only

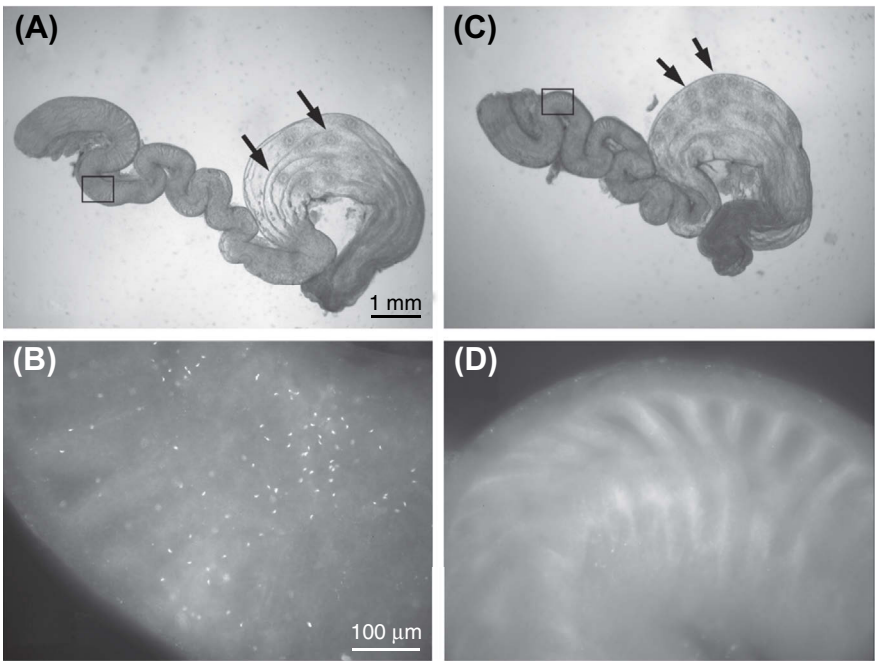

FIGURE 5.13 Comparison of oviductal colonization by sperm from *Clgn–/–* and *Clgn+/–* male mice. Both homozygotes and heterozygotes produced sperm tagged with enhanced green fluorescent protein in their acrosomes, due to insertion of a transgene into the line. *Arrows* indicate oocytes within the oviductal ampullae. (A) Whole mount of an oviduct from a female mated with a *calmegin* +/– male. The *box* indicates the isthmic region shown in (B). (B) Fluorescent illumination of the boxed area from (A) shows sperm within the oviductal isthmus. (C) Whole mount of an oviduct from a female mated with a *calmegin* –/– male. The *box* indicates the isthmic region shown in (D). (D) Fluorescent illumination of the boxed area from (C) reveals no sperm in the isthmus. *Source: From Ref. 10.*

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wild-type sperm could be found within the oviduct.[10] This indicates that normal morphology and motility are not sufficient for enabling sperm to enter the oviduct. An additional factor, likely a sperm surface protein or proteins (such as ADAM3), is required by each sperm in order to pass through the junction. In videos of the behavior of mouse sperm in the uterotubal junction, the sperm do appear to attach transiently to the mucosal lining of the junction (Suarez, personal observation); however, the nature of this interaction remains largely unknown.

To identify other proteins dependent on CLGN for placement in the sperm plasma membrane, 2D gels of Triton X114-extracted proteins from sperm of *Clgn* knockout mice were compared with extracts from wild-type mouse sperm. Two proteins were identified, PMIS1 and PMIS2. When the genes for these proteins were knocked out, *Pmis1*–/– mice were fertile, but *Pmis2*–/– males showed the same infertility phenotype as Clgn–/– males. ADAM3 was missing from the sperm of *Pmis2*–/– males and PMIS2 was severely reduced in sperm of *Adam3*–/– males.[16] Consequently, it was proposed that either PMIS2 or ADAM3 or another protein, perhaps complexed with PMIS2 or ADAM3, is directly responsible for the sperm interaction with the uterotubal junction that enables sperm to pass into the oviduct.

Rapid Transport of Sperm Through the Junction

Sperm have been recovered from the cranial reaches of the ampulla only minutes after coitus or insemination in humans[115] and several other species of mammals.[51,124,162] This phenomenon has been termed rapid sperm transport.[51] Rapid transport of sperm into the oviduct would seem to counter the proposed model of sperm swimming one by one through the uterotubal junction. Nevertheless, when the conditions of rabbit sperm recovered from the cranial ampulla shortly after mating were evaluated by Overstreet and Cooper,[51] they found that most sperm were damaged and immotile. They proposed that the waves of contractions that are stimulated by insemination force some sperm all the way to the cranial ampulla; however, those sperm are unlikely to fertilize because they are damaged by the sheer stress of rapid transport. The contractions might serve primarily to draw sperm into the cervix and/or uterus, but some sperm are forced beyond the uterus. Later, motile sperm gradually pass through the uterotubal junction to establish an oviductal population capable of fertilizing. Rapid transport resulting in sperm damage has not been studied this carefully in other species, but it is likely to apply in cases where sperm are found near the ovary within a few minutes of coitus.

Oviductal Sperm Reservoir

The Reservoir Stores Sperm and Prevents Polyspermy

Many sperm that enter the oviduct do not immediately continue to the site of fertilization but are instead held in a storage reservoir. The mammalian oviductal sperm reservoir was first reported in hamsters by Yanagimachi and Chang[163] and has since been reported to exist in a variety of species (mice,[9] hamsters,[164] rabbits,[51,165] cows,[119] pigs,[66] and sheep[166]).

The oviduct provides a safe haven for sperm. Unlike the vagina, cervix, and uterus, the oviduct does not respond to insemination with an influx of leukocytes.[167] However, the oviduct seems to be more than simply a safe haven for sperm; in addition, it somehow acts to maintain the fertility of sperm between the onset of estrus and the time of ovulation. Sperm fertility and motility are maintained longer in vitro if the sperm are incubated with oviductal epithelium (bovine,[168,169] porcine,[145] human,[170] and canine[171]).

The holding of sperm in the oviductal isthmus may also serve to prevent polyspermic fertilization by allowing only a few sperm at a time to reach the oocyte in the ampulla. Sperm numbers have been artificially increased at the site of fertilization in the pig by surgical insemination directly into the oviduct,[172,173] by resecting the oviduct to bypass the reservoir,[174] or by administering progesterone into the muscularis to inhibit smooth muscle constriction of the lumen.[175,176] Each of these treatments raised the incidence of polyspermic fertilization.

How Sperm are Held in the Reservoir

There is strong evidence from multiple species that the oviductal reservoir is created in eutherian mammals when sperm bind to oviductal epithelium. Motile sperm have been observed to bind by their heads to the oviductal epithelium in cattle (Figure 5.14),[145] mice,[9] hamsters,[178] pigs,[144] horses,[179] and dogs.[180] The opportunity for binding could be enhanced because the mucus-filled, narrow lumens of the uterotubal junction and isthmus slow the sperm and increase their contact with the epithelium.

Guinea pig sperm have been observed stacked in large rouleaux in pockets in the wall of the uterotubal junction 1–12 h after copulation,[30,31] indicating that sperm-to-sperm binding also plays a role in sperm storage in some species.

Rabbit sperm flushed from the oviductal isthmus with oil showed only sluggish motility, but could be revived by dilution in medium. This led to the proposal that motility suppression is also a mechanism for trapping and storing sperm.[181–183]

In hamsters[184] and mice,[9] immotile sperm have been seen in the central part of the isthmic lumen; however,

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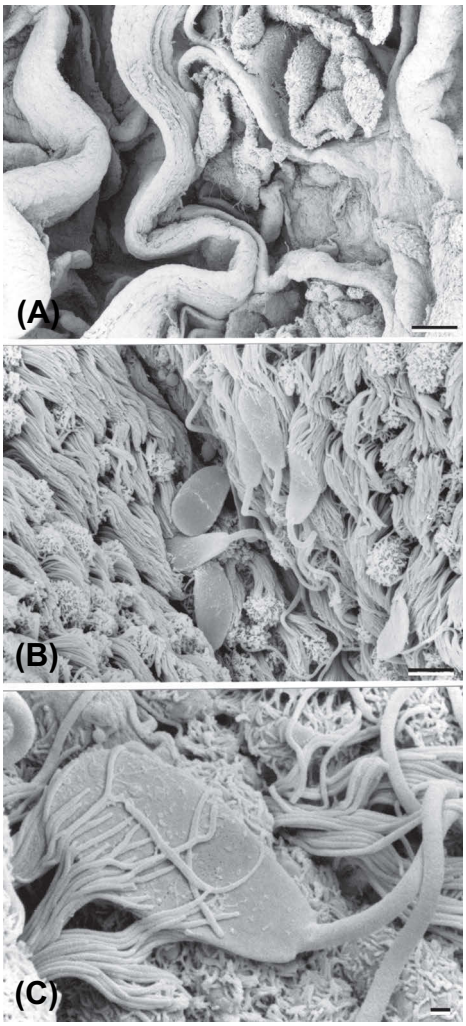

FIGURE 5.14 Scanning electron micrographs of bovine sperm attached to epithelium of the oviductal isthmus. Sperm are located in grooves created by mucosal folds. (A) A low magnification view of the isthmus (bar = 75 μm). (B) A higher magnification of a mucosal groove (bar = 5 μm). (C) A high magnification view of a sperm cell associated with the cilia of the epithelium (bar = 1 μm). *Source: From Ref. 177.*

when hamster oviducts were flushed 2–8 h after mating, most of the unattached sperm recovered from the central lumen were dead, whereas most of the sperm that were attached to epithelium were motile.[184] In this case, binding of sperm to the epithelium seemed to be required for maintenance of viability during storage of sperm in the oviduct.

Sperm Bind to Carbohydrates on Oviductal Epithelium

Sperm binding to oviductal epithelium involves carbohydrate recognition. Fetuin and its terminal sugar, sialic acid, were found to competitively inhibit binding of hamster sperm inseminated directly into oviducts, whereas desialylated fetuin did not.[185] The sperm could be labeled over the rostral portion of the head by colloidal gold-tagged fetuin, indicating that they bind fetuin in the same region by which they bind to epithelium. Gold-tagged fetuin also labeled certain protein bands on western blots of membrane extracts from hamster sperm.[185] These observations indicate that there is a carbohydrate-binding molecule on the heads of hamster sperm that binds sialic acid and is responsible for attachment of sperm to the epithelium.

Binding of other species of sperm to conspecific epithelium was also reported to be competitively inhibited by specific carbohydrates. Binding of stallion sperm to explants of oviductal epithelium was inhibited by asialofetuin and its terminal sugar, galactose.[186,187] Bull sperm binding was blocked by fucoidan and its component fucose.[90] Pretreatment of bovine oviductal epithelium with fucosidase, but not galactosidase, also reduced sperm binding.[90]

The binding of bull sperm to oviductal epithelium was the first to be more fully characterized. Fucose in an *alpha* 1–4 linkage to *N*-acetylglucosamine, as in the trisaccharide Lewis-a, inhibited binding to oviductal epithelium more efficiently than fucose in monosaccharide form or other linkages. Furthermore, Lewis-a tagged by conjugation to fluorescein-labeled polyacrylamide bound to the heads of live bull sperm.[188]

Recently, the development of glycan arrays enabled rapid and detailed identification of glycan motifs to which boar sperm bound. Boar sperm bound directly to variants of Lewis-X trisaccharide and also to a branched structure with a mannose core and antennae terminating in sialylated or unsialylated lactosamine.[189] Mass spectrometric analysis of extracts of isthmic epithelium from porcine oviducts identified these motifs in *N*- and *O*-linked glycans and glycolipids. Sialylated lactosamine was identified by SNA lectin on apical surfaces of mucosal epithelium in both the isthmus and ampulla of the porcine oviduct. Labeled sialylated lactosamine bound to heads of boar sperm; labeling was reduced after capacitation, indicating that loss of sialylated lactosamine groups from sperm heads could play a role in release of sperm from the reservoir.[189]

Altogether, studies of carbohydrate involvement in sperm binding to epithelium indicate that the phenomenon is widespread among eutherian mammals, although the particular carbohydrate moiety comprising the binding site varies according to species. These species differences may not seem so unusual when one considers that a single amino acid residue can determine the ligand specificity of a lectin[190,191] and that closely related animal lectins have different carbohydrate specificities.[192]

Sperm Ligands and Oviductal Receptors

In order to identify the ligand on bull sperm that binds them to oviductal receptors, the oligosaccharide that blocked sperm binding to oviductal epithelium, Lewis-a,

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was used to affinity purify potential ligands from extracts of bull sperm plasma membranes. A protein of approximately 16.5 kDa was pulled down from the extracts and identified by amino acid sequencing as BSP1 (Binder of SPerm 1, formerly known as PDC-109), which is a secretion of bovine seminal vesicles that becomes adsorbed onto sperm (Figure 5.15 (A–D)).[194] BSP1 purified from seminal plasma competitively inhibited bull sperm binding to oviductal epithelium. Bovine epididymal sperm lack BSP1 on their membranes (Figure 5.15) and, indeed, show only minimal binding to oviductal epithelium in vitro. However, when epididymal sperm were incubated with purified BSP1, then washed to remove unadsorbed protein, and then added to explants of oviductal epithelium, incidence of sperm binding was raised to the level of ejaculated sperm.[193]

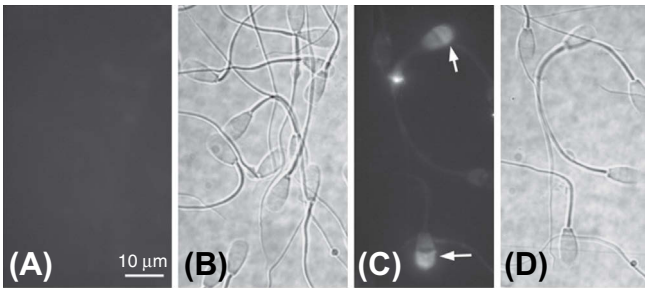

FIGURE 5.15 Bovine epididymal sperm adsorbed BSP1 over the acrosomal region of the head plasma membrane, shown using anti-BSP1. (A) Epididymal sperm without BSP1 show no antibody label. (B) Brightfield image of (A). (C) Epididymal sperm previously treated with BSP1 are labeled over the acrosomal region (*arrows*). (D) Brightfield of (C). *Source: From Ref. 193.*

BSP1 is the most abundant protein in bovine seminal plasma, present at concentrations of 15–20 mg/ml.[195] It occurs in two forms: BSP-A1 and BSP-A2. BSP-A1 possesses a single trisaccharide, NeuNAc-Gal-GalNAc, that is *O*-linked via GalNAc to threonine on residue number 11 in the N-terminal domain,[196] while BSP-A2 lacks the trisaccharide. The N-terminal domain is followed by two fibronection type II (FN2) domains.[197] BSP1 binds to choline phospholipids via short hydrophobic sequences within its fibronectin domains.[198] This is thought to be the mechanism by which it adsorbs onto epididymal sperm when they are exposed to secretions of the seminal vesicles.[199,200]

It was later reported that two other BSP proteins, BSP3 (formerly known as BSPA3) and BSP5 (formerly known as BSP30K), each acting alone, can also induce sperm binding to oviductal epithelium.[201] BSP1, BSP3, and BSP5 are each comprised of a unique N terminal domain, followed by two FN2 domains with heparin and phospholipid binding sites (Figure 5.16).[201] BSP3 and BSP5 are also produced exclusively by the seminal vesicles, but in concentrations only about one-tenth that of BSP1.[202] Because each BSP can act alone to bind sperm to epithelium, it is not required that they form a complex in order to do so. If each BSP acts alone, each may play a slightly different role in holding sperm in the reservoir, maintaining sperm functionality during storage in the reservoir, or in regulating the release of sperm from storage.

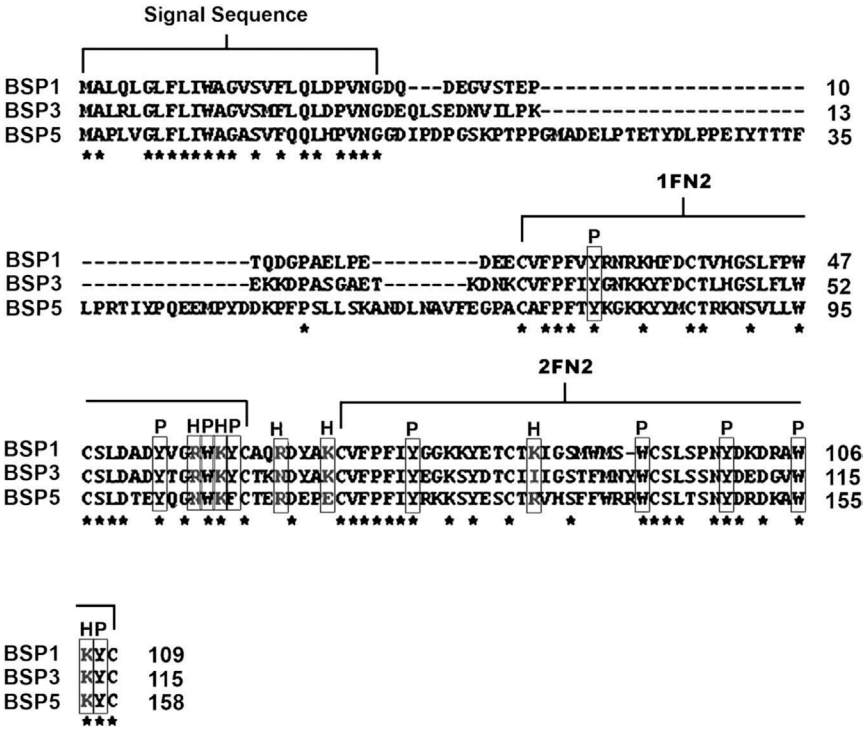

FIGURE 5.16 Amino acid sequence alignments of BSP1, BSP3, and BSP5. Residues conserved among all three proteins are indicated by asterisks. The FN2 domains are indicated above the sequences. P, aromatic residues that bind phosphorylcholine; H, heparin-binding residues. *Source: From Ref. 201.*

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Homologs of the bovine BSP proteins have been identified in a wide array of mammalian species, including rodents, ruminants, and humans. The homologs of humans and mice are expressed in the epididymis rather than in the seminal vesicles and would therefore coat epididymal sperm.[203]

Oviductal receptors for the BSP proteins on sperm were tentatively identified by using each BSP protein as bait in affinity columns to pull proteins from extracts of apical membranes of bovine oviductal epithelium. Oviductal protein bands of approximately 34 and 38 kDa were obtained from the columns and identified by tandem mass spectrometry as annexins (ANXA) 1, 2, 4, and 5. Antibodies to each of the four ANXA proteins blocked sperm binding to explants of oviductal epithelium and labeled the apical surfaces and cilia of the mucosal epithelium in histological sections of bovine oviduct.[204] Western blots confirmed the presence of annexins in apical plasma membranes of oviductal epithelium.[204]

Preserving Sperm Fertility during Storage

The oviductal mucosa protects sperm against aging damage during storage. Sperm incubated with oviductal epithelium in vitro remain viable longer than when they are incubated in medium alone (porcine,[145] equine,[205] human,[170] canine[171]) or with tracheal epithelium (bovine[168]). Viability can even be extended by incubating sperm with vesicles prepared from the apical membranes of oviductal epithelium (rabbit,[206] equine,[207] human[208]), indicating that the epithelium can produce the effect by direct contact with sperm. It was reported that equine sperm binding to epithelium or membrane vesicles maintained low levels of cytoplasmic Ca^{2+} when compared with free-swimming sperm, sperm attached to Matrigel, or sperm incubated with vesicles made from kidney membranes.[207] Equine and human sperm incubated with oviduct membrane vesicles also capacitated more slowly than sperm incubated in capacitating medium alone, when capacitation was assessed by chlortetracycline fluorescence patterns.[207,208] Possibly, sperm viability is maintained by inhibition of capacitation and its concomitant rise in cytoplasmic Ca^{2+}. The mechanism for preventing rises of cytoplasmic Ca^{2+} in sperm are not known, but one suggestion is that catalase, which is present in the oviduct, serves to protect against peroxidative damage to the sperm membranes,[209] which could increase the permeability of membranes to Ca^{2+}.

The oviductal binding protein on bull sperm, BSP1, probably preserves sperm by acting to stabilize sperm plasma membranes. By adding spin-labeled lipids to membranes in order to assess membrane fluidity by electron spin resonance spectroscopy, it was determined that addition of BSP1 to phospholipid membranes reduces membrane fluidity and immobilizes cholesterol in artificial membranes and in membranes of epididymal sperm.[210,211] BSP1 can also contribute to membrane stability by inhibiting the activity of phospholipase A_2.[212,213] Thus, BSP1 may play a role in preserving sperm fertility while they are stored in the reservoir.

Release of Sperm from the Reservoir

Theoretically, sperm could be released from the reservoir either by loss or alteration of receptors on the epithelium or by loss or alteration of ligands on sperm. Changes in the hormonal state of oviductal epithelium related to impending ovulation were not found to affect the density of oviductal binding sites for bull sperm[177]; therefore, it appears that epithelium does not release sperm by reducing available binding sites, at least in cattle. Instead, current evidence indicates that a change in the sperm brings about its release.

As part of the complex process of capacitation, which prepares sperm to fertilize oocytes, sperm undergo two changes that may play a role in their release from the reservoir: modification of the plasma membrane and motility hyperactivation (see Chapter 4). Modification of proteins on the surface of the plasma membrane overlying the acrosome could reduce binding affinity for receptors on the oviductal epithelium. Motility hyperactivation is a change in the flagellar beating pattern of sperm that is characterized by increased flagellar bend amplitude and asymmetry of the beat (Figures 5.17 and 5.18).[214,215] Hyperactivation could provide the force necessary for overcoming the adhesion between sperm and oviductal epithelium.[215]

Hamster sperm that had undergone both capacitation and hyperactivation in vitro did not bind to epithelium when infused into hamster oviducts, while uncapacitated/unhyperactivated sperm did bind.[178] When bull sperm were incubated in bovine sperm capacitation medium[216] (a buffered balanced salts medium containing energy substrates, bovine serum albumin, Ca^{2+}, bicarbonate, and heparin) and were then added to explants of oviductal epithelium, binding was significantly reduced.[217] In this case, the sperm were capacitated in the sense that they could undergo physiological acrosome reactions, but they were not hyperactivated; therefore, it was concluded that capacitation reduces binding affinity of sperm for epithelium. Taken together, these observations indicate that capacitation-induced changes in the surface of the sperm head are responsible for loss of binding affinity, although the pulling force produced by hyperactivation can enhance the ability of bound sperm to detach from the epithelium.

Oviducts removed from mated mice can be transilluminated in order to examine the behavior of sperm within the reservoir. Under these conditions, it was noted that only hyperactivated mouse sperm detached from epithelium.[218] Hyperactivated mouse sperm appeared

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to use a rocking motion to tear themselves off of the cilia on the epithelial surface.[219]

Hyperactivation of sperm can also assist their escape from the reservoir by propelling them through mucus in the oviductal lumen. Mucus that fills the uterotubal junction also extends into the isthmus in rabbits,[142,143] pigs,[144] dairy cattle,[138,145] and humans.[146] Hyperactivated sperm penetrate artificial mucus, such as viscoelastic solutions of long-chain polyacrylamide or methyl cellulose, far more effectively than nonhyperactivated sperm[220–222]; therefore, they are better equipped to swim through oviductal mucus (Figure 5.17).

Even in the absence of mucus, hyperactivation can assist escape from the reservoir, because it endows sperm with greater flexibility for turning around in the pockets formed by folds in the oviductal mucosa (Figure 5.18).[214,215,223,224]

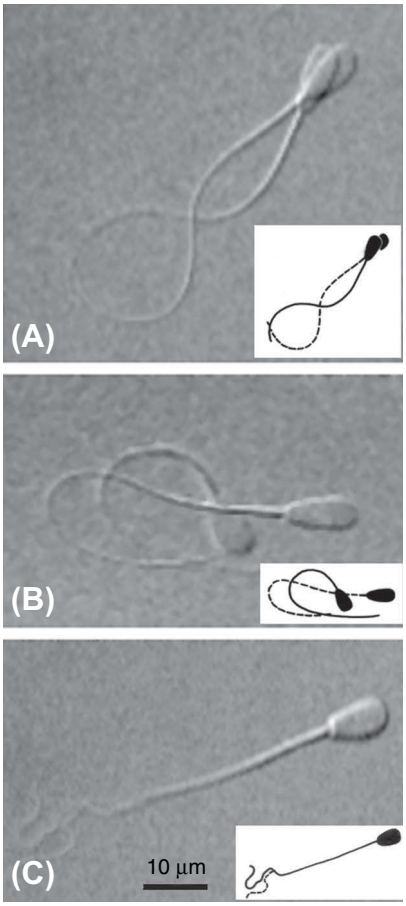

FIGURE 5.17 Video images of swimming patterns of bull sperm on glass slides. Sperm were illuminated by flashes from a xenon stroboscope at 60 Hz and images were captured at 30 Hz. Traces of the images are shown in the insets. (A) The symmetrical flagellar beat pattern of activated (progressive) bull sperm in aqueous medium. (B) The asymmetrical beat pattern of a hyperactivated sperm. (C) The progressive swimming of a hyperactivated sperm through a thick, viscoelastic solution of long-chain polyacrylamide. *Source: From Ref. 214.*

While evidence is lacking for a release mechanism involving reduction in binding sites on the epithelium, the epithelium could play a role in sperm release by secreting factors that affect sperm. For example, hormonal signals that induce ovulation or signals from the preovulatory follicle could stimulate the oviductal epithelium to secrete factors that hasten sperm capacitation and trigger hyperactivation, thereby bringing about sperm release. Soluble oviductal factors have been reported to enhance capacitation of bull sperm in vitro.[225,226]

As sperm undergo capacitation, the carbohydrate-binding molecules responsible for binding sperm to the epithelium may be lost from the sperm surface or modified so as to lose affinity for specific receptors on

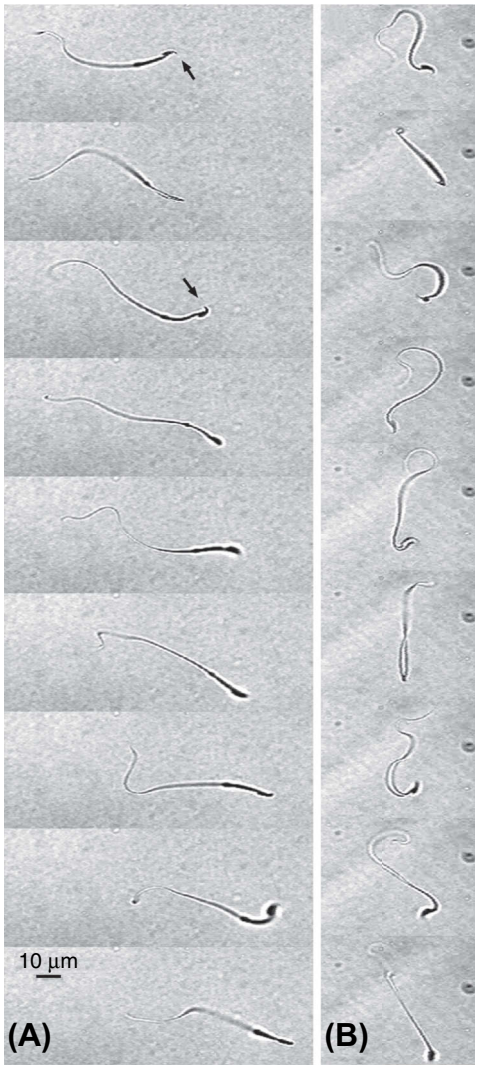

FIGURE 5.18 Phase contrast sequential video images of (A) activated (progressive) and (B) hyperactivated mouse sperm. Consecutive images were collected at 30 Hz. Each series begins at the top of the figure. Rotation of the head (rolling) can be envisioned by noting the position of the hook of the head. Note the increased flexing of the hyperactivated sperm. *Source: From Ref. 214.*

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the oviductal epithelium. Hamster sperm capacitated in vitro no longer labeled over the acrosomal region with fetuin,[185] indicating that they had lost the ability to bind to oviductal epithelium via sialic acid. Similarly, electrophoretically resolved proteins extracted from capacitated hamster sperm showed diminished fetuin labeling on blots, compared with proteins extracted from uncapacitated sperm.[185]

Capacitated bull sperm showed reduced binding to oviductal epithelium,[217] as well as to fucose, as measured using fluorescein-labeled fucosylated bovine serum albumin.[194,227] The loss of binding affinity for the oviductal epithelium could be accounted for by a loss of the adsorbed BSP proteins from the sperm head. When BSP1 coats sperm by binding to choline phospholipids in the plasma membrane, it forms homodimers with heparin-binding sites exposed.[196,228] Heparin is used to capacitate bull sperm in vitro,[216,229,230] and there is some evidence that BSP1 is lost from the sperm surface during heparin-induced capacitation.[231] BSP3 and BSP5 also have heparin binding sites.[201] Addition of heparin to cocultures of bovine sperm and oviductal epithelium enhance the release of sperm from epithelium.[232] In vivo, as the time of ovulation approaches, an increase of heparin-like glycosaminoglycans in oviduct fluid could serve to release sperm. Oviduct fluid collected from cows in the periovulatory period showed a peak in heparin-like biochemical activity, as well as a peak in capacitation-inducing activity.[229]

Although BSP1, BSP3, and BSP5 can each attach sperm to oviductal epithelium, their response to capacitation and the specific roles they may play in holding and releasing sperm could vary due to differences in their amino acid sequences. The N-terminal domain of each of the three BSP proteins is unique. The two FN2 domains, each of which contains binding sites for membrane phospholipids and heparin, differ somewhat in sequence.[203] In fact, like many proteins specific to the reproductive system, members of the BSP protein family are undergoing rapid evolutionary change.[203] It was proposed that reduced binding to oviductal epithelium is due in part to loss of BSP proteins during capacitation. Because of differences in amino acid sequences, it was predicted that each BSP would respond differently to capacitating conditions. To test whether all three BSP proteins were lost from sperm during capacitation and whether the kinetics of loss differed among the three bovine BSP proteins, ejaculated bull sperm were incubated under various capacitating conditions, and then the amounts of BSP proteins remaining on the sperm were assayed by western blotting. Capacitation was assayed by analysis of protein tyrosine phosphorylation. While loss of BSP1 was not detected in these experiments, most BSP5 was lost from sperm even during incubation under minimally capacitating conditions. Surprisingly, antibodies against BSP3 detected a new protein band of reduced molecular mass in extracts of incubated sperm. Its identity was confirmed as BSP3 by mass spectrometry, indicating that BSP3 undergoes modification while on the sperm surface.[233] Because BSP3 is not glycosylated, the reduction in size cannot be attributed to deglycosylation. Metalloproteases and serine proteases have been identified on the surface of sperm[233,234]; therefore, the mass reduction could be due to proteolytic cleavage. Altogether, the different responses of the three BSP proteins on bull sperm indicate that a complex process is involved in the release of sperm from the reservoir.

The development of Acr-EGFP mutant mice that produce sperm with fluorescent acrosomes[11] facilitated the study of sperm movement into the ampulla of the oviduct. Periovulatory wild-type female mice were mated with males who produced the fluorescent sperm and the oviducts were removed 3 h later for observation of sperm. Hyperactivated sperm in the isthmic reservoir detached frequently from the epithelium and then reattached. Unexpectedly, most of the few sperm found in the ampulla were also attached to epithelium, often for several minutes (Figure 5.19). Some sperm detached from epithelium in both the ampulla and isthmus during strong contractions of the oviduct. Blockage of oviduct contractions with nicardipine, however, did not prevent sperm from reaching the ampulla, indicating that contractions are not required for moving sperm. These observations show that sperm continue to bind to oviductal epithelium as they move up the oviduct,[219] and they agree with earlier reports that bull sperm can bind to ampullar epithelium.[144,177,179]

Summary: Entrapment and Release of Sperm in the Reservoir

In summary of what is known about sperm movement in the oviduct, the following picture emerges. The sperm reservoir forms in the uterotubal junction and/or the

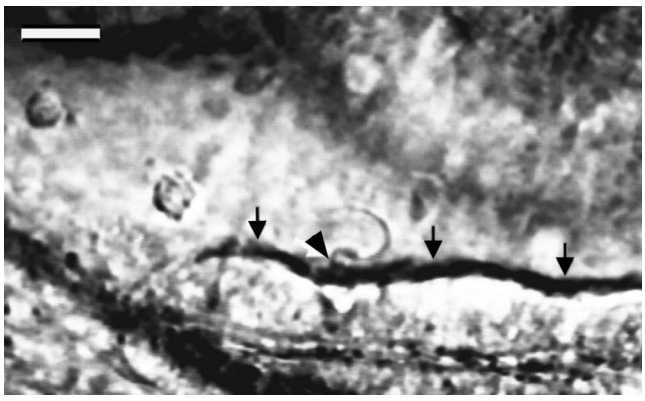

FIGURE 5.19 A mouse sperm attached by the hook of the head (*arrowhead*) to cilia on a ridge of epithelium (*arrows*) in the oviductal ampulla. Bar = 20 µm. *Source: From Ref. 219.*

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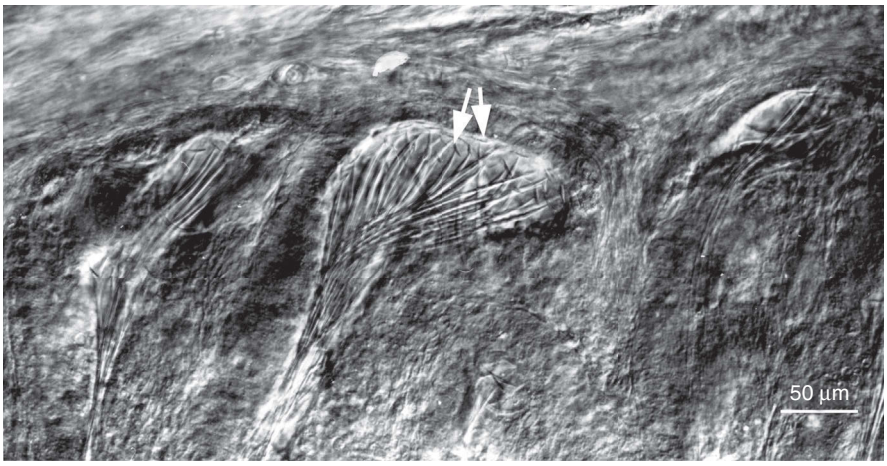

FIGURE 5.20 Sperm in mucosal crypts in the isthmic region of an oviduct from an Australian marsupial, *Sminthopsis crassicaudata*. *Arrows* point to some sperm heads. Nomarski optics of a fresh specimen. Methods described in Ref. 237. *Source: Provided by J. Michael Bedford.*

caudal isthmus when a carbohydrate-binding molecule on the sperm head adheres to a glycosylated receptor on the oviductal epithelium. The narrowness of the oviductal lumen and mucus within the lumen can enhance sperm binding by slowing their progress and increasing their contact with the epithelial surface. Binding to the mucosal epithelium prolongs sperm survival and delays capacitation. As the time of ovulation approaches, capacitation is likely initiated by secretions in the oviduct fluid. The coating of oviduct-binding molecules on the surface of sperm is modified during capacitation, thereby changing binding affinity of sperm for the epithelium. As the binding affinity of sperm is modified, hyperactivation provides them with the force necessary to pull away from the oviductal epithelium. Nevertheless, sperm continue to bind intermittently to oviductal epithelium as they move toward the site of fertilization.

Oviductal Sperm Storage in Marsupials and Insectivores

In some marsupial mammals,[235,236] sperm are stored in special mucosal crypts in the oviduct (Figures 5.20 and 5.21). However, sperm within the crypts do not attach to the epithelium. Many of the sperm in the crypts of the marsupial *Sminthopsis crassicuadata* were observed to be immotile[237]; therefore, motility suppression may serve to keep sperm in the crypts until ovulation. In other marsupials, however, sperm appear to attach to oviductal epithelium.[239]

In primitive eutherian mammals in the order Insectivora, some species of shrews and moles possess isthmic sperm storage crypts,[240] while others store sperm in bubble-like outpocketings of the ampullar wall (Figure 5.22).[241,242] Sperm found within these structures do not bind to the epithelium. Perhaps the most complex oviductal sperm storage system is that of the white-toothed shrew (*Crocidura russula*) and the African shrew (*Myosorex varius*). In these insectivores, sperm inhabit crypts in the isthmus prior to ovulation and move up to bubble-shaped outpocketings in the ampulla at about the time of ovulation (Figure 5.23). Sperm seen in the isthmic crypts have slowly beating flagella; whereas, sperm in the ampullar structures have rapidly beating flagella.[243,244]

Another insectivore, the African pygmy hedgehog (*Atelerix albiventris*), lacks distinctive oviductal sperm storage structures, but little is known about how it stores sperm.[245] More advanced eutherian mammals, such as mice, cows, and pigs, also lack distinctive storage structures. In the "advanced" species, sperm bind to epithelium primarily in grooves created by folds of mucosa. It is curious that distinctive storage structures would be lost and sperm binding would evolve to replace them as a mechanism of sperm storage.

Sperm Storage in Women

So far, no conclusive evidence has emerged for a distinct oviductal sperm reservoir in humans.[246] While associations of human sperm with oviductal epithelium have been observed in vitro, the sperm do not seem to bind as quickly or as tightly to the epithelium as those of nonprimate mammals.[208,247–250] On the other hand, human sperm viability is maintained by incubation with oviductal epithelium,[208] as it is in species in which there is immediate and strong binding of sperm to epithelium.[168,169,171]

The human cervix has been designated a sperm reservoir; however, as discussed previously, the evidence is not robust. Leukocytic infiltration of the uterus becomes significant several hours after coitus, with leukocytes outnumbering sperm at about 24 h postcoitus.[251] Unless sperm are protected from phagocytosis (and they might be!), it is unlikely that they could be stored for more than a few hours in the cervix and still reach the site of fertilization in the oviduct.

Human sperm could be stored for long periods of time in the oviduct, but not in a distinct reservoir and not as a result of binding tightly to the mucosal surface. The

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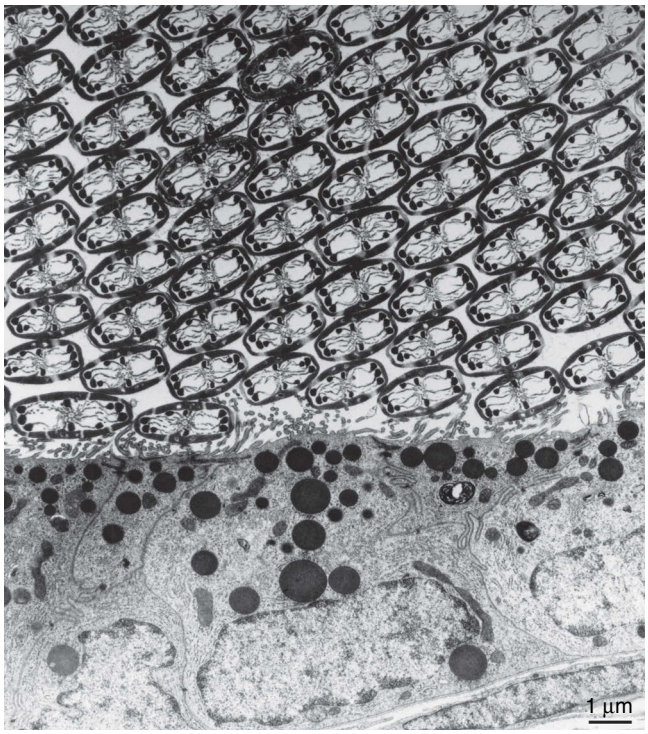

FIGURE 5.21 Transmission electron micrograph of sperm in an isthmic crypt of a recently mated Australian marsupial, *Sminthopsis crassicaudata*. Cross sections of tightly packed sperm tails are seen above sections of isthmic epithelium. *Source: From Ref. 238.*

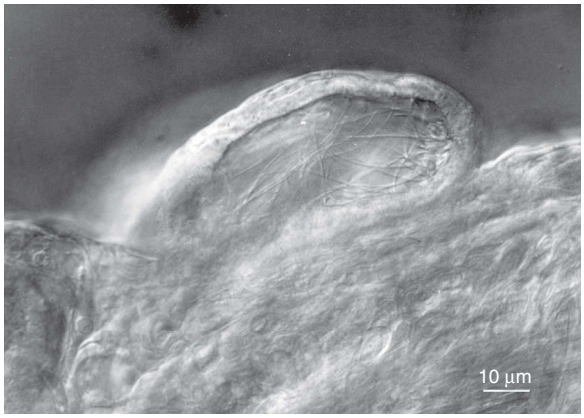

FIGURE 5.22 A transilluminated bubble-shaped outpocketing of the wall of the oviductal ampulla of the insectivore *Cryptotis parva*. The flagella of the sperm within the pocket were beating vigorously before they were fixed in 2% glutaraldehyde for the purpose of photography. *Source: From Ref. 241.*

mucosal folds of the human oviductal lumen increase in height and complexity toward the ovary (Figure 5.24), thereby producing increasingly greater obstacles to the advancement of sperm. Sperm progress could also be slowed by the mucus in the lumen[146] and by sperm sticking lightly to the epithelium.[248,249] So, rather than having a distinct reservoir, human sperm advancement to the

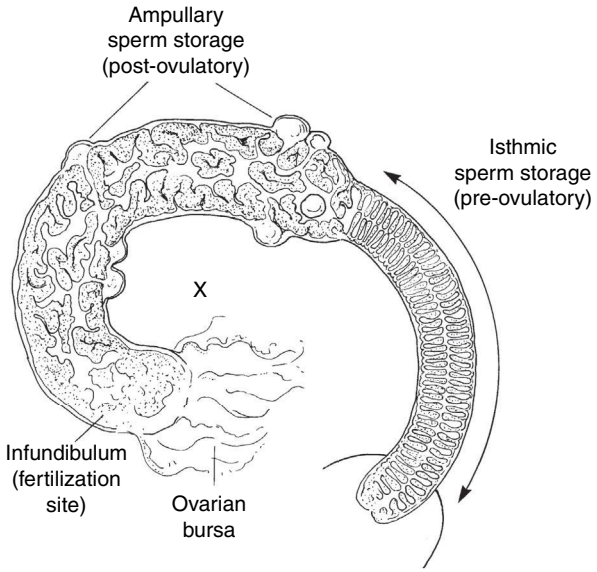

FIGURE 5.23 Diagram of the main features of the oviduct of the shrew, *Crocidura russula* monacha. Crypts are seen in the wall of the isthmus, while bubble-shaped outpocketings are seen on the surface of the ampulla. The oviduct is approximately 5 mm long. (X) indicates the location of the ovary. *Source: From Ref. 243.*

site of fertilization could be slowed in such a manner so as to increase the chances that a few will be present at the site of fertilization when ovulation occurs hours to days after insemination. Muscular contractions and secretions at the time of ovulation could move or activate sperm and increase chances of encountering the oocyte.

Reports of sperm distribution in the oviducts of women have not provided a clear picture of the events of sperm transport. Numbers of sperm recovered at various times in different regions of the human oviduct have varied so much that the data do not permit the construction of a model for the pattern of sperm storage and transport in women.[246] Perhaps fertilization is a relatively inefficient and unregulated process in humans, because coitus has taken on an additional role of promoting long-term pair bonding in addition to promoting fertilization success.

The Fate of Nonfertilizing Sperm in the Oviduct

After fertilization, mammalian sperm may be phagocytosed by isthmic epithelial cells (Figure 5.25)[243,253,254] or may be eliminated into the peritoneal cavity[255] and then phagocytosed. Phagocytosis within the oviduct may be employed by species, such as mice, which have an extensive ovarian bursa that would limit passage of sperm into the peritoneal cavity.

Sperm Chemotaxis in the Oviduct

Chemotaxis is the directional movement of a cell or organism along a gradient of an attractant produced

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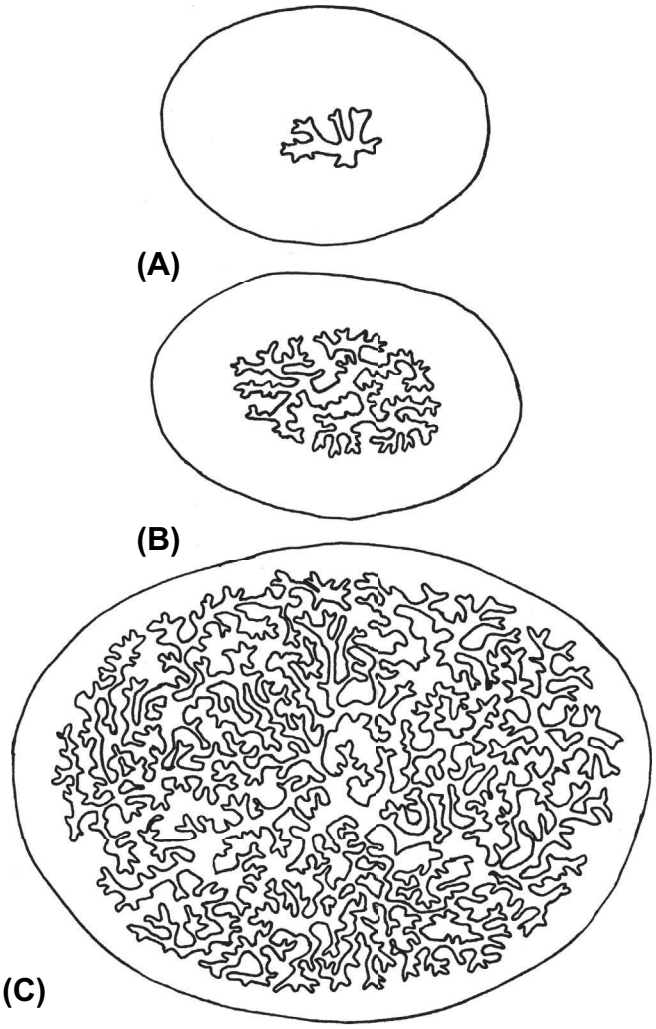

FIGURE 5.24 Diagram illustrating the increasing complexity of the human oviductal lumen from the uterotubal junction (A) to the isthmus (B) to the ampulla (C). *Source: Illustration by C. Rose Gottlieb.*

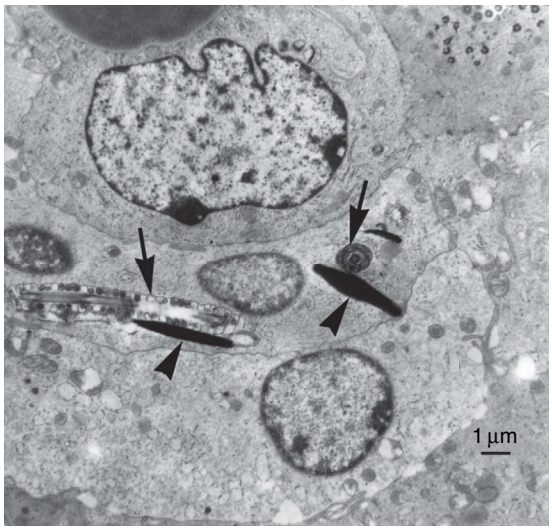

FIGURE 5.25 Phagocytosis of surplus sperm by isthmic epithelium. Transmission electron micrograph of mouse isthmic epithelium, taken 15–22 h after coitus. Flagella (*arrows*) and sperm heads (*arrowheads*) can be seen in the cytoplasm of the epithelial cells. Methods described in Ref. 253. *Source: Provided by Joana Chakraborty.*

by another cell or organism. In vitro, human and rabbit sperm have been reported to turn toward or accumulate in a gradient of follicular fluid.[252,256–259] Progesterone, which is present in follicular fluid and also secreted by the cells of the cumulus oophorus, has been proposed to be a chemoattractant for human and rabbit sperm.[260,261]

Odorant receptors unique to sperm have been localized to a spot on the base of the flagellum of canine,[262] rat,[263] and human sperm.[264] Mark Spehr's group screened components of a complex mixture of 100 odorants to see if any evoked a physiological response in human sperm. Eventually, they identified the floral odorant "bourgeonal" as a potential chemoattractant for human sperm.[264] The results of their first experiments have been validated; however, neither bourgeonal, nor an equivalent odorant compound with sperm chemoattractive activity, has been identified in mammalian eggs or reproductive tracts.

Sperm are equipped with mechanisms for turning. One is that they can switch back and forth between symmetrical flagellar beating and the asymmetrical flagellar beating of hyperactivation. Hyperactivation is triggered by a rise in cytoplasmic Ca^{2+} and has been reversed in mouse sperm in vitro.[265] Reversing and reinitiating hyperactivation by lowering and raising cytoplasmic Ca^{2+} levels would enable sperm to alternate between turning and swimming straight ahead. A second mechanism for turning has been observed in mouse sperm, that is, the switching of the orientation of the deep asymmetrical bends of hyperactivation from one side of the flagellum to the other. In vitro, treatment of mouse sperm with procaine or 4-amino pyridine to trigger Ca^{2+} influx induces hyperactivation characterized by the formation of deep bends in the same orientation as the hook of the sperm head; whereas, treatment with thimerosal to trigger release of Ca^{2+} from internal stores induces formation of deep flagellar bends in the direction opposite to the orientation of the hook of the sperm head.[219] Nevertheless, the identities of signal or signals from the egg or oviduct that trigger switching between symmetrical and asymmetrical flagellar beating patterns, or switching the direction of the deep asymmetrical flagellar bends, have not been established.

Chemotactic guidance of mammalian sperm toward oocytes, if it exists, is likely to be limited to short distances within the oviduct, because gradients established in oviductal fluids would be disrupted over longer distances by the loops of the oviduct, the folds in the lining of the oviduct, muscular contractions, and ciliary

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currents. In the well-established example of chemotaxis of sperm of the sea urchin *Arbaci punctulata* in gradients of resact, a peptide from the jelly layer of the egg, the calculated effective range of resact diffusing radially out from the egg is only about 0.4 cm, even though the sperm can respond to as little as a single molecule of resact.[266] If translated to humans, this effective distance would not reach the lower isthmus from the fertilization site. Babcock[267] suggested that human sperm could be guided into the cumulus mass from nearby mucosal pockets in the oviductal ampulla. In other species, such as mice, large cumulus masses fill the width of the ampullar lumen (Figure 5.13). In these species, chemoattraction could serve to guide sperm from the edge of the cumulus mass to the oocytes deep within the mass, if the oocyte released an attractant that formed a gradient within the cumulus.

Chemotaxis has been well established in several species of marine invertebrates. This is because the phenomenon is easily observed in vitro. When a gradient of chemoattractant is introduced into a dish containing high concentrations of sperm, a swarm of sperm can immediately be seen to move up the gradient.[266] No movies or images of this kind of behavior have yet been published for mammalian sperm. It has been proposed that the reason for this is that only a small fraction of sperm is capable of responding to chemoattractant at any given time. If this hypothesis is correct, then it is challenging to obtain an adequate sample size to detect small but significant differences between control and treatment samples. Recently, block bootstrapping statistical procedures have been adapted for detecting differences in the distribution of angles of sperm swimming direction with respect to the orientation of a chemotactic gradient. These procedures were used to detect chemotactic responses of human sperm to progesterone in gradients where the maximum progesterone concentrations were 10 and 100 pM.[268]

On the other hand, the very low percentages of chemotactic responses reported for samples of mammalian sperm could be due to the conditions of the experiments performed in vitro. Some experiments have been carried out at room temperature, rather than the temperature of the oviduct, and this could reduce the ability of sperm to respond. Other experiments have been carried out using putative attractants under conditions that could induce acrosome reactions and therefore cause sperm to stick to the walls of the chemoattractant chamber in the regions of high concentrations. Still other experiments have been carried out in chambers in which chemotaxis is assayed by the number of sperm that pass through a screen into a separate chamber. These experiments could be measuring entrapment rather than chemotaxis. If the agent being tested triggers continuous hyperactivation, rather than true chemotaxis, sperm that become hyperactivated in the separate chamber could become trapped there. Lastly, it has been reported that sperm must be capacitated in order to respond to chemoattractants. In vitro, sperm do not capacitate synchronously, which could explain the low incidence of chemotaxis detected in experiments. Nevertheless, the synchronicity of capacitation of sperm in the oviductal reservoir is unknown at present.

In addition to possible chemoattractants already mentioned, a variety of other potential chemoattractants have to date been put forth in a number of publications. As improved chambers and statistical methods become available to test for chemotaxis, one or more of the proposed attractants may turn out to be true physiological chemoattractants.

An alternative, or additional, mechanism for guiding sperm to the site of fertilization is that of chemical guidance provided by molecules on the mucosal surface of the oviduct. For example, three different BSP proteins have been reported to bind sperm to the oviductal wall, and four different annexin proteins have been tentatively identified as receptors for the BSP proteins. Because the composition of the coat of BSP proteins changes during capacitation in vitro,[233] the interaction of sperm with the oviductal epithelium would change concurrently, perhaps resulting in release and then reattachment of sperm further up the oviduct. Sperm have been observed to attach to epithelium in both the isthmus and ampulla.[144,177,179] In the mouse oviduct, sperm have been observed to detach from and reattach to the oviductal epithelium as far up the oviduct as the ampulla.[219] These observations provide preliminary evidence for a solid phase chemical guidance system for sperm.

To date, the reports of chemotaxis in mammalian sperm have been intriguing and have justifiably stirred up excitement; however, much remains to be learned about whether and how the chemotaxis occurs.

In addition to chemical attractants, the movement of sperm could be guided by physical factors. The tendencies of sperm to move along walls and against gentle fluid flows could be utilized by the female to guide sperm to and through the oviduct. Microchannels designed on the basis of observed sperm interactions with wall geometries have succeeded in guiding the movement of human sperm (Figure 5.26).[22] Evidence has been presented to demonstrate that sperm are thermotaxic; however, there is controversy about whether sperm could be guided by thermotaxis in vivo.[269] Physical factors might function less efficiently than chemical gradients but could still be sufficient to ensure that enough sperm reach the oocytes in a timely manner.

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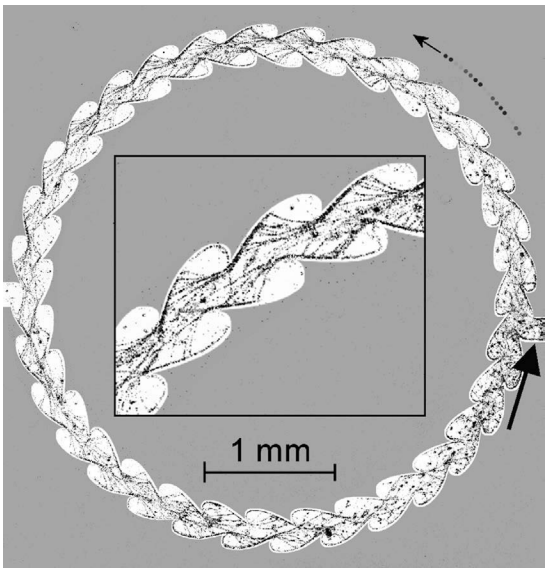

FIGURE 5.26 Human sperm in a microchannel designed to funnel them to swim in a counterclockwise direction (*curved arrow*). The gray shading indicates the position of the walls, but is placed 10–20 μm back from the actual walls so as not to interfere with viewing the tracks of the sperm. The input channel for sperm is on the right (*large, straight arrow*). The insert shows a higher magnification of tracks of sperm swimming within the microchannel. The dots represent locations of sperm heads. Images were collected at four frames per second, and the location of sperm heads in 200 consecutive images were summed to produce this figure. Notice that sperm tend to swim in a certain pattern in the channel. *Source: From Ref. 22.*

OOCYTE AND EMBRYO TRANSPORT IN THE OVIDUCT

In many mammalian species, oocytes progress from prophase of the first meiotic division to metaphase of the second meiotic division just before ovulation (see Chapter 1). Most then arrest in the second metaphase until fertilization activates completion of meiosis.[270] Oocytes are usually fertilized soon after entering the oviduct. If mating or artificial insemination is delayed until the time of ovulation or after, the incidence of pregnancy is reduced and the occurrence of developmental anomalies is increased.[271] The most common anomalies detected after delayed insemination are polyspermy (multiple sperm fertilizing a single oocyte), polygyny (failure to complete the second meiotic division and extrude the second polar body), and disintegration of the oocyte chromosomes.[271,272] Aged unfertilized oocytes may undergo a spontaneous, though abnormal, form of activation, eventually leading to cell death.[273,274] Aging reduces developmental competence of oocytes by altering Ca^{2+} homeostasis and signaling, including reduction of Ca^{2+} levels in the endoplasmic reticulum and of IP_3-induced release of Ca^{2+} from intracellular stores.[275] Mitochondrial function and actin distribution in the oocyte cytoplasm are also altered by aging.[275]

Female mice inseminated 10 h after ovulation had shortened gestation length and decreased litter size. Their pups had a higher perinatal death rate, growth retardation, and other developmental delays.[276] In the CF-1 mouse strain, oocyte abnormalities begin to appear about 4 h after ovulation or about 16 h after hCG administration,[273] although the fertile lifespan of oocytes in Swiss Webster mice may be 15 h or more.[272]

Early reports indicated that the fertile lifespan of oocytes is about 6 h in the rabbit,[277] 12 h in the rat,[278] 9 h in the golden hamster,[279] and 20 h in the guinea pig.[280] Ferret (*Mustela* sp.) oocytes may be fertilizable for 30–36 h.[281,282]

Oocyte Pickup

Muscle and Ciliary Action in Oocyte Pickup

There are two steps involved in oocyte transport. The first is the picking up of the oocyte from the surface of the ovary or from the ovarian bursa by the fimbria. The second is transport of the oocyte through the oviductal ampulla.

Richard Blandau's pioneering films of ovulation and oocyte pickup in rabbits inspired an appreciation of the process. The films revealed that the mesosalpinx (the segment of the broad ligament of the uterus that suspends the oviduct in the pelvic cavity) contracts rhythmically during ovulation, causing the fimbria to slide over the surface of the ovary.[7] Smooth muscle in various regions of the broad ligament moves the oviduct and ovary to aid in the positioning of the fimbria over the ovary, as do contractions of smooth muscle in the wall of the fimbria.

In some species, such as rats and hamsters, the fimbria are small and cannot sweep over the surface of the ovary. In these species, the space between the ovary and oviductal ostium is nearly completely enclosed by the mesovarium and mesosalpinx, which together form a bursa. The cumulus mass is actually ovulated into the bursa, where it is jostled by movement of the ovary and oviduct until it comes into contact with cilia on the fimbrial surface and is picked up.[7,283,284] At ovulation in the hamster, follicular contents are extruded as a long, sticky strand of cumulus, matrix, and oocyte. The strand soon makes contact with the cilia on the surface of the fimbria, drawing the rest of the mass away from the surface of the ovary. The mass is then rapidly transported over the fimbrial surface toward the ostium (Figure 5.27). Upon reaching the ostium, the mass churns in the entrance and becomes compacted before it enters the ampulla.[284,285]

Adhesive Interactions

Ciliary currents alone cannot sweep an object the size of a cumulus mass or even an oocyte into the ostium of the ampulla; such currents can only move small particles, such as *Lycopodium* spores.[286] In the eutherian

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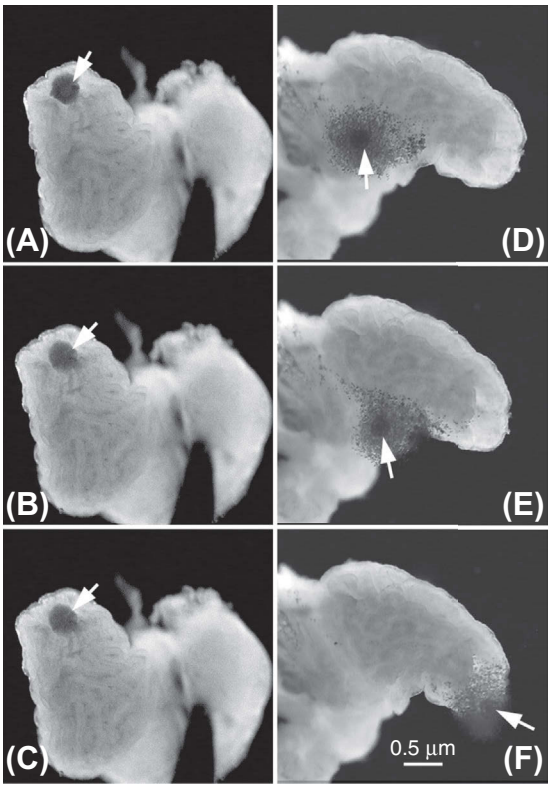

FIGURE 5.27 Video images of unexpanded and expanded hamster oocyte cumulus complexes interacting with infundibula. (A–C) The unexpanded oocyte–cumulus complex (shown 0, 3, and 6 min after being placed in the ostium) did not adhere to the surface of the infundibulum and did not undergo normal pickup. (D–F) In contrast, the expanded oocyte–cumulus complex moved across the surface of the infundibulum to the entrance into the ampulla in about 10 s. *Source: From Ref. 284.*

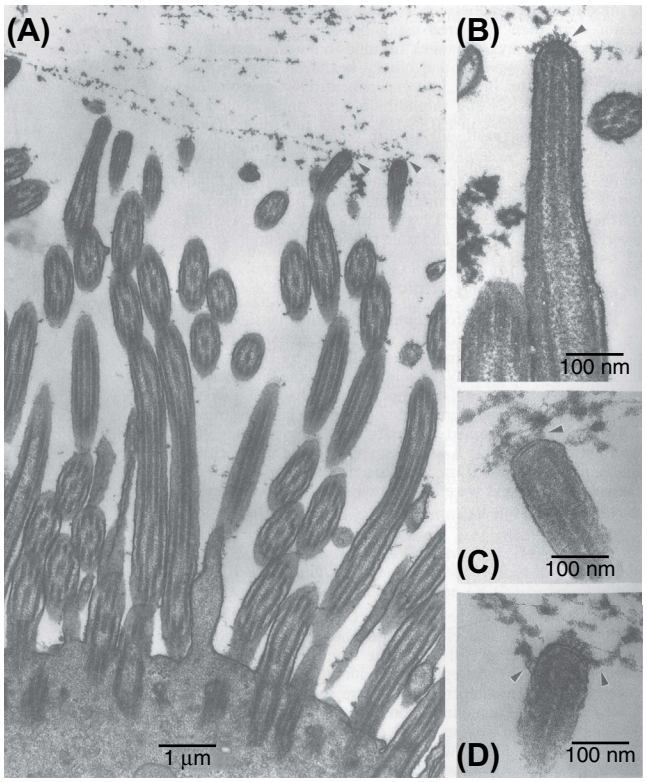

FIGURE 5.28 Transmission electron micrographs showing the interaction of infundibular cilia with the matrix of the cumulus oophorus. (A) Tips of cilia in contact with the cumulus matrix at its periphery (*arrowheads*). (B) Higher magnification of the interaction, showing the glycocalyx of the tip of a cilium (*arrowhead*). (C) Higher magnification showing granules and filaments of the matrix (*arrowhead*) associating with the tip of a cilium. (D) Another high magnification view of matrix components associating with the tip of a cilium. *Source: From Ref. 284.*

mammals studied so far, adhesion of the cumulus mass to the cilia is required for successful pickup. The sites of interaction on the surface of the fimbria are the tips of its cilia (Figure 5.28).[284,287]

Adhesion between the cilia and cumulus-enclosed oocyte is mediated by the negative charge of sialic acid glycosyl moieties on the surface of the cilia. Polycationic molecules block oocyte pickup in the rabbit and hamster.[283,286,287] Neuraminidase pretreatment of hamster fimbria to remove sialic acid reduces pickup.[283]

On the oocyte side of the adhesive interaction, presence of cumulus is required for pickup. In rabbits and hamsters, if the cumulus and its matrix are removed from the oocyte, it is not picked up by the fimbria.[283,284]

Hyaluronic acid is the chief component of the extracellular cumulus matrix; however, it is unlikely to be the molecule directly responsible for sticking the cumulus to the fimbrial cilia. When placed on the surface of hamster fimbria, hyaluronate gel was not picked up. Furthermore, a solution of hyaluronate did not block pickup, nor did pretreatment of the cumulus with hyaluronidase prevent pickup. Cumulus-free oocytes were not picked up, even if they were coated with Na-hyaluronate.[283] In contrast, coating the cumulus-free oocytes with egg white restored pickup.[283]

Fertility of female mice was severely reduced by targeted disruption of the bikunin gene.[288] Bikunin is the light chain of proteins in the IαI family (inter-alpha-trypsin inhibitors). Heavy chains in the family, called SHAPs (serum-derived hyaluronan-associated proteins) bind directly to hyaluronic acid. IαI proteins formed from the light and heavy chains link hyaluronic acid and associated molecules together to form gels. In the mutant mice, cumulus formation was abnormal but could be significantly improved if bikunin was infused into the peritoneal cavity.[288]

TNFAIP6 (hyaladherin tumor necrosis factor-α-induced protein 6) binds to hyaluronan and forms a complex with IαI proteins.[289] It acts as a catalyst to covalently link the heavy chains of IαI to hyaluronan. Disruption of the gene encoding TNFAIP6 also severely reduced fertility of female mice. Fertility was significantly improved by injecting mouse recombinant TNFAIP6 protein into the peritoneal cavity at the time of ovulation induction

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by hCG.[289] Similarly, disruption of the gene for PTX3 (pentraxin 3) resulted in abnormal cumulus formation and female subfertility. PTX3 binds cumulus matrix protein to TNFAIP6.[14,15] In vitro fertilization was normal in *Ptx3*–/– mice, indicating that while a normal matrix is not required in vitro, it is critical in vivo. PTX3 is also present in human cumulus matrix.[15] The exact roles of these molecules in adhesion to the fimbria are unknown. They may either interact directly with molecules of the ciliary plasma membranes or they may assemble other cumulus matrix molecules into the proper molecular structure for adhesion to the cilia.

In hamsters, the adhesive attraction between cumulus mass and oviductal cilia increases 10- to 40-fold when the mass reaches the ostium. After compaction of the cumulus in the ostium, adhesive strength of the interaction decreases precipitously.[284]

While the cumulus is undoubtedly important for egg pickup in the eutherian mammals studied, a cumulus oophorus has not been found in marsupial mammals. The granulosa cells do not accompany the oocyte at ovulation.[235,238,290] In some shrews, which are considered primitive eutherian mammals, the cumulus does not have a visible matrix at the time of ovulation, although a matrix may be produced after fertilization, when the zygote is in the oviduct.[241,291]

Oocyte Transport in the Oviduct

Ovulatory Products Fit the Oviductal Lumen

Bedford noted that the diameter of the central lumen of the oviduct matches the size of the ovulatory products in mammals.[290] In most eutherian mammals, there is a large, expanded cumulus mass surrounding the oocytes, which fills a segment of the ampulla or the central channel of the ampullar lumen (Figure 5.29). Cumulus expansion is accomplished by the secretion of hyaluronic acid and other matrix materials, followed by hydration. In marsupial mammals, there is no cumulus surrounding the ovulated egg and the ampulla is narrower than those of eutherian mammals.[238] In shrews, an intermediate situation exists; that is, there is a cumulus around the ovulating oocytes, but it is not expanded.[241,243,291] Correspondingly, the ampullar lumen of shrews is intermediate in diameter and fits closely around the compact cumulus mass (Figure 5.29). So, in all cases, the oocyte and its vestments fit snugly in the ampullar lumen or central channel. These observations raise an interesting question: why did the vestments of the oocyte increase and expand? Bedford and Kim have proposed that the expanded cumulus evolved to serve to sequester sperm.[292]

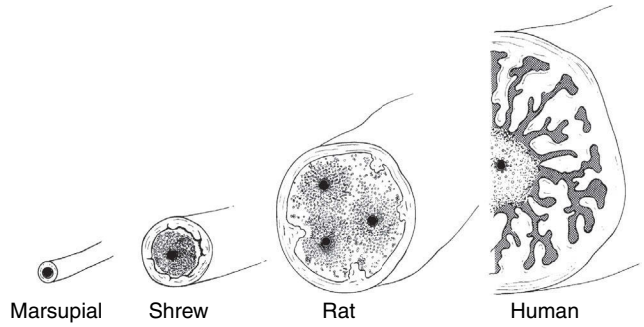

FIGURE 5.29 Diagram of the spatial relationship between the egg or egg–cumulus complex and the site of fertilization in the oviduct of various representative mammals. Where the oviduct is much larger than the egg, then the ability of the cumulus to fill the space is maximized by a variable degree of cumulus expansion. *Source: From Ref. 290.*

Muscle and Ciliary Action in Oocyte Transport

In eutherian mammals, once the cumulus mass containing one or more oocytes enters the ampulla, it moves rapidly to the ampullary-isthmic junction. Potential effectors of this movement are the oviductal musculature and the cilia.

Cilia lining the oviductal ampulla of dairy cows, sheep, guinea pigs, rats, and rabbits beat toward the uterus.[293,294] When rabbit oviductal smooth muscle contractions were blocked by isoproterenol, an agonist of beta-adrenergic receptors, the net rate of transport of cumulus-oocyte masses down the ampulla (about 0.12 mm/s) was not affected.[295] Isoproterenol was also tested in rats, where it inhibited muscle contractile activity without affecting ciliary activity or transport of surrogate ova (polystyrene microspheres suspended in egg white).[296] These observations indicate that cilia alone can move the cumulus/oocyte mass to its destination. When muscular contraction was allowed, back-and-forth motion of the mass was observed in the lower ampulla, although net movement was toward the uterus. When muscular contractions were blocked by isoproteronol, the mass moved smoothly down the ampulla.[296] Thus, while the overall rate of transport is not affected by inhibiting muscular action, the pattern of transport is affected. The back-and-forth movement could serve to enhance infiltration of the cumulus matrix with ovarian secretions or to initiate the process of cumulus removal.

In the transparent oviduct of the mouse, movement of oocytes in a cumulus mass has also been reported to move in a back-and-forth fashion through the ampulla toward the uterus. However, in contrast to the aforementioned descriptions of oocyte movement in rabbits and rats, smooth muscle contractions in the wall were shown to be required for oocyte transport. When contractions of the smooth muscle in the oviduct wall were inhibited by nicardipine, a Ca^{2+} channel blocker, movement of cumulus oocyte complexes ceased; however movement of 15–25 micron particles of apparent cellular

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debris were unaffected.[297] This indicates that, while cilia are capable of moving small particles down the oviduct, ciliary motion alone is not sufficient to move cumulus oocyte complexes at normal rates in mice. The contrasting reports of the relative roles of cilia and smooth muscles could be attributed to species differences or to differences in the spectrum of effects produced by nicardipine and isoproteronol.

There is evidence that the pendular muscle contractions of the mouse oviduct are controlled autonomously by interstitial cells of Cajal in the muscular layer of the oviductal wall, because treatment with tetrodotoxin to block nerve input did not inhibit contractions in mouse oviducts, whereas contractions were inhibited by treatment with nicardipine.[297]

Some women diagnosed as having Kartagener's syndrome (immotile cilia syndrome) are infertile,[298,299] while others are fertile.[300] This suggests that cilia are not absolutely necessary for oocyte transport in humans. Nevertheless, Kartagener's syndrome is a genetically heterogenous disorder, and some affected women have been reported to have some motile cilia.[298,299]

Embryo Transport in the Oviduct

In most eutherian mammals, the cumulus disperses soon after fertilization, while the zona pellucida remains intact. The fertilized ovum usually pauses at the ampullary-isthmic junction before resuming transport toward the uterus.[301] At the time of resumption of transport, the amplitude of isthmic contractions decreases in the rabbit, detected by insertion of a pressure-sensitive microballoon.[302]

Oviductal transport of embryos differs from that of unfertilized oocytes in some species. In the mare, unfertilized oocytes remain in the oviduct for several estrous cycles, while embryos pass into the uterus 5–6 days after ovulation.[303,304] In rats, unfertilized oocytes reach the uterus about 72 h after ovulation, while fertilized oocytes take about 96 h.[305] These observations indicate that the early embryo interacts differently with the oviductal mucosa than the unfertilized oocyte in some species, perhaps initiating signaling pathways that modulate its transport to the uterus.

In horses, there is evidence that early embryos secrete prostaglandin E2 (PGE_2), which acts to hasten the transport of embryos into the uterus. Infusion of PGE_2 into the oviducts of pregnant mares hastened the transport of both oocytes and embryos into the uterus.[306,307]

Platelet activating factor (PAF) bioactivity was detected in early hamster embryos, and PAF antagonists were found to prevent hastening of embryo transport into the uterus.[308] PAF is also produced by human embryos,[309] and receptors for PAF have been detected in the mucosal epithelium of human oviducts.[310]

The evidence supporting roles for estradiol (E2), PAF, and PGE_2 indicate that smooth muscle contraction plays a role in transport of the early embryo toward the uterus. The isthmus of the oviduct is generally lined by far fewer ciliated cells than the ampulla[293] and possesses a thicker muscular tunic.

In contrast to these findings, porcine ova fertilized by intrauterine insemination were not found to traverse the isthmus and reach the uterus any faster than unfertilized oocytes in uninseminated sows.[311]

In the rat, the time of embryo transport into the uterus is 72–94 h from the approximate time of fertilization.[312] Systemic administration of E2 at noon on day 1 of the estrous cycle in mated rats reduced the time of transport to 11–23 h.[312] Evidence indicates that E2 acts on the oviduct via both genomic and nongenomic pathways.[313–315] Nongenomic effects mainly depend on conversion of E2 to 2-methoxyestradiol, which activates cAMP-PKA and PLC-IP_3 signaling pathways in the oviduct.[315,316] Microarray analysis of gene expression revealed that the effects of E2 on the oviduct change after mating, decreasing activation of the nongenomic pathway and increasing gene expression through the genomic pathway.[316]

Rabbit embryos acquire a visible mucoid coat as they move down the oviduct.[317] The oviductal epithelium of many species of eutherian mammals secretes an oviduct-specific group of glycoproteins with mucin-like domains; these glycoproteins adhere to the zona pellucidae of oocytes and early embryos.[318–320] As marsupial embryos roll down the oviduct, they are wrapped in strands of mucin that form concentric layers.[321] However, the role mucoid coats play in embryo transport is unknown.

Little is known about the passage of the embryo through the uterotubal junction, although relaxation of the smooth muscle is likely to open the lumen for passage of the embryos. In dairy cows the sigmoid flexure at the junction must relax and the vascular plexus surrounding the junction must drain fluids out of the wall to open the lumen.[134,137]

Cigarette Smoke Interferes with Oocyte and Embryo Transport

Epidemiological data have revealed a correlation between smoking and ectopic pregnancy in women.[322,323] In a hamster model, the soluble components of mainstream cigarette smoke inhibited ciliary activity in the oviduct and oocyte pickup.[324–326] Of the components of cigarette smoke, nicotine, pyridines, and pyrazine were found to interfere with oviductal functions. Pyridines and pyrazine and some of its derivatives were effective in picomolar or nannomolar doses at reducing oocyte pickup rate, ciliary beat frequency, and smooth muscle contractions in vitro in fresh preparations of hamster oviductal infundibula.[327,328]

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Exposure of female hamsters to mainstream or sidestream cigarette smoke delays the arrival of embryos into the uterus.[329] Some of the delay could be attributed to impaired oocyte transport as well as embryo transport.

CONCLUSION

What has been learned to date about gamete transport can be summarized as follows. Sperm are deposited at coitus into the anterior vagina or uterus. Those deposited in the vagina swim through the cervix or are drawn through by uterine contractions. Muscular contractions assist in moving sperm through the uterine cavity. Then, a few thousand sperm enter the uterotubal junction. While still within the junction, or upon reaching the caudal isthmus, sperm are trapped by binding to the mucosal epithelium, forming a storage reservoir. As the time of ovulation approaches, sperm become capacitated and hyperactivated, which enables them to release from the epithelium and escape from the reservoir. Meanwhile, oocytes, surrounded in some species by cumulus cells in a sticky, viscoelastic matrix, are released from the ovary. The cumulus mass adheres lightly to cilia on the mucosal surface of the fimbria and is transported into the oviductal ampulla and then toward the ampullary-isthmic junction by ciliary action. During this time, a few sperm reach the cumulus mass. Fertilization occurs soon thereafter, as sperm penetrate the cumulus, reach and penetrate the zona pellucida, and finally fuse with the oocyte plasma membrane. After pausing at fertilization, the embryo resumes transport, probably by the actions of smooth muscle in the oviductal wall, and reaches the uterus in a few days.

Much remains to be learned about molecules and physical factors that regulate and guide transport of gametes. If the female tract is indeed adapted for enabling sperm transport to the oocyte while impeding the ascent of infectious microorganisms, elucidating these adaptations could inspire development of more effective nonhormonal contraceptives, as well as improved diagnosis and treatment of infertility. In the past, attention has been directed at the roles of female immune responses; however, more remains to be learned about innate and adaptive immune responses, as well as structural adaptations at the micron scale that could guide sperm. Another largely unknown story is how sperm in the uterus find the entrance to the uterotubal junction and why they require certain surface proteins to move through the junction. Yet another set of questions revolves around the oviductal sperm reservoir: how does binding to the oviductal epithelium benefit sperm and what triggers their release to continue ascending the oviduct? Once sperm are released from the reservoir, are there chemical and/or physical mechanisms that guide them to the cumulus-oocyte complex and to the oocyte? If so, what are they?

Transport of the oocyte into the oviduct and down the ampulla has been closely observed in a few species; however, we have only scratched the surface of understanding the chemical and physical interactions between the cumulus-oocyte complex and the oviduct. Of particular interest is how molecular interactions between the ovulatory products and the oviduct change with fertilization and with activation of the zygote genome. Also, although this subject is beyond the scope of this chapter, much more needs to be known about how the embryo benefits from interaction with the oviductal fluid and epithelium. This information could provide critical improvements to embryo culture prior to embryo transfer.

The species diversity seen in the details of gamete and embryo transport is wonderful to see and yet challenging for researchers who are trying to use animal models to study basic mechanisms. Recent advances in the technology of gene modification, such as the injection of CRISPR/Cas9 (clustered regularly interspaced short palindromic repeat/CRISPR-associated proteins) or TALENs (transcription activator-like effector nucleases) into zygotes to generate mutants in a single generation,[330] opens up the opportunity of efficiently creating mutants in a broad array of mammalian species. Applications of these new technologies to the study of gamete transport in a variety of species have great potential to uncover critical new information.

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CHAPTER

6

Preimplantation Embryo Development and Primordial Germ Cell Lineage Specification

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INTRODUCTION

Key Concepts

Historically, preimplantation embryo development has been poorly understood; early scientists and philosophers vigorously debated whether development originated via enlargement of fully preformed tiny beings delivered by the father as originally promoted by Pythagoras and later by Leeuwenhoek or whether epigenesis (the derivation of form from the egg) was the central tenet of development as first suggested by Aristotle.[1] Much of the debate was settled during the Renaissance when diverse Italian embryologists produced elaborate drawings of the oocyte to embryo transition and subsequent fetal stages of development that suggested a gradual and predictable change in form most consistent with epigenesis.[2] Since these early discoveries, more mechanistic details of preimplantation embryo development, including the major developmental stages and metabolic requirements of early embryogenesis, have been uncovered by several individuals working with different mammalian species.[3–7] These and subsequent studies discussed in greater detail later have shown substantial similarities in preimplantation development between species as well as significant species-specific differences such as the timing of embryonic genome activation (EGA) or the frequency of chromosomal abnormalities. Our most current understanding of preimplantation development has been enhanced by technological advances in imaging, molecular, genetic, and epigenetic analyses and the conceptual strides integrally linked to fundamental principles of developmental and stem cell biology.[8–13] Scientists have attempted to tackle tough questions regarding when symmetry in embryonic developmental potential is first disrupted[14,15] and what factors might contribute to success and failure of embryos to thrive.[8,16] In recent years, the derivation of pluripotent stem cells has also provided a new system to model human embryo and germ line development for the first time.[17]

Purpose and Scope

In this chapter we review the fundamentals of mammalian preimplantation embryogenesis, including epigenetic reprogramming, genetic (chromosomal) instability, timing and mechanisms of embryonic gene activation, early miotic divisions, compaction, and cavitation. We then examine lineage specification, first with a discussion of trophectoderm (TE) formation and later with a focus on germ line development. This subject matter then naturally leads to consideration of the derivation of human embryonic stem cells (hESCs), their biological properties, and the closely related induced pluripotent stem cells (iPSCs) derived via nuclear reprogramming of a nonpluripotent cell type, typically an adult somatic cell. Finally, we return to the beginning—the formation of the

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germ line, its properties, and the use of pluripotent hESCs and iPSCs to probe fundamental aspects of germ line formation and gametogenesis. The scope is thus focused on events that initiate during the first two weeks of human development and track with the germ line through gametogenesis. Note that other chapters provide greater detail of gametogenesis, while the focus here is on understanding gametogenesis in light of stem cell biology. We conclude by discussing the clinical need for knowledge of early human development as it relates to infertility, assisted reproduction, and regenerative medicine.

Overview of Mammalian Preimplantation Development

Mammalian preimplantation embryo development begins under transcriptional silence with an oocyte-to-embryo transition that encompasses the migration and fusion of the maternal and paternal pronuclei at the one-cell stage, genetic and epigenetic reprogramming, activation of the embryonic genome, and a series of mitotic, or cleavage, divisions that result in the formation of a compacted morula, which further develops into a compartmentalized blastocyst primed for implantation (Figure 6.1). Details regarding mammalian preimplantation development have been extrapolated from studies in model organisms, predominantly the mouse.[18,19] Additional insight has been provided by an examination of other mammalian species, particularly as conditions for in vitro culture of preimplantation embryos were established and improved.[20–22] Moreover, early characterization of human embryos following natural conception[3,4] and more recent studies in the context of in vitro fertilization (IVF) have also afforded an understanding of human embryogenesis.[23,24] While mammalian embryos may appear morphologically similar at this stage of development, it is clear that certain aspects, such as the inheritance of maternal and/or paternal factors, timing of EGA, patterns of gene expression and epigenetic modifications, and susceptibility to chromosomal instability are likely species-specific and may limit extrapolation between species. Nonetheless, early pioneering studies[3,4,18,19] and recent technological advances in imaging, genomic and molecular analyses,[8–13] have greatly contributed to our knowledge of mammalian preimplantation development. It is important to note that the mammalian preimplantation embryo does not necessarily develop by itself and there are several extrinsic cues provided by the oviduct in vivo or replaced by culture media in vitro.[25] However, this section will primarily focus on the major events and underlying mechanisms regulating proper growth and development of the embryo itself throughout the pre- and peri-implantation period as discussed in further detail as follows.

Overview of Germ Line Development

The establishment of early germ cells and their successful maturation are complex processes that require continual changes in physiology, location, and transcriptional profiles of involved cells. In lower organisms such as flies (*Drosophila melanogaster*) and worms (*Caenorhabditis elegans*), primitive germ cell formation first arises within the heterogenous cells of the developing embryo by the segregation of cells destined to be germ cells from cells destined to become the soma.[26] In the worm, this mechanism occurs after four asymmetric divisions resulting in primordial germ cells (PGCs), while in flies, the segregation of PGCs occurs during embryogenesis to the poles of the developing embryo.[27] Despite the slight differences, it is considered that the embryonic material contains instructive signals for germ line specification (germ "plasm") and that the subset of cells containing the germ plasm commit to a PGC-like fate. Concurrently, the coordinated suppression of transcriptional programs by RNA polymerase II inhibition in PGCs prevents their entry into somatic lineages. In this manner, germ cell formation is endowed upon physically segregated cells, specifically, in the fly and worm.

In contrast, germ line establishment in mammals occurs via "inductive" signaling involving specific signaling molecules that instruct a germ cell fate in cells of the epiblast.[28–30] In rodents and humans, the first glimpses of PGC formation are observed in the embryo after implantation and gastrulation, when the epiblast, endoderm, mesoderm, and ectoderm are first established (Figure 6.2, right).[31] At this time, in response to molecular cues including bone morphogenetic proteins (BMP4 and BMP8a) from the yolk sac, a population of pluripotent stem cells is segregated from the inner cell mass (ICM) and set physically apart from the extraembryonic ectoderm or yolk sac of the embryo (Figure 6.2, middle).[32–34] While BMPs provide the inductive signal to epiblast stem cells to supply PGCs, it is not clear what signals control the size of the PGC founder population or what molecules signal the termination of PGC specification. BMPs are crucial to specification of PGCs due to their activation of the ALK2 receptor and Smad-1/5 signaling pathways, as evidenced in mice.[35] Importantly, the function of BMPs in inducing PGC formation is conserved in the early human embryo. Subsequently, mouse and human PGCs embark on a remarkable physical journey, encompassing important events such as migration via the hindgut and arrival at the primitive genital ridge (Figure 6.2, left). During this remarkable migratory process, PGCs repress their somatic gene program and undergo genome-wide DNA demethylation, histone modifications, and establishment of sex-specific imprints. After sexual divergence of the indifferent fetal gonad, acquisition of spermatogonial and oogonial

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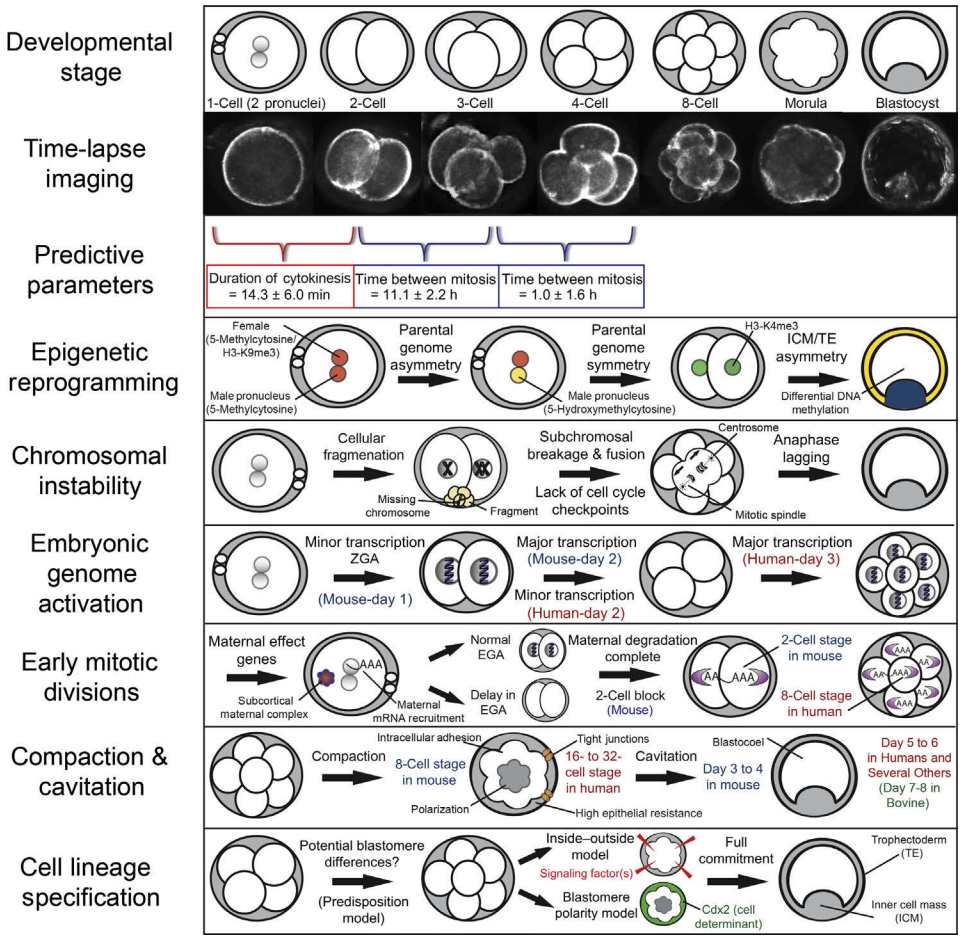

FIGURE 6.1 Summary model of fundamental aspects of mammalian preimplantation development. Recent technological advances in embryology, including time-lapse imaging, whole genome and transcriptome approaches, and single cell molecular analysis have provided insight into key characteristics of mammalian preimplantation development. Assessment of each embryonic developmental stage via time-lapse imaging has shown that the timing windows of certain cell cycle parameters, namely the duration of the first cytokinesis, time between the first and second mitosis, and time between the second and third mitosis is highly predictive of which embryos will successfully reach the blastocyst stage. This oocyte-to-embryo transition is characterized by two phases of epigenetic reprogramming and asymmetry: the asymmetry of epigenetic marks in maternal and paternal pro-nuclei at the zygote stage, which become more symmetrical beginning at the 2-cell stage, and the asymmetry in species-specific DNA methylation patterns between the inner cell mass (ICM) and trophectoderm (TE) at the blastocyst stage. There are several underlying factors that contribute to the generation of chromosome instability, particularly in human embryos, including cellular fragmentation, subchromosomal breakage and fusion, a lack of cell cycle checkpoints, and chromosomal lagging during anaphase. While the major wave of embryonic genome activation (EGA) occurs in the mouse at the 2-cell stage, the major wave of human EGA begins on day 3 at approximately the 8-cell stage and is independent of cell number. In preparation for EGA, maternal effect genes, which were synthesized during oogenesis, are passed to the embryo during the early mitotic divisions. Besides individual maternal proteins, multiprotein complexes, including the subcortical maternal complex (SCMC), are important for embryonic progression beyond the 2-cell stage. Without these maternally provided cytoplasmic components, mouse embryos may undergo a delay in EGA and subsequently exhibit a phenomenon known as the "in vitro 2-cell block". A select group of maternal mRNAs are recruited via poly(A) tail adenylation for translation following fertilization, while the remaining maternal mRNAs are degraded, a process that is essentially complete by the 2-cell stage in mouse embryos and the 8-cell stage in human embryos. The processes of compaction, intracellular adhesion, and polarization result in the formation of a morula at the 8-cell and 16- to 32-cell stage in mouse and human embryos, respectively. The majority of mammalian species, including humans, undergo cavitation to form a fluid-filled cavity called a blastocoel between days 5 and 6, whereas mouse embryos begin blastulation earlier between days 3 and 4, and bovine embryos later between days 7 and 8. It remains unclear as to when differences in blastomere developmental potential are first observed, however, it is thought that blastomeres become fully committed to form either the ICM or TE cells at the morula stage. Two models of cell lineage specification have been proposed: the inside-outside model, which suggests that the location of cells within the embryos determines the signal(s) they receive; and the blastomere polarity model, whereby cell determinants such as Cdx2 are inherited or precluded from symmetric versus asymmetric divisions to confer cell fate. *Source: Shawn Chavez, unpublished.*

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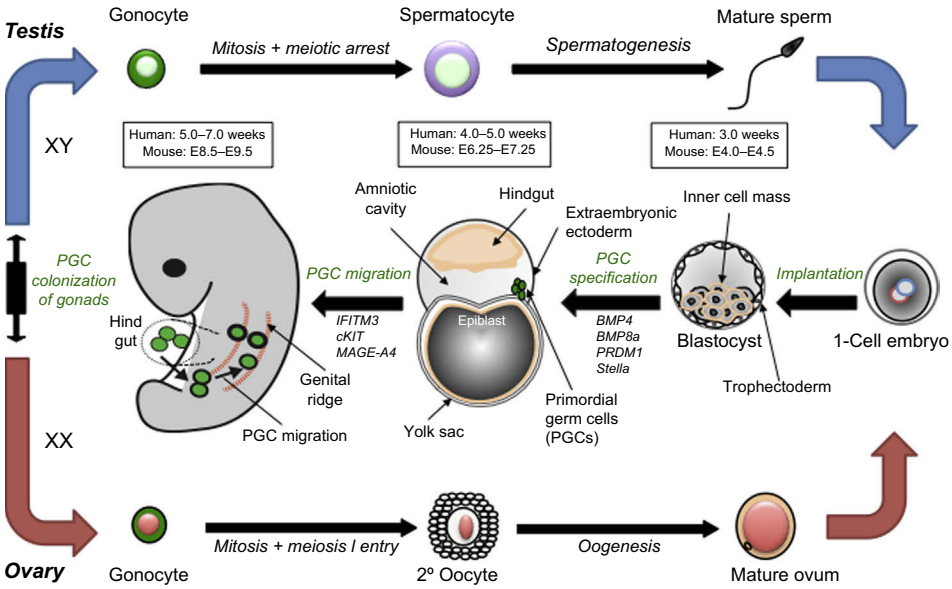

FIGURE 6.2 Life cycle of mammalian germ cells from PGC specification to fertilization. Fertilization of an oocyte by a sperm promotes the formation of a 1-cell zygote that undergoes several cell divisions to form a specialized embryo called a blastocyst. The outer layer of blastocyst will give rise to the trophectoderm, whereas the inner cell mass (ICM) will form the embryo proper later in development. Following implantation, the embryo undergoes gastrulation (E6.25–7.25 in mouse; 4.0–5.0 weeks in human), to form the epiblast, which will differentiate into the three embryonic germ layers, as well as the germ line. Primordial germ cells (PGCs) are specified at the base of the allantois and localize near the extraembryonic ectoderm. Once specified, the PGCs migrate from the invaginating hindgut to the genital ridges (arrows), which eventually develop into fetal gonads. The primitive gonads are formed when PGCs are encircled by somatic cells to form either testicular cord-like structures in the male or primitive follicular structures in the female. PGCs enclosed in either the testis or ovary undergo sex-specific development and maturation to male and female gonocytes, respectively. While male gonocytes arrest meiosis and undergo spermatogenesis to form mature sperm, female gonocytes enter meiotic prophase I and begin oogenesis, which is completed upon ovulation of a mature ovum. At the time of fertilization, the mature germ cells together transmit genetic information to the next generation, thus completing the cycle. Molecules that are involved in regulation of each PGC specification stage are listed. XY = male lineage, XX = female lineage. *Source: Adapted from Ref. 17.*

features and transcriptomes sets the stage for sex-specific mitotic expansion and meiosis of the resultant gametes (Figures 6.2 and 6.3). In this chapter, the processes outlined will be broadly defined with focus on PGC specification in the developing embryo, PGC migration, and developmental remodeling of PGCs. We will briefly discuss studies on gonadal stem cells in the mouse and human models with emphasis on human development. A summary of efforts using human fetal tissue and germ cells derived in vitro to overcome lack of access to early human tissue is also presented.

EMBRYOGENESIS

Epigenetic Reprogramming

Epigenetics is the study of alterations in gene expression or cellular phenotype caused by other mechanisms besides changes in DNA sequence; it was first described by C.H. Waddington as an explanation of how cells with the same genetic makeup could express different genes to determine cell fate.[36] Epigenetic reprogramming, or the erasure, conversion, and reestablishment of epigenetic marks, is initiated in mammals during early embryogenesis and continues through gametogenesis.[37,38] Following fertilization, the paternal and maternal genomes are predominantly erased and/or epigenetically modified and subsequently reset around the time of implantation[39–41] (Figure 6.1), which is thought to be required for the totipotency of the newly formed embryo.[42] The two main types of epigenetic mechanisms are DNA methylation and histone modifications (please refer to Chapter 52), which influence gene expression and chromatin structure in a potentially heritable manner without altering DNA sequence.[43–45] DNA methylation is mediated by a family of DNA methyltransferases (DNMTs) that catalyze the transfer of a methyl group to the 5′-position of cytosine residues within CpG dinucleotides, usually resulting in effective gene silencing through the recruitment of methyl-CpG-binding proteins and inhibition of transcription factor binding.[46] Histone modifications include, but are not limited to, the phosphorylation of serine residues, acetylation of lysine residues, and the methylation of either lysine or arginine residues, all of which are mediated by distinct histone-modifying enzymes. As covalent modifications, these histone marks can alter the interaction between DNA and

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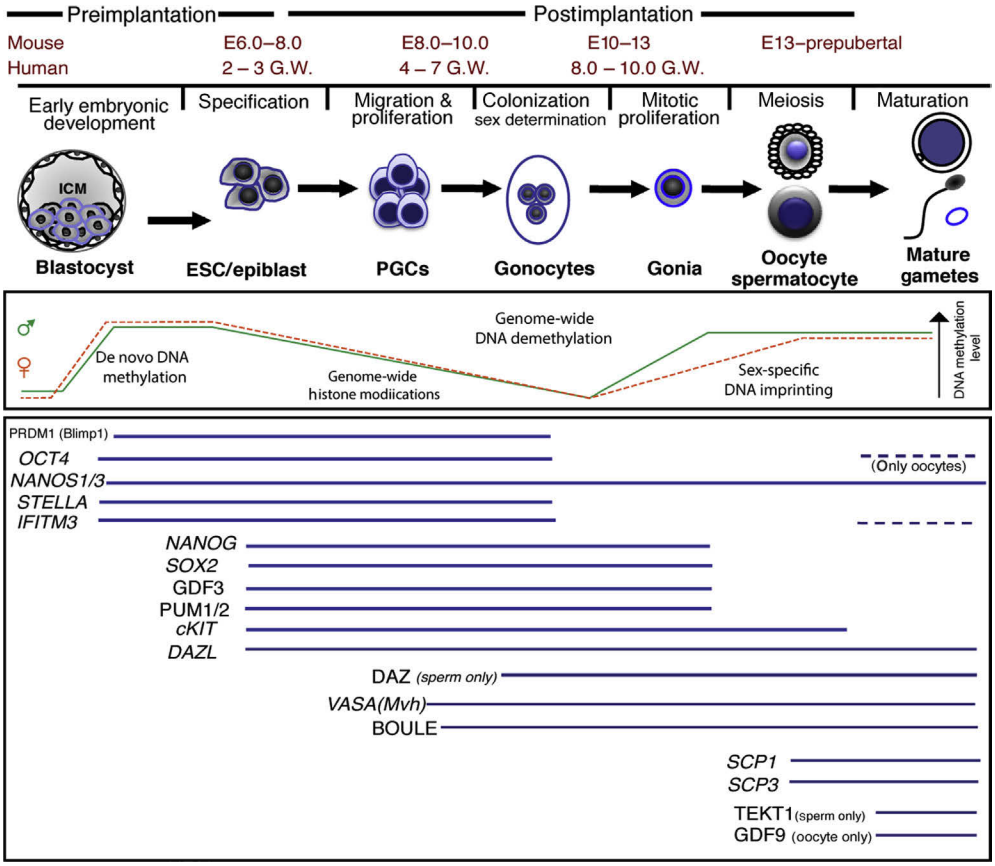

FIGURE 6.3 **Timeline of germ line specification with stage-specific germ cell marker expression.** A temporal representation of the different stages of human and mouse germ line differentiation during pre- and postimplantation in vivo. The approximate time in development for both humans and mice is indicated at the top with the major stages pictured below. An examination of global DNA methylation as assessed by five methylcytosine levels in both males and females demonstrates a period of de novo methylation for male (continuous line) and female (broken line) occurring in the blastocyst prior to, and during, implantation. This is followed by genome-wide DNA demethylation in the ESCs and epiblast stages and proceeding to PGC specification. In the mouse, and largely true for the human, genome-wide changes in histone modifications occur concurrently with DNA demethylation between the time of primordial germ cell (PGC) specification, migration, and colonization of the gonads. Sex-specific DNA methylation and imprinting are reestablished later during male and female gametogenesis, albeit at different times. Specific molecular markers for germ cell identification are indicated on the left with lines depicting the duration of expression from the blastocyst stage to the formation of mature gametes. Genes that are italicized are present in both mouse and human germ cells. *Source: Adapted from Ref. 17.*

nuclear proteins and, together with DNA methylation, ensure appropriate gene activation during preimplantation development.[47]

Besides being associated with the epigenetic silencing of genes, DNA methylation and histone modifications have also been shown to be important for normal embryogenesis, X-chromosome inactivation, and genomic imprinting.[26,48–50] Unlike the vast majority of autosomal genes, whereby gene expression occurs from two alleles simultaneously, a subset of approximately 80 or more known imprinted genes vary in expression according to the maternal or paternal origin and escape the epigenetic reprogramming that occurs during this period of development.[51] Genomic imprinting is thought to represent a barrier to parthenogenesis and has been shown to play an important role in normal embryo growth and development, placental function, as well as neurobehavioral processes.[52] As development of the mammalian zygote proceeds, the paternal genome must undergo dramatic structural and epigenetic changes such as chromosome decondensation, protamine replacement by histones, and rapid DNA demethylation. It was originally thought that the methylation of DNA is almost completely erased by the morula stage of development, with the exception of maternal and paternal DNA imprints.[40,53] However, more recent studies have shown that 5-methylcytosine, a marker of global DNA methylation levels, is actually converted to 5-hydroxymethylcytosine in the paternal pronucleus to directly or indirectly influence transcription and/or DNA replication, whereas the maternal pronucleus is protected from this conversion in several mammalian species[41] (Figure 6.1). In conjunction with the asymmetric DNA methylation reprogramming of the parental genomes, certain histone modifications such as

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histone H3 lysine 9 methylation mark the female pronucleus during the oocyte-to-embryo transition.[54] This epigenetic asymmetry of parental genomes is thought to remain until at least the two-cell stage when other histone marks, including histone H3 lysine 4 methylation, begins to appear more similar between the two genomes.[55] By the time of implantation, DNA methylation is subsequently reestablished during a period of de novo methylation at the blastocyst stage, and a second wave of asymmetric epigenetic marks is observed to coincide with the commitment of blastomeres to form either ICM or TE cells.[37] Additionally, cells of the early female embryo display marks of X-chromosome inactivation, such as X-inactive specific transcript (*XIST*) and histone H3 lysine 27 methylation, at or around the time implantation.[56,57]

Despite conservation of certain epigenetic marks between species during preimplantation development, there are clear species-specific distinctions in DNA methylation and histone modification dynamics in different mammalian embryos. For instance, there are different degrees at which the paternal genome is demethylated and/or modified upon fertilization, and the ICM and TE cells of blastocysts are known to exhibit differential methylation levels across mammalian species[58–62] (Figure 6.1). This may be related to distinctions in developmental timing, activation of the embryonic genome, cellular differentiation, and/or the chromosomal status of the embryo. Future work should enhance our understanding of how epigenetic mechanisms are cooperatively regulated to achieve normal embryogenesis and how perturbations in this process during preimplantation development may contribute to adverse outcomes such as birth defects.

Chromosomal Instability

Studies using whole genome approaches have demonstrated that aneuploidy, or an abnormal number of chromosomes, is present in the blastomeres of between 50% and 80% of cleavage-stage human embryos, including those from fertile couples and regardless of cryopreservation.[12,16,63] The high incidence of human embryonic aneuploidy observed in vitro may also be reflected in vivo considering that approximately 30% of natural human conceptions result in live births and chromosomal abnormalities have been reported in as many as 70% of spontaneous miscarriage cases.[64–70] In several instances, the embryo may successfully implant but does not develop further and results in early miscarriage, often before detection of pregnancy, and is historically termed a blighted ovum.[71] This is in contrast to other mammalian species, particularly the mouse, which is estimated to exhibit less than 1% embryonic aneuploidy rates at this stage of development.[72] However, arrays for the detection of copy number variants are far more utilized for humans than other mammalian species due, in part, to preimplantation genetic screening (PGS) of human embryos in IVF clinics and genetic testing centers.[73–75] Thus, it will be interesting to determine the overall aneuploidy rates via similar cytogenetic approaches in preimplantation embryos from additional species, especially those more closely related to humans.

It is thought that chromosomal errors most commonly occur on the meiotic spindle during oogenesis or the mitotic spindle during embryonic cleavage divisions,[76] which could be perpetuated due to the apparent lack of cell cycle checkpoints in cleaving human embryos,[13,77] as well as anaphase lagging, or the failure of one or more chromosomes to connect to the spindle during mitosis at the blastocyst stage[78] (Figure 6.1). Indeed, estimates of meiotic error rates in humans are relatively high at 5–20% of oocytes compared with other species such as the mouse, whereby only 0.05–1% of oocytes are typically aneuploid.[79] Therefore, it is not surprising that maternal meiotic errors are much more frequent than paternal meiotic errors in human embryos determined to be chromosomally abnormal.[63] However, it is important to note that the centrosome and potentially other spindle components, which mediate the first mitotic division(s) in embryos, are paternally inherited in the majority of mammalian species.[80,81] In contrast, it appears that the centrosome is maternally inherited in the mouse embryo,[82] the only known exception to date. This may help explain the relatively low aneuploidy rates observed in mouse embryos[72] and suggests that paternal contribution to human embryonic aneuploidy is likely greater than expected and invokes consideration of paternal age as maternal age-related aneuploidy does.[63,76] Additional studies have supported this notion by demonstrating that the generation of chromosomal errors during early human embryo development is very often mitotic in origin.[12,37] Moreover, Chavez and colleagues also revealed via single cell analysis of whole embryos that the high frequency of human embryonic aneuploidy may have contributions from chromosome-containing embryonic micronuclei that arise via cellular fragmentation, particularly during or following the first mitotic division[16] (Figures 6.1 and 6.4). While the cause of cellular fragmentation has been disputed for several years and was previously thought to represent anucleate cytoplasmic components,[83] it is estimated to occur in greater than 50% of human embryos cultured in vitro[84] and is distinct from the DNA fragmentation that can occur following cell death later in preimplantation development.[85–87] There is also evidence to suggest that cellular fragmentation occurs in human embryos in vivo, indicating it is not just a consequence of in vitro culture[88,89] and that it may negatively correlate with implantation potential.[90,91] It is also important to note that not all fragments contain

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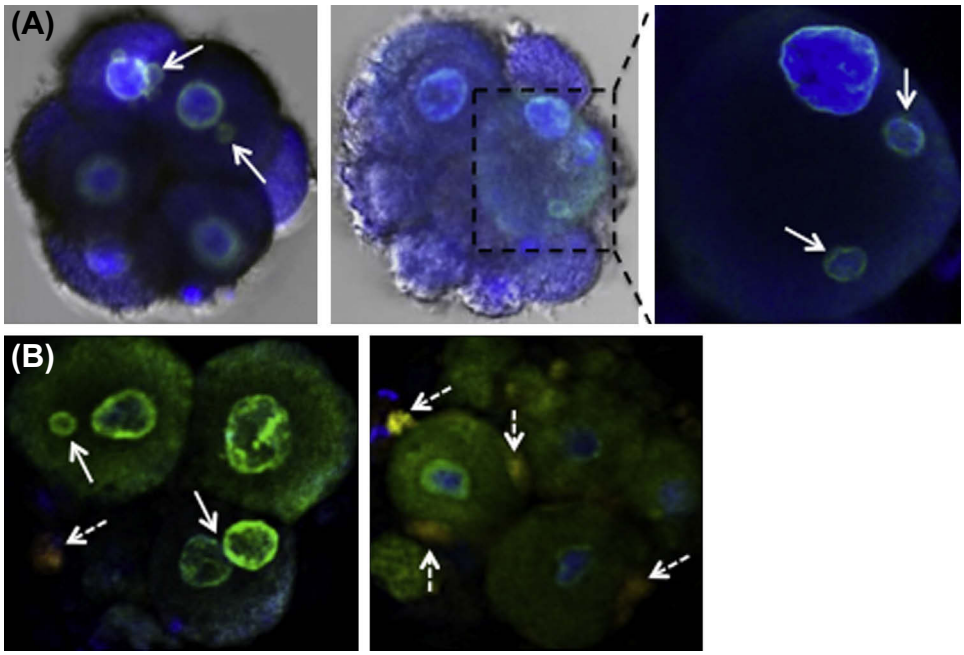

FIGURE 6.4 Detection of embryonic micronuclei in cleavage-stage human embryos. This figure is reproduced in color in the color plate section. (A) Expression of the nuclear envelope marker, LAMIN-B1 (green), in 4′,6-diamidino-2-phenylindole (DAPI)-stained (blue) cleavage-stage human embryos visualized by immunofluorescent confocal microscopy and differential interference contrast (DIC) imaging at 20× (left and middle) and 40× (right) reveals the presence of several embryonic micronuclei (indicated by white solid arrows) distinct from primary nuclei in human blastomeres. (B) Similarly evaluated additional cleavage-stage human embryos with low (left) and high (right) fragmentation also immunostained for centromere protein E (CENP-E; orange) in the absence of DIC imaging identifies multiple missing chromosomes in cellular fragments (indicated by white dashed arrows) adjacent to blastomeres at this stage of development. *Source: Adapted from Ref. 16.*

missing chromosomes (Figures 6.1 and 6.4), which may partially explain how certain fragmented embryos can still result in successful pregnancy outcome.[83] Regardless of its origins, cellular fragmentation is observed, although to a lesser extent, in preimplantation embryos from other mammalian species,[92,93] and it will be important to determine the mechanism(s) by which it occurs and how dynamic events such as the timing, degree, and resorption of chromosome-containing fragments contributes to the generation of embryonic aneuploidy.[16,94–96]

Notably, aneuploid human embryos at the cleavage stage have also been shown to exhibit frequent chromosome segmental deletions, duplications, and/or amplifications that are balanced between presumptive sister blastomeres.[12,16] Not only does this suggest that chromosome breakage and fusion often arises during early cleavage divisions but it also indicates that there is a relationship between aneuploidy and subchromosomal instability in human embryos (Figure 6.1). There is some evidence to suggest that chromosomal correction can occasionally occur during embryo development based on PGS and biopsy of an aneuploid blastomere on day 3, followed by euploid TE cells on day 5.[97–99] While blastocysts may exhibit less mosaicism, defined as the presence of two or more cell populations with different chromosomal makeup, and contain fewer aneuploid cells than cleavage-stage embryos,[100] frequent findings of complex mosaicism, whereby multiple chromosomes are affected, in embryos without a single euploid blastomere indicate that it is unlikely that most chromosomal abnormalities can be corrected through development.[12,16] Nevertheless, some groups have proposed that euploid cells may exhibit advantageous growth over aneuploid cells in embryos or, perhaps, there is preferential contribution of chromosomally normal cells to the ICM and chromosomally abnormal cells to the TE layer.[97,101,102] More recent studies, however, have shown no preferential distribution of chromosomally abnormal cells between the ICM and TE.[103,104] Thus, while ICM and TE chromosomal asymmetry may not explain the so-called "embryo self-correction," there remains the possibility that alternative mechanisms such as blastomere exclusion or DNA replication in the absence of cell division, called endoreduplication, followed by abnormal mitotic divisions with multipolar spindles may play a role and future studies will assist in deciphering this phenomenon.[103]

Embryonic Genome Activation

Perhaps the most compelling species-specific difference between mammalian preimplantation embryos is the developmental stage at which EGA occurs. The

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mouse preimplantation embryo exhibits the earliest transition from maternal to embryonic transcriptional control at the beginning of the 2-cell stage with a few mRNAs being transcribed at the 1-cell stage and is therefore an event often referred to as zygote gene activation (ZGA)[105] (Figure 6.1). Rat EGA begins at the 2- to 4-cell stage,[106] the 4-cell stage in pigs,[107] and in humans on day 3 at the 4- to 8-cell stage,[108] which is similar to nonhuman primates as EGA occurs at the 6- to 8-cell stage in rhesus macaque embryos.[109] For other mammalian species, including cow, rabbit, and sheep, EGA begins later in preimplantation development at the 8- to 16-cell stage[110–112] and may coincide with delayed implantation timing. Regardless of the stage, all mammalian embryos begin preimplantation development under relative transcriptional silence, and it is clear that the duration of this quiescence differs between species. Thus, the mammalian preimplantation embryo must rely predominantly on maternally-derived RNAs and proteins for the first day(s) of life in order to survive, although previous findings have demonstrated the importance of a small population of paternal transcripts inherited from the sperm beginning at the 3- to 4-cell stage in human embryos.[113,114]

In preparation for ZGA/EGA, the majority of maternal mRNAs and proteins are degraded, a process that is largely complete by the 2-cell stage in mouse embryos and the 8-cell stage in human embryos.[11,115,116] These maternally stored factors are subsequently replaced by newly synthesized embryonic gene products,[117] which are important for establishing the totipotency of each blastomere in the cleavage-stage embryo.[118] Thus, ZGA is required for the oocyte-to-embryo transition[119] as well as development to the 4-cell stage at least in mouse embryos.[120] Failure of embryos to progress beyond the 2-cell stage is referred to as the "in vitro 2-cell block".[121] It had long been assumed that this block in early murine development was due to the failure of ZGA based on the timing of ZGA as described before and frequent findings of embryo arrest in certain mouse strains at the 2-cell stage. The utilization of blocking and nonblocking mouse strains, however, has shown that embryos with 2-cell block still initiate ZGA, and it is therefore not due to the inability to begin ZGA. More specifically, Qui et al. demonstrated via analysis of the transcription-requiring complex (TRC), a subset of proteins whose expression is a hallmark of ZGA; it is a delay in ZGA that contributes to the 2-cell block phenomenon in the mouse.[122] Nevertheless, additional studies have identified specific genes, including heat shock 70 kDa protein 1 (*Hsp70.1*),[123] eukaryotic initiation factor-IA (*eIF-IA*),[124] as well as zinc finger and SCAN domain containing 4 (*Zcan4*)[125] that are actively transcribed at the onset of ZGA.

While it is generally accepted that the human embryo activates its genome on day 3, data from a recent study suggesting early waves of transcriptional activation at the 2-cell stage has prompted disagreement as to when EGA actually begins in the human preimplantation embryo.[126] Although a few genes may be expressed as early as the 2-cell stage, genome-wide transcriptional analyses of human preimplantation embryos have shown that the major wave of EGA does indeed occur on day 3 at the 4- to 8-cell stage[9–11] (Figure 6.1). Further support for this time course is provided by early studies demonstrating via uridine radiolabeling that nucleolar RNA synthesis takes place no earlier than the fourth cell cycle and embryonic cleavage divisions are not sensitive to inhibition of transcription by alpha-amanitin until after the 4-cell stage.[108,127] More recently, the first whole transcriptome analysis of preimplantation human embryos demonstrated that the expression of approximately 1800 mRNAs is modulated during the first three days of development and that the vast majority of transcripts were targeted for destruction and downregulated.[9] However, Dobson and colleagues also showed that a small group of mRNAs were upregulated between days 1 and 2, and a much larger group of transcripts increased in abundance on day 3. The authors suggested that the increase in mRNA levels prior to day 3 likely represented minor transcriptional activity or preferentially stable transcripts in a pool of rapidly degrading maternal mRNAs. In addition, they demonstrated that the major wave of EGA was independent of cell number since embryos that arrested with fewer than eight cells still exhibited EGA on day 3.[9] Additional studies using global genome assays have revealed similar findings of major EGA in human embryos at the 4- to 8-cell stage,[10,11] at the 6- to 8-cell stage in rhesus monkey embryos,[128] and at the 8-cell stage in bovine embryos[129] (Figure 6.1). Thus, it remains unclear whether apparent changes in gene expression prior to the 4- to 8-cell stage are the result of maternal mRNA degradation, actual gene activation, or associated with embryo arrest and/or other aspects of abnormal embryogenesis, which may affect embryo quality and the interpretation of the results.[126] Given that blastomeres of the cleavage stage human embryo appear to develop cell autonomously, divergent findings may also be attributed to differences in gene expression between whole embryos and single blastomeres.[8,11] Nevertheless, more extensive transcriptional analysis of individual embryos and blastomeres throughout mammalian preimplantation development are still warranted and together with confirmation at the protein level, may help determine the importance of dynamic gene expression patterns at different stages.

Early Mitotic Divisions

Following fertilization, the 1-cell zygote undergoes a series of mitotic cell divisions to produce an increasing number of progressively smaller cells without changing

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the overall size of the embryo.[119] Notably, several studies have shown that male mammalian preimplantation embryos may cleave faster than female embryos cultured in vitro,[130–132] although other studies have shown no difference in the sex ratios between early or late-cleaving human embryos.[133,134] This suggests that potential sex-related differences in the cleavage rate of male versus female human embryos does not occur until later in development or is a phenomenon that is restricted to only certain mammalian species. Nevertheless, early cleavage appears to be a strong indicator of embryo viability as human embryos that undergo the first mitotic division sooner have greater potential for successful implantation and possible pregnancy outcome.[134] A more recent study has demonstrated that it is the duration of the first mitosis together with the time between the first and second as well as second and third mitotic division that is highly predictive of which human embryos will reach the blastocyst stage[11] (Figure 6.1, Predictive Parameters), which is further discussed in a later section. Whether the first three mitotic divisions are similarly predictive for other mammalian species besides the human remains to be determined, but an examination of early mitotic timing in mouse[135] and bovine embryos[136] has suggested that this may be the case.

As described earlier, cellular fragmentation has been observed to occur most often in human embryos during or following the division from one cell to two cells.[16,96] In many instances, this emergence of fragments is resolved by the 8-cell stage or later in development, which may be explained by frequent findings of fragment resorption in cleavage-stage human embryos.[10,94–96] Therefore, it is not surprising that the incidence of cellular fragmentation itself is an unreliable indicator as to whether an embryo will successfully develop to the blastocyst stage.[18,137] Given the poor prognostic value of fragmentation in human embryos and findings that removal of acellular debris promotes blastocyst hatching in mouse models,[138] the practice of fragment removal via laser-assisted microsurgery was introduced.[139–141] However, as discussed earlier, cellular fragments can contain genetic information, and, thus, the elimination of fragments may potentially be deleterious for an embryo.[10] Regardless of micronuclei formation, subsequent findings have demonstrated that fragment removal provides no benefit to the developing embryo at least to the blastocyst stage,[140] and, as such, surgical removal of cellular fragments is generally no longer promoted.

Besides varying degrees of cellular fragmentation, as discussed earlier, another hallmark of early cleavage divisions in mammalian preimplantation embryos is the degradation of maternally deposited factors in preparation for the oocyte-to-embryo transition. There must be, however, a certain amount of selectivity in the destruction of maternal mRNAs and proteins in order to facilitate the successful shift from maternal to embryonic control of development.[142] In support of this, it was shown that several maternal mRNAs are recruited via polyadenylation of their poly(A) tails for translation following fertilization but prior to the activation of the embryonic genome[143] (Figure 6.1). Further evidence has been provided by findings that the injection of cytoplasm from a nonblocking mouse strain into a strain with 2-cell block rescues the defect,[144] suggesting that the block is mediated by a cytoplasmic component. Additional studies have demonstrated that rescue occurs in the absence of maternal chromosomes to indicate that stored, rather than newly synthesized factors, are responsible for overcoming this block in development.[145] Although the identities of the specific maternally provided cytoplasmic proteins are still largely unknown, factors that are thought to originate from the oocyte and persist in the embryo are termed maternal effect genes and are transcribed during oogenesis. However, some maternal effect genes are not expressed until after activation of the embryonic genome to complicate the differentiation between maternal and embryonic effects.[117] Several maternal effect genes have been identified and include factors involved in maternal mRNA degradation, transcription, chromatin remodeling, and DNA methylation. In addition to individual maternal proteins, multiprotein complexes such as the subcortical maternal complex (SCMC) have also been shown to be important for embryonic progression beyond the 2-cell stage in mouse embryos.[146] One of the first members of this complex to be reported was maternal antigen that embryos require (Mater/Nlrp5), which by definition is not expressed by the embryonic genome.[147] Thus, the depletion of *Mater* prevents SCMC formation and results in preimplantation embryonic lethality in both mice[146] and monkeys.[148] A similar phenotype is observed in mouse embryos lacking factor located in oocytes permitting embryonic development (*Floped/Ooep*), another member of the SCMC complex. The absence of other SCMC proteins such as *Filia (RIKEN)*, on the other hand, results in less pronounced phenotypic effects.[149] Given the conservation of the SCMC genes in humans, it will be important to determine whether these maternal effect proteins play an analogous role in human preimplantation development.

Compaction and Cavitation

Mitosis continues during preimplantation development until the embryo undergoes compaction, or intracellular adhesion, as first described by Mulard[150] and further characterized by several others in different mammalian species,[151–153] to form a morula (Figure 6.1). The formation of a morula represents the first morphological indication of disruption in radial symmetry, or

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the symmetrical arrangement of components emanating from a central point, in the embryo during preimplantation development. It is thought that compaction is essential for later morphogenetic events such as lineage specification, but how the process of compaction is initiated is not well understood.[154,155] In conjunction with cell adhesion, intracellular polarization occurs, whereby the outward-facing (apical) domains of each blastomere become distinct from inward-facing (basolateral) regions. More specifically, the cell nuclei move basolaterally, whereas actin and microtubules accumulate apically concomitant with differential localization of membrane and polarity protein complexes.[156–158] While cell–cell contact does appear to be important for establishing the orientation of polarity since blastomeres polarize along the axis perpendicular to the cell contact and apical poles form farthest from the contact point, additional mechanisms are likely to be involved.[151,159] Nevertheless, once the morula has compacted and polarized, subsequent cell divisions are influenced by the orientation of the cleavage plane so that polarity is inherited in the daughter cells. If a blastomere divides at an angle perpendicular to its axis of polarity, both daughter cells will be polar and remain on the outside of the embryo. In contrast, if a blastomere undergoes mitosis parallel to its axis of polarity, one daughter cell will be polarized and the other apical, and thus they will be located on the outside and inside of the embryo, respectively.[160,161] These symmetric versus asymmetric cleavage divisions result in the generation of two distinct cell populations: the cells on the inside will become a part of the ICM and the cells on the outside will contribute to the TE layer.

The processes of compaction and polarization together with symmetric versus asymmetric cell division eventually lead to the formation of a blastocyst that comprises a fluid-filled cavity called a blastocoel and an ICM surrounded by TE cells beginning on day 4.5 during human preimplantation development.[162] While the ICM will eventually give rise to the embryo proper, the TE will form the fetal portions of the placenta later in development. Blastocoel formation is initiated almost immediately after compaction by the assembly of tight junctions, which includes occludin, cingluin, as well as other components, and the establishment of high epithelial resistance in TE cells until the 32-cell stage.[163–165] As the blastocoel forms, which is essential for proper ICM development,[166] water may enter the embryo via osmosis until tight junctions in TE cells are fully functional to prevent fluid loss.[164,167] In preparation for implantation into the uterus, the blastocyst "hatches" from the zona pellucida, a glycoprotein layer that encapsulates and protects the oocyte during fertilization and transition to an embryo. Blastocyst hatching allows for increased embryo growth and development as well as TE cell access to the lining of the uterine wall to allow adhesion to occur. Just prior to implantation, which occurs on day 7 of human embryonic development, a second lineage decision is made, whereby the ICM of the blastocyst differentiates into either early epiblast or primitive endoderm to form the future fetus and parietal/visceral endoderm, respectively, the latter of which will contribute to the yolk sac.[168]

Much of what we know about embryo compaction and cavitation has been derived from studies in the mouse, and relatively little is known of these processes in other mammalian species, including humans. Although in vitro studies have shown that different preimplantation embryos closely resemble each other at this stage of development, there are a few notable species-dependent exceptions. For example, compaction often occurs much earlier in mouse embryos at the 8-cell stage than in humans, which begins at the 16-cell stage.[162,169] Thus, it remains unclear how the timing of compaction may affect polarization and asymmetric cell divisions in the human embryo.[168] In addition, human embryos are likely to undergo at least one additional round of cell division to form a ~256-cell blastocyst, whereas mouse blastocysts typically comprise ~164 cells.[170] However, morphological changes such as compaction or cavitation have been shown to be a function of timing in development rather than cell number, at least in the human preimplantation embryo. Finally, human embryos show delayed blastocyst development compared with mouse embryos; the mouse blastocyst forms between days 3 and 4 (Figure 6.1). Analogous to humans, the vast majority of other mammalian species, including rhesus, ovine, porcine, rabbit, and rat, form blastocysts on days 5 to 6,[171–174] but bovine embryos exhibit blastocyst formation on days 7 to 8,[175] which might be due to differences in embryo energy requirements and metabolism and/or the extent of placental invasion.[176,177]

TE Lineage Specification

The first cell lineage to be specified in the mammalian preimplantation embryo is the TE layer, but how this decision is made is not entirely clear. In the mouse embryo, blastomeres become fully committed to form either the TE or ICM lineage at the 32-cell stage.[166,178] Based on the location of TE cells on the outside and the ICM on the inside of the embryo, as well as the observation that changing the position of a cell may change its fate, the inside–outside model of lineage specification was initially proposed[179] (Figure 6.1). According to this model, cells on the outside would receive different signaling factors, including components of the Hippo signaling pathway such as Nf2/Merlin, than cells on the inside of the embryo and signal to the downstream factors, large tumor suppressor homolog1 (Lat1), Lat2, and yes-associated protein 1 (Yap1), to result in differences

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in cell fate. Both Lat1 and Lat2 are serine–threonine kinases that are functionally redundant, whereas Yap1 is a transcriptional activator that is required by the transcription factor, TEA domain family member 4 (Tead4), for TE specification.[180,181] Once blastomere polarization was discovered, however, the cell polarity model was established and the inheritance of polarity was suggested to determine TE fate.[160] For this model, certain cell fate determinant(s) such as caudal type homeobox 2 (*Cdx2*) may be restricted to or separated from polarized cells during cell division. Since it localizes to the apical region of blastomeres at the 8-cell stage and is necessary for murine trophoblast development, Cdx2 is thought to play a role in cell fate determination at least in the mouse.[14,182] However, more recent studies have shown that the initiation of TE lineage specification is independent of both maternal and zygotic *Cdx2* mRNA[183] and that with the use of an endogenous Cdx2 fusion protein, outer TE cells may change their fates by migrating inward and losing Cdx2 expression as late as the 32-cell stage.[184] Thus, regardless of whether TE lineage specification is due to cell position and/or blastomere polarity, additional signaling pathways and transcription factors are likely required to determine cell fate. Indeed, other factors such as suppressor of defective silencing 3 (Suds3) and TCFAP2C/AP-2γ as well as Hippo-independent pathways via angiomotin have recently been shown to play an important role in early lineage specification.[185–188] How all these factors are temporally, spatially, and/or cooperatively regulated throughout preimplantation development, and whether there is differential regulation at the mRNA and protein level or between mammalian species, should be the focus of future studies.

Besides the uncertainty regarding the molecular mechanism(s) mediating cell fate determination, it is also unclear as to how early in development individual cells of the mammalian embryo acquire the first developmental differences predictive of lineage specification. While certain studies have suggested that lineage allocation is decided stochastically by cell–cell interactions at the 16- to 32-cell stage of mammalian development as previously described with the inside-outside and blastomere polarity models,[15,189,190] others have argued that cells are predisposed to form either the ICM or TE cells beginning at the 4-cell stage to suggest a potential third model of cell lineage specification[14,191] (Figure 6.1). In this model, the levels or kinetics of Cdx2 and the pluripotency-related transcription factor, octamer-binding transcription factor 4 (Oct4)/Pou5f1, in individual blastomeres may promote symmetric versus asymmetric cell divisions in the embryo, which ultimately determines cell fate. However, whether molecular differences between blastomeres are the cause or a consequence of lineage commitment is not known and continues to be debated.[192] Nevertheless, there remains the possibility that intrinsic differences between cells, irrespective of mechanism, stage, or lineage-defining factor, may be reflected from particular epigenetic modifications in individual blastomeres as previously suggested.[193,194]

As expected, it is likely that the timing and developmental stage of distinctions in blastomere potential are species specific. Similar to the mouse, cell fate has been suggested to be determined as early as the 4-cell stage in human embryos, even without transcription, based on the localization of TE markers such as human chorionic gonadotropin (hCG) to particular blastomeres[195,196] (Figure 6.1). In contrast, subsequent studies using whole-genome approaches or single cell quantitative-PCR analysis demonstrated that human blastomeres are transcriptionally similar up to the 8-cell stage and do not express TE-associated genes.[8,11] Wong et al., however, did observe differences in the molecular programs of maternal mRNA degradation and EGA between individual blastomeres to suggest that they develop by themselves.[8,11] Moreover, species-dependent differences have been noted not only for the expression of CDX2 but also for OCT4 during human, rhesus monkey, bovine, and mouse preimplantation development. More specifically, both human and bovine embryos exhibit prolonged OCT4 and CDX2 co-localization in the TE layer,[197,198] as compared to mouse and rhesus embryos,[190,199,200] suggesting a lack of evolutionary conservation in the formation of ICM and TE. There is also evidence to suggest that species-specific mechanisms exist for the specification of epiblast and primitive endoderm lineages later in development. For instance, mouse and bovine embryos require fibroblast growth factor (FGF)/mitogen-activated protein kinase (MAPK) signaling for the formation of primitive endoderm cells, whereas the development of primitive endoderm in human embryos is unaffected by FGF and MAPK inhibition.[201–203] More detailed analysis of the molecular mechanisms mediating lineage restriction in the early human embryo will undoubtedly lead to a better understanding of human preimplantation development as well as assist in the determination of the similarities and differences between humans and other mammalian species.

Recent Advances in Understanding Embryology

As discussed in detail earlier, new developments in whole genome and transcriptome analysis of individual embryos and single blastomeres have greatly contributed to our knowledge of mammalian preimplantation development.[8–13] In addition, the implementation of time-lapse imaging to monitor human embryos throughout development has provided a noninvasive approach to potentially assess embryo viability[8,94,204,205] (Figure 6.5(A) and (B)). Once it was confirmed that time-lapse

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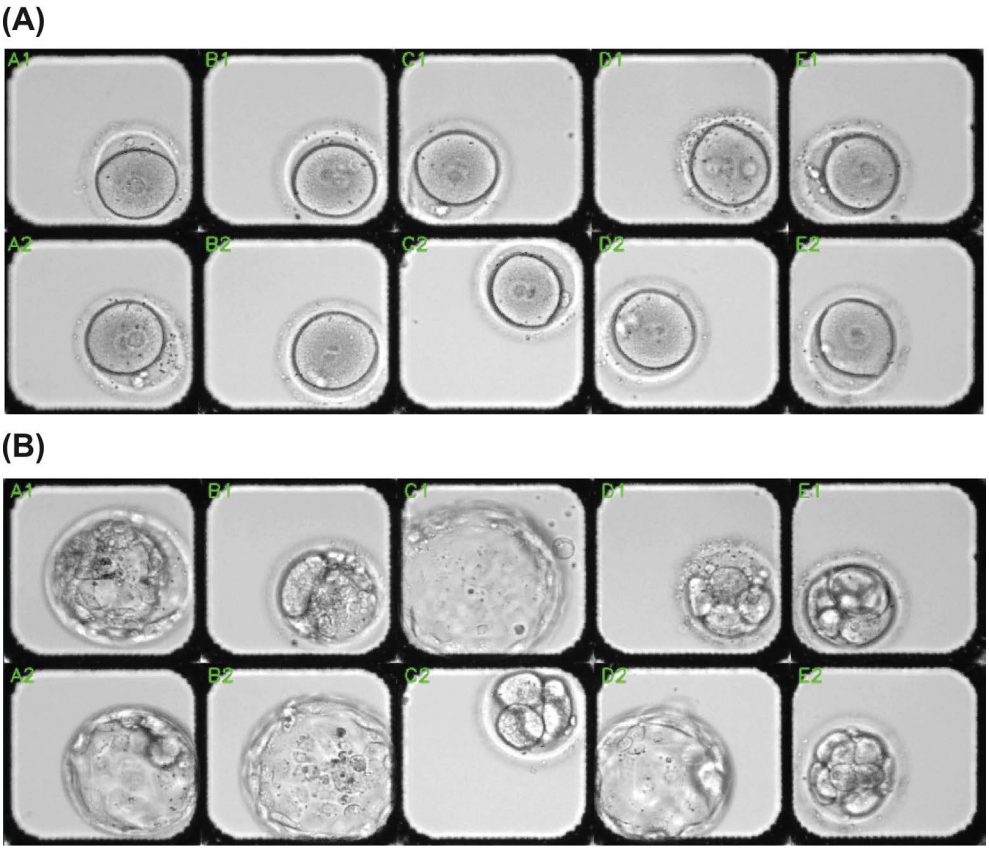

FIGURE 6.5 Time-lapse image analysis of human preimplantation development. (A) 10 human zygotes were thawed on day 1 and cultured together in alphabetically and numerically labeled microwell-containing Petri dishes. (B) Time-lapse imaging was performed in a standard incubator until day 6 when the majority of embryos had reached the blastocyst stage. Embryo imaging behavior was monitored by measuring dynamic imaging parameters between the first and last frame of an image sequence compiled into a time-lapse movie with well identification labels and time stamps. *Source: Brooke Friedman and Shawn Chavez, unpublished.*

imaging was not detrimental to embryo development, several groups began conducting morphological, spatial, and temporal studies to determine if there were correlates between imaging behavior and embryo quality.[8,206,207] In 2010, Wong and colleagues demonstrated that time-lapse image analysis can be used to predict blastocyst fate prior to EGA by measuring the duration and time between the first three mitotic divisions. By evaluating the correlation between imaging and gene expression analysis, it was determined that embryos with normal gene expression patterns followed strict cytokinesis and mitotic division timing, whereas arrested embryos exhibited abnormal gene expression profiles and one or more parameter values outside the timing windows of normal embryos[8] (Figure 6.1). More recently, Chavez et al. demonstrated that the timing of the first three mitotic divisions, in conjunction with dynamic cellular fragmentation analysis, may also be used to distinguish euploid from aneuploid human embryos.[16] Obviously, clinical validation of these findings is required, and recent retrospective studies of patient embryos have confirmed the importance of these and additional cell cycle parameters in predicting embryo viability and/or aneuploidy risk.[208,209] Given the significance of preimplantation embryo development in human biology and pathology, further investigation of the cellular dynamics, underlying molecular programs, as well as genetic and epigenetic profiling of normal and abnormal human development is clearly warranted.

EMBRYONIC STEM CELLS

Biology of Embryonic Stem Cells

Questions about human origins and human development have always been important centerpieces of scientific and public discourse. The significance and impact of embryonic stem cells (ESCs) on the public has fluctuated considerably over the last 50 years, but always invigorated the scientific community. Early observations with mouse teratocarcinomas and embryonal carcinomas demonstrated the sheer developmental power of stem cells to become virtually any other cell type. However, interest remained limited until scientists isolated and harnessed the potential of mouse embryonic stem cells (mESCs) for the generation of genetically modified mice. With the advent of hESCs isolation from discarded embryos, the scientific and public community increasingly shifted their attention to ESC biology. Moreover, recent innovations in resetting cells to an embryonic-like state developed by Nobel prize recipient Shinya Yamanaka and colleagues incorporated 50-year-old concepts developed by his Nobel corecipient, John Gurdon (2012 Nobel Prize

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in Physiology or Medicine), about somatic cell cloning and have refocused a multidecade topic on the prospect of stem cell-based therapies for regenerative medicine. More importantly, the use of ESCs and the aforementioned iPSCs have helped us understand the molecular control of self-renewal and introduced new concepts in preimplantation human development such as trophoblast function, germ line specification, and neuronal lineage formation. Some of these historical breakthroughs encompass the analysis of primitive embryonic tumors, germ cell tumors, and ESCs and are discussed next.[210]

Early efforts at understanding pluripotency in the mammalian embryo began with rather simple experiments in which pieces of mouse germ cell tumors known as teratocarcinomas were grown in culture and a variety of pluripotent cell types emerged.[210] One cell type, embryonal carcinoma cells, produced all three germ layers as well as the original tumorigenic cells when isolated and transplanted into host mice. This established, for the first time, the pluripotent nature of embryonic cells. mESCs from the preimplantation embryo initially form within the ICM. In studies by Evans and Kauffman and others, cells of the ICM were isolated from late blastocysts and grown in culture.[211,212] These cells proliferated and demonstrated the capacity for self-renewal. The subsequent addition of leukemia inhibitory factor (LIF) promoted the self-renewal of mESCs in culture by acting through the Stat3 pathway.[213] Further studies showed that the ICM-derived cells could, if reintroduced into an embryo lacking an ICM, recolonize and form all three germ layers of the embryo, including the germ line.[214] mESCs are characterized by the strong and persistent expression of important pluripotency genes such as POU domain, class 5, transcription factor 1 (*Pou5f1/Oct4*), SRY-containing box-2 (*Sox2*), NANOG homebox protein (*Nanog*), and transcription factor 4 or 8 (*Tcf-4/Tcf8* among others).[215] In general, mESCs are considered to have a more naïve ground state of pluripotency, meaning their potential for differentiation to all cell lineages, including the epiblast, is greater than hESCs.[216,217] However, mESCs still possess important differences from cells of the mouse ICM in their hypermethylated DNA composition and their capacity for self-renewal.[217,218] Meanwhile, hESCs share some properties with mouse epiblast stem cells (mEpiSCs) (pluripotency) but may be more restricted than mESCs.[219] This may be the case because current hESC derivation methods from the ICM could actually select for cells in a different developmental stage than that from the mouse ICM.[216]

hESCs are derived in a very similar manner to mESCs from explants of ICM of in vitro, fertilized blastocysts that bear features of a transient epiblast-like state.[220] Thomson et al. first isolated hESCs from preimplantation human blastocysts and analyzed their morphology and expression of pluripotency genes.[221] Although they express high levels of pluripotency genes, hESCs share a morphology that is more consistent with that of early mEpiSCs. Specifically, they are "flattened", possess large nuclear to cytoplasmic ratios, and respond less favorably to single cell clonability in contrast to mESCs.[219,222] In addition, they depend on basic FGF (bFGF) and activin signaling for self-renewal. While the signaling pathways of the TGFβ superfamily (TGFβ/activin/nodal) are undoubtedly important for hESC pluripotency, the molecular mechanisms underlying these observations have yet to be explained. Nevertheless, the transcription factors OCT4, NANOG, and SOX2 are clearly all involved in hESC pluripotency, and more recently identified molecules such as the WNTs (wingless-type MMTV integration site family members) have been implicated to drive hESC self-renewal through canonical signaling pathways.[223] The functions of OCT4, NANOG, and SOX2 are regarded as indispensable for hESC self-renewal as their expression typically reduces when hESCs differentiate. Additionally, activation of activin and nodal signaling pathways are thought to be essential for controlling hESC cell fate. Specifically, the high expression of activin and nodal is thought to either directly maintain pluripotency via their downstream receptors, ACTRIIB and ALK4 receptors and SMAD coactivators, or prevent the spontaneous differentiation of hESCs to neuroectodermal pathways.[157] Finally, hESCs and mESCs are similar in that they both express a repertoire of cell surface proteins that distinguish them from other cell types, including the stage-specific embryonic antigen-3 and -4 (SSEA-3 & SSEA-4) and the proteoglycan receptors TRA1-60 and TRA1-81. However, mESCs and mouse iPSCs (miPSCs) express SSEA1 and SSEA3, but not SSEA4. Together with OCT4, NANOG, and SOX2 protein expression, these markers are routinely used for characterization of hESCs, mESCs, and iPSCs (see following) cell lines. Additionally, it has been observed that ESCS and embryonic germ cells (EGCs) share several biomarkers, making it difficult to distinguish between the embryonic, pluripotent, and primitive germ cells.[224,225] The intimate relationship that exists between the germ line and ESCs in mice and humans is critically important to decipher in the future and will greatly inform current and future efforts to derive "artificial" gametes. In fact, derivation of functional gametes from pluripotent stem cells in the mouse has been accomplished with considerable success and is discussed later in this chapter. However, insights into human germ line specification are lagging behind those of the mouse field largely because more efficient models of human PGC specification from pluripotent stem cells are still being designed and tested. Nevertheless, there is an overall emphasis for derivation of male and female gametes in order to advance our understanding of the genetic and nongenetic origins of human infertility.

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Nuclear Reprogramming and Induced Pluripotency

A set of landmark studies conducted by Yamanaka and Gurdon and supported by work from other laboratories revealed that just four factors normally produced in the embryo are sufficient to reprogram differentiated, non-embryonic cells into pluripotent stem cells. This important finding enables many of the ethical issues associated with the use of human embryos to be bypassed and also evades potential problems with immune rejection that might affect patients upon stem cell transplantation. The four Yamanaka reprogramming factors—*Oct4, Sox2, Klf4*, and *C-myc*—when introduced into differentiated cells in excess amounts, change cell fate by inducing these cells to revert back to a progenitor-like or ESC-like state. It is believed that this is a stochastic process whose mechanisms lie in the ability of these four transcription factors to induce epigenetic restructuring of the genome, thereby enabling expression of previously silenced genes.[217,226] These artificially created pluripotent stem cells were first described by Takahashi and Yamanaka in rats in 2006.[227] The same researchers also demonstrated that they could derive iPSCs from adult human cells, a finding subsequently confirmed by others.[228–231]

A major concern with iPSCs is the level of genomic disruption that occurs with viral delivery of the reprogramming factors using retroviral or lentiviral vectors. Theoretically, these viral vectors, by integrating randomly into the host genome, may cause chromosomal translocations or transform reprogrammed cells into malignant cells. There are now several initiatives underway that seek to understand the degree of genome-wide mutations and changes in gene function that occur in iPSCs.[232] The art of generating pluripotent cells now involves making iPSCs with reprogramming proteins using techniques that both avoid the use of embryos and the downstream hazards of viral vectors. Additionally, it is now possible to employ just the reprogramming proteins of these factors without the use of viral vectors to create iPSCs.[233] Several gene delivery systems that have been described, including those that employ excisable polycistronic cassettes,[234,235] *piggy*BAC introduction,[236] transfection and/or nucleofection of plasmids,[237] and most recently, mRNA and miRNA delivery.[238] There is also evidence that cocktails of small chemicals including forskolin, valporoic acid, and CHIR99021 (a glycogen synthetase kinase 3 inhibitor) can be used as agents to induce reprogramming in somatic cells.[239] As per these reports, mechanisms by which small molecules induce pluripotency may include the initial activation of a transcription factor, sal4, which may assist in oct4 promoter demethylation and activation of oct4 transcription. Finally, direct reprogramming methods wherein cells from one lineage are directly transdifferentiated to another lineage (e.g., fibroblast to neural-like lineage) are gaining in popularity. Specifically, terminally differentiated cells like skin fibroblasts can be exposed to a cocktail of factors that simultaneously enable a potential reversal of cell fate and induce differentiation to the second lineage.[240–242] It is not clear if these cells transit through an ESC-like state before acquisition of their eventual cell fate. The use of these exciting approaches further highlights the plasticity in the fate of somatic cells that was previously treated as irreversible and suggests that acquisition of complete pluripotency may not be a requisite for full function of the desired cell type.[243] In sum, the use of human, patient-specific iPSCs in cell lineage tracing, cell fate alteration, and cell-based therapies has set the stage for revolutionary clinical breakthroughs in regenerative medicine.

Ethical and Technical Concerns in the Use of Stem Cells

Among the many ethical, technical, and safety concerns associated with the clinical application of stem cells, the primary concern surrounds the stem cell source. hESCs have generated the most controversy because the procedure of deriving a hESC line involves the sacrifice of a donated human embryo, even though the donated embryos would have likely been discarded (reviewed in Refs 244,245). Therefore, alternative sources of stem cells, such as iPSCs, which might achieve similar therapeutic goals, could be very valuable and bear fewer ethical issues than hESCs. Since one of the standard measures of a pluripotent stem cell is that it reliably forms teratomas when transplanted into animal models, careful long-term analysis and follow-up after transplantation will be needed in animal models before stem cells or directed differentiated cells are applied to humans. Yet, hESCs still represent the best model system with which to study early human development because of their robust differentiation potential and relatively permissive epigenetic status, and therefore remain the standard for studying stem cell properties. However, their suitability for transplantation studies is questionable mainly because of the risk of immune rejection from host human tissues.[246] In contrast, patient-autologous iPSCs are considered more suitable for clinical transplantation due to better immunological suitability, but their differentiation profiles, epigenetic status, and potential for tumor formation are far less characterized than are hESCs.[247] Therefore, it is imperative that studies with hESCs continue to allow better definition of the true potential of all other stem cell sources, including human iPSCs. Fortunately, there is currently considerable research devoted to deciphering the molecular mechanisms that underlie the cell fate reprogramming process. In parallel, many laboratories are now developing iPSC-based disease models

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for human degenerative diseases and cancers. These in vitro studies should provide the research community with insight into the mechanisms of disease progression and illuminate avenues of therapeutic potential. Consequently, hESCs will likely remain the gold standard stem cell type for developing basic scientific and clinical therapeutic protocols, and to which iPSCs and adult stem cells will be carefully compared for their therapeutic potential.

GERM LINE DEVELOPMENT

PGC Specification

As defined in the introduction, germ line establishment in mammals occurs via "inductive" signaling, in contrast to lower organisms such as flies and worms where the germ cell identity is transmitted via the inheritance of germ plasm.[28–30] In rodents and humans, PGC formation is first observed in the proximal epiblast region and near the extraembryonic ectoderm (ExE) region of the embryo (E6.5 in mouse) after implantation and gastrulation (Figure 6.2).[30,31] Coincident with the formation of epiblast, endoderm, mesoderm, and ectoderm, molecular cues including BMP4 and BMP8a are produced from the yolk sac. BMP signals induce a population of pluripotent stem cells to be segregated from the ICM and set physically apart from the extraembryonic ectoderm or yolk sac of the embryo.[32–34] Additional mechanisms that may assist germ line specification are the activation of molecules and pathways that either promote PGC survival such as the cytokines, fibroblast growth factor 9 (FGF9), LIF, and kit ligand (*KITLG*), or via modulation of signaling molecules such as AKT kinase and interleukin 6 pathways. Germ line specification can also be controlled by the inhibition of molecules that promote somatic differentiation of PGCs including the WNT/beta-catenin pathway and p53 activation. These mechanisms have been greatly elucidated in the mouse and are comprehensively reviewed in Ref. 248. Although several of these molecules and pathways are conserved in humans, their contribution to germ line specification is yet to be confirmed. As such, mouse and human PGCs retain expression of several biomarkers of pluripotency, including OCT4, NANOG, and SOX2, which underlies their close resemblance to other pluripotent stem cells (Figure 6.3).[225,249,250] Interestingly, the transcription factor SOX2 is expressed in both mouse and hESCs, but unlike in the mouse, human SOX2 expression is diminished when PGCs migrate to the fetal gonad.[251] Concomitant with the timing of germ line specification in the mouse (E7.5), PGCs near the extraembryonic ectoderm begin to express germ cell-specific markers such as *Blimp-1* (*PRDM1* in human), *Stella* (*DPPA3* in human) and deleted-in-azoospermia-like (*DAZL*) as well as acquire alkaline phosphatase (AP) activity (Figure 6.3). BLIMP-1/PRDM-1 is a transcriptional repressor whose activity is restricted to the germ lineage and appears to be critical for maintaining a PGC fate. There is strong evidence to suggest that in the mouse, BLIMP-1 actively represses the somatic fate of PGCs by inhibiting expression of key somatic regulators during development.[35,252,253] It is unclear whether PRDM1 carries out a similar function in human germ line formation. Once cell–cell communication has been established, the interferon-induced transmembrane proteins (IFITM1 and IFITM3) and STELLA/DDPA3 proteins promote further development of PGCs and may do so in a similar fashion as BLIMP-1 in mice.[30,254]

While several studies have documented how mESCs give rise to the PGC lineage in the mouse embryo, the lineage tracing of hESCs is difficult. Specifically, molecular data documenting the induction and specification of PGCs is severely affected by the lack of pregastrulation embryos. Nevertheless, early efforts of tracing human PGC development utilized three-week-old embryos in which a cluster of extragonadal PGC-like cells were observed as dense, rounded cells that were distinct from somatic cells (reviewed in Ref. 255). However, to remedy the lack of early lineage markers and developmental events of human PGCs, several groups have embarked on in vitro-based strategies to derive PGCs from human ESCs and iPSCs and then use these in vitro-derived germ cells for marker analysis (Figure 6.3). These efforts will be discussed in detail later in this chapter. Interestingly, hESCs may share some features with PGCs, and a subset of hESCs may be "primed" for a germ cell fate.[224,225] This observation has further underlined the unique biology of human germ cell development.

To develop more sustainable models for the study of molecular mechanisms of early germ cell development, considerable efforts have been made to culture mouse and human PGCs and gonocytes in vitro. In the case of mouse germ cells, the addition of endogenous factors known to affect germ cell development such as BMPs, RA, LIF, and forskolin have produced mixed results in maintaining PGCs in culture. For example, adding LIF enhanced PGC survival, but the observations with the use of other factors is not as clear. PGCs may also behave erratically in culture (showing low survival rates and nonsex-specific behavior) because of the lack of a normal somatic environment.[256] However, a landmark study has recently shown that supplementation of LIF in a "2i" cocktail for mESC growth can induce an epiblast-like cell stage of mESCs and set the stage for PGC development.[257] The 2i cocktail consists of serum-free medium and the use of two small molecules, PD0325901 and CHIR99021, that inhibit activation of MAP kinase and glycogen synthetase kinase 3, respectively. These inhibitors modulate cell

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turnover and differentiation potential in favor of overall stability of mESC cultures. Efforts to culture human fetal gonocytes by Shamblott et al., Turnpenny et al., and Tu et al. resulted in a mixture of cellular phenotypes. Some gonocytes appeared rounded, while others took on an elongated or spindly appearance. In addition, they appeared to have different proliferation rates.[258–260] Currently, it is not at all clear that these cells resemble their germ cell counterparts in vivo, but improvements in culture conditions and the cellular microenvironment will certainly help in this regard. The difficulties in growing these cells have led several researchers to turn attention to the isolation and in vitro culture of prospective spermatogonial stem cells (SSCs) from human tissue, which will be discussed later in this chapter.

Germ Cell Colonization of Gonads

Migration of germ cells to the gonad begins at E8.5 in the mouse and during weeks 4–6 of human gestation (first trimester). At this stage, PGCs accumulating at the base of the allantois exit the extraembryonic ectoderm and begin migration to the developing gonads, also known as the genital ridge (Figure 6.2). Specifically, PGCs are brought to the genital ridges by way of invagination of the hindgut and passage through the dorsal mesentery (Figure 6.2, left, arrows). Because of the remarkable journey undertaken by PGCs through several somatic tissue environments, it is plausible that PGCs are responsive to several different signaling environments that instruct their motility. It is thought that IFITM1 and/or IFITM3 are largely responsible in initiating PGC migration into the endoderm and to the future hindgut (Figures 6.2 and 6.3). However, mouse knockout studies have yielded conflicting results on the role of this molecule.[254,261] Other molecules expressed during migration include the CXCR4 receptor on the surface of mouse PGCs and the proto-oncogene c-KIT and its ligand, KITL, in both mice and humans.[262,263] DAZL is also expressed on migrating PGCs in mice[264] and on postmigratory human PGCs in the testis.[265,266] In support of a model of directed migration of PGCs in mouse and human embryos, it is considered that extracellular proteins such as collagen I/II/IV, fibronectin, and laminin all play a passive role in guiding PGCs to their destinations. Accordingly, it follows that adhesion molecules such as integrin α and β receptors on PGCs and in the cells of the hindgut are important guidance cues.[267,268] In the mouse, migratory PGCs display pseudopodia that may assist in movement through the hindgut, and it is plausible that human germ cells behave similarly since they are also observed in the hindgut during migration.[269,270] Indeed, studies using electron micrographs of 4–6-week-old human embryos revealed irregular structures that resembled pseudopodia on PGCs.[270,271] Various somatic tissues interact with PGCs during the migratory path to the gonad. It is therefore likely that these tissues express molecules and factors that guide or "cue" the PGCs and help maintain their survival. Several candidate molecules have been identified in the mouse, including receptors such as β-1 integrin and extracellular matrix components such as collagen I.[272,273]

Although migration of PGCs is less well understood in human development, germ cells have been histologically observed during the late first trimester, when they undergo migration to the hindgut.[271,274,275] In another study, histological evaluation of first-trimester fetuses revealed that human PGCs intimately contact with and migrate along nerve fibers and Schwann cells to arrive at the gonadal ridge.[276] Male and female PGCs have been isolated from 10-week old fetuses and were observed to express AP, a marker of PGCs.[275] The morphology of human PGCs also resembles the rounded shape of mouse PGCs. Female-specific germ cells have also been visualized at the ultrastructural level during gonadal development in human fetuses.[277] Finally, recent studies by Kerr et al. with human fetal gonads (testis and ovary) provide a detailed analysis of pluripotency and germ cell-specific markers. The germ cells of the fetal testis are OCT4+/NANOG+/C-KIT+ from week 7–15, after which these cells become localized to the periphery of the testis. Meanwhile, the presumptive gonocytes in the week-15 testis show strong expression of pumilio RNA-binding family member 2 (PUM2), VASA, a cytoplasmic protein that is implicated in translational regulation and DAZL and express low to no pluripotency markers (OCT4, NANOG, C-KIT, TRA1-60, TRA1-81).[265] In the human fetal ovary, as in the testis, the expression of pluripotency markers peaks by week 8 and then declines after week 9, as oocytes enter meiosis. Interestingly, the cell surface markers SSEA-1 and SSEA-4, which are also negative and positive cell surface markers of hESCs, respectively, are co-expressed on the female germ cells from week 5 onwards, although only SSEA-1 is restricted to the germ cell lineage.

Upon arrival at the genital ridge, mouse and human germ cells begin to express VASA (Figure 6.3). The gene encoding VASA expression, *DDX4* (*Mvh* in mouse), is highly conserved among species and is expressed exclusively in both male and female pre-meiotic germ cells.[278] This finding underlines the importance of the VASA protein in germ line function and makes it an attractive candidate for further study. Along with VASA, other factors produced in the mouse are germ cell nuclear antigen-1 (GCNA-1) and E-cadherin. The sex-specific character of the developing gonad is controlled by the chromosomal constitution of gonadal somatic cells as discussed in Chapter 7. In particular, the *Sry* gene expressed on the Y chromosome in mammals is thought to be an essential regulator of various downstream targets, including the *Sox9* gene that controls male gonadal development.[279,280] Once within the

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gonad, germ cells associate with Sertoli cells to form testis cords, and this interaction induces the expression of VASA in postmigratory PGCs. VASA expression is induced in both male and female PGCs and persists until these cells enter meiosis, after which its levels diminish.[281,282]

Developmental Remodeling of PGCs

During embryonic and germ line development, an extensive remodeling of the epigenetic landscape occurs (Figure 6.3). This takes place by global DNA demethylation at the transition from morula to blastocyst stages of embryogenesis followed by reestablishment of DNA methylation in the blastocyst during implantation as discussed earlier. When PGCs are specified from ESCs or epiblast at about 2 to 3 gestational weeks in human and E6.0–E8.0 in mice, they begin to undergo global DNA methylation erasure.[250] Postimplantation, de novo methylation of DNA continues exclusively in PGCs during their specification period. Finally, migrating PGCs undergo a genome-wide DNA demethylation period, presumably induced by the local environmental changes around PGCs in the hindgut and genital ridges.[283–285] This phase occurs only in germ cells and may help to prime them for sex-specific DNA remethylation, when their developmental programs are established.[286] In the mouse, the reestablishment of imprints takes place prior to birth in the male prospermatogonia (E15) and only after birth in oocytes.[287] In addition to DNA methylation changes, male and female germ cells also undergo posttranslational histone modifications such as increased methylation of histone H3 at lysines 27 and decreased methylation at lysine 9 on histone H3, which may regulate transcriptional regulation of germ line-specific genes. Additionally, RNA-mediated silencing and X chromosome inactivation also participate in epigenetic remodeling of male and female germ cells (reviewed in Refs 250,288).

Hypomethylated PGCs in primitive gonads undergo sex-specific methylation changes during oogenesis and spermatogenesis. However, establishment of male and female imprints does not take place until after gonadal differentiation and at different time points in male and female development (Figure 6.3).[289] The DNMT enzymes DNMT1, DNMT3A, and DNMT3B are considered to be the principal catalysts of DNA methylation of cytosines at the 5- position (5-methylcytosine). The specific roles of DNMTs in human PGCs is not clear due to the lack of access to migratory PGCs, but mouse studies have yielded important insights about DNMT1 maintaining methylation globally, while DNMTs 3A and 3B serve as de novo methylators of DNA (reviewed in Ref. 283). Recently, the modification of 5-methylcytosine to 5-hydroxymethycysotosine (5-hmC) by the TET family of enzymes has illuminated an additional level of regulation of DNA methylation erasure in PGCs.[290] The precise role of 5-hmC as an intermediate modification or source of additional molecular recognition is being actively explored. Meanwhile, core histone modifications may also aid in the DNA demethylation process. Specifically, dimethylation of histone H3 at lysine 9 (H3K9), a repressive mark, appears to be reduced in PGCs over time, while the permissive marks of H3K27 trimethylation and H3K4 dimethylation increase up until the eventual G2 arrest of PGCs in gonads (Figure 6.3). Therefore, a working model of epigenetic remodeling is a passive mechanism involving de novo methylation followed by widespread DNA demethylation at imprinted and nonimprinted loci, resulting in activation of sex-specific gene expression and commitment to the spermatogenesis or oogenesis pathways.

The successful passage of germ cells through meiosis is a unique and highly rigorous process as detailed in Chapter 1. However, in general, between male and female embryos, the timing and mechanisms of meiotic entry bear similarities and key differences.[266] Male germ cells are restricted from entering meiosis, while female germ cells enter meiosis within the embryo. Female germ cells destined to become oocytes eventually arrest during meiosis I prior to fetal birth and will only resume meiosis upon receiving hormonal signals during adulthood.[291] Meanwhile, male germ cells transition from primordial status to the gonocyte stage, stop proliferating, and remain quiescent in the fetal seminiferous tubules until after birth. The postnatal gonocytes then commit to a SSC fate and amplify through self-renewal or enter meiosis to initiate spermatogenesis. In both male and female germ cells, the synaptonemal complex proteins (SCPs) SCP-1, SCP-2, and SCP-3 are critical components of the meiotic machinery during chromosomal segregation (Figure 6.3).[292–294] The completion of meiosis signals that germ cells have matured into haploid male and female gametes. At this stage, oocytes exclusively express growth differentiation factor-9 (GDF9) and spermatocytes express tektin 1 (TEKT1).

GONADAL STEM CELLS

Guided by the premise that male gametes must be replenished, several investigators have sought the identity of germ line stem cells (GSCs) in the testis. In females, a finite supply of follicles endowed upon birth in women and in mice argues against the existence of oogonial stem cells, although this concept has been revisited recently.[295–297] Moreover, the pluripotency potential of EGCs and their similarity to ESCs has fueled the speculation about the similarities between gonadal stem cells such as SSCs and ESCs in vitro and in vivo. There is compelling data proving the existence and plasticity of testicular GSCs in the mouse and more recently the human testis. Functional evidence for stem cells in the rodent

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adult testis was first provided by Brinster and colleagues in 1994 when they transplanted a putative SSC population extracted from a prepubertal mouse testis into completely sterile *W/W* recipient mice and observed chain-like colonies of donor-derived cells, indicating complete spermatogenesis with transplanted cells.[298] Brinster and colleagues followed up their studies with transplantations of primate SSCs and human testicular cell suspensions into seminiferous tubules and observed colonization and survival of donor cells two months after transplantation, which solidifies the use of this assay for testing SSC function.[299,300] Recent research has sought to characterize the true stem cell potential of early spermatogonia within the seminiferous tubule. Kanatsu-Shinohara et al. first reported the generation of pluripotent stem cells from the mouse testis.[301] In their study, a population of cells termed GSCs was isolated from neonatal mouse testis and propagated in culture.[302] Subsequently, they found that cell colonies with morphology quite distinct from GSCs, termed ESC-like cells, also appeared in culture. The formation of both GSCs and ESC-like cells depended on a GDNF signal, and only ESC-like cells expanded in regular mESC media. The expression profile and imprinting profiles indicated that the ESC-like cells had gene expression and imprinting profiles that closely matched those of mESCs. Interestingly, transplantation of ESC-like cells into seminiferous tubules gave rise to teratomas, while GSC transplants led to spermatogenesis without teratomas.

In 2006, pluripotent stem cells were derived from the adult mouse testis.[303] With the introduction of a *Stra8-eGFP* reporter in which GFP expression was regulated by *Stra8* promoter activity, ES-like cells were separated from a heterogenous population of testicular cells. When these isolated ES-like cells were grown on mouse embryonic fibroblasts or cultured in medium containing LIF, they evolved into a subpopulation of cells that the authors termed multipotent adult germ line stem cells (maGSCs). Another transplantation study by Nakagawa et al. in 2007 employed the neurogenin 3 (*Neurog3*) promoter to induce GFP expression and thereby enrich for two populations of undifferentiated spermatogonia.[304] The researchers took advantage of the expression of NEUROG3 on undifferentiated spermatogonia, and, from their sorting studies, the first population of cells maintained the SSC progenitor pool through self-renewal while the second population revealed a pool of SSCs that diminished with age. In parallel to these studies of mice multipotent GSCs, several groups have reported a collection of genes and biomarkers that are nonexclusively expressed by mouse SSCs.[305,306]

Motivated by the successful discovery of SSCs in the adult rodent testes, several investigators have attempted the same types of studies in the human. In a landmark study, Conrad and colleagues harvested THY1-positive SSCs from adult human testes and subsequently reprogrammed them into pluripotent cells.[307] The single-cell suspension was then cultured in vitro in the presence of the self-renewal factor, GDNF. SSCs were purified from the mixed population by magnetic-activated cell separation with CD49-bound beads. Overall, the authors observed significantly different expression profiles in human maGCSs (hmaGSCs) compared to the parent spermatogonial cells. They also noted that hmaGSCs acquired expression profiles similar to hESCs. Although the foregoing study represents a complete analysis of a novel cell population in the human testis, several caveats to this research exist. Entire testicles were used to derive spermatogonial cells, bringing into question the feasibility of this approach for clinical use.[308] In addition, the global gene expression patterns of pluripotency markers obtained from these hmaGSCs have been challenged as being more closely associated with fibroblasts than hESCs.[309] Nevertheless, the above experiments were corroborated by other groups, who used THY1 as a marker for isolation of prospective SSCs.[310,311]

To address the issue of limited availability of starting material, two similar approaches were used for generating human pluripotent stem cells from testicular biopsies as reported by Kossack et al. and Golastaneh et al.[312,313] After enzymatic digestion of the adult testis biopsy, cells were plated with or without mouse feeder cells, and hESC-like colonies were observed after about 7–10 days. Subsequently, hESC-like cells in colonies were propagated by manual dissection onto fresh mouse embryonic fibroblasts in hESC culture conditions, supplemented with FGF. Under these conditions, the spermatogonial cells maintained an hESC-like morphology, were karyotypically normal, expressed both pluripotency and early germ cell markers, and contained a generally hypomethylated DNA pattern consistent with hESCs rather than somatic cells. However, hmaGSCs isolated in this study did not successfully form large teratomas in vivo. Therefore, even with limited substrate, this study suggested that hmaGSCs, unlike true hESCs, might be only multipotent and not fully pluripotent. In more recent experiments, at least two other markers, SSEA4 and the CD9 receptor, have been used to test for the presence of SSCs in the human testis. CD9 is a matrix metalloproteinase that binds to extracellular matrix molecules and is expressed on the surface of human spermatogonia. Following transplantation of CD9-positive human spermatogonia into mouse testes, long-term survival of donor cells was observed.[314] Meanwhile SSEA4-positive cells isolated from human testicular biopsies were enriched for SSCs as evidenced by their survival and localization to the basement membrane after transplantation to mouse seminiferous tubules.[315] Collectively, these studies highlight the existence of a multipotent stem cell population that bears resemblance to mouse SSCs by virtue of colonization of the seminiferous tubules and their clonogenic properties.

The exploration of GSCs in the human ovary has gained interest recently because of findings that challenged

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the long-held notion that women are endowed with a finite supply of oocytes. For instance, Johnson and colleagues observed that a small population of GSCs reside in the ovarian surface epithelium in the mouse and can undergo self-renewal of the follicular pool within 24 h.[316] The source of these GSCs was proposed to be from the bone marrow and via the vasculature.[317] Despite the proof of successful transplantation in this study, they failed to demonstrate meiosis in the oocytes generated from ovarian stem cells (OSCs) or bone marrow-derived stem cells.[318] Furthermore, it was not clear whether these OSC-derived oocytes could be artificially fertilized. Based on these findings, the existence of GSCs in the adult ovary remained unknown until a follow-up study by the same group in 2012. In their latest efforts, Tilly and colleagues established a method by which adult human OSCs can be isolated using a FACs-based approach to target the N-termini of VASA.[319] They went on to characterize the germ cell behavior of OSCs, their meiotic competence, and potential for follicle formation in vivo following xenotransplantation with human ovarian cortical tissue into mouse ovaries. Yet, questions remain as to the efficiency of OSC derivation, their ability to mature in vitro, and, more importantly, the veracity of their transformation in vivo after their exposure to artificial in vitro manipulation.[320] If OSCs can be isolated from adult ovarian biopsies, as these studies claimed, it seems reasonable to expect that immature ovaries from neonatal or prepubertal mice or adolescent girls would produce far greater numbers of OSCs by the methods described.[319,321] However, the studies by Tilly and colleagues did not examine this possibility, and if the future strategy is to preserve OSCs from young women or adolescent girls prior to extensive follicle loss or before chemotherapeutic exposure, then it seems worthwhile to quantify and document the OSCs at those stages. Nevertheless, these proof-of-principle studies are illuminating about the possible existence of an ovarian progenitor cell type.

GENERATION OF GAMETES FROM PLURIPOTENT STEM CELLS

Our knowledge about mouse embryo and germ line development from mESCs has allowed for in vivo and in vitro modeling of germ line development. However, very limited access to early stage human embryos and especially human PGCs has greatly reduced the ability to conduct similar investigations into human germ line development, let alone modeling it in vitro (Figure 6.6).

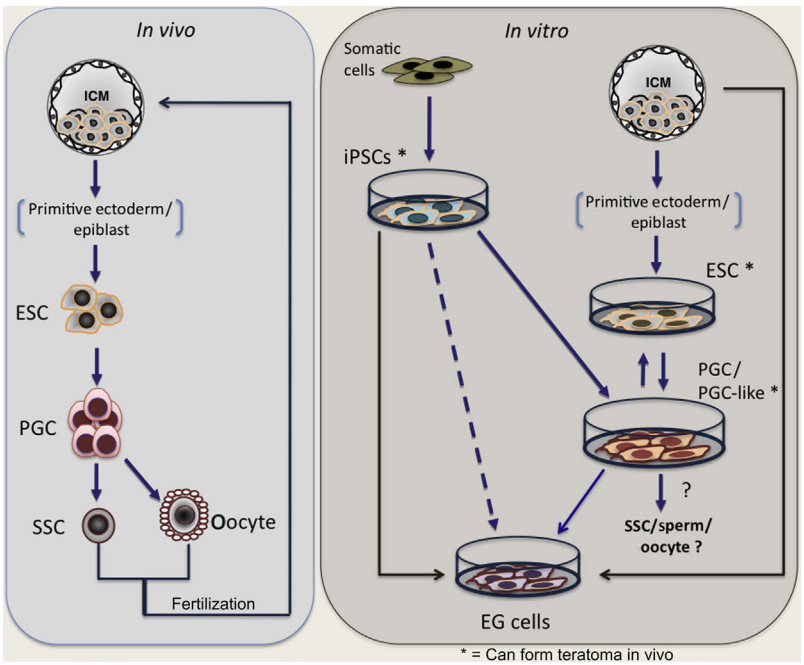

FIGURE 6.6 Comparison of in vivo germ line development and in vitro germ cell derivation from ESCs and iPSCs. A depiction of in vivo development of germ cells and gametes from the mammalian embryo is shown on the left to demonstrate that embryonic stem cells (ESCs) originate from primitive ectoderm (epiblast) by way of the inner cell mass (ICM). Primordial germ cells (PGCs) then migrate to gonads where they undergo self-renewal in the form of spermatogonial stem cells (SSCs) in the testis or further differentiate to oocytes in the ovary. On the right is a schematic of the different cell lineages that can be derived in vitro. ESCs cultured in dishes can be differentiated to PGCs via methods summarized in Table 6.1. PGCs cultured in vitro, at least in the mouse, can transform to embryonic germ cells (EGCs). There is also evidence to suggest that a subpopulation of hESCs cultured in vitro behave like EGCs. Finally, somatic cells can be reprogrammed to iPSCs with addition of the transcription factors, OCT4, SOX2, KLF4, and MYC to induce a stem cell-like fate. iPSCs can then be induced to form PGC-like cells or EG-like cells by in vitro and in vivo methods. Those cell types marked with an asterisk indicate that ESCs, PGCs, and iPSCs can all form teratomas when injected in vivo as demonstration of their pluripotency. *Source: Adapted from Ref. 17.*

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This section reviews the highlights of experiments conducted during the last decade using in vitro model systems of mouse and human germ line development from mESCs and hESCs (Table 6.1).

Derivation of Germ Cells from Mouse and hESCs

The mouse has proven to be a robust and dependable system for analysis of germ line development. Studies conducted in vivo during early mouse development have greatly provided information for subsequent in vitro investigations and have generated promising prospects for creating gametes from nongonadal stem cells. The earliest in vivo evidence for ESC-derived germ cells was suggested from experiments performed by Bradley et al. in 1984. They generated germ line chimeras after injecting mESCs into mouse blastocysts.[214] This work suggests that mESCs, if cultured under the right conditions, could be differentiated to PGCs. We now know that mESC share many developmental features and markers with PGCs, which further supports the use of mESCs as a starting point. This is most convenient as mouse PGCs are difficult to grow and differentiate in vitro.

The first report on the creation of putative germ cells in vitro was reported in 2003. In this study, mESCs were transfected with a reporter green fluorescent protein (GFP) under the control of an oct4 enhancer sequence.[322] The withdrawal of LIF, a factor that promotes self-renewal in mESCs, promoted PGC development and resulted in an increase in GFP-positive cells that resembled a follicle-like appearance. Tooyaka et al. used a mouse VASA homologous gene promoter *Mvh* to drive GFP or LacZ expression in mESCs, which then spontaneously differentiated to embryoid bodies (EBs) with supplementation of BMP4 and in the absence of LIF. EBs were subsequently dissociated and cells expressing GFP were separated from the bulk population using fluorescence-activated cell sorting (FACS) and found to be positive for Mvh. Subsequently, MVH-driven LacZ+ cells were derived, aggregated with mouse embryonic gonadal tissue, and transplanted under a mouse neonatal testis capsule. These cells differentiated to give rise to LacZ-positive spermatozoa.[323] These two landmark studies set the stage for further investigation of deriving gametogenesis from mESC.

In 2004, Geijsen et al. derived male gametes from mouse EBs in retinoic acid-enriched medium. These germ cells were capable of entering meiosis and fertilizing a mouse oocyte.[324] Other studies have employed slightly different approaches to derive putative oocytes such as cocultures of PGCs made from cultured EBs with fetal ovarian granulosa cells.[325] In an alternative approach developed by Salvador et al., a *Gdf9*-driven GFP reporter transgene was introduced into mESC. The mESCs were then differentiated to EBs and GFP-positive cells were isolated from the EBs to isolate PGCs.[326] It was later shown by Kerkis et al. that the divergence of male and female germ cell programs during spontaneous differentiation of EBs in vitro was dependent on the length of culture time.[327] Several attempts at oocyte or follicle derivation from mESC have been made using the EB-based approach with various culture and attachment conditions, all of which yielded oocyte-like cells that lacked critical structural features such as the zona pellucida or expression of meiotic molecular machinery.[328,329] In summary, these latter studies may have derived primitive oocytes from mESCs but failed to accomplish meiotic entry in the final cell products. An alternative approach to deriving female germ cells was employed by Nicholas et al. using a germ cell-specific reporter *ΔPE:Oct4:GFP*.[330] GFP-positive cells exhibited a higher expression of germ cell and oocyte markers, and when aggregated with fetal ovarian tissue and transplanted under a mouse kidney capsule, gave rise to mature oocytes in vivo.

The first study to show virtually complete gametogenesis in vitro was conducted by Nayernia et al. in 2006. A two-stage approach was employed to first derive pre-meiotic PGCs from mESCs with a *Stra8*-driven GFP reporter induced with retinoic acid (RA) addition.[331] GFP-positive cells were then separated by FACS, grown in RA-supplemented medium, and transfected with a second reporter, *Prm1-Dsred*. The second reporter enriched for PGCs that had undergone meiosis and formed red-fluorescing, haploid cells. After further RA treatment, the haploid sperm-like cells were then injected into mouse oocytes by intracytoplasmic sperm injection (ICSI) to test the functionality of the derived germ cells. Indeed, blastocysts formed and viable offspring were born that eventually succumbed to developmental complications and died prematurely. Potentially, the epigenetic reprogramming of the derived germ cells may not have been complete. Nevertheless, these efforts provided functional proof of gametogenesis in vitro.

To further demonstrate in vitro spermatogenesis, researchers have also employed the use of organ culture systems. This is best exemplified by a very recent study by Sato et al. in which functional mouse sperm were generated in vitro by employing a novel organ culture method.[332] Specifically, they improved on existing organ culture methods by placing neonatal (mouse pup) testis fragments in serum-free or FBS-soaked agar plugs in culture. Within these plugs, they had the ability to control the gas:liquid interface while also adding exogenous factors such as BMPs, activin A, hepatocyte growth factor, and testosterone. Their study conclusively showed through an analysis of reporter expression before and after meiosis that functional haploid spermatozoa were generated that could fertilize oocytes and give rise to viable, healthy pups. Moreover, the generated pups

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TABLE 6.1 A Summary of the Recent Efforts to Derive Gametes from ESCs and iPSCs

Source of Cells	Germ Line Differentiation Strategy	Endpoint			Reference	Reference #
		Cell Types Formed	Gametes	Fertilization		
mESC—XY, XX	Spontaneous differentiation in adherent culture and selection with *OCT4-GFP* reporter	Follicle & oocyte-like cells, parthegenote-like cells	N	ND	Hubner et al., 2003	288
mESC—XY	BMP-releasing EB formation, selection with *Mvh-GFP* reporter	*Mvh*-positive PGCs, formed sperm when transplanted in vivo	N	ND	Toyooka et al., 2003	289
mESC—XY	EB formation with RA addition, selection of SSEA-1 positive cells	Haploid spermatids capable of fertilization	Y	Y	Geijsen et al., 2004	290
mESC—XY	EB formation with testis-conditioned medium	Immature oocyte-like cells, oocyte gene expression	N	ND	Lacham-Kaplan et al., 2006	294
mESC—XY	EB formation and adherent culture differentiation	Follicle & oocyte-like cells	N	ND	Novak et al., 2006	295
mESC—XY	EB formation, selection with *Stra8-GFP* and subsequently with *Prm1-dsRed*; RA addition	Haploid, SSC-like cells which showed motility and form sperm	Y	Y, ICSI	Nayernia et al., 2006	297
mESC—XY	EB formation with RA addition	Oocyte-like and sperm-like cells	N	ND	Kerkis et al., 2007	293
mESC	EB formation and coculture with ovarian granulosa cells	Oocyte-like cells, oocyte genes expressed	N	ND	Qing et al., 2007	291
mESC—XX	EB formation and adherent culture differentiation, selection with *Gdf9-GFP* reporter	Follicle-like structures, oocyte genes expressed	N	ND	Salvador et al., 2008	292
mESC—XX	Adherent culture differentiation with Oct4:PE-GFP reporter and transplantation of GFP+ cells into kidney capsule aggregated with ovarian fetal tissue	Female PGCs formed in vitro and oocyte-like structures formed in vivo	Y	ND	Nicholas et al., 2009	296
mESC—XY miPSC—XY	Transplantation of PGCs formed from male epiblast-like cells (EpiLCs)	EpiLCs, PGCs, and spermatozoa	Y	Y, IVF	Hayashi et al., 2011	226
mESC—XY miPSC—XY	Transplantation of PGCs formed from female epiblast-like cells (EpiLCs)	EpiLCs, PGCs and oocytes	Y	Y, IVF	Hayashi et al., 2012	300
mESC—XY miPSC—XY	Overexpression of PRDM14, STELLA and TFAP2C	EpiLCs, PGCs, and spermatozoa	Y	Y, IVF	Nakaki et al., 2013	301
hESC—XX, XY	EB formation	Immature male/female germ cells	N	ND	Clark et al., 2004	194
hESC—XX	EB formation with BMP addition	VASA+ germ cells	N	ND	Kee et al., 2006	298
hESC—XX	EB formation and adherent culture differentiation	VASA+ follicle-like structures	N	ND	Chen et al., 2007	300
hESC—XY	Adherent culture on MEF feeders	Immature germ cells	N	ND	West et al., 2008	303

(Continued)

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TABLE 6.1 A Summary of the Recent Efforts to Derive Gametes from ESCs and iPSCs—cont'd

Source of Cells	Germ Line Differentiation Strategy	Endpoint			Reference	Reference #
		Cell Types Formed	Gametes	Fertilization		
hESC	Low-density adherent culture differentiation with laminin substrate	CXCR4+ germ cells and Sertoli cells	N	ND	Bucay et al., 2008	305
hESC—XX	EB formation and adherent culture differentiation, selection of SSEA-1 positive cells	Immature germ cells with PGC-like imprinting status	N	ND	Tilgner et al., 2008	301
hESC—XX, XY hiPSC—XY	Coculture of hESCs and hiPSCs with human fetal gonadal cells, selection with SSEA-1 and c-Kit	Double-positive germ cells with PGC-like imprinting status (only with hESCs)	N	ND	Park et al., 2009	302
hESC—XX, XY	Overexpression of DAZ, DAZL, BOULE and adherent culture differentiation +/− BMPs	Haploid germ cells expressing germ line markers and PGC-like imprinting	Y	ND	Kee et al., 2009	306
hESC—XY	Adherent culture differentiation on KITL KO/WT feeders with addition of BMP4	Early germ-like cells expressing VASA and OCT4	N	ND	West et al., 2010a	304
hESC—XY	Adherent culture differentiation on MEF feeders	VASA+/OCT4+ clonally expanded early germ-like cells	Y	ND	West et al., 2010b	307
hESC—XX, XY hiPSC—XX, XY	*VASA-GFP* reporter or overexpression of DAZ, DAZL, BOULE and adherent culture differentiation + BMPs	Haploid, sperm-like germ cells expressing germ line markers and PGC-like imprinting	Y	ND	Panula et al. 2010	310
hESC—XX, XY hiPSC—XX, XY	Overexpression of VASA and DAZL and adherent culture differentiation + BMPs	Haploid, sperm-like germ cells expressing germ line markers and incomplete imprinting	Y	ND	Medrano et al., 2011	308

mESC, mouse embryonic stem cells; hESC, human embryonic stem cells; EB, embryoid body; BMP, bone morphogenetic protein; Mvh, mouse Vasa homolog; RA, retinoic acid; ICSI, intracytoplasmic sperm injection; IVF, in vitro fertilization; MEF, mouse embryonic feeders; Y, Yes; N, No; ND, not determined.

were also shown to be naturally fertile as adults. In summary, attempts to generate germ cells from mESCs have yielded cells that resemble sperm or oocytes in gene expression, ploidy, epigenetic reprogramming, and fertilization ability. However, these studies have also revealed irregularities in the duration of meiosis in culture, the morphological features of oocytes, the viability of generated offspring, and in the germ line transmission of exogenous genes to the offspring.[333] Saitou and colleagues recently achieved the most advanced derivation of functional male and female gametes from mECS and miPSCs.[257,334] In these studies, mESCs were induced into an epiblast-like cell stage (EpiLCs), enriched by selection for the STELLA germ cell marker and then further induced to produce PGCs. When transplanted to empty mouse seminiferous tubules of infertile male mice (*W/W* strain), PGCs made in vitro differentiated and formed spermatozoa. Similarly, to produce oocytes, PGCs derived in vitro were co-aggregated with embryonic gonadal somatic cells (that were depleted of PGCs) and transplanted under the ovarian bursa of neonatal recipient mice. These "reconstituted" ovaries enabled proliferation of female PGCs, meiotic entry, and completion of oogenesis, which when fertilized in vitro yielded healthy pups.[334] In both male and female ESC-derived gametes, the mating of donor cell recipient mice led to successful germ line transmission to progeny mice that appeared normal. Most recently, the same group has uncovered a mechanism by which mouse PGC formation from EpiLCs can be enhanced by the overexpression of STELLA (PRDM1), PRDM14, and TFAP2C transcription factors.[335] These fascinating results have provided

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an impressive system with which to query germ cell developmental stages in mice.

The evidence for germ line differentiation from hESCs lags severely behind the progress made in the mouse system; as such, literature is more preliminary than that from the mouse primarily due to the low efficiencies in derivation of human germ cells. Furthermore, doubts regarding the extent of pluripotency in hESCs versus mESCs and the ethical constraints of testing the functionality of human germ cells are legitimate concerns of this research. Despite this, there is a tremendous need to understand human germ line formation, the genes that control spermatogenesis and oogenesis, and, from a clinical standpoint, the requirement for in vitro-derived functional gametes. Efforts to derive human germ cells began with a study by Clark et al. in 2004 in which spontaneously differentiated hESCs were used to create EBs.[225] Within the mixed cell population of the EBs, they identified several known germ cell markers such as DAZL, STELLA, and c-KIT. Additionally, they observed that VASA levels were induced within a putative PGC population, and other markers of meiosis were also upregulated. Collectively, their data indicated that PGCs had formed but had not entered meiosis. An important facet of their study was the identification of germ line–associated genes such as DAZL and c-KIT, which are expressed at low levels in undifferentiated hESCs. The methods of Clark et al. were optimized in a follow-up study in which BMP-4, -7, and -8 were added exogenously during EB formation from hESCs, and higher VASA expression was observed in the putative PGC population.[336] Therefore, similar to the mouse model, the role of BMPs in human germ line specification may be important. More recent studies using mESCs in vitro by Young et al. have illustrated how BMP2 and BMP4 but not BMP8b are important inductive agents for specifying the germ cell lineage in EBs.[337] These results help to clarify our understanding of BMP action in the germ line as the goal of deriving haploid germ cells is pursued.

Chen et al. and Tilgner et al. also derived human PGCs from substrate EBs in independent studies. They observed that their EBs contained small structures that resembled ovarian follicles that tested positive for c-KIT and expressed low amounts of VASA protein.[338] They also observed these follicle-like structures in hESCs that were attached on matrigel-coated tissue culture plates and then spontaneously differentiated. This finding was important in suggesting that EB formation may not be essential for germ cell differentiation from hESCs, at least in the female lineage. Meanwhile, Tilgner et al. used a more sophisticated strategy to separate germ cells from EBs and adherent cultures through FACs sorting for cells expressing the surface marker SSEA-1, a protein associated with very early germ cells.[339] Despite evidence of complete epigenetic reprogramming in these early germ cells, the authors did not observe meiosis. In 2009, Park et al. built upon the findings of Tilgner et al. by differentiating iPSCs and hESCs and isolating putative germ cells enriched in SSEA-1 as well as c-KIT expression. However, they used an alternative differentiation strategy and cocultured hESC colonies with stromal cells from the human fetal gonad.[340] In this model, the paracrine factors produced from fetal stromal cells would ideally resemble those made in vivo, thereby theoretically inducing hESCs and iPSCs to differentiate into PGCs. Their efforts were successful, yielding SSEA-1/c-KIT-positive PGCs that expressed numerous germ cell markers such as VASA, PRDM1, and DAZL, and revealed evidence of partial epigenetic reprogramming.

Based on the findings of spontaneous differentiation of hESCs to PGCs, researchers have entertained the possibility that a small population of cells within a typical hESC population constitutes a primitive germ cell population or a subpopulation that is developmentally "primed" to become germ cells (Figure 6.6). Based on this hypothesis, several groups have explored whether simply optimizing culture conditions of hESCs can promote or induce germ line differentiation. Stice and colleagues first reported that by the simple addition of bFGF to hESCs growing on inactivated MEF feeder cells they could produce approximately 69% VASA-positive cells with increased expression of germ line and meiotic markers.[341] These data seem rather optimistic given that most other groups have reported much lower germ cell numbers under similar culture conditions. In recent studies, West et al. explored the effect of modulating KIT ligand (KIT-L) and BMP4 activities in culture of hESCs and observed that these are required for early germ-like cell (GLC) derivation.[342] Concurrently, Bucay et al. modified their culture conditions by lowering the confluency of hESC colonies and observed a subpopulation of cells expressing CXCR4, a membrane receptor implicated in PGC migration.[343] Upon purification, the CXCR4-expressing cells could be grown on a laminin substrate and gave rise to putative PGCs and Sertoli cells.

A major milestone in germ line differentiation of both male and female human PGCs is the initiation and completion of meiosis. Deriving haploid gametes in vitro is a significant feat and one that researchers have struggled with for years in human germ line differentiation. Kee and colleagues derived human haploid gametes for the first time in 2009 by introducing three major germ cell-specific genes of the deleted-in-azoospermia family—*DAZ2*, *DAZL*, and *BOLL*— that encodes for the protein BOULE in hESCs, and then differentiating the cells spontaneously in adherent culture conditions.[344] Although a low number of haploid cells were observed (~2%) 7 days after differentiation, they expressed numerous germ cell-specific markers, and chromosomal spreads indicated the presence of SCP3, a key member of the

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synaptonemal complex that forms during meiosis. These data also reinforce the importance of the DAZ family of genes for early (*DAZL*) and late (*BOLL*, *DAZ*) PGC formation. Since this work was published, another group has demonstrated meiosis in in vitro-derived PGCs; West et al. had previously shown that they could isolate early VASA-positive GLCs and were able to subclone three populations of these cells from their mixed cultures and propagate them through at least 50 serial passages.[345] These cells were then differentiated with FBS into a more homogenous population, and the majority of cells entered meiosis (~71%) while also expressing germ line and meiotic markers. Taken together, these studies offer alternative and compelling strategies for derivation of PGCs from hESCs. While the latter study suggests that GLCs are a natural clonal subpopulation of hESCs that can be propagated in vitro, the former argues in favor of an active, gene-driven induction of germ line differentiation with the addition of BMPs. The concept of genetic control of germ cell induction has been further explored through the derivation of germ cells from hESCs by the overexpression of the RNA-binding proteins DAZL and VASA.[346]

The composition of medium used for differentiation of hESCs is also important, as was revealed by a recent study in which hESCs were exposed to a medium composition that is known to promote mouse SSC survival and self-renewal in vitro.[347] Without the use of genetic modifications and reporter-based enrichment, this unique approach revealed that hESCs adapt to conditions favoring spermatogonial development and that a subpopulation of hESCs appeared to differentiate to germ cells capable of entering and completing meiosis in vitro.

Germ Line Differentiation from Human iPSCs

Ongoing advances in reprogramming of human somatic cell to iPSCs or direct reprogramming of somatic cells to specific cell lineages are legitimate avenues for autologous, patient-derived stem cell therapies (Figure 6.6; Table 6.1). By applying similar genetic approaches used for hESCs, iPSCs can now be directed to give rise to cells of the germ cell lineage such as PGCs (iPGCs). Moreover, the use of iPSCs derived from infertile, azoospermic men offers a viable avenue by which to explore patient-specific developmental changes during germ line development. We summarize here some of the recent efforts in this growing area of exploration. The use of human iPSCs to derive gametes was first shown by Park et al. in a study that also compared the derivation efficiency to hESCs.[340] In short, they grew iPSCs on a feeder layer of fetal gonad stromal cells and subsequently isolated SSEA-1+/c-KIT+ cells, which they characterized as a putative PGC population. These human iPGCs expressed several germ line markers but did not show evidence of imprinting erasure, a key feature of early PGCs. Further evidence for the creation of haploid iPGCs from male and female iPSCs was provided recently by Panula et al. wherein they used a GFP reporter under the control of the *VASA* promoter to select for putative germ cells from spontaneously differentiated cultures.[348] The *VASA*-GFP transduced human iPSCs were differentiated adherently in feeder-free conditions in the presence of BMPs, giving rise to approximately 5% GFP-positive PGCs. These PGCs expressed a number of germ cell markers such as *PRDM1A*, *ACTC*, *GATA6*, *PELOTA*, and *IFITM3*. More importantly, the GFP-positive iPGCs expressed the meiotic marker SCP3 in both punctate and elongated patterns, indicative of early and late meiosis, respectively. Interestingly, when compared to PGCs derived from both male or female hESCs, the efficiency of iPGC derivation appears higher, suggesting that iPSCs may be a better starting substrate for germ line differentiation. The roles of RNA-binding proteins VASA and DAZL were further explored as modulators of germ cell fate by overexpressing them in human iPSCs and inducing germ cell formation with BMPs.[346] The derivation of low numbers of putative haploid germ cells from these experiments confirmed the importance of genetic regulation of germ cell differentiation in humans. Despite offering novel insights into mechanisms of germ cell differentiation, the efficiency of human PGC derivation remained low. In summary, the above investigations provide encouraging results about the ability to use iPSCs to derive gametes in vitro. Although the differentiation potential of iPSCs may vary greatly from one cell line to another, they appear to have strong potential as a genetic model system to study human germ cell development.

Clinical Need and Applications for In vitro-Derived Gametes

Currently, many men suffer from nonobstructive azoospermia, as described earlier, that results in complete sterility. In addition, in the United States alone, there are 55,000 childhood cancer survivors annually, many of whom were unable to bank sperm before receiving sterilizing treatments. Similarly, women have a finite supply of reproductively competent oocytes, while others undergo premature ovarian failure, making childbearing difficult or impossible. Alternative options for parenthood in these cases are not always simple or inexpensive. Although sperm donation is economical, oocyte donation is quite expensive and the use of donated embryos is unusual.[250] For men, recent strategies such as extracting low numbers of testis sperm after

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prior localization with fine needle aspiration mapping offer the hope of fatherhood for some candidates.[349,350]

Although such strategies are gaining in popularity, the pregnancy success rate remains at about 30–40% using sophisticated IVF techniques (Centers for Disease Control, 2008). In addition, with these reproductive technologies, there remain important safety concerns about de novo sex chromosome disorders, birth defects, and imprinting-related disorders such as Beckwith–Wiedeman syndrome in offspring.[256] Therefore, there is an obvious clinical need for high quality, in vitro-derived gametes. However, the prospect of employing current protocols and reagents to derive human gametes from hESCs appears unlikely at this time. On the other hand, the use of iPSCs and alternative stem cell sources such as adult stem cells hold great promise in this regard. In the foreseeable future, patients who have undergone chemotherapy and/or patients with genetic disorders affecting fertility could theoretically donate somatic cell biopsies that could then be reprogrammed into iPSCs, genetically modified if required, and subsequently utilized for gametogenesis in vitro.

In sum, the low efficiency of meiotic potential in vitro and the incomplete epigenetic reprogramming of PGC-like cells have thwarted studies of germ line derivation from hESCs. A new avenue of exploration is required, and current efforts are focused on utilizing existing xenograft technologies to derive PGCs from human iPSCs and hESCs in vivo in the environment of the mouse gonad or kidney capsule. These efforts, if successful, will pave the way for better xenograft models that resemble human physiology more accurately and subsequently lead to cell-based therapies for infertile men and women.

CONCLUSION

Humans have long pondered the mechanisms and meanings that underlie the formation of a new individual. Since the days of debate between preformation and epigenesis, several scientific discoveries have addressed many complexities in human development. More specifically, the underlying programs mediating epigenetic erasure and reestablishment of methylation and histone modification marks, which genes are first expressed during development, and the timing of cell lineage differentiation have been revealed in recent years. In addition, systems and technologies such as whole genome and epigenome analysis, as well as imaging and transplantation techniques to probe fundamentals of embryo and germ line development, have been extensively elaborated and hold promise to provide the means to discover new molecules and mechanisms.

Future Challenges

Although much has been accomplished and revealed in the fields of human embryology and gametogenesis in recent years, many challenges and questions surrounding early human development still persist. In particular, the possible origins and consequences of embryonic chromosomal abnormalities, the long-term effects of epigenetic changes during early embryogenesis, and whether the completion of human germ cell meiosis in vitro or in vivo following xenotransplantation will ever be attainable remain elusive. As noted before, most studies of embryo development are conducted in the mouse or non-mammalian species, requiring extrapolation of results to human development. However, given extensive species-specific differences, even comparison between closely related mammalian species may be difficult. Moreover, most data on human embryo development by necessity is limited to that obtained from infertility clinics and embryos produced with germ cells that may be compromised. Thus, while such studies have proven to be invaluable, the interpretation of results and what should be considered "baseline" must be approached cautiously. Further, data specific to segments of human populations, or cohorts, and the similarities or differences between populations that may translate to disease risk or other factors is limited. In large part, these limitations are linked to a lack of tools, historically, to probe imaging, molecular, genetic, and epigenetic determinants of human development especially on a small scale such as at the single embryo or blastomere level. It is likely that as tools for single cell analysis continue to be developed, along with computational means to integrate different types of data, and continued improvements in the differentiation of stem cells to germ cells in vitro and/or in vivo, that understanding of human embryo and germ line development will be dramatically improved and translate to clinical applications.

Perspectives on the Future of Reproductive Medicine

Infertility is common, with IVF and other forms of assisted reproduction increasingly being used to treat this disorder. Fundamental problems in identifying embryos that are likely to be successful in development, reducing embryo loss, and examining development at the cellular basis should be a focus of studies in human reproduction over the next several years. It is envisioned that with knowledge of human embryonic preimplantation development, three outcomes might be realized in assisted reproduction: (1) Predominant transfer of single embryos, (2) improvements in success rates, and (3) reduction in adverse outcomes associated with multiple embryo transfer/births.

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In summary, over the last decade human preimplantation development has been the subject of much inquiry, resulting in greatly increased knowledge of fundamentals. The coming decade is likely to witness genetic and epigenetic studies at the single embryo and/or single cell level that further improve our understanding of basic human development, including the genes and proteins required for reprogramming through the oocyte to embryo transition and activation of the embryonic genome, clinical applications in reproductive medicine that allow transfer of single embryos cultured under optimal conditions, and translation to other pluripotent cell types important to stem cell biology and regenerative medicine.

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CHAPTER

7

Sex Determination and Differentiation

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INTRODUCTION

Sex determination is the fundamental step that an animal takes to become male or female. Although this decision is common to most species, the mechanism that determines sexual dimorphism is highly diverse. The question of how sex is determined has puzzled scientists and philosophers for centuries.

Aristotle predicted in 335 BC that sex was determined by environmental factors—specifically, by the temperature of both partners during intercourse. The theory that environmental sex determination occurred in all species remained popular until the turn of the twentieth century. In 1891, it was observed that wasp sperm cells contained unequal numbers of chromosomes. Some of the sperm cells contained an unidentified 12th chromosome, which was named the X element, and later the accessory chromosome. In 1902, two separate papers hypothesized that this chromosome was involved in sex determination.[1,2] Further observations led to the realization in 1910 that this unequal distribution of sex chromosomes only occurred in some animals; in others, the same number of chromosomes appeared, although one set was morphologically different in males and females.[3] This was the first observation of the XY sex determination system that is found in humans. In the mid twentieth century, experiments demonstrated that a "testis determining factor" present on the Y chromosome was critical for male development in mammals. This factor was finally identified in 1990,[4] cementing the modern view of mammalian sex determination.

In mammals, sex determination is classically divided into three hierarchical, irreversible stages. The first step is the establishment of chromosomal sex at fertilization. For the most part, the initial stages of fetal development do not show any morphological differences between the sexes until the gonad develops. The second step in sexual differentiation is the choice of the bipotential gonad to become an ovary or a testis. The third step is the development of the sexual phenotype of the fetus, some aspects of which are controlled by diffusible substances produced by the gonads. In recent years, advances in genomics have resulted in an influx of data on sex determination in many species, providing insights into the dynamic and rapid evolution of this process. Furthermore, recent data have challenged the simple stepwise model of sex determination, indicating that sex in mammals is more plastic than previously thought; indeed, sex can be altered even into adulthood. This chapter will discuss the three stages of sex determination and differentiation, focusing primarily on mammals, and examining how mouse models and human disease have expanded and challenged our understanding of the meaning of sex.

CHROMOSOMAL SEX

Mammalian species rely on genetic factors to determine sex. In eutherian (placental) mammals such as humans and mice, heterogametic chromosomes determine sex. Homogametic individuals—those possessing two X chromosomes—are genetically female, while heterogametic individuals possessing one X and one Y chromosome are male. This is due to the presence of a single gene on the Y chromosome, *SRY* (Sex-determining region on the Y chromosome), which is necessary and sufficient to determine male development.[5,6]

Evolution of Heteromorphic Sex Determination

Heterogamety is often viewed as the standard mode of chromosomal sex determination. As such, heteromorphism—a difference in morphology between male and female members of the same species—is evident in many of the model species studied by developmental biologists, including mice, rats, chicks, and *Drosophila*.

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In most cases, male heterogamety is the norm, the notable exception being birds, in which the female exhibits ZW heterogamety. Heteromorphic chromosomes have evolved on multiple occasions. Evidence from lower vertebrate groups such as the teleosts and amphibians, in which both male and female heterogamety have evolved in separate populations, suggests that environmental rather than genetic pressure may drive evolution of heteromorphism.[7,8] However, the reasons for evolution of male versus female heterogamety remain unknown.

Heteromorphic sex chromosomes like the X and Y are thought to have evolved from an identical pair of autosomes. Several evolutionary theories propose that the first step in the evolution of sex chromosomes is the appearance of an autosomal gene with two different alleles, where homozygosity leads to the development of one sex and heterozygosity to the other. Once this occurs, there is a suppression of recombination between the chromosomes, probably initiated by the reduced crossing over at the sex-determination locus due to chromosomal rearrangements such as inversions around the sex-determining locus. This leads to a degeneration of the sex-linked loci in the heterogametic sex[9] (Figure 7.1(A)). This process may be driven by sexual antagonism. In this model, an allele on the same chromosome as the sex-determining locus that is beneficial to the heterogametic sex will become fixed into the population even if this allele has no effect or is disadvantageous to the other sex. This happens because natural selection will favor suppression of recombination between the chromosomes to keep the sex-determining locus and the sexually antagonistic locus together. Thus, in XY species, an allele may become fixed in the population even if it is harmful to the female. For example, in the fruitfly, *Drosophila melanogaster*, genes are fixed in the population that are beneficial to male fertilization rates but disadvantageous to female ability to reproduce.[10] This model of sex chromosome evolution is complicated by unequal distribution of the chromosomes between the sexes, and thus cannot be determined by a straightforward weighing up of advantage versus harm. This was demonstrated by recent experiments that showed that a novel gene with the above properties could become fixed into a population of *D. melanogaster*, despite the allele conferring twice as much harm to the female as benefit to the male.[11] To date, however, little is known about the mechanisms by which sexual antagonism acts.

Evolution of heteromorphic chromosomes due to suppression of recombination should in theory lead to the accumulation of deleterious mutations on the Y/W chromosome, and even gene deletion. This is indeed observed on the mammalian Y. It is far smaller than the X chromosome and consists of two main regions. One is a relatively short region, named the pseudoautosomal region, which is found at either end of the chromosome and is identical to the corresponding regions of the X chromosome. This is the only place where the X and Y chromosomes still recombine during male meiosis, ensuring sequence identity (discussed in detail in Chapter 1).[12] The remainder of the chromosome, the male-specific region (MSY), comprises 95% of the chromosome and is Y-specific (Figure 7.1(B)). Deletion of deleterious genes is thought to be counteracted by gene conversion of necessary testis-specific genes within the MSY, leading to regions of duplication within the Y chromosome. Indeed, this region is a mosaic of heterochromatin—highly repetitive DNA that is thought to be nonfunctional—and euchromatin sequences.[13] In some species, such as certain types of mole vole, deleterious

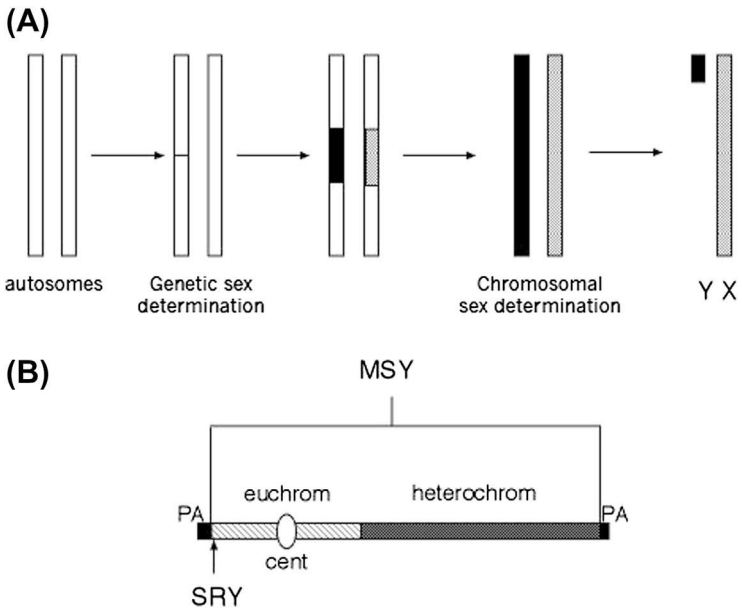


FIGURE 7.1 (A) Proposed mechanism for the evolution of sex chromosomes from a pair of autosomes. (B) Structure of the human Y chromosome indicating the position of the SRY gene. PA, pseudoautosomal region; MSY, male-specific region of the Y; euchrom, euchromatic region; heterochrom, heterochromatic region; cent, centromere. *Source: (Adapted from Ref. 248).*

mutation and subsequent gene deletion occurs to such an extent that the entire Y chromosome is lost.[14]

These evolutionary theories of heterogametic emergence and subsequent gene deletion indicate that it should be possible to date divergence of heteromorphic chromosomes. The determination of the physical map of the Y chromosome and the sequencing of the euchromatic portion of MSY provided evidence for the model that the human X and Y originated about 300 million years ago with the emergence of *SRY* as the primary sex determinant.[13,15,16] X and Y recombination was then suppressed in a series of steps, probably through chromosomal inversions, which led to the differentiation of each chromosome. Selection then occurred on the Y chromosome for a group of testis-specific genes that enhanced male fertility, which are maintained by Y–Y gene conversion.[17]

Dosage Compensation

An inevitable consequence of heteromorphic sex determination is that in the homogametic sex, twice as many alleles will be expressed from the X/Z chromosome as the Y/W. Thus, dosage compensation is critical to maintain normal regulation of genes on sex chromosomes. This is not well conserved evolutionarily, and as such is achieved in different ways in different organisms, as exemplified by comparison of model organisms. In mammals, silencing the transcription of genes from one X chromosome occurs when two are present, a process called X inactivation.[18] In *Caenorhabditis elegans*, instead of silencing one chromosome, gene expression from both is downregulated in the XX hermaphrodite.[19] In the *D. melanogaster* fruitfly, gene expression from the male X chromosome is upregulated by a male-specific dosage compensation complex. Although all three organisms modulate sex-linked gene expression in different ways, all use chromatin remodeling to achieve a similar result.

A major component of silencing in humans is the non-protein-coding *XIST* gene, which maps to the X chromosome inactivation center, Xic, and is preferentially expressed by the inactive X chromosome.[20,21] *Xist* RNA expression has been shown to be necessary for X inactivation in mouse models.[22] It appears that localization of *Xist* RNA to the Xic may be mediated by binding to the nuclear scaffold, via the nuclear scaffold protein SAFA.[23] Circumstantial evidence suggests that silencing then "spreads" along the chromosome from the Xic via long interspersed element 1 (LINE1) repeats, nuclear "relay" elements, which are enriched on the X chromosome.[24] This hypothesis is supported by mouse data showing that misexpression of *Xist* on an autosome causes only partial silencing.[25] Furthermore, all genes on the X chromosome should be subject to X inactivation, yet reports show that up to 15% of X-linked genes in humans might escape this control[26,27]; these "escaper" genes tend to cluster together in areas that have fewer than average LINE1 elements. LINE1 elements are not sufficient for complete silencing,[28] however, and thus other as yet undetermined factors must also be involved. The mechanism by which *XIST* triggers X inactivation is not fully understood and is a subject of intense study. It is thought likely that transcriptional machinery is excluded from the Xic, although how this is achieved is unknown. Subsequently, two repressive complexes, apparently acting redundantly with one another, are recruited to the chromosome.[29,30] These complexes contain polycomb group proteins, which have the ability to remodel chromatin in order to impose epigenetic silencing on target regions of the genome. The final result is the formation of facultative heterochromatin, which is transcriptionally repressed and epigenetically silenced down the cell lineage.

Inactivation of the X chromosome in XX embryos occurs at different stages in different species, and in different cell types within each species. In the mouse, cells initially undergo paternal X inactivation at the four-cell stage.[31] This situation is temporarily reversed in inner mass cells that will give rise to the embryo, while extraembryonic cells escape reversal.[31,32] The embryonic cells then undergo random X inactivation at the blastocyst stage.[33,34] In humans, X inactivation is believed to occur after the blastocyst stage, although the exact timing has not been defined (reviewed in Ref. 35). After the blastocyst stage, the majority of murine embryonic cells maintain their inactivated status throughout life. The exceptions to this rule are the XX primordial germ cells (PGCs), which undergo X reactivation at some point between E7 and E10.5, during which time they migrate to the genital ridge.[36–38] Once the cells undergo meiosis they become haploid and thus X inactivation is no longer a requirement.

Sex Chromosome-Related Disease

Disorders of sexual development (DSDs) are among the most common congenital disorders in humans. DSDs can encompass a wide range of phenotypes, from mild androgen insensitivity in males or primary ovarian insufficiency in females to complete sex reversal (termed XX,46 DSD in the case of genetic females who are phenotypically male, and XY,46 DSD in the opposing case). As sexual development is not identical in humans and mouse models, mutations that cause complete sex reversal in humans may be associated with intermediate phenotypes in the mouse, or vice versa. Many of the genes associated with DSDs are found on the sex chromosomes.

SRY

Inactivating mutations in the testis-determining gene, *SRY*, are associated with XY,46 female sex reversal in humans. The study of DNA from XX male individuals

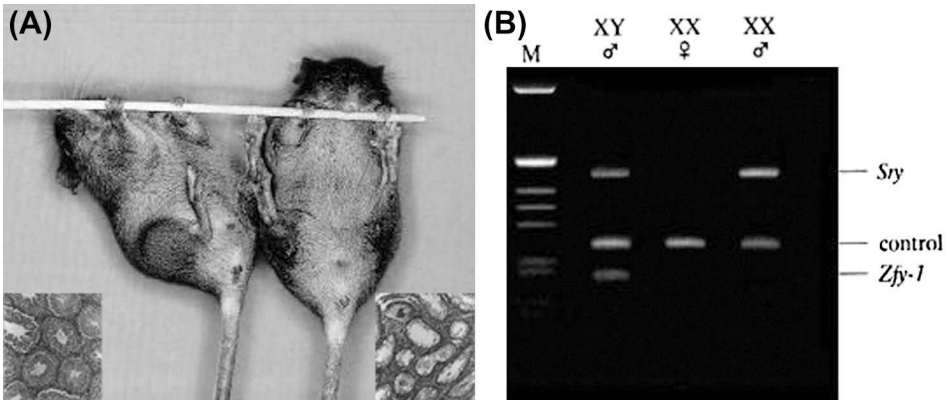


FIGURE 7.2 XX male sex reversal with an *Sry* transgene. (A) On the left is a control XY male and on the right is his littermate, an XX transgenic carrying a 14-kb genomic fragment containing the mouse *Sry* gene. The external and internal genitalia are indistinguishable except for the testis, which is smaller in the transgenic animal due to the lack of germ cells (see insets, in which cross-sections through the testis of each mouse are shown). (B) PCR analysis of genomic DNA from these mice as well as an XX sibling control. Bands for *Sry* and the DNA loading control (myogenin) are present in the XX transgenic animal but the Y chromosome marker *Zfy-1* is missing. M, size marker. *Soure: Adapted from Ref. 6.*

identified small regions of the Y chromosome that had transferred onto the X chromosome by abnormal X-Y interchange during male meiosis. The *SRY* gene was found within the minimum region of Y-specific DNA required for male development.[4] Confirmation of its role was found by the analysis of XY female patients with mutations within this gene.[5,39]

The mouse has been an important working model to study the process of mammalian sex determination. As in the case of human patients, XX mice carrying the *Sxr* mutation develop as males and it was shown that the X chromosome in these mice carried a small portion of the Y chromosome containing the *Sry* gene.[40] Conversely, a mouse mutation that produced XY females was shown to have an 11-kb deletion on the Y chromosome that included the *Sry* gene.[41] Direct evidence that *SRY* is the only gene on the Y chromosome to be necessary and sufficient for establishing male development came from transgenic experiments in the mouse. XX mice carrying a 14-kb fragment of Y chromosome DNA that contains the mouse *Sry* gene and no other gene developed as males[6] (Figure 7.2). These transgenic animals were infertile, however, because of a block in spermatogenesis due to the presence of two X chromosomes and the lack of other Y-linked genes. The SRY protein contains an evolutionarily conserved HMG box type of DNA-binding domain found in a number of other transcription factors. This is believed to be important for SRY function because almost all mutations found in human XY females cluster within this region.[39,42]

SRY is thought to have evolved around 300 million years ago due to duplication of SOX3, resulting in the primary sex determinant present in mammals today.[43] The two proteins share a highly similar HMG box domain.[44] Despite its location on the X chromosome, *Sox3* is not required for normal sex determination in mice and loss of function mutations have not been documented in DSD patients.[45] However, misexpression of *Sox3* in the gonads of XX mice causes sex reversal. In vitro experiments indicate that SOX3 is capable of activating downstream targets of *Sry*.[46] Analysis of 16 XX male sex reversal cases revealed three patients with chromosomal rearrangements in and around the *Sox3* locus.[46] Furthermore, a separate case has since been reported,[47] implying that *Sox3* mutations may well be a clinically relevant cause of XX male sex reversal. Together, these data suggest that SOX3 can act in a manner analogous to that of SRY in mammals.

The master regulator role of *Sry* has been used in mouse models to genetically differentiate between chromosomal and gonadal regulation of sexual characteristics. In the four core genotypes (FCG) model, generation of a Y chromosome lacking *Sry* (Y^-)[48] and a transgenic *Sry* expressed from an autosome[49] allows separation of gonadal and chromosomal male determination. Thus, mice can be one of four "core" genotypes: phenotypic female with XX or XY (abbreviated to XYF) chromosomes, or phenotypic male with XX*Sry* (abbreviated to XXM) or XY-*Sry* (XYM) chromosomes (Figure 7.3(A)).[50] The sexual traits controlled by gonadal sex can be established by comparison of animals of different phenotypic sexes (XXF/XYF versus XXM/XYM). Conversely, sexual traits governed by chromosome complement can be elucidated by observation of differences between XX and XY animals, regardless of *Sry* status (Figure 7.3(B)). The FCG model should therefore also determine traits in which both chromosomal and gonadal sex have a role to play. However, caution must be used when interpreting the data gained from these mouse models, as animals of the same phenotypic sex still have some differences; for example, XXM mice are azoospermic (lacking in sperm production), as spermatogenesis requires genes on the

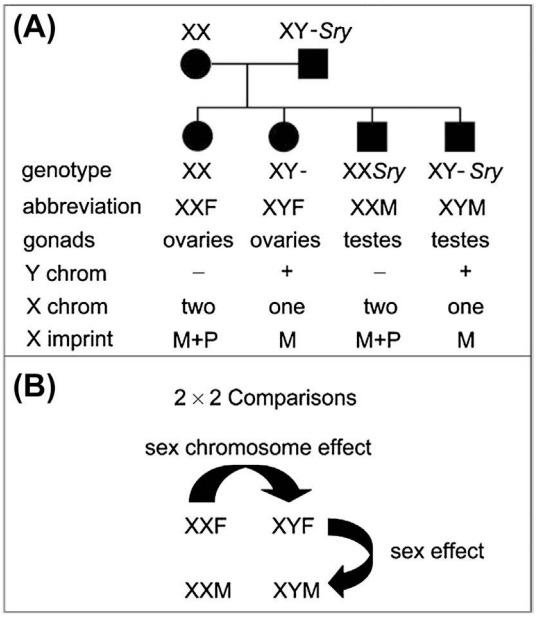


FIGURE 7.3 The four core genotypes (FCG) model separates the effects of chromosome complement from those of gonadal sex. (A) Breeding scheme for generation of FCG mice. XX female mice are crossed with XY male mice in which *Sry* has been deleted from the Y chromosome (Y^-) and reinserted on an autosome (*Sry*). Progeny of four different genotypes are generated. Their phenotype, chromosome complement, and X imprinting status are listed below. (B) A 2 × 2 schematic demonstrates how the FCG mice can be used to determine the cause of phenotypic differences observed. *Source: Figure reprinted with permission from Ref. 249.*

Y chromosome. Furthermore, these mice have different genetic imprints. For example, XXF mice have both maternal and paternal imprints, while XYF mice have only one maternally imprinted X chromosome. As genetic imprinting can determine expression of certain genes, this status needs to be taken into account when comparing phenotypically similar mice. Experiments using FCG mutants have shown that, although *Sry* is necessary and sufficient for male sex determination, X chromosome dosage plays a role in multiple aspects of sexual dimorphism despite X inactivation[50–52]—perhaps not surprising as the "escaper" genes on the inactive X are expected to be expressed at a higher level in XX mice than XY.[26,53]

Y-Specific Genes

Many of the genes on the Y chromosome are involved in male fertility, in particular in the regulation of spermatogenesis. Consistent with this, most XX,46 men are azoospermic and therefore infertile. Microdeletions in three regions of the long arm of the Y chromosome (azoospermia factor, AZF, a, b, and c) have been associated with reduced sperm production (oligospermia) or azoospermia in humans.[54] Due to the large repeats and multicopy gene sequences in this region, it has been difficult to establish which genes are responsible for the spermatogenic failure in each case. The variable phenotype of patients with AZF microdeletions implies that multiple genes in each region are involved in male fertility. Deletions of regions of the Y chromosome in the mouse also lead to a block in spermatogenesis. These animal models have been used to define multiple genes on Y that are involved in spermatogenesis. For example, deletion of the long arm of Y, MSYq, revealed that genes in this region are required for sperm differentiation.[55,56] Deletion of smaller regions on this arm results in reduced fertility, and an increase in transcription from sex chromosomes in spermatids.[55,57] Transgenic mice harboring siRNA constructs were used to identify which candidate genes within this region were responsible for the infertility phenotype. Reduction in levels of the *Sly* genes led to defects in sperm differentiation, resulting in reduced fertility. Furthermore, *Sly* was found to drive repression of genes from sex chromosomes following meiosis in the germ cells.[58] This type of targeted gene disruption will be important in dissecting the roles of other multicopy genes on the Y chromosome.

X-Dosage

XO

In humans the loss of one X chromosome gives rise to Turner syndrome. In most cases XO fetuses are lost, but some survive, with a frequency of 1 in 2500 to 3000 female births. The phenotypes of the affected individuals include short stature, a webbed neck, broad chest, and cognitive defects. Genes escaping X inactivation have been proposed to be responsible for these defects, as only one copy of these genes is expressed in these individuals. Consistent with this, fewer genes in the mouse escape X inactivation and XO mice have a less severe phenotype.[59] Turner patients show gonadal dysgenesis, which is characterized by the loss of oocytes. XO mice also show a reduced number of oocytes at birth, and analysis of these mice suggests that this is due to the lack of chromosome pairing of the single X during meiosis.[60,61]

XXY

Males with Klinefelter syndrome are characterized by an XXY genotype, increased stature and infertility. Loss of germ cells in these individuals occurs prior to puberty, and a similar situation is seen in the mouse. It has been proposed that the loss of germ cells in these individuals is due to the presence of two X chromosomes. Prior to entry into meiosis, germ cells with two X chromosomes undergo X chromosome reactivation, giving rise to a double dose of X-linked genes. If this occurs in the testis, the X chromosome dosage acts to impair germ cell development.[62]

GONADAL SEX

The action of SRY that determines male sex differentiation occurs within the developing gonad. The gonads are part of the urogenital system, which is derived from the intermediate mesoderm of the embryo. They initially develop as thickenings on the ventromedial surface of the mesonephros on either side of the midline at around 4 weeks in the human fetus and 9.5 days in the mouse. The somatic cells of the gonad are derived from the mesonephros and the coelomic epithelium that lines the coelomic cavity. The primordial germ cells originate at earlier stages of embryogenesis at the base of the allantois, and migrate through the hindgut to join the gonad as it develops. This gonadal primordium is a unique aspect of embryogenesis as it is bipotential; it has the potential to become either male or female. Adrenal cortical cells also derive from this primordium, which is therefore called the adrenogonadal primordium (AGP).[63]

This initial development is identical between males and females but at early stages, around E10.5–11 in the mouse, SRY acts in the XY embryo to initiate testis development. Without SRY the gonad will develop as an ovary; therefore the mammalian embryo is programmed to develop as a female and the male-specific pathway is imposed on this basic body plan.

Sex Determination

Sry was first identified as a regulator of sex determination in 1990. Studies in the mouse have shown that *Sry* is expressed almost exclusively in the somatic cells of the gonad of XY embryos as it begins to develop. Transcript levels reach a peak around E11.5 and then decline to undetectable levels by E12.5.[64,65] This transient pattern of expression suggests that *SRY* acts as a trigger for testis differentiation but is not required for the subsequent development of the organ or maintenance of testis-specific gene expression.

As discussed above, *Sry* contains an evolutionarily conserved HMG box domain, believed to be important for its DNA binding activity. Most human *SRY* mutations implicated in DSDs cluster in the HMG box.[39,42] However, other domains within the protein have also been implicated in its function. Experiments using transgenic mice in which stop codons were introduced into different regions of *Sry* revealed that the HMG domain alone was not sufficient for male sex differentiation in XX mice. Furthermore, insertion of a stop codon that caused production of a truncated protein lacking only a glutamine-rich region at the C-terminal of SRY produced the same phenotype.[66] This domain may be required for protein interactions in the mouse, although it is not conserved in other mammals. Alternatively, this mutation may simply decrease the stability of the protein. Transgenic expression of human or goat *SRY* in mice can drive male sex determination in XX mice.[67,68] Although this may implicate the well-conserved HMG domain in SRY function, an alternative explanation is that the other regions, although not conserved in sequence, may have similar functions. This is supported by evidence indicating that, as in the mouse, human SRY must be full-length to function properly.[69]

It has been proposed that the main role of SRY is to activate the expression of *Sry-related HMG box-containing gene 9 (Sox9)* within the male gonad. SOX9 expression leads to the differentiation of Sertoli cells, which then direct the differentiation of other male-specific cell lineages within the gonad.[70] Transgenic mice studies identified a 3.2-kb testis-specific enhancer element (TES) that was sufficient to promote initiation and maintenance of *Sox9* expression.[71] This was subsequently narrowed down to a 1.4-kb region known as the testis-specific core element, TESCO, to which SRY directly binds via the HMG box. Initiation of *Sox9* expression is induced by the synergistic action of SRY and steroidogenic factor 1 (SF1), an orphan nuclear hormone receptor (Figure 7.4(A) and (B)).[71] The TESCO enhancer was reported to be well conserved in mammals. Subsequently, an 180-bp region of TESCO was found to be conserved in mammals, reptiles, birds, and amphibians, suggesting that *Sox9* expression in the gonad is regulated in a similar manner in many vertebrates.[72]

Studies in chimeric mice derived from aggregations of early XX and XY embryos showed that Sertoli cells were the only cell type in the testis to have a bias towards an XY genotype. Consistent with this, cell fate studies in transgenic mice have shown that *Sry* is expressed in Sertoli cell precursors.[73,74] These data show that *Sry* initiates the differentiation of the Sertoli cell lineage within the developing gonad and that these cells direct the differentiation of the rest of the cell lineages along the male pathway.[70]

Genes Implicated in Sex Determination and Their Associated Defects

There is a window of time during gonad development when SRY needs to achieve a certain level of expression to ensure testis differentiation. If this is not the case, testis development is delayed and ovary development will occur. In some cases this will give rise to the development of ovotestes, where testicular cords are found in the middle portion of the gonad surrounded by ovarian tissue on either side (Figure 7.5). This property of sex determination implies that genes that act to affect the levels of expression of SRY or interfere with SRY action, either directly or indirectly, are also involved in the process of sex determination.

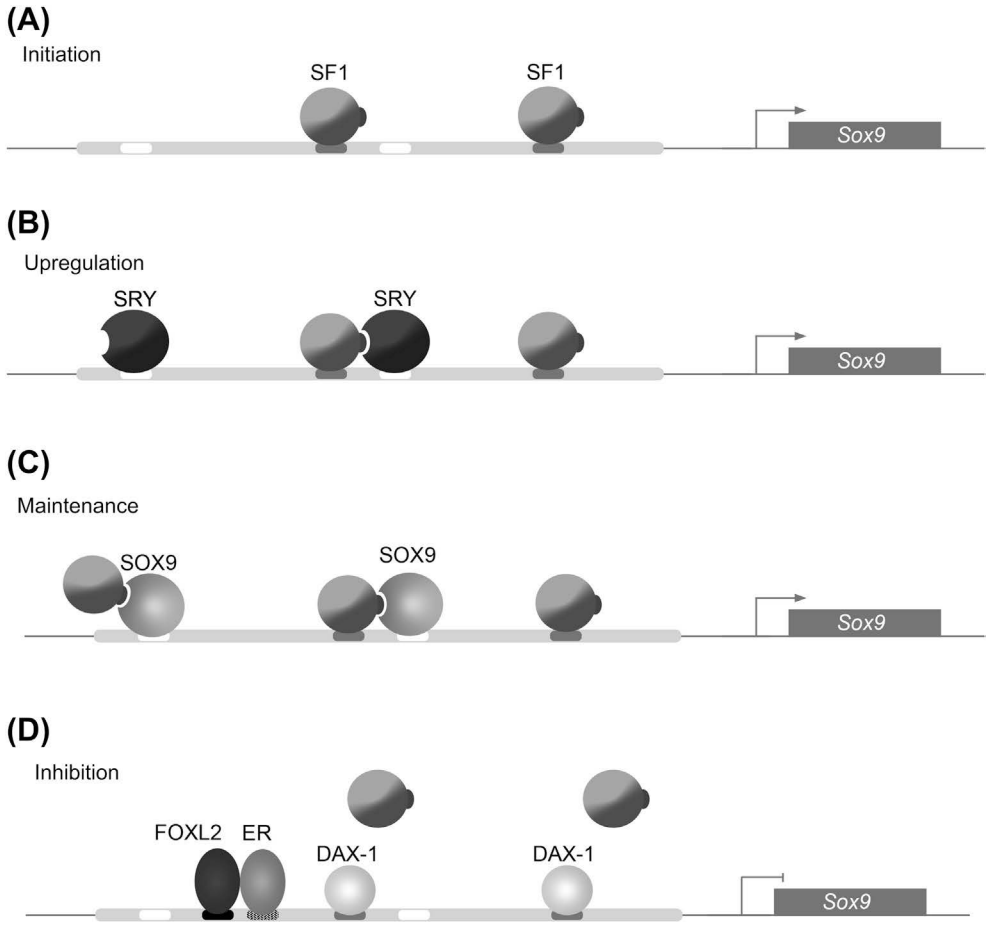


FIGURE 7.4 Regulation of *Sox9* expression at the TESCO enhancer. (A) Binding of SF1 to the TESCO enhancer is sufficient for initiation of *Sox9* expression. (B) Expression is subsequently upregulated by synergistic action of SF1 and SRY, which may physically interact at the enhancer. (C) Once SRY expression ceases, *Sox9* expression is maintained by an autoregulatory loop in which SOX9 itself binds to TESCO and interacts with SF1 to promote expression. (D) In female embryos, FOXL2 and estrogen receptor (ER) bind to TESCO, acting synergistically to inhibit *Sox9* expression. In addition, DAX1 competes with SF1 for binding sites on TESCO, preventing *Sox9* expression. *Source: Adapted from Sekido and Lovell-Badge, 2008.*[251]

GENETIC BACKGROUND AND TDA LOCI

A well-studied sex reversal system in mice is that of the *Sry* allele derived from a subspecies of *Mus musculus domesticus* from the Val Poschiavino in Switzerland. This allele (Sry^{POS}) is not able to trigger testis differentiation as efficiently as other alleles and causes a 14-h delay in the activation of the testicular pathway within the gonad. On a particular inbred mouse strain background, C57BL/6, Sry^{POS} is unable to initiate proper testis development and the gonads of these animals develop as ovotestes, with both ovarian and testicular components, or as ovaries[75] (Figure 7.5). Eicher et al. have mapped the C57Bl/6 loci involved in this effect to three regions: testis determining autosomal 1 (tda1) on chromosome 4, tda 2 on chromosome 2, and tda 3 on chromosome 5.[76] The genes associated with these loci have not yet been identified but it is likely that they encode factors that are involved in the regulation of *Sry* expression and/or that promote ovary development.[77] It has been posited that *Sf1* and Wilm's tumor 1 (*Wt1*) may be good candidates for tda2, and *Wnt4* for tda1.[78] These genes map to the tda loci, and are known to be dose-sensitive determinants of sex.

These studies highlight the importance of genetic background on the process of sex determination. Consistent with this, several mutant strains of mice, including those carrying mutations in *Fgf9* and *Dax1*, only show a sex reversal phenotype when the genetic background is derived from the C57Bl/6 background. This suggests that allelic variations that might have a small effect on available levels of active SRY could have a significant impact on phenotype due to the delicate balance that controls the decision to become male or female. Indeed, transcriptome analysis of E11.5 XY gonads in C57Bl/6J and 129S1/SvImJ mice revealed that the C57Bl/6J gonads had a more "female-like" transcriptome, explaining the propensity for XY sex reversal on this genetic background.[79]

Comparisons between human and mouse suggest that this balance is set up slightly differently between the two species such that mutations in the same gene can give different phenotypes, as mentioned below in the case of *WT1*. Thus it may be that analysis of sex determination in mouse models is best performed with the C57Bl/6 background, which may better mimic the state in humans.

WT1 AND CITED2

WT1 was first identified as one of the genes mutated in nephroblastomas found in newborns. This gene was

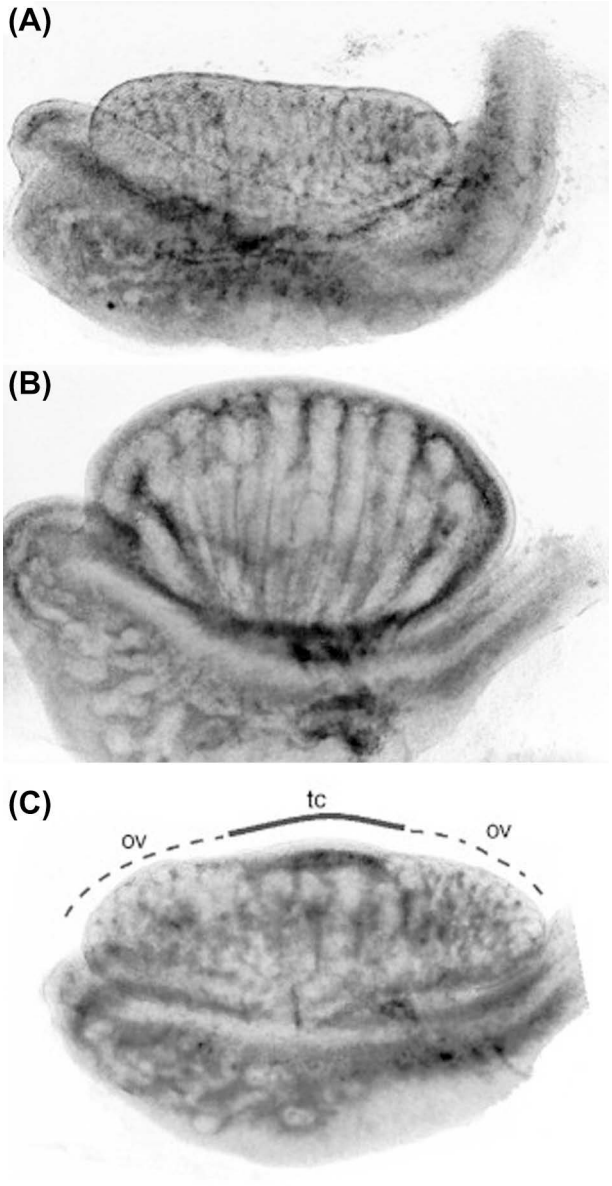


FIGURE 7.5 XY^{POS} mice on a C57Bl/6 background exhibit ovotestis formation. Brightfield images of wild-type female (A) and male (B) gonads from embryos at E14.5. Testicular cords are clearly visible in the male gonad at this stage. (C) Ovotestis formation in XY^{POS} mice on a C57Bl/6 background. Male testicular cords can be seen in the central portion of the gonad while the ovarian portion is found at both poles. *Source: Pictures taken from Ref. 250.*

also associated with defects in urogenital development in both mouse and man. During embryo development, WT1 is expressed throughout the intermediate mesoderm at early stages of urogenital development including the gonad.[80,81] Mice with a disrupted *Wt1* gene show absence of gonad and adrenal and arrested kidney development.[82] Studies on genetically modified mice have shown that not only is WT1 important in the development of the early genital ridge in both sexes but it also has a role in sex determination in the XY gonad.

The WT1 locus encodes a series of variant transcripts due to alternative splicing, alternative transcriptional start sites and RNA editing. These variants encode two main proteins containing a zinc finger domain that differ in the presence or absence of three amino acids (KTS) between two zinc fingers (designated +KTS or −KTS isoforms). Cell culture studies showed that these two proteins had different functions within the nucleus.[83] The −KTS isoform was associated with transcriptional regulation while the +KTS isoform co-localized with splicing factors and could bind RNA. Hammes et al. generated two strains of mice with mutations in the *Wt1* gene that inhibit the splicing events that give rise to the different isoforms such that the modified mice only expressed one variant or the other.[84] These two mouse strains showed different phenotypes in the gonad. The mice lacking the −KTS isoform showed a lack of proper gonad development in both sexes, while the mice lacking the +KTS isoform showed relatively normal gonad development but the XY gonad developed as an ovary. *Sry* expression in the gonad was significantly reduced in the latter case, accounting for the sex reversal phenotype.[85] These data suggest that WT1 regulates the levels of *Sry* within the gonad, possibly post-transcriptionally, through the action of the +KTS isoform.

Human patients with Frasier syndrome carry a heterozygous mutation in the splice site of WT1 that prevents the production of the +KTS isoform, shifting the ratio of the two isoforms within cells.[86] Consistent with the mouse studies, the XY Frasier individuals develop as females. However, the sex reversal phenotype observed in mice that lack the +KTS isoform is only observed in the human when the mutation is present in a homozygous state. Differences between human and mice phenotypes have been found for mutations in various genes involved in early sexual development, suggesting that the sensitivities to the levels of different factors are particular to each species.

CITED2 is a transcriptional cofactor that activates *Sry* via genetic interaction with WT1 and SF1. This is achieved by binding to WT1 and regulating SF1 levels within the AGP. *Cited2*$^{-/-}$ mice exhibit adrenal aplasia.[87] Similarly, compound mutant mice lacking *Cited2* and heterozygous for *Wt1* or *Sf1* lack adrenal glands and in addition exhibit partial XY sex reversal. Furthermore, loss of *Cited2* on an *Sry*^{POS} background produces complete XY sex reversal.[88] Thus, a WT1/CITED2/SF1 regulatory cascade mediates *Sry* expression.

SF1

SF1 is an orphan member of the nuclear receptor superfamily. *Sf1* expression in the early embryo is restricted to the genital ridge and adrenal and is the earliest marker of these tissues. Mice with a mutation

in *Sf1* lack gonads and adrenals and develop as female embryos.[89,90] This early phenotype prevents easy analysis of the role of SF1 in sex determination and gonad differentiation. Deletion using tissue-specific Cre drivers has allowed researchers to overcome this early phenotype to dissect the roles of SF1. Using this technique, it has been shown that *Sf1* is critical at multiple stages of sex determination and differentiation. As described above, SF1 acts with SRY to activate *Sox9* expression, triggering the male cascade.[71] Subsequent to this, *Sf1* expression in Sertoli cells is required for regulation of anti-Müllerian hormone (AMH), leading to degeneration of the Müllerian ducts in male embryos.[91] SF1 is a master regulator of steroidogenesis, required for normal steroid production in the Leydig cells of the testis, the stromal and theca cells of the ovary, and the cortical cells of the adrenal[92] (reviewed in Ref. 93). In addition, SF1 expression is required for normal differentiation of the ovary.[94] SF1 has been shown to be dosage sensitive, as mice heterozygous for *Sf1* exhibit impaired testis differentiation on an Sry^{POS} genetic background,[78] while sexual development occurs normally on a mixed background.

In accordance with the critical role of SF1, its expression is stringently regulated at both the gene and protein level. As discussed above, WT1 and CITED2 interact to regulate expression of *Sf1*.[87,88] POD1, a basic helix-loop-helix transcription factor, is also reported to regulate *Sf1* expression in the developing gonad.[95] DAX1, a female fate-promoting protein discussed in depth below, interacts antagonistically with SF1 at the protein level.[96] At the post-translational level, SUMOylation of SF1 has been shown to be required for normal gonadal and adrenal development. Inhibition of SUMOylation causes a change in the set of genes regulated by SF1 expression, resulting in an under-differentiated and mixed phenotype in the gonad and adrenals.[97] Regulation of *Sf1* expression is reviewed in more detail elsewhere.[98–100]

This requirement for stringent regulation is reflected in the clinical importance of normal SF1 expression. Heterozygous *Sf1* mutation in human disease was first observed in a patient with XY sex reversal.[101] Since this identification, many human SF1 mutations have been reported.[102–104] Although a small number of patients exhibit complete sex reversal, the majority fall elsewhere within the DSD spectrum (reviewed in Ref. 104).

INSULIN RECEPTOR FAMILY

The insulin receptor family is a set of receptor tyrosine kinase (RTK) proteins. Signals received by the insulin receptors are transduced by phosphoinositide-three kinase. Targeted mutagenesis in the mouse has uncovered a role for the insulin family signaling pathway in sex determination. XY mice with simultaneous mutations in all insulin receptors (*Insr, Igf1r*, and *Irr*) show complete sex reversal, with gonads developing into ovaries.[105] *Sry* expression during gonad development in these mice was found to be significantly reduced, accounting for the sex reversal phenotype. However, cell proliferation and the size of the gonads in both sexes was reduced in these mutant animals. Cell proliferation has been shown to be important in sex determination, as inhibition of proliferation at particular stages of gonad development can prevent testis determination.[106] Therefore the phenotype observed in the mutant mice was probably due to a defect in proliferation of cells of the gonad at the time when *Sry* is acting to determine testis development. Recently, it has been shown that mice with mutations in both *Igfr* and *Insr* have the same phenotype, indicating that *Irr* may not be required for sexual development.[107] Gonads remain undifferentiated until E16.5, when female-specific gene expression is detected, supporting the hypothesis that defects in these mutants are due to abnormal proliferation.

GATA4/FOG2

Members of the GATA family of transcription factors are important in many processes during embryogenesis. These factors contain a zinc finger DNA binding domain and interact with FOG (Friend of GATA) proteins that contain multitype zinc finger domains. Expression analysis of *GATA* and *FOG* genes during urogenital development show that GATA4 and FOG2 are expressed in the somatic cells of gonad.[108,109] Mouse XY embryos with a mutation in *Fog2* show sex reversal.[110] Analysis of the gonads of these mutant mice shows a reduction in *Sry* expression when compared to wild-type XY embryos. Ovary development was also seen in XY embryos with a mutation in *Gata4* that abrogates the interaction of GATA4 with FOG2, showing that both proteins are required for sex determination.[110] Mutations in GATA4 that prevent binding with FOG2 have been found in patients with XY,46 female DSDs.[111] Studies are underway to determine a direct role for GATA4 in the regulation of *Sry* transcription.

MAPK SIGNALING

RTK signaling is of critical importance in a large number of developmental settings. Recent research has revealed that RTK signaling via the Ras/Mapk pathway is critical for normal male development.[112,113] p38-MAPK signaling lies upstream of *Gata4* and *Sry* in murine sex determination.[112] This cascade is regulated by the stress-response protein GADD45G, which is expressed in a pattern similar to, but earlier than, that of *Sry*. Mice lacking *Gadd45g* exhibit reduced *Sry* expression and XY sex reversal. These phenotypes are mimicked in mice lacking *Map3k4* and *p38α+β*. GADD45G has been shown to functionally interact with MAP3K4 to activate p38-MAPK signaling.[114,115] Although the exact mechanism by which this cascade acts to activate *Sry* is unknown, it has been shown that demethylation of the *Sry* promoter, which precedes *Sry* gene expression by several hours,

still occurs in *Gadd45g* mutants; thus *Sry* is primed for activation, but lacking the stimuli required for expression.[114] Mutation in *MAP3K1* results in XY DSDs in both humans and mice.[113,116] Although a relationship between MAP3K1 and GADD45G has not yet been proven, this data implicates MAPK signaling as a clinically relevant factor in human sex determination.

CHROMATIN REMODELING FACTORS

Two factors involved in chromatin domain organization, M33 and ATRX, have been implicated in sex determination. M33 is a member of the Polycomb Group Gene family, and a general repressor of transcription. XY mice mutant for this gene show sex reversal.[117] However, the phenotype was variable and gonad development was retarded. Microarray analysis of these mutants indicated that *Sry* and other downstream male-specific genes were downregulated in M33 null mice. Forced expression of *Sry* rescued the sex reversal defects, although development of the gonad was still somewhat retarded. Thus, M33 acts upstream of *Sry*, although additional roles in gonad development are also probable.[118] Human XY individuals with mutations in the X-linked gene *ATRX* show sex reversal.[119,120] The role of this factor in gonad development has not yet been studied in any detail. Interestingly a homolog of the *ATRX* gene was found on the marsupial Y chromosome.[120] This gene was found to have a testis-specific expression, in contrast to *ATRX*, that is expressed in many tissues, suggesting that it has a role in marsupial testis development.

Testis and Ovary Development

The undifferentiated gonad is made up of four bipotential cell types: the supporting cells, interstitial cells, PGCs, and connective tissue (Figure 7.6). Upon sex

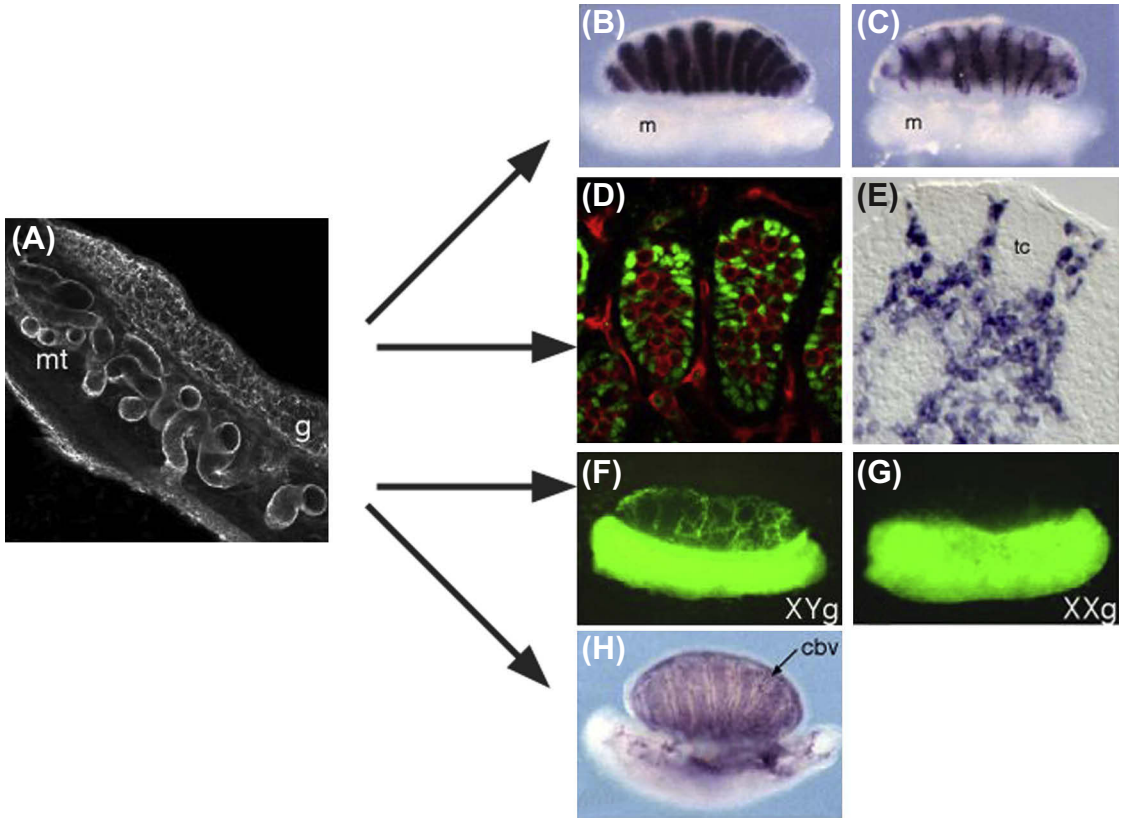


FIGURE 7.6 Differentiation of the embryonic testis from a bipotential precursor. This figure is reproduced in color in the color plate section. (A) Confocal image of a gonad and mesonephros stained with an antibody to laminin at a stage before morphological changes take place (E11.5). (B–H) Morphology of the embryonic testis. (B, C) The Sertoli and Leydig cells of the E13.5 gonad are marked by whole-mount in situ hybridization for the expression of the Sertoli cell marker AMH (B) and the Leydig cell marker *Cyp11a1* (C). The structure of the testicular cords is seen in the confocal image of an E12.5 gonad that was stained with an antibody against SOX9 in green (D). Germ cells are found within the testicular cords as marked by the antibody stain for PECAM in red (D). In the interstitium, epithelial cells, as marked by the antibody stain for PECAM in red (D), and Leydig cells, as marked by the section of the whole mount in situ hybridization on a gonad from a E14 embryo for the expression of *Cyp11a1* (E), are found. (F, G) Mesonephric cell migration is observed in the XY (F) but not the XX (G) gonad, as shown by organ culture samples where the mesonephros but not the gonad is derived from transgenic mice that express GFP ubiquitously. (H) The formation of male-specific vasculature as shown by a whole-mount antibody stain to PECAM, which marks endothelial cells, of gonad and mesonephros from an E14 embryo. mt, mesonephric tubules; g, gonad; m, mesonephros; tc, testis cord; cbv, coelomic blood vessel. *Source: Adapted from Ref. 252; confocal image (D) courtesy of Blanche Capel.*

determination, these cells types differentiate, assuming sex-specific fates. The supporting cells are the first male cell type to undergo differentiation, to give rise to the Sertoli cells. In the female, differentiation occurs postnatally, at which time the supporting cells give rise to the granulosa cells of the ovary. The interstitial cells of the male undergo differentiation to give rise to Leydig cells, while the same cells give rise to theca cells in the female at postnatal stages. The germ cells become sperm or egg cells (oocytes), while the connective cells encompass the rest of the cell types such as ovarian stromal and testicular peritubular myoid cells and the differing patterns of vasculature in the male or female gonad. In this section we will describe in detail the differentiation of each of the four cell types, discussing the cellular and molecular pathways that are activated in the developing male and female gonad.

Supporting Cell Differentiation

As the name suggests, the supporting cells are vital to nurture the germ cells during their development until their release. The Sertoli cells are also believed to direct Leydig cell differentiation and hence testicular development. Initiation of differentiation along a male or female path is determined by expression of SRY, or lack thereof, respectively. Subsequently, expression of different genes mediates supporting cell differentiation and maintenance.

SOX9 IS REQUIRED FOR SERTOLI CELL DIFFERENTIATION

Differentiation of the supporting cells into Sertoli cells is the first stage of male gonadal differentiation. This process is mediated by *Sox9*, which must be activated by the synergistic action of SRY and SF1 between E10.5 and E12.5 in order to direct the gonad to become a testis, rather than an ovary.[71,74] Expression analyses in the mouse have shown that *Sox9* is one of the first genes upregulated in the male gonad compared to the female, at around E11.5, and its expression is then associated with Sertoli cells throughout testis development.[121,122] Indeed, SOX9 is vital for definition and maintenance of Sertoli cell identity. In humans, heterozygous mutations in *SOX9* give rise to XY female sex reversal associated with the severe dwarfism syndrome campomelic dysplasia,[123,124] while translocation or mutation 5′ of *SOX9* has been reported to cause XY female sex reversal with or without the associated syndromes in several cases.[125–128] Mice that lack *Sox9* in the developing testis showed defects in testis differentiation, giving rise to ovotestis-like gonads.[129] Transgenic experiments in mice showed that SOX9 can induce Sertoli cell differentiation and testis development in XX gonads.[130] In one case, a chance integration of a transgenic construct 1.3 Mb from the *Sox9* locus resulted in the induction of the expression of this gene within the embryonic XX gonad.[131] Consistent with this, duplications of the *SOX9* locus in humans are associated with XX male sex reversal.[132,133]

Binding of SRY and SF1 at the TESCO enhancer initiates *Sox9* expression (Figure 7.4(A) and (B)), but *Sry* is only expressed for a brief period. Therefore, maintenance of *Sox9* expression must involve other transcriptional activators. In vitro, SF1 can recruit SOX9 to the TESCO element (Figure 7.4(C)), where the proteins are capable of acting in synergy to promote expression of *Sox9* itself. Thus it appears that maintenance of *Sox9* is achieved via an autoregulatory feed-forward loop. FGF9 has been implicated in maintenance of *Sox9* expression in Sertoli cells. *Fgf9* is expressed in the bipotential gonad at E11.5, but becomes restricted to the XY gonad by E12.5.[134] Expression begins at the center of the gonad and radiates out, in a manner similar to that of *Sry* and *Sox9*.[135] Loss of *Fgf9* causes XY sex reversal in mice, a phenotype that arises due to failure of Sertoli cell differentiation, measured by lack of desert hedgehog (*Dhh*) and *Amh* expression.[134,136] *Sry* expression is normal in these mutants, indicating that *Fgf9* is downstream of *Sry* in sex determination. In most mutants, *Sox9* expression is lost, but a small proportion retain a low level of *Sox9* expression, implying that FGF9 is not required for initiation of *Sox9* expression.[137] In cell culture, FGF9 promotes expression of *Sox9*, indicating that FGF9 is involved in maintenance of gene expression.[134] Furthermore, expression of *Fgf9* is lost in *Sox9*$^{-/-}$ gonads. Thus, *Fgf9* and *Sox9* act in a feed-forward loop in which SOX9 activates *Fgf9* expression, which then promotes continued expression of *Sox9*. FGF9 appears to act through FGFR2, which is expressed in the coelomic epithelium—where FGF9 promotes proliferation specifically in the male—and in Sertoli cell precursors. Deletion of *Fgfr2* in mouse models causes XY sex reversal and a decrease in proliferation within the coelomic epithelium.[138]

Prostaglandin D2 (PGD2) has been implicated as a paracrine positive regulator of *Sox9* in Sertoli cells. PGD2 is capable of inducing *Sox9* expression in XX gonads in organ culture, while *Fgf9* is not,[139] implying that *Sox9* is downstream of PGD2. This was supported by in vivo data demonstrating that mice lacking the enzyme responsible from production of PGD2, lipocalin prostaglandin D synthase (L-PGDS), exhibit downregulation of *Sox9*.[140] Furthermore, SOX9 was found to be required for *L-pdgs* expression. Thus, PGD2 and SOX9 also function in a positive regulatory loop. Analysis of *Fgf9*$^{-/-}$ and *L-pgds*$^{-/-}$ mice indicated that the two loops are independent from one another, and act additively to promote *Sox9* expression.[140]

The defects observed in *Sox9* mutant mice were shown to be more severe when these mice also lacked the related gene *Sox8*, which is upregulated in Sertoli cells of the developing gonad slightly after *Sox9*.[141]

Duplication of chromosome 22q13, where *SOX10* is located in humans, causes XX male sex reversal.[142] A gain-of-function mutation in *Sox10* in mice was shown to cause dose-dependent sex reversal, the severity of the phenotype corresponding to the level of *Sox10* expression.[143] This suggests that these *Sox* genes may have similar functions and therefore are part of a reinforced redundant system to ensure the differentiation of Sertoli cells, implying that conservation of *Sox9* function is vital for differentiation.

EVOLUTIONARY CONSERVATION OF REGULATION OF SERTOLI CELL DEVELOPMENT

SOX9 protein is related to SRY in that it also contains an HMG box type of DNA binding domain. Outside this domain it differs from SRY in that it contains a strong transcriptional activation domain. Evolutionary studies have shown that the *SOX9* gene is highly conserved in vertebrates and in many cases its expression is associated with early testis differentiation.[121,122,144–146] This suggests that there is a common pathway for ensuring Sertoli cell differentiation in vertebrates and that different sex determination mechanisms have evolved to control this process.[147] Consistent with this model, conserved members of the DMRT family of proteins are found in the developing gonad of all vertebrates that have been studied.

DMRT1 is a transcription factor expressed in the germ and Sertoli cells of the developing mouse testis.[148] DMRT1 homologs, those proteins possessing a zinc finger-like DM DNA-binding domain, are highly conserved factors in sexual determination and differentiation. Indeed, DM domain proteins play a role in these processes in virtually all metazoans analyzed (although their role is not always limited to regulation of sexual dimorphism). In the vast majority of metazoans, DM proteins appear to act immediately downstream of sex determination, at the top of the sexual differentiation cascade. This produces what may be termed an "evolutionary bottleneck" in sexual development. Many different mechanisms act upstream of primary sex determination in different species. Downstream of *Dmrt1*, once again a wide variety of signaling pathways act to enforce varying sexual dimorphisms on the different species, such as horns, plumage, mating behaviors, and many more.[149]

In humans, *DMRT1* is located on chromosome 9p24.3, a locus whose deletion results in DSDs and often sex reversal.[150,151] In mice, *Dmrt1* deletion does not cause congenital sex reversal,[152] although *Dmrt1* expression is required for normal adult development of both the Sertoli and germ cells.[153,154] As the deletions reported in humans affect multiple genes, it is possible that another gene at this locus is responsible for the resulting phenotypes. However, there are several alternative explanations for this difference; differences in the role of DMRT1 between species or a genetic background effect in the mouse, for example. Interestingly, in non-mammalian vertebrates, DMRT1 homologs have evolved multiple times to represent the top of the sex determination cascade. In both teleosts and some amphibians, duplication of *Dmrt1* has given rise to a primary sex determinant much like the mammalian *SRY*.[155–157] Thus, *Dmrt1* is important for sexual development throughout the animal kingdom. Together the genetic and functional conservation of *Sox9* and *Dmrt1* in vertebrates, and in particular in mammals, indicates that regulation of Sertoli cell development is critical for normal development of the testis.

DIFFERENTIATION OF FOLLICLE CELLS

Until fairly recently it was assumed that the default pathway for sexual development was female, and that SRY, as the primary male determinant was the only factor preventing this fate. While it is true that SRY is necessary and sufficient for male development, it has become apparent that the female pathway is also active, rather than default, and that the two fates repress one another. Activation of the female pathway in the absence of SRY causes differentiation of supporting cells into granulosa cells that, together with the interstitial theca cells, will make up the follicles within which germ cells are located. Normal folliculogenesis is vital to support development of oocytes from PGCs.

The exact stage at which follicle differentiation occurs is ill defined, as reorganization into female-specific structures is not evident by histological analysis until as late as E18.5, although female-specific markers such as the forkhead transcription factor *Foxl2* are expressed in the supporting cells of the XX gonad as early as E12.5.[158,159] *Foxl2* is evolutionarily well conserved in vertebrates.[160] Deletion in mice causes ovarian failure; thus the gene has been implicated as a primary female-determining gene.[158,159,161] However, *Foxl2* mutation does not cause complete sex reversal in humans or mice. FOXL2 has been shown in vitro to bind to the TESCO enhancer of *Sox9*, preventing upregulation of *Sox9* in Sertoli cells (Figure 7.4(D)).[162] Mice lacking *Foxl2* show sex reversal postnatally, implicating FOXL2 in maintenance of female sex programs via *Sox9* inhibition. This phenotype is discussed in more detail below. Together these data indicate that *Foxl2* is unlikely to be the primary determinant of female development. An alternative candidate is the Wnt signaling pathway.

Canonical Wnt signaling promotes female sexual differentiation via *Wnt4* and *R-spondin1 (Rspo1)*. R-spondins are a group of four secreted ligands that act to stabilize beta-catenin in a manner analogous to that of canonical Wnt ligands. Mice lacking either *Wnt4* or *Rspo1* show partial XX sex reversal.[163–165] In the case of *Rspo1* this was demonstrated to be due to alteration in beta-catenin signaling.[163] XY mice in which *Wnt4* is overexpressed undergo improper patterning of the vasculature in the testis, and abnormal Leydig cell recruitment, indicating that *Wnt4* alone is not a primary sex-determining

factor.[166,167] In humans, mutation of *Rspo1* has been documented in complete 46,XX male sex reversal.[168]

While loss of Wnt signaling affects female development, normal regulation of canonical Wnt signaling is also important for male development. In humans, duplication of chromosome 1p, where both *Wnt4* and *Rspo1* are located, results in complete XY,46 sex reversal.[169] This requirement was further demonstrated in mice in which a stabilized form of beta-catenin was expressed under control of an *Sf1Cre* driver. Stablilization of beta-catenin in this manner caused constitutive activation of canonical Wnt signaling in the somatic cells of the bipotential gonad. The XY mutant embryos showed development of follicles and upregulation of female-specific genes, including *Foxl2*.[170] Despite this phenotype, some aspects of initial testis formation were observed, including formation of the coelomic vessel. This indicates that Wnt signaling likely acts downstream *Sry* expression. Indeed, downregulation of *Sox9* was observed from E11.5 onwards. Data suggest that beta-catenin, like FOXL2, acts to induce female development by repressing binding between SF1 and the TES enhancer of *Sox9*.[171] Thus it is probable that canonical Wnt signaling in the XX gonad acts to modulate the effect of SRY on *Sox9*, repressing the male and activating the female fate.

BALANCING MALE VERSUS FEMALE GENE EXPRESSION

The above data show that canonical Wnt signaling via *Wnt4* plays an active role in female development, rather than female fate being a default pathway. WNT4 and the male-promoting FGF9 have been shown to antagonize one another upstream of *Sox9* expression. Although expression of these genes is ordinarily sex-specific from E12.5 onwards, *Fgf9* and *Sox9* are expressed in *Wnt4*$^{-/-}$ XX gonads, and *Wnt4* and Rpso are expressed in *Fgf9*$^{-/-}$ XY gonads.[134,172] In embryonic gonad organ culture, addition of exogenous FGF9 can induce *Sox9* expression and cause downregulation of *Wnt4*.[134] Furthermore, deletion of *Wnt4* in *Fgf9* or *Fgfr2* null XY mutant mice rescues the sex reversal phenotype, although the reverse is not true of the partially sex reversed XX *Wnt4* null mice.[172] Together, these data indicate that *Wnt4* and *Fgf9* are responsible for inhibition of one another once sex is determined by expression (or lack thereof) of *Sry*.

Although many genes are characteristically viewed as male- or female-promoting, the role of the X-linked gene *DAX1* (Nr0b1) has long puzzled researchers. *DAX1* has been implicated in sex determination and gonad and adrenal development. *DAX1* is an unusual member of the nuclear hormone receptor family because it lacks the classic zinc finger DNA binding domain but contains instead a novel domain with a repeated motif.[173] In many respects, *DAX1* is a classic female fate-promoting gene. *Sox9* is downregulated in transgenic mice overexpressing *Dax1*, and overexpression of *Dax1* in *Sox9*$^{+/-}$ mice results in partial XY sex reversal. However, the role of *Dax1* in sex determination is thought to be complex. XY mice with a targeted mutation in this gene showed defective testis cord development. Moreover, in certain genetic backgrounds these mutant mice showed complete male to female sex reversal.[174,175] *Sry* expression was not affected in these sex-reversed mice, suggesting that this gene acts downstream of *Sry* in the gonad. Mutations in *DAX1* in XY human individuals gave rise to adrenal deficiencies and hypogonadotrophic hypogonadism, where their testes were reduced in size but no sex reversal was observed.[176,177]

Human XY individuals with a duplication in the region of the X chromosome that contains *DAX1* show sex reversal, called the dosage-sensitive sex reversal syndrome.[178] Consistent with this, transgenic XY mice with extra levels of *Dax1* show sex reversal, although only in certain genetic backgrounds.[179] These studies show that although DAX1 is required for testis development, at high levels it can act antagonistically to SRY and promote sex reversal.

Tissue culture studies showed that DAX1 can act as a repressor of transcriptional activation by other classic nuclear hormone receptors.[177,180,181] DAX1 expression within the early gonad correlates with that of SF1. In vitro evidence indicates that the sex reversal observed in *Sox9*$^{+/-}$*; Dax1TG* mice is due to competition between DAX1 and SF1 at the TESCO enhancer of *Sox9* (Figure 7.4(D)).[96] Thus DAX1 promotes the female pathway via antagonistic interactions with SF1.

Cell Migration and Vasculature Formation

Once sex determination has occurred, the size of the male gonad increases relative to the female gonad, due to FGF signaling acting downstream of SRY in the gonad.[73,136,138] In addition, cell migration and vasculature formation occur in a sex-biased manner (Figure 7.6(F) and (G)).

The characterization of the cellular processes involved in gonad development has been possible due to the development of in vitro organ culture systems. Tissues from genetically modified mice have been used in recombinants to determine the molecules involved in the different processes. This type of study has shown that cells from the mesonephros migrate into the gonad during development, and that this only occurs in the male gonad.[182,183] The migrating cells are endothelial cells that instruct testis cord formation; blocking this process in organ cultures impairs the proper formation of cords.[184,185] Several growth factors, including FGF9, VEGF, and PDGFs, were able to induce migration of mesonephric cells into an XX gonad in culture.[137,186,187] These data were subsequently supported by in vivo

work. Repression of FGF signaling by the gain-of-function mutation in the RTK inhibitor *Sprouty2* in mice inhibited migration from the mesonephros, and prevented normal testis cord formation.[188]

Normal endothelial cell migration is a requirement for formation of the male vasculature. Testis cord formation in the XY gonad is accompanied by the formation of a prominent blood vessel running along the periphery of the testis, just under the coelomic epithelium. Although vascular formation is an active process in the gonad of both sexes during development, this coelmic blood vessel (CBV) is only found in the male. The endothelial cells associated with this vessel were found to express arterial markers, suggesting that a male-specific circulatory system that promotes the export of testosterone (T) produced by Leydig cells needs to form to ensure the masculinization of the embryo during its development.[189] Mice lacking PDGFRa show a defect in CBV assembly and branching in the testis.[186] VEGF inhibition in gonad culture also prevents normal vasculature formation due to lack of endothelial migration. PGDF signaling is proposed to act downstream of VEGF in this process, as addition of PDGF to VEGF-inhibited gonads can rescue the proliferation, but not the vascular formation defects.[187] In addition, mice lacking the gene inhibin beta B *(Inhbb)*, which encodes activin B, exhibit mild defects in CBV formation. *Inhbb* expression is sex-biased from E12.5, when it is detectable only in XY gonads.[190] The phenotypes observed, such as a thin CBV, differ from those seen in the PDGFR and VEGF mutants, and likely represent a different pathway.

The XX gonad does not normally form a CBV. An active repressive pathway is present in the ovary to prevent the migration of endothelial cells from the mesonephros and the formation of a CBV. Mice with mutations in the genes encoding either WNT4 or follistatin (*Fst*) showed the presence of an ectopic CBV in the ovary, similar to that found in the normal XY gonad.[166,191] WNT4 was found to act upstream of *Fst* in this process. Loss of *Inhbb* in $Wnt4^{-/-}$ or $Fst^{-/-}$ mice prevented formation of the CBV, indicating that activin B is responsible for ectopic CBV formation in these mutants.

Interstitial Cell Differentiation

The indifferent interstitial cells of the bipotential gonad are believed to give rise to the steroid producing cells of the adult reproductive organs; the Leydig or theca cells in the male or female, respectively. In the male gonad, the Leydig cells arise from the interstitium in the days succeeding Sertoli cell differentiation. Differentiation of the interstitial cell population is stimulated by signals from the forming testis cords. Subsequently, the Leydig cells produce enzymes that catalyze the conversion of cholesterol to androgen. Gonadal steroidogenesis is discussed in depth in Chapter 8. Theca cell differentiation is less well understood and occurs after birth. Interstitial cells surrounding the follicle are believed to differentiate into theca cells upon ill-defined signals from the granulosa cells during folliculogenesis. The theca cells then surround the follicle and produce androgens, which are subsequently converted to estrogens by the underlying granulosa cells (see Chapter 28 for details).

Adrenal cortical cells, which also produce steroids, are, like the testis and ovaries, derived from the AGP.[63] These cells arise when the genital ridge first forms, and separate into adrenal and gonadal populations as development proceeds. Although the expression of genes involved in steroidogenesis is observed in the fetal adrenal cells at early stages, it is not until after cord formation that this gene expression is seen in the testis. Steroidogenesis is not apparent until postnatal stages in the ovary. Either different pathways must activate the steroidogenic cascade in each organ, or different control mechanisms release the brakes on these pathways at different times, permitting differentiation of the interstitial population and subsequent steroidogenesis. Sex-biased roles in interstitial cell differentiation have been documented for the hedgehog signaling pathway.

The cell-signaling molecule DHH is a candidate factor for the activation of Leydig cell differentiation. DHH is produced by Sertoli cells at early stages of testis development, and mice that lack this gene showed a severe reduction in Leydig cell numbers during development in certain genetic backgrounds.[192,193] Furthermore, on a particular mixed background, the mutant testis cords were disorganized; therefore Dhh is required for normal cord formation. Downstream targets *Patched1*, *Gli1*, and *Gli2* are expressed not in the Sertoli cells but throughout the interstitium, indicating that hedgehog signaling is transduced in the Leydig and myoid cells of the testis rather than the Sertoli or germ cells.[193,194] DHH and its downstream targets are not expressed in the developing XX gonad.[194,195] Both Leydig cell differentiation and testis cord formation were affected in *Patched1* mutant mice, suggesting that hedgehog signaling has a general role during testis development.[193] Consistent with this, human individuals with mutations in DHH show variable gonadal phenotypes.[196] $Sf1^{+/-}$; $Dhh^{-/-}$ mice undergo XY external sex reversal. Testis cords and germ cells are present, but Leydig cells fail to differentiate, as measured by expression of *Cyp11a1*, a gene encoding the steroidogenic enzyme p450SCC.[197] Constitutive activation of Smoothened, a transducer of hedgehog signaling, in SF1-positive cells in the XX gonad resulted in the differentiation of steroidogenic cells but not of other testicular cell types.[198] This indicates firstly that hedgehog signaling can instruct differentiation of fetal Leydig cells, and secondly that hedgehog signaling is disruptive to normal female gonadal development.

Although absent during embryogenesis, it is possible that the hedgehog signaling pathway is involved in female steroidogenesis postnatally. DHH and its sister ligand Indian hedgehog (IHH) are both expressed in the granulosa cells of the adult ovary and are thought to signal to the theca cells, in which *Patched* and *Gli* expression has been documented.[199,200] Inhibition of hedgehog signaling with cyclopamine in organ culture experiments did not appear to affect the ovary, although the short duration of culture might be insufficient for phenotypes to arise. Therefore, any functional role for this signaling pathway in theca cells remains undefined.[200] Furthermore, other signals responsible for theca cell differentiation are also poorly understood. Proteins implicated include IGF1 and luteinizing hormone, as well as factors produced by the granulosa cells.[201] Addition of these proteins to an interstitial cell culture system induced differentiation of theca cells and steroidogenesis. However, this in vitro result has yet to be confirmed in vivo. Together, these data provide some insight into the potential cues governing thecal cell differentiation, but much more work is needed to achieve a good understanding of this process.

Although thecal cell development remains ill defined, Leydig cell differentiation has been more thoroughly studied. Mutation in the X-linked aristaless related homeobox (*Arx*) gene in both XY mice and humans results in similar phenotypes to those documented in DHH mutation.[202] *Arx* is expressed in interstitial and peritubular myoid cells of the developing testis, and Leydig cell differentiation is severely affected in the mutant mouse embryos. However, *Arx* is not expressed in Leydig cells, indicating that the differentiation and compartmentalization of the interstitial cells during development is important for the induction of steroid production. In humans, mutations in *ARX* are associated with the XLAG (X-linked lissencephaly with abnormal genitalia) syndrome, as this gene also has a function in forebrain development.[202]

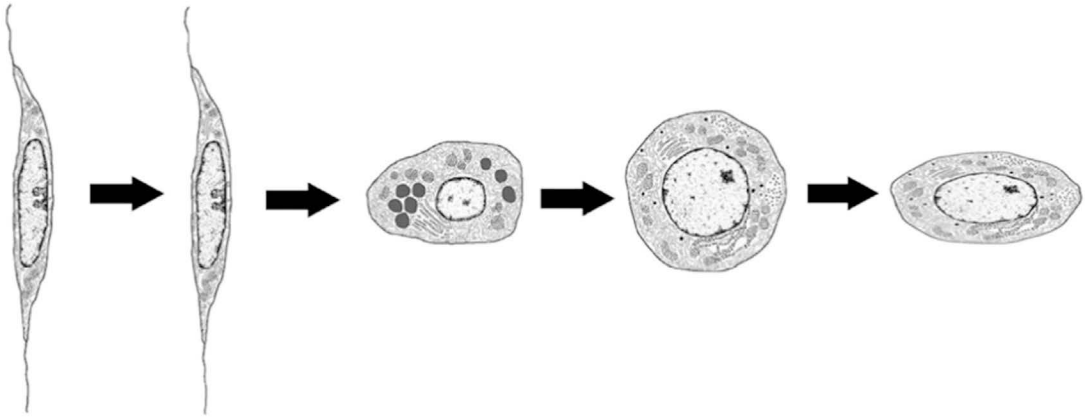
Notch signaling has also been implicated in Leydig cell differentiation. The Notch genes are expressed in a sex-biased manner in the XY gonad during development. Downstream targets *Hairy/Enhancer-of-split1 (Hes1)* and *Hes5* are expressed in the developing interstitium and Sertoli cells, respectively. Inhibition of Notch signaling results in an increase in the number of Leydig cells, while Sertoli cell number is unaffected. Conversely, constitutive activation of Notch signaling in the mouse causes a decrease in Leydig cell number, due to maintenance of a progenitor interstitial cell population in preference to differentiation into Leydig cells. Notch was not capable of reverting differentiated Leydig cells to a progenitor phenotype, indicating a specific requirement in the process of fetal Leydig cell differentiation.[203]

The genes described above have largely been studied during embryogenesis, and thus have been shown to be required for fetal Leydig cell differentiation. However, fetal Leydig cells are believed to be a separate population from adult Leydig cells. It is thought that fetal Leydig cells originate from two sources. Lineage tracing using Mitotracker dye showed that Leydig cells can derive from both the coelomic epithelium and the gonad-mesonephros border.[204] It is unclear whether the Leydig cells deriving from the gonad-mesonephros border represent true Leydig cells or the so-called adrenal-like cells, a population of steroidogenic cells in the fetal testis that express genes involved in adrenal steroidogenesis.[205]

The origin of adult Leydig cells remains unclear, although an adult Leydig stem cell population likely exists in the testis (see also Chapter 14). Studies suggest that onset of differentiation of the adult Leydig stem cell population occurs at different stages in different organism (reviewed in Ref. 206). Hardy described passage of the stem Leydig cell (SLC) through two intermediate stages to give rise to a terminally differentiated Leydig cell (Figure 7.7).[207] The SLC population proliferates to give rise to spindle-shaped progenitor Leydig cells (PLC), which begin to produce some enzymes associated with steroidogenesis, such at 3β-hydroxysteroid dehydrogenase (3β-HSD) (encoded by the gene *Hsd3b*).[208,209] Over time, the PLCs assume a rounded morphology, characteristic of Leydig cells, and upregulate expression of steroidogenic genes such as *Hsd3b* and *Cyp11a1*.[209,210] This population is referred to as the immature Leydig cell population (ILCs). Finally, one cell division in each ILC gives rise to the full complement of terminally differentiated adult Leydig cells. Although these stages of development have been documented histologically, they are hard to define on a stage-by-stage basis, as there are no known markers that distinguish each stage. Indeed, since the process was described almost 20 years ago, little data has been produced to improve our understanding of the process. Multiple attempts to characterize the adult Leydig stem cell population have been published.[211–213] However, contradictory evidence and a lack of markers mean that the population has yet to be defined.

Germ Cells

In mammals, germ cell fate is determined by induction; that is, signals from the surrounding environment induce multipotent embryonic stem cells to become germ cells. Thus, up until the blastocyst stage any cell is capable of becoming a germ cell if it is placed in the correct environment. This is in contrast to animals such as *Drosophila* and zebrafish, in which maternal factors predetermine which cells will become germ cells, a mechanism known as preformation. Once specified, germ cell fate is maintained by stringent transcriptional



Cellular attributes	Stem Leydig cell	Progenitor Leydig cell	Immature Leydig cell	Adult Leydig cell	Aged Leydig cell
Mitotic activity	Low	high	Once	Probably none	Probably none
Morphology	Spindle	Spindle	Round with large lipid droplets	Round with small lipid droplets	Round with small lipid droplets
Smooth ER	Low	Low	High	Highest	High
Testosterone metabolism	None	High	Highest	Low	Low
Testosterone synthesis	None	Low	Intermediate	High	Intermediate
Age when cell is typical	7 days	21 days	35 days	90 days	20 months
LH receptor number	None	Low	Intermediate	High	Intermediate
Androgen receptor number	?	High	High	Low	?

FIGURE 7.7 Stages of adult Leydig cell development. Pictorial representations of the morphology of the developing adult Leydig cell, with defining characteristics tabulated below. *Source: Taken from Ref. 253.*

repression, via chromatin modification and, in some species, repression of RNA polymerase. These processes are reviewed in detail elsewhere.[214]

In the mouse, germ cells migrate into the gonad from the proximal epiblast after specification. Upon arrival, transcriptional repression is lifted somewhat in order to allow a process known as licensing, which allows germ cells to respond to sex-specific signaling, and so enter meiosis or mitotic arrest. Although the signals determining a female versus male fate are extrinsic, the ability of the germ cell to respond is an intrinsic property. Intrinsic licensing is controlled by an RNA-binding protein, Deleted in Azoospermia-Like (DAZL). *Dazl* expression is restricted solely to germ cells.[215] In *Dazl* null mice, the germ cells maintain the primordial state normally found during migration. Thus the cells remain sexually undifferentiated and do not enter meiosis.[216,217]

Germ cells within the developing mammalian gonad follow a sexually dimorphic pathway. In the ovary, they enter meiosis at around E13.5, but in the male, they arrest in mitosis at about the same time and remain in G0 until neonate stage. This difference is dependent not upon the chromosomal sex of the germ cells but upon the somatic environment of the gonad.[218] The factor controlling entry into meiosis is the helix-loop-helix protein stimulated by retinoic acid 8 (STRA8). In the XX mouse, deletion of *Stra8* causes loss of germ cells by E16.5, as the cells cannot complete the transition to meiotic prophase, although they appear normal until E13.5.[219] The same phenotype is observed postnatally in the male mutants.[220]

Evidence indicates that *Stra8* expression in the gonad is downstream of retinoic acid (RA) signaling.[221] However, recent work has indicated that other pathways are also capable of activating *Stra8* in this setting, as deletion of *Raldh* genes does not result in a loss of *Stra8* expression in the early female gonad.[222] In the female gonad, RA signaling from the mesonephros occurs from E12.[221,223] In the male gonad, CYP26B1 in Sertoli cells degrades retinoic acid, thus inhibiting expression of *Stra8* and delaying entry into meiosis. *Cyp26b1* is not expressed in the female gonad at this time. After birth, *Cyp26b1* is downregulated in the male, permitting expression of *Stra8*. In XY mice lacking *Cyp26b1*, the germ cells enter meiosis at the same time as those of wild-type females.[223]

Interestingly, analysis of *Cyp26b1* mutants revealed that the level of *Stra8* expression was higher in XX than XY gonads, indicating the likely presence of an additional, male-promoting pathway.[224] FGF9 signaling through FGFR2 acts independently of RA signaling to inhibit *Stra8* expression and thus prevent meiosis in the embryonic male gonad. On some genetic backgrounds, loss of *Fgf9* causes germ cell apoptosis in the XY gonad.[225] Furthermore, treatment of XX gonads with FGF9 in culture induced expression of male germ cell markers including *Nanos2*, indicating that FGF9 actively promotes a male fate in the germ cells.[224] NANOS2 itself is a meiotic repressor in male germ cells.[226] Nodal/activin signaling has recently been proposed to act as a downstream target of FGF9, mediating expression of *Nanos2*. Loss of nodal/activin signaling causes apoptosis in male germ cells, a phenotype also seen in *Nanos2* mutants. This signaling cascade also appears to act to inhibit RA signaling.[227]

The data above indicate that the formation of male versus female germ cells is an active process with antagonistic/agonistic signaling pathways converging on the same target. Thus, progenitor cells would be expected to be unbiased. However, transcriptome-wide expression analysis of germ cells has recently revealed that prior to E13.5 this population is primed towards a male fate, indicating that the process may be incompletely understood.[228] Indeed, *DMRT1*, traditionally viewed as a "male" gene, has been implicated as a direct transcriptional activator of *Stra8* in the XX gonad, contrary to its role as an inhibitor of *Stra8* in the postnatal male testis.[229]

Plasticity of Gonadal Sex

Classically, sex determination in metazoans has been viewed as a largely irreversible process. Gonadal sex is determined embryonically and phenotypic sex cements the gonadal phenotype. However, recent data have challenged this dogma.

In 2011, it was shown that XY *Dmrt1*$^{-/-}$ mice exhibit feminization of the somatic and germ cells of the gonads postnatally. In the four weeks after birth, the somatic cells of the gonad switch off *Sox9* and instead begin to express *Foxl2* and other ovary-enriched genes. The germ cells adopt an oocyte-like phenotype. Furthermore, inducible deletion of *Dmrt1* in adult males causes the same phenotypes to arise.[230] As DMRT1 is capable of binding upstream of both male and female fate-promoting genes, these data suggest a model whereby DMRT1 activates male programming while inhibiting female-promoting gene expression throughout adult life.

Foxl2 has been implicated as a primary female-determining gene, as *Foxl2* deletion causes ovarian failure.[158,159,161] Furthermore, mutation of the gene has been implicated in XX male sex reversal in goats.[231] However, *Foxl2* mutation does not cause complete sex reversal in humans or mice. Loss of function mutation in humans leads to blepharophimosis/ptosis/epicanthus inversus syndrome associated with premature ovarian failure in females.[232,233] Mice with loss-of-function mutations in *Foxl2* exhibit impaired ovarian development, including loss of the granulosa cells.[158,159,161] In these mice, female sex reversal as such is not documented during development, although testis-specific genes are upregulated in the ovary.

Although not a master regulator during embryogenesis, conditional ablation of *Foxl2* in the adult ovary can cause masculinization.[162] In these mice, testis cords form from the somatic cells of the follicles, and Leydig cells are observed that produce levels of T equivalent to that of XY littermates. These are posited to derive from non-cell autonomous lineage reprogramming of the female steroid-producing theca cells, which did not express the *Cre* driver. *Sox9* upregulation was observed soon after deletion of *Foxl2* in these mutants, suggesting that FOXL2 might repress *Sox9*. Several lines of experimentation in vitro support this hypothesis, indicating that FOXL2 negatively regulates *Sox9* by direct binding to the TESCO enhancer. FOXL2 appears to be physically and genetically interacting with estrogen receptors (ERα and β mutation), which causes similar, but less severe phenotypes in the adult ovary.[234] ERβ and ERβ also have binding sites in the TESCO, and in vivo mutation of both the FOXL2 and the ERβ binding site in the TESCO enhancer is required for complete sex reversal in the adult ovary (Figure 7.5(D)).[162]

Together, these data from *Dmrt1* and *Foxl2* mutant mice indicate that sex in mammals is much more plastic than previously thought, with maintenance of sex vital after determination and differentiation have taken place (Figure 7.8). The extent to which this is true remains to be determined; it seems likely that other genes that are involved in initial sexual differentiation, such as *Sox9* in the XY gonad and components of Wnt signaling in the XX gonad, may continue to play a role in maintaining these phenotypes in the adult. Temporal deletion

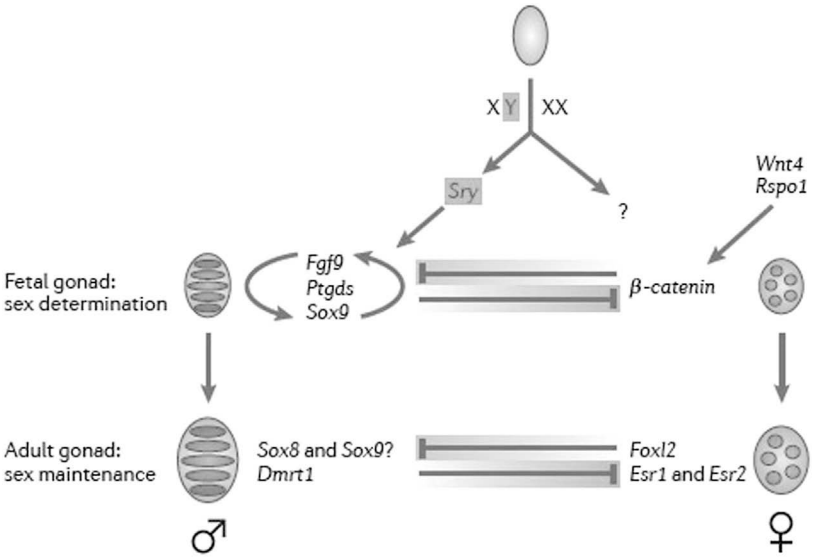


FIGURE 7.8 Initiation and maintenance of sex-specific gonadal phenotypes. Initially, a male versus female gonadal fate is dependent upon expression of *Sry* from the Y chromosome, and genes including *Sox9*, *Fgf9*, and *Pgd2* promote testis organogenesis. Male-promoting genes negatively regulate female-promoting genes including members of the Wnt pathway, and vice versa. In the adult, negative regulatory loops remain, and are now thought to involve female fate-promoting *Foxl2* and *ERα/β* genes, and male-specific *Dmrt1*. *Source: Taken from Ref. 149.*

of these genes in the mouse will be required to define the signaling cascades involved in maintenance of sex through adult life.

PHENOTYPIC SEX

Sexual Differentiation of Reproductive Organs

Experiments conducted by Jost in the 1950s on rabbit fetuses showed that male embryos that have been castrated develop as females. Jost postulated that secretions from the fetal testis induced the development of male sexual characteristics.[235] Today we know of at least three diffusible factors produced by the fetal testis with these properties: AMH, insulin-like factor 3 (Insl3), and T (Figure 7.9). As mentioned above, AMH is produced by Sertoli cells and its role is to activate the regression of the Müllerian duct, which gives rise to the oviduct, uterus, and the vagina in the female. Leydig cells produce Insl3 that, together with T, regulates the descent of the testis from a location next to the kidney to the region where the scrotum is developing.[236,237]

The development of most male-specific structures is dependent on T produced by the Leydig cells of the embryonic testis. T regulates three main aspects of male phenotypic development: the conversion of the Wolffian ducts into the epididymis, vas deferens, and seminal vesicles; the formation of the male urethra and prostate from the urogenital sinus; and the formation of the phallus and scrotum from the genital tubercle. In some of these target tissues, T is not the acting hormone but is converted to a more potent androgen, dihydrotestosterone (DHT), through the action of the enzyme 5α reductase found in these tissues. In marsupials it was found that the male-specific circulating androgen during the stage of development of male-specific organs was not DHT or T but a 3 α reduced derivative of DHT, 5 α-androstane-3α, 17β-diol (5α-adiol). However, 5α-adiol is considered a weak androgen and therefore it is thought that it converts back to DHT to induce virilization.[238]

Both T and DHT act through the androgen receptor, an X-linked member of the nuclear hormone receptor family, with DHT having approximately a 10-fold higher affinity.[239] Mutations in the androgen receptor gene and in genes encoding any of the enzymes required for DHT synthesis give rise to defects in the development of all male-specific structures in both mouse and man.[240] These defects can be highly variable depending on the nature of the mutation and the importance of the gene involved in the process. Analysis of these mutations showed that not all of male-specific structures require DHT for development as human individuals with 5α reductase deficiencies show virilization of the Wolffian duct but absence of other male-specific structures.[240] In the mouse, males lacking both isoforms of the 5α reductase enzyme showed proper internal and external genitalia, although the prostate and the seminal vesicles were smaller, showing that T is able to induce most male sexual development in this species.[241]

The development of sexual reproductive organs in the female is less dependent on diffusible factors made by the gonad. The exact role of steroids in female development has been hard to determine, because although the ovary does not produce steroids during embryogenesis, the fetus is bathed in female hormones derived from the mother. Mice lacking both ERα and ERβ develop proper

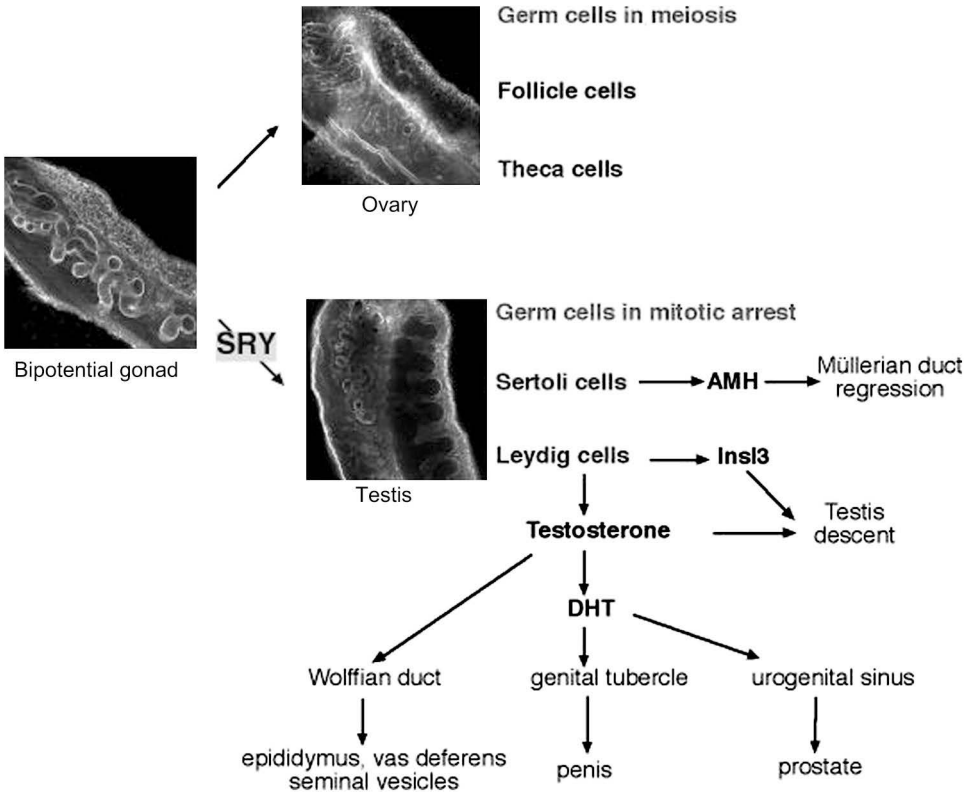


FIGURE 7.9 Sexual differentiation of the internal and external genitalia. The bipotential gonad develops into an ovary in the absence of SRY or a testis if SRY is present. Three main sexually dimorphic cell types are found in the gonad: germ cells, supporting cells (Sertoli cells and follicle cells), and steroidogenic cells (Leydig and theca cells). In the male, products from the testis, AMH, Insl3, and testosterone, induce the differentiation of the male-specific internal and external genitalia. *Source: Confocal images of laminin stained gonads were adapted from Ref. 254.*

Müllerian duct derivatives and ovary development is not affected initially. However, in adulthood these animals show uterine hypoplasia and testis-like cords within the ovary, showing that estrogen does play a role in late development of female reproductive organs.[234] As expected from Jost's postulate, female embryos that have been treated with androgens show virilization of their reproductive organs. A similar situation occurs in XX human individuals with congenital adrenal hyperplasia. These individuals carry mutations in the genes encoding enzymes in the final steps of adrenal steroid synthesis, the most common of which is a mutation in *CYP21*. This mutation causes steroid 21-hydroxylase deficiency, which results in a reduced level of corticosteroid production. This in turn activates feedback mechanisms in the pituitary, causing release of ACTH. This high level of ACTH stimulates the adrenal cortex, increasing production of steroidogenic intermediates with androgenic properties.

Sexual Differentiation of the Nervous System

Androgens have been shown to have a role in the sexual differentiation of the nervous system. Several regions of the rodent brain including the sexually dimorphic nucleus of the preoptic area (SDN-POA), the anteroventral periventricular nucleus (AVPV), and the spinal nucleus of the bulbocavernosus (SNB) show sexual differences, which are dependent on the presence or absence of androgens during fetal or neonatal stages.[242–244] In some cases it was found that androgens were not acting through the androgen receptor but were converted into estrogen in the brain by the enzyme aromatase, which was acting through the estrogen receptor. Consistent with these differences in brain structure between the sexes, behavior can be dependent on the level of androgens during fetal life. This was first demonstrated in guinea pigs, where females were exposed to T in utero and this interfered with their female pattern of reproductive behavior in adulthood.[245] Detailed descriptions of the role of steroids in different aspects of reproductive function and behavior are found in other chapters of this book.

Although many of the sex differences found in the brain are due to the effect of steroids, evidence has shown genetic contributions to these differences. Most of these differences are expected to be derived from the sex chromosomes, as these will contribute to gene differences between sexes. For example, females lack Y-linked genes; males may have lower doses of X-linked genes that escape X inactivation; and as males only inherit their X chromosome from their mothers, they will lack paternal imprinting effects. Experiments using the FCG mice were designed to uncover the effects of sex chromosomes independently of the sex of the gonad[50] (discussed in more detail in Chapter 47). Using these mice it was shown that the density of vasopressin innervation of the lateral septum was different between XX and XY animals independently of

the phenotype of the gonad. A difference was also seen in the number of dopamine neurons in cultures from embryonic mesencephalic cells.[246] Further characterization is needed to determine the importance of genetic effects in the development of brain structure and function and what effects these might have on behavior.

CONCLUSION

The study of sex determination and differentiation has provided great insight into different aspects of reproductive biology. The analysis of disorders of human sexual differentiation has been essential in the identification of genes regulating this process. In addition, the mouse has been an important model system with the advent of genetic technology such as targeted mutagenesis and transgenesis. In the last decade, the increasing use of genomics has proved a powerful tool for identification of evolutionarily conserved factors in sex determination, as well as for dissecting aspects of the process that are unique to mammals, leading to an influx of new discoveries in the field. However, many questions remain to be answered.

- New genes involved in sex determination continue to be discovered each year, yet many patients suffering from DSDs still have unknown mutations. New techniques must be employed in order to identify the full complement of genes involved in human DSDs. Improvements in genomics have already helped uncover new candidate genes, such as *Sox3*.
- It is still unclear how each lineage in the gonad is specified. Could induced pluripotent stem (iPS) cell technology be used to define the pathways involved in lineage specification? Such trials are currently underway.
- With the discovery of new genes involved in DSDs comes the challenge of characterizing their role in sex determination, and of understanding how their mutation causes disease. Generation of iPS cell lines from DSD patients could be used to study unknown mutations, or to analyze genetic modifiers that are likely partially responsible for the spectrum of DSDs seen in patients. These cell lines could also be used in drug discovery and testing. These techniques have already been used in research into heart disease and disorders of the nervous system (reviewed in Ref. 247).
- Recent data has revealed that the gonad is more plastic than previously realized. Exactly how is sex maintained in the adult gonad? Further analysis of gene function in the adult testis and ovary is likely to pinpoint more genes critical in maintaining each sex.

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CHAPTER

8

Human Steroid Biosynthesis

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INTRODUCTION

Heinrich Wieland received the Nobel Prize in Chemistry 1927 for determining the chemical structure of bile acids, and Adolf Windaus received the Nobel Prize in Chemistry 1928 for the chemical structure of cholesterol and sterols related to vitamins. Although their structures, alas, were not quite correct, this work prompted intense efforts to isolate similar compounds responsible for the biological activities of certain endocrine glands and their extracts. In the mid-1800s, Addison found that adrenal destruction was fatal in human beings, and Brown-Sequard showed that adrenalectomy was fatal in laboratory animals. Consequently, there must be a factor, which was essential for life, derived from the adrenal cortex.

Rosenheim and King determined the correct structure of the cyclopenanoperhydrophenanthrene steroid nucleus in 1932 (Figure 8.1(A)). In the ensuing years, Leopold Ruzicka and Adolf Butenandt isolated and determined the structures of estrone (E1), progesterone (P4), and androsterone, and these feats were likewise recognized with the Nobel Prize in Chemistry 1939. Their findings, which showed a diversity of hydrocarbon hormones bearing a common general structure, began to codify the field of steroid biology and to beg the question of how such a repertoire of compounds could be formed efficiently. Many compounds with the basic steroid structure were found in the adrenal gland, and epinephrine, which was the first hormone isolated from the adrenal, did not prevent death in adrenalectomized animals. Thus began the quest that ultimately led to the discovery of cortisone.

The elucidation of the structures of the first steroids inspired the work of Henry Kendall at Mayo Clinic; W.W. Swingle and J.J. Pfiffner at Princeton and later Columbia University; G.F. Cartland and M.H. Kuizenga at Upjohn; and Tadeus Reichstein in Zurich. These laboratories extracted massive amounts of animal adrenal cortex, painstakingly isolating and characterizing an alphabet soup of 29 adrenal steroids, which differed primarily on the location of a few hydroxyl and ketone groups. Once cortisone was isolated and synthesized, Phillip Hench at Mayo Clinic treated rheumatoid arthritis patients with cortisone and showed dramatic and unprecedented responses. Given the validation of cortisone (actually cortisol, formed in the liver from cortisone) as a hormone and vital factor, attention turned to its genesis.

In reviewing Reichstein's 29 compounds, one could logically propose some precursor–product relationships based on chemical thermodynamics. The more heavily oxidized or hydroxylated the compound, the lower its free energy, and the farther downstream in a pathway. The Δ^4-steroids with the enone structure are more stable than Δ^5-steroids and likely to be downstream in these pathways. Clues to the order of the adrenal pathways derived from patients with defects in specific reactions. The first such disorder was Luigi de Crecchio's description of a "man" with genital ambiguity at birth who lived as a male, but following death during a diarrheal illness of an apparent adrenal crisis, he was found to have female internal reproductive structures and enlarged adrenals at autopsy. He probably had a 46,XX karyotype and 21-hydroxylase deficiency (21OHD), the most common form of congenital adrenal hyperplasia (CAH). Joseph Jailer and Seymour Lieberman,[1] as well as Albert Bongiovanni in independent work, studied steroid production in similar patients in the 1950s. By identifying the steroids, which were unusually high or low, they correctly deduced that these patients had a defect in the steroid 21-hydroxylase enzyme, the most common human genetic enzyme deficiency. Similarly, Bongiovanni found high 11-deoxycortisol in patients

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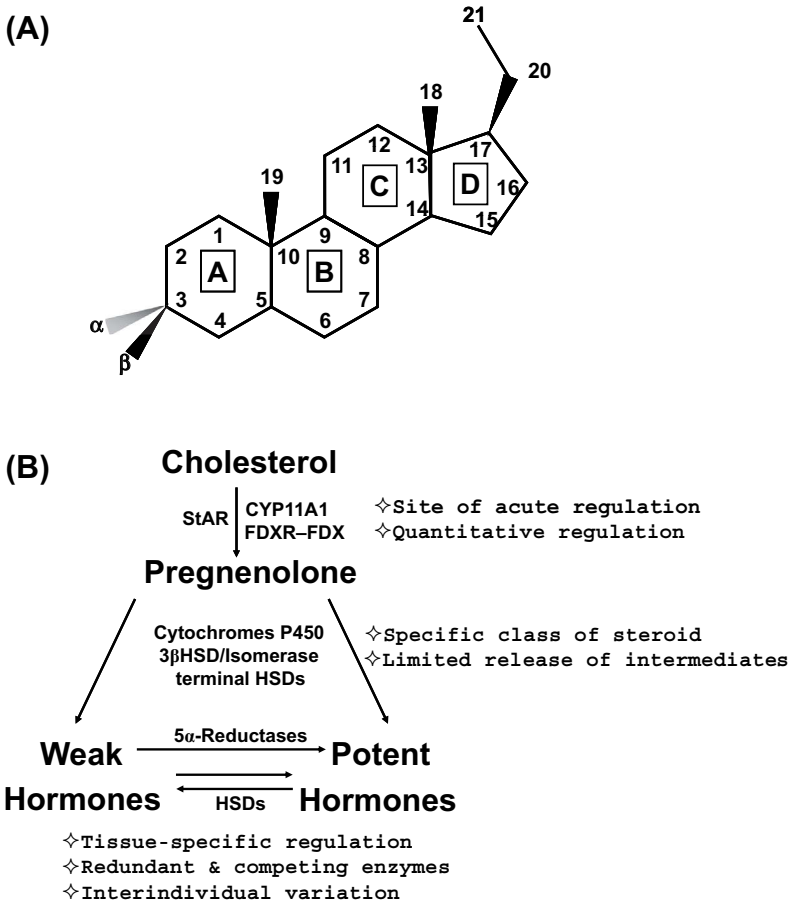

FIGURE 8.1 Conceptual model for steroidogenesis. (A) Cyclopentanoperhydrophenanthrene steroid nucleus, with boxed letters indicating rings A–D and numbers for carbon atoms 1–21. Substituents (see carbon 3) projecting in front of the page (dark wedge) are β-configuration, and those projecting behind the page (light wedge) are α-configuration. (B) The conversion of cholesterol to pregnenolone is the quantitative and acutely regulated step, involving StAR and CYP11A1. The downstream enzymes determine the major steroid products of each steroidogenic cell. The hydroxysteroid dehydrogenases dominate the terminal reactions in the steroidogenic cells and in peripheral tissues and target cells. Additional enzymes, including the sulfotransferases, 5α-reductases, and UGTs, participate in various biosynthetic and degradative processes, including enzymes not traditionally considered steroidogenic, such as CYP3A4.

with a hypertensive form of CAH and therefore ascribed this disorder to 11β-hydroxylase deficiency,[2] and he also described 3β-hydroxysteroid dehydrogenase/isomerase deficiency based on high Δ^5-steroids but low Δ^4-steroids in these patients.[3]

The formal birth of the field of biochemistry was in 1897, when Eduard Buchner showed that a yeast culture killed by grinding with sand continued to produce carbon dioxide—for which he too won a Nobel Prize in 1907. The concept of enzymes as biological catalysts was well advanced by 1950, following the purification of pepsin and urease in the 1940s. The important steroid-metabolizing enzymes, however, are mostly intrinsic membrane proteins, which dampened the interest of biochemists of that day, who were more comfortable studying soluble proteins. The first progress in the elucidation of the nature of the 21-hydroxylase enzyme came in the early 1960s, when David Cooper and Otto Rosenthal in Ron Estabrook's laboratory showed that carbon monoxide inhibited this activity in adrenal microsomes.[4] Around this time, Omura and Sato showed that the carbon monoxide–binding pigment of liver microsomes was responsible for oxidative xenobiotic metabolism, which became known as the cytochrome P450 enzymes. Radiolabeled steroids became commercially available in the 1950s, which facilitated experiments to confirm precursor–product relationships. Despite these initial advances, further progress was slow, due to low abundance of enzymes, the need to obtain animal adrenals as the source, the tedious nature of the assays, and the inability to purify the enzymes, which limited the interpretation of messy experiments.

By the mid-1960s, detergents were being used to solubilize proteins from membranes, and some enzymes retained activity despite this previously unthinkable manipulation. In 1969, Anthony Lu in Jud Coon's laboratory described the components of the P450 system: the P450 protein, the flavoprotein reductase, and phospholipid.[5] They showed that these components could be solubilized, purified, and reconstituted to restore activity with NADPH and oxygen. This work paved the way for the isolation of the steroidogenic P450 enzymes, which was still a daunting task, so investigators instead relied mainly on crude microsomal and mitochondrial preparations for many years. The DNA double helix was described in 1953, and the field of molecular genetics had developed the model that one gene encodes one enzyme by the 1960s. Consequently, by the early 1970s, the concept that 21OHD was a defect in the 21-hydroxylase enzyme due to a mutation in the 21-hydroxylase gene

was well accepted. This simple paradigm, however, does not account for several bizarre features of human steroidogenesis, which took many years to resolve, including:

1. discrepancies between activities and pathways in animal models and human beings;
2. multiple activities of one enzyme;
3. multiple enzymes catalyzing the same reactions;
4. similar enzymes with different directional preferences in intact cells;
5. activity regulation via cofactor proteins;
6. how a mutation in one protein can affect several enzymes;
7. how a single biochemical phenotype can result from mutations in three different proteins; and
8. the difference in clinical phenotype between severe and partial deficiency states.

With this framework, one of the seminal advances in steroidogenesis came at the end of the 1970s when Shizuo Nakajin and Peter Hall showed that the 17-hydroxylase and 17,20-lyase activities required for androgen biosynthesis co-purified from neonatal pig testis.[6] The reconstitution of 17-hydroxylase activity required phospholipid and flavoprotein reductase, while Makodo Onoda with Hall showed soon afterwards that restoration of maximal 17,20-lyase activity also required addition of cytochrome b_5.[7] As the field of molecular biology was developing during this time, purification and partial sequencing of porcine 17-hydroxylase/17,20-lyase led to cloning of partial and then complete cDNAs. Mario Zuber and Michael Waterman showed the expression of a single cDNA in non-steroidogenic COS-1 cells conferred both 17-hydroxylase and 17,20-lyase activities,[8] validating beyond any doubt the biochemical studies from Hall's laboratory. The cloning of other cDNAs and genes for the steroid hydroxylases followed (for review, see Ref. 9).

Ferdinand Labrie's group in Canada, including Van Luu-The, Jacques Simard, and many other talented investigators, spent over a decade during the 1980s and 1990s characterizing the molecular genetics and biochemistry of the multiple hydroxysteroid and dehydrogenases,[10,11] which turned out to be far more complex than anyone imagined (at least 15 17β-hydroxysteroid dehydrogenases have been identified to date). The first crystal structures of steroidogenic enzymes were of hydroxysteroid dehydrogenases (HSDs) and were solved in the early 1990s. Deb Ghosh and Bill Duax solved the structure of a bacterial 3α,20β-hydroxysteroid dehydrogenase[12] and with Shin Lin the structure of human placental estrogenic 17β-hydroxysteroid dehydrogenase.[13] Trevor Penning and Mitch Lewis solved the structure of rat liver 3α-hydroxysteroid dehydrogenase,[14] which revealed a second type of structural fold for these enzymes, the β-barrel. The structures of the steroid hydroxylases and other important proteins are still under study.

With the identification of the genes encoding different enzymes and ancillary proteins, diseases could be assigned to specific gene mutations, such as 21OHD, 5α-reductase type 2 deficiency, steroidogenic acute regulatory protein (StAR) deficiency, which is called lipoid CAH, and most recently cytochrome P450-oxidoreductase (POR) deficiency, which was reported in 2004.[15,16] Our understanding of pathways and key enzymes has led to the development of targeted therapies, such as aromatase inhibitors in breast cancer and 17-hydroxylase/17,20-lyase inhibitors in prostate cancer. This chapter will explore the basic principles and intricacies of human steroidogenesis, which is a fundamental and indispensable component of reproductive physiology. Additional details and references are found in recent comprehensive reviews of steroidogenesis[17] and its history.[18]

GENERAL CONCEPTS

Cholesterol is the 27-carbon precursor of all steroids, which share the common four-ring perhydrocyclopentanophenanthrene skeleton with sterols. The first committed step in steroidogenesis is the cleavage of cholesterol to 21-carbon pregnenolone, the common precursor of all other steroids. The capacity to convert significant amounts of cholesterol to pregnenolone is restricted to the cells of the adrenal cortex, the testicular Leydig cells, the ovarian theca and granulosa cells, and placental trophoblast cells. Some other cells might also produce enough pregnenolone for autocrine generation of specific steroid hormones acting on that cell. The first step is also the site of acute regulation and under most circumstances the rate-limiting step, thus determining quantitatively how much steroids are made. In contrast, the downstream enzymes determine the characteristic products of a given cell, or which steroids are made (Figure 8.1(B)).

Steroidogenic enzymes and other key proteins can be heuristically divided into three basic categories (Table 8.1). The first category contains the cytochromes P450, which are the steroid hydroxylases. In human beings, three P450s are mitochondrial and three are located in the smooth endoplasmic reticulum (microsomal). The mitochondrial enzymes use as electron transfer proteins the flavoprotein ferredoxin reductase (FDXR) and the iron–sulfur protein ferredoxin (FDX1), while the microsomal enzymes use only the flavoprotein POR, which contains both a flavin adenine dinucleotide (FAD) and a flavin mononucleotide (FMN). In addition, CYP17A1 (P450c17, steroid 17-hydroxylase/17,20-lyase) employs cytochrome b_5 (CYB5A) for the 17,20-lyase reaction. The second

TABLE 8.1 Classes of Human Steroidogenic Enzymes and Other Proteins

1. Cytochromes P450
   a. Mitochondrial: CYP11A1, CYP11B1, CYP11B2
   b. Microsomal: CYP17A1, CYP21A2, CYP19A1
   c. Redox proteins: FDX1, FDXR, POR, CYB5A
2. Oxidoreductases
   a. Hydroxysteroid dehydrogenases: 17βHSDs, 11βHSDs, 3βHSDs, 3αHSDs
      – Short-chain dehydrogenase/reductases
      – Aldo-keto reductases, includes 5β-reductase
   b. 5α-Reductases
3. Conjugating enzymes and transport proteins
   a. Steroidogenic acute regulatory protein
   b. Sulfotransferases and sulfatases
   c. Uridyldiphosphate glucuronyltransferases

category, the oxidoreductases, is dominated by the HSDs, named for the carbon atoms for which their activity is best characterized and the order of their discovery. The HSDs are structurally grouped as short-chain dehydrogenase/reductases (SDRs), with a β-α-β weave,[12,13] or as aldo-keto reductases (AKR), with a β-barrel or triosephosphate isomerase (TIM-barrel) fold.[14] Steroid 5β-reductase (AKR1D1), which also participates in bile acid synthesis, is an AKR enzyme,[19] while the 5α-reductases are a unique subgroup of oxidoreductases. I have arbitrarily grouped all of the other key proteins in the third category, including the conjugating enzymes and StAR, which mobilizes cholesterol into the steroidogenic pathways.

Most steroid-metabolizing enzymes have K_m values near 1 μM, which is much higher than the circulating concentrations of most steroids. Consequently, the high intracellular concentration of upstream steroids generated in these specialized cells is critical for the completion of steroid synthesis. One corollary of this principle is that negligible amounts of circulating steroids enter the adrenal cortex and gonads for further metabolism, but large tissues such as the placenta, liver, and skin have greater capacity to metabolize steroids present in the circulation in low concentrations. In addition, target tissues might further metabolize circulating steroid to more potent or less active products. Testosterone (T) 5α-reduction in prostate to 5α-dihydrotestosterone (DHT) is a classic example of intracellular hormone amplification via an enzymatic conversion.

Most of the steps in steroidogenesis are irreversible transformations, including the cytochrome P450-catalyzed oxygenations, the 3βHSD/$\Delta^5 \rightarrow \Delta^4$-isomerase (3βHSD) reactions, and the 5α- and 5β-reductase reactions proceed only in one direction. The 3α-, 3β-, 11β-, 17β-, and 20αHSD reactions are reversible both in vitro and in vivo, although these reactions have a strong directional preference in intact cells.[20,21] A composite pathway is shown in Figure 8.2; however, this complete repertoire of enzymes is never present in any one cell. Instead, individual pathways leading to specific products are optimized within individual steroidogenic cell types. These specific pathways will be developed in subsequent sections.

THE CONVERSION OF CHOLESTEROL TO PREGNENOLONE AND MITOCHONDRIAL STEROID METABOLISM

In addition to being the step subjected to acute regulation, the conversion of cholesterol to pregnenolone is ordinarily the rate-limiting step. The cholesterol side-chain cleavage enzyme (P450scc, CYP11A1) catalyzes the three oxygenations required for this transformation in the mitochondria. CYP11A1 resides on the inner mitochondrial membrane (IMM) with FDXR, while FDX1 is a soluble protein in the mitochondrial matrix. The outer mitochondrial membrane (OMM) contains a pool of cholesterol, which is available for steroidogenesis within the specialized mitochondria of these cells. The access of CYP11A1 to this cholesterol pool requires transfer across the intercisternal space. The StAR protein mediates this transfer of cholesterol from the OMM to the IMM (Figure 8.3), which enables the acute regulation of steroid production within minutes of the tropic stimulus.[22] In most cells of the adrenal cortex and gonads, cyclic AMP (cAMP) is the second messenger responsible for StAR activation. In the zona glomerulosa (ZG) cells, the stimulus is intracellular calcium; in the placenta, steroidogenesis is StAR independent. The rate of StAR-stimulated steroidogenesis is about sevenfold above the StAR-independent rate.[23]

The episodic activation of cholesterol transfer via StAR accounts for the pulsatile secretion of testosterone by Leydig cells following each pulse of luteinizing hormone (LH) and the rapid increase in cortisol following a stress-induced adrenocorticotropin (ACTH) rise or during a cosyntropin (synthetic ACTH[1–24]) stimulation test. The acute rise in the end-product steroids rather than pregnenolone demonstrates that the downstream enzymes are present already and that these subsequent transformations are more rapid than pregnenolone generation.

The mechanism of StAR action has been studied extensively, and several features are now known. StAR expression increases acutely with the rise in cAMP, but the augmented expression is not fast enough to account for its action. StAR phosphorylation is probably the most rapid event and might initiate the action of preformed StAR,[24] which nascent StAR then amplifies. StAR is imported into the mitochondrion and degraded, which

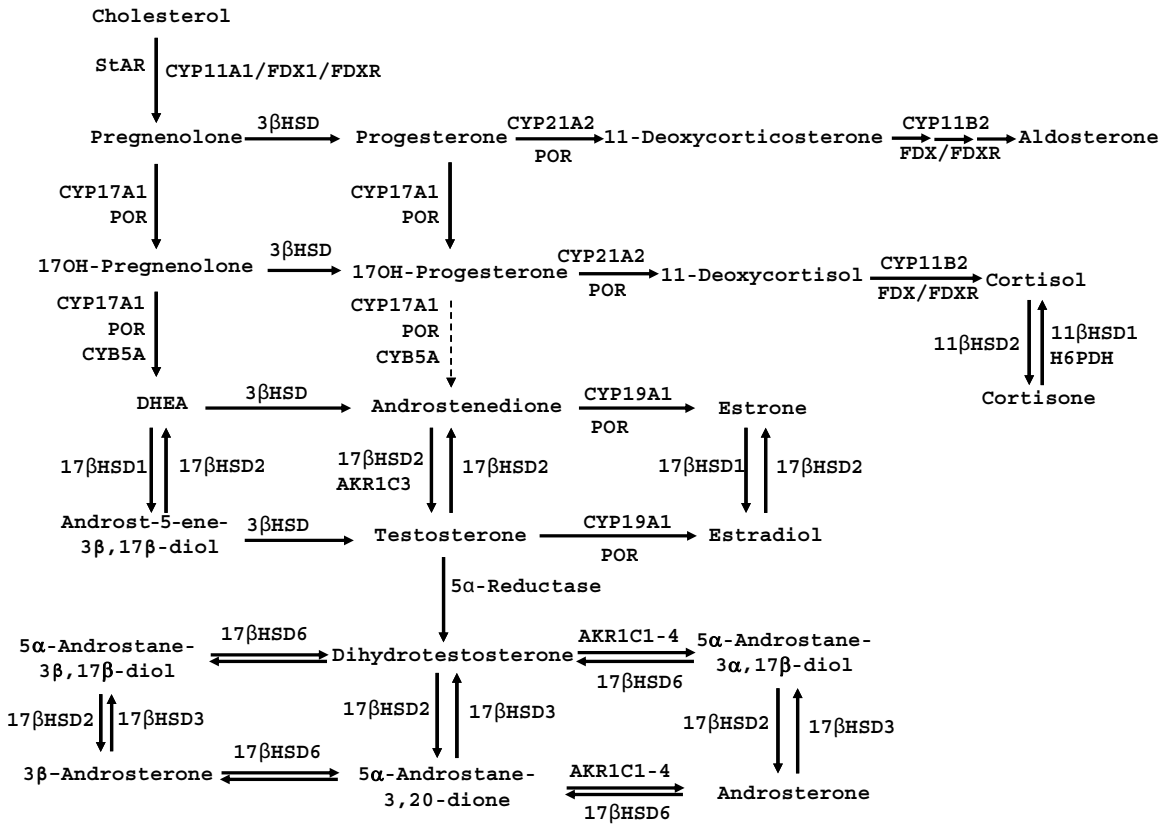

FIGURE 8.2 **A canonical, composite steroidogenic pathway.** In this rendering, progesterone and aldosterone pathways are at the top, cortisol synthesis is in the second row, and androgen and estrogens are in the lower half. The major enzyme is shown with each arrow, although additional enzymes might catalyze individual reactions. The dashed arrow indicates minor pathway.

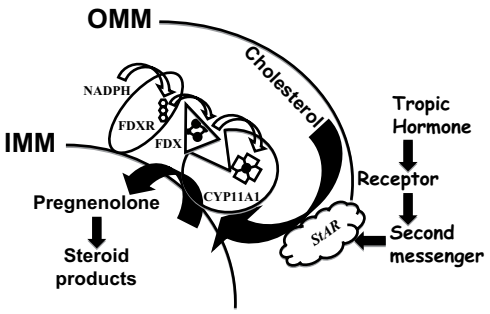

FIGURE 8.3 **The conversion of cholesterol to pregnenolone.** The StAR protein (cloud-shaped image) is required to transfer cholesterol in the outer mitochondrial membrane (OMM) to the inner mitochondrial membrane (IMM), where CYP11A1 resides. Electrons flow (open block arrows) from NADPH to the flavin (three fused hexagons) of FDXR (oval) to the iron–sulfur cluster (rhombus with filled circles at vertices) of FDX1 (triangle) to heme (Maltese cross with central filled circle, representing iron atom) of CYP11A1 (circle with wedge-shaped indentation), to support catalysis.

terminates its action. Considerable evidence suggests that StAR acts at the OMM and that its residence time is proportionate to its cholesterol transfer activity.[25] The structure of the START domain of MLN64, a StAR homolog, contains a molecule of bound cholesterol in a pocket lined with β-sheets,[26] which suggests that StAR binds and transports cholesterol to the IMM and CYP11A1. StAR appears to function catalytically, transporting many molecules of cholesterol per molecule of StAR. The exact route of cholesterol movement and the kinetic details of StAR action, as well as other participating proteins, are incompletely understood.

The side-chain cleavage reaction is somewhat more complex than the typical mitochondrial cytochrome P450 oxygenation in that it is actually three consecutive reactions, which ultimately yields isocaprioaldehyde and pregnenolone. During each catalytic cycle, one molecule of both NADPH and oxygen are consumed. The electrons from NADPH pass first to FDXR and then to FDX1 before flowing one at a time to CYP11A1 according to the canonical P450 reaction cycle (Figure 8.4). In the X-ray crystal structure of the CYP11A1 complex with FDX1,[27] cholesterol binds at about a 45° angle with the heme ring. The side chain is extended across the heme near the steroid D-ring. This structure is consistent with order of reactions, first the formation of 22(*R*)-hydroxycholesterol, then 20(*R*),22(*R*)-dihydroxycholesterol, and finally the pregnenolone product. Only a fraction of the hydroxycholesterol intermediates accumulate during turnover, suggesting that they rarely dissociate from

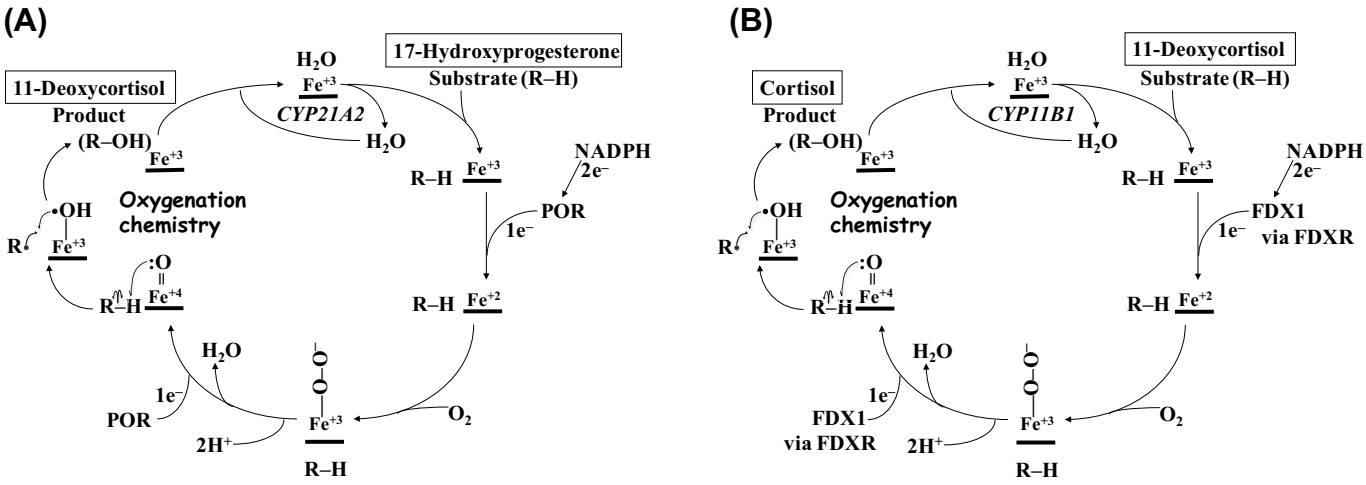

FIGURE 8.4 The cytochrome P450 reaction cycle. Examples shown here are the final two steps in the biosynthesis of cortisol. (A) CYP21A2-catalyzed 21-hydroxylation of 17-hydroxyprogesterone (substrate in box, "R–H") to 11-deoxycortisol (product in box, "R–OH"), a microsomal P450 reaction. (B) CYP11B1-catalyzed 11β-hydroxylation of 11-deoxycortisol (substrate in box, "R–H") to cortisol (product in box, "R–OH"), a mitochondrial P450 reaction. The two reaction cycles are identical except for the electron transfer proteins, which are POR for CYP21A2 and FDX1 (which receives electrons from FDXR) for CYP11B1. In each case, the heme is shown as a thick, short horizontal line with Fe above. In the resting state, the sixth ligand of the ferric iron (Fe^{+3}) is water. Substrate binding (R–H) displaces this water and lowers the redox potential of the heme, which favors one-electron reduction via POR or FDX1. In the ferrous (Fe^{+2}) state, the iron binds molecular oxygen avidly, and the oxyferrous P450 receives a second electron. Protonation of the iron-peroxide allows cleavage of the O–O bond, releasing water, and generating the reactive iron-oxide species with radical or odd-electron character. Electrons are shown as dots (i.e., R·, :O), and single-barbed arrows or "fishhooks" show single-electron movements, such as cleavage of R–H bond to form R· and ·H. The reactive heme-oxygen species performs a hydrogen atom abstraction, removing H· from substrate and forming R· and an iron-bound hydroxide radical (Fe–·OH). The final step of the hydroxylation chemistry is radical recombination, in which R· reacts with ·OH to form R–OH, the hydroxylated product, 11-deoxycortisol or cortisol, respectively. The mechanism is the same for all steroid hydroxylation reactions. The mechanisms of the carbon–carbon bond cleavage reactions, which CYP11A1, CYP17A1, and CYP19A1 all catalyze, probably share some similarities with the normal reaction cycle but must also account for the unique products and transformations.

the enzyme before subsequent reactions. This phenomenon is used experimentally to estimate P450scc activity, because the hydroxycholesterols bypass StAR and are directly converted to pregnenolone.[23]

A similar process occurs via one cycle for the CYP11B1-catalyzed 11β-hydroxylase reaction and three times for the CYP11B2-catalyzed aldosterone synthase sequence, one oxygenation at C-11 and two at C-18. These other mitochondrial P450s are required for cortisol and aldosterone synthesis and are expressed almost exclusively in the zona fasciculata (ZF) and ZG of the adrenal cortex, respectively. The cognate genes of these enzymes, which share 93% amino acid identity, are located in tandem on chromosome 8q (Table 8.2). Their proximity and similar activities explains the origin of the disease familial hyperaldosteronism type 1 (FHA1), also known as glucocorticoid-remediable aldosteronism. An unequal crossing over links the ACTH-responsive regulatory elements of CYP11B1 with a hybrid enzyme bearing aldosterone synthase activity.[28]

Both CYP11B1 and CYP11B2 enzymes catalyze the 11β-hydroxylation of 11-deoxycorticosterone (DOC) to corticosterone (B), and CYP11B1 has a small amount of 18-oxygenase activity. The X-ray crystal structure of human CYP11B2 with bound DOC illustrates the proximity of the hydrogen atoms at C-11 and C-18 to the heme iron.[29] Biochemical assays with purified proteins suggest that the intermediate 18-hydroxycorticosterone (18OHB) is a poor substrate for CYP11B2, whereas some DOC and B substrates are converted to aldosterone. The explanation for these data is that all the aldosterone product of CYP11B2 derives from 18OHB, which has not dissociated from the active site prior to the final 18-oxidation reaction.[29] These observations might explain why circulating concentrations of 18OHB exceed those of aldosterone by at least a factor of two under most circumstances.

STEROID HYDROXYLATION AND THE MICROSOMAL P450 ENZYMES

Nascent 21-carbon pregnenolone in a steroidogenic cell has only two primary fates (Figure 8.2). The first route is conversion to progesterone (P4) via 3β-HSD. This pathway limits flux to P4, as occurs in the corpus luteum or placenta, and to mineralocorticoids and glucocorticoids, with low flux to androgens and estrogens. The other route is 17-hydroxylation via CYP17A1 to 17-hydroxypregnenolone (17Preg), a cortisol precursor, and if CYB5A is present, the 17,20-lyase activity of CYP17A1 produces the 19-carbon steroid dehydroepiandrosterone (DHEA) from 17Preg. Viewed in this manner,

TABLE 8.2 Properties of Human Steroidogenic Enzymes and Other Proteins

Protein	Gene Size (kb)	mRNA Size (kb)	Chromosome Locus	Molecular	
				Exons	Weight (kDa)
StAR	8	1.6	8p11.2	8	32
CYP11A1	30	2.0	15q23–q24	9	60
CYP11B1	9.5	4.2	8q21–22	9	58
CYP11B2	9.5	4.2	8q21–q22	9	58
CYP17A1	6.6	1.9	10q24.3	8	57
CYP21A2	3.4	2.0	6p21.1	10	56
CYP19A1	130	1.5–4.5	15q21.1	10	58
3βHSD1	8	1.7	1p13.1	4	42
3βHSD2	8	1.7	1p13.1	4	42
11βHSD1	7	1.6	1q32–q41	6	32
11βHSD2	6.2	1.6	16q22	5	44
17βHSD1	3.3	1.4–2.4	17q11–q21	6	35
17βHSD2	63	1.5	16q24.1–q24.2	5	43
17βHSD3	67	1.2	9q22	11	35
17βHSD6	24.5	1.6	12q13	5	36
AKR1C1	14.3	1.2	10p14–p15	9	37
AKR1C2	13.8	1.3	10p14–p15	9	37
AKR1C3	13.0	1.2	10p14–p15	9	37
AKR1C4	22.1	1.2	10p14–p15	9	37
5α-Reductase 1	36	2.4	5p15	5	29
5α-Reductase 2	56	2.4	2p23	5	28
SULT2A1	17	2.0	19q13.3	6	34
PAPSS2	85	3.9	10q24	13	70
POR	69	2.5	7q11.2	16	77
FDX1	35	1.0–3.2	11q22	5	19
FDXR	11	2.0	17q24–q25	12	54
CYB5A	32	0.9	18q23	5	15
H6PDH	36.5	9.1	1p36	5	89

the presence of CYP17A1 activities regulates the classes of steroids made in a given cell.

CYP17A1 is a typical microsomal P450 of approximate molecular mass 57 kDa. Its catalytic cycle involves one molecule of NADPH and oxygen for each turnover, similar to the mitochondrial P450s, but rather than FDXR and FDX1, it is POR that funnels electrons from NADPH to the microsomal P450s. The human *CYP17A1* gene is expressed in both the adrenal cortex and gonads, yielding the same protein in both glands.[30] Human CYP17A1 performs the 17-hydroxylase reaction well with both pregnenolone and P4 substrates, as well as the 5α-reduced homologs 5α-dihydroprogesterone and allopregnanolone.[31] With P4 substrate, 20–25% of the product is 16α-hydroxylated,[32] due to the presence of alanine at residue 105,[33] and a trace of 21-hydroxylation also occurs, yielding DOC.[34] Unlike bacterial P450 enzymes, which are much faster catalysts, C–H bond breaking is substantially rate limiting for the overall hydroxylation reactions.[34]

The 17,20-lyase reaction is not as robust or promiscuous as the CYP17A1-catalyzed hydroxylations. Human CYP17A1 performs the 17,20-lyase reaction about 50 times more efficiently with 17Preg than with 17-hydroxyprogesterone (17OHP), favoring the Δ^5-pathway to 19-carbon steroid formation (Figure 8.2),[35,36] and 17-hydroxyallopregnanolone (5α-pregnan-3α,17α-diol-20-one, Pdiol) is the best substrate yet identified.[31] The discovery of mutations in CYP17A1[37,38] or POR,[39] which minimally impair the 17-hydroxylase activity but markedly impair the 17,20-lyase activity and lead clinically to isolated 17,20-lyase deficiency, illustrates the vulnerability of the latter activity to subtle changes in enzyme structure. In reconstituted systems, the 17,20-lyase activity is very sensitive to phospholipid composition,[40] and phosphorylation also regulates this activity.[41,42] Most significantly, the presence of CYB5A stimulates the 17,20-lyase reaction 10-fold with 17Preg and 17OHP[35] and 3-fold with Pdiol.[31] This phenomenon, which was first demonstrated in recombinant systems in vitro,[43] has been substantiated with the discovery of patients having isolated 17,20-lyase deficiency due to mutations in CYB5A.[44,45] Although CYB5A functions as an alternate electron donor for the second electron transfer of some P450-catalyzed reactions, apo-CYB5A lacking the heme redox center stimulates the 17,20-lyase of human CYP17A1 as well as holo-CYB5A, suggesting an allosteric mechanism of activation.[35] The CYB5A mutation E48G+E49G retains normal electron transfer kinetics but fails to stimulate 17,20-lyase activity,[46] suggesting that residues E48 and E49 of CYB5A are essential for this stimulation and supporting an allosteric mechanism. In the presence of CYB5A, a small amount of pregnenolone and allopregnanolone are directly cleaved to Δ^{16}-19-carbon products without the intermediacy of the 17-hydroxylation products.[47]

The most similar steroidogenic enzyme to CYP17A1 is CYP21A2, steroid 21-hydroxylase. The two enzymes share 29% amino acid identity and up to 50% identity near the C-terminus, very similar hydropathy profiles, identical gene structures, and common substrates, yet their catalytic strategies and functions in human physiology are nearly

orthogonal. CYP21A2 hydroxylates the electron-deficient 21-methyl group of 17OHP and P4 (Figure 8.2). With P4 substrate, a trace of 16α-hydroxylation occurs,[34] and mutations to progressively reduce the bulk of V359 (V359A, V359G) increase the fraction of 16α-hydroxylation.[48] As with CYP17A1, C–H bond breaking is also partially rate determining for CYP21A2.[34]

Although its enzymology is more limited than CYP17A1, the molecular genetics of CYP21A2 are unique, which makes 21OHD the most common form of CAH. The classic or severe form of 21OHD occurs in 1:16,000 live births worldwide,[49] while the nonclassic or partial form is 10 times more common or even more prevalent in some populations. The reason for the high prevalence of 21OHD is the juxtaposition of the *CYP21A2* gene in a duplicated locus with the *CYP21A1P* pseudogene.[50] The vast majority of 21OHD cases arise from the process of gene conversion, in which all or part of the *CYP21A1P* pseudogene replaces the corresponding region of the *CYP21A2* gene.

The final microsomal P450 of steroid biosynthesis is CYP19A1, commonly named aromatase (P450aro) because it forms the aromatic A-ring of 18-carbon estrogens from 19-carbon androgens (Figure 8.2). CYP19A1, like CYP11A1, performs three cycles of oxygenation, ultimately releasing the C-19 methyl group as formic acid with the estrogen product. Unlike the restricted expression of CYP21A2 to the adrenal cortex and of CYP17A1 to the adrenal cortex and gonads, CYP19A1 is expressed in biologically significant amounts in many cells and tissues.[51] The enormous 130 kb *CYP19A1* gene contains at least five distinct promoters, which orchestrate the diverse regulation of its expression in tissues such as brain, bone, adipose, placenta, and ovary.

The X-ray crystal structures of modified human CYP17A1, human CYP19A1, and bovine CYP21A2 have all been recently solved and described. For CYP19A1, androstenedione (AD) fits tightly in the pocket above the heme ring with the A-ring and C-19 methyl group closest the iron center.[52] The positioning of the steroid is only slightly different than that of DOC in the CYP11B2 structure,[29] as the chemistry is shifted along the steroid by two rings, about 4 Å. This similarity explains why isomers of fadrozole bind to and inhibit both enzymes. If AD is removed from the structure of CYP19A1, clinically used aromatase inhibitors such as anastrozole and letrozole do not fit in the pocket where steroid was found. This observation suggests that the enzyme retains considerable flexibility and that the active site undergoes substantial conformational changes depending on ligand. The crystal structure of CYP17A1[53] was obtained on a modified CYP17A1 protein in a complex with abiraterone acetate, a steroidal azole inhibitor used in the treatment of prostate cancer,[54,55] which tightly binds to the iron of the heme. The steroid nucleus drapes along the I-helix, which contains residues critical for catalysis. Although a structure with bound pregnenolone has not been published, a pregnenolone molecule fits in the space occupied by abiraterone, and this orientation places the 17α-hydrogen atom near the heme iron.[53] The structure of bovine CYP21A2 contains two molecules of bound 17OHP.[56] One molecule is bound distant from the heme and is unlikely to be the molecule undergoing hydroxylation, but a structural or allosteric function is possible. The second 17OHP molecule is likely the catalytically relevant steroid. The steroid nucleus of this 17OHP molecule is positioned nearly orthogonal to the heme ring with the A-ring farthest away and the 21-methyl group dangling above the iron center. This orientation restricts chemistry only to the 21-position, as other more reactive hydrogen atoms are too far away to undergo hydroxylation without expansion of the active site.[48]

HSDs, THE TERMINAL STEPS, AND PERIPHERAL METABOLISM

The HSDs catalyze the terminal steps of steroidogenesis and dominate steroid metabolism in peripheral tissues and target organs. For each conversion, a pair of enzymes drives steroid flux in one direction or the other, with one enzyme favoring hydroxysteroid oxidation to ketosteroid and the other favoring ketosteroid reduction to hydroxysteroid. Often, more than one enzyme catalyzes the same reaction, such as AD reduction to T via 17βHSD3 and AKR1C3 and estradiol (E2) oxidation to E1 via 17βHSD2 and 17βHSD10. Some HSDs catalyze reactions at more than one site on steroids, but all of these enzymes have a strong directional preference in intact cells.

All of the HSDs catalyze both oxidation and reduction reactions in vitro, depending on the pH and nicotinamide cofactor(s) present, but in intact cells, each enzyme has a strong directional preference. In cell culture models, the reactions reach a steady-state or pseudoequilibrium, in which individual steroid molecules continue to undergo oxidation and reduction at the same rate, without any net flux of steroids.[20,21] The main reason for this discrepancy between reactions in intact cells and in vitro is that the nicotinamide cofactor (NAD[P][H]) abundance and usage determines the directional preference in intact cells.[57] In normal physiology, steroid concentrations are in the nanomolar to micromolar range, whereas the nicotinamide cofactors inside cells are at millimolar concentrations. In the cytoplasm of respiring cells with a normal glucose supply, [NADPH] exceeds [$NADP^+$] by three orders of magnitude, because cells regenerate NADPH via glucose-6-phosphate dehydrogenases and isocitrate dehydrogenases, subsequently using NADPH as a common source of electrons for various intracellular

reductions. Conversely, [NAD^+] exceeds [NADH] by a similar factor, because NADH provides reducing equivalents to molecular oxygen via the mitochondrial electron transport chain during oxidative phosphorylation. Consequently, HSDs that use NADP(H) favor ketosteroid reduction by NADPH via mass action, and HSDs that use NAD(H) favor hydroxysteroid oxidation with abundant NAD^+ cofactor.

For both SDR and AKR enzymes, a single residue provides the major structural feature favoring NADP(H) usage. In the crystal structure of 17βHSD1,[13] amino acid R38 forms a salt bridge with the 2′-phosphate, which distinguishes NADP(H) from NAD(H). Mutation of this R38 to G or D neutralizes and reverses, respectively, the positive charge of this residue and alters in parallel the directional preference for E1 reduction to E2 in intact cells.[58] The affinity of wild-type 17βHSD1 for NADP(H) is less than a factor of 10 stronger than for NAD(H), and these R38 mutations alter this preference only slightly, yet markedly alter steroid distributions in the steady-state.[58] Similarly, AKR1C9 has much greater affinity for NADP(H) than for NAD(H) compared to 17βHSD1, and this preference is driven by R276, which forms a salt bridge with the 2′-phosphate of NADP(H)[14] and is important for cofactor binding.[59] Mutation R276G changes the directional preference little, while introduction of a negative charge in mutation R276E reverses the directional preference to oxidation in intact cells.[60]

A similar attenuation and reversal of directional preference occurs when cells are depleted of NADPH. Glucose deprivation with 2-deoxyglucose in glucose-free medium, which lowers but does not eliminate NADPH regeneration, reduces the directional preference for cells expressing 17βHSD1[58] but not AKR1C9. Addition of methylene blue, which severely depletes cells of NADPH, reverses steroid flux from ketosteroid reduction to hydroxysteroid oxidation for both 17βHSD1 and AKR1C9.[60] Intracellular compartmentalization is another mechanism to favor hydroxysteroid oxidation. The lumen of the endoplasmic reticulum is strongly oxidative, favoring disulfide bond formation and other oxidation reactions, and the catalytic domains of some HSDs with oxidative directional preference such as 17βHSD2 are found in this environment.[61] The exception is 11βHSD1, which favors cortisone (E) reduction to cortisol (F) in this oxidizing compartment.[62] The mechanism of this paradox is via hexose-6-phosphate dehydrogenase (H6PDH), which regenerates NADPH within the endoplasmic reticulum lumen and supplies NADPH to 11βHSD1.[63,64]

In human physiology, the SDR isoenzymes are all membrane bound, the most active HSDs, and the forms associated with disease when deficient. The tissue-specific expression of these isoforms also parallels the sites of synthesis or inactivation known to be important endocrinologically. Mutations in the genes encoding 11βHSD1[65] or H6PDH,[66,67] 11βHSD2,[68] and 17βHSD3[69] all cause disease, whereas disease-causing mutations in 17βHSD2 and 17βHSD6 have not been described. Combined mutations in AKR1C2 and AKR1C4, however, have been found in two kindred with 46,XY disorder of sex development (DSD).[70] The catalytic efficiencies of the SDR enzymes are much higher than for the AKR enzymes, because the rates of both catalysis and product release from the AKR1C1-4 isoenzymes is very slow,[71] even compared to the rat homolog AKR1C9.[72]

One important HSD activity upstream of several of the steroid hydroxylases in most pathways is the 3βHSD reaction, which is coupled to the $\Delta^5 \rightarrow \Delta^4$-isomerase activity. There are two human 3βHSD isoenzymes[10]: the type 2 enzyme, which is expressed in the adrenal cortex, testis, and ovary—and is defective in 3βHSD deficiency leading to DSD in both males and females[73]; and the type 1 enzyme, which is expressed in the placenta as well as peripheral tissues such as liver and skin. The 3βHSD enzymes belong to the SDR family and share 94% amino acid identity.

The K_m of 3βHSD2 for the composite reaction is 1–5 μM using its three major substrates: pregnenolone, 17Preg, and DHEA.[74] When the dehydrogenase and isomerase activities are assayed individually using proteins engineered to be soluble, the K_m values of 3βHSD1 for the dehydrogenase reaction with these substrates is lower than for 3βHSD2 by an order of magnitude,[75] suggesting that 3βHSD1 is a high affinity/low capacity enzyme suitable for peripheral conversion, where steroid concentrations are not as high as in the adrenal cells. The 3βHSD1 isoenzyme also has higher affinity for inhibitors such as trilostane than 3βHSD2. The isomerase activity is linked to the dehydrogenase activity, in that the bound nascent NADH activates this second activity of the enzymes. The second, peripheral 3βHSD1 isoenzyme is responsible for the metabolism of orally ingested DHEA to T and explains why girls with 3βHSD2 deficiency have androgen excess in the face of cortisol deficiency.

STEROID SULFONATION

Steroids containing one or more free hydroxyl groups are subject to conjugation reactions, yielding steroid conjugates. One important reaction in the adrenal cortex is steroid sulfonation, which the enzyme SULT2A1 catalyzes. Over 99% of DHEA in the circulation exists as the sulfate conjugate (DHEAS), which is the major product of the zona reticularis (ZR). This sulfotransferase activity is also abundant in liver, which explains why orally administered DHEA is rapidly converted almost completely to DHEAS.[76] Sulfonation increases protein binding and prolongs the half-life of steroids; consequently,

the circulating concentration of DHEAS has little diurnal variation unlike free DHEA. The majority of the other Δ^5-steroids, such as pregnenolone, also circulate as their sulfates, although these conjugates are not normally measured in clinical laboratories. Another consequence of sulfonation is that it blocks conversion to the corresponding Δ^4-steroid. DHEA is metabolized to androgens in peripheral tissues, but DHEAS requires desulfation to DHEA first. This sulfatase activity has been described but appears to be low in human beings, or at least the steady-state balance for the two opposing reactions far favors the sulfonated form.[77]

The co-substrate of the SULT2A1 reaction is phosphoadenine phosphosulfate (PAPS), the common sulfate donor of biochemical reactions. PAPS is synthesized from two molecules of adenosine triphosphate (ATP) and sulfate in two steps. In many species, ATP sulfurylase converts one molecule of both ATP and sulfate to adenine phosphosulfate (APS) and pyrophosphate, and APS kinase completes the synthesis of PAPS. In human beings, two isoforms of one enzyme, phosphoadenine phosphosulfate synthetase (PAPSS), catalyze both reactions. PAPSS2 is the major isoform in the adrenal gland and is responsible for PAPS generation for SULT2A1 in the adrenal cortex. Incomplete deficiency of PAPSS2 causes a form of premature adrenarche—the appearance of axillary and pubic hair—and childhood androgen excess,[78] because impaired DHEA sulfonation causes enhanced conversion of DHEA to androgens. Complete PAPSS2 deficiency has been described in kindred afflicted with dwarfism and skeletal dysplasia called spondyloepimetaphyseal dysplasia, Pakistani type, also called autosomal recessive brachyolmia. Patients with partial PAPSS2 deficiency have evidence of milder skeletal abnormalities.

STEROID DEGRADATION AND EXCRETION

In addition to interconversion via HSDs between weak and potent hormones, steroids undergo extensive hepatic metabolism, which leads to their permanent inactivation and excretion in urine. The 5α-reduction of T to DHT is an amplification reaction in prostate and genital skin,[79] but all Δ^4-steroids are substrates for the two 5α-reductase isoenzymes as well. In the liver, 5α-reductase type 1 converts cortisol, progesterone, and other hormones to their 5α-reduced dihydro (or *allo*-) derivatives. These intermediates are now substrates for the AKR1C enzymes, which then catalyze 3α-reduction to the *allo*-tetrahydrosteroids. Also in liver, AKR1D1, which is steroid 5β-reductase, performs the first step in conversion to tetrahydrosteroids, such as cortisol to tetrahydrocortisol. In general, the 5β-route of catabolism accounts for more steroid degradation than the 5α-route, with some interindividual variation.

The hydroxyl group at the 3-position in the (*allo*)-tetrahydrosteroids and Δ^5-steroids allows for not only sulfonation but also glucuronidation, similar to the "Phase II" conjugation reaction common in drug metabolism.[80] Several uridine diphosphate glucuronyltransferase (UGT) enzymes use hydroxysteroids as substrates, and this glucuronide moiety is a signal for excretion via anion transporters/solute carrier proteins in the kidney. A major fraction of the steroids synthesized in the endocrine organs is thus eliminated as the glucuronide and sulfate conjugates. Of the ~10 mg of cortisol synthesized daily, for example, only 0.1–0.3% occurs in the urine as free cortisol, while the remaining 99+% is glucuronides and sulfates of tetrahydrocortisols, tetrahydrocortisones, and other metabolites. The UGT enzymes are also polymorphic within the population, which in part accounts for some of the interindividual differences in urinary steroid excretion patterns.

Certain common alleles of UGT2B7 appear to be the major enzymes for glucuronylation of glucocorticoid and mineralocorticoid metabolites.[81] The UGT2B4, UGT2B7, UGT2B15, and UGT2B17 enzymes all catalyze the glucuronylation of androgens and their metabolites such as androsterone and etiocholanolone (the major androgen metabolites in urine), as well as the androstanediols.[82] Androgen glucuronylation has been studied in detail due to its relevance to prostate cancer and to androgen doping in sports. UGT2B17 is the nearly exclusive enzyme that catalyzes T glucuronylation at the 17β-position, whereas epitestosterone (epiT, the 17α-epimer of T), DHT, and other androgen metabolites are substrates for both UGT2B15 and UGT2B17.[83] An allele containing a ~150 kb deletion, lacking the entire *UGT2B17* gene but not affecting the *UGT2B15* gene, is commonly distributed in the population,[84] particularly in Asia, where roughly 70% of the population has no functional UGT2B17 enzyme. As a consequence, exogenous T administration manifests as a high T/epiT ratio in urine, except for homozygotes for the *UGT2B17* deletion allele, for whom little T-glucuronide appears in the urine. After administration of a 500 mg T-enanthate injection, only half of homozygotes for the *UGT2B17* deletion allele have a urinary T/epiT ratio >4 and are considered a positive screen for exogenous T use, while all individuals with one or two wild-type copies of the *UGT2B17* allele have positive screens.[85] Consequently, homozygotes for the *UGT2B17* deletion allele taking exogenous testosterone might not be identified using the urinary T/epiT ratio testing. Estrogens and catechol estrogens also undergo UGT2B7-catalyzed glucuronylation, and these 18-carbon steroids are also substrates for some of the UGT2A enzymes.

Additional hepatic metabolism of steroids contributes to their inactivation and also to extra-adrenal conversion to active steroids. Both CYP3A4 and CYP2C19 are progesterone 21-hydroxylases, which yields DOC as a minor product with other hydroxysteroids.[86] CYP3A4 catalyzes 6β-hydroxylation of progesterone, cortisol, and testosterone; this reaction accounts for a considerable amount of hormone inactivation, particularly when orally administered. This phenomenon is important, because patients taking CYP3A4 inducers and inhibitors on cortisol replacement might require dose adjustment. The most profound effect is observed with adrenocortical cancer patients receiving mitotane therapy. Mitotane potently induces CYP3A4 expression and leads to more than 10-fold increase in 6β-hydroxycortisol production,[87] which mandates hydrocortisone dose increases in these patients.

PATHWAYS

Composite diagrams of steroidogenic pathways, such as Figure 8.2, can be misleading, because no steroidogenic cell type contains all of these enzymes and pathways. This section will dissect the major pathways of the different zones of the adrenal cortex, the placenta, and the different cells of the testis and ovary. The simplest pathways are those of the corpus luteum and the placenta, which terminate at P4. Only two enzymes—CYP11A1 and 3βHSD—are required, and there are no branch points or side products. The placenta does not even use StAR for cholesterol mobilization. The 3βHSD isoenzymes are the type 1 in the placenta and the type 2 in the corpus luteum.

In progressing through the adrenal cortex, the major product of the outermost ZG is the mineralocorticoid aldosterone. Low serum potassium concentrations and angiotensin II stimulate steroidogenesis in this zone, which act to elevate intracellular calcium and activate enzyme expression via calmodulin.[88] This zone also contains CYP11A1 and 3βHSD2, as well as CYP21A2, which converts P4 to DOC. CYP11B2 is uniquely expressed in the ZG, and its three sequential 11β-hydroxylase, 18-hydroxylase, and 18-oxidase activities metabolize DOC to B, 18OHB, and aldosterone (Figure 8.5(A)). Because DOC is a substrate for CYP11B2 and P4 is not, the ZG also has a single major pathway with no branch points, but as mentioned earlier, a significant amount of the 18OHB intermediate is released into the circulation. Basal steroidogenesis is also very low in the absence of angiotensin II; otherwise, hypertension would result as in primary aldosteronism.

The adrenal ZF contains CYP11A1, 3βHSD2, and CYP21A2 like the ZG. Unlike the ZG, which expresses CYP11B2, the ZF contains CYP11B1 and CYP17A1 instead. ACTH binds to and activates the melanocortin type 2 receptor (MC2R), which is a G-protein coupled receptor. The resultant increase in cAMP activates StAR, maintains expression of the four P450 enzymes, and drives cortisol production. The presence of three steroid hydroxylases with somewhat broad substrate specificity downstream of 3βHSD2, however, leads to branch points, ambiguity, and potential for additional products (Figure 8.5(B)). CYP11B1 also has low 18-hydroxylase activity, leading to some 18OHB synthesis.[89] Cortisol synthesis is normally very efficient, with circulating concentrations of the trihydroxysteroid cortisol 10–1000 times higher than those of its intermediates. Only small amounts of side products such as the dihydroxysteroids B and 21-deoxycortisol (21dF) are normally produced; however, when one of the steroid hydroxylases is absent or inhibited, the precursors are diverted to these ordinarily minor products. In 17-, 21-, and 11β-hydroxylase deficiencies, impaired cortisol synthesis leads to accumulation of DOC and B,[90] 17OHP and 21dF,[91] or DOC and 11-deoxycortisol,[92] respectively. These products are predictable, based on the known activities of these enzymes and inspection of Figure 8.5(B). Additional minor pathways are also present but not shown in the figure.

The ZR also produces steroids under ACTH stimulation of MC2R, but its enzymatic machinery differs considerably from that of the ZG. Rather than three steroid hydroxylases downstream of pregnenolone, the ZR has only one, CYP17A1. In addition, the ZR expresses abundant CYB5A,[93] which activates the 17,20-lyase activity of CYP17A1 and allows 19-carbon DHEA synthesis, and the ZR is deficient in 3βHSD2, which limits steroids to the Δ^5-pathway. SULT2A1 is also highly expressed in the ZR; consequently, the majority of DHEA formed is sulfonated, using PAPSS2-derived PAPS, and exported as DHEAS (Figure 8.5(C)). The ZR makes small amounts of T relative to DHEAS, due to small amounts of 3βHSD2 and the presence of AKR1C3,[94] which has low 17βHSD activity to convert AD to T.

The Leydig cell of the testis is similar to the ZR in that it has high CYP17A1 and CYB5A expression but no other steroid hydroxylases, which funnels pregnenolone to 19-carbon steroids. Elevated cAMP also drives steroidogenesis in the Leydig cell, which occurs when LH binds to its G-protein coupled receptor, which is also the receptor for chorionic gonadotropin (LH/CG receptor). Unlike the ZR, however, the Leydig cells lack SULT2A1 and uniquely express 17βHSD3,[69] which efficiently reduces 19-carbon ketosteroids to the corresponding hydroxysteroids. In addition, the Leydig cells express 3βHSD2; consequently, the major product is T, although several routes are possible due to the branch points seen in Figure 8.6(A). Most likely, the majority of synthesis follows the pathway

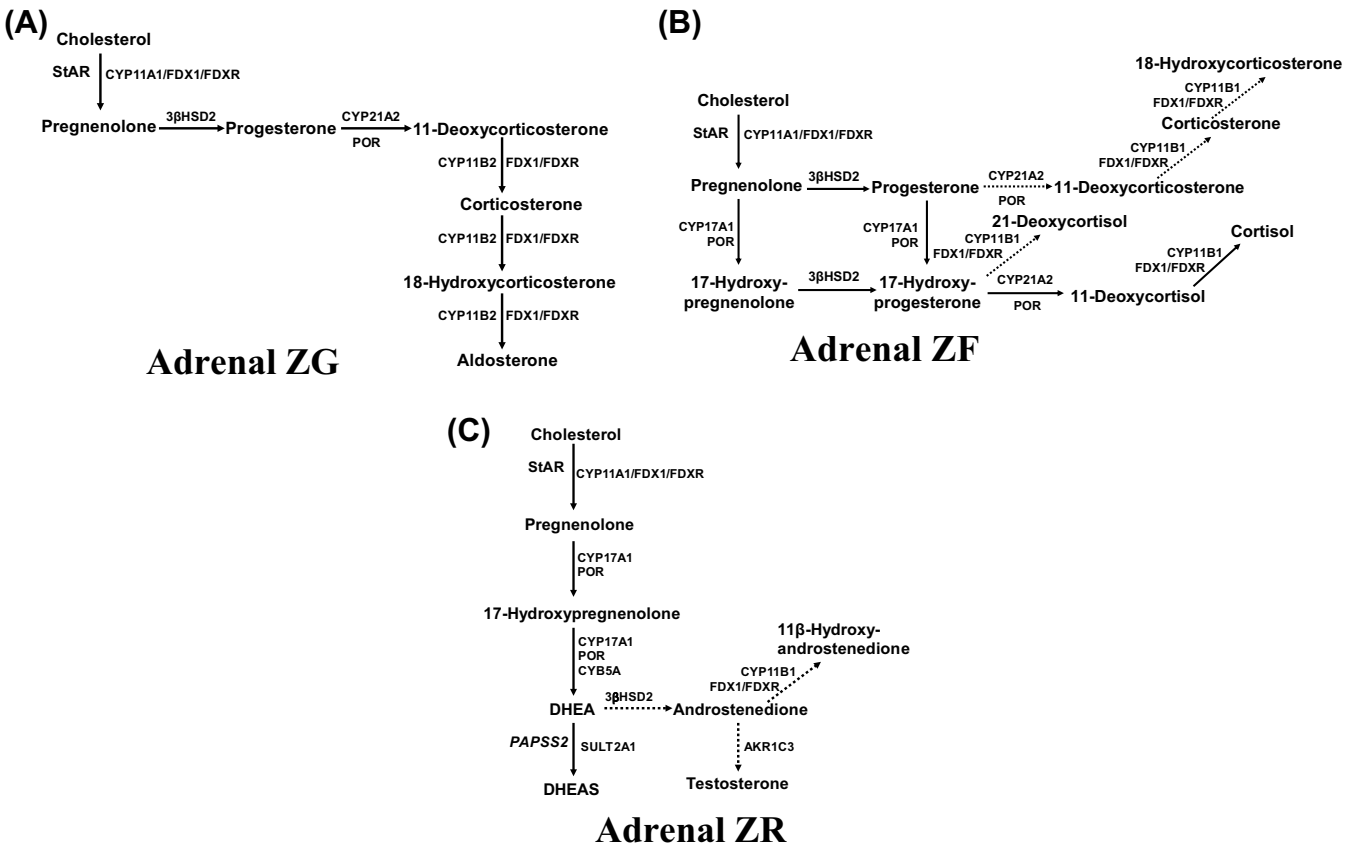

FIGURE 8.5 Steroidogenic pathways of the adrenal. (A) The zona glomerulosa makes aldosterone and few other products. (B) The zona fasciculata makes cortisol using three steroid hydroxylases. Minor products and pathways are shown with dotted lines, although these pathways become significant in disease states such as CAH. (C) The zona reticularis makes primarily DHEAS, yet a small amount of testosterone and other 19-carbon steroid are variably produced as well.

pregnenolone to DHEA via CYP17A1, 17βHSD3 reduction to androst-5-ene-3β,17β-diol, and finally 3βHSD2 oxidation to T.[36] This route employs the much higher 17,20-lyase activity of human CYP17A1 with 17Preg than with 17OHP, whereas the 3βHSD2 and 17βHSD3 activities are similar at each branch point.

The ovary uses a two-cell system to synthesize E2, the granulosa cells, and the theca cells. Follicle development and enzyme expression in the granulosa cells is primarily driven by follicle-stimulating hormone binding to its receptor, which increases intracellular cAMP. Elevated cAMP induces progesterone production and CYP19A1 (aromatase) expression. However, granulosa cells lack CYP17A1 and therefore cannot metabolize progesterone to C-19 steroids, which are substrates for aromatase. Theca cells, under LH stimulation, convert cholesterol to C-19 aromatase substrates.[95] Consequently, 19-carbon steroid synthesis proceeds to AD, but only a small amount is converted to T via AKR1C3. Instead, AD moves back to the granulosa cells, which surround the developing oocyte and contain both CYP19A1 and 17βHSD1 to complete the pathway AD to E1 to E2 (Figure 8.6(B)). Following ovulation, the granulosa cells undergo luteinization to the corpus luteum, which produces P4 via CYP11A1 and 3βHSD2 (Figure 8.6(C)).

The placenta has two disconnected steroidogenic pathways (Figure 8.6(D)). P4 synthesis is the same two-enzyme process as in the corpus luteum, and this process maintains the pregnancy after the luteo-placental shift at about six weeks of gestation. The placenta is also the site of estrogen synthesis during pregnancy, but CYP17A1 expression in the placenta is very low. Instead, the fetal adrenal is the source of 19-carbon steroid precursors to estrogen. DHEAS and 16α-hydroxyDHEAS from the fetus are desulfated by steroid sulfatase, oxidized to AD and 16α-hydroxyAD by 3βHSD1, aromatized to E1 and 16α-hydroxyE1 by CYP19A1, and reduced to E2 and estriol (E3) by 17βHSD1. The E2 and E3 are thus products of the fetoplacental unit rather than just the fetus or placenta.

The most recently described pathway is a route to DHT, which does not involve DHEA, AD, or T as intermediates and instead uses androsterone as the initial 19-carbon steroid, called the alternate or "backdoor pathway" to DHT.[96] Originally described in the neonatal tammar wallaby testis,[97] this pathway requires the

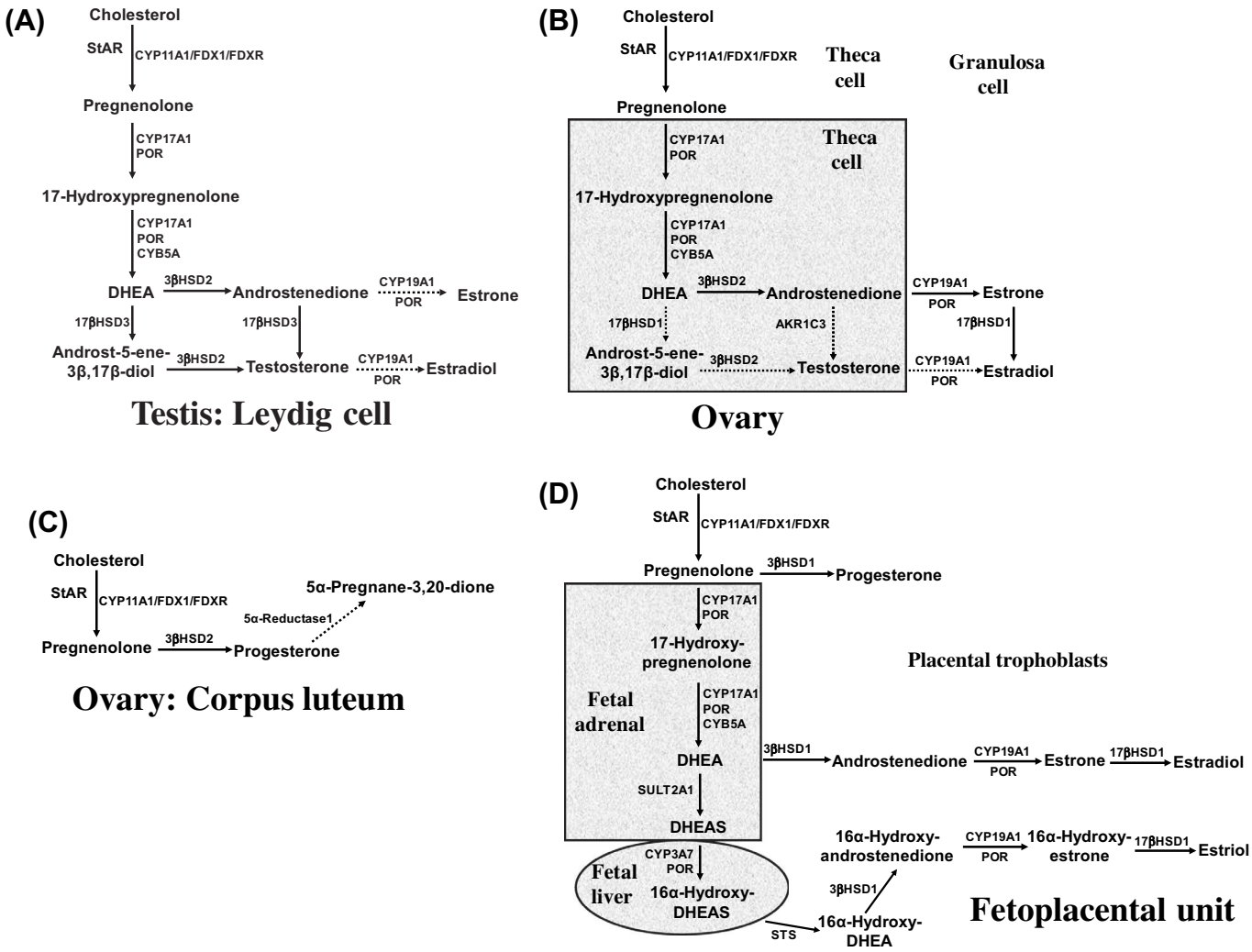

FIGURE 8.6 Steroidogenic pathways of the gonads and placenta. (A) Steroidogenesis in the testis (Leydig cell) yields T, although several sequences of intermediates are likely. (B) E2 production in the ovary uses two cell types. The granulosa cells generate pregnenolone and complete the conversion of androgens to estrogens (reactions in clear area outside box), while the theca cells convert pregnenolone to androstenedione (reactions inside textured box). Theca cells also convert cholesterol to pregnenolone (label in white area adjacent to this reaction). (C) In the corpus luteum, steroidogenesis proceeds only to P4 using two enzymes CYP11A1 and 3βHSD2, and because the corpus luteum also expresses 5α-reductase type 1, a significant amount of P4 is reduced to 5α-pregnane-3,20-dione. (D) Placental steroidogenesis has two pathways. P4 production in the placenta mimics that of the corpus luteum, except using 3βHSD1 (not type 2). E2 and E3 synthesis in the fetoplacental unit is a complex interplay of three compartments. The fetal adrenal (textured square) generates the required 19-carbon precursor DHEAS, some of which is 16α-hydroxylated in the fetal liver (textured oval) via CYP3A7 to 16α-hydroxyDHEAS. The placental steroid sulfatase (STS) and 3βHSD1 enzymes remove the sulfate and oxidize/isomerize the steroid to yield 16α-hydroxyandrostenedione; parallel reactions also occur with DHEAS to form AD (STS reaction not shown in figure to avoid clutter). These 19-carbon, Δ^4 steroids are substrates for aromatase (CYP19A1), and the products—16α-hydroxyestrone and estrone—are substrates for 17βHSD1, yielding the potent estrogens E3 and E2, respectively.

presence of 5α-reductase activity in the steroidogenic cell. In this pathway, P4 or more typically 17OHP is both 5α- and 3α-reduced, forming Pdiol, which is the substrate for the 17,20-lyase activity of CYP17A1 (Figure 8.7). Human CYP17A1 catalyzes the cleavage of Pdiol to androsterone even more efficiently than the conversion of 17Preg to DHEA, and the former reaction is about as rapid in the absence of CYB5A as the latter reaction in the presence of CYB5A.[31] The backdoor pathway contributes proportionately greater to androgen flux in pathologic states characterized by 17OHP accumulation, as first described in POR deficiency.[15,98] This route explains why 46,XX newborns with POR deficiency can be born partially virilized, when postnatal androgen synthesis is poor. Similarly, this pathway might be responsible for the severe virilization in 46,XX newborns with 21OHD and explain why, in contrast, those with 3βHSD2 deficiency—who produce even more DHEA than in 21OHD but cannot make 17OHP in the adrenal—are only slightly virilized late in gestation.[96] In addition, the description of two families with 46,XY DSD and combined deficiencies of AKR1C2

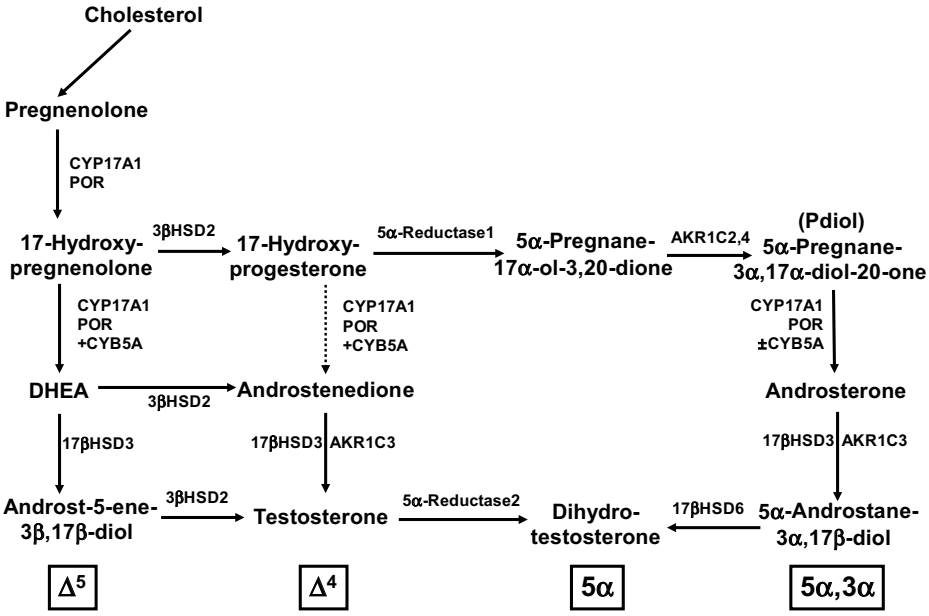

FIGURE 8.7 The alternate or "backdoor pathway" to DHT. This pathway requires that 5α-reductase activity is present and abundant 17OHP. The canonical pathways are also shown for comparison.

and AKR1C4,[70] which probably catalyze one or more steps of this pathway in human beings, suggests that the backdoor pathway might also be important for normal male development, at least for some individuals.

Variations in these canonical pathways occur frequently in disease states. Blocks in the normal pathways to cortisol in 21OHD cause accumulation of 17OHP and 21dF, but in addition, high 17OHP shunts steroids to androgens via the backdoor pathway in the first year of life[99] and possibly during fetal life. Unusual combinations of enzymes occur in tumors, such as prostate cancer cell lines, in which 3βHSD1 and 5α-reductase type 1 are the dominant isoforms unlike normal prostate or testis. These cells convert DHEA to DHT via that pathway DHEA→AD→5α-androstane-3,17-dione (5αdione)→DHT, entirely bypassing T.[100]

COMPARISON WITH OTHER SPECIES

Many of the enzymes, intermediates, and pathways found in human steroidogenesis are common to most other species, such as the cholesterol side-chain cleavage and the 3βHSD reactions as the earliest steps. For the latter steps and products, which diverge significantly among species, steroidogenesis in human beings is more similar to that of other large mammals such as cows and primates rather than rodents. For example, the major glucocorticoid product of the human adrenal cortex is cortisol, which bears hydroxyl groups at the 11β-, 17α-, and 21-positions, as is the case in monkeys, cows, and dogs. In contrast, rodent adrenals synthesize primarily corticosterone (11β- and 21-hydroxylated), because mouse and rat adrenals are deficient in CYP17A1 and thus lack significant 17-hydroxylase activity. For this same reason, the rodent adrenal makes little DHEA or DHEAS, and only the adrenals of large mammals make 19-carbon steroids.[101] The preferred pathway of the 17,20-lyase reaction is also variable, with the human and bovine CYP17A1 enzymes favoring the Δ^5-pathway,[35] the rodent enzymes favoring the Δ^4-pathway,[102,103] and the pig enzymes roughly equal for both pathways.[6] In some species of fish, two CYP17A1 isoenzymes exist, one with 17,20-lyase activity and the other without.[104]

The HSD reactions are also quite different amongst species. Human beings contain only two functional 3βHSD genes—*HSD3B1* and *HSD3B2*—whereas rodents express at least six 3βHSD isoenzymes from a gene cluster.[105] The differences in the 17βHSD isoenzymes are even more variable.[106] All mammals have an equivalent of "estrogenic" 17βHSD1 and "androgenic" 17βHSD3, although the numbering, which generally reflects order of cDNA cloning rather than any physiologic organization, might also vary amongst species.

The pathways and physiology of the fetoplacental unit and pregnancy is perhaps the aspect of steroidogenesis that is the most highly divergent amongst species. In the rabbit, the corpus luteum produces P4 throughout pregnancy without a shift to placental dominance, which is why a *CYP11A1* deletion rather than StAR mutations causes lipoid CAH in a strain of rabbits.[107] The contributions of the human fetal adrenal to steroidogenesis in pregnancy, by providing the 19-carbon steroid substrate, are rather unique among eutherian mammals. In sheep, the fetal adrenal produces cortisol but not DHEA derivatives late in gestation, which induces

CYP17A1 expression in the placenta, thus providing substrate for aromatase and estradiol synthesis toward term.[108] Withdrawal of P4 action is critical for parturition in many species, but the mechanism of this withdrawal varies from decreased production to increased catabolism to reduced sensitivity amongst various species (see Chapter 42).

CONCLUSION

Steroidogenesis in the adrenal, gonads, and placenta is a directional process designed to synthesize one or a few final steroid products unique to each cell type. The specific enzymes present in each cell type determine the major products of that cell. Regulation of steroidogenesis is complex and redundant, although the consequences of an enzyme deficiency or blockade are usually apparent from inspection of the general pathway scheme. Once one leaves these traditional steroidogenic glands, the pathways and enzymes are difficult to predict and can change depending on a variety of factors.

Strongly held beliefs and dogma about human steroid biosynthesis have been dashed many times in the last 50 years, and new enzymes, pathways, and diseases continue to be described. Among the remaining unsolved problems are the molecular details of StAR action; the structural features of the steroid hydroxylases, which convey their unique substrate selectivity and regiochemistries; the contributions of the various HSDs to peripheral androgen and estrogen synthesis; and the biochemistry of the H6PDH-11βHSD1 enzyme system. Steroidogenesis in the nontraditional tissues such as the brain and colon has been recognized for decades, but the contribution of de novo synthesis from cholesterol compared to conversion of circulating precursors remains unknown. Some steroids such as tetrahydrodeoxycorticosterone or allopregnanolone bind to and modulate the activities of ion channels, and these steroids also elicit behavioral or electrophysiological changes. The route of synthesis from circulating 21-carbon precursors is predictable from the principles discussed previously, via sequential 5α- and 3α-reduction of DOC or P4, respectively. The evidence supporting their synthesis from cholesterol in the brain, however, is controversial. A similar debate rages in prostate cancer, where the source of androgens driving its receptor in advanced disease is controversial. Although many of the genetic diseases of steroidogenesis are rare, disordered steroidogenesis occurs commonly in prevalent diseases such as polycystic ovarian syndrome and primary aldosteronism. A better understanding of milder defects such as these will enable major advances in the treatment of infertility and reproductive senescence.

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CHAPTER

9

Gonadal Steroid Action

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INTRODUCTION

Steroid hormones comprise a large class of bioactive cholesterol-derived compounds defined by a central four-carbon-ring system. They comprise the *sex steroids* or *gonadal steroids*, which encompass estrogens, progestins, and androgens (AR) (Figure 9.1), and the *adrenal steroids*, principally glucocorticoids and mineralocorticoids. While the adrenal steroids have certain functions that relate to reproduction, the scope of this chapter will be limited to the gonadal steroids. The mechanism of action of gonadal steroids is best characterized with regard to their interaction with members of the nuclear receptor (NR) superfamily of transcription factors, which, upon binding their cognate ligands, engage specific sequences in the regulatory regions of target genes to regulate their transcriptional output.[1] A heterogeneous class of molecules termed coregulators mediates many of the diverse mechanistic functions of the gonadal steroids and their receptors, and contributes to the integration of steroid signals with those of parallel cellular signaling pathways to effect tissue-selective patterns of transcriptional regulation.[2,3] In addition to their NR-dependent modes of action, gonadal steroids signal through membrane receptors to directly engage cellular kinase cascades, culminating in both transcriptional and nontranscriptional functional endpoints.[4] In the realm of mammalian reproductive physiology, gonadal steroids support numerous aspects of mammalian reproductive physiology, encompassing growth and differentiation of the uterus, ovary, mammary gland, prostate, and testis; the maintenance of pregnancy in the female and spermatogenesis in the male; and mediation of the development of secondary sexual characteristics in the musculoskeletal system and other organs. Moreover, these hormones have myriad effects on tissues of the central nervous system (CNS) that themselves exert profound effects on reproductive organs in both sexes.

The purpose of this chapter is not to provide a catalog of molecular pathways activated by each major class of steroids in the various reproductive tissues that are sensitive to these hormones, but rather to provide an overview of the types of molecular and transcriptional responses that steroid hormones utilize to effect their physiological actions. In this way, the reader will be introduced to a framework of general concepts regarding steroid hormone action that will serve as a point of departure to explore more select aspects of testosterone (T), estradiol (E2), and progesterone (P) actions as they relate to the physiology of reproduction. The chapter opens with a discussion of historical aspects of gonadal steroid action and goes on to emphasize mechanistic aspects of their cellular functions, both genomic, resulting in the direct regulation of target genes, and nongenomic, mediated through cytoplasmic effectors.

The Gonadal Steroids

Figure 9.1 shows the principal bioactive gonadal steroids, which have tissue-selective effects in most organs and cell types in the body, including the gonads themselves. In the female, the ovary is the site of synthesis of the three classes of gonadal steroids, namely, the C18 estrogens, the C19 androgens, and the C21 progestins, all of which are derived from the entry of cholesterol to the steroidogenic pathway. A detailed discussion of ovarian steroid synthesis is beyond the scope of this chapter and can be found elsewhere in this volume (see Chapter 8). In brief, ovarian steroids are synthesized in the granulosa (estrogens) and thecal (progestins and AR) cell layers of the ovarian follicle, and the corpus luteum (progestins). Ovarian steroid biosynthesis engages enzymes in the cytochrome P450 and oxidoreductase classes, genes encoding that are under transcriptional control by the pituitary gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone. All three classes of

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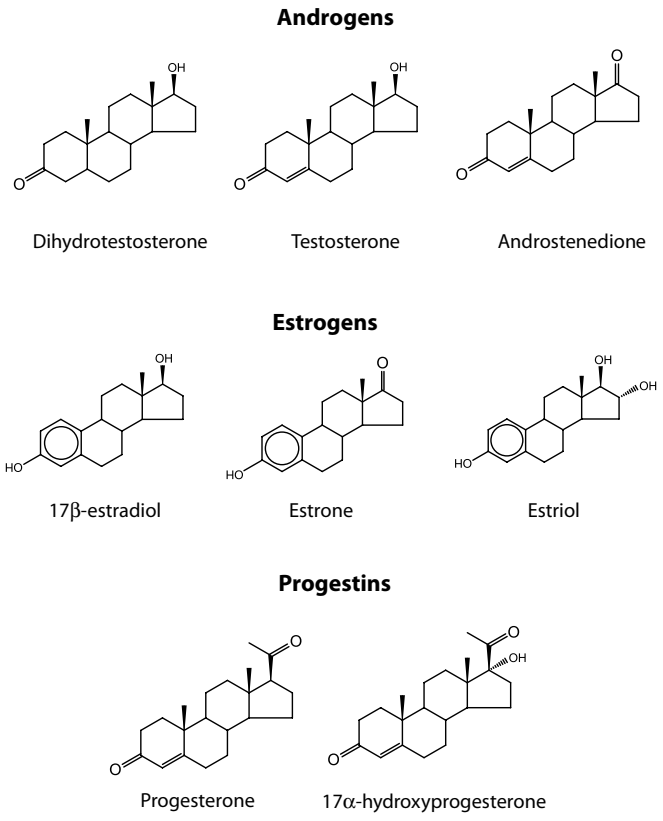

FIGURE 9.1 Gonadal steroids. 17β-estradiol (E2), progesterone (P), testosterone (T), and dihydrotestosterone (DHT).

ovarian steroids are converted in the ovary to metabolites of uncertain function. Testicular steroidogenesis initially involves the production of T from cholesterol by the interstitial Leydig cells of the testis. In brief again, this process is driven by one of two pathways: the Δ4 pathway in the rat and mouse, and the Δ5 pathway in humans and higher primates. T undergoes conversion in the Leydig cells, or in the prostate, by 5α-reductase to 5α-dihydrotestosterone (DHT), the most potent bioactive androgen. Genes encoding enzymes in androgen biosynthesis are under the control of LH.

Historical Perspectives

It has been nearly six decades since the influence of gonadal steroids on enzyme activity, and de novo protein synthesis was first characterized in the context of regulation of cellular metabolism. The rapid uptake of E2 in target tissues gave rise to the appreciation at a relatively early stage that the mode of action of steroid hormones, namely, diffusion across the cell plasma membrane, differed fundamentally from that of peptide hormones.[5] Steroid target tissues were defined by their ability to bind and retain hormone, giving rise to the concept of specific cellular receptors for these molecules that mediated their function, at least in part. By the mid-1970s, characterization of these receptors had defined their existence in specific multiprotein complexes that alternated between larger forms, of the order of 8S as estimated by sucrose density gradient centrifugation, to smaller 6S and 4S forms.[6,7] It became clear that the binding of hormone to the receptor had profound implications, including the induction of conformational changes in the complexes[8] and, in some cases, translocation to the nucleus.[9] The development of these early mechanistic models proceeded alongside seminal functional studies of steroid biology, such as those by Gordon Tomkins, who demonstrated specific inhibition in rat metabolic tissues of diphosphopyridine nucleotide dehydrogenase by P, E2, T, and other steroids.[10]

Experiments in the early 1960s demonstrating a stimulatory effect of E2 on the rate of RNA synthesis were the first to demonstrate a conceptual link between E2—and steroid hormones as a class—and the regulation of gene expression. The concept of a "nuclear acceptor", while initially nebulous, represented an appreciation that the nuclear effects of steroids could be ascribed at least in part to their engagement of specific nuclear entities to mediate their downstream functions. Subsequent experiments by Yamamoto and Alberts[7,11] germinated the concept of the existence of high-affinity DNA sequences (termed hormone response elements) to which the hormone–receptor complex bound, and these were paralleled by studies demonstrating E2-induced increases in the rate of ovalbumin mRNA synthesis in chick oviduct.[12,13] Subsequent to the purification of receptors for P, E2, glucocorticoids, and AR, a model emerged in the late 1970s and early 1980s in which hormone entered the target cell, bound to receptor, translocated to the nucleus, engaged chromatin-binding sites, and initiated the synthesis of specific mRNA and protein species.[14]

TRANSCRIPTIONAL ACTIONS OF GONADAL STEROIDS

Introduction

While this section will highlight mechanistic principles of classical steroid action, many of these principles apply also to members of the broader NR superfamily. NRs comprise a large superfamily of conserved transcription factors that are activated by their steroid hormone ligands and play essential roles in diverse biological processes. For example, the estrogen, P, and androgen receptors are important in reproduction; glucocorticoid receptors are important in glucose metabolism and stress; the thyroid hormone receptor is important in oxidative metabolism; and peroxisome proliferator-activated receptors (PPARs) are important in lipid and energy metabolism.[15] Coregulators are

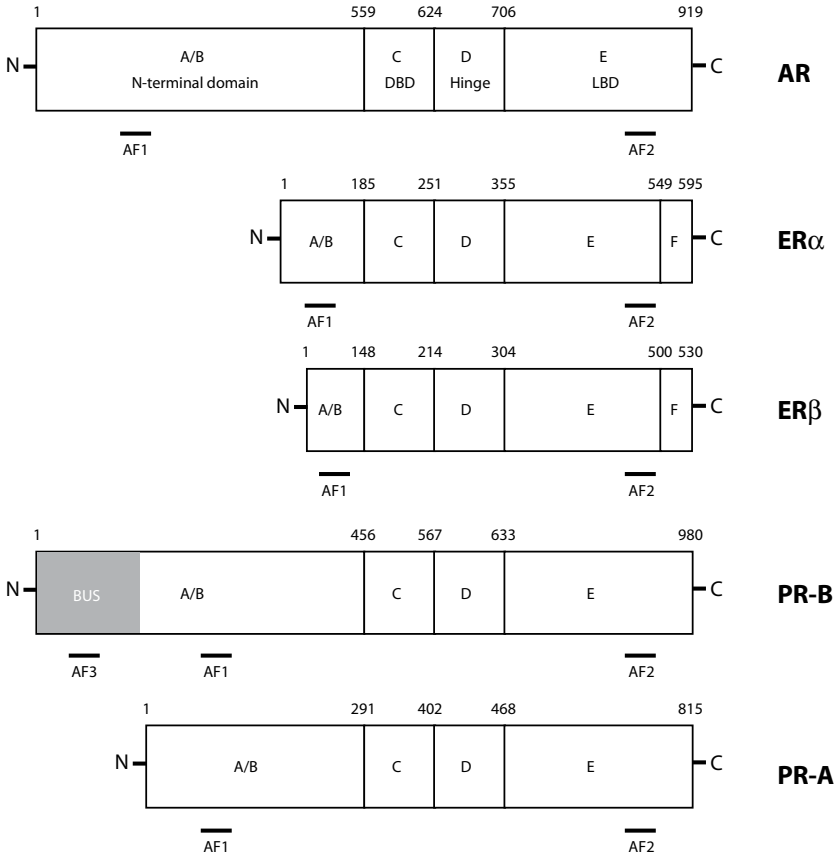

FIGURE 9.2 The gonadal steroid receptors. Androgen receptor (AR) and estrogen receptors α (ESR1) and β (ESR2) share a number of common domains, including an N-terminal A/B domain (containing activation function 1 (AF1)), a DNA-binding domain (DBD; C), a hinge domain (D), and a C-terminal ligand-binding domain (LBD; E) containing AF2. ESRs possess a C-terminal F domain, and PGR-B contains an N-terminal B-upstream segment (BUS) containing a third activation function, AF3.

molecules recruited by ligand-bound activated NRs (or other DNA-binding transcription factors) to effect enhanced (coactivators) or repressed (corepressors) gene expression. In contrast to NRs, which are structurally conserved, their coregulators are diverse, both structurally and in the way they regulate transcriptional process, namely, through a diverse array of enzymatic activities such as acetylation–deacetylation, methylation–demethylation, ubiquitination, and phosphorylation, or as chromatin remodelers.[2,16] Overall, through their opposing actions, a balance of the actions of coactivators and corepressors contributes to the remarkable spatiotemporal diversity of gonadal steroid action.

The early biochemical experiments discussed in the introductory section above were followed in the 1980s by a series of experiments that availed themselves of advances in recombinant DNA technology, and that resulted in the cloning of receptors for E2 (ESRs), P (PGR), and AR. These receptors collectively represent the classical gonadal steroid receptors, referred to here as SRs (reviewed in detail in Ref. 17). Based on significant amino acid sequence identity in various subdomains, SRs and other NRs were designated as a family of evolutionarily related transcription factors, the NR superfamily. These subdomains are now designated as defining structural features of members of this superfamily (Figure 9.2). On a general level, NRs are composed of an amino-terminal activation function (AF1) (A/B domain), the DNA-binding domain (DBD) (C), a hinge region (D), and a carboxy-terminal ligand-binding domain (LBD) (E). An F domain in the human ER is thought to exert a repressive effect on receptor dimerization in the presence of E2.[18] Mutational analysis of the E domain led to the designation of a second activation function, AF2, which is indispensable for proper ligand-dependent activation by NRs. Other functions have been ascribed to the E domain, including ligand binding, heat shock protein (HSP) interactions (6), and nuclear localization. These functional domains reflect an intricate, but well-characterized, ligand-mediated receptor activation pathway. This multistep process involves activation of receptor by binding of the cognate hormone, a change in receptor structure and dissociation of several HSPs,[19] nuclear translocation of the activated receptor, and dimerization and apposition of the transformed receptor to its DNA response elements. More recent years have seen the identification of a constellation of proteins that are important for the regulation of gene expression by NRs, the coregulators,[3] although the sequence of events by which activated, DNA-bound receptor achieves transcriptional regulation in a tissue-selective manner remains only vaguely characterized. Implicit

in this discussion is the fundamental role played by the specific engagement of ligand by the ligand-binding domain, coordinating nuclear translocation, DNA binding, and the recruitment of coregulators to direct an appropriate transcriptional response to the hormonal signal. I draw the reader's attention to the fact that in this chapter, I have chosen not to discuss steroid receptor coactivators and corepressors as distinct molecular entities, since abundant evidence has demonstrated that they have many overlapping and complementary roles and, in some cases, both functions exist within the same protein.[2] If there is one tenet that is taken away from this chapter, it is that steroid receptor regulation of gene expression is a highly contextual process and that principles gleaned from one experimental or physiological context might not necessarily find application in another—for example, due to distinct complements of coregulators in different cells.

Gonadal Steroid Receptors

Regulation of cell and nuclear function by T and DHT is mediated by the AR, which is remarkable for a long polyglutamine tract in its N-terminal region that has been implicated in Kennedy's disease due to its effect on ligand binding.[20] The classical effects of E2 on gene expression are mediated by ESR1 (ERα) and ESR2 (ERβ), which share 94% amino acid homology in the DBD and approximately 55% homology in the LBD, although significant sequence divergence exists in the N-terminal, hinge, and C-terminal regions of the ESRs.[21] Moreover, numerous variants of both isoforms have been reported in a number of different species, cell types, and physiological events. ESRs contain dual activation functions (AFs) that have been shown to interact with distinct groups of coactivators. The N-terminal activation function 1 (AF1) mediates ligand-independent activation of the ESR, whereas the conformation and activity of AF2, located in the LBD, are determined by ligand binding.[21] As might be anticipated from the relatively low sequence homology in the LBD, the ESRs differ in their affinity for physiological and synthetic ligands, and although they have many coregulators in common, some are specific to one or the other receptor subtype. In general, the effects of estrogen in a given tissue are a function of the expression ratio of the isoforms of ESR, the complement of coregulators expressed in the cell, the activity of parallel afferent signal transduction pathways, as well as patterns of epigenomic modification of the promoters of their cognate target genes. As with AR, these coregulatory molecules may influence the response in both a ligand- and promoter-dependent context, which in turn may be influenced by other signaling pathways.

The DBD of PGR is flanked by an upstream N-terminal region that contains the ligand-independent AF1 and a downstream hinge region and C-terminal LBD. Two PGR isoforms, PGR-A and PGR-B, differ in an additional 164 amino acids in the extreme N-terminal region of PGR-B known as the B-upstream segment (BUS; Figure 9.2),[17] which contains a third activation function, AF3. T47D human breast cancer cells have served as the workhorse for study of PGR signaling, and in these cells, synthesis of the PGR-A and PGR-B isoforms has been shown by Pierre Chambon's group to be driven by alternative transcriptional initiation from two distinct promoters[22] located at −711 to +31 and +464 to +1105 of the transcription start site, respectively. Several different transcripts encode PGR and, in addition to containing translational start sites for PGR-A and PGR-B, they also encode a minor N-terminally truncated receptor, PGR-C. In general, PGR-A and PGR-B are expressed at comparable levels in P target tissues, including the mammary gland and uterus, although their ratio is known to differ in breast tumors. The major factors that regulate PGR mRNA levels in a variety of cells and tissues are E2 or related estrogens (Figure 9.1) bound to ERs. PGRs are E2 regulated in the uterus, including the endometrium; in the pituitary and brain; in the mammary gland and breast; and in other tissues.[23]

Steroid Receptor Transcriptional Coregulators

A remarkable aspect of steroid signaling is the functional diversity of transcriptional coregulators with which steroid receptors, activated by either ligand binding or phosphorylation by kinase cascades (see the section Nontranscriptional Effectors of Gonadal Steroids), interact to effect transcriptional regulation (Figure 9.3). These include recruitment of histone acetyltransferase (HAT) and methylase activities in the form of nuclear coactivators (NCOAs) and protein methyltransferases (PRMTs), the kinase activities of the mitogen-activated protein kinase (MAPK) family of proteins, and two additional enzyme activities, adenosinetriphosphatase (ATPase) and glycosylation. This section focuses on factors associated with the transcriptional effects of gonadal steroids, followed by a discussion of the cross talk between NRs and other classes of DNA-binding transcription factors, and concludes with a summary of selected classical transcriptional targets of gonadal steroids.

Histone Acetylases and Deacetylases

The organization of eukaryotic chromosomes into nucleosomal subunits confers a thermodynamic barrier to the activation of gene expression. The DNA–histone salt interactions that maintain nucleosomal structure can be offset to some extent by the acetylation of key basic lysine moieties, a reaction catalyzed by cellular HATs. The overall loss of compact nucleosomal structure that

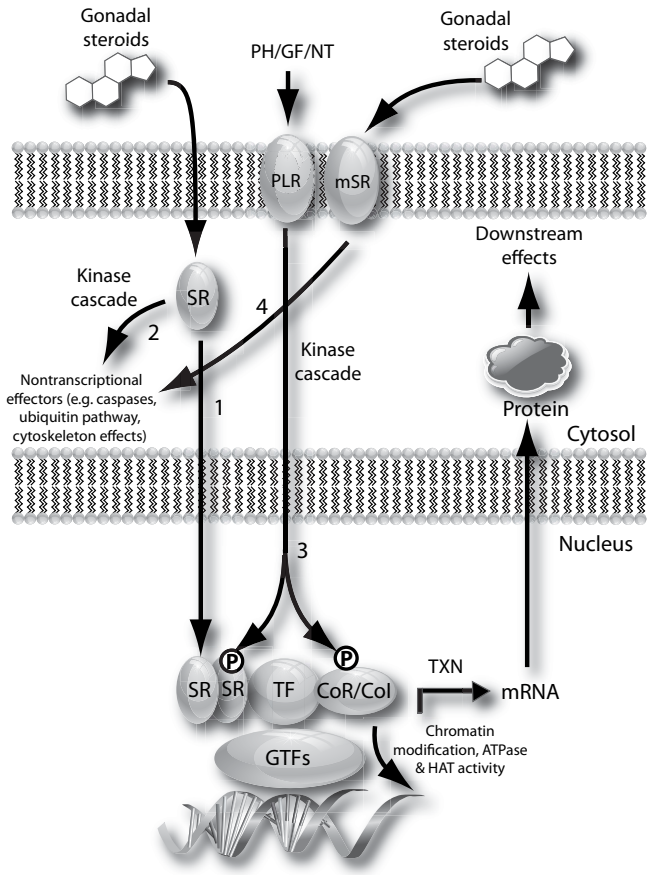

FIGURE 9.3 General models for signaling by gonadal steroids and SRs. (1) In the classical model of steroid-dependent, SR-dependent, genomic action, gonadal steroids bind directly to SRs in the cytoplasm, inducing the recruitment of nuclear coregulators (CoR) and cointegrators (CoI) and directly modulating expression of target genes (TXN), often in association with other classes of transcription factors (TFs). (2) In steroid-dependent, SR-dependent, nongenomic signaling, gonadal steroids have also been reported to elicit rapid cellular effects via cross talk of SRs with cellular kinase cascades. (3) In the steroid-independent, SR-dependent, genomic-signaling model, the binding of a variety of peptide hormones (PH), growth factors (GF) and neurotransmitters (NT) to their receptors (peptide ligand receptors (PLRs)) also touches off a variety of kinase cascades targeting steroid receptors (SRs) and coregulators (CoRs), which collectively modulate target gene expression via general transcription factors (GTFs), leading to de novo protein synthesis. (4) Finally, in the steroid-dependent, SR-independent signaling model, steroids are also thought to interact with nonclassical membrane steroid receptors (mSRs) with rapid functional endpoints in the cytoplasm and nucleus. Phosphorylation events are represented by an encircled P. *Source: Adapted from Ref. 24. Copyright 2012, The Endocrine Society.*

ensues from acetylation facilitates access of transcriptional mediators to the promoter template. Conversely, recruitment of histone deacetylases (HDACs) is thought to result in loss of the acetyl groups, reestablishing the coherence of the nucleosomal structure and restricting access of transcription factors to the promoter. Emerging evidence suggests that initial models casting acetylases as transcriptional coactivators and deacetylases as transcriptional corepressors are oversimplified and that the functions of these classes of transcriptional coregulators are context dependent,[3] and for this reason they will be discussed here in tandem.

Members of the (NCOA) family,[25] including NCOA1 (nuclear receptor coactivator 1), nuclear receptor coactivator 2 (NCOA2)/GRIP1, and nuclear receptor coactivator 3 (NCOA3)/p/CIP, which contain intrinsic histone acetyltransferase activity, act as general transcriptional coregulators for SRs. Members of this family also possess a basic helix–loop–helix (bHLH) domain, linking them to other members of this large family of transcription factors, and suggesting that although they do not bind DNA directly, they may have had that function at some point during their evolution. The possession by NCOA family members of Per–Arnt–Sim (PAS) domains also points to some commonality of function between them and the proteins originally shown to contain this motif (i.e., the period circadian protein, the aryl hydrocarbon receptor, and single-minded protein).[26] NCOA family members bind NRs directly through LXXLL motifs, or NR boxes, and stimulate their transcriptional AF in a hormone-dependent fashion as has been shown for AR,[27,28] ESRs,[29–31] and PGR.[32,33]

Active gene repression was originally thought to be a property of a subclass of nonsteroid NRs, which are tethered to their target gene promoters in the absence of ligand, and the discovery that gene silencing was also a property of SRs opened up an entirely new facet in gonadal steroid signaling. In addition to the well-documented interactions of SRs with proteins generally considered positive regulators of transcription, SR function also encompasses transcriptional corepressors that, analogous to the HAT activity of coactivators, possess intrinsic HDAC activity and recruit other HDACs to effect repression at target gene promoters. Nuclear receptor corepressor 1 (NCOR1) was originally shown to cooperate with unliganded retinoic acid receptor and thyroid hormone receptor to effect repression by bringing about conditions of chromatin condensation and inhibiting access to the target gene promoters of general transcription factors (see the section General Transcription Factors). As is the case with many other factors that regulate eukaryotic transcription, NCOR1 is a component of a ternary complex that includes other HDACs and negative nuclear factors such as SIN3A (SIN3 transcription regulator homolog A) and SIN3B (SIN3 transcription regulator homolog B), mammalian homologs of the yeast transcriptional repressor, Sin3p. Inhibition of activated SRs by NCOR1 has been demonstrated in the case of AR,[34] PGR,[35] and ESR,[36] pointing to a possible role for NCOR1 in negative-feedback regulation at SR-regulated promoters. Closely related to NCOR1 is NCOR2/SMRT, originally cloned as the silencing mediator of retinoid and thyroid receptors (SMRT), which is, like NCOR1, a conserved transcriptional repressor that

participates in silencing of eukaryotic gene promoters with a host of transcription factors.[37] As with NCOR1, SMRT has been shown to mediate the repression of gonadal steroid-regulated promoters.[5,38]

In addition to being shown to be members of the multiprotein complex recruited by NCOR1 and NCOR2, members of the HDAC–acuc–alpha family of proteins[39] act as AR corepressors in their own right, and like NCOR1 and NCOR2, may bridge between different transcription factors to integrate silencing by multiple transcription factors. HDAC1 interacts with retinoblastoma 1 (RB1), another characterized AR coregulator, to play the role of a key element in the regulation of cell proliferation and differentiation. Evidence indicates that AR, KAT5 (K(lysine) acetyltransferase 5)/TIP60 (a histone acetyltransferase), and HDAC1 participate as components of a trimeric complex to regulate expression of the promoter of the "gold-standard" androgen-regulated gene, prostate-specific antigen (PSA), suggesting that a finely controlled balance between acetylation and deacetylation is an important component of androgen regulation of gene expression.[40] Other lines of evidence indicate that principles of transcriptional regulation originally thought to be specific to non-SR members of the NR superfamily also extend to pathways that mediate gonadal steroid action. 5′TG3′ interacting factor (TGIF1)[41] is a member of the evolutionarily conserved three-amino-acid loop extension (TALE) class of homeodomain proteins that was originally shown to repress signaling by RXRalpha, a non-SR member of the NR superfamily, and which has been shown to function as a transcriptional silencer of the transcription factor SMAD2 (Sma- and Mad-related proteins (SMAD) family member 2). TGIF has been shown to interact with Sin3A and to repress AR-mediated transcription in an androgen-independent manner.[42]

In addition to acetylation and deacetylation, a host of other enzymes and proteins that effect specific posttranslational modifications have been shown to be important components of androgen action. The protein arginine N-methyltransferase (PRMT) family includes CARM1 (coactivator-associated arginine methyltransferase 1) and PRMT1, and posttranslational methylation of nuclear proteins by proteins in this family is known to play an important regulatory role in many biological processes through myriad transcription factors, including AR[43] and PGR.[44] Conversely, demethylation of histones in the vicinity of target gene promoters has also been tied to transcriptional regulation by gonadal steroids. The mouse mammary tumor virus (MMTV) promoter has been a model for induction of gene expression via signaling by P and other steroid hormones since the mid-1980s. Miguel Beato's group has recently detailed the intricate interplay between liganded PGR and members of the large transcriptional coregulatory complexes ASCOM (ASC2 complex) and NURF (nucleosome remodeling factor) in the regulation of this promoter.[45] Interactions between liganded PGR and several intrinsic and ancillary enzymatic components of this complex, including the H3K4 demethylase KDM5B (lysine (K)-specific demethylase 5B), effect displacement of histone H1 during the first minute of MMTV promoter activation.[45]

Transcriptional Cointegrators

See Figure 9.3. SRs have been shown to recruit a number of proteins referred to as transcriptional cointegrators, which are large, highly conserved multifunctional proteins that integrate the functions of different transcription factors and signaling pathways at target gene promoters (Figure 9.3). CREB-binding protein (CREBBP or CBP) was originally identified as protein localized in the nucleus that was recruited by cyclic adenosine monophosphate (cAMP) response element-binding protein (CREB),[46] and has since been demonstrated to mediate important facets of cell growth in a wide variety of physiological settings, including reproduction. In addition to associating with CBP as a member of a multi-subunit nuclear complex, CBP has been shown to possess intrinsic histone acetyltransferase (HAT) activity to target the function of many nuclear proteins in addition to histones, and also acts as a scaffold to stabilize additional protein interactions with the transcription complex. CBP has been shown to be an important player in gonadal steroid action by functioning as a coactivator for AR[47] and ESR.[48]

Transcriptional interference between SRs and other transcription factors for limiting amounts of CBP in the cell is thought to be an important facet of the appropriate distribution of cellular signaling inputs between distinct afferent signaling conduits. Extensive regions of CBP, including the bromodomain, the cysteine-rich region, and the HAT domain, share significant regions of very high sequence similarity with EP300, the adenovirus E1A-associated cellular p300 transcriptional coactivator protein.[49] While often implied as synonyms for a single functional entity, these are distinct proteins with distinct functions, albeit with a considerable degree of functional redundancy. Similar to CBP, the HAT activity of EP300 participates in the regulation of transcription and chromatin remodeling by diverse transcription factors, including CREB and hypoxic-inducible factor 1 A (HIF1A), and is a critical player in the regulation of cell proliferation and differentiation. Given the close functional connections between CREBBP and EP300, the identification of the AR as a direct target of p300[50] and ESRs[51] is intuitive. Another group of highly conserved factors that have been shown to participate in classical genomic androgen signaling is the Mediator (MED) complex.[52] One of the critical steps in transcriptional regulation is at the nexus of regulatory factors and the

initiation of transcription by RNA polymerase II, and the MED complex is known to be a critical player at this interface. As with CBP and p300, members of this complex participate in signaling by many transcription factors, including specificity protein 1 (SP1). Chromatin immunoprecipitation (ChIP) assays have demonstrated that MED1 is recruited to the PSA promoter in ligand-stimulated LNCaP cells, indicating that the MED complex has a critical role in modulating signaling by AR,[53] and similar observations have been made in the case of ESR and E2.[36]

DNA and Chromatin Modification

Eukaryotic DNA is packaged into a naturally repressed three-dimensional network supported by numerous intricate, hierarchical protein–DNA interactions, and this default silenced state requires the mediation of large, energy-consuming protein complexes involved in DNA and chromatin modification (see Figure 9.3). These complexes carve out functional niches in the vicinity of target genes to effect access for transcriptional regulatory complexes. Members of the ATP-dependent chromatin remodeling complex SNF–SWI (sucrose nonfermentable and Switch), including SMARCAs (SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily a), SMARCDs, and SMARCEs, are recruited by activated ESR[54–56] and PGR[57–59] to bring DNA helicase and ATPase activities to bear on local chromatin structure and facilitate transcriptional activation. TDG (thymine–DNA glycosylase),[60] a member of the TDG–mug DNA glycosylase family, strips thymine moieties from mismatched G–T pairings that occur during DNA replication and recombination. It achieves this through hydrolysis of the carbon–nitrogen bond in the backbone of the DNA double helix and the mispaired thymine, thereby playing an important role in mitigating against spontaneous deamination of 5-methylcytosine and cytosine.

Consistent with recent studies demonstrating the importance of DNA breakpoint repair in gonadal SR function,[61,62] two proteins representing important classes of DNA maintenance proteins have been implicated as coregulators of PGR action. High-mobility group (HMG) proteins[63] constitute members of the nonhistone chromosomal HMG protein family that are widely expressed and present in the nuclei of higher eukaryotic cells. By virtue of their ability to induce bending in DNA and the formation of DNA circles, as well as mediate breakpoint repair and ligation, HMG family members have been speculated to be involved in long-distance interactions between regulatory DNA elements, and transcription regulatory factors HMG1 and HMG2 have been demonstrated to increase the DNA-binding affinity of PGR and to stimulate the transcriptional activity of PGR in target cells.[64] It can be reasonably speculated that they endow PGR with the ability to activate or repress transcription over long genomic distances, conceivably through the de novo creation and repair of breakpoints, as has been shown in the case of AR.[62] Moreover, long-range looping interactions between the promoter and enhancer during prolactin and glucocorticoid regulation of the beta-casein promoter are inhibited by liganded PGR.[65] XRCC6 (X-ray repair complementing defective repair in Chinese hamster cells 6), also known as KU70, is a member of a nuclear DNA protein kinase-containing complex that functions as a DNA- and ATP-dependent helicase whose activity is required for double-strand break repair. The KU70 complex has been shown to interact with PGR in vitro,[66] suggesting a potential role for this holoenzyme in P regulation of gene expression.

RNA Helicases

RNA–protein interactions play central roles in the posttranscriptional regulation of gene expression, serving as the basis for feedback regulation of the mRNA output of a specific promoter by direct binding of an RNA-binding protein to its own RNA,[67] or for mediating the actions of microRNAs.[68] A major class of RNA-binding proteins is the RNA helicases, which represent the largest group of enzymes in RNA processing metabolism, and are critically required for all cellular processes relating to RNA. Members of the DEAD box subfamily proteins of ATP-dependent RNA helicases,[69] defined by the presence of the eponymous conserved Asp–Glu–Ala–Asp (DEAD) motif, have been linked to a variety of cellular processes involving modifications of RNA secondary structure, including transcript splicing, translation initiation, and the assembly of ribonucleoprotein complexes. Members of this family, DDX5 and DDX17, have been shown to coregulate ESR-dependent regulation of gene expression.[36] While the concept of regulation of mRNA levels has been a central component of the model of steroid hormone action since the early 1970s, it was only with the discovery of an interaction between members of the U2AF family of RNA-binding proteins and ESR[70] that the notion of qualitative regulation of promoter output gained credence. U2AF is a bi-subunit protein that is recruited to exon–intron boundaries in nascent mRNA transcripts, and also forms specific interactions with specific intronic polypyrimidine sequences during spliceosome assembly in a manner thought to stabilize its overall structure.[71]

General Transcription Factors

Initiation of transcription at RNA polymerase II-regulated genes is a complex process (Figure 9.3), requiring the mediation of over 60 distinct factors, arguably the most important of which is transcription factor IID (TFIID), a multi-subunit factor composed of the

TATA-binding protein (TBP) and an array of highly conserved proteins known as TBP-associated factors, or TAFs.[72] TFIID binds to core regions of gene promoters to serve as a platform to stabilize the construction of additional members of the transcriptional pre-initiation complex. In addition to the wealth of coregulators that participate in estrogen signaling, in part by recruitment of general transcription factors (GTFs) (Figure 9.3), the direct interaction of activated ESR with TFIID[73] suggests that it and possibly other SRs retain the intrinsic ability to directly contact basal transcription factors to coordinate the assembly of the protein complexes required to initiate transcription.

Transcription Factor Cross Talk

Genomic steroid signaling regulates cellular functions indirectly through modulating the expression of target genes via the classical gonadal SRs. As has been discussed here, this can occur by direct binding of the receptor homodimer to estrogen (EREs), P (PREs), or androgen response elements (AREs) in the vicinity of their target genes. A second mode of action, known as regulation in *trans*, is by tethering indirectly to other promoter sites through protein–protein interactions with members of other classes of transcription factors such as Sp1 and Fos–Jun (AP1), a phenomenon that presumably acts to integrate the multiple stimuli to which cells are subjected from endocrine, paracrine, and autocrine inputs. FOXA1 (forkhead box A1) and FOXO1 (forkhead box O1) are members of the forkhead class of DNA-binding proteins,[74] which are defined by possession of a unique forkhead domain, and they have been shown to play a role in tethering a variety of transcription factors to DNA promoters. Both have been shown to act as modulators of AR-[75,76] and ESR[77,78]-mediated transcription, suggesting that the interaction of activated SRs with their target genes may not exclusively rely upon interactions between the DBD and HREs for these receptors, as has historically been held to be the case.

The importance in estrogenic signaling of *trans* interactions between ESR and transcription factors engaged by other cellular signaling transduction pathways is becoming increasingly well understood, and has prompted a radical revision of our notion of what constitutes an ESR target gene. Building on earlier observations,[77,78] Myles Brown's laboratory has shown that FOXA1 is required for the majority of DNA-binding events in E2 stimulated MCF7 cells.[79,80] The interaction of ESR with hepatocyte nuclear factor 4 alpha,[81] a critical factor in the development of the hepatic, renal, and digestive systems, highlights the importance of the interactions of ESR, not only with other classes of transcription factors but also with other members of the NR superfamily. The overarching principles to emerge are that the requirement for interaction of activated ESR with EREs in its target genes is an obsolete notion, and that target genes are defined less by their physical properties and more by the specific signaling context and the complement of ancillary transcription factors expressed and appropriately activated in a given cell.

Inflammatory processes are fundamental to the regulation of cellular proliferation, and many proinflammatory stimuli, such as those initiated by cytokines and microbial products, are mediated by nuclear factor kappa beta (NFκB).[82] When released from a repressive complex in its activated form, this transcriptional regulator undergoes nuclear translocation and stimulates the expression of a large number of genes. Transcriptional interference between AR and RelA, one of the subunits of NFκB,[83] suggests that mutual antagonism between these transcription factors may be due to competition for a common coactivator such as CBP, and is an important mechanism for load balancing between these transcription factors.[83] The mammalian SMAD family of proteins is evolutionarily related to *Drosophila* "mothers against decapentaplegic" proteins (Mad) and function as signal transduction factors and modulators of gene expression for myriad signaling pathways.[84] A broad spectrum of biological responses is subject to regulation by SMAD proteins, including cell proliferation and death, cell–cell communication, morphogenesis, and immunological responses. The identification of functional interactions between androgen-activated AR and SMAD1,[85] SMAD3,[86] and SMAD4[87] suggests that cooperation between this family of proteins and AR, and conceivably generally across the NR superfamily, represents a broad thematic element in the regulation of eukaryotic gene expression.

Two other transcription factors shown to be recruited by ESR in its mediation of estrogenic signaling are SP1 and X-box binding protein 1 (XBP1). SP1 is a well-characterized bifunctional (activation and repression) zinc finger transcription factor implicated in multiple processes in cellular physiology, including cell growth and differentiation, apoptosis, DNA damage repair, and remodeling of chromatin.[88] SP1 has been identified, in addition to FOXOA1, as another important transinteracting factor that mediates ERE-independent interactions with target genes.[89] XBP1, another basic leucine zipper domain (bZIP) transcription factor, is thought to play a role in the processing of unfolded proteins in the endoplasmic reticulum, and has been shown to contribute to ligand-independent activation of ESR.[90]

The transcription factor BRCA1 plays a major role in the maintenance of genomic stability and has been exhaustively characterized as a tumor suppressor protein whose functional derangement contributes to the progression of a large percentage of hereditary breast cancers.[91] BRCA1 has been shown to be present in a large multi-subunit complex, referred to as BASC (BRCA1-associated genome surveillance complex),

which contains DNA damage sensors and other signal transducers and which is thought to be involved in DNA repair of double-stranded breaks and recombination. BRCA1 has been shown to cross talk with AR signaling,[92] an observation that echoes more recent work documenting the role of DNA breakage and repair in the long-range regulation of transcription by activated AR complexes.[62] The appearance of translocation breakpoints during SR-mediated transcriptional regulation has been attributed to the binding of liganded AR to specific intronic regions and the induction of DNA-modifying enzyme complexes,[62] phenomena that trigger the rapid intra- and interchromosomal interactions that are required for translocations.

While better characterized in the case of AR and the ESRs, the theme of transcription factor cross talk extends to PGR, and is reflected in its functional interactions with basic leucine zipper transcription factor 3 (BATF3),[93] Kruppel-like factor 9 (KLF9; aka BTEB1),[94] and BPTF.[45] BATF3 is a member of the basic leucine zipper transcription factor family, deficiency of which has been associated with deficits in the development of lymphocytes, antigen cross-presentation, and cytotoxic T lymphocyte responses to viral infection. BATF3, also known as JDP1, has been shown to bind to the PGR DBD in a hormone-independent manner, suggesting a possible mechanistic basis for the historically well-established role of P signaling in modulation of the immune system during pregnancy. The protein encoded by the KLF9 gene is a Kruppel-like transcription factor that interacts with GC box elements in the promoters of its target genes to alternately potentiate or repress mRNA expression. KLF9 has been shown to interact discriminately with the two major PGR isoforms, PGR-A and PGR-B, potentiating ligand-dependent PGR-B-mediated transactivation and augmenting PGR-A inhibition of PGR-B activity.[94] The ubiquitin ligase activity of BRCA1 has been shown to be involved in regulating the rate of PGR protein turnover,[95] thereby controlling the rate of PGR recruitment to target genes. Moreover, by mediating ubiquitination of histone H2A, BRCA1 modulates transcriptional repression of PGR-regulated genes. It has been speculated that mutations in BRCA1 would relieve the repression of PGR target gene expression and provide for an environment in which proliferation of the mammary gland epithelium—a risk factor for breast cancer—can proceed.

Steroid Transcriptional Targets

Activated SRs regulate the tissue-specific transcription of a large number of genes, the diversity of whose functions reiterate the crucial role that gonadal steroids play in coordinating physiological responses in a large number of tissues. This section discusses selected transcriptional targets of the three SRs to afford the reader an insight into some of the myriad cellular processes that are subject to regulation by gonadal steroids.

The *TMPRSS22* gene[96] encodes a protein with a number of different functional domains, including a type II transmembrane domain, a receptor class A domain, a scavenger receptor cysteine-rich domain, and a protease domain. TMPRSS2 has been shown to be robustly induced by AR[97,98] and is downregulated in androgen-independent prostate tumors, and a fusion of the TMPRSS2 gene with the erythroblast transformation-specific (ETS)-related gene, a member of the ETS family of transcription factors, is one of the best characterized genetic lesions in prostate cancer. Another strongly induced androgen-signaling target gene, *FKBP5* (FK506 binding protein 5),[99] encodes a *cis–trans* prolyl isomerase that is a member of the immunophilin family of proteins, whose members play critical functions in immunoregulation. Along with HSP90 and HSP23, FKBP5 has been shown to interact functionally with mature hetero-oligomeric PGR in the absence of their ligand, hinting at a role for mutual antagonism between AR and PGR in the regulation of their respective target gene networks. Moreover, the role of an AR-regulated gene in immune processes is redolent of empirical observations of a sex bias in autoimmune disease.[100] In terms of androgen repression of target gene expression, members of the ADAMTS (a disintegrin-like and metallopeptidase (reprolysin type) with thrombospondin type 1 motif) family,[101] including ADAMTS1 and ADAMTS15, are downregulated by AR.[97,98] Members of the ADAMTS family share several distinct protein modules, including propeptide, metalloproteinase, and disintegrin-like domains and a thrombospondin type 1 (TS) motif. Expression of ADAMTS15 has been shown to be predictive for survival in certain tumors, inviting speculation that downregulation of this gene by AR is an important component of their stimulation of prostate cancer growth.

Estrogen-regulated gene expression programs have been exhaustively studied in cancer cells in recent years using a combination of expression microarrays, RNA-Seq, and genome-wide location analysis (GWLA). Collectively, these studies have reported different numbers of E2/ESR-regulated genes in MCF7 (Michigan Cancer Foundation 7) breast cancer cells, ranging from ~500 to ~3000 genes, depending on the duration of ligand treatment and the techniques to quantify expression levels. These and previous studies (for references, please refer to the Transcriptomine database[97]) have defined a group of gold-standard E2-regulated genes, including trefoil factor 1 (*TFF1*), growth regulation by estrogen in breast cancer 1 (*GREB1*), *CCND1* (cyclin D1), insulin-like growth factor-binding protein 4 (*IGFBP4*), adenosine A1 receptor (*ADORA1*), and *MYC* (v-myc avian myelocytomatosis viral oncogene homolog). TFF1 is a member of the trefoil family of proteins, which contain one or

more copies of the trefoil motif, a 40-amino-acid residue domain that contains three conserved disulfide bonds. TFF1 has been shown to suppress transcription of the CDH1 gene to enhance cell migration and invasiveness.[102] GREB1 is a rapidly induced E2 target gene that has been shown by numerous studies[103,104] to stimulate cell growth and proliferation in E2 target tissues such as the mammary gland. CCND1 and MYC are key players in the regulation of the cell cycle, CCND1 being a cyclin whose function is required for the G1/S transition,[105] and the transcription factor oncoprotein MYC being a well-established regulator of multiple genes in cell cycle progression and cellular transformation.[106] In addition to primarily nuclear proteins, E2 also induces genes encoding proteins that participate in pro-proliferative cellular kinase cascades. ADORA1 is the adenosine A1 receptor,[107] a member of the G-protein coupled receptor (GPCR) superfamily of receptors, and IGFBP4 binds to and potentiates the function of insulin-like growth factors (IGFs), which are well-known signals for stimulation of cell growth. Collectively, the functions of these effectors of estrogenic signaling are reflective of the role of estrogen in enhancing cell growth, proliferation, motility, and survival.

Activation by P signaling of the IHH (Indian hedgehog), WNT (wingless-type MMTV integration site), and BMP (bone morphogenetic protein) pathways in the epithelial and stromal compartments of the uterus mediate embryo attachment and implantation, subsequent decidualization of the underlying stroma, as well as uterine glandular development (see Chapter 24). A variety of genes have been implicated as effectors of P-induced, PGR-dependent cellular proliferation, morphogenesis, and differentiation, including Wnt4, ID4 (DNA-binding protein inhibitor), receptor activator of nuclear factor kappa-B ligand (RANKL), IHH, and heart- and neural crest derivatives-expressed 2 (HAND2). Members of the Wnt protein family play widespread roles in the regulation of differentiation and cell fate. Wnt activity on target cells involves interaction with frizzled (Fz), a transmembrane receptor that sets off a series of intracellular events, culminating in the formation by β-catenin of complex with TCF, a transcription factor that mediates induction of proliferative downstream genes.[108] Expression of Wnt4 in the mammary epithelium increases during pregnancy to initiate ductal proliferation and morphogenesis through induction of cyclin D1 and p21. ID4 is a member of the basic helix–loop–helix (HLH) family of Id proteins, which function as corepressors of other bHLH transcription factors to inhibit differentiation.[109] ID4 is acutely induced by P4 in the mammary gland of ovariectomized adult mice and is induced during pregnancy in ESR+/PGR+ luminal epithelial cells, which increase dramatically and transiently during early pregnancy; this increase was restricted to ESR+/PGR+ luminal epithelial cells, indicating that ID4 may be involved in the early proliferative expansion phase of P signaling-dependent morphogenesis. RANKL, which is acutely induced by P in the mammary gland in a PGR-dependent manner, is a cytokine member of the tumor necrosis factor alpha (TNFα) superfamily that signals through a transmembrane receptor to activate signaling cascades and upregulate target genes, including the NFκB target gene cyclin D1. RANKL-null mutants are deficient in cellular proliferation and alveologenesis of the mammary gland during pregnancy. *Ihh*, a rapidly induced target gene, is a member of the hedgehog (Hh) signaling pathway that has been shown to be critical for development. Finally, induction of HAND2 by P mediates the inhibition of estrogen-induced epithelial proliferation in the uterus.[110]

NONTRANSCRIPTIONAL EFFECTORS OF GONADAL STEROIDS

Cell Kinase Cascades

In addition to the classical steroid-dependent, SR-dependent genomic model of SR action (Figure 9.3), substantial evidence exists for rapid actions of cytoplasmic SRs engaging effector proteins associated with cellular kinase cascades, in what is referred to as the steroid-dependent, SR-dependent nongenomic model of steroid action (Figure 9.3). Reciprocal functional interplay between cellular kinase pathways, in particular those regulated by growth factors, and estrogenic signaling was suggested as long ago as 1981 by Ferdinando Auricchio's group, who furnished biochemical evidence for its regulation by cytoplasmic phosphorylation and subsequent nuclear dephosphorylation.[111] Since that time, the discovery that an abundance of proteins that participate in these kinase cascades are in fact cytoplasmic SR effector proteins sketches a mechanistic basis for these pioneering discoveries.

The serine–threonine protein kinase encoded by the v-akt murine thymoma viral oncogene homolog 1 (*AKT1*) gene[112] has been shown to be activated by a pathway regulated by phosphatidylinositol 3-kinase and the platelet-derived growth factor, illustrating the cross talk that occurs through phosphorylation by cellular kinase pathways of AR and other SRs by cellular kinases.[113,114] AKT is an important regulatory factor in the developing nervous system, suggesting a potential role in regulation of androgen action in the CNS. AKT1 has also been shown to inactivate components of the apoptotic machinery by phosphorylation and has also been shown to downregulate androgen-induced apoptosis through phosphorylation and inhibition of the AR,[115] as well as integrating with ESR signaling.[116] The calmodulin 1 (CALM1) gene encodes a member of the EF-hand

calcium-binding protein family,[117] which has been demonstrated to interact with other members of this family as a multi-subunit, reversible phosphorylase–kinase complex, and is also known to recruit AR and modulate androgen signaling.[118] The regulation of intracellular calcium storage and release is a critical component of cellular signaling. The multifunctional calreticulin protein[119] is a major player in the binding and storage of Ca(2+) binding in the endoplasmic reticulum lumen. Interestingly, calreticulin has been shown to bind to the synthetic peptide sequence KLGFFKR, which bears a high degree of similarity to a peptide sequence in the DBD of members of the NR superfamily. Reflecting this, data indicate that calreticulin acts as a coregulator of the transcriptional AFs of modulator of AR and other SRs.[120]

Peptide ligand receptors (PLRs; Figure 9.3) are cell surface transmembrane glycoproteins that are activated by the binding of peptide ligands, which induce receptor dimerization and tyrosine autophosphorylation and activation of downstream kinase cascades targeting steroid receptors and coregulators. EGFR (epidermal growth factor receptor) is a member of the ErbB family of receptors, which includes HER2/neu, and whose members transduce signals that profoundly and rapidly stimulate cellular growth and proliferation and repress apoptosis.[121] Unsurprisingly, dysregulation of the function of these receptors has been consistently linked to myriad human cancers. ESR has been shown to be activated by EGFR phosphorylation[122] in a manner independent of its DNA-binding ability, indicating that the pro-proliferative and anti-apoptotic effects of this interaction may be attributable, at least in part, to tethering of ESR to other DNA-bound transcription factors.

A-kinase anchor proteins (AKAPs)[123] interact with and sequester the regulatory subunit of PKA (protein kinase A) to specific regions within the cell. One member of this family, AKAP13A, exists in a variety of splice variants containing a patchwork of functional motifs, including the dbl oncogene homology (DH) and pleckstrin homology (PH) domains. By effecting guanine nucleotide exchange activation of the Rho–Rac family of GTP-binding proteins, the DH domain drives conversion of the inactive GTPase to the active signal transduction-competent form. Evidence suggests that by interacting with components of different pathways, AKAP13A and other AKAPs serve as scaffolding proteins to coordinate flux through parallel afferent signaling pathways. Evidence casting AKAP13A as a physical and functional partner of ESR[124] invites conjecture that in doing so, it provides a nexus between ESR and cellular kinase pathways. Another growth factor receptor tyrosine kinase, IGF1R,[125] binds insulin-like growth factor to exert anti-apoptotic effects and is robustly overexpressed in a variety of malignant tissues, where it enhances cell survival. The binding of the ESR1, but not ESR2, isoform with IGF1R[126] demonstrates that ESR2 is required for full function of the IGF1R regulated signaling pathway. MAPKs, also referred to as extracellular signal-regulated kinases (ERKs), are intracellular kinases that act as molecular gates to integrate numerous afferent signaling inputs from the host of cell surface receptors amassed on the surface of eukaryotic cells.[127] Signals conveyed by kinase-activated MAP kinases ultimately regulate the activity of transcription factors in the nucleus to modulate cellular gene expression patterns, through which they impinge upon practically every known cellular function, including proliferation, differentiation, and apoptosis. Numerous studies have documented the phosphorylation and functional regulation of ESR by members of the MAPK family, including MAPK1,[128] MAPK3,[129] and MAPK14,[130] an observation that has also been made for PGR.[131]

Downstream of MAPKs are members of the MKNK (MAP kinase interacting serine–threonine kinase) family of proteins, which are activated through interactions with MAPKs.[132] In addition to conserved DLG (Asp–Leu–Gly) and ENIL (Glu–Asn–Ile–Leu) motifs, MKNK2 contains an amino-terminal region containing multiple basic amino acid residues. This motif engages importin A, a cytoplasmic–nuclear shuttle protein, and phosphorylates the translation factor scaffold factor eIF4G during the initiation of mRNA translation. A role for MKNK2 in protein processing is also hinted at based on its interaction with members of the CBC (VHL (Von Hippel–Lindau)) ubiquitin ligase E3 complex, including VHL, ring-box 1 (Rbx1), and cullin 2 (CUL2). The identification of MKNK2 as an ESR-interacting protein[133] reflects previous work identifying ubiquitin ligases as important coregulators of estrogenic signaling, and reiterating the importance of efficient processing of receptors for optimum signal transduction. Providing further evidence of the importance of receptor recycling in estrogen-mediated signal transduction, ESR has been shown to recruit COPS5 (COP9 complex homolog subunit 5)–Jab1, a member of the E3–ubiquitin ligase-associated COP9 signalosome, in an interaction that increases the rate of ligand-induced ESR degradation.[134]

Two proteins upstream of MAPKs that are involved in the regulation of cell growth and apoptosis, GADD45B and GNAI1, have been characterized as functional partners in signaling by ESR. GADD45B (growth arrest and DNA-damage-inducible, beta)[135] is induced in response to cellular stress, such as that which follows treatment with DNA damage agents, and in turn it activates MAP3K4 (mitogen-activated protein kinase kinase kinase 4, or MEKK4) an activator of the p38–JNK (Jun amino-terminal kinases) pathway. While GADD45B plays a variety of context-specific roles in the cell, it is, along with related proteins such as GADD45G (growth arrest and DNA-damage-inducible, gamma; also called

cytokine-responsive protein (CR6)), primarily a negative regulator of cell growth. It was identified as an interacting partner of ESR in a yeast two-hybrid screen, a method commonly used during the early characterization of ESR-interacting proteins.[136] While the yeast two-hybrid screen platform has generated a daunting amount of data, it should be borne in mind that there is a fundamental distinction between interactions of which a given protein is *capable* and those that are physiologically *meaningful*. In order to fully validate an interaction for a given protein, therefore—in this case, coregulation of ESR—comprehensive in vivo validation in terms of gain and loss of function must be demonstrated.

As is the case with androgen and E2 signaling, P-mediated signaling exhibits a high degree of codependency with cellular kinase cascades, and abundant evidence suggests multiple points of vertical integration of these signaling axes, exemplified by the identification of the tyrosine kinase SRC (v-src avian sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog, not related to SRC1, the name by which NCOA1 was originally cloned) and FGFR2 (fibroblast growth factor receptor 2) as coactivators of PGR-dependent regulation of gene expression (see steroid-dependent, SR-independent nongenomic signaling; Figure 9.3). SRC bears a high degree of similarity to the protein encoded by the v-src gene of Rous sarcoma virus and has been implicated as a proto-oncoprotein with a role in the development of a variety of human tumors. Motifs within SRC, referred to as SRC homology 3, are found in a variety of cytoplasmic signaling molecules.[137] Work in the laboratory of Dean Edwards identified a polyproline motif in the amino terminus of the human PGR involved in the formation of P-dependent complexes between PGR and the SH3 domains of HCK (hemopoietic cell kinase), GRB2 (growth factor receptor-bound protein 2), and SRC itself. Mutational analysis showed that rapid P signaling was dependent on the integrity of this motif, implicated in the accelerated transduction of P-initiated membrane signaling to cytoplasmic and nuclear targets.[138] Upstream of SRC tyrosine kinases are membrane receptors for a variety of peptides and polypeptides that regulate the functions of eukaryotic cells. FGFR2 is a member of the conserved fibroblast growth factor receptor family, members of which comprise an extracellular region composed of three immunoglobulin-like domains, a hydrophobic membrane domain, and a cytoplasmic tyrosine kinase moiety. The interaction of the extracellular region of the protein with fibroblast growth factor 2 initiates a cascade of cytoplasmic phosphorylation event signals with nuclear and cytoplasmic endpoints in the regulation of cell growth, proliferation, and differentiation. A complex functional interaction between FGFR2, STAT5 (signal transducer and activator of transcription 5A), and PGR has been shown to potentiate PGR regulation of gene expression,[139] exemplifying the ability of PGR to tap into these diverse signaling pathways at the level of both the growth factor receptor and the transcription factors.

Cell Cycle Proteins

The interaction of SRs with a variety of factors with functions related to regulation of the cell cycle points to the profound effect of steroids on the proliferative capacity of their target tissues. Somewhat counterintuitively, given the role of AR and ESR1 in the progression of prostate cancer and breast cancer, respectively, several of these factors function as tumor suppressor proteins. RB1, for example, one of the first genes to be identified as a tumor suppressor gene,[140] is a negative regulator of the cell cycle and has been shown to mediate stabilization of cellular heterochromatin. RB1 has been shown to interact with and regulate the function of AR,[141] suggesting that it induces the transcriptional activation potential of AR in prostate cells, and postulating a link between negative regulation of growth and AR function, potentially as part of a feedback loop.

Several other cell cycle regulators are important modulators of SR function. Cyclin D1 and cyclin E1 belong to the highly conserved cyclin family, expression of which is subject to sensitive regulation during the cell cycle.[142] Cyclins are known to regulate the functions of a number of CDKs (cyclin-dependent kinases), members of the Ser–Thr protein kinase family that have been shown to function as part of large protein complexes, and whose activity is required for cell cycle G1/S transition. Coordinating of mitotic timing and regulation of mitotic checkpoints is thought to be a function in large part of the variable expression patterns of cyclins during distinct phases of the cell cycle. Cyclin D1[143] and cyclin E1[144] have been shown to interact with and regulate the function of AR, reflecting a close connection between the phosphorylation status of AR and regulation of the cell cycle. Moreover, a direct connection between CDKs and AR function has been demonstrated for CDK1,[145] CDK6,[146] CDK7,[147] and CDK9.[148] The numerous functional interactions between AR and cell cycle regulatory proteins provide a mechanistic window into the regulation by AR of a variety of "immediate early" genes involved in cell cycle regulation, including FOS (FBJ murine osteosarcoma viral oncogene homolog) and JUN (jun proto-oncogene) (for references, see the Transcriptomine database of NR-regulated genes[97]).

Ubiquitin Pathway

The spindle checkpoint assembly plays an important role in chromosomal surveillance and in ensuring appropriate chromosomal segregation. CUEDC2 (CUE domain

containing 2),[149] a ubiquitin-binding motif-containing protein, is a cell cycle regulator known to be involved in spindle checkpoint inactivation, and cellular depletion of this protein has been shown to cause a checkpoint delay in the metaphase–anaphase transition. CUEDC2 has been shown to act as a corepressor of PGR and to increase its rate of degradation by the ubiquitin proteasome system,[150] hinting at a functional link between P signaling and regulation of the cell cycle. Furthermore, expression of the *UBR5* gene, which encodes a member of the HECT (homology to E6-AP carboxyl terminus) family of E3 ubiquitin-protein ligases, is induced by P. UBR5 has been shown to interact with and potentiate the transcriptional activity of PGR,[151] hinting at a possible role for this protein in a feed-forward mechanism to potentiate cellular sensitivity to P signaling. While ubiquitination targets proteins for degradation, other posttranslational modifications act as molecular postcodes, directing target proteins to specific cellular destinations or to carry out specific spatiotemporal functions. SUMO1 (small ubiquitin-like modifier 1) is a member of the small ubiquitin-like modifier class of proteins, which stages target proteins for functions in processes as diverse as nuclear transport, regulation of gene expression, apoptosis, and the modulation of protein stability.[152] The characterization of PGR and the pan-coregulator NCOA1–SRC-1 as molecular targets of SUMO1, and the potentiation of PGR transcriptional activity that occurs pursuant to sumoylation, cast this protein as a critical modulator of PGR-dependent gene expression.

Members of the PIAS (protein inhibitor of activated STAT) family of transcriptional coregulators have been shown to function as modulators of androgen signaling.[153] PIAS3, for example, is a SUMO–E3 ligase that mediates the covalent attachment of a SUMOylated protein to specific target substrates. PIAS1, which contains a zinc-binding domain and a region rich in acidic amino acid residues, has been shown to inhibit transcriptional regulation by members of the STAT family of transcription factors, not only reiterating the importance of coregulators in integrating signaling across distinct SRs, but also encompassing multiple distinct classes of transcription factors.

The Cytoskeleton: Cell Trafficking, Motility, and Cell–Cell Communication

A large number of steroid receptor effector proteins tie into functions associated with the cellular skeleton, including cell–cell communication, cell motility, and subcellular trafficking. Two proteins with functional connections to the cytoskeleton and subcellular trafficking, major vault protein (MVP)[154] and integrin beta 3 binding protein (ITGB3BP),[155] have been demonstrated to function as ESR effector proteins. MVP[156] is a member of the vault complex, a series of large RNA–protein structures thought to be involved in transport between the nucleus and the cytoplasm, and has been shown to be the target of a variety of cellular kinase cascades, including phosphoinositide 3-kinase (PI3K)–Akt, Janus kinase (JAK)–STAT, and MAPK-mediated pathways. MVP has been shown by a variety of studies to be a potential prognostic marker in multiple distinct tumor types. The encoded protein may play a role in multiple cellular processes by regulating the MAPK, JAK–STAT, and signaling pathways. Integrins are cellular receptors that, as their name implies, are integrated into the plasma membrane of eukaryotic cells, which play an important role in cell–cell interactions. Integrin-binding proteins, such as ITGB3BP,[157] bind to the cytoplasmic domain of these receptors to mediate the transduction of signals to downstream effector proteins, thereby communicating the status of the extracellular environment to signaling pathways within the cell. ITGB3BP is also present in the centromere-specific histone H3 variant nucleosome associated complex (CENP-NAC), hinting at a possible role in the regulation of mitosis. As well as being shown as a coactivator for ESR and other members of the NR superfamily, including TRs and RXRs, ITGB3BP silences transcription by NFκB-regulated transcription factors. This Janus-faced functionality (i.e., a given protein acting as an activator or coactivator in one context and a repressor or corepressor in another) is a recurring theme in NR coregulator biology and, in addition to illustrating the remarkable economy of eukaryotic cellular physiology, reiterates the need to avoid classifying coregulator function solely on the basis of the strictly defined experimental conditions in which they were initially characterized. The *FHL2* gene encodes a member of the four-and-a-half-LIM-only protein family, containing two characteristic, highly conserved, tandemly arranged zinc finger domains in which four highly conserved cysteine residues chelate a zinc atom in each of the zinc fingers. FHL2,[158] a cardiac-specific protein, is thought to have a role in the assembly of extracellular membranes, and was identified in a yeast two-hybrid based screen for ESR-interacting proteins in a human heart cDNA library.[159]

The existence in the genome of high-affinity binding sites for SRs has been known since early biochemical experiments on steroid hormone biology, and the discovery of coregulators has shed additional light on these seminal observations. SAFB[160] is a DNA-binding protein with high specificity for scaffold or matrix attachment region DNA elements (S/MAR DNA), which are eukaryotic sequences where chromosomes anchor to the surrounding nuclear matrix. Members of the scaffold attachment factor family have been postulated to act as molecular foundations on which large transcriptional complexes of the type known to be involved in androgen signaling are assembled on target gene promoters.

SAFB, which has been implicated in the etiology of breast cancer, has been shown to function as an ESR corepressor,[161] although, as with other corepressors, this does not exclude the possibility that it can function as a coactivator in different physiological or signaling contexts. Another member of the SAF family, SAFB2, has also been shown to function as an ESR corepressor.[162] While SAFB1 is primarily localized to the nucleus, SAFB2 is also distributed in the cytoplasm and has been shown to interact with vinexin, a protein implicated in integrating cellular signaling with cytoskeleton components. Collectively, these results reinforce the importance of communication between ESR signaling and the cytoskeleton and shed additional light on the intimate connection between estrogenic signaling and cellular mobility.

In opposition to the tumor suppressors that regulate AR function is the oncoprotein and scaffolding factor CAV1 (caveolin 1), an important component of the caveolae membranes, which has been shown to play a role in cell–cell communication by mediating functional interactions between integrin subunits and the Ras–ERK pathway to promote progression through the cell cycle.[163] Using a mammalian two-hybrid assay system, it has been shown that AR interacts with the N-terminus of CAV1,[164] a protein that promotes cell survival and angiogenesis, thereby giving some insight into a mechanism for the role of AR as a tumor promoter, at least in the case of prostate cancer.[165]

ESR cell biology is closely linked to the regulation of cell division and cellular motility and, by extension, the cytoskeleton, and this is reflected in the involvement of several coregulators with the cytoskeleton. ESR has been shown to interact with catenin β, which is encoded by the *CTNNB1* gene,[166] and which was originally identified as a component of a complex of factors that form adherens junctions, which are required for the establishment and maintenance of mechanical attachments between epithelial cells. CTNNB1 has been shown to play an anchoring function in connection with actin components of the cytoskeleton, and a role for this protein has been intimated in transducing contact inhibitory signals that are involved in bringing a halt to cell division upon completion of the epithelial cell layer. A functional relationship between ESR and CTNNB1 was first demonstrated during cross talk between ESR and signaling by the Wnt family of proteins,[167] which are conserved signaling factors that coordinate cell–cell interactions.

Caspases

Members of the caspase family of proteins exist in inactive pro-enzymatic forms, which undergo processing via proteolysis at select, evolutionarily conserved aspartic residues to produce large and small subunits that undergo dimerization to generate the active enzymatic form of the protein and play a central role in cellular apoptosis.[168] Enzymes in the caspase family carry out proteolytic cleavage to activate the precursor form of interleukin 1 (IL1), a factor involved in a variety of processes related to cellular inflammation, and have been implicated in the etiology of neurodegenerative diseases. Several members of the CASP family, including CASP1, CASP3, CASP7, and CASP8,[169] have been shown to interact with and regulate the function of AR.

STEROID SIGNALING VIA MEMBRANE RECEPTORS

Genomic steroid signaling originates either via activation of SRs through interactions with their cognate ligands or by activation of SRs via phosphorylation in the cytoplasm, followed by nuclear translocation (Figure 9.3). In contrast, rapid (within minutes of hormone exposure) signaling of gonadal steroids through a variety of membrane receptors (mSRs) activates myriad kinase-driven signaling cascades (PI3K, phospholipase C, MAPK, ERK, PKA/C, etc.) that can affect cell function directly—for example, through phosphorylation of cytoplasmic and membrane proteins, or indirectly through modulation of gene expression by targeting transcription factors (Figure 9.3). The body of work on mSRs notwithstanding, the focus of this chapter is largely on signaling by classical steroid receptors, and for a discussion of mSRs the reader is referred to current reviews on the topic.[4]

Of the three classes of gonadal steroids, signaling via mSRs has been characterized most comprehensively in the case of the estrogens, including proteins such as ER-X, GPR30,[170] and Gq-mER, whose functions have been demonstrated to be important for rapid estrogenic signaling in the brain, particularly the hypothalamus and cortex. ER-X is a high-affinity estrogen receptor that bears sequence homology to classical ESRs, and that is associated with caveolar-like regions in neocortical neurons.[171] GPR30 is a GPCR, which also has high affinity for E2 and which is thought to mediate expedited actions of E2 in reproductive tissue through the ERK pathway. GPR30 is also expressed in the hypothalamus, with peaks of expression in the supplementary cortex, the piriform cortex, the supraoptic nucleus, the paraventricular nucleus of the thalamus, and other areas,[172] although its function in these regions is not fully understood. Gq-mER, another membrane E2 receptor classified as a GPCR, was originally characterized in the hypothalamus, and has been suggested to engage a number of rapid signaling pathways through PKC and interacting protein 3 (IP3).[173] While membrane PGRs have been claimed to exist in some species, better characterized is rapid signaling of P through

interactions of cytoplasmic PGR with elements of the SRC pathway (see the section Cell Kinase Cascades). Nonclassical signaling by AR during spermatogenesis has been shown to take place via a variety of methods, including rapid T-mediated calcium influx, activation of a (GPCR), and subsequent activation of the PLC pathway, and via the direct interaction of the classical AR with the SH3 domain of SRC, which culminates in activation of the MAPK cascade.[174]

COREGULATORS AND GONADAL STEROID PHYSIOLOGY

One of the interesting aspects of SR coregulators is the mechanistic basis they provide for historical observations of steroid physiology. For example, abundant historical evidence links estrogenic signaling in multiple facets of the immune system, including resistance to infection and the stimulation of the growth of important classes of immune cells.[175] LCK, a member of the Src family of protein tyrosine kinases (PTKs), was originally identified as an important factor in the thymocyte development in the immune system.[176] LCK appears to be subject to extensive posttranslational modification such as palmitoylation and myristylation, and also contains Src homology domains that govern interactions with phosphotyrosine- and proline-rich motifs, respectively. Given the recruitment by ESR of LCK,[177] possibly to functionally engage cell surface receptors such as CD4 and CD8, a role for LCK can be envisaged in communicating the signaling status of other immunoregulatory signal transduction pathways to the ESR and vice versa. ESR-regulated transcriptomes in the immune system are further expanded through its interactions with CEBPB, a bZIP transcription factor that, in addition to homodimerizing to regulate transcription of its target genes, forms heterodimers with other members of the CEBP family.[178] CEBPB has well-defined roles in the regulation of gene networks involved in the immune and inflammatory responses. E2-dependent repression by ESR of IL6, a gene involved in mediating bone resorption by stimulating the maturation of immature osteoclasts, has been shown to be mediated through its interaction with NFκB and CEBPB, and demonstrates a clear dichotomy between the mechanisms of ESR transactivation and transrepression.[179]

The beneficial effects of estrogen on lipid metabolism in humans have been well characterized in the literature.[180] Estrogen replacement therapy, for example, is known to have beneficial effects on the incidence of cardiovascular disease, an effect that has been attributed to maintenance of optimal serum concentrations of low-density lipoprotein (LDL) cholesterol concentrations. The identification of two key players in lipid metabolism, SREBF1 and PPARGC1, as ESR coregulators sheds some light on the mechanistic basis for this effect, and reiterates the importance of coregulators in integrating multiple afferent signaling inputs to maintain homeostatic control over multiple organ systems. SREBF1, a member of the basic helix–loop–helix–leucine zipper (bHLH-Zip) transcription factor family, has been shown to regulate the expression of the LDL receptor gene, in addition to a variety of genes involved in sterol biosynthesis. E2 has been shown to regulate the expression of the high-density lipoprotein receptor (HDLR) scavenger receptor class B type I (SRBI) gene[181] in part through direct interaction with SREBF1, identifying this transcription factor as a coregulator of ESR signaling. PPARGC1 was first identified as a cold-inducible coactivator of PPARG, a master regulator of adipose tissue and a fatty acid-activated orphan member of the NR superfamily. It has since been shown to mediate transcriptional regulation by other members of the NR superfamily, in addition to other transcription factors such as CREB and nuclear respiratory factors. PPARGC1 is now known to be a critical player in whole-body energy homeostasis, with functions as diverse as regulation of mitochondrial biogenesis, specification of muscle fiber–type determination, regulation of blood pressure, and coordination of cellular cholesterol homoeostasis. The characterization of PPARGC1 as an ESR coactivator[182] identifies a key convergence point between PPARG and ESR signaling in the regulation of fat metabolism.

Xenobiotics, including dioxin and polycyclic hydrocarbons such as bisphenol A, are environmental or industrial compounds that impact the physiology of living organisms, and, as such, organisms have evolved a variety of mechanisms to process and excrete these compounds. The activation of a battery of genes encoding enzymes that catalyze their catabolism is a common mechanism among mammals, and one of the primary transcription factors that orchestrates the expression of these genes is the aryl hydrocarbon (Ah) receptor (AhR).[183] In the absence of ligand, cytosolic Ah receptor is sequestered with HSP90, but upon binding of xenobiotic ligands, nuclear translocation of the ligand-binding subunit occurs, followed by interaction with xenobiotic response elements in the promoters of its target genes. A coregulator for AhR, ARNT, has also been identified as a coregulator for ESR[184] and has been postulated as a mechanistic basis for transrepression by ESR of AHR-dependent regulation of gene expression. Interestingly, ARNT is a subunit of a heterodimeric transcription factor, adding further complexity to an already intricate model for interactions between ESR and other classes of transcription factors. AHR, also known as CUL4B, has been linked to ESR signal transduction directly, its E3 ubiquitin ligase functionality having been shown

to target ESR and other gonadal steroid receptors for degradation.[185]

INFORMATIC APPROACHES AND TRANSCRIPTIONAL REGULATION BY GONADAL STEROIDS

The advent of global discovery-driven data acquisition, or "-omics", platforms, especially transcriptomic and GWLA, has afforded researchers the opportunity to shed light on aspects of gonadal steroid action that, while characterized on an empirical level, have lacked a molecular basis that would place the effects of these hormones in the context of the wider systemic physiology of their target tissues.

Global transcriptomic platforms such as microarrays and, more recently, RNA-Seq have demonstrated themselves to be of considerable use in opening novel vistas for gonadal steroid biology, by virtue of the fact that an a priori hypothesis relating to a specific gene or gene network is not required. While extrapolation of data points from a single cell culture model to all steroid target tissues must be exercised with caution, and tissue-specific epigenomic factors ultimately influence the complement of genes regulated in any one tissue, these studies do provide a starting point on which hypotheses for androgen action in other tissues can be based. The NR field has been extremely active in the production of transcriptomic data sets, which have documented the regulation by gonadal steroids of a variety of target genes in models representative of historically well-characterized steroid-regulated tissues, chiefly the LNCaP, MCF7, and T47D cell lines. Given that several public databases of steroid-regulated genes have existed in the past but have since been taken offline, there is a current dearth of web-based databases for mining gene expression data that are relevant to gonadal steroids, with the exception being the Nuclear Receptor Signaling Atlas (NURSA) Transcriptomine resource[97] at www.nursa.org/transcriptomine.

While expression microarrays provide a valuable global perspective on the collective transcriptional response to steroid hormones, they are not designed to distinguish between direct and indirect regulation of genes by SRs, or the contribution, if any, of ligand functions involving cytoplasmic effectors in a given tissue. The relatively new discipline of GWLA encompasses a group of chromatin immunoprecipitation-based techniques that provide data on the specific genomic locations of NRs and coregulators in specific signaling contexts, and that have demonstrated tissue-specific patterns of DNA binding by SRs.[186] The term "cistromics" coined by the laboratory of Myles Brown—recognized as the pioneer in the field—in the latter part of the last decade describes the complete set of *cis*-acting targets of a transacting factor across the genome. Early adopters of GWLA used affinity purification of protein–DNA mixtures (chromatin IP, or ChIP) coupled to solid-phase tiled arrays (ChIP–chip), but this technique has been largely supplanted by the advent of massively parallel sequencing platforms in the form of ChIP-seq. The GWLA waters are muddied to an extent by the fact that, as has been discussed, DNA-bound SRs can influence transcriptional events at promoters far distant from their sites of occupancy, both proximal and distal, such that tying transcription of a specific gene to a specific DNA-binding event can for now be done only tentatively. As has also been alluded to here, Brown's groundbreaking papers demonstrated unequivocally that cross talk on DNA of ERα with transcription factors such as FoxA1 was an early event in the regulation of ERα target genes in MCF7 cells.[80] A more recent variant on the GWLA theme is GRO-Seq (global run-on-Seq), a deep sequencing approach that acts as a measure of direct transcriptional output by mapping the location and orientation of RNA polymerase II.[187] In this study, Lee Kraus's laboratory applied the technique to E2 stimulation of MCF7 cells, opening up the possibility of defining the time course of regulation of transcripts directly regulated by E2.

In technical terms, the field of gonadal steroid action is very well positioned to accumulate the informatic raw data from which novel testable hypotheses can emerge. Transcriptomic surveys are increasingly being complemented by next-generation sequencing approaches, and advances in GWLA make possible the integration of data sets from different platforms to provide additional context and increase confidence in the functional relevance of the data provided by each. In addition, the subdiscipline of epigenomics profiles the myriad posttranslational modifications of chromatin-associated proteins such as histones that an increasing body of evidence is linking to tissue-specific regulation of gene expression. Proteomics technologies such as mass spectrometry add detail both upstream and downstream of transcriptional events regulated by steroid hormones. Upstream, they report on patterns of post-translational modifications of receptors and coregulators and provide insights into how these might dictate the elaboration of tissue- and ligand-specific transcriptomes.[24] Downstream, proteomic studies define the identity and function of de novo proteins and protein complexes that give effect to gonadal steroid actions in their target tissues. Finally, the relatively nascent (in the reproductive endocrinology field, at least) discipline of metabolomics reports on the impact in terms of target cell biochemistry, and of the exposure of a given target cell to a specific gonadal steroid. In combination with the use of conventional and conditional gene knockout and knockdown models, which have already shown

themselves to be powerful tools in discerning the contribution of gonadal steroid receptors and isoforms to their tissue-selective effects, the technologies that these disciplines encompass are poised to provide vast amounts of new data in addition to what have already been generated.

CONCLUSION

Gonadal steroid action encompasses the complex interplay of classical and nonclassical steroid signaling pathways in numerous target tissues, including the mammary gland, uterus, ovary, and CNS, with effects in many other physiological contexts that are beyond the scope of this chapter, but that nevertheless ultimately impact the reproductive homeostasis of both sexes. Common principles have emerged from the study of signaling by the individual gonadal steroids—arguably, the most important of which is the diverse constellation of functions that coregulators bring to bear in the regulation of gene expression by these hormones, and upon which this chapter has largely focused. Moreover, abundant evidence has demonstrated that the lines of demarcation between gonadal steroid receptors and other classes of transcription factors are arbitrary at best. The appreciation is growing that a full understanding of gonadal steroid biology requires a perspective on the numerous points of integration between gonadal steroid signaling axes and those under the control of peptide hormones, growth factors, cytokines, and neurotransmitters acting through their cognate receptors (Figure 9.3), among other signaling molecules.

In comparison to its prodigious generation of data, however, the discipline of reproductive endocrinology has been slow to embrace the importance of archiving raw data sets in specialist public repositories. It is only when data sets are archived, consistently annotated, integrated, and exposed for data mining for the entire reproductive endocrinology community that the full value of the financial and scientific investment in these data sets can be realized. In technical terms, our field is firmly in the twenty-first century but is served by a model of data dissemination that remains in the twentieth century and in many ways has still not shrugged the vestiges of the nineteenth. The discipline of reproductive endocrinology holds great promise for increasing our understanding of human physiology as well as for setting the stage for the development of novel therapeutics, but the speed at which it does this will be determined ultimately by the extent to which scientists and clinicians from diverse subdisciplines find common ground in the rich mine of data points that the field has produced and will continue to produce in the coming decades.

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CHAPTER

10

Gonadotropes and Gonadotropin-Releasing Hormone Signaling

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INTRODUCTION

Gonadotropin-releasing hormone (GnRH, also known as GnRH I or luteinizing hormone (LH)-releasing hormone) is synthesized and stored in neurons within the medial basal hypothalamus and the preoptic area of the brain. The human gene (*GNRH1*) is located on chromosome 8 and encodes a 92-amino-acid pre-prohormone. This is processed within GnRH neurons to produce the linear decapeptide that is secreted by regulated exocytosis from nerve terminals in the median eminence, and then passes via the hypothalamo–hypophyseal portal circulation to the anterior pituitary gland. In the context of gonadotrope cell function within the anterior pituitary gland, the hypogonadal (HPG) mouse provided genetic evidence of the required role of GnRH in the regulation of the reproductive axis. The HPG mouse harbors a single nucleotide insertion within the gene encoding GnRH, resulting in a loss-of-function mutation.[1] This mutation results in a loss of GnRH stimulation of gonadotropes, a critical step in the control of the synthesis and secretion of gonadotropins. Consistent with this finding, the HPG mouse reproductive phenotype could be rescued by exogenous administration of GnRH. Moreover, several labs carried out transplantation of hypothalamic neurons to rescue the hypogonadal phenotype.[2–4] Human mutations leading to abnormal migration of GnRH neurons from the olfactory bulb to the hypothalamus (Kallman's syndrome)[5–16] or within the GnRH receptors (GnRHRs)[17–19] were linked to hypogonadotropic hypogonadism, suggesting that normal migration of GnRH neurons to specific nuclei within the hypothalamus or appropriate expression of the GnRHR in the plasma membrane of gonadotropes was critical for the gonadotrope to convert a hypothalamic signal (GnRH) into a systemic response (the secretion of follicle-stimulating hormone (FSH) and LH) governing fertility. These mouse models and human disease conditions underscore the importance of the pituitary and its adjacency to the hypothalamus and infundibulum to establish effective neuroendocrine communication between the brain and pituitary within the reproductive axis.

GnRHRs are G-protein coupled receptors (GPCRs) that mediate the effects of GnRH on the synthesis and secretion of LH and FSH. These are heterodimeric glycoprotein hormones that share a common α-glycoprotein hormone subunit (αGSU) but have distinct β subunits (LHβ and FSHβ). LH stimulates testosterone production in males and ovulation as well as corpus luteum formation and function in females, whereas FSH stimulates spermatogenesis in males and ovarian follicle formation as well as estrogen secretion in females. The gonadal steroids (estrogen, progesterone, and testosterone) exert feedback effects, acting centrally to influence GnRH secretion and at the pituitary to influence gonadotrope responsiveness to GnRH. Thus, GnRH is a hypothalamic decapeptide that mediates central control of reproduction, but this simple statement rests on several decades of research[20–40] establishing, amongst other things, the nature of the interaction between the brain

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and humoral regulation of reproduction and the identity of the hypothalamic releasing factor. This paved the way for detailed understanding of the physiological role of GnRH as well as its mechanism of action, all of which has informed the therapeutic targeting of its receptors in treatment of reproductive disorders and hormone-dependent cancers.

Milestones in the discipline of neuroendocrinology include the discoveries of "neurosecretion" in fish and insects and that the brain controls the endocrine system by regulated patterns of peptide hormone synthesis and release.[41–43] Several groups embarked on the purification of putative hypothalamic releasing factors, notably the two competing groups of Roger Guillemin and Andrew V. Schally who, given the low yields of existing techniques, were faced with the Herculean task of isolating peptides from millions of sheep and pig brains collected from the slaughterhouse. Both groups eventually isolated the GnRH decapeptide, identified its structure, and synthesized it.[26,44–50] The identical peptide was subsequently found in numerous species (including humans) and was shown to control secretion of both LH and FSH.[26] The importance of this work was recognized in 1977 when Guillemin and Schally (along with Rosalind Yalow) were awarded the Nobel Prize in Medicine for their "discoveries concerning the peptide hormones of the brain". Even before its chemical identity had been established, clinical studies with purified GnRH had demonstrated its ability to stimulate LH and FSH release in humans.[51–53] However, knowledge of the structure and the availability of synthetic GnRH led to more extensive work and to the development of GnRH analogs (with agonist or antagonist activity) that are now used extensively for assisted reproduction and for the treatment of hormone-dependent cancers and other hormone-dependent conditions such as endometriosis. The discoveries that GnRH secretion is pulsatile and that its effects on gonadotropes are dependent on stimulation paradigms have been pivotal for therapeutic manipulation of the system. Thus, Ernst Knobil and colleagues first noted pulses in levels of LH in rhesus monkey circulation[54] and demonstrated their importance by showing that hypothalamic lesions prevented menstrual cyclicity and LH pulses in monkeys, and that menstrual cycles could be restored by injection of GnRH at sufficient frequency.[55,56] When the frequency of LH pulses caused by GnRH injection was too low or too high, LH secretion was suppressed,[57,58] providing the rationale for therapeutic inhibition of gonadotropin secretion by GnRH agonists as well as antagonists. Pulsatile LH secretion has subsequently been shown in many species and is attributed to pulsatile electrical activity in brain regions containing GnRH neurons, driving pulsatile secretion of GnRH into the portal circulation. Notably, Knobil's group monitored multiunit activity (MUA) in the monkey mediobasal hypothalamus and found that LH pulses correspond to periodic MUA volleys in individual monkeys.[59–61] Moreover, Clarke's group was able to monitor pulses of GnRH within the portal circulation of ewes,[62] opening the way for "ultra-frequent" portal sampling and the increasing evidence that each LH pulse is driven by a pulse of GnRH in the portal circulation.[63,64] Recent studies have greatly advanced our understanding of the nature of the GnRH pulse generator and its regulation by gonadal steroids, by higher centers, and by other environmental inputs (such as photoperiod, nutritional status, and stress). These are dealt with elsewhere in this book, whereas mechanisms of GnRH action and GnRH pulse frequency decoding by its target cells are covered in the section Pituitary Actions and Mechanisms of Action of GnRH.

In addition to the in vivo approaches outlined in this section, GnRH action has been extensively explored in vitro. Pituitary explants and enzymatically dispersed pituitary cell cultures proved to be valuable models for studying GnRH signaling in vitro, but an obvious issue is that gonadotropes form only a small subset (typically, 5–15%) of the cells within the pituitary.[65,66] This can complicate experiments and necessitate monitoring of gonadotrope-specific experimental measures (e.g., gonadotropin secretion or GnRHR expression), single-cell measurements (e.g., Ca^{2+} concentration or electrophysiological study of identified gonadotropes), or gonadotrope purification. Nevertheless, the development of gonadotrope-lineage cell lines[67,68] provided an important advance facilitating the study of GnRH effects on gonadotropin expression and (perhaps most importantly) the cloning of mouse GnRHR.[69,70] This was rapidly followed by the cloning of GnRHRs from humans[71–73] and other species,[74–79] which enabled extensive work on heterologously expressed GnRHRs in academic and industry research labs. It also ushered in the current era where molecular and genetic techniques enable detailed exploration of specific receptors and effectors in health and disease, as well as more holistic approaches to understanding the molecular and cellular effects of GnRH and its role in reproductive endocrinology, at the systems level. This chapter is intended to provide a broad overview of GnRH and its target cells, starting with a brief coverage of gonadotropes and their development (see the section Gonadotropes) and leading into more detailed coverage of GnRH action. Emphasis is placed on effects of the hormone on pituitary gonadotropes (see the section Pituitary Actions and Mechanisms of Action of GnRH), although additional regulators of these cells and additional GnRH target cells are also briefly considered (see the section Autocrine and Paracrine Regulation of Gonadotropes). Similarly, emphasis is placed on molecular and cellular mechanisms, although physiological, therapeutic,

and pathophysiological relevance is also highlighted where appropriate. Given the breadth of the topic, a vast amount of important work is necessarily excluded. We hope to convey some of the excitement of recent developments in the field and apologize in advance to friends and colleagues whose work could not be incorporated.

GONADOTROPES

Early Insights into Pituitary Organogenesis, the Gonadotrope Lineage, and Fertility

H. Rathke first described the developmental origins of the pituitary gland in the 1830s. The developmental placode and invagination of the oral ectoderm that give rise to the adult anterior pituitary bear his name (Rathke's pouch) to the present day. A careful anatomical description of the pharyngeal end of Rathke's pouch was provided by E. Frazer in 1911,[80] outlining morphological changes within the developing embryo. These early studies provided clear anatomical evidence of the adjacency between the neural and oral ectoderm. Further, thickening of the placode suggested proliferation of cell populations leading to expansion of Rathke's pouch. In 1926, early ablation studies by P.E. Smith[81] demonstrated that removal of the pituitary gland in rats resulted in loss of ovarian function and in ovarian atrophy; this outcome could be reversed with replacement therapy in the form of pituitary transplantation, leading to insight into the role of the pituitary in producing "activities" that could regulate distal organs such as the gonads. Compelling work from F.L. Hisaw and his colleagues (S.L. Leonard and H.L. Fevold) in the early 1930s characterized two important activities from the adult pituitary gland in rabbits, labeling them follicular- and corpus luteum–stimulating activities[82,83] and providing initial evidence that two gonadotropic hormones (FSH and LH) were produced and released by the pituitary gland to control the development of ovarian follicles, ovulation, and thus fertility. These studies were later extended in elegant studies by R.O. Greep and colleagues of pituitary stalk transections in male and female rats leading to infertility[84,85]; however, the studies by Greep and colleagues speculated that the stalk transections resulted in a loss of blood flow to the anterior pituitary rather than loss of hypothalamic input. It was reported by Geoffrey Harris in a series of articles that helped to provide more definitive insight into the role of the hypothalamus in control of anterior pituitary function.[86–88] In these studies, surgical transection did indeed result in infertility, and recovery from transection via regeneration of the portal vasculature restored estrous cycles in rats. This restoration could be blocked effectively if transections were carried out with placement of a physical barrier (e.g., a small disk) between the transected stalk and the adenophypophysis, blocking regeneration of the portal vasculature. Thus, loss of hypothalamic signals to the pituitary gland through surgical transection resulted in hypogonadotropic hypogonadism in these animals, suggesting that control of pituitary gonadotropins was indeed governed by activities (releasing peptides) within the hypothalamus.

The anterior portion of the pituitary is characterized by five distinct endocrine cell types giving rise to six peptide hormones involved in the regulation of metabolism, growth, reproduction, lactation, and accommodation to stress. These cell lineages include the lactotrope (prolactin producing), the somatotrope (growth hormone (GH) producing), the thyrotrope (thyroid-stimulating hormone (TSH) producing), the adrenocorticotrope (adrenal corticotropic hormone (ACTH) producing) and the gonadotrope (FSH and LH producing). Loss of function of any of these cell lineages is linked to diseases ranging from altered long-bone growth and dwarfism to hypogonadotropic hypogonadism and infertility. Important genetic studies in early mouse models help to crystallize the role of a number of transcription factors and secreted molecules working in a paracrine manner to support pituitary development and cell lineage specification (extensively reviewed in Refs 89–97). Early characterization of the Snell and Ames dwarf mice[98–101] led to a remarkable understanding of the role of the pituitary gland in normal postnatal growth governed by GH. In both of these mutant mouse lines, deficiency of GH production was characteristic, and analyses of the genetic causes led to the discovery of causal mutations in homeobox containing transcription factors POU1F1 (POU domain, class 1, transcription factor 1; pituitary-specific transcription factor 1 (Pit1) and growth hormone factor 1 (GHF1) in the Snell dwarf) and Prophet of Pit1 (PROP1 in the Ames dwarf) underlying the GH deficiency. PROP1 induces transcriptional control of POU1F1 (Pit1), while POU1F1 binds to and transactivates the GH and prolactin gene promoters in somatotropes and lactotropes, respectively.[98,102–112] Thus, POU1F1 is thought of as a key transcription factor required for the terminal differentiation of cell lineages within the anterior pituitary, and this discovery set the stage for analyses of other transcription factors and secreted molecules that control pituitary organogenesis. More in-depth understanding of the so-called Pit1-dependent lineages (somatotropes, lactotropes, and thyrotropes) can be found in Chapter 12. Here, we focus broadly on lineage determination and specific mechanisms of gonadotrope differentiation during fetal development.

Inductive Model of Pituitary Organogenesis

Development of the pituitary gland requires important embryological tissue interactions via elaborate paracrine-signaling systems and an extensive transcription factor network that are critical for the pituitary anlagen to give rise to the diverse cell lineages within the anterior pituitary. This system is an excellent model of the study of organogenesis. In this section, we outline the key signaling systems and transcriptional mechanisms that underlie the ability of five distinct endocrine cell types to differentiate essentially side by side to control so many diverse physiological functions.

The anterior and intermediate lobes of the pituitary emerge initially from a thickening of oral ectoderm at the roof of the presumptive oral cavity (as early as E7.5 in the mouse) that eventually gives rise to the emergence and expansion of Rathke's pouch; the posterior portion of the pituitary gland is derived from neural ectoderm from the diencephalon overlying the oral ectoderm. Thus, two distinct embryological compartments give rise to the adult gland. The close physical proximity of the oral ectoderm and the neural ectoderm of the diencephalon is critical for the formation of the gland and essentially establishes a lifelong relationship between the anterior pituitary and the hypothalamus. Studies using coculture of these two embryological epithelia from rats provide important evidence that appropriate specification of cell lineages within Rathke's pouch (from oral ectoderm) results from secretion of diffusible factors from the diencephalon (neural ectoderm) functioning in a paracrine manner.[113–115] These secreted factors appear to control the differentiation of progenitor cells in the oral ectoderm into discrete cell lineages.

Much of what we understand currently about pituitary cell proliferation and specification is the result of perturbations of signaling molecules and transcriptional regulators in naturally occurring mouse models or from genetically modified mice. This section will largely focus on mouse model systems illustrating key points. Such notables as M.G. Rosenfeld, H. Westphal, J. Drouin, and S.A. Camper (and their remarkable trainees[89–97]) have contributed greatly to the field, and the reader is directed to this rather large body of important literature. Focusing on such model systems, Westphal and colleagues[116] describe the induction and formation of Rathke's pouch as a multistep process. In the mouse, the initial event in the inductive model is the formation of the rudimentary pouch at ~E8.5, which is characterized by a thickening of the oral ectoderm at the point where the oral and neural ectoderm come into close physical proximity.[117] This is followed over the next several days by an involution and expansion of Rathke's pouch toward the diencephalon.[118] The culmination of this event is the formation of a definitive pouch, with the diencephalon evaginating toward the oral ectoderm (Figure 10.1). The definitive pouch is formed by E12.5 and is marked by a complete detachment from the remaining oral ectoderm[119,120] and the appearance of hormone-secreting cell lineages defining the anterior lobe. Thus, tissue interactions between the oral and neural ectoderm via paracrine factors are essential for the induction and proliferation of Rathke's pouch. In addition, cell lineage determination within the anterior lobe requires the presence of important mesenchymal-expressed paracrine factors derived from the neural crest that surround the definitive pouch. Key paracrine factors from both the diencephalon and the surrounding mesenchyme are outlined throughout the rest of this section.

Paracrine Factors and Pituitary Development

Bone morphogenic protein (BMP4): The diencephalon is an important source of secreted signaling molecules governing pituitary development and cell lineage determination. As outlined in this chapter, the close physical proximity of the diencephalon and the oral ectoderm is central to the inductive model of pituitary organogenesis. At E8.5, BMP4 begins to be secreted from the diencephalon, reaching a peak at E9.5. By E11.5, BMP4 expression decreases to a nadir.[93,121] Several pieces of evidence underlie the importance of the window of BMP4 expression in the establishment of the placode within the oral ectoderm. Perhaps most compelling of these studies is the knockout of BMP4 in mice where the pouch placode within the oral ectoderm fails to form.[118] Consistent with this, misexpression of noggin (a BMP antagonist) using a Pitx1 promoter (directing expression in the oral ectoderm, as outlined in this chapter[118]) results in similar defects. Concurrent with the BMP4 expression domain in the diencephalon, sonic hedgehog (Shh) initially is uniformly expressed within the ventral diencephalon and oral ectoderm; however, Shh expression is specifically excluded from the region of the developing placode and invaginating Rathke's pouch, suggesting that the Shh expression domain (or lack thereof) defines the limits of the invaginating pouch. Repression of Shh expression within the oral ectoderm destined to become the pouch appears to be linked to BMP4 expression from the diencephalon.[93,95,122,123] Deletion of Shh or Gli family members (downstream targets of Shh signaling) in mice results in abnormal pituitary development (variable to complete absence of the gland).[121,124–127] Since the notochord is a primary source of Shh controlling or shaping midline structures in development, it is difficult to know definitively that the loss of Shh leading the disruption of pituitary development is direct or a function of more generalized abnormal midline development within embryos lacking Shh.

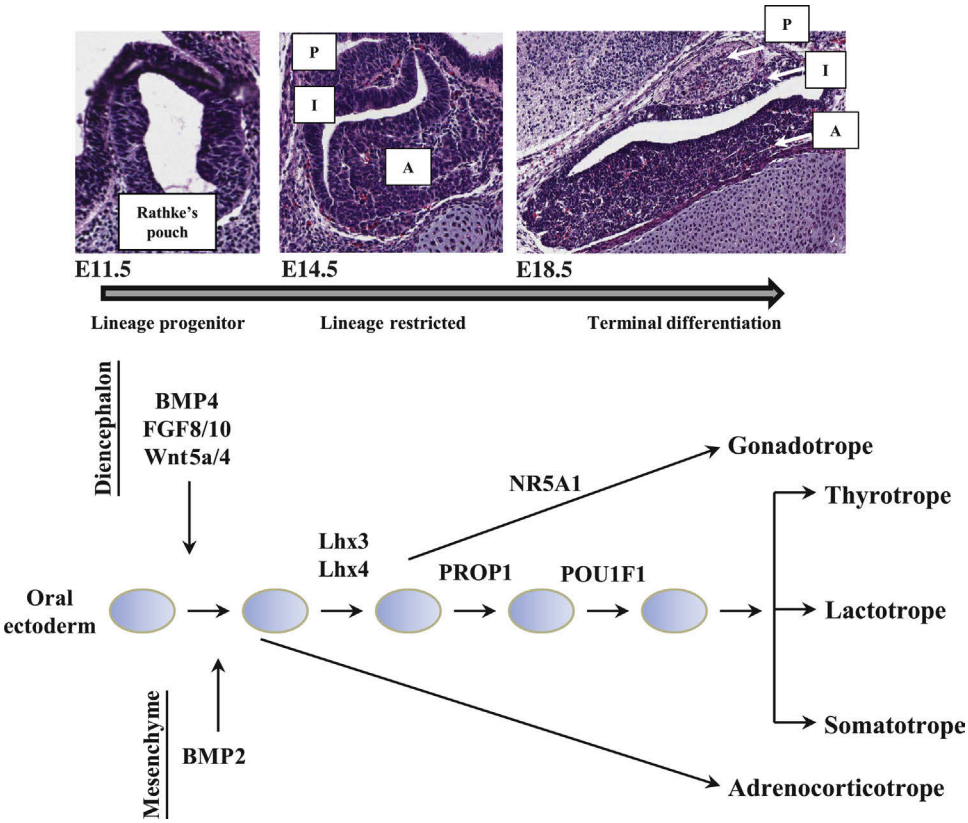

FIGURE 10.1 Pituitary development. This figure is reproduced in color in the color plate section. The pituitary gland emerges from important interactions between the oral and neural ectoderm (diencephalon). Early expansion of the oral ectoderm appears to be functionally related to paracrine signals (such as bone morphogenic proteins (BMPs) and fibroblast growth factors (FGFs)) originating from the neural ectoderm and affecting proliferation and early differentiation of the oral ectoderm. The resulting expansion of tissue forms Rathke's pouch, the anlagen of the presumptive lineage progenitors of the anterior and intermediate lobes of the gland. In addition to signals emerging from the diencephalon, the surrounding mesenchyme provides additional paracrine signaling from BMPs, further restricting lineage identity. Differentiation of the five endocrine cell types within the anterior lobe of the pituitary is controlled by concise expression of a series of transcription factors that serve to restrict cell identity and drive terminal differentiation of the anterior pituitary. For specification of the gonadotrope cell lineage, early expression of Lhx3 (controlled by FGF signaling) and later expression of NR5A1 are central. Interestingly, Lhx3 and NR5A1 play central roles in the regulation of the four gonadotrope signature genes that define gonadotrope function in the adult animal. Sagittal tissue sections shown are from the mouse (unpublished data from M.S. Roberson and A.M. Navratil; e11.5 represents embryonic age in days post conception; P: posterior; I: intermediate; A: anterior).

Fibroblast growth factor (FGF): In addition to BMP4, FGF8 and FGF10 are also expressed and secreted from the ventral diencephalon and appear to be critical in the expansion of Rathke's pouch. FGF10 or FGF receptor 2IIIb (FGFR2IIIb) knockout mice initially form Rathke's pouch; however, the pouch quickly undergoes marked cell death. As a result, FGF10$^{-/-}$ and FGFR2IIIb$^{-/-}$ animals display pituitary agenesis by E14.5,[128,129] suggesting that activation of FGFR2IIIb plays an important role in cell survival in the developing gland. FGF8 expression begins at E9.25, slightly later than BMP4. The FGF8$^{-/-}$ mouse dies at E8.5, prior to pituitary organogenesis[121,130]; as such, this precludes examining the direct role of FGF8 on the development of Rathke's pouch. Evidence for the importance of FGF8 comes from in vitro studies examining the impact of the ventral diencephalon and FGF8 on Rathke's pouch explants. Coculture of ventral diencephalon with Rathke's pouch explants leads to expression of the Lin11, Isl1 and Mec-3 (LIM) homeodomain transcription factor Lhx3 within the oral ectoderm.[121,130] Similar increases in Lhx3 occur when pouch explants are placed in proximity to FGF8-soaked beads, suggesting that the action of the ventral diencephalon on Lhx3 expression in the pouch is dependent on FGF8. Importantly (as outlined in this chapter), in Lhx3 null mice, Rathke's pouch forms but does not develop into a definitive gland.[118] These studies support the conclusion that FGF8 from the ventral diencephalon is required for upregulation of Lhx3. Lhx3 also plays a key role in the expression of a number of gonadotrope-specific genes in the adult animal (described in this chapter), supporting the conclusion that at least some of the transcription factors required for normal pituitary organogenesis retain a functional role in gonadotrope cell behavior following birth.

More definitive clues regarding the role of FGF8 and Lhx3 came from the knockout of Nkx2.1, another homeobox protein expressed in the diencephalon. The Nkx2.1$^{-/-}$ mouse is characterized by pituitary aplasia[131] and provides direct evidence that a transcription factor expressed exclusively within the diencephalon is required for development of the adjacent oral ectoderm. The expression domain of FGF8 in Nkx2.1$^{-/-}$ mice is essentially absent, while the BMP4 expression domain remains intact.[118,132] Thus, the Nkx2.1$^{-/-}$ model provided an opportunity to directly examine the role of FGF8 in vivo. The loss of FGF8 within the diencephalon in the Nkx2.1 null mouse resulted in a loss of Lhx3 expression within the developing pouch, providing important corroboration for in vitro studies demonstrating a requirement of FGF8 signaling for normal upregulation of Lhx3 within the developing pouch. Collectively, BMP4 expression within the diencephalon is required for rudimentary pouch formation, while FGF8 has a clear role in upregulation of Lhx3 and expansion of the rudimentary pouch into a definitive pouch through either induction of proliferation or increased anterior pituitary cell survival. The presence of FGF8 (and likely other FGFs) is proposed to create a dorsoventral polarization of the Lhx3 expression domain and provides a mechanism for continued proliferation of dorsal cell populations in closest proximity with the FGF signal source.

Wnts: Wnt5a and Wnt4 and presumably β-catenin signaling have been linked to the development of the pituitary gland. Wnt5a is expressed within the ventral diencephalon, while Wnt4 expression appears within Rathke's pouch.[133] The Wnt5a$^{-/-}$ mouse is characterized by abnormal pouch morphology and elevated numbers of adrenocorticotropes; however, this pituitary phenotype appears to be relatively mild. The Wnt4$^{-/-}$ mouse displays mild pituitary hypoplasia with reduced numbers of gonadotropes. The Wnt5a–Wnt4$^{-/-}$ double mutant shows both of these phenotypes, suggesting that signaling through Wnt5a and Wnt4 occurs independently in different cell lineages.[93]

BMP2: The paracrine effectors derived from the ventral diencephalon and the emerging pouch appear to play critical roles in establishing signaling centers and secreted factor gradients involved in the regulation of cell proliferation, cell survival, and differentiation. However, as the definitive pouch develops and detaches from the oral ectoderm, the developing gland is surrounded with mesenchyme derived largely from the neural crest. This mesenchymal compartment adds to the signaling complexity of the developing gland as a source of BMP2 beginning at E10.5. Expression of BMP2 appears to induce ventral markers of pituitary cell lineages, including the homeobox gene Isl1 and the α subunit of the glycoprotein hormones (αGSU). Isl1 is expressed within the oral ectoderm beginning at E9.5 and is later (E10.5) restricted to the ventral portion of the gland, coincident with increased BMP2 expression from the mesenchyme. Isl1$^{-/-}$ animals die in midgestation (E10) due to fetal abnormalities not linked to the pituitary; however, at E9.5, a hypoplastic pouch does form, suggesting that Isl1 may be involved in proliferation and/or survival of cells within the developing pouch.[118,121] αGSU is a common subunit to the glycoprotein hormone family, including LH, FSH, and TSH, and is expressed in the ventral portion of the pouch eventually associated with the thyrotrope and gonadotrope cell lineages.

Expression of BMP2 from the mesenchyme surrounding the ventral portion of the developing pouch provides a ventral signaling gradient in opposition to the more dorsal FGF8 gradient arising from the diencephalon and infundibulum.[93,121,134] These two signals clearly play differential roles in the induction of transcription factors involved in cell lineage determination. As outlined in this chapter, FGF8 is required for the expression of Lhx3, while BMP2 appears to be necessary for expression of Isl1. These opposing gradients regulate the emergence of cell lineages by E12.5, with corticotropes appearing initially and more closely aligned with the dorsal portion of the pouch, associated with FGF8 signaling, while thyrotropes, gonadotropes, and lactotropes reside initially in the more ventral portion of the developing gland. These observations support the hypothesis that opposing FGF8 and BMP2 signaling gradients create a spatially and temporally restricted pattern of lineage specification.[119,120] Perturbing these gradients can alter pituitary cell lineage specification. For example, ectopic expression of a dominant-negative form of the BMPR1A receptor in αGSU-expressing cells leads to retarded differentiation of lactotropes, thyrotropes, and somatotropes and a hypoplastic gland, suggesting that BMP2 expression from the mesenchyme regulates important differentiation mechanisms through BMPR1A. Conversely, shifting the signaling polarity with overexpression of FGF8 throughout the pouch appears to induce hyperplasia within the pouch and reduce cell differentiation within the anterior pituitary.[121] These studies suggest that FGF8 supports cell proliferation and/or cell survival while inhibiting some differentiation programs. The end result is the establishment of five endocrine cell identities, each defined by a set of signature genes that confers cell identity. For the gonadotrope, these signature genes include αGSU, the β subunits for LH and FSH, and expression of GnRHR to provide a mechanism for hypothalamic control of the synthesis and secretion of these gonadotropic hormones. For the pituitary in general and each cell lineage during development, specific sets of transcription factors regulate cell identity and are in large part regulated by the signaling network outlined in this chapter.

Also outlined in this chapter are the core transcription factors that contribute to early and late aspects of pituitary organogenesis.

Transcription Factor Network Regulating Pituitary Development

As discussed here, Nkx2.1 is a homeobox factor, and expression of Nkx2.1 is detectable at E8.5 within the ventral diencephalon and is not expressed within the oral ectoderm. Targeted deletion of Nkx2.1 in mice results in failed development of the anterior and intermediate lobes of the pituitary, products of the oral ectoderm.[135] Nkx2.1 appears to be required for expression of FGF8 within the diencephalon; FGF8 is necessary for the upregulation of Lhx3 (described here) within the developing pouch.

Both Pitx1 and Pitx2 play important roles in establishing the early pituitary primordia. Pitx1 is expressed at E9.5 uniformly in oral ectoderm and continues to be expressed within Rathke's pouch. Pitx1 and Shh have overlapping expression domains within the oral ectoderm. Coincident with increased BMP4 expression from the ventral diencephalon, Shh expression is extinguished within the rudimentary pouch; however, Pitx1 continues to be expressed. Pitx1$^{-/-}$ mice display a modest pituitary phenotype with decreased expression of the β subunits to the gonadotropins as well as TSH.[123] Pitx2 appears to play a more prominent role in pituitary-specific activity than Pitx1. Pitx2 gene deletion in mice results in apparently normal establishment of the definitive pouch; however, the pouch fails to proliferate and, based upon in vitro studies, this may be regulated by Wnt signaling (as described here). Pitx2$^{-/-}$ pituitaries are characterized by the presence of only the adrenocorticotrope lineage, the earliest lineage to emerge in development. Both Pitx1 and Pitx2 appear to play a regulatory role in the expression of Lhx3, and in somatotropes and lactotropes, Pitx2 is critical for expression of Pit1 (outlined in this chapter). Interestingly, Pitx1 and Pitx2 appear to bind to and transactivate the gene promoters of αGSU, the gonadotropin β subunits, TSHβ, GH, and prolactin, making the importance of this bicoid factor quite far-reaching in terms of pituitary function.[136]

The LIM homeodomain-containing transcription factors Isl1, Lhx3, and Lhx4 are also obligate early determinants of pituitary development. Isl1 expression is initiated within Rathke's pouch at E8.5 and appears throughout Rathke's pouch at E9.5. By E10.5, Isl1 expression is limited to the ventral portion of the pouch in proximity to the BMP2 gradient.[121] The effects of Isl1 appear to be most critical early in establishment of the primitive pouch. Isl1 null mice display a pouch epithelium characterized by hypoplasia, suggesting that Isl1 supports early progenitor proliferation.[118] Lhx3 is initially expressed at E9.5 within Rathke's pouch.[137] By E12.5, Lhx3 expression is more restricted to the dorsal portion of the pouch[121] and Lhx3 expression persists in the adult pituitary gland. In the Lhx3 null animal, the pouch forms initially but fails to proliferate, leading to inappropriate cell differentiation. Loss of Lhx3 has been linked to elevated levels of apoptosis within the early pouch, leading to hypoplasia.[138] This lesion results in a loss of nearly all differentiated cell lineages within the anterior pituitary with the exception of adrenocorticotropes producing ACTH,[96] a condition similar to human patients with genetic mutations within the LHX3 gene.[96,139,140] Lhx4 also has an important impact on the developing gland; however, this effect is less robust than that of Lhx3, and Lhx4 clearly cannot rescue the Lhx3 null phenotype. Lhx4 is expressed at E9.5 in an expression domain consistent with Lhx3; however, unlike Lhx3, the Lhx4 expression domain becomes restricted by E15.5. Lhx4$^{-/-}$ mutants display elevated cell death within the developing pituitary by E12.5 and E14.5[97,137,141]; by near term, the Lhx4 mutants have reduced numbers of somatotropes and lactotropes. The compound Lhx3–Lhx4 double null is embryonically lethal and fails to form a definitive pouch.

Transcription Factor Network and Lineage Specification within the Pituitary

The individual transcriptional activators outlined in this chapter play fundamental roles in organizing early events of pituitary organogenesis and shared functions to regulate specific cell lineage determination; however, definitive lineage specification requires additional transcription factors to fully define the gene program that delineates the function of a specific cell lineage. Perhaps the most clearly defined example of lineage specification within the anterior pituitary comes from regulation of PROP1 and POU1F1 (Pit1) ultimately giving rise to the so-called Pit1 lineages, somatotropes, lactotropes, and thyrotropes. PROP1 is initially expressed by E10.5, peaks at E12.5, and is largely extinguished by E14.5–15.5.[142] The naturally occurring Ames dwarf mouse is characterized by a missense point mutation (S83P) within PROP1, leading to reduced DNA-binding activity of PROP1 at target genes.[99,101] PROP1 expression appears to precede that of POU1F1, consistent with its name "Prophet of Pit1". In vitro and in-tissue chromatin immunoprecipitation studies support the conclusion that PROP1 can bind to and transactivate the POU1F1 gene promoter. Reconstituting the S83P mutation in vivo gives rise to animals with combined pituitary hormone deficiency (CPHD) with a loss of GH, prolactin, and TSH-producing cells. Interestingly, mutations in PROP1 also give rise to a reduction in gonadotrope cell populations; however, the mechanisms associated with this loss have not yet been elucidated.

The Snell and Jackson dwarf mouse lines are also characterized by a loss of the Pit1 cell lineages and CPHD. The Snell mouse has a point mutation within the DNA-binding domain (W261C) of POU1F1, leading to reduced DNA-binding affinity at target genes.[98,143,144] The Jackson dwarf also lacks POU1F1; however, this appears to be a consequence of a larger scale chromosomal rearrangement. Loss of POU1F1 in genetically engineered mouse models results in a similar CPHD phenotype. Consistent with a role in cell lineage specification, POU1F1 expression initiates later in development (E13.5) and appears just prior to the extinguished expression of PROP1. POU1F1 expression is restricted to the caudomedial portion of the developing gland and is a definitive marker for lactrotropes, somatotropes, and thyrotropes; these lineages are completely absent in POU1F1-deficient mice. Interestingly, the absence of POU1F1 also results in expanded corticotrope and gonadotrope populations, suggesting that one role of POU1F1 is potentially to repress these latter two lineages. Elegant molecular endocrinology and biochemical studies have demonstrated that POU1F1 directly binds to and transactivates the GH, prolactin, TSHβ, and GH-releasing hormone receptor genes, providing important key mechanistic information for how POU1F1 is capable of controlling these lineages via direct regulation of a cohort of signature genes that defines lactotropes, somatotropes, and to a certain extent thyrotropes.[93]

NR5A1 and Specification of the Gonadotrope Lineage

Cell lineage commitment reflects the genetic signature required to define the differentiated character of the specific cell type. The gonadotrope must be able to respond to hypothalamic stimuli via GnRH and produce gonadotropin hormones to control gonadal function. Thus, the gonadotrope cell lineage is defined by at least four signature genes: the gonadotropin subunits (αGSU, LHβ, and FSHβ) and GnRHR.[38] The gonadotrope is the last of the cell lineages to become fully differentiated during pituitary organogenesis; however, expression of αGSU (one of the earliest markers of pituitary development) is a requisite component of this lineage. At E10.5, αGSU expression appears coincident with expression of Lhx3, other ventral markers (Isl1), and the BMP2 expression from the mesenchyme surrounding Rathke's pouch. Due to the overlapping requirement of αGSU in the thyrotrope and gonadotrope cell lineage, true gonadotrope lineage commitment is often associated with the expression of the orphan nuclear receptor NR5A1 (nuclear receptor subfamily 5, group A, member 1 (also known as steroidogenic factor 1 or SF1) at E13.5. NR5A1$^{-/-}$ mice die perinatally (at p8) due to adrenocorticoid insufficiency.[145,146] The phenotype of this mouse is actually remarkable, demonstrating that NR5A1 expression in vivo is required for normal development of the ventromedial hypothalamus and the adrenals and gonads. Specific conditional deletion of NR5A1 in pituitary gonadotropes using the αGSU–Cre deleted strain revealed that loss of NR5A1 in gonadotropes and thyrotropes resulted in hypogonadotropic-hypogonadal infertility linked to deficiencies in the αGSU and β subunits to LH and FSH along with reduced or absent GnRHR expression.[147] This striking hypogonadal phenotype was effectively rescued by administration of exogenous gonadotropins and, interestingly, by GnRH administration. Thus, NR5A1 is absolutely required for the regulation of the four gonadotrope signature genes; however, the definitive role of NR5A1 on the regulation of GnRHRs may be only partial.

NR5A1 mouse models have provided important in vivo details regarding the role and requirement of this orphan nuclear receptor in the development and function of the hypothalamic–pituitary–gonadal axis and the specific importance of NR5A1 on gonadotrope lineage specification. Equally important have been observations of naturally occurring mutations of NR5A1 in human populations and their impact on fertility.[12,13,148–150] NR5A1 is a 461-amino-acid protein with several discrete and important domains, including activator function domains and DNA-binding domains (DBDs). DBD is characterized by the presence of two zinc fingers at the amino termini of the protein. A patient presenting with primary adrenal failure and XY sex reversal was characterized with a G35E substitution mutation in NR5A1. This mutation occurred within the P box of the first zinc finger in NR5A1, rendering the protein incapable of DNA binding and transactivation of target genes linked to steroidogenesis and, perhaps more relevant to this discussion, the β subunit to LH in the gonadotrope. This human phenotype was entirely consistent with observations made in mouse models and underscored the importance of NR5A1 on gonadotrope cell function (as well as steroidogenic tissues) in human patients.

The importance of NR5A1 is potentially far-reaching as a determinant of the gonadotrope lineage and gonadotrope cell biology since this orphan nuclear receptor has been shown to interact with a rather large number of chromatin-remodeling complexes that serve as coactivators, corepressors, and transcription factors.[150] It is perhaps not surprising that as an orphan steroid hormone receptor, protein–protein interactions and recruitment of chromatin-remodeling machinery to target genes are fundamental components of NR5A1 action in steroidogenic tissues. In the context of NR5A1-dependent promoters of steroidogenic enzymes (cytochrome P450 (CYP)11A1, CYP17, and CYP21), NR5A1 recruits coactivators such as cyclic adenosine monophosphate (cAMP)

response element-binding protein (CREB)-binding protein (CBP)/p300, p300/CBP associated factor (PCAF), and glutamate receptor interacting protein 1 (GRIP1) to these promoters, effectively modulating chromatin structure via histone acetylation.[151–153] Conversely, corepressor activity associated with C-terminal binding protein 1 (CtBP1), receptor-interacting protein 140 (RIP140), and the silencing mediator of retinoid and thyroid hormone receptors (SMRT) can also be recruited by NR5A1 complexes bound to CYP17 and CYP21 gene promoters to modulate steroidogenic activity via chromatin repression.[153–160] Interestingly, NR5A1 interactions with GRIP1 and SMRT depend upon the phosphorylation state of NR5A1 at S.[161] S[161] is a likely substrate for extracellular signal-regulated kinase (ERK) signaling (a key signaling system induced by GnRHR activation) and appears to be necessary for NR5A1 to assume an active confirmation in the absence of a ligand. This suggests that in some instances, the activation and phosphorylation state of NR5A1 controls protein–protein interactions and that higher order complexes controlling chromatin structure and gene transcription at NR5A1 target gene promoters may be critical for NR5A1 action in gonadotropes.

NR5A1 forming complexes with other transcriptional regulators appears to be critical to the specific regulation of gonadotropin subunit genes. As indicated here, NR5A1 plays a critical role in the regulation of the four gonadotrope signature genes during development, and the specific mechanisms of transcriptional activation of these gene promoters are addressed in other sections of this chapter. Here we simply point out the breadth of NR5A1 interactions with various factors to illustrate the diverse mechanisms of action of NR5A1, specifically in the gonadotrope. NR5A1 interacts directly with early growth response factor 1 (Egr1) and Pitx1 to regulate the activity of the rat LHβ subunit gene promoter.[162,163] Egr1 is an immediate early-response gene induced by GnRHR activation and is critical within the GnRH signaling network for LHβ subunit gene transcription.[164,165] Egr1$^{-/-}$ females are infertile due to a specific deficiency in LHβ subunit gene transcription via signaling through ERK1 and ERK2.[38,166] Pitx1 and Pitx2 were identified early in this chapter as key regulators of pituitary development and lineage determination. Both Pitx isoforms are bicoid-related homeodomain-containing transcription factors. Interaction between NR5A1 and Pitx1 results in a synergistic activation of the bovine LHβ gene promoter; overexpression of Egr1 or GnRH treatment enhances this activity further, suggesting that higher order complexes between these factors are critical for LHβ gene transcription. From a mechanistic perspective, interactions between the amino termini of NR5A1 and the carboxyl termini of Pitx1 appear to reconstitute full NR5A1 activity in the absence of a ligand-binding domain. These observations support speculation that interaction between NR5A1 and Pitx1 during gonadotrope development may occur without the need for a putative NR5A1 ligand.[162]

NR5A1 has also been shown to interact with androgen receptor (AR), leading to repression of the rat LHβ subunit gene promoter.[167] Androgens have important negative-feedback effects in the hypothalamic–pituitary–gonadal axis, and the direct interaction between androgens, AR, and NR5A1 has important implications regarding negative feedback directly at the level of the gonadotrope. Interestingly, association between NR5A1 and AR occurs through the ligand-binding domain of NR5A1 and the DNA-binding domain of the AR. Furthermore, the addition of Egr1 or Pitx1 to these binding complexes competes for NR5A1–AR interactions and, as such, relieves the androgen-mediated repression of the LHβ promoter. In other studies, the small nuclear RING finger (SNURF) protein also interacts with AR and as such serves as a putative coactivator at the LHβ subunit promoter.[168] The effects of SNURF are associated with direct interaction between SNURF and NR5A1, and this complex appears to blunt or reverse the repressive effects of androgens and ARs on the LHβ promoter.

Nuclear factor Y (NFY) and NR5A1 form a binding complex associated with regulation of the FSHβ subunit gene promoter.[169] Using a GST pulldown approach, NFYA (but not NFYB or NFYC) was shown to specifically interact with NR5A1. The importance of this interaction is presently not clear; however, the *cis* regulatory elements binding NFYA and NR5A1 within the FSHβ promoter do establish these transcription factors as critical for its full transcriptional expression in gonadotropes.[170]

Collectively, NR5A1 plays a central role in gonadotrope cell lineage determination largely via contributions to transcriptional activation to four gonadotrope signature genes. Importantly, NR5A1 can physically interact with a large number of chromatin-remodeling proteins identified in the context of steroidogenic enzyme gene promoter regulation, supporting speculation that similar complexes may be involved in the regulation of gonadotrope-specific genes. Moreover, a number of transcription factors have also been shown to interact with NR5A1 in the context of control of gonadotropin β subunit gene promoters or in conjunction with GnRH action. While not specifically examined within the pituitary anlagen during development, it seems highly plausible that NR5A1 interactions with some, if not all, of these transcription factors may contribute to gonadotrope lineage determination and coordination of gonadotrope-specific gene regulation during pituitary organogenesis. Clearly, NR5A1 plays a critical and continued role in the expression of cell-specific genes within the gonadotrope in the postnatal animal and into adulthood. Control of the gonadotrope signature genes also

includes regulation by a number of other transcription factors critical for development of the pituitary gland and, in particular, the gonadotrope. These include the Pitx family members and the LIM homeodomain factors such as Lhx3.

Changes in Biosynthesis of Gonadotropin Subunits and the GnRHR during the Estrous Cycle

A central outcome of an estrous cycle is the temporal coordination of sex behavior and timing of ovulation with uterine receptivity to establish successful implantation and pregnancy. This is accomplished through coordination of the ovarian and uterine cycles through GnRH and gonadotropin secretion regulating follicle and gamete maturation and ovulation and the production of gonadal steroid and peptide hormones. During the 4-day estrous cycle of the rodent, ovulation is preceded by a preovulatory surge of GnRH and LH that occurs on the evening of proestrus. GnRH-dependent FSH also surges at this time; however, elevated levels of FSH persist in circulation through the morning of estrus, and inhibin appears to regulate the latter portion of this biphasic FSH surge.[171–177]

The sensitivity of the gonadotrope leading up to the LH surge is regulated by increased expression of transcripts encoding the GnRHR. During the afternoon of proestrus, GnRHR mRNA is markedly increased, leading to increased numbers of GnRHRs within hours of the LH surge.[171,178] As preovulatory follicles undergo late-stage maturation, increased synthesis and secretion of estradiol are critical in coordinating increased expression of GnRHRs as well as sex behavior at estrus. Transcripts for the β subunit of LH increase coordinately with elevated GnRHR mRNA prior to the LH surge that is presumably associated with increased GnRH responsiveness of the gonadotrope and the need for high relative levels of LH biosynthesis in anticipation of the surge. FSHβ mRNA also increases during the proestrus period; however, this increase occurs later relative to LHβ and persists into the morning of estrus, consistent with FSH in circulation. Interestingly, transcripts for the α subunit to the glycoprotein hormones do not change appreciably during the proestrus period, suggesting that this common subunit to the gonadotropins is not rate limiting in gonadotropin biosynthesis leading up to the preovulatory surge. Collectively, it appears that estradiol-dependent increases in sensitivity to GnRH are coupled with increased GnRH-dependent increases in the β subunits to LH and FSH that are necessary for a preovulatory gonadotropin surge. In addition, factors such as inhibin (and likely activin) specifically modulate FSH biosynthesis following the surge.

Relationships involved in the gonadal control of negative-feedback mechanisms governing the regulation of GnRHR and gonadotrope subunit transcripts within the gonadotrope may be best demonstrated by examining the responses to removal of the gonad and the loss of gonadal steroid hormones and inhibin. Castration in both genders results in an increase in the secretion of both LH and FSH related to the removal of these gonadal steroid–linked negative-feedback mechanisms.[166,179] In female rats, replacement of estradiol following castration blunts the castration-induced rise in gonadotropin secretion and elevation in gonadotropin subunit mRNAs.[180] Interestingly, in estrogen receptor α (ESR1) null animals, gonadotropin subunit transcripts are elevated in gonadotropes without castration,[181] again providing important evidence that estradiol feedback via ESR1 is central to controlling gonadotrope cell function.

Response of the gonadotropin subunit mRNAs to castration varies with gender. In some recently published studies using quantitative polymerase chain reaction (PCR) to assess pituitary mRNA content 7 days following castration in male and female mice, αGSU, LHβ, and FSHβ mRNAs all increased in both genders. However, the relative increase in transcript abundance was greater in females relative to males of similar age,[166] although in these studies the stage of the estrous cycles in females was not standardized. Interestingly, in these mice, transcripts for the GnRHR in male and female mice were unaffected (males) or slightly diminished (females) with castration. This observation is in contrast to male and female rats where castration resulted in elevated GnRHR transcript abundance.[75,182] This may reflect differences in species or in the methodologies used to quantify transcript levels. The use of castration in many of these experiments focused on the response of the gonadotrope to a physiologically relevant elevation of GnRH pulse stimuli with the ablation of the gonad. The response of increased GnRH pulse frequency to regulate αGSU and LHβ subunit mRNAs was dependent upon intracellular signaling between the GnRHR and the ERK pathway. This pathway is dealt with in more detail in the section Mitogen-Activated Protein Kinases but it is important to note here that postcastration elevation in αGSU and LHβ subunit mRNAs was greatly blunted or abolished in male and female mice that harbored a conditional gonadotrope-specific deletion of ERK1 and ERK2. These were the first studies to demonstrate a specific requirement for GnRH-induced ERK signaling mediating the postcastration response of gonadotropin subunits.[166] Postcastration-induced LH secretion was also greatly blunted with the loss of ERK signaling in male and female mice, suggesting fidelity between transcription and biosynthesis; in contrast, the loss of ERK signaling failed to affect FSH secretion in either gender.

PITUITARY ACTIONS AND MECHANISMS OF ACTION OF GnRH

GnRH Receptors

Prior to their cloning, GnRHRs were extensively characterized as binding sites mediating potent GnRH effects, notably on synthesis and secretion of gonadotropins and on receptor regulation.[20–24] Early radioligand-binding studies revealed a single class of binding sites with classical receptor characteristics (high affinity and specificity as well as saturability) in pituitaries from several species.[183–185] The dissociation constant (K_d) for binding of GnRH to pituitary GnRHRs has been estimated (for several species) to be between 2 and 5 nM, whereas the K_d for the GnRH agonist (des-Gly^{10}-D-Ala^6)*N*-GnRH-*N*-ethylamide is between 0.2 and 1 nM.[183–189] Radioligand binding to GnRHRs in cell membranes varies markedly under different physiological conditions (during development and through puberty, the estrous cycle, pregnancy, and lactation), and this reflects changes in receptor number rather than affinity.[20,189–191] GnRHR number is also altered in primary cell cultures and in gonadotrope-derived cell lines by numerous treatments, including gonadal steroids and GnRH itself.[20,37,76,188–194] The number of rat or mouse GnRHRs has been estimated at 20,000–80,000 sites per cell in various models,[188,193,195] although calculating the number of receptors at the cell surface is complicated by the fact that the number of gonadotropes also varies under different conditions and because the enriched membrane preparations used for binding experiments likely contain membranes other than the plasma membrane. Nevertheless, the effects of GnRH are dependent upon the GnRHR number in many models, and the observed physiological changes in the number of pituitary GnRHRs immediately implies that GnRH expression and/or compartmentalization are regulated, as discussed in the section Desensitization and Receptor Internalization.

Cloning of GnRHRs revealed them to be members of the rhodopsin-like GPCR family (family A), and their identification as GPCRs is consistent with the fact that the GnRHRs of gonadotropes and gonadotrope-lineage cells act primarily via $G_{q/11}$ to activate phospholipase C (PLC). Consequently, they mediate elevation of cytoplasmic [Ca^{2+}] and activation of protein kinase C (PKC) isozymes, both of which are important for GnRHR-mediated effects on gonadotropin synthesis and secretion.[21,23–25,27,34–36,39,40,187,196] The cloned vertebrate GnRHRs can be broadly divided into three groups. All of the cloned mammalian GnRHRs are in groups I or II,[27,197] and the type I GnRHRs of humans, rats, mice, pigs, sheep, and horses have >80% amino acid sequence homology.[35] There are also multiple forms of GnRH peptide, although reptiles, birds, and mammals express only GnRH I and GnRH II (pGlu–His–Trp–Ser–His–Gly–Trp–Tyr–Pro–Gly–NH_2). The latter is widely distributed in the central nervous system (CNS),[198–200] where it may act as a neurotransmitter or neuromodulator, whereas GnRH I acting via pituitary type I GnRHRs is the essential cognate pairing for central control of mammalian reproduction. Type II GnRHRs are selectively activated by GnRH II and bind GnRH I poorly. In spite of the fact that GnRH II is ubiquitously expressed, the type II GnRHR is silenced in several species, including some primates. Thus, although marmosets express GnRH I and GnRH II as well as GnRHR I and GnRHR II, in humans there is a frameshift mutation and premature stop codon in the GnRHR II (pseudo)gene, so the two GnRH ligands likely act via a single type I GnRHR.[27,39,197,201] In addition to expression in the pituitary, GnRHRs are found (often along with GnRH) in many other tissues, including those of the brain and the reproductive system.[25,27,30,36,39,197,202] Most interest in extrapituitary GnRHRs stems from the discovery that they are expressed by numerous cancers, including those of the mammary, prostate, and ovary. In cells derived from such cancers GnRH analogs (or cytotoxic derivatives) often exert antiproliferative and/or pro-apoptotic effects,[30,39,40,161,202–236] raising the exciting possibility that such direct action may contribute to the therapeutic effects of GnRH analogs in cancer treatment.

Type I GnRHRs are proteins of 327 (or, in humans, 328) amino acids. Their crystal structure is unknown, so molecular models rely on predictions from the solved structure of rhodopsin or, more recently, those of other GPCRs, including the β1 and β2 adrenergic receptors.[25,27,31] This indicates that GnRHRs, like other GPCRs, have the characteristic seven transmembrane domains (TMDs) linked by three intracellular (IC1-3) and three extracellular (EC1-3) loops. There is a 35-amino-acid extracellular amino-terminal domain, and receptor structure is stabilized by disulfide bridges between EC1 and EC2 and between EC2 and the amino-terminal domain. There are also glycosylation sites within the N-terminal domain, at Asn18 in the human (h)GnRHR and Asn4 and Asn18 in the mouse (m)GnRHR. Mutation of these sites reduces cell surface GnRHR number (without altering receptor binding affinity), implying that glycosylation is important for GnRHR trafficking and/or stability, but not for conformation of the ligand-binding pocket.[25,27,31,39,237,238] Many point mutations have been introduced into GnRHRs, and functional analysis has yielded increasingly detailed maps of the predicted GnRHR tertiary structures annotated for amino acids involved in ligand binding and effector activation. This is reviewed elsewhere[25,27,30,31,34,35,238–240] and will not be considered in detail here, although type I GnRHRs have a number of atypical or unique structural features that certainly warrant mention. Firstly, there is a highly conserved Asp–Arg–Tyr sequence

toward the cytoplasmic end of TM3 in most GPCRs, which is replaced in GnRHRs by Asp–Arg–Ser, but this alteration is thought to have little or no effect on binding or signaling.[25,241] Secondly, there is an Asn at the position of a highly conserved Asp in TMD2 as the opposite change in 7TMD where the consensus Asn–Pro–XX–Tyr motif is Asp–Pro–XX–Tyr in GnRHRs. This interchange of conserved residues raised the possibility that they interact, as supported by the discovery that when Asn87 is replaced with Asp, there is no detectable binding, but when Asp318 is replaced with Asn (as well as replacement of Asn87 with Asp), binding is rescued. This recovery of high-affinity binding by reciprocal mutation (Asp87 and Asn318 GnRHR) supports the possibility that the two residues are adjacent to one another in space and helped confirm and refine models for structure of the TMD helix bundle.[25] A third, quite remarkable feature of GnRHR structure is that type I mammalian GnRHRs lack the intracellular carboxyl-terminal tail region (C-tail) found in all other GPCRs. The fact that this structure is present in all type II and III GnRHRs and in nonmammalian type I GnRHRs implies a period of rapidly accelerated molecular evolution with the advent of mammalian type I GnRHRs.[27,30,31,197,242,243] The C-tails of GPCRs have important roles in the regulation of receptor trafficking, desensitization, and signaling, and the functional relevance of their absence from mammalian type I GnRHRs is discussed here.

Although it was initially assumed that GPCRs function as monomers, it is increasingly evident that they can act as dimers or higher order multimers that may be homomeric or heteromeric.[244,245] For some GPCRs, oligomerization is thought to be associated with biogenesis in the endoplasmic reticulum (ER), whereas for others it can occur at the plasma membrane in association with ligand activation and/or ligand-induced internalization.[244–246] For GnRHRs, early studies using antibody to cross-link ligands suggested that small-scale receptor–receptor interaction (i.e., dimerization) was stimulatory; indeed, it is now thought that "microaggregation" of GnRHRs is a central and early event leading to productive calcium signaling and secretion of gonadotropins.[247,248] As more advanced biophysical techniques were developed, such as fluorescence resonance energy transfer (FRET) and fluorescence recovery after photobleaching (FRAP), greater details emerged regarding association of the GnRHR within the plasma membrane.[249,250] Such studies revealed that ligand occupancy of the GnRHR caused a marked reduction in the lateral diffusion kinetics of the receptor within the membrane as well as GnRH-dependent energy transfer between GnRHRs fused to different fluorophores, suggesting they indeed could self-associate within the plasma membrane of the gonadotrope. Importantly, administration of receptor antagonists (such as antide) in similar studies did not result in energy transfer between GnRHRs, suggesting that only GnRH agonists would promote the receptor oligomerization that is correlated with productive signaling. These experiments provided additional support to the microaggregration hypothesis and revealed the importance of receptor-associated membrane dynamics on GnRH action (sections Desensitization and Receptor Internalization and Lipid Rafts).

Heterotrimeric G-Proteins, Ca^{2+} Metabolism, and Acute Regulation of Gonadotropin Secretion

The endogenous GnRHRs of pituitary gonadotropes and gonadotrope-lineage cell lines signal primarily via the G_q and/or G_{11}. These are heterotrimeric G-proteins containing α, β, and γ subunits, of which the α subunits bind either guanosine-5′-triphosphate (GTP) or guanosine diphosphate (GDP).[251] Activated GPCRs are guanine nucleotide exchange factors for their cognate heterotrimeric G-proteins, stimulating the exchange of GDP for GTP. The GTP-bound α subunits separate from the βγ subunits (that remain bound to one another) and regulate enzyme or ion channel target proteins. In the case of G_q and G_{11}, the main target is phosphatidylinositol-specific phospholipase C (particularly PLC β1), which is activated primarily by the α subunits but also by βγ subunits. PLC cleaves membrane inositol phospholipids (particularly phosphatidylinositol-4,5-bisphosphate (PIP_2)) to generate inositol-1,4,5-trisphosphate (IP_3) and diacylyglycerol (DAG). IP_3 is a hydrophilic molecule that diffuses away from the membrane and acts via intracellular receptors that are ligand-gated Ca^{2+} channels on intracellular Ca^{2+} stores, mediating mobilization of Ca^{2+} and elevation of the cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$). Diacyclglycerols are lipophilic molecules that stay in the membrane, where they can activate PKC isozymes.[251] The PKCs are serine–threonine protein kinases divided into three families with characteristic domain structures and activation mechanisms. Thus, the conventional forms (cPKCs: α, βI, βII, and γ) are acutely activated by Ca^{2+} and DAG, the novel forms (nPKCs: δ, ε, θ, and η) are activated by DAG but not Ca^{2+}, and the atypical forms (aPKCs: ζ and ι/λ) are not activated by DAG or Ca^{2+}. The cPKCs are largely cytoplasmic in unstimulated cells, but Ca^{2+} binding induces their translocation to the membrane where they can be activated by DAG.[251] Similar translocation occurs with the nPKCs but is slower because they lack the C2 Ca^{2+}-binding domain needed for this Ca^{2+}-dependent increase in affinity for membranes.

There is abundant evidence from numerous models that GnRHRs engage the classical bifurcating signaling pathway that is outlined here[21,23–25,37,76,187,252–257] and illustrated in Figure 10.2. This includes the demonstration that radioligand binding to GnRHRs is modulated

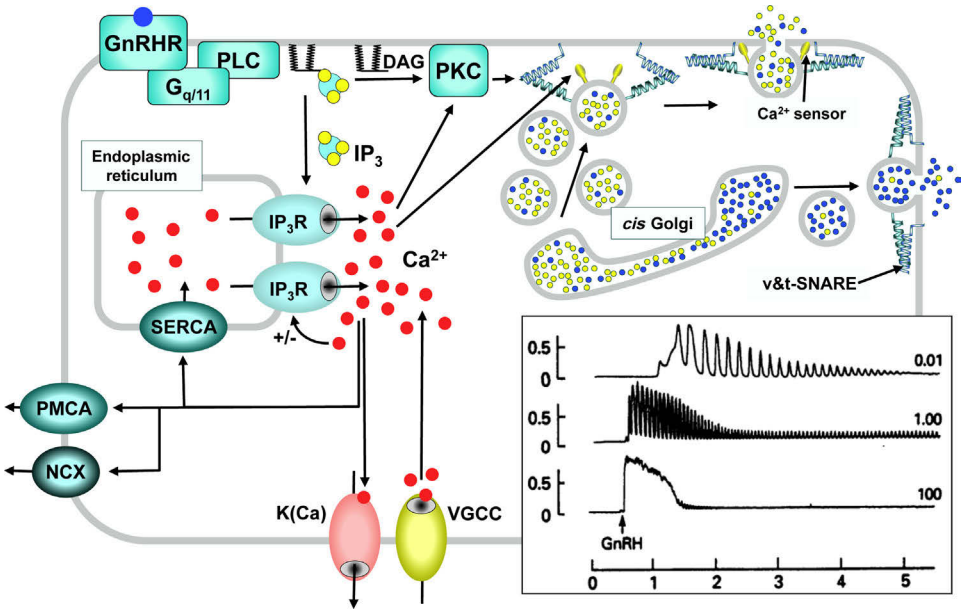

FIGURE 10.2 Acute effects of GnRH on Ca^{2+} and gonadotropin secretion. This figure is reproduced in color in the color plate section. GnRH acts via Gq/11 coupled GPCRs to activate PLC, with consequent IP_3-mediated Ca^{2+} mobilization and Ca^{2+}–DAG-mediated activation of PKC. The interplay of Ca^{2+} mobilization from intracellular stores and Ca^{2+} entry across the plasma membrane increases $[Ca^{2+}]_i$. This response may be oscillatory or more sustained, often with a spike–plateau-type profile. The response elicited by GnRH varies according to cell type and stimulus intensity but is also variable from cell to cell, even within isogenic cell populations. The GnRHR-mediated increase in $[Ca^{2+}]_i$ drives the secretion of LH and FSH by regulated exocytosis, a process likely involving interaction of a vSNARE (i.e., synaptobrevin) and a t-SNARE (i.e., syntaxin) as well as regulation by a Ca^{2+} sensor (i.e., synaptotagmin). PKC can also stimulate gonadotropin secretion, acting as a positive modulator of Ca^{2+}-driven exocytosis. In addition to regulated exocytosis, gonadotropins may be secreted via the constitutive pathway. The proportion of FSH (blue circles) secreted constitutively is higher than that of LH (yellow circles), and this could reflect sorting into distinct secretory vesicles formed at the Golgi. This principle is illustrated here by the presence of a Ca^{2+} sensor on LH-enriched vesicles but not on FSH-enriched vesicles. The inset shows μM $[Ca^{2+}]_i$ in individual cells stimulated with the indicated concentration of GnRH (plotted against time in min) and is adapted from Ref. 258.

by guanine nucleotides, which is indicative of G-protein binding,[259] as well as GnRH-induced downregulation of α_q and α_{11},[260] which is a characteristic consequence of their activation. PLC activation has been documented by showing GnRH-induced reduction in PIP_2 and associated increases in DAG and IP_3. Similarly, GnRH causes an increase in [^{3}H]inositol phosphate (IP) production in cells pretreated with [^{3}H]inositol (in order to label inositol phospholipids), an effect that can be prevented by inhibition of PLC.[252,261–264] PKC activation was initially documented with assays of enzymatic activity, showing that GnRH stimulates PKC translocation from the cytoplasm to the plasma membrane in vitro and in vivo.[265,266] In the latter case, the effect was inhibited by Ca^{2+} channel antagonists,[266] implying activation of one or more cPKC isozymes. Gonadotrope lineage αT3-1 and LβT2 cells express members of all three PKC families (α, β, δ, ε, θ, ι/λ, and ζ),[37,267–271] and translocation indicated activation of α, β, ε, and ζ isoforms by GnRH in αT3-1 cells.[37,267,270,271] In LβT2 cells, GnRH reduces levels of PKCδ, ε, and θ, which provides indirect evidence for their activation[272] and is consistent with the observation that PKCδ and PKCε can mediate GnRH-stimulated adenylyl cyclase activity in these cells.[273]

As expected for a $G_{q/11}$-coupled GPCR, activated GnRHRs mediate rapid increases in $[Ca^{2+}]_i$.[24,25,27,32,34,36–38,187,192,196,257,274–281] In some models, including gonadotrope lineage cell lines, high concentrations of GnRH cause a pronounced biphasic elevation of $[Ca^{2+}]_i$, with an initial spike followed by a more or less sustained plateau response, but in other models, including primary cultures of rodent gonadotropes, GnRH causes an oscillatory increase in the $[Ca^{2+}]_i$ or a spike response followed by $[Ca^{2+}]_i$ oscillations.[24,257,278–284] The spike phase of the response is attributed to IP_3-mediated Ca^{2+} mobilization from the ER, and its transience reflects the reduced $[Ca^{2+}]$ in the lumen of the ER as well as removal of Ca^{2+} from the cytoplasm by sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA) pumps, mitochondrial uptake, as well as Ca^{2+} ATPase pumps and Na^+–Ca^{2+} exchangers in the plasma membrane.[251]

To understand subsequent response phases, it is important to recognize that gonadotropes (like other endocrine anterior pituitary cells) are excitable cells in which electrically driven Ca^{2+} signals resemble those of neurons.[257,281] Gonadotropes show spontaneous electrical activity, in which gradually increasing membrane

potential reaches a threshold at which voltage-gated Ca^{2+} channels (VGCCs) are activated. This not only gives rise to the spiking depolarization but also drives voltage-gated Ca^{2+} influx. Where GnRH causes spike–plateau-type elevation of $[Ca^{2+}]_i$, the plateau response is rapidly reversed on transfer to Ca^{2+}-free medium, demonstrating dependence on ongoing entry across the plasma membrane, and this likely reflects depolarization and activation of voltage-gated Ca^{2+} influx and/or capacitative Ca^{2+} entry.[251,257,281,285] Where GnRH causes oscillatory increases in $[Ca^{2+}]_i$, these reflect the interplay of two oscillators. Here, key observations are that GnRH-induced $[Ca^{2+}]_i$ oscillations continue after the transfer of cells to Ca^{2+}-free medium and when membrane potential is clamped to prevent voltage gating, and the findings that oscillations can be caused by microinjection of metabolically stable IP_3 analogs or prevented by SERCA pump inhibition.[257,281,286–288] A mathematical model has been developed in which this oscillation is due to fast activation of IP_3 receptors (by Ca^{2+} and IP_3) and their slow inactivation (by Ca^{2+}). A fundamental feature of this model is the fast activation by Ca^{2+}, providing positive feedback for the upstroke of the oscillation, and the slow inactivation by Ca^{2+}, providing negative feedback for the downstroke.[257,281] This closed-cell oscillation model mirrors experimental observations but also predicts that a proportion of the Ca^{2+} mobilized in each oscillation will be pumped out of the cell. The driving force for IP_3 receptor–mediated Ca^{2+} mobilization is thereby reduced, so that oscillations will reduce in frequency and amplitude over time and will eventually terminate.[257] However, in an open system, influx of Ca^{2+} across the plasma membrane may prevent this run-down by enabling refilling of intracellular Ca^{2+} stores between pulses. The importance of this refilling is illustrated by experiments in which GnRH-stimulated Ca^{2+} oscillations run down in cells clamped at −60 mV to prevent Ca^{2+} entry via Ca_v, but increase as membrane potential is increased toward −20 mV to increase Ca^{2+} entry.[286,289] This demonstrates that oscillatory Ca_v-mediated Ca^{2+} entry is not needed for Ca^{2+} oscillations, but the bidirectional coupling of the ER and plasma membrane actually does provide coordinated ER and plasma membrane oscillations. Mobilization of Ca^{2+} from the ER leads to stimulation of Ca^{2+}-activated SK channels that contribute to hyperpolarization of the gonadotrope plasma membrane, and is followed by a burst of electrical oscillations. Each Ca^{2+} spike activates a Ca^{2+}-activated K^+ current that terminates or interrupts this electrical spiking. Thus, there are antiphasic oscillators, with membrane potential oscillations causing the Ca^{2+} entry needed for refilling of intracellular stores and Ca^{2+} oscillations that periodically interrupt the membrane oscillations and drive Ca^{2+}-dependent processes.

Ca^{2+} acts via sensors and effectors to influence a vast number of cellular activities, and in the case of GnRH, most emphasis has been placed on its role in mediation of exocytotic gonadotropin secretion as well as transcriptional regulation. Indeed, some of the earliest work on GnRH signaling revealed the importance of Ca^{2+},[23,24,183,190,192,290–293] showing not only that GnRH-stimulated gonadotropin secretion was markedly reduced in Ca^{2+}-free medium but also that the effect of GnRH on secretion could be mimicked by manipulations used to increase the $[Ca^{2+}]_i$ without GnRHR activation (Ca^{2+} ionophores, membrane depolarization, etc.). The spike–plateau phases of Ca^{2+} mobilization are mirrored by GnRH-stimulated gonadotropin secretion,[23,24,294] demonstrating the close relationship between $[Ca^{2+}]_i$ and exocytosis from gonadotropes. It is generally thought that global increases in $[Ca^{2+}]_i$ mediate GnRH-stimulated exocytosis, as opposed to the localized high concentrations of Ca^{2+} that mediate neurotransmitter secretion into the synapse,[281] and that the pattern of Ca^{2+} responses is not critical for secretion, as it can be evoked by either spike–plateau or oscillatory Ca^{2+} signals.[281,284] The Ca^{2+} effectors mediating GnRH-stimulated gonadotropin secretion have not been described in detail, although it has long been known that GnRH causes rapid translocation of the ubiquitous Ca^{2+} sensor calmodulin from the cytoplasm to the plasma membrane and that pharmacological inhibition of calmodulin can inhibit GnRH-stimulated gonadotropin secretion.[291,295–298] It is also well established that GnRHR-mediated PKC activation can be rapid (as revealed by rapid translocation to membranes) and that PKC activation can rapidly increase gonadotropin secretion. However, early PKC depletion experiments[274,299] revealed that the phorbol ester-sensitive PKC isozymes are not absolutely required for GnRH-stimulated gonadotropin secretion (whereas elevation of the $[Ca^{2+}]_i$ is required), implying that GnRH-stimulated gonadotropin secretion is Ca^{2+} mediated and PKC modulated.[192,274,299–301] This is in accord with data showing that PKC activation increases secretion from gonadotropes by increasing the sensitivity of the secretory machinery to Ca^{2+}(rather than by increasing the size of the releasable hormone pool) and that prior PKC activation can increase the potency of GnRH and Ca^{2+} ionophores at stimulation of gonadotropin secretion.[302,303] In other models, the small G-protein Rab3 tethers secretory vesicles to the plasma membrane, facilitating the NSF-primed assembly of vesicle membrane and target membrane proteins (i.e., syntaxin and synaptobrevin, complexed with synaptosomal-associated protein 25 (SNAP25)) that docks the granule to the membrane.[251] This docking machinery interacts with the Ca^{2+}-activated synaptotagmin that drives the fusion of docked vesicles with their target membranes. To our knowledge, the nature of this secretory machinery has

not been explored in detail in gonadotropes, although it is known that SNAP, soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE), and Rab3 family proteins are expressed in gonadotropes and that GnRH can increase SNAP25 mRNA expression in αT3-1 cells.[304] Here, it is also important to recognize that although Ca^{2+} mediates stimulation of regulated secretion, a significant proportion of gonadotropin secretion is via the constitutive pathway (Figure 10.2), such that pulses of GnRH-stimulated gonadotropin secretion are superimposed over significant levels of basal secretion.[34] Indeed, the proportion of FSH secreted constitutively exceeds that for LH,[34] and examination of hypophyseal and peripheral blood has revealed a high degree of synchrony between pulses of GnRH and LH, whereas FSH pulses are associated with only a small proportion of GnRH pulses.[305,306] Since LH and FSH are present in the same gonadotrope,[307,308] this requires sorting of the gonadotropins into distinct vesicles, and it is noteworthy that only vesicles containing LH are associated with the storage protein secretogranin II and that the amount of LH stored in the pituitary can be 10–50 times higher than that of FSH.[34,309,310] Thus, GnRH-stimulated Ca^{2+} transients drive regulated exocytotic release of storage vesicles containing LH, but have a less pronounced effect on FSH secretion because it is less abundant in these vesicles and is, instead, directed largely for constitutive secretion.[34]

Although GnRHRs characteristically mediate activation of G_q and/or G_{11}, there is good evidence for activation of other heterotrimeric G-proteins and for G-protein-independent GnRHR signaling. Early work with pituitary cells revealed that GnRH can stimulate an increase in cAMP levels,[311] although this does not necessarily reflect activation of G_s in gonadotropes. Moreover, GnRHRs have been shown not to increase cAMP or to do so by mechanisms other than G_s activation in various models.[273,312,313] Nevertheless, Arora et al. (1998) showed that GnRHRs expressed in COS cells mediate cAMP elevation and identified amino acids in IC1 required for the cAMP response but not for stimulation of [^{3}H]IP accumulation, uncoupling that argues against the cAMP response occurring downstream of PLC.[314] GnRH can also increase cAMP accumulation in LβT2 gonadotropes,[273,315] an effect that has been attributed to sequential activation of G_q and adenylyl cyclase (i.e., PKC-mediated activation of adenylyl cyclases 5 and 7[273]) or to direct activation of G_s.[315] In the latter case, GTP loading provided direct evidence for G_s activation. As noted here, GnRHRs are expressed in a number of nonpituitary sites, and the most compelling evidence for activation of multiple G-proteins by endogenous GnRHRs comes from work with immortalized GnRH-producing neurons.[316,317] In these cells, GnRH causes activation of G_s, G_i, and G_q as revealed by agonist-induced release of activated G-protein subunits from membranes and associated functional responses. Temporal differences were observed in G-protein activation, and a molecular switch was described with G_s being activated at lower GnRH concentrations than G_i and G_q.[316,317] There is also considerable evidence for GnRHR-mediated activation of G_i in cancer cell lines as, for example, in JEG-3 human choriocarcinoma cells, and in the human benign prostate hyperplasia (BPH) epithelial cell line (BPH1),[318] where GnRH inhibits forskolin-stimulated cAMP accumulation, and this effect is blocked by pertussis toxin, implying mediation by G_i. Moreover, in several models pertussis toxin has been shown to prevent antiproliferative effects of GnRHR ligands, again suggesting action via G_i.[318] In general, it appears that G_i is an important mediator of the antiproliferative effects of extrapituitary GnRHRs, whereas G_q is the primary mediator of pituitary GnRHR effects on gonadotropin synthesis and secretion.[39] Together, these studies demonstrate the potential for GnRH to activate distinct heterotrimeric G-proteins and to do so in a manner that is dependent upon cell context.

Multiple Lipid-Derived Messengers

Membrane phospholipids are a major source of signaling molecules generated in response to receptors activating distinct phospholipases that differ in terms of their preferred substrates and the phospholipid bonds that they hydrolyze. The phosphoinositide-specific PLCs are divided into five main groups: PLCβ, γ, δ, ε, and ζ. They have distinct domain structures and activation mechanisms but all have PIP_2 as the preferred substrate, and they cleave this to generate IP_3 and DAG.[251] There are two forms of phospholipases D (PLD); PLD1, which is associated with vesicles of the endosomal–lysosomal pathway, and PLD2, which is mainly located at the plasma membrane. Both act mainly on phosphatidylcholine to produce phosphatidic acid (PA) and polar alcohol. At least 20 phospholipase A2 (PLA2) enzymes have been cloned in mammals, all of which release long-chain unsaturated fatty acids, including arachidonic acid (AA), oleic acid, and linoleic acid, from the sn-2 position of membrane phospholipids.[37,319] They are divided into the small secretory forms ($sPLA_2$) that are activated by mM Ca^{2+}, the cytosolic forms ($cPLA_2$) that are activated by serine phosphorylation and by μM Ca^{2+}, and the calcium-independent group ($iPLA_2$). The AA produced by PLA_2 can directly regulate target proteins, can be converted to prostaglandins (PGs) and thromboxanes via cycloxygenase 1 (COX1) or COX2 and PG synthase, or via lipoxygenases can be converted to lipid mediators, including leukotrienes.[37] GnRHRs characteristically mediate rapid (within 5–30 s) activation of PLCβ, which may be followed (after approx. 5–15 min) by activation

of PLD and PLA_2. The PA produced by PLD can be converted to DAG, so its delayed activation may mediate a second, more sustained wave of PKC activation.[37] Conversely, GnRHRs have been shown to cause a c-Src-mediated activation of diacylglycerol kinase (DGK)-ζ, an enzyme that phosphorylates DAG to produce PA. PA is itself able to alter activity of PAK1 and RasGAP as well as DNA synthesis and actin polymerization,[320] so PLD and DGK-ζ may both promote PA-mediated signaling.

Lipoxygenase products of AA metabolism are implicated in control of GnRH secretion and synthesis,[37] and its PG products have been shown to selectively inhibit GnRH-stimulated LH secretion.[37] Whereas GnRHR-mediated PLCβ stimulation involves direct activation by subunits of $G_{q/11}$, activation of PLA_2 and PLD is less direct. Many GPCRs activate PLD via the small G-protein ARF and RhoA, and this effect appears dependent on the presence of an Asn–Pro–XX–Tyr motif in TMD7 (and, as noted above, this is Asp–Pro–XX–Tyr in GnRHRs). Consequently, they do not cause ARF-mediated PLD activation, but they do so if the Asp is replaced by Asn.[321,322] Instead, it is thought that PLD activation occurs downstream of PLC and PKC, as it is reduced by inhibition of PLC and by depletion of PKC or inhibition of Ca^{2+} entry via VGCCs.[24,323,324] Whilst considering the hierarchical activation of phospholipases, it is also important to recognize that GnRHRs cause a PKC-mediated activation of epidermal growth factor (EGF) receptors in some models (see the section Mitogen-Activated Protein Kinases) and might therefore be expected to activate PLCγ, a well-established effector for EGF receptors. Moreover, since all of the PI-specific PLCs (β, γ, δ, and ε) are Ca^{2+} sensitive,[251] the potential exists for their activation as a consequence of GnRHR-mediated Ca^{2+} mobilization. PLA_2 activation by GnRH has not been explored extensively, although a recent study showed that LβT2 cells express $cPLA_2$ and $iPLA_2$ transcripts and (rather surprisingly) that GnRH caused AA release by activation of the Ca^{2+}-insensitive $iPLA_2$.[319]

Although much work on GnRH signaling focuses on PIP_2 as a source of signaling intermediates, PIP_2 is itself an extremely important signaling molecule that directly regulates effectors, including transient receptor potential channels (TRPV1 and TRPM7), K^+ channels (ATP-sensitive, HERG, and KCNQ K^+ channels), and PLD,[251] although the potential relevance of such effects for GnRH signaling has not, to our knowledge, been explored. Moreover, enzymes of the PI3-kinase (PI3K) family phosphorylate PIP_2 to generate $PI3,4,5P_3$, the main second messenger in the PI3K signaling pathway that influences a wide range of cellular processes, including glycogen metabolism, gene transcription, and cell growth and apoptosis.[251] Tyrosine kinase receptors can feed into this pathway by p85-mediated activation of class 1A PI3K, whereas GPCRs can do so by βγ subunit–mediated activation of class 1B PI3K.[251] Both phosphorylate PIP_2 to generate $PI3,4,5P_3$, which directly activates several important target proteins, including PLCγ and PKCs δ and ζ, as well as the serine–threonine protein kinase Akt–protein kinase B (PKB) that has important effects on cell fate and monomeric G-proteins (Rac, Rho, and Cdc42) that regulate cytoskeletal architecture.[251] Roles of the PI3K signaling pathway in GnRH signaling have not been extensively explored, although GnRH has been shown to activate PI3K via transactivation of EGF receptors in GT1-7 cells (immortalized GnRH-expressing neurons),[325] whereas it inhibits IGF-I-stimulated activation of Akt–PKB in αT3-1 cells[326] and PI3K has been identified as a negative regulator of FSHβ and LHβ gene expression in LβT2 cells.[327]

Mitogen-Activated Protein Kinases

Mitogen-activated protein kinases (MAPKs) are Ser–Thr protein kinases with pivotal roles in control of many cellular activities, including proliferation, survival, differentiation, growth, and apoptosis. The MAPK modules contain three protein kinases—an MAPK kinase kinase (MAPKKK), an MAPK kinase (MAPKK), and MAPK—that phosphorylate and activate one another in sequence, as well as numerous upstream regulators and downstream effectors[251,328–330] (Figure 10.3). The four major mammalian cascades are designated according to their MAPKs, which are ERK1 and ERK2 in the ERK cascade, c-Jun N-terminal kinases 1–3 (JNK1–3) in the JNK cascade, p38α–δ in the p38–stress-activated protein kinase, and big MAPK (BMK) in the ERK5 cascade.[251] The best characterized of these is the ERK cascade (where the term ERK is used to mean ERK1 and/or ERK2), in which a Raf protein kinase (i.e., the MAPKKKs Raf1, A-Raf, and B-Raf) phosphorylates and activates MEK (i.e., the MAPKKs MEK1 and MEK2). Activated MEK then phosphorylates ERK on Thr and Tyr residues of a TEY activation loop. ERK is a Ser–Thr kinase that phosphorylates substrates in a consensus Pro-X-Ser/Thr–Pro sequence. It has numerous substrates, including cytoplasmic proteins such as cPLA2, B-Raf, and SOS (Son of Sevenless), and transcription factors such as c-Fos, c-Jun, Elk1, STAT1, CREB, and Egr1.[251] ERK is mostly cytoplasmic in unstimulated cells, but activation causes its translocation to the nucleus, facilitating access to transcription-regulating substrates and thereby mediating effects on cell fate.[331–335] ERK does not contain nuclear localization or export signals, and it therefore relies on association with other proteins for appropriate cellular compartmentalization.[336] Thus, cytoplasmic and nuclear binding proteins influence ERK distribution by altering the proportion of ERK available for movement to or from the nucleus. Notably, MEK is a major cytoplasmic scaffold of ERK. When MEK phosphorylates

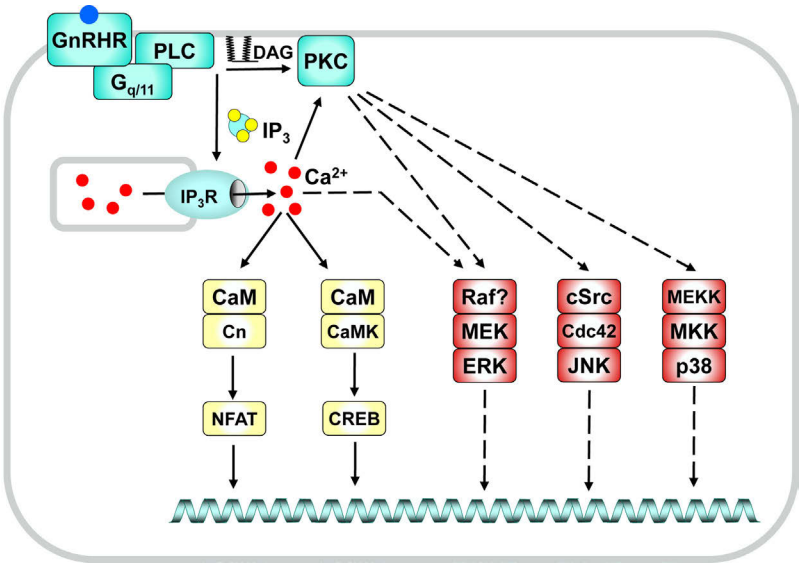

FIGURE 10.3 GnRH signaling from the plasma membrane to the nucleus. This figure illustrates pathways by which GnRHR can mediate effects on gene expression. One of the key Ca^{2+} targets implicated in GnRH action is calmodulin (CaM), which has the potential to activate the Ca^{2+}-sensitive transcription factor NFAT by activation of the protein phosphatase calcineurin (Cn), and to activate the transcription factor CREB by activation of calmodulin-dependent protein kinase (CaMK). The other key Ca^{2+} target illustrated is PKC, which acts (by numerous mechanisms) as a positive regulator of ERK, JNK, and p38 MAPK cascades, each of which influences gene expression by phosphorylation of targets, including transcription factors. This illustrates only a small proportion of the known mechanisms. For the sake of simplicity, we focus solely on chronic responses following the acute effects shown in Figure 10.2 and have not included cross-talk, convergence, compartmentalization, or kinetics.

ERK, this causes its liberation from MEK and facilitates its interaction with other proteins, including those of the nuclear pore complex. Consequently, MEK activation characteristically increases the proportion of ERK in the nucleus, and this effect is dependent upon the TEY motif but not on catalytic activity of ERK.[337–344] Other proteins that can cause cytoplasmic retention of ERK include PEA15, dual-specificity phosphatase 6 (DUSP6), β-arrestins, and Sef.[335,345–350] In contrast, expression of nuclear DUSPs 2, 4, and 5 can mediate dephosphorylation and nuclear anchoring of ERK.[351–354]

ERK binds to a large number of proteins (>400, including substrates, scaffolds, and phosphatases) in a dynamic and highly specific manner,[336] and this is dependent upon docking domains. The common docking (CD) motif in ERK binds to Lys- and Arg-rich sequences called D (docking) domains in its binding partners.[355] Another docking site in ERK protein partners is termed the DEF (docking site for ERK: F/Y-X-F/Y-P) motif, and this binds to a DEF-binding pocket (DBP) in ERK.[356,357] ERK protein partners can contain D and/or DEF domains,[356,358–360] and release of ERK from MEK exposes these docking sites for interaction with other binding partners during stimulation.

The classic mechanism for ERK activation involves stimulation of tyrosine kinase receptors that dimerize to facilitate autophosphorylation on tyrosines and consequent recruitment of transducers. Thus, for example, EGF-activated ErbB1 receptors recruit an adapter protein (Grb2) via its SH2 domains, and Grb2 binds SOS via the SH3 proline-rich domain. SOS is a guanine nucleotide exchange factor for the monomeric G-protein Ras, and the active (GTP-bound) form of Ras activates the MAPKKK Raf.[251] However, many other stimuli activate ERK by a range of mechanisms that may or may not involve transactivation of tyrosine kinase receptors. GnRH has been shown to activate ERK in many systems, and the mechanisms appear to be dependent upon cell context. The endogenous GnRHRs of rat pituitary cells and gonadotrope lineage αT3-1 or LβT2 cells mediate ERK activation that is dependent upon PKC and on Ca^{2+} influx via VGCCs,[37,38,361–366] which is in accord with evidence showing that PKC can feed into activation of Raf and that ERK activation is often Ca^{2+} dependent.[251] However, the absolute role of Raf in ERK activation by GnRH is not completely clear since conditional deletion of c-Raf kinase in mouse gonadotropes did not cause a reproductive phenotype.[367] GnRH-mediated ERK activation was also shown to be dependent upon c-Src and Ras in αT3-1 cells and upon c-Src in LβT2 cells, supporting a model in which PKC causes a c-Src-mediated activation of Ras.[37,361,362] As an alternative mechanism, the endogenous GnRHRs of GT1-7 cells cause PKC-mediated transactivation of EGF receptors and consequent activation of ERK. EGF receptor transactivation could reflect c-Src-mediated phosphorylation of the EGF receptor, or the shedding of soluble EGF receptor ligands such as heparin-binding EGF.[368] In the

latter case, matrix metalloproteinases (MMPs) release the soluble ligand to activate EGF receptors and feed into the canonical activation pathway. In GT1-7 cells, GnRH causes a PKC-mediated activation of MMPs and an EGF receptor–mediated activation of ERK,[368] whereas GnRHR-mediated ERK activation has been reported to be independent of EGF receptors in αT3-1 and LβT2 cells[361,362] or to be dependent on MMP2 and MMP9 and EGF receptor transactivation in αT3-1 cells.[369]

In addition to activation of ERK, GnRH can activate the JNK, p38, and ERK5 cascades in various cell models with variable kinetics (Figure 10.3). The canonical JNK cascade is activated in response to osmotic stress and redox stress that activate monomeric G-proteins (Rac, Cdc42, and Rho), which activate MAPKKKs, including MEKK1, MLK1, TAK1, ASK1, and TPL2. These phosphorylate and activate the MAPKKs MEK4 and MEK7, which, in turn, phosphorylate and activate JNKs 1–3.[251] The JNKs influence cell fate (particularly cell division and apoptosis) by phosphorylation of effectors, including c-Jun, Jun-B, Jun-D, ATF2, and Elk1. GnRH causes JNK activation that has been reported to be dependent upon PKC, c-Src, CDC42–Rac1, and MEKK1 in αT3-1 cells,[370] whereas others have reported dependence on Ca^{2+} but not PKC in these cells,[371] or independence from PKC and Ca^{2+} in LβT2 cells.[365] The canonical p38 cascade is activated under osmotic stress and by Fas ligand, which cause receptor-mediated activation of the MAPKKK TAK1, and by some cytokines that cause Rac- and Cdc42-mediated activation of TAK1. TAK1 then phosphorylates and activates the MAPKK MEK3, which in turn activates and phosphorylates the p38α–δ MAPKs.[251] The p38 MAPKs mediate effects on cell function (notably apoptosis, cytokine release, and cell cycle arrest) by phosphorylation of cytoplasmic targets, including MNK, MSK, MK3, and MK5, and nuclear targets, including ATF2, Ets, SAP1, and CHOP. GnRH activates p38 MAPK in αT3-1 and LβT2 cells, an effect that is mimicked by a PKC-activating phorbol ester and prevented by PKC depletion, implying mediation by PKC.[269,362] Finally, several mitogens and cell stress activate the ERK5 cascade via an upstream transducer (LAD1) that activates the MAPKKKs MEKK2 and MEKK3. These then activate the MAPKK MEK5, which phosphorylates and activates ERK5. ERK5 activates a number of transcription factors, including MEF2, SAP1, and Fra1. These mediate ERK5 effects on cell proliferation and stress responses,[37] and recently, GnRH was shown to activate ERK5 in LβT2 cells.[372]

In contrast to the information available on activation mechanisms, much less is known about inactivation of MAPKs after GnRH stimulation, an issue that is particularly germane with physiologically relevant pulsatile stimulation (see the section Extrapituitary GnRHRs and Context-Dependent GnRHR Signaling and Ligand Bias). This is a complex area as the human genome has many phosphatases involved in signal transduction, including 23 Ser–Thr phosphatases and a protein tyrosine phosphatase (PTP) family with 106 members.[373–375] The PTPs can be divided into four subfamilies on the basis of amino acid sequence in the catalytic domains, and the first of these (type I Cys-based PTPs) is further divided into the classical PTPs and the dual-specificity phosphatases (DUSPs). MAPKs can be dephosphorylated and inactivated by multiple Ser–Thr phosphatases and PTPs,[374] so the number of enzymes that inactivate MAPKs by dephosphorylating one or both of the phosphosites in their activation loops is much greater than the number of activating kinases.[373,374] In the case of GnRH signaling, most work has focused on the DUSPs, enzymes characterized by their ability to dephosphorylate both the Tyr and the Ser–Thr residue within the same substrate. The catalytic pocket of DUSPs is shallower than that of other PTPs, and it is thought that this enables them to simultaneously accommodate both phosphosites in the substrate.[374] Two major groups of DUSPs are the MAPK phosphatases (MKPs) and the closely related atypical DUSPs.[374] The three groups of MKPs acting directly on MAPKs are (1) the nuclear-inducible MKPs (DUSP1–MKP1, DUSP2–PAC1, DUSP4–MKP2, and DUSP5–VH3), which all dephosphorylate ERK and (with the exception of DUSP5) also dephosphorylate JNK and/or p38; (2) the cytoplasmic ERK MKPs (DUSP6–MKP3, DUSP7–PYST2, and DUSP9–MKP4), which preferentially target ERK; and (3) the JNK–p38 MKPs (DUSP8–VH5, DUSP10–MKP5, and DUSP16–MKP7), which preferentially target JNK–p38.[353,354,373–378] In addition, the atypical DUSPs lack obvious MAPK-targeting motifs and dephosphorylate a broad group of substrates. However, some members of this group are known to act directly on MAPKs.

Many stimuli cause an ERK-mediated increase in expression of nuclear-inducible MKPs that can influence the amplitude, duration, and cellular compartmentalization of MAPK activation. Indeed, early studies showed that GnRH increases the expression of two nuclear-inducible MKPs (DUSP1 and DUSP4) in αT3-1 cells.[379–381] In this model, the increased expression of MKPs caused by sustained stimulation with GnRH exerted negative feedback on JNK but had little or no effect on the ERK response. Subsequent work in a GnRHR-transfected HeLa cell model again revealed induction of DUSP1 and DUSP4 (as well as DUSP2) by GnRH.[382] In this model, endogenous ERKs were knocked down with small interfering RNA (siRNA), and recombinant adenoviruses were used to add back an ERK2–GFP reporter at a concentration similar to that of the endogenous kinase in normal cells.[353,354,382] This approach enables mutant constructs to be used, and it was found that GnRH has a greater effect on ERK activation when a D319N mutation is introduced into ERK2 in order to prevent

D-domain-dependent association with binding partners, including DUSPs.[382] Similar amplification of responses was seen when protein neosynthesis was prevented with cycloheximide,[382] and a siRNA screen targeting all of the expressed DUSPs revealed effects of GnRH to be influenced by knockdown of DUSP from each MKP class (DUSPs 3, 5, 9, 10, and 16). Nevertheless, knockdown of DUSPs 1, 2, and 4 (alone or in combination) did not alter GnRHR effects. Thus, GnRH-mediated ERK responses are modulated by DUSPs and are dependent on both protein neosynthesis and D-domain binding, but the nuclear-inducible DUSPs appear not to shape ERK signaling in this model,[382] as has been reported for DUSP1 and DUSP4 in αT3-1 cells.

This section has been intended primarily to describe the fundamental pathways and mechanisms of MAPK activation by GnRHRs. However, much work focuses on the role of these pathways in mediating GnRH effect on gene expression, and this is dealt with in the section Regulation of Gene Expression. Similarly, more recent work on the compartmentalization of signaling, the spatial and temporal aspects of ERK activation, and the roles of MAPKs in mediation of pulsatile GnRHR signaling are dealt with in the sections Lipid Rafts and Extrapituitary GnRHRs, Context-Dependent GnRHR Signaling, and Ligand Bias.

Regulation of Gene Expression

The GnRH-Induced Gene Network: A Genomewide Perspective

The Sealfon group has been instrumental in examining the genomewide impact of GnRH on models of the pituitary gonadotrope.[383–387] These studies were carried out in the LβT2 gonadotrope-derived cell line examining changes in transcript abundance at 1 and 6 h following application of GnRH. Custom cDNA microarrays containing 956 clones of transcripts identified via literature search or from input from collaborating laboratories were used. These studies were some of the initial efforts to describe genomewide responses to GnRH, and they identified a relatively large group of transcripts that helped to identify the immediate early gene repertoire, including a group of transcription factors and modulators of cell signaling.[383] Based upon the cDNA microarray, transcript abundance for transcription factors such as c-Fos, Egr1, and ATF3 was markedly upregulated from 1.3- to 30-fold and formed the foundation for later analyses of regulation of the gonadotrope signature genes. For example, the activator protein 1 (AP1) heterodimer (a c-Fos–c-Jun heterodimer) plays an important role in the regulation of FSHβ and αGSU promoters,[170,388] Egr1 plays an essential role in the regulation of the LHβ gene promoter,[164,389] and ATF3 was linked to expression of the human αGSU gene promoter.[388,390] The signaling and transcriptional networks induced by GnRH in this cell model are reviewed extensively in Refs 391,392. These types of genomewide analyses have not yet been reported in vivo using mouse models to confirm the important findings of the LβT2 cell model.

The genomewide micro-RNA (miRNA) transcriptome was also investigated by the Sealfon group in LβT2 cells using microarrays to examine the possibility that miRNAs may play an important role in modulating responses to GnRH in gonadotropes.[393] Several hundred miRNAs were detected in this cell model, were validated beyond the microarray using quantitative PCR (qPCR), and were largely present independent of GnRH action. Two miRNAs (miR-132 and miR-212) were regulated by GnRH, but these miRNAs did not respond to specific changes in GnRH pulse frequency. Follow-up experiments by the Webster group[394] demonstrated that miR-132 and miR-212 targeted p250 Rho GAP, a GTPase-activating protein. Using knockdown strategies, p250 Rho GAP appears to play an important role in the GnRH-induced modification of LβT2 cell morphology. Loss of p250 Rho GAP resulted in increased activity of the small GTPase Rac, leading to increased formation of neurite-like cellular processes and a marked increase in cell motility in vitro. Collectively, these studies suggest important genomewide alterations in the RNA and miRNA transcriptomes in LβT2 cells, setting the stage for these types of analyses of these potential changes induced by GnRH and potentially GnRH pulse frequency in vivo.

Transcriptional Regulation of the Gonadotrope Signature Genes

This chapter has outlined mechanisms associated with the development of pituitary cell lineages during organogenesis. This complex process is critically dependent upon specific transcriptional regulators such as Lhx3, NR5A1, and Pitx family members. Cell differentiation largely reflects the ability of specific transcriptional regulators to control cell-specific gene expression networks. This scenario is not necessarily specific to developmental programs and can often reflect mechanisms that persist into the adulthood of a living animal. For example, NR5A1 and Pitx family members contribute to the regulation of GnRHR, αGSU, LHβ, and FSHβ subunit gene promoters, while Lhx3 appears to regulate GnRHR, αGSU, and FSHβ subunit gene promoters. Furthermore, expression of the four gonadotrope signature genes during the estrous cycle and following castration reflects physiologically relevant input by factors such as gonadal steroids and GnRH pulsatility directly on the gonadotrope that modulates "inducible" gene regulation appropriate to physiological needs, such as during the transition from the prepubertal to sexually mature state and during portions of the estrous cycle leading up to the preovulatory surge of LH and ovulation. The

following section explores the transcription factors and signaling systems that contribute to the regulation of the GnRHR, αGSU, LHβ, and FSHβ subunit gene promoters (Figure 10.4). Where relevant, we explore mouse gene knockout models that provide critical in vivo evidence for these relationships.

Regulation of the GnRHR Gene Promoter

In the context of the mammalian estrous cycle, changes in GnRHR gene transcription lead to crucial changes in receptor numbers at the plasma membrane of the gonadotrope. Increased receptor numbers alter the sensitivity and responsiveness of the gonadotrope during physiologically relevant situations such as changes leading to the preovulatory surge of LH and ovulation. GnRH and estradiol are thought to be key regulators of GnRHR gene expression.[395–400] Moreover, the GnRHR gene promoter is also a target of activin signaling in the gonadotrope.[401–407] In some instances, GnRH and activin may function together to regulate GnRHR transcription. Alternatively, regulation of GnRHR transcript abundance is likely to be independent of gene transcription and can be regulated at the translational level.[408] Here, we focus primarily on discussions of regulatory mechanisms of the murine GnRHR since much of what we know of the gene regulation of the GnRHR comes from this species, from both murine clonal cell lines and gene knockout models. The reader should bear in mind that important species similarities and differences exist; due to space constraints, these species differences are not covered to any great extent here.

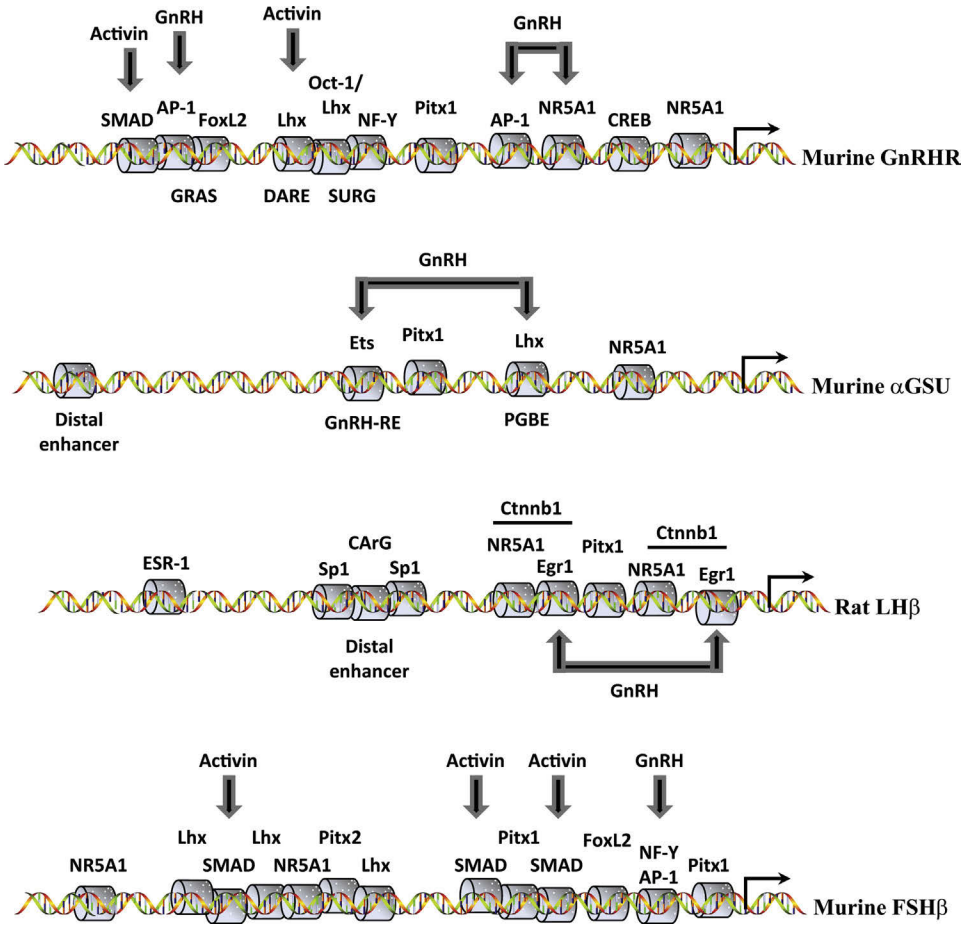

FIGURE 10.4 Transcriptional control of the gonadotrope signature gene promoters. The diagram depicts the 5′ flanking sequences from the four signature genes that define the gonadotrope cell lineage; the murine GnRHR, the murine glycoprotein hormone α subunit (αGSU), the rat LHβ subunit, and the murine FSHβ subunit. Individual *cis* regulatory elements and associated transcription factors are depicted within the promoter upstream of the transcription start site (depicted by the black arrow). Spatial organization of these elements is not drawn to scale but reflects the relative placement of these elements within the promoter. Single and combinations of elements involved in regulation by GnRH and activin are identified by the gray and black arrows. In some instances, single or combinatorial elements are identified: GRAS: GnRHR-activating sequence; DARE: downstream activin regulatory element; SURG: sequence underlying responsiveness to GnRH; GnRH-RE: GnRH responsive element; PGBE: pituitary glycoprotein hormone basal element.

In the mouse, basal and GnRH-regulated control of the GnRHR promoter requires 1.2–1.9 kb of the 5′ flanking sequence. Gonadotrope-specific expression and induction by GnRH were seen in transgenic mice harboring reporters controlled by either of two GnRHR promoter fragments.[397,409,410] Similar observations were made using clonal gonadotropic and heterologous cell lines. Within this region of the murine GnRHR gene promoter, a number of *cis* regulatory elements have been identified, including the GnRHR activating sequence (GRAS),[411,412] sequences underlying responsiveness to GnRH (SURG),[397,413] the downstream activin regulatory element (DARE),[414] an AP1 site, a NR5A1-binding site,[412] and a Pitx1-binding site.[415,416] The GRAS element is a complex combination of an SMAD, AP1, and forkhead factor–binding sites; mutations within GRAS, the AP1 site, and the NR5A1 element each have a marked impact on basal regulation of the murine GnRHR promoter; and mutations in all three of these elements essentially abolish promoter activity. The engagement of the AP1 and NR5A1 elements appears to be critical for basal and GnRH-stimulated activity of this promoter, and these data provide corroborative evidence for the importance of NR5A1 on GnRHR regulation, consistent with the NR5A1 null mouse described in this chapter.[146,147] However, since gonadotropin secretion can be partially rescued by exogenous GnRH treatment in the NR5A1$^{-/-}$ mouse, it seems likely that the role of NR5A1 in gonadotropes is in combinatorial context with other important regulators of GnRHR promoter activity. The role of the AP1 site is clearly central to GnRH regulation of the GnRHR, and this finding helps to substantiate findings that GnRH action to activate the c-Jun N-terminal pathway and Fos–Jun family members is required with this role.[400] Interestingly, gonadotrope-specific conditional deletion of ERK1 and ERK2 does not appear to have a strong impact on GnRHR transcript levels in the mouse gonadotrope in vivo,[38,166] suggesting that GnRH-induced JNK activity is central to this AP1 site.

Activins play an important role in the regulation of both the GnRHR and FSHβ subunit (described further in this chapter) gene promoters in gonadotropes. Activins are TGFβ family members composed of inhibin βA and βB dimers.[170,417] Originally identified along with inhibin from gonadal extracts, activin is now known to be synthesized and secreted by gonadotropes to function in an autocrine and/or paracrine manner. Given the fact that activin has important effects of GnRHR gene transcription, the overall impact of activin action on gonadotropes likely goes well beyond regulation of FSH synthesis and secretion. In the context of the GnRHR promoter, the GRAS binding site along with the DARE element appears to be critical for activin regulation; and GRAS together with the AP1 site facilitates synergistic activation by GnRH and activin.[411] The SMAD binding site within GRAS is central to activin action. Activin induces recruitment of SMAD3 and SMAD4 to the SMAD-binding site in GRAS; SMAD association with this complex is required for the recruitment of SMAD3.[411] Administration of activin alone in αT3-1 cells does not affect GnRHR promoter activity; however, in combination with GnRH, activin induces a synergistic transcriptional response. A direct effect of activin on the murine GnRHR was better defined by culturing gonadotrope cells in the presence of follistatin, a known inhibitor of FSH secretion through inhibition of activin action.[170,414] These studies suggested that follistatin effectively interferes with endogenous activin produced by gonadotropes and with activin inhibition, GnRHR promoter activity was induced. Using this strategy, these authors were able to more critically define the *cis*-acting regulatory elements necessary for activin action. This included identification of the DARE element consisting of a binding site for a putative homeobox protein likely in the Lhx family; Lhx2 or Lhx3 are likely candidates; however, this has not been confirmed.[414] Thus, activin action on the murine GnRHR required GRAS and DARE and the combined actions of GRAS, DARE and the AP1/NR5A1 sites working as an enhanceosome facilitate synergist activation of the GnRHR by activin and GnRH.

The third component to bind to the GRAS element is the forkhead factor, FoxL2.[411,418] Interaction between FoxL2 and the proximal end of the GRAS element was demonstrated using standard DNA binding assays along with definitive interactions shown using a yeast two-hybrid system. Interestingly, FoxL2 was also shown to physically interact with both AP1 and SMAD4 and optimally with a SMAD3/4 dimer. FoxL2 is first expressed in the anterior pituitary coincident with αGSU during development, and expression persists in gonadotropes and thyrotropes into adulthood.[418] Interestingly, disruption of FoxL2 by mutation in human patients or by knockout in mice results in sterility due to gonadal dysgenesis reminiscent of premature ovarian failure.[419,420] Thus, the impact of FoxL2 is clear at multiple levels of the hypothalamic–pituitary–gonadal axis.

The SURG element within the murine GnRHR promoter lies in close proximity to the GRAS element, and together with the AP1 site, the SURG element facilitates basal and GnRH-stimulated GnRHR promoter activity.[396,397] Like the GRAS element, SURG is a composite element consisting of binding sites for Oct1 and NF-Y; mutations in these elements reduce basal expression of the promoter, and combined SURG and AP1 site mutations abolish GnRH action on the GnRHR promoter. Also important to this mechanism is Pitx1 binding to the proximal murine GnRHR promoter.[416] Pitx1 in combination with the SURG element and the AP1 site, and in particular specific interactions between c-Jun and Pitx1, facilitate GnRH-induced GnRHR promoter activation.

Given the importance of estradiol in GnRHR regulation during the estrous cycle, the presence of an estradiol responsive element within the GnRHR gene promoter would not be surprising; however, this is not the case. The effects of estradiol on GnRHR are directly at the level of the gonadotrope[421–423] based on studies using primary culture of pituitary cells in the absence of hypothalamic input. Original predictions included the possibility that since estrogen receptor α (ESR1) and Fos–Jun family members could functionally interact in complexes associated with chromatin-remodeling enzymes, the effects of estradiol via ESR1 were through the AP1 site within the GnRHR promoter.[424] More recently, using ovine pituitary cells, Nett and colleagues[398] demonstrated that estradiol action on the ovine GnRHR was robust and could be recapitulated with estradiol–bovine serum albumen (BSA) conjugates, suggesting that the effects of estradiol were not through direct genomic actions but were indirect through membrane-associated ESR1. Defining this unique pathway will likely provide important clues for how estradiol functions as a critical regulator of GnRHR gene transcription during the mammalian estrous cycle.

Transcriptional regulation of GnRHRs within gonadotropes is critical in facilitating changes in gonadotrope sensitivity to GnRH during the mammalian estrous cycle. However, not all changes in GnRHR levels are via gene expression, and key mechanisms may include modulation of GnRHR protein levels by modulating translation efficiency of the GnRHR transcripts.[408] Using αT3-1 cells as a model system, chronic exposure to high relative levels of GnRH resulted in a depletion of ~75% of cell surface receptors by 24 h. Interestingly, GnRHR transcript levels were unchanged in the paradigm. Isolation of the transcripts and subsequent injection into *Xenopus* oocytes revealed that these GnRHR transcripts were greatly compromised in their ability to generate cell surface GnRHRs capable of mediating membrane-associated electrical currents. Translational inhibition appeared to be attributed to compromised translational efficiency on polyribosomes. Thus, chronic exposure to GnRH pharmacologically or potentially during the preovulatory GnRH–LH surge downregulates GnRHRs without apparent changes in GnRH-induced gene transcription but altered translational efficiency.[408]

Regulation of the Glycoprotein Hormone α Subunit Gene Promoter

The glycoprotein hormones (LH, FSH, TSH, and chorionic gonadotropins in primates and equines) are heterodimers consisting of a common αGSU and a hormone-specific β subunit. The β subunits of LH and FSH appear to be rate limiting in the formation of these heterodimeric hormones and confer individual hormone specificity.[425] Conversely, the αGSU is common to all of these hormones within a species and does not appear rate limiting in the assembly of the gonadotropins. A great deal of research has focused on the cell type–specific regulation of the gonadotropin subunit gene promoters due to their central importance to the regulation of gonadal function and fertility.[38] Moreover, analysis of the αGSU has represented an important model system for cell-specific transcriptional regulation since this same gene is regulated differently in gonadotropes, thyrotropes, and placental trophoblasts.[426–432] This includes a rather complex analysis of the contributions of combinations of *cis* regulatory elements in a so-called combinatorial code that control cell-specific regulation.[428]

In the mouse, ~0.5 kb of a 5′ flanking sequence is required for basal gonadotrope-specific expression of the αGSU promoter; in humans, a 0.88 kb fragment has been central to these studies. In mouse models in vivo, ~4.3 kb of the mouse promoter has been used to direct Cre recombinase expression to gonadotropes and thyrotropes.[166,433] The central regulator of αGSU gene transcription appears to be GnRH, where loss of hypothalamic GnRH stimuli results in a marked reduction of αGSU transcript abundance[4,434–436]; GnRH replacement in these models rescues this phenotype. These studies are complicated by the fact that αGSU is also expressed in thyrotropes, which contributes to the overall measurement of these transcripts. In addition to GnRH, androgens have been implicated as a modulator of negative feedback on αGSU gene transcription specifically at the level of the gonadotrope.

Basal regulation of the αGSU is regulated through the actions of three elements within the mouse promoter; the pituitary glycoprotein hormone basal element (PGBE), a Pitx1, and NR5A1-binding sites. The PGBE was originally identified in a mutagenesis screen of the promoter where mutations in this site dramatically reduced basal expression and, to a lesser extent, interfered with GnRH-induced expression of αGSU.[426,427,437] Expression cloning of a λGT11 library using a multimer of the PGBE yielded the complete open reading frame of a LIM class homeobox-containing protein, Lhx2.[437] Lhx2 was shown to be expressed abundantly in αT3-1 cells and in cell lines derived from the thyrotrope lineage.[438] Despite this in vitro evidence, Lhx2 expression does not appear within the developing anterior portion of the pituitary gland, and identification of Lhx2 appeared to identify an important class (LIM domain containing) of transcription factors but likely not the factor specific to αGSU.[89] Subsequent to these experiments, Lhx3 and Lhx4 were identified as key regulators of pituitary organogenesis (as outlined in detail in this chapter), and Lhx3 is a more likely candidate as the binding factor regulating the effects of the PGBE in mature gonadotropes.[93,137,140] The PGBE is conserved in the human αGSU promoter, where it likely plays a similar role in maintenance of

basal promoter activity.[439–441] The Pitx1-binding site also supports basal activity of the mouse αGSU promoter.[123,442] Interestingly, Lhx3 and Pitx1 appear to cooperate in the regulation of αGSU, and Pitx1 may indeed lie upstream of Lhx3 since disruption of Pitx1 results in a reduction in both Lhx3 and αGSU transcript expression in gonadotropes. The NR5A1-binding site is also critical for gonadotrope-specific regulation.[443] As indicated here, NR5A1 orchestrates the expression of a large cohort of steroidogenic enzymes, and it is critical to the development of the hypothalamic–pituitary–gonadal axis.[145,147] Conditional deletion of NR5A1 results in a marked reduction in αGSU expression, supporting the conclusion that NR5A1 plays a key role in gonadotrope gene regulatory mechanisms associated with αGSU.

GnRH induction of αGSU gene expression in αT3-1 cells is mediated by both the PGBE and a putative Ets factor–binding site termed the GnRH–responsive element (RE[426,427,444]). Multimers of the GnRH–RE were not sufficient to support basal transcription; however, these multimers were responsive to GnRH and phorbol esters used to activate PKC isozymes in transfection studies. In contrast, PGBE multimers supported basal transcription but were unresponsive to GnRH. Reconstituting the GnRH–RE and PGBE into the same reporter construct restored basal expression and augmented GnRH inducibility of this reporter. These studies suggested that the combined actions of the cell type–specific Lhx factor were permissive in conferring hormone responsiveness to the promoter via Ets factors whose expression was likely ubiquitous. Ets factors represent a large family of transcriptional regulators characterized by a conserved Ets domain, a DNA-binding domain favoring a 5′-GGAA central core binding motif.[444] Family members include Ets1 and Ets2 along with Elk1, an Ets-related factor associated with a serum response element shown to be critical for cellular responses to growth factors via receptor tyrosine kinases mediating ERK and JNK signaling. The GnRH–RE was identified using a mutagenesis screen where the mutation overlapped a motif consistent with an Ets factor–binding site. The DNA-binding domain of Ets2 was sufficient to specifically bind the GnRH–RE, and overexpression of a dominant-negative form of Ets2 could block GnRH induction of the αGSU reporter. These data suggested that an Ets family member is likely to be involved; however, the specific family member expressed in gonadotropes has not yet been determined. The possibility that GnRH signaling to the αGSU functions through a target of ERK signaling is supported by observations that gonadotrope conditional deletion of both ERK isoforms results in a marked reduction in basal and GnRH-stimulated accumulation of αGSU in vivo. This was corroborated by a sharp reduction in LH in serum in female double ERK knockouts.[166] A definitive mechanism coupling GnRH, αGSU, and putative Ets factors in vivo has yet to be elucidated.

The human αGSU gene promoter contains several elements conserved in the mouse promoter (the PGBE-, Pitx1-, and NR5A1-binding sites). In addition, other specific elements have been characterized in the human promoter, including two basal elements termed αBE1 and αBE2 and a cluster of elements (the trophoblast specific element (TSE), an α activating sequence (αACT), two consensus cAMP response elements (CREs), a junctional regulatory element, and a cytidine–cytidine–adenosine–adenosine–thymidine (CCAAT) box). Based upon mutational analysis, αBE1 and αBE 2[431] appear to be important regulatory sequences controlling basal expression of the human αGSU promoter in gonadotropes but not trophoblasts; however, little is known regarding the transacting factors that occupy these elements. The TSE, αACT, and the dual CREs play important regulatory roles in gonadotropes as well as trophoblast cells based upon scanning mutations. Of these, the dual CREs are likely the most prominent in the context of GnRH-induced expression of the αGSU.[388] Transcript profiling of immediate genes induced by GnRH reveals marked upregulation of activator protein 3 (ATF3). ATF3 has been identified as an immediate early response gene in genomewide studies of GnRH action. Expression of the human αGSU in αT3-1 cells is enhanced markedly by administration of GnRH. This induction could be abolished by pharmacological blockade of GnRH-induced ERK and JNK signaling and required the dual CREs. ATF3 upregulation could also be abolished using ERK and JNK pharmacological inhibitors, and overexpression of ATF3 in gonadotropes mimicked the effects of GnRH on this promoter. These studies supported the hypothesis that GnRH-induced ATF3 mediated increased human αGSU expression via ATF3 at the dual CREs. A similar mechanism was also reported for GnRH-induced secretogranin II promoter activity via consensus CREs.[390] Secretogranin II is a member of the chromogranin family of proteins found in secretory vesicles along with LH in gonadotropes. Finally, androgens have been demonstrated to repress human αGSU via the dual CREs and sequences comprising the αBEs.[445] The ability of androgens to function as negative-feedback inhibitors directly at the gonadotrope was associated with physical interactions between the ligand-bound androgen receptor and c-Jun–ATF2 heterodimers at the CREs. Again, these studies focused attention on the important role of the dual CREs of the human αGSU on both upregulation by GnRH and repression by androgens.

Regulation of the LHβ Subunit Gene Promoter

As indicated here, the β subunit to LH confers biological activity to the LH heterodimer.[425] Much of what we know regarding the transcriptional regulation of

the LHβ gene promoter comes from work done in the rat with confirmation from important knockout mouse models. Like αGSU, the LHβ gene promoter is sensitive to stimulation by GnRH and negative-feedback mechanisms associated with androgens and estradiol. In the rat, *cis* regulatory elements within ~0.5 kb of the transcription start site are sufficient to confer basal and GnRH-regulated expression on this gene in gonadotrope cell lines such as LβT2 cells. These elements include two specificity protein 1 (Sp1) sites, a CArg box, two NR5A1-binding sites, two Egr1 sites, and a Pitx1-binding site.

Responsiveness of the rat LHβ promoter to estradiol requires sequences further upstream within the promoter.[446] SP1 sites and the CArG box reflect an enhancer that lies more distal to the transcription start site of the promoter, and these sites appear to support basal regulation as well as control by the androgen receptor.[447] Importantly, the NR5A1, Egr1, and Pitx1 elements are highly conserved in the rat, bovine, equine, and human, suggesting an evolutionary importance to these elements in the control of LHβ in gonadotropes. The conservation of these sites is completely consistent with the role and requirement for these same transcription factors in coordinating promoters regulating the GnRHR, αGSU, LHβ, and FSHβ (detailed in this chapter).

Mutations in the NR5A1, Egr1, and Pitx1 sites all reduce basal expression of the LHβ gene promoter.[448–452] These three elements and their cognate-binding proteins form complex interactions leading to transcriptional activity of the LHβ promoter. For example, NR5A1 and Egr1 *cis* elements functionally interact in the regulation of LHβ,[163,448,451,453] and this interaction appears to facilitate synergistic activation of this promoter when stimulated by GnRH. Recently, GnRH-induced β-catenin (Ctnnb1) was shown to also participate in the interaction between NR5A1 and Egr1.[454] In those studies, association between Ctnnb1 and NR5A1 was required to facilitate the NR5A1–Egr1 interaction at the LHβ promoter. Knockdown of Ctnnb1 blunted GnRH-induced LHβ promoter activity in LβT2 cells. Ctnnb1 is thought to serve as a coactivator to NR5A1 activity at this promoter; however, this effect appears to be independent of glycogen synthase kinase 3, a critical regulator of Ctnnb1 activity since GnRH receptor activation results in inactivation of this kinase.[455–457] NR5A1, Egr1, and Pitx1 have also been shown to form a tripartite complex mediating GnRH-stimulated LHβ transcription.[162,163] The more distal Sp1 sites also appear to contribute to GnRH responsiveness since mutations within these elements blunt GnRH-induced activation of this promoter in vitro. Moreover, mutations within the Sp1 elements along with disruption of NR5A1 and Egr1 elements essentially abolish GnRH response of LHβ in in vitro cell models.[389,458]

Perhaps the most compelling evidence for the role of NR5A1 and Egr1 in LHβ subunit gene regulation comes from genetically modified mouse models. As discussed here, conditional deletion of NR5A1 in the pituitary results in the specific loss of LHβ subunit transcripts, providing important evidence that NR5A1 is required for the regulation of this gene in vivo.[147] The caveat here is that NR5A1 is also required for normal GnRHR expression, suggesting that both direct and indirect effects of NR5A1 and GnRH are plausible. Equally compelling is the role of Egr1 on LHβ transcript abundance in mouse knockout models.[164] Egr1 is an immediate early response gene induced by GnRH in both in vitro and in vivo model systems.[38,166,383,391] The regulation of the Egr1 gene is primarily mediated by the serum response element and Elk1 binding within the 5′ flanking sequences on the Egr1 gene promoter and appears to be the target of signaling from a wide variety of receptor systems, including GnRH.[459–465] Given the diversity of conditions shown to upregulate Egr1 as an immediate early gene via the serum response element, it was somewhat surprising that the Egr1 knockout mouse was born at expected frequencies and one of the primary lesions of this mouse model was female-specific infertility due to the specific loss of LHβ subunit gene regulation in the gonadotrope.[164] Ovariectomy in this mouse model resulted in the specific inhibition of castration-induced upregulation of LHβ without having a significant impact on the regulation of FSHβ subunit transcript abundance. Indeed, GnRH-induced Egr1 is absolutely required for LHβ gene expression. Further, Egr1 regulation by GnRH requires signaling through the ERK pathway, and specific conditional deletion of both ERK1 and ERK2 from gonadotropes results in a loss of Egr1 upregulation coincident with a loss of LHβ transcripts.[166] Collectively, these studies underscore the importance of Egr1 in the regulation of LHβ by GnRH–ERK signaling in gonadotropes in vivo.

The specific regulation of Egr1 is complicated by a well-characterized intracellular feedback loop involving Egr1-binding partners Ngfi-A-binding proteins 1 and 2 (NAB1 and NAB2).[453,466–470] In vitro, GnRH clearly induces NAB1 and NAB2 with kinetics slightly delayed compared to Egr1.[467] NAB1 and Egr1 physically interact, and this interaction results in inhibition of Egr1 action on target genes; however, this observation is controversial when, in specific situations, NAB1 and NAB2 have been observed to enhance Egr1 action or have little effect.[467–474] In the context of GnRH effects on LHβ in LβT2 cells, NAB1 and NAB2 appear to serve as GnRH-inducible repressors of Egr1 action, establishing an important intracellular negative-feedback system within the gonadotrope.[467,468] However, in rats examined in vivo and rat pituitary cells in primary culture, GnRH pulses did not affect NAB1 accumulation, suggesting that species and or in vitro model systems may vary in the role of NABs in the regulation of LHβ.[468]

Steroid hormones have been shown to impact LHβ subunit promoter activity. Androgens have a negative effect on LHβ promoter activity; however, the specific *cis* regulatory elements involved in this action are not entirely clear.[167,475] Inhibition by androgens is likely to involve interaction of the AR with components of the NR5A1 and Egr1 sites within the proximal LHβ promoter. AR can interact with both NR5A1 and Egr1, and these observations support speculation that this interaction blunts responsiveness of the promoter, particularly to GnRH stimulation in gonadotropes. AR suppression of LHβ has also been linked to suppression of NR5A1 activity on the LHβ promoter along the more distal Sp1 sites.[167] Given the central role of the NR5A1-, Egr1-, and Pitx1-linked enhancer, it seems plausible that ligand-bound AR forms a critical interface with this tripartite enhancer to modulate LHβ promoter activity.

Estradiol also has an indirect effect on mouse LHβ gene expression within gonadotropes. Initial studies examining the impact of estradiol on rat LHβ promoter activity identified an estrogen-responsive element that lies considerably upstream of the NR5A1, Egr1, and Pitx1 sites. Using an in vitro heterologous system (GH3 cells of lactotrope origin) known to be estradiol responsive, estradiol administration increased LHβ promoter activity several-fold.[476] In later studies using LβT2 cells (of gonadotrope origin), the direct effects of estradiol on the mouse LHβ promoter were only observed to augment GnRH action through increased levels of Egr1 following combined administration of estrogen and GnRH.[477] Given the novel role of estradiol action at the plasma membrane of pituitary gonadotropes described in the context of the GnRHR promoter,[398] it is presently difficult to parse out genomic versus nonclassical ESR action on the LHβ promoter.

Regulation of the FSHβ Subunit Gene Promoter

Regulatory mechanisms governing the control of the FSHβ subunit promoter arguably are the most complex of the gonadotropin subunits and perhaps the least understood. This is based on the complexity of the GnRH pulse parameters and variations in GnRH pulsatility controlling the FSH and LHβ subunit promoters, together with additional control of FSHβ by activin–inhibin and follistatin. In addition, a relative paucity of cell lines derived from the gonadotrope lineage that endogenously express FSHβ with fidelity to the in vivo situation contributes to this problem. Moreover, marked species differences appear to exist in the transcriptional control of FSHβ, and in some cases it is difficult to reconcile in vitro approaches in clonal cell lines and in vivo evidence just beginning to emerge from mouse model systems. The details of these mechanisms have been reviewed recently in very comprehensive fashion by Bernard.[170] Due to space constraints, the reader is directed to this[170] and other reviews[478,479] for a more detailed examination of FSHβ gene regulation.

Consistent with other gonadotrope signature genes, FSHβ promoter activity is regulated by *cis* regulatory elements binding NR5A1, Lhx3, and Pitx family members. In addition, binding sites for SMAD proteins are central to regulation by activin. Approximately 4 kb of the human FSHβ subunit gene promoter is sufficient to convey cell type–specific regulation in the transgenic mouse[480]; in the sheep, ~5.5 kb of a 5′ flanking sequence was sufficient for gonadotrope-specific expression in vivo.[481,482] The *cis* regulatory elements described here all are found within ~0.5 kb of the transcription start site, and their importance has been identified primarily using luciferase reporter constructs in the LβT2 cell line; these genomic promoter fragments are considerably smaller than those examined in transgenic mice.

The role of NR5A1 on FSHβ subunit regulation is best exemplified by the gonadotrope-specific conditional knockout of NR5A1 in mice[147]; these animals display markedly reduced levels of FSH immunoreactivity in the anterior pituitary. However, these studies are confounded by a loss of the GnRHR, with conditional deletion of NR5A1 making a clear interpretation of the specific role of NR5A1 on FSHβ expression somewhat difficult to determine.[170] Consistent with this notion, the direct effects of NR5A1 alone on FSHβ transcription appear to be relatively modest and likely dependent upon binding partners central to basal regulation.[169] For example, NR5A1 and NFY can physically and functionally interact in the context of the two NR5A1 and single NFY *cis* elements in the mouse FSHβ promoter in the LβT2 cell line. Mutations in at least one of the NR5A1 sites combined with an NFY binding site mutation was required to blunt basal expression of the promoter. Furthermore, this combination of elements and transcription factors also confers gonadotrope-specific expression. NR5A1 and NFY each have been shown to interact with and recruit chromatin-remodeling complexes to gene promoters,[151–153,483,484] supporting the possibility that higher order complexes incorporating histone acetyl transferases and potentially deacetylases may function to control gonadotrope-specific expression.

Development of the anterior pituitary is dependent upon Lhx3 in the mouse.[97,116,137] Human patients presenting with CPHDs have also been shown to have genetic loss-of-function mutations in Lhx3 correlated with CPHD, including deficiency in FSH production and secretion. While these reports do not provide a direct link to loss of Lhx3 function at the FSHβ gene promoter, they do underscore the importance of Lhx3 as a critical regulator of pituitary cell function and potentially as a transcriptional activator of the human FSHβ subunit promoter.[485–488] Multiple Lhx3-binding sites have been identified on the human FSHβ promoter, and

overexpression studies suggest that Lhx3 is sufficient to transactivate this promoter.[485] The porcine FSHβ promoter has also been characterized with multiple Lhx3-binding sites, several of which appear to be conserved with the human promoter.[485] The potential role of Lhx3 in FSHβ promoter activity is consistent with the required role of Lhx3 in the regulation of the αGSU and a presumed role in the regulation of the GnRHR gene promoters in the fully differentiated gonadotrope.

Pitx1 and Pitx2 also play functional roles in the regulation of the rodent and human FSHβ subunit promoter in LβT2 cells.[489,490] Using mutational analyses and DNA-binding studies, the effects of Pitx family members occur via a conserved *cis* element to support FSHβ transactivation. Using Pitx dominant-negative overexpression and knockdown approaches, studies support the conclusion that Pitx1 and Pitx2 are required for basal and activin-stimulated FSHβ (described in this chapter).[490] As indicated, Pitx proteins form complexes with a number of transcriptional regulators, including the association between Pitx, NR5A1, and Egr1,[162,163] supporting speculation that similar types of protein–protein interactions may support basal and GnRH- or activin-stimulated expression of the FSHβ gene; however, this has not been characterized.

The hypogonadal mouse with a loss of GnRH signaling provides definitive evidence for the role of GnRH in the regulation of FSHβ in rodents.[2–4,434] This conclusion is also supported by studies of hypothalamic-pituitary disconnection studies in sheep resulting in a hypogonadotropic-hypogonadal phenotype.[435,436,491] In both instances, exogenous administration of GnRH rescues gonadotropin subunit mRNA levels, including FSHβ, in these animal models. Despite the profound effects of GnRH on FSH biosynthesis, relatively little is known regarding molecular mechanisms involved in this regulation. In rat and human FSHβ promoters, half sites for AP1 appear to mediate the effects of GnRH in some instances.[492,493] For example, using mutational analysis of an AP1 half-site and overexpression studies of dominant-negative forms of AP1 in the mouse FSHβ promoter, loss of this site or interference by the dominant-negative AP1 resulted in a blunted response of the promoter to GnRH in LβT2 cells. The presence of the AP1 site predicts a role for GnRH-induced ERK activation governing this mechanism; indeed, pharmacological disruption of the ERK and the p38 MAPK pathways blocked the stimulatory effects of GnRH in the human promoter in LβT2 cells. These in vitro studies are difficult to reconcile with recent studies of a conditional gonadotrope-specific knockout of ERK1 and ERK2 in the mouse.[166] While loss of ERK signaling in gonadotropes in vivo resulted in female-specific infertility, this deficiency was attributable to a loss of GnRH-induced αGSU and, in particular, LHβ subunit expression. This genetic ERK lesion had very little effect on GnRH induction of FSHβ, circulating levels of FSH, or the distribution of ovarian follicles in the ERK conditional double knockout. Thus, while the AP1 site is likely to play a key role in GnRH induction of FSHβ, the signaling pathways leading to AP1-mediated transactivation are likely to include novel mechanisms potentially independent of GnRH-induced ERK signaling.

The effects of AP1 on the rat and mouse FSHβ promoters may also include physical interaction with the adjacent NF-Y binding site.[493] The close proximity of the NF-Y and AP1 site along with the observation that NF-Y-A can physically interact with c-Jun in the context of an AP1 complex support this idea. An overlapping E-box that binds basic helix–loop–helix proteins in the upstream stimulatory factor family is also likely to be involved since mutations within this site blunt GnRH-induced FSHβ subunit transcription in vitro.[494] Alternatively, and perhaps not surprisingly, the AP1 site in the rat FSHβ promoter can also bind CREB.[495] Using LβT2 cells, these studies provide important evidence that the AP1 site can indeed bind CREB, and the use of a dominant-negative CREB (but not dominant-negative AP1) and CREB knockdown suggests that it is CREB rather than AP1 heterodimers that mediates GnRH action on the FSHβ promoter. Moreover, the ability of the FSHβ promoter to discriminate differential GnRH pulse frequency may depend upon competition between CREB and inducible cAMP early repressor (ICER) binding at this site. FSHβ transcription is favored by slow relative GnRH pulsatility, while rapid GnRH pulsing favors LHβ and αGSU transcription.[496,497] Rapid pulsing also favors ICER accumulation that can directly compete for CREB binding at the AP1 site and blunt transactivation by GnRH. The potential involvement of CREB rather than AP1 may also be consistent with GnRH-induced signaling to the mouse FSHβ promoter independent of the ERK pathway.

Along with GnRH action, FSHβ gene transcription is also controlled by activin signaling. Activins, along with inhibin and follistatin, are members of the transforming growth factor β (TGFβ) family and have been described thus far in the context of control of SMAD activity at the GnRHR gene promoter. Administration of activin to gonadotrope cells results in a marked upregulation of FSHβ transcript abundance,[498–502] while inhibin and follistatin inhibit this action through interference between activin and the activin receptor or by sequestering activin away from the receptor by direct binding.[502] The actions of activin are largely mediated via the activin receptor ACVR2[405,503]; this knockout mouse model provides strong support for the role of activin signaling in the gonadotrope directing expression of FSHβ and the GnRHR. Activin and follistatin are both expressed in gonadotropes; follistatin is also expressed in other pituitary cell lineages, including

folliculostellate cells,[504] and their actions to control activin signaling to FSHβ are thought to be autocrine and/or paracrine within the anterior pituitary.

Activin signaling via ACVR2 (the type II receptor) occurs in collaboration with the type I receptors ALK4 and/or ALK7 in gonadotropes[505–508]; however, it appears that ALK4 may have higher importance in activin signaling based upon pharmacological studies in LβT2 cells and the ALK7 knockout mouse.[170] Activin engagement of the activin receptor complex results in rapid phosphorylation of SMAD2 and SMAD3, followed by nuclear localization of SMAD2/3 and SMAD4 complexes in LβT2 cells.[509] SMAD complexes engage the FSHβ gene promoter via multiple SMAD-binding sites in the mouse promoter; however, the presence of consensus SMAD-binding sites is not conserved across species.[170] Mutations that interfere with SMAD binding to these elements blunt activin-induced FSHβ gene transcription in LβT2 cells. Interestingly, mutations that interfere with Pitx1 and Pitx2 binding to the mouse FSHβ promoter blunt the responsiveness of the promoter to activin or expression of SMAD2/3/4.[490] Furthermore, SMAD3 can physically interact with Pitx1 and Pitx2, suggesting that activin-induced FSHβ promoter activity requires association between at least SMAD3 and Pitx family members.

The forkhead factor FoxL2 appears to play a key role in the regulation of FSHβ promoter activity.[510] Developmentally, loss of FoxL2 does not appear to impact the development of the gonadotrope lineage; however, FoxL2-deficient gonadotropes have marked reduction in FSHβ transcript expression. The role of FoxL2 in governing FSHβ appears to be linked to the interaction between FoxL2 and activin signaling.[511–514] In the human and mouse FSHβ subunit promoter, FoxL2 appears to interact with the promoter and FoxL2 is required for FSHβ responsiveness to activin; however, the FoxL2-binding sites alone are not sufficient to confer activin responsiveness.[511,514] In other studies of the mouse FSHβ promoter, a specific FoxL2-binding site was demonstrated, and the actions of FoxL2 and SMADs were shown to induce a synergistic transcriptional response.[513] Thus, the regulation of FSHβ in the mouse by activin includes the activation of SMAD2/3 coupled with SMAD4. Interactions between FoxL2 and SMAD complexes in this gene promoter initiate high-level promoter activity within the gonadotrope. The caveat here is that there is clear evidence of species variation in the context of FSHβ regulation by activin and the role of FoxL2 in this process.[170]

Activin signaling at the gonadotrope through local production and secretion of activin also contributes to how the gonadotrope responds to a GnRH stimulus in the context of FSHβ promoter activity. For example, activin plays an important role in the regulation of the GnRHR, creating the opportunity for activin to contribute to the sensitivity of the gonadotrope to a GnRH stimulus. Furthermore, the action of GnRH along with activin appears to induce a synergistic activation of the mouse and rat FSHβ gene promoter in LβT2 cells independent of GnRH-induced ERK pathway activation but was observed to depend upon GnRH-induced p38 MAPK activity.[515,516] A similar transcriptional synergy between activin and GnRH signaling occurs on the human FSHβ promoter.[492] In these studies, GnRH-induced activity of AP1 via ERK and p38 MAPK activation and blockade of these pathways or the use of a dominant-negative Fos protein or mutation of AP1 sites within the human promoter provided strong support for this mechanism. Thus, across multiple mammalian species, response of the FSHβ promoter to GnRH pulses is greatly affected by activin levels within the local pituitary milieu. The ability to specifically titrate FSHβ gene regulatory mechanisms in the apparent absence of LHβ regulation provides important clues to how these two gonadotropin subunit genes are regulated in complex physiological situations such as during the estrous cycle.

Androgens and glucocorticoids have been shown to stimulate FSHβ transcript abundance and FSH secretion in primary cultures of pituitary cells obtained from male rats.[517] Furthermore, glucocorticoid or androgen treatment augmented the effects of activin in these studies. Interestingly, these steroids have diverse effects on follistatin production from the same culture. For example, androgens suppressed follistatin mRNA levels, while dexamethasone enhanced follistatin transcript levels; the addition of activin to dexamethasone-treated pituitary cell cultures further augmented upregulation of follistatin. These experiments suggest that control of FSHβ by androgens and glucocorticoids may occur by diverse mechanisms, including a direct effect of androgens and an indirect effect of glucocorticoids via changes in follistatin abundance. The combined effects of glucocorticoids and activin on FSHβ were also recapitulated in the LβT2 cell model, where the combined actions of both agonists resulted in synergistic activation of the mouse FSHβ promoter.[518] This synergistic transcriptional response was promoter specific in LβT2 cells, was inhibitable by the addition of follistatin, and required SMAD2/3 binding to this promoter. Collectively, these studies reflect the importance of local activin signaling on the responsiveness of the gonadotrope to GnRH and gonadal and adrenal steroid hormones. This illustrates the importance of the local pituitary milieu with regard to activin and follistatin on regulation of the FSHβ subunit gene promoter.

Desensitization and Receptor Internalization

As noted here, pulsatile release of GnRH is crucial for gonadotropin biosynthesis and secretion in vivo.[55,56] Pulses of GnRH can raise circulating gonadotropin and

gonadal steroid levels, and this is exploited in assisted reproduction and in treatment of hypogonadotropic hypogonadism, whereas with sustained and/or high doses of agonists (particularly metabolically stable agonist analogs of GnRH), the initial secretory response is followed by reduced levels of gonadotropins and gonadal steroids. This is exploited with subcutaneous depot formulations of GnRH agonists that are used to treat central precocious puberty, uterine fibroids, endometriosis, and gonadal steroid-sensitive cancers, including those of the breast and prostate.[22,26,519] This can be viewed in light of the fact that all cells adapt to their environments in order to be able to function under a wide range of environmental conditions. Very often, cells adapt to low-level stimulation (by hormones, neurotransmitters, growth factors, etc.) by increasing sensitivity to the stimulus (sensitization), and they adapt to intense stimulation by reducing sensitivity to the stimulus (desensitization).[251] Such adaptation may be homologous (where exposure to a stimulus alters responsiveness to the same stimulus) or heterologous (where exposure to a stimulus alters responsiveness to another stimulus). Intense stimulation of gonadotropes with GnRH causes pronounced desensitization of GnRH-stimulated gonadotropin secretion, whereas brief GnRH stimulation can actually increase responsiveness to a later stimulus in a process known as "self-priming" that is thought important for generation of the preovulatory gonadotropin surge.[520–522] Moreover, gonadal steroids can also act directly on gonadotropes or gonadotrope-derived cell lines to increase or reduce responsiveness to GnRH, so these cells show sensitization and desensitization that may be homologous or heterologous.

Since therapeutic applications of GnRH agonists revolve around exploitation or avoidance of desensitization, most work in this area has inevitably focused on homologous desensitization. Here, it is important to recognize that intense stimulation typically causes desensitization of 7TM-mediated responses, and that changes in receptor coupling provide the rapidest and best described cause of such desensitization.[523,524] Activation of 7TM receptors is typically followed by their desensitization and internalization. These processes involve receptor phosphorylation that is stimulated by agonist binding, is typically rapid (occurring in seconds to minutes), and may be mediated by specific G-protein receptor kinases (GRKs), by second messenger-regulated kinases (e.g., PKC or PKA), or by casein kinases.[525–527] This phosphorylation may facilitate association with arrestins 2 or 3, which block G-protein binding and thereby prevent effector activation.[523,524] Since this effect is seen within seconds to minutes and is specific to the activated receptor, it is termed rapid homologous receptor desensitization. Arrestins also act as adapters, binding to proteins such as AP2 and clathrin and thereby targeting desensitized 7TM receptors for internalization.[523,524,527–529] These receptors are internalized from the cell surface by endocytosis, most often via clathrin-coated vesicles. The formation of these vesicles is typically controlled by a dynamin collar, which separates the nascent endosome from the plasma membrane.[530,531] After internalization, the receptors are either recycled to the cell surface (promoting resensitization) or targeted to lysosomes for degradation (promoting receptor downregulation).[532] In addition, it is now well established that arrestins can act as scaffolds for assembly of complexes containing 7TM receptors and effectors. These can include Src, MAPKKKs, and MAPKs, and arrestins can mediate G-protein-independent activation of these effectors.[347,348,524,533–537] Consequently, mechanisms previously thought of in terms of switching receptor signaling off may actually be switching between two distinct modes of receptor signaling (from G-protein mediated to arrestin dependent).

It is well established that sustained exposure to GnRH causes desensitization of GnRH-stimulated gonadotropin secretion and that this is associated with GnRHR downregulation and internalization of GnRHRs via coated pits,[274,538,539] so it could have been anticipated that GnRHRs would follow the model outlined here. However, the amino acids phosphorylated to facilitate arrestin binding are most often located within the C-terminal tails of 7TM receptors, and this raised the possibility that the absence of C-tails in type I mammalian GnRHRs might cause resistance to rapid desensitization. Indeed, this was demonstrated in early functional studies using GnRH-stimulated [^{3}H]IP accumulation (in cells prelabeled with [^{3}H]inositol and stimulated in the presence of LiCl) as a readout for GnRHR-mediated PLC activation of αT3-1 cells.[540] In this study, the initial rate of [^{3}H]IP accumulation was maintained for at least 90 min, and, since this is typically not maintained beyond 1 min with desensitizing receptors, it clearly implied that the endogenous mouse GnRHRs of these cells do not rapidly desensitize. These data have been confirmed and extended in numerous studies showing that mammalian GnRHRs without C-tails do not undergo rapid homologous desensitization or agonist-induced phosphorylation and do not bind arrestins.[194,541–554] They do undergo agonist-induced internalization, but this is relatively slow and is neither arrestin dependent nor associated with arrestin translocation to the plasma membrane or to endosomes containing internalized GnRHRs. Here, it is important to recognize that although type I mammalian GnRHRs lack C-tails, nonmammalian GnRHRs do have such tails with potential sites for phosphorylation and arrestin binding. This has facilitated comparative studies with wild-type or chimeric receptors, and several groups have shown that activation of nonmammalian GnRHRs with C-tails does cause their rapid

homologous desensitization and internalization, and that these processes are associated with and dependent upon agonist-induced receptor phosphorylation and arrestin binding. Moreover, studies with truncated nonmammalian GnRHRs (i.e., catfish GnRHRs with C-tail deletions), chimeric GnRHRs (i.e., type I rat, human, or sheep GnRHRs with added *Xenopus laevis* or catfish C-tails), or nonmammalian GnRHRs with potential C-tail phosphorylation sites removed support the idea that phosphorylation within the C-tails causes arrestin-mediated desensitization and rapid homologous desensitization of GnRHRs that have C-tails.[543,545–550,553]

The data outlined here are consistent with the suggestion[540] that absence of C-tails in type I GnRHRs underlies their atypical resistance to desensitization. However, GnRHR-mediated gonadotropin secretion certainly does desensitize, so other mechanisms must be involved. Indeed, gonadotropes may well provide a unique opportunity for investigation of downstream adaptive responses to 7TM receptor activation, in the absence of receptor desensitization. In exploring this, we have found that sustained stimulation of αT3-1 cells with GnRH causes a pronounced desensitization of Ca^{2+} responses,[194,541] an effect that was associated with and apparently dependent upon a GnRH-stimulated reduction in the IP_3 receptor number.[552] Sustained activation of several PLC-activating GPCRs has been shown to cause IP_3 receptor downregulation, an effect attributed to the ligand-stimulated ubiquitinylation and consequent proteolysis of the activated IP_3 receptors.[555–557] However, this effect is typically slow and modest as compared to the pronounced and rapid effect of GnRH (80% reduction within 20–30 min). Thus it appears that this receptor, which is atypically resistant to desensitization, is unusually efficient at provoking this form of postreceptor desensitization.[552] It is important to recognize, however, that the relevance of this effect to desensitization of gonadotropin secretion has not been established. Other effects, such as depletion of releasable gonadotropin pools,[558,559] downregulation of GnRHRs, upregulation of RGS proteins, desensitization of VGCCs,[560] and reduced gonadotropin synthesis (section Regulation of Gene Expression), may well prove to be more important for long-term desensitization of gonadotropin secretion in vivo.

Heterotrimeric G-Protein-Independent Signaling

As noted here, arrestins can act as scaffolds, facilitating the interaction of phosphorylated 7TM receptors with other regulatory proteins. The arrestin-mediated coupling of GPCRs to clathrin, AP2, and NSF directs GPCRs for internalization via clathrin-coated vesicles (CCVs), and the binding to kinases such as JNK3, ASK1, Src, ERK, and Raf can mediate MAPK signaling in a heterotrimeric G-protein-independent manner.[347,348,529,533–537] One of the earliest indications of a signaling role for receptor-associated arrestins was the demonstration that β2 adrenergic receptor stimulation not only activated the tyrosine kinase c-Src but also caused it to bind to the receptor-associated arrestin 2. The arrestin-associated c-Src could then phosphorylate and activate dynamin 1, causing dynamin-dependent receptor internalization. This raised the possibility that a similar paradigm would be applicable to GnRHRs; that is, arrestin binding to the C-tail of nonmammalian GnRHRs would target them for Src and dynamin-dependent internalization. In accord with this, internalization of a *X. laevis* (X)GnRHR is dynamin sensitive (inhibited by expression of the GTPase-deficient, dominant-negative, K44A dynamin 1), whereas internalization of the hGnRHR is not.[548,551,554] Moreover, addition of the XGnRHR C-tail to the C-terminus of the hGnRHR, either alone or in addition to exchange of the third intracellular loop regions, directed the receptor for arrestin and dynamin-dependent internalization.[554] Although these chimeric receptors have comparable affinity and ligand specificity to the wild-type hGnRHRs, the dual chimera did not signal (as judged by the lack of effect on Ca^{2+} or cAMP), and inhibition of Src had no effect on internalization of any of these constructs. These data suggest that arrestin mediates signaling of C-tailed GnRHRs to dynamin, but the β2 adrenergic receptor paradigm is not entirely applicable because internalization is independent of Src (and apparently does not require receptor activation). As an alternative, we considered the possibility that arrestin scaffolding might mediate ERK activation by C-tailed GnRHRs and found that hGnRHR and XGnRHR mediated ERK activation with similar kinetics (rapid and transient increases in ppERK to maxima at 5–15 min) in HeLa cells.[366,561] These responses were not sensitive to blockade of dynamin-dependent receptor internalization or to coexpression of arrestins or a dominant-negative truncated arrestin. The increase in ppERK mediated by both receptors was not attenuated by inhibition of EGF receptor transactivation, PI3K, or Src, but was dependent on PKC. We therefore reassessed the arrestin dependence of GnRHR-mediated ERK activation in the presence of a PKC inhibitor and found that the XGnRHR-mediated increase in ppERK was indeed blocked by the dominant-negative arrestin, whereas the hGnRHR-mediated effect was not.[366,561] In addition, the chimeric receptor (the full-length hGnRHR with the added XGnRHR C-tail) mediated ERK activation, and introduction of an A261K mutation within the third intracellular loop prevented G-protein activation by this construct without completely blocking ERK activation.[40] Accordingly, these data provide the first direct demonstration of arrestin-mediated (heterotrimeric G-protein-independent) GnRHR signaling and raise the intriguing possibility that the evolution of GnRHRs has restricted

their signaling repertoire. The modulation and mediation of signaling by arrestins may be characteristic of GnRHRs with C-tails, and the absence of such effects for type I mammalian GnRHRs may enable them to faithfully transduce GnRH signal into intracellular G-protein activation. This could have adaptive advantages in situations such as the preovulatory gonadotropin surge, where there is an increase in GnRH pulse frequency or a sustained increase in GnRH[63,562–564] and where a corresponding surge in downstream signaling might otherwise be prevented by receptor desensitization.

Further evidence for GnRHR-mediated and heterotrimeric G-protein-independent activation of ERK has been obtained with GnRHR-transfected HEK293 cells in which GnRHRs mediate cytoskeletal reorganization and GnRHR-mediated ERK activation is prevented by cytoskeletal disruption.[565] Indeed, GnRHRs mediate ultrastructural changes in several models, including the HEK293 cell model in which they cause the formation of numerous spinelike plasma membrane protrusions.[320,565] These contain focal adhesions, and GnRHR activation causes rapid activation and translocation of Pyk2 to these processes.[320,565] Pyk2 is a proline-rich protein tyrosine kinase member of the focal adhesion kinase (FAK) family that mediates ERK activation by 7TM receptors. In GnRHR-expressing HEK293 cells, GnRH-stimulated ERK activation was not Pyk2 mediated[566] but was dependent upon assembly of an ERK–FAK–c-Src signaling complex within focal adhesions and was resistant to blockade by inhibitors of phospholipase C or PKC.[565] It was, however, dependent upon Rac and was therefore attributed to a novel monomeric G-protein-dependent pathway and not to the classical heterotrimeric G-protein–phospholipase C pathway.[565] However, these mechanisms are controversial as others have reported that PKC inhibition prevents GnRHR-mediated ERK activation in GnRHR-expressing HEK293 cells.[566] Moreover, calmodulin mediates GnRH-stimulated ERK activation in αT3-1 cells,[567] and GnRHR-mediated Pyk2 activation does contribute to GnRH-stimulated ERK activation in the αT3-1 model.[567]

Intracellular GnRHRs

Another fundamental feature of GnRHRs related to the C-tail is that a large proportion of these receptors are actually intracellular.[195,539,568–576] This was highlighted by work on hGnRHR mutants that cause infertility and were found to be nonfunctional because of impaired trafficking (as opposed to deficient signaling).[568,569,572,575,576] It then became clear that even wild-type hGnRHRs are relatively poorly expressed at the cell surface. This can be viewed in light of the fact that at least 50% of newly synthesized protein fails to meet quality control criteria for ER exit. Instead, proteins may enter the ER-associated protein degradation (ERAD) system in which misfolded proteins traffic to the cytoplasm and are polyubiquitinated, targeting them to proteasomes for degradation.[577,578] This certainly applies to 7TM receptors,[579–582] as, for example, less than 40% of delta opioid receptors exit the ER after appropriate glycosylation and folding.[581,582] Although the features controlling ER exit for 7TM receptors are not well known, they certainly include glycosylation because only 7TM receptors with mature glycosylation patterns typically traffic to the cell surface. For hGnRHRs, the presence of a primate-specific Lys191, the absence of a second N-terminal glycosylation site, and the absence of a C-tail are all implicated in poor cell surface expression.[195,237,568,570,572,573,575,576,583] Indeed, when we quantified HA-tagged GnRHRs at the cell surface and within cells,[195,570,571,573] we found that at least 95% of hGnRHRs were intracellular after heterologous expression in hormone-dependent cancer cells (MCF7, T47D, PC3, and LnCAP cells). In MCF7 cells, less than 1% of hGnRHRs were at the cell surface, and this remarkably small proportion was increased as much as 10–50-fold for hGnRHRs with C-tails (in comparisons of a *X. laevis* GnRHR with the human GnRHR and of a chimeric type I mammalian GnRHR with added XGnRHR C-tail). The proportion of hGnRHRs at the cell surface was also approximately fivefold higher in gonadotrope-derived cell lines (LβT2 and αT4) than in hormone-dependent cancer cells, demonstrating that GnRHR compartmentalization is dependent upon the cellular context.[570,573] Cell permeant GnRHR ligands are being developed as potential orally active GnRHR antagonists[584] and we found that the proportion of hGnRHRs at the cell surface was also increased (10–20-fold) by a nonpeptide indole GnRHR antagonists (IN3). This compound had been shown elsewhere to rescue signaling by the trafficking-impaired hGnRHR mutants noted here.[568,569,572,575] It is thought to act as a pharmacological chaperone, aiding the folding of ER-resident GnRHRs into a conformation needed to meet ER exit quality control criteria, and thereby increasing GnRHR trafficking to the cell surface. In the absence of reliable antibodies to hGnRHRs, these studies have been performed primarily with tagged receptors. It is therefore important to recognize that the chaperone effects on hGnRHR trafficking are paralleled by effects on receptor function (i.e., rescue of signaling via trafficking-deficient mutants and enhanced signaling via wild-type hGnRHRs) and that these effects can also be seen with epitope tag-free GnRHRs.[195,568–573,575,576] The caveat remains, however, that the number and proportion of native receptors at the cell surface have not been defined for hGnRHRs in pituitary or extrapituitary sites. Moreover, although a great deal is known about control of GnRHR expression and internalization, we know of no work addressing the possibility that the proportion of hGnRHRs failing ER

exit quality control criteria is regulated to meet physiological demand.

The idea that intracellular GnRHRs are primarily located in the ER is supported by imaging studies[195,568–573,575,576,585] and by work showing that proteins involved in ER exit quality control also influence the number of GnRHRs at the cell surface.[586–588] However, not all intracellular GnRHRs are ER residents; they are also presumably located in transport vesicles of the exocytotic, endocytotic, and lysosomal degradation pathways. Moreover, binding studies have revealed GnRHRs in nuclear fractions of several cell types,[589–592] and this has been confirmed recently by immunohistochemical work showing that epitope-tagged hGnRHRs are located in the nuclear envelope[585] as well as the ER. In contrast, an XGnRHR was more effectively expressed at the cell surface and was not found in the nuclear envelope. This is not surprising given the fact that the ER and nuclear envelope are contiguous, but it was also shown that GnRH could stimulate the acetylation and phosphorylation of histone 3 in purified nuclei.[585] An obvious question is how or whether such receptors could be ligand activated, and it was speculated that they could be subject to intracrine regulation in cells producing both GnRH and GnRHRs.[585] Accordingly, GnRHRs are among a small number of 7TM receptors known to exist in the nuclear envelope, and a functional role in this location may well be specific for type I mammalian GnRHRs.

Lipid Rafts

In addition to trafficking to and from the cell surface, it is well established that compartmentalization within the plasma membrane influences the activity of many receptors and effectors. Early work focused on the aggregation (patching and capping) of GnRHRs at the cell surface that precedes agonist-induced GnRHR internalization[548,554,593,594] and on the possible relevance of mobility within the plasma membrane for formation of GnRHR multimers. This section examines the ability of the GnRHRs to aggregate and associate within discrete membrane compartments and how this membrane localization may affect productive signaling within the gonadotrope. Early studies by Naor and Catt[595] made use of rhodamine-derivatized agonists of GnRH to track ligand–receptor complexes on the surface of gonadotropes. Despite the observation that rhodamine-derivatized GnRH displayed reduced receptor affinity and ability to induce LH secretion, these studies revealed that upon ligand binding, the GnRHR transitions from diffuse distribution across the membrane to points of focal aggregation followed by internalization. Microaggregation of the GnRHR appears to be a central and early event leading to productive calcium signaling and secretion of gonadotropins.[247,248]

From the early 1970s to the early 1990s, plasma membrane dynamics was characterized by the fluid mosaic model of lipids and proteins within a uniform bilayer.[596,597] This early view supported the notion that the multiplicity of lipids within the plasma membrane simply played a passive role that formed the structure of the membrane; membrane-associated proteins were essentially adrift within the bilayer. Association of proteins within the lipid bilayer could occur via membrane-spanning domains such as with 7TM receptors or via lipid modifications (e.g., myristoylation) of proteins effectively linking them to the plasma membrane.[598] Since that time, a great deal has been learned regarding the lipid and protein composition and dynamics of the plasma membrane and how the biophysical properties of the membrane may provide a unique contribution to the organization of receptors and signaling systems within the membrane. Here we focus on the membrane raft hypothesis, which posits that various lipids and sterols present within the plasma membrane have variable affinities for each other as well as for specific integral membrane and membrane-associated proteins. The high-affinity association between lipids and proteins is able to form (transiently) stable and biochemically distinct domains within the membrane nucleating foci (or rafts) where signaling intermediates can concentrate[598–604] (Figure 10.5).

Biochemically distinct domains within the membrane appear to be dependent upon the lipid composition within these domains. For example, GPI-anchored proteins were recoverable from plasma membrane in sucrose density gradients if these preparations were made in the presence of low levels of nonionic detergents such as Triton X-100 at low temperatures.[605] The lipid composition of these detergent-resistant membranes was enriched in cholesterol and sphingolipids and gave rise to a so-called liquid-ordered domain due to the tight packing of cholesterol and sphingolipids within the domain or raft relative to the more liquid-disordered state of the surrounding plasma membrane.[598,604] The unique composition of the sphingolipid–cholesterol-enriched raft is thought to include (or exclude) specific proteins in this compartment, effectively assembling (for example) receptor populations with intracellular signaling intermediates to form platforms or complexes to facilitate cell signaling.[38,606] The importance of these rafts is to facilitate rapid response kinetics by preassembling signaling complexes that could respond quickly upon receptor activation. While this is an attractive hypothesis, the fact that these detergent-resistant membranes required isolation at low temperatures raised significant controversy as to whether these membrane compartments existed in normal cell membranes at physiological temperatures or were simply artifacts of the conditions necessary to recover the rafts.[598,604,607] Visualization of these dynamic

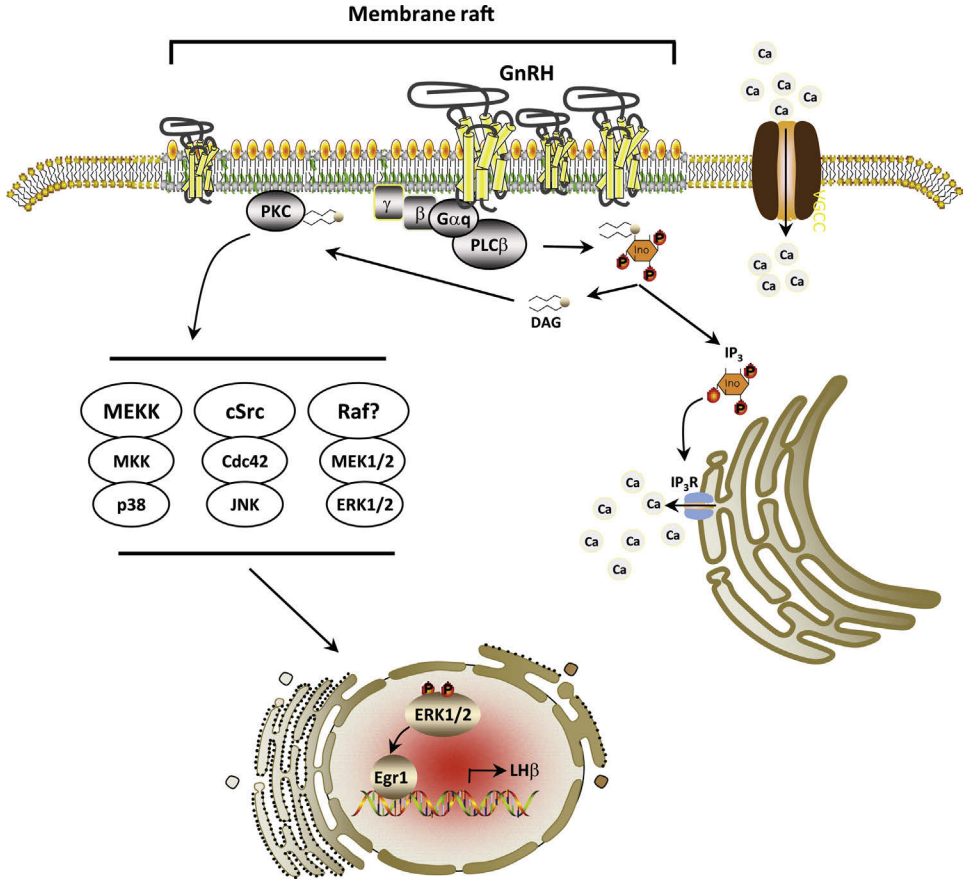

FIGURE 10.5 Membrane rafts as GnRHR signaling platforms. Proposed model for membrane raft-associated GnRHR facilitates signaling to the mitogen-activated protein kinase (MAPK) pathways in gonadotropes. The G-protein coupled GnRHR initiates signaling via Gαq (and associate β and γ subunits) and activation of PLCβ. The GnRHR is a constitutive and exclusive occupant of a detergent-resistant, low-buoyant-density membrane compartment, the membrane raft. Gαq also resides within the raft but can be isolated from nonraft membrane fractions. Activation of PLCβ results in the elaboration of two second important messengers, DAG and IP_3. DAG (along with cell calcium) induces activation of PKC isozymes, while IP_3 promotes mobilization of intracellular calcium via interaction with IP_3 receptors (IP_3R) within the endoplasmic reticulum. Membrane depolarization also induces an important source of intracellular calcium via an influx of extracellular calcium through VGCCs within the membrane. VGCCs are not specifically associated with the membrane raft compartment. Activation of PKC isozymes appears to be critical for activation of multiple MAPK pathways, particularly activation of the ERK cascade. ERKs are co-localized with the GnRHR within rafts, and phosphorylated (activated) ERKs are raft localized following GnRH treatment in αT3-1 cells, suggesting that this compartment may serve as an activating platform for GnRH signaling. Once activated, ERKs nuclear translocate to phosphorylate transcription factors in the nucleus and can affect gene transcription such as the activation of the LHβ gene promoter by Egr1. Interestingly, while Raf kinase appears to be a canonical activator of MAPK–ERK kinases (MEKs) and is localized to membrane rafts along with the GnRHR, recent evidence suggests that at least c-Raf kinase is not required for GnRH-induced ERK activation.[367] A detailed understanding of the role of p38 MAPK and JNK pathway signaling via rafts awaits analyses. *Source: Adapted from Ref. 38.*

compartments in living cells has been elusive; however, recent advances in high-resolution fluorescent imaging have begun to provide convincing evidence of the existence of membrane rafts as small (20–200 nm), highly dynamic, sterol- and sphingolipid-enriched domains capable of facilitating protein–lipid and protein–protein interactions to form signaling platforms.[608] Importantly, the size of membrane rafts and the time scale in which they coalesce and dissociate are thought to be extremely small and rapid, giving rise to a remarkably dynamic system that can be affected by and have great impact on the inclusion or exclusion of receptors and intracellular signaling systems in the membrane compartments.

As indicated earlier, microaggregation of GnRHRs following ligand binding was an early observation suggesting that receptor compartmentalization within the plasma membrane may be important in the context of hormone action.[249,250] Compartmentalization of intracellular signaling from the murine GnRHR may be equally important. This possibility is underscored from studies examining the roles and requirement for specific calcium signaling in GnRH-induced activation of specific MAPK pathways.[363,371] In those studies, GnRH activation of the ERK and JNK pathways was clearly separable based on inhibition of two different Ca^{2+} pools, both induced by GnRH: intracellular calcium release induced by IP_3

and influx of extracellular calcium through VGCCs. Disruption of VGCC activity using dihydropyridine receptor antagonists (like nifedipine) resulted in the specific inhibition of GnRH-induced ERK (but not JNK) activation. These studies supported speculation that local or compartmentalized calcium signaling may play an important role in signaling between the GnRHR, VGCC calcium, and the ERK pathway. Furthermore, this was consistent with the notion that the local signaling environment associated with GnRHR microaggregation may be necessary if calcium influx via VGCCs is maintained in microdomains adjacent to the plasma membrane.[363]

The microaggregation hypothesis was further substantiated by the observation that the murine GnRHR was a constitutive and exclusive occupant in membrane rafts within gonadotropes.[38,606,609–611] Solubilization of αT3-1 cells in nonionic detergent at 4 °C, and their subsequent fractionation in discontinuous sucrose gradients, resulted in enrichment of the GnRHR in detergent-resistant membrane fractions with low buoyant density. In addition to these biochemical characteristics, raft compartments within the plasma membrane have also been defined by specific marker proteins. For example, caveolae are a form of rafts characterized by invaginations within the membrane to form flasklike deflection marked by the presence of caveolins 1–3.[598,612,613] Importantly, caveolins interact directly with cholesterol-enriched membranes, forming scaffolds that structurally support rafts and the signaling intermediates within them.[614,615] While specific cell types are critical in the formation of raft compartments, caveolin isoforms are not expressed within gonadotropes (in αT3-1 cells or in murine pituitary cells) and thus do not appear to play a role in raft compartments harboring the GnRHR.[606,609,616] Flotillins 1 and 2 appear to be ubiquitously expressed and also mark raft compartments; however, unlike caveolae, enrichment of flotillins forms rafts that do not change membrane topology. Both isoforms of flotillin are expressed within gonadotropes and mark raft compartments specifically associated with the murine GnRHR.[38,606,609–611]

Enrichment of cholesterol, sphingolipids, and the ganglioside GM1 in the membrane raft has been useful in our understanding of how the GnRHR functions within these liquid-ordered domains. Binding of fluorescently labeled cholera toxin B, which selectively binds to GM1 in living cells, has been used to mark raft compartments within the plasma membrane and colocalize with the GnRHR in the plasma membrane of gonadotropes.[609,611] GnRHR–GM1 colocalization studies using confocal microscopy have helped to corroborate the biochemical fraction studies described here. Manipulating plasma membrane cholesterol content has been used to examine the requirement of the raft domain on signaling via the GnRHR. Depleting cholesterol from the plasma membrane using methyl β-cyclodextrin results in an uncoupling of the GnRHR to activation of the ERK pathway; repletion of cholesterol in cholesterol-depleted plasma membrane effectively rescues this signaling phenotype.[38,611] These studies provide important evidence that raft occupancy by the GnRHR is essential for hormone action, likely by virtue of the receptor's proximity to signaling complexes also residing within the raft. This hypothesis is supported by the additional observations that a number of signaling intermediates also are present within flotillin-positive rafts. These include Gαq, c-Raf kinase, MEK, ERK1 and ERK2, calmodulin, and 14-3-3 adapter protein isoforms. Furthermore, it appears that ERK protein levels are stabilized within the raft upon GnRH administration to gonadotropes, and GnRH-induced ERK phosphorylation and activation occur rapidly within the raft compartment.[38,606,609,611,617] Based on these colocalization and functional studies, it is entirely plausible that since all of the intermediates leading to ERK activation by GnRH reside within the raft, disrupting the raft by cholesterol depletion would lead to uncoupling the ability of the GnRHR to activate the ERK pathway. While these studies provided compelling evidence for a requirement for intact raft compartments for GnRHR signaling, cholesterol depletion–repletion studies require very careful interpretation since loss of cholesterol within the plasma membrane can be damaging in general to cellular homeostasis. The repletion of cholesterol to depleted membranes to recouple GnRH-induced ERK signaling helps to obviate this important concern. Overall, these studies suggest that the constitutive occupancy of the GnRHR in flotillin-marked membrane rafts in gonadotropes is critical to GnRHR signaling.

An important feature of GnRHR and membrane raft association is the defining characteristics or domains within the receptor and raft that facilitate constitutive occupancy within the membrane compartment. Unfortunately, very little is known regarding the biochemical basis for how this receptor partitions into rafts constitutively and engages the ERK pathway. As described in this chapter, a unique feature of the type I GnRHR is an apparent lack of a C-tail. Fusing the C-tail of the nonraft associated LH receptor onto the GnRHR results in a repartitioning of the hybrid GnRHR into nonraft domains within the plasma membrane.[610] These hybrid GnRHRs could bind GnRH and productively signal for ERK activation. Repartitioning of GnRHRs into nonraft compartments was dependent upon the specific C-tail used in the chimeras, providing evidence that the C-tail alone does not dictate raft localization. Another important possibility for raft targeting of the GnRHR is the glycosylation state of the receptor. As presented earlier, mutations at Asn4 and Asn18 reduce trafficking of the murine GnRHR to the plasma membrane.[27] Although speculative, it is entirely plausible that the glycosylation

state at either or both of these residues may facilitate partitioning of the GnRHR into membrane rafts. Additional studies will be necessary to determine if such a mechanism may exist.

Decoding GnRH Pulse Frequency

GnRH effects on its target cells depend upon pulse frequency, as illustrated by early studies showing that constant GnRH suppresses LH and FSH secretion, whereas restoration of GnRH pulses restores gonadotropin secretion.[55–58] Changing GnRH pulse frequency is also the primary means by which the body alters its reproductive status during development, with an increase in GnRH frequency driving the increased gametogenesis and gonadal steroid production at puberty,[29] and stimulation paradigm is crucial for therapeutic manipulation of this system.

In a signal transduction cascade, upstream responses may have rapid onset and decay and therefore track pulsatile inputs closely. In contrast, downstream responses with slower onset and/or offset may not have returned to baseline before repeat stimulation. This is illustrated in Figure 10.6(A), where sequential activation of three effector proteins is considered (i.e., three tiers in a signal transduction cascade), and where high pulse frequency and/or slow response kinetics generates cumulative sawtooth-type responses (lower right panel of Figure 10.6(A)). In essence, this generates a curvilinear relationship between pulse frequency and system output (as measured by the area under the curve for the response traces) that is dependent upon activation and inactivation kinetics for the output measured and can yield near-maximal responses at submaximal pulse frequency (Figure 10.6(B)). This can provide an extremely efficient means of signaling, and such efficiency may be particularly pertinent for central control of reproduction, as it is mediated by the GnRH secreted by a remarkably small number (hundreds) of hypothalamic neurons. Another important consequence of these relationships is that

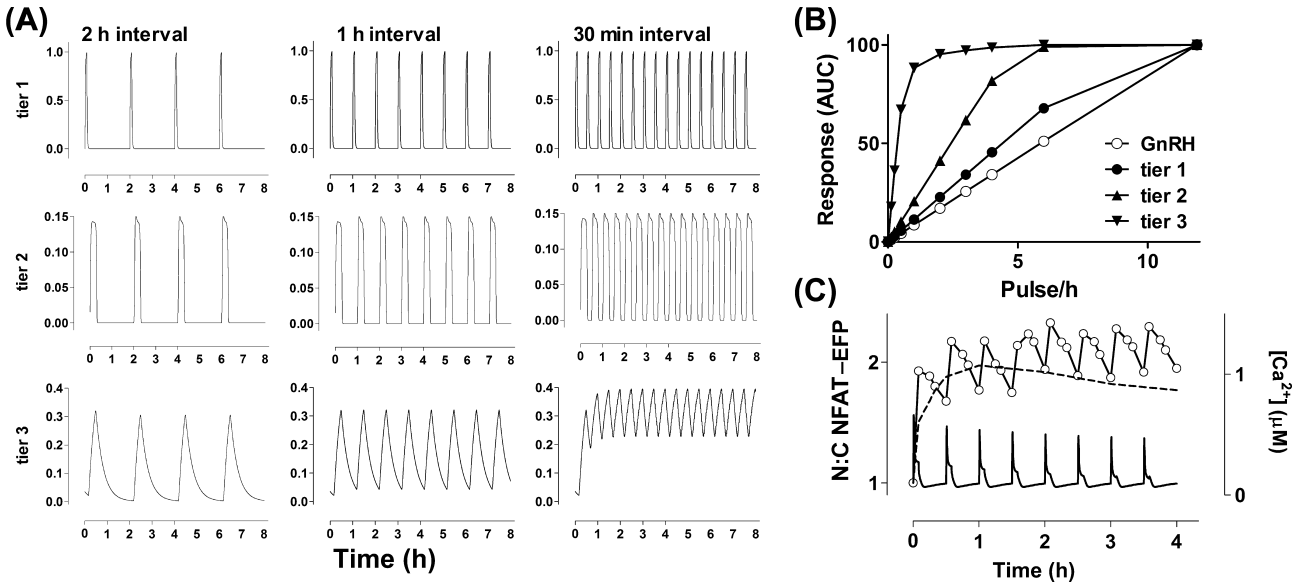

FIGURE 10.6 GnRH pulse frequency dependence. Panel (A) illustrates possible responses to 5 min GnRH at 2 h, 1 h, or 30 min pulse intervals, considering the sequential activation of three effector proteins (a three-tier signaling pathway). With pulsatile stimulation, upstream responses may have rapid onset and offset, following the input closely (tiers 1 and 2), but downstream responses with slower activation and/or inactivation may not have returned to baseline values before repeat stimulation. This can cause cumulative sawtooth responses that do not return to basal levels between pulses when pulse frequency is sufficiently high (tier 3). This can increase the efficiency of signaling, and the different kinetics for activation and inactivation at each tier can yield differences in frequency dependence. This is shown in panel (B), where the relationship between pulse frequency and response is estimated using the area under the curve for the input signal (GnRH) or responses at three sequential tiers of a signaling pathway (tiers 1–3), again using 5 min stimuli. Note that maximal activation at tier 1 requires continuous stimulation (12 pulses/h), whereas near-maximal activation occurs at 1 pulse/h in tier 3. Note also that the outputs are all increasing monotonic relationships, so these processes do not explain the bell-shaped frequency–response relationships seen for GnRH effects on gonadotropin subunit and GnRHR expression. Such maximal activation at submaximal pulse frequency is thought to require positive or negative feedback or feedforward loops or cooperative convergent signaling, as shown in Figure 10.7. Panel (C) shows wet-lab data for the effect of pulsatile GnRH (5 min pulses of 1 nM at 30 min intervals) on the nuclear–cytoplasmic ratio of NFAT–EFP (open circles), a Ca^{2+}-dependent transcription factor used as a downstream readout for Ca^{2+} mobilization. Also shown are the mathematically modeled transient increases in $[Ca^{2+}]_i$ that drive the NFAT translocation (lower solid trace) and the NFAT–EFP ratio in cells receiving constant stimulation with 1 nM GnRH (dotted trace). Note that the NFAT translocation response is slower in onset and offset than the $[Ca^{2+}]_i$ input, yielding a cumulative sawtooth increase in N:C ratio, and that the area under the curve for the response to pulsatile GnRH is comparable to that for constant GnRH, demonstrating the efficiency increase with pulsatile stimulation. *Source: Figure adapted from Refs 618,619.*

they can provide output specificity, as a given pulse frequency could drive a low, intermediate, or near-maximal response for different biological outputs, dependent on the output-specific time delays in activation and inactivation (Figure 10.6(B)). These principles are illustrated by work with fluorescent reporters for activation of the Ras–MEK–ERK and Ca^{2+}–calmodulin pathways.[620,621] Here, GnRHR-expressing HeLa cells were stimulated with GnRH pulses, and live cell imaging was used to monitor cytoplasmic-to-nuclear translocation of ERK2–GFP (reflecting ERK activation) and NFAT–EFP (reflecting Ca^{2+}–calmodulin–calcineurin-dependent activation of the Ca^{2+}-dependent transcription factor, NFAT). Five-minute pulses of GnRH cause rapid, transient, and reproducible translocation of ERK2–GFP and NFAT–EFP from the cytoplasm to the nucleus, but the latter responses were slower in onset and decay so that at high pulse frequency (2 pulses/hour), sawtooth responses were seen for the NFAT–EFP response but not for the ERK2–GFP response (Figure 10.7(A)). Indeed, the integrated NFAT–EFP translocation response was comparable for sustained GnRH and pulsatile GnRH (Figure 10.6(C)), whereas this is not the case for the ERK2–GFP translocation response, illustrating the additional economy and specificity provided by pulsatile GnRH in this system.

The other obvious reason for use of GnRH pulses in biological systems is that the pulses convey specific information. Theoretically, pulse height, width, shape, and frequency and interpulse GnRH concentration could all encode information, but research emphasis has been on pulse frequency and the mechanisms by which it is decoded by gonadotropes. Here, it is important to recognize that, for the data in Figure 10.6, the outputs all increase to a maximum at maximal pulse frequency (or constant stimulation), whereas for some effects of GnRH this is not the case. It is now well established that transcriptional effects of GnRH are dependent upon pulse frequency[35,36,75,182,495,496,622–633] and that GnRH pulse frequency–response relationships are dependent upon the endpoint measured. This is seen in work with luciferase reporters for gonadotrope signature genes, where it was shown[622] that optimal GnRH pulse frequencies for expression of LHβ, FSHβ, αGSU, and GnRHR reporters differed, with 20 h transcriptional responses of LβT2 cells being maximal with constant stimulation or with pulses of 2, 0.5, and 1 h intervals, respectively.[622] In essence, GnRH can elicit bell-shaped pulse frequency–response relationships (i.e., maximal responses at submaximal pulse frequency), and this has been described as genuine frequency decoding (as opposed to the simple frequency dependence shown in Figure 10.6). It is thought to require more complex signal architecture involving feedback or feedforward loops[634] and is exemplified by the bell-shaped frequency–response relationships seen for effects of GnRH on transcription reporters containing LHβ, FSHβ, or GnRHR promoters.[622] However, the nature of these loops is unclear, and three distinct possibilities are highlighted in the literature. The first is that rapid (transcription-independent) negative feedback reduces information transfer from the cytoplasm to the nucleus at high GnRH pulse frequency (shown schematically as black feedback loops in Figure 10.7(C)). Rapid homologous receptor desensitization can be excluded as a potential mechanism because type I mammalian GnRHRs do not show this behavior (see the section Desensitization and Receptor Internalization). GnRH does, however, cause downregulation of cell surface GnRHRs (see the section Desensitization and Receptor Internalization again), and a recent mathematical model of GnRH signaling predicts pulse frequency–dependent desensitization of upstream signals as a consequence of GnRHR downregulation.[635] The second possibility is that frequency decoding reflects the interplay of transcription factors, as exemplified by work on Egr1 and a co-regulator (Nab-2) at the LHβ promoter. In this model, low pulse frequency causes transient Egr1 expression, driving expression of Nab2, which then inhibits LHβ expression, whereas at high pulse frequency more sustained increases in Egr1 quench Nab2 and increase LHβ transcription.[467] For the FSHβ promoter, a similar interplay between c-Fos and the co-regulator TGIF has been proposed to underlie preferential activation at low GnRH pulse frequency. CREB and ICER may be involved as high pulse frequencies preferentially induce ICER, causing transcriptional repression by competing for a CRE site in the FSHβ promoter.[495,636] The third possibility is that transcription-dependent negative feedback on upstream pathways occurs at high GnRH pulse frequency (red feedback loops in Figure 10.7(C)). This could include GnRHR-mediated induction of the regulator of G-protein signaling 2 (RGS2), which has the potential to inhibit all responses downstream of $G_{q/11}$,[391,392] or induction of MAPK phosphatases (MKPs) with the potential to modulate GnRHR-mediated MAPK signaling.[372,637] The live cell imaging experiments described here provide some insight, as the ERK2–GFP and NFAT–EFP translocation responses were both reproducible with repeated GnRH pulses (Figure 10.7(A)). This lack of desensitization argues against upstream negative feedback attenuating the signals passing from the cytoplasm to the nucleus. Moreover, Egr1-responsive and NFAT-responsive luciferase reporters were also used as transcriptional readouts for ERK and NFAT activation in these experiments, and both showed maximal responses at maximal GnRH pulse frequency.[620,621] Importantly, luciferase reporters for LHβ and FSHβ showed maximal responses at submaximal pulse frequency in the HeLa cell model used, demonstrating that genuine frequency-decoding behavior is not restricted to gonadotropes.[620,621]

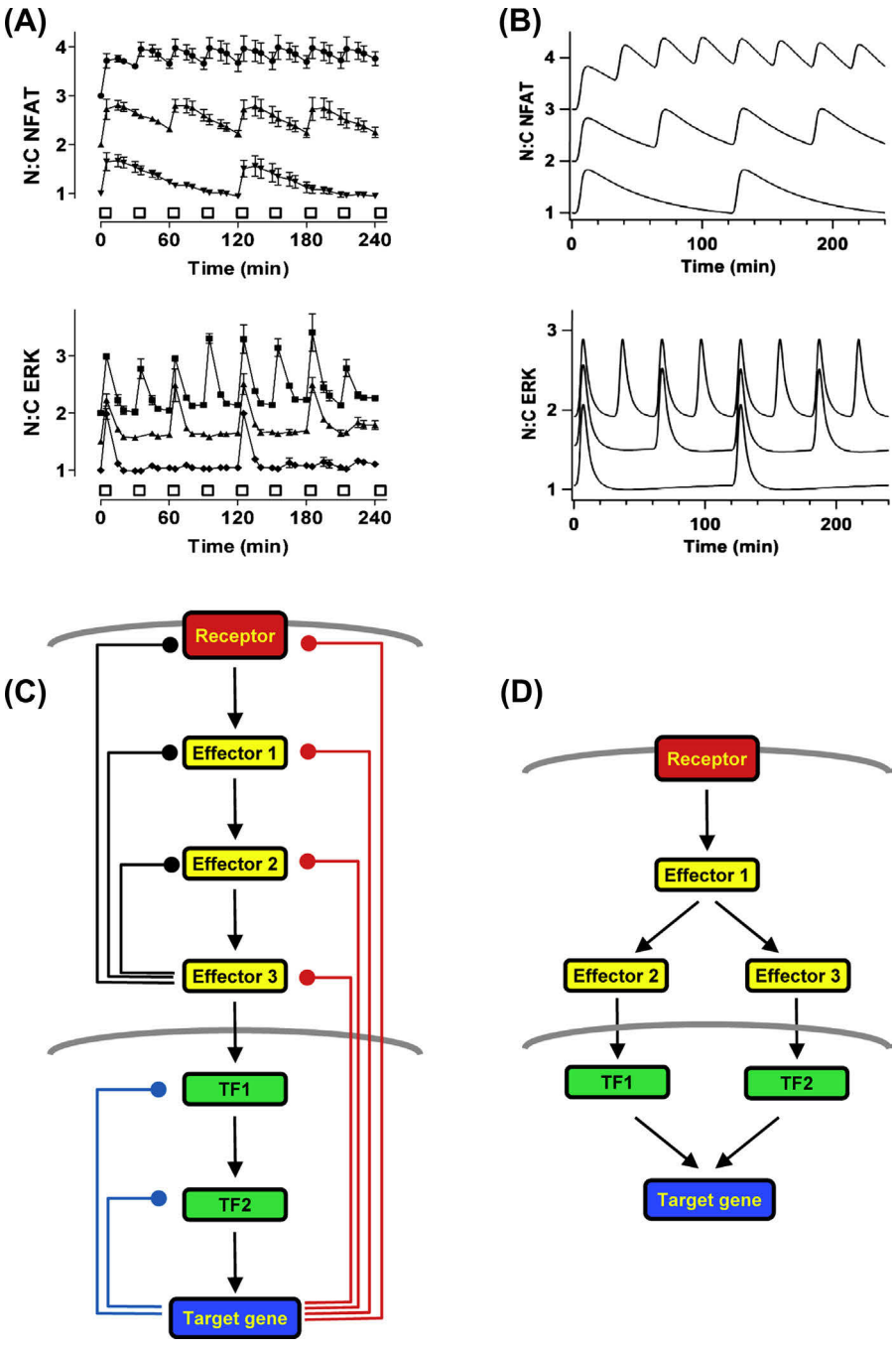

FIGURE 10.7 GnRH pulse frequency decoding, part I. This figure is reproduced in color in the color plate section. Panel (A) shows the effects of pulsatile GnRH (1 nM pulses of 5 min duration and varied intervals) on the nuclear:cytoplasmic ratio (N:C) of NFAT2–EFP (upper chart) or ERK2–GFP (lower chart). In each case, the data are normalized to the prestimulation value and are offset on the vertical axis for clarity. Note that neither of the responses shows desensitization from pulse to pulse and that NFAT–EFP shows a cumulative sawtoothed response at the highest pulse frequency, whereas the ERK2–GFP response is not sawtoothed at any frequency (because it is rapidly reversed on removal of GnRH). Panel (B) shows data from an ordinary differential equation–based mathematical model that mirrors the empirical data for NFAT–EFP and ERK2–GFP translocation. Panel (C) shows a schematic signal transduction cascade in which a receptor in the plasma membrane causes sequential activation of three cytoplasmic proteins (effectors 1–3), and the last of these drives sequential activation of two transcription factors (TF1 and TF2) within the nucleus, which are upstream of a target gene. Also illustrated are multiple negative-feedback loops that could be restricted to the cytoplasm (black) or could involve transcription-dependent feedback to cytoplasmic proteins (red) or nuclear proteins (blue). Panel (D) shows a schematic network in which the receptor activates a bifurcating signal transduction pathway involving three cytoplasmic effectors and two nuclear transcription factors and where these transcription factors converge on the target gene. The empirical and modeled data in panels (A) and (B) suggest that the signal passing from the cytoplasm to the nucleus does not desensitize with pulsatile GnRH, arguing against the upstream negative-feedback pathways shown in black or red in panel (C). *Source: The data in panels (A) and (B) are adapted from Refs 618,620,621.*

A more recent study used mathematical modeling to explore possible frequency-decoding mechanisms. A dynamical model was generated that differs from other GnRH-signaling models[372,635,638] in that it incorporates the Ca^{2+}–calmodulin–calcineurin–NFAT and Raf–MEK–ERK pathways, includes nuclear and cytoplasmic compartments, and lacks negative feedback. The model predictions accurately mirrored empirical data for effects of GnRH pulses on translocation of NFAT–EFP and ERK2–GFP (compare Figure 10.7(A) and (B)), so these could be used as inputs to the transcriptome (shown schematically in Figures 10.7(D) and 10.8(B)). In doing so, it was assumed that two transcription factors (TF1 and TF2) act at distinct sites on a common gene promoter (using NFAT as TF1 and an undefined ERK-dependent TF as TF2). Three distinct logic gates were considered: an "and-gate" where the TF effects are additive, an "or-gate" where only one TF is needed, and a "cooperative gate" where the two TFs act synergistically. This model predicted that bell-shaped frequency–response curves could be achieved when the two TFs are activated with different kinetics and when the two TFs

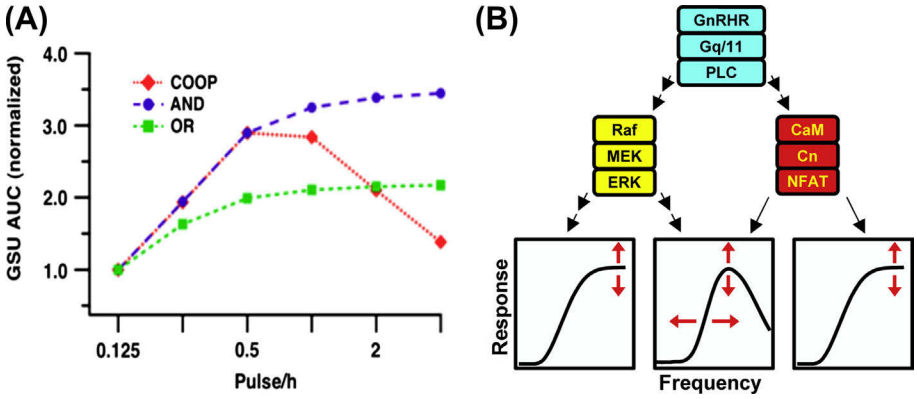

FIGURE 10.8 GnRH pulse frequency decoding, part II. For panel (A), the modeled data in Figure 10.7(B) were used as inputs to the transcriptome, assuming convergent signaling via an "and-gate", an "or-gate," or a "cooperative gate" and activation of an undefined target (termed GSU for gonadotropin subunit). Modeling outputs (area under the curve for GSU expression over time) under different stimulation conditions predicted increasing monotonic relationships for pulse frequency–dependent gene expression with the and-gate and the or-gate but a nonmonotonic relationship with the cooperative gate. Further work predicted that altering the amount of key model parameters (i.e., a concentration of effectors such as ERK or NFAT) would influence response amplitude with the and-gate or the or-gate, but with the cooperative gate could also influence the pulse frequency at which maximal outputs are obtained (panel (B)).

act cooperatively.[618,639] The characteristic feature of maximal response at submaximal frequency was never seen with the and-gate or with the or-gate (Figure 10.8(A)). The kernel of the model is the assumption of Michaelis–Menten kinetics driving the association of two distinct TFs with their target DNA and (in the cooperative scenario) the association of these TF–DNA complexes with one another.[618,639] Accordingly, the cooperative gate differs from conventional computational logic gates but may well have widespread relevance for biological systems, such as transcription regulation by assembly of multimeric protein–DNA complexes potentially like those described for the regulation of the gonadotrope signature gene promoters (see the section Regulation of Gene Expression).

This modeling assumed a simple network with a GnRHR–G_q–PLC module upstream of Raf–MEK–ERK and Ca^{2+}–calmodulin–calcineurin–NFAT modules (Figure 10.8(B)), both of which drive transcription. This is clearly a vast oversimplification of transcriptional regulation by GnRH, as it excludes many known upstream pathways and interactions. It also excludes the real complexity of the combinatorial signals mediating transcriptional effects of GnRH (also in the section Regulation of Gene Expression) and only considers regulation of a generic gene target (termed GSU and not equated to any specific β or α subunit). Nevertheless, the model does illustrate the potential for the ERK and NFAT pathways to decode frequency when acting on a shared target. Since LHβ and FSHβ promoters are both responsive to NFAT and ERK, it predicts that genuine GnRH frequency decoding will be absent for an ERK-responsive reporter or an NFAT-responsive reporter but may occur with FSHβ and LHβ, and this is supported by the work with luciferase reporters outlined here. However, the real value of the model is that it shows how a simple network could yield relatively complex input–output behaviors. The model predicts bell-shaped frequency–response relationships as a likely consequence of the activation of two effectors, as long as they have different kinetics and converge in a cooperative manner on a common target (Figures 10.7(D) and 10.8(B)). This architecture is abundant in signaling pathways, so the implication is that genuine frequency decoding is a likely emergent feature of signaling networks rather than just a function of specific proteins or modules within the network. In the case of GnRH, it illustrates the possibility that such behavior could be generated without negative feedback (note the absence of feedback in Figure 10.7(D)). Indeed, we suggest that pulsatile GnRH secretion and the resistance of type I mammalian GnRHRs to desensitization both serve to minimize negative feedback and thereby place increasing reliance on such downstream combinatorial mechanisms.

Another important aspect of the mathematical modeling approach is that manipulation of model parameters suggests a surprising degree of plasticity.[618,639] Thus, the model outlined here predicts not only that cooperative convergence of the Raf–MEK–ERK and Ca^{2+}–calmodulin–calcineurin–NFAT pathways could cause maximal transcription responses at submaximal GnRH pulse frequency, but also that the optimal pulse frequency may be dependent on model parameters such as the concentrations of ERK and NFAT as well as the kinetics of their activation and inactivation (Figure 10.8(B); McArdle and Tsaneva-Atanasova, unpublished data). To our knowledge, the control of these parameters has not been explored, except in the case of ERK-signaling

kinetics. Comparison of hGnRHR and XGnRHR signaling suggests that receptor desensitization and internalization (which differ markedly for these receptors without and with C-tails) are not important determinants of the kinetics or magnitude of whole-cell ERK phosphorylation.[561] However, ERK responses are modulated by many ERK-interacting proteins, including dual-specificity phosphatases (DUSPs) induced by GnRH, some of which can both scaffold and inactivate ERKs. Using siRNA to target DUSPs, we found that 12 of the 16 DUSPs expressed in HeLa cells influenced ERK responses to sustained stimulation with GnRH or a PKC activator.[353,354,640,641] Moreover, GnRH can increase expression of nuclear-inducible MKP family DUSPs,[353,366,382,640] and a recent computational model illustrated the potential for pulse frequency decoding by MAPK pathways and inducible phosphatases.[372] To our knowledge, the possibility that optimal GnRH pulse frequencies (i.e., for regulation of LHβ and FSHβ expression) vary under different physiological conditions or as a function of ERK inactivation kinetics have not been explored experimentally. Nevertheless, the possible occurrence of coordinated regulation of GnRH pulse frequency and frequency–response relationships is certainly intriguing.

Extrapituitary GnRHRs, Context-Dependent GnRHR Signaling, and Ligand Bias

Pituitary GnRHRs are targeted with agonists and antagonists to cause the medical castration that underlies their efficacy against steroid hormone-dependent neoplasms,[26,519] but GnRHR expression is not restricted to the pituitary. Indeed, GnRHRs are found (often along with GnRH) in many cancers of reproductive tissues.[30] These extrapituitary GnRHRs are of particular interest in light of the fact that GnRH analogs, or cytotoxic derivatives of such analogs, can inhibit proliferation, stimulate apoptosis, and inhibit migration in cell lines derived from such cancers.[30,39,40,161,202–236] Investigation of these systems has revealed major functional differences between GnRHRs in pituitary and extrapituitary sites. In radioligand binding studies,[208] pituitary GnRHRs have high affinity for ligands such as Buserelin (nM K_d values), whereas much of the work on extrapituitary GnRHRs reveals low affinity binding (μM K_d values). In the pituitary, GnRHRs couple via $G_{q/11}$ to PLC, causing an IP_3-mediated mobilization of Ca^{2+} and a PKC-mediated activation of MAPKs (see the section Mitogen-Activated Protein Kinases), but there is little evidence for PLC activation by native extrapituitary GnRHRs. Instead, G_i-mediated activation of protein phosphatases and inhibition of MAPK activity may underlie at least some of the antiproliferative effects mediated by GnRHRs in these cancer cells.[208,209,211,215,216,219,236] A further distinction is that ligands acting as competitive antagonists of pituitary GnRHRs can sometimes mimic the effects of agonists in cancer cells, implying that the agonist–antagonist dichotomy established for pituitary GnRHRs may not be applicable to extrapituitary GnRHRs.[208,216,219,220] The simplest explanation for differing pharmacology with pituitary and extrapituitary GnRHRs would be that they are mediated by distinct receptors, but the type I hGnRHR transcripts expressed in pituitary and extrapituitary sites are identical.[642] The cloning of type II GnRHRs in primates[79] and the demonstration that GnRH II has potent antiproliferative and/or apoptotic effects[217,221,224,225,228,234] raised the possibility that the functional differences reflected mediation by type I and type II receptors. However, humans express a type II GnRHR pseudogene that does not encode functional GnRHRs.[27,39,197,201] Moreover, effects of GnRH I and GnRH II can both be prevented by depletion of type I GnRHRs,[234] confirming action via a single GnRHR. Consequently, it is generally thought that both GnRH I and II effects are mediated by type I GnRHRs in humans.[31,201]

The second obvious possibility is that type I hGnRHR function is dependent upon the cell type in which it is expressed. Identical receptors could presumably signal differently in different cellular environments because of differences in access to effectors, either because whole-cell expression levels of receptors and/or effectors differ or because the proteins are differentially insulated from one another (in space or time). To explore this, we made use of the fact that subclones of hormone-dependent cancer cell lines often differ from one another. Thus, the endogenous GnRHRs of breast cancer (MCF7) and prostate cancer (PC3) cells can mediate direct antiproliferative effects of GnRHR ligands, but in our hands these cells did not express measurable GnRHRs, as judged by binding and functional assays.[213,218,223] However, when recombinant adenovirus was used to express type I GnRHRs in these cells (at levels similar to those found in gonadotropes), the heterologously expressed receptors were very similar (in terms of binding affinity, ligand specificity, and $G_{q/11}$ coupling) to those of the native GnRHRs in gonadotropes. Moreover, activation of these receptors did reduce proliferation, with effects apparently mediated by G_q rather than G_i.[213,218,223] Such experiments with heterologous GnRHRs are clearly consistent, with a role for GnRHR in the mediation of direct effects on cell fate. Nevertheless, it remains unclear why the native type I GnRHR of GnRHR-positive breast and prostate cancer cells should mediate proliferation inhibition by a distinct mechanism to the type I GnRHR expressed heterologously in GnRHR-negative versions of the same cells.

With the protocols used here, it was possible to vary GnRHR number (by varying the adenoviral titer) and to express modified GnRHRs (i.e., chimeric type I GnRHRs with an added type II GnRHR C-tail), and this revealed

the importance of receptor number and function as determinants of efficacy for proliferation inhibition.[223] The data implied that the resistance of type I GnRHRs to desensitization might enable them to effectively mediate proliferation inhibition and that manipulations increasing GnRHR expression might increase the direct antiproliferative effect. In this regard, it is also important that a large proportion of hGnRHRs are intracellular, whereas GnRHRs from other species can be expressed more effectively at the cell surface (see the section Intracellular GnRHRs). Indeed, when fluorescence microscopy was used to explore this,[195,570] it was found that <1% of HA-tagged hGnRHRs were at the cell surface in two hormone-dependent cancer cell lines (MCF7 and DU145). This was increased 7–18-fold by treatment with a small-molecule GnRHR antagonist (the pharmacological chaperone IN3) that was actually able to increase GnRHR-mediated antiproliferative and pro-apoptotic effects. Interestingly, the proportion of hGnRHRs at the surface was at least fivefold higher in gonadotrope-lineage LβT2 cells than in the hormone-dependent cancer cell lines, and the pharmacological chaperone caused a more modest 1.5–3-fold increase in the proportion of receptors at the cell surface.[223,570] This provides a clear demonstration of a cell context-dependent GnRHR function. It also raises the possibility that compartmentalization may underlie some of the reported functional differences between pituitary and extrapituitary GnRHRs. Thus, binding studies with crude plasma membrane preparations (that were developed largely for work with rodent tissues and cells) may be inappropriate for detection of hGnRHRs that are predominantly intracellular in cancer cells. Similarly, rapid responses such as Ca^{2+} mobilization and [^{3}H] IP accumulation may be more readily detected when a large proportion of GnRHRs are located in the plasma membrane (as is the case for rodent GnRHRs in gonadotropes), whereas chronic effects on proliferation may provide time for intracellular receptors to traffic to the surface for signaling (favoring the detection of responses mediated by hGnRHRs in cancer cells).

Until recently, it was generally thought that any given 7TM receptor would have one active conformation and couple to one G-protein (e.g., G_s or G_q) with one effector (i.e., adenylyl cyclase or PLC), but it is now evident that some 7TM receptors act via multiple G-proteins to control multiple effectors.[643–645] Such divergent signaling can reflect the existence of multiple active conformations preferentially coupled to distinct effectors, in which case specific ligands may preferentially stabilize different active conformations and bias signaling to distinct effectors.[643–645] Accordingly, a third possibility is that cell context-dependent effects of GnRH analogs reflect ligand bias,[40] which is also known as functional selectivity, biased receptor signaling, pluridimensional efficacy, biased agonism, or ligand-induced selective signaling.

Ligand bias requires the existence of at least two distinct active receptor conformations that are preferentially stabilized by different agonists and preferentially coupled to different effectors. In reality, however, the system is much more complex, with numerous different tertiary structures predominantly occurring in related groups of preferred conformations known as receptor ensembles.[643–645] Ligands are then thought to alter the thermodynamics of receptor ensemble formations, and the distinct conformations within them have the potential to differentially activate distinct effectors. In this way, ligand efficacy reflects stabilization of ensembles of receptor conformations and is specific for the effectors activated by such ensembles. It is unlikely that any two ligands would produce identical receptor ensembles or that different receptor ensembles would activate effectors identically,[645] so ligand bias is now seen as a likely (possible inevitable) feature of 7TM receptor function. For type I mammalian GnRHRs, the absence of agonist-induced receptor phosphorylation and arrestin-mediated signaling may limit the number of GnRHR conformations and the effector repertoire, but even with these receptors there is good evidence for multiple active receptor conformations and ligand bias for upstream signaling pathways (i.e., G_q versus G_s), global downstream readouts (i.e., inhibition of proliferation), as well as receptor trafficking (to and from the plasma membrane).

It is important to recognize that the cell context-dependent effects of GnRHR ligands may largely be due to differences in effector abundance and signal architecture, rather than ligand bias. Indeed, the fact that GnRHRs mediate activation of G_s in LβT2 cells[315] and of G_i in some hormone-dependent cancer cells,[219,220,232,318] but apparently have neither effect in αT3-1 cells,[312,313] does not necessarily reflect stabilization of different active GnRHR conformations (or conformation ensembles). Similarly, the cell context-dependent mechanisms underlying GnRHR-mediated ERK activation[646] do not require activation of different GnRHR conformations. However, other differences cannot be easily explained without invoking distinct types of GnRHR or distinct active conformations of a single type of GnRHR. Thus, it is well known that the peptide "antagonist" cetrorelix is a pure antagonist of GnRH effects on [^{3}H]IP accumulation and gonadotropin secretion in pituitary cells, but it actually mimics antiproliferative effects of GnRH in some models.[214,225,318] Similarly, GnRH I is more potent than GnRH II at stimulation of IP accumulation by type I GnRHRs in pituitary cells, which is the reverse of the situation for inhibition of proliferation in some models.[30,39,221,225,232,548] Since this reversal of potency or efficacy appears not to reflect action via different receptors (as discussed here), ligand bias remains the most likely explanation for much data showing differences in ligand specificity when native GnRHR-mediated effects have been compared in different cell types.[40]

More direct evidence for ligand bias comes from work with different ligands and non-native GnRHRs. For example, we found that XGnRHRs expressed in HeLa cells had higher affinity for GnRH II than for Buserelin (a type I mammalian GnRHR-selective agonist) and that PKC activation increased the affinity of the XGnRHRs for GnRH II but not for Buserelin.[647] This was paralleled by an increase in Ca^{2+} mobilization by GnRH II (but not Buserelin), and, interestingly, the C-tail was shown to be crucial for this effect as chimeric receptors (type I sheep or hGnRHRs with an added XGnRHR C-tail) showed the same PKC-mediated increase in binding to GnRH II but not Buserelin. These data demonstrate a novel form of "inside-out signaling" and the existence of distinct active conformations of GnRHRs,[647] which is an essential prerequisite for ligand bias at these receptors. Further evidence for ligand bias comes from work comparing the effects of GnRH analogs on different type I mammalian GnRHR-mediated responses. It was found[318] that a series of GnRHR ligands all inhibited proliferation in JEG-3 cells and BPH1 cells (both with native hGnRHRs) and in SCL60 cells (which have exogenous rat GnRHRs). They all apparently activated G_i and caused Gi-dependent (PTX-sensitive) inhibition of proliferation.[318] Marked ligand bias was observed because GnRH I stimulated [^{3}H]IP accumulation; activated ERK, p38, and JNK; and inhibited proliferation, whereas a GnRH analog (135-25) mimicked all other GnRH I effects but failed to increase [^{3}H]IP accumulation.[318] In the SCL60 cells, GnRH I is more potent at stimulation of [^{3}H]IP accumulation (EC_{50} < 1 nM) than for inhibition of proliferation (EC_{50} approximately 100 nM), whereas GnRH II has comparable potency for both experimental endpoints (EC_{50} values 10–100 nM). There are only three amino acid differences between GnRH I and II, and subsequent work revealed that Tyr^8 of GnRH II is the main determinant of its bias toward proliferation inhibition. Here, the idea is that interaction of Arg^8 of GnRH I with Asp^{303} of the GnRHR stabilizes a conformation that preferentially activates G_q, whereas no such interaction occurs with ligands containing Tyr.[8] Instead the Tyr^8 of GnRH II faces away from Asp,[303] and the ligand stabilizes a different receptor conformation that preferentially activates G_i.[648] More recently, domain swapping (human–catfish and human–chicken GnRHR chimeras) was used to demonstrate the importance of extracellular domains as determinants of ligand specificity, and provided further evidence that GnRH I and GnRH II stabilize distinct active GnRHR conformations.[649]

Ligand bias at GnRHRs is also evident in work on GnRHR localization and trafficking. As noted in the section Intracellular GnRHRs, nonpeptide pharmacological chaperones can increase GnRHR trafficking to the cell surface, providing an additional endpoint for consideration of ligand bias. Indeed, the work with such chaperones provides a marked example of ligand pluridimensional efficacy as nonpeptide ligands can act as antagonists in terms of cell surface GnRHR signaling, but as agonists in terms of anterograde trafficking. This raises the intriguing possibility that ligands could be developed that are selective for these effects. Indeed, when we compared three nonpeptide antagonists (IN3, NBI42902, and NBI54048), we found marked differences in potency for inhibition of cell surface GnRHR signaling but not for effects on cell surface expression (C. McArdle, unpublished data), implying that these effects may be selectively manipulated. These data also indicate that the cell surface and intracellular GnRHRs have different conformations, which is not unexpected as most GnRHRs within the cell have apparently failed quality control criteria for ER exit, whereas those at the cell surface evidently have not.

In assessing the effects of pharmacological chaperones on GnRHR expression, control experiments were performed with two peptide antagonists (antide and cetrorelix). These membrane-impermeant GnRH ligands would not be expected to access intracellular GnRHRs and, as expected, had no effect on the proportion of hGnRHRs at the cell surface in MCF7 cells.[570] However, when the XGnRHR C-tail was added to the hGnRHR in order to increase cell surface expression, it was noted that the peptide antagonists further increased cell surface expression of h.XGnRHRs. Although the effect was modest, it raised the possibility that the peptides might act at the surface to increase GnRHR number by slowing internalization, and subsequent work showed that this is indeed the case.[573] Indeed, a pronounced synergism can occur when a nonpeptide chaperone is used to increase GnRHR trafficking to the cell surface and a peptide antagonist (cetrorelix) is used to slow internalization from the cell surface. This effect was seen with hGnRHRs and mGnRHRs and in gonadotrope-lineage LβT2 cells, as well as in HeLa cells. Cetrorelix is not known to stimulate signaling in these models, and similar data were obtained using the signal-defective A261K mutant of the hGnRHR.[573] Thus, although the mechanisms are not known, this work clearly demonstrates that the cetrorelix-occupied hGnRHR is functionally distinct from the unoccupied receptor and that cetrorelix can be a pure antagonist for GnRH I-stimulated [^{3}H]IP accumulation and Ca^{2+} signaling, and an inverse agonist for GnRHR internalization. Importantly, this form of ligand bias was seen with a compound that is used clinically and in gonadotropes, the only proven targets for GnRHR-directed therapy.

Ligand bias has a number of implications for understanding and manipulating GnRHR signaling. It provides a likely explanation for early studies showing reversal of potency or efficacy when comparing the effects of GnRHR ligands in different cell types. It may

also prove crucial for understanding the physiological relevance of the two GnRH ligands acting via a single type of GnRHR in humans. Moreover, it highlights the need to qualify classical pharmacological descriptors; for example, cetrorelix is well established as a full antagonist of GnRHR-mediated gonadotropin secretion from pituitary cells but is an inverse agonist for downregulation of cell surface GnRHRs in others (as discussed here). Similarly, nonpeptide ligands that are pure antagonists for GnRHR-mediated IP accumulation and Ca^{2+} signaling are also full agonists for GnRHR trafficking to the cell surface, providing complex pharmacological profiles dependent upon relative potency for the two effects and receptor location before ligand exposure. Most importantly, however, it raises the exciting possibility of developing ligands that more selectively engage therapeutically beneficial effectors and responses. Here, an obvious strategy would be to develop GnRHR ligands that are antagonists for G_q-mediated stimulation of gonadotropin secretion from the pituitary and agonists for direct G_i-mediated antiproliferative effects on hormone-dependent cancers. However, this remains highly speculative, and a major challenge is now to understand the pharmacological relevance of biased GnRHR signaling in vivo.

AUTOCRINE AND PARACRINE REGULATION OF GONADOTROPES

The vast majority of work on gonadotrope cell signaling focuses inevitably on molecular mechanisms of GnRH action. However, these cells sense and respond to numerous additional extracellular stimuli, including the gonadal steroids 17β estradiol, progesterone, and testosterone, each of which can act centrally to influence GnRH secretion but can also act directly at the pituitary to modulate GnRH effects on gonadotropes. They are also targets for numerous local regulators for which physiological roles are largely unknown. The earliest evidence for such local regulation is anatomical. Thus, in 1970, Nakane[650] showed that GH, prolactin, and ACTH were stored in different cell types, whereas many gonadotropes were immunoreactive for both LH and FSH, and also noted that the cells were not randomly distributed; somatotropes were often closely associated with corticotropes, just as lactotropes were often associated with gonadotropes. In fact, gonadotropes are often found in clusters that may be tightly juxtaposed to blood vessels and/or with lactotropes or folliculostellate cells.[651] The field was advanced with the development of techniques for partial purification of the different cell types from mixed-cell populations of dispersed anterior pituitaries. It was found, for example, that when enriched gonadotropes were stimulated with GnRH, they released surprisingly high amounts of FSH, implying that the microenvironment of gonadotropes within mixed-cell populations restricts FSH secretion.[651,652] When gonadotrope-enriched cell populations were remixed with gonadotrope-poor populations (prolactin-positive cells), this again influenced the FSH:LH ratio secreted.[651,653] It has since become clear that gonadotropes are influenced by many local regulatory factors that mediate communication between gonadotropes and between different cell types, particularly between gonadotropes, lactotropes, and folliculostellate cells, in the anterior pituitary.[651] More recently, sophisticated 3D imaging techniques have revealed that cells thought to be dispersed throughout the gland actually form extensive networks that coordinate cell population responses to stimulation and remodel under different physiological conditions.[654–657] Such studies highlight the question of how such homotypic or heterotypic communication is mediated. The autocrine and paracrine regulation of gonadotropes has been thoroughly reviewed by Carl Denef, who lists over 100 extracellular signaling molecules that have been identified in the anterior pituitary.[651] Some of the paracrine regulators of pituitary organogenesis are described in see the section Paracrine Factors and Pituitary Development. Here we highlight a few additional systems, selected to illustrate different signaling paradigms.

Activins, Inhibin, and Follistatin

Activin and inhibin are members of the TGF-β family. They were first characterized as proteins that activate and inhibit FSH expression (respectively), but this has led to a broader understanding of the roles played by several family members (including BMPs and TGFs) in the control of pituitary development (in the section Paracrine Factors and Pituitary Development) as well as the differential control of gonadotrope signature gene expression (see the section Regulation of Gene Expression). Inhibins were first characterized as gonadal feedback regulators of FSH secretion, and analysis of side fractions from inhibin purification led to the discovery of activins and the realization that they were members of the TGF-β family. Inhibins have an α subunit combined with one of two β subunits (A or B) to produce inhibin βA or βB. Activins are dimers containing only the β subunits (i.e., activin A is a dimer of βA subunits, activin B is a dimer of βB subunits, and activin A/B is a dimer of βA and βB subunits). These proteins play important roles in the hypothalamo–pituitary–gonadal axis, with gonadotropes being a site of synthesis of α and β inhibins, as well as the main site of activin synthesis in the mammalian pituitary.[478,479,651,658,659] Activin is an important local regulator of FSH synthesis, and this action is one of the fundamental reasons why GnRH has selective effects on

LH and FSH secretion under different physiological conditions. It is thought that gonadotrope-derived activin B exerts a tonic stimulatory effect on FSH synthesis that can be prevented by inhibin.[660] Evidence for such a role has been obtained with immunoneutralization experiments showing that activin can mediate the rise in FSH seen after ovariectomy in the rat. In physiological settings, activins are thought to mediate the increased expression of FSH in estrus (i.e., the secondary rise occurring after the GnRH-driven preovulatory gonadotropin surge) and also contribute to the juvenile FSH surge occurring as the gonadotrope cell population expands in 1–2-week-old female rats.[651]

TGF-β family ligands act via cell surface receptors with intrinsic serine–threonine kinase (RSK) activity. They are divided into two groups, type I receptors (collectively termed ALKs for activin receptor-like kinases) and type II receptors, each of which functions as a heteromeric protein complex.[478,479,658,659] Complexity is added by the fact that ligands characteristically bind more than one type of receptor and that their receptors signal cooperatively. Activins act via two of the five mammalian type II receptors (ActRII and ActRIIB) and one of the seven type I receptors (ActR1B–ALK4). In the female rat, ActRII is expressed in many anterior pituitary cell types, whereas ActRIIB is apparently only expressed by gonadotropes.[661,662] A current activation model is that ligand binding brings type I and type II receptors together so that the constitutively active type II receptor can phosphorylate specific residues in the type I receptor. The phosphorylated type I receptors then recruit and activate SMADs. These are transcription factors with an N-terminal MH1 domain containing DNA binding and nuclear localization sequences, and a C-terminal MH2 domain needed for transactivation.[479] The situation is a little more complex with inhibins because their signaling requires expression of a betaglycan termed TβRIII (originally characterized as a TGF-β2 co-receptor). Here, the idea is that binding of inhibin to TβRIII promotes high-affinity binding of the ligand to type II receptors (ActRII, ActRIIB, and BMPRII). The TβRIII–inhibin–type II receptor complex does not lead to type I receptor recruitment and importantly sequesters type II receptors, so that inhibin not only fails to activate the cells but also prevents activin from doing so. This explains why activin and inhibin typically behave as functional antagonists and why the inhibitory effect of inhibin is only seen in cells expressing the TβRIII proteoglycan.[479]

Effects of activins can also be opposed by follistatins, cystein-rich monomeric proteins that bind and neutralize activins at a molar follistatin–activin ratio of 2:1. Inhibition is achieved because the two follistatin molecules bind activins with high affinity, occluding the type I and type II receptor–binding sites.[478,479,651] Follistatins are often coexpressed with the ligands with which they bind, and many of their effects are attributed to autocrine or paracrine neutralization. In the pituitary, follistatins are thought to be produced by both gonadotropes and folliculostellate cells.[651] Fluctuations in pituitary follistatin levels through the estrous cycle are generally suggestive of a role in control of FSH expression, and its production is influenced by GnRH and gonadal steroids, providing a mechanism for hormonal modulation of locally produced activin B.[651]

Pituitary Adenylyl Cyclase-Activating Polypeptide

In addition to GnRH, gonadotropes are targets for a large number of 7TM receptor-activating ligands.[651] These include oxytocin, endothelin-1, galanin, β-endorphin, neuropeptide Y, nucleotides (discussed further here), and pituitary adenylyl cyclase-activating polypeptide (PACAP), a highly conserved member of the vasoactive peptide (VIP)–secretin–glucagon peptide superfamily. PACAP is widely distributed in the nervous, immune, gastrointestinal, cardiac, and endocrine systems but was originally isolated from hypothalamic extracts based on its ability to stimulate cAMP production by primary cultures of rat pituitary cells.[663,664] The major forms are a 38-amino-acid peptide (PACAP38) and a C-terminally truncated 27-amino-acid form (PACAP27) that is less prevalent in most tissues.[651,663–666] It acts via three distinct types of 7TM receptors: $VPAC_1$ and $VPAC_2$, which have similar affinity for VIP and PACAP, and PAC_1, which is highly selective for PACAP.[665–668] PAC_1 receptors have the potential to couple directly to both G_s and $G_{q/11}$ and exist as splice variants differing in the presence or absence of sequences termed "hip" and "hop" in the third intracellular loop (i.e., one, two, or neither of these sequences is present).[651,666,668–670] Early work showed that (for most PAC_1 variants) PACAP38 and PACAP27 had comparable potency for stimulation of cAMP production, whereas PACAP38 was much more potent than PACAP27 for stimulation of [^{3}H]IP accumulation.[671] This is of particular interest in terms of ligand bias (see the section Decoding GnRH Pulse Frequency), as it provides an early example of receptor function that could not be explained by existing models; these functional features immediately suggested that the two ligands activated different conformations of the same receptor and that these conformations activated effectors differentially.

The major secretory cells and folliculostellate cells of the anterior pituitary all express at least one form of PACAP receptor.[651] Notably, PACAP activates PAC_1, causing a G_s-mediated stimulation of cAMP production and a $G_{q/11}$-mediated increase $[Ca^{2+}]_i$ in gonadotropes and gonadotrope cell lines.[277,313,651,665,668,672–675] Moreover, there is increasing evidence that PACAP is an important regulator of both gonadotropin secretion and

expression of signature genes in gonadotropes, acting either alone or by virtue of its ability to modulate GnRH effects.[651,665,668,674,676–681] Since PACAP was initially purified from hypothalami and PACAP concentration is higher in the portal circulation than in the periphery,[651] PACAP is seen as a hypothalamo-hyophysiotropic hormone that uniquely appears to target both gonadotropes and folliculostellate cells.[651] However, there is also evidence for a local role within the anterior pituitary. Notably, PACAP has been detected in rat and human pituitaries (by immunoassay) and in rat gonadotropes (by immunohistochemistry), whereas PACAP mRNA has been detected in folliculostellate cells.[651,682–686] Interestingly, it has been shown that PACAP increases follistatin expression by gonadotropes and folliculostellate cells,[675,687,688] raising the possibility that it modulates activin signaling in the pituitary.[665] Finally, when considering the possible effects of GnRH on cAMP signaling in gonadotropes, it is worth bearing in mind that maximal activation of PAC_1 receptors on gonadotrope-derived cell lines causes much greater elevation of cAMP than GnRH does,[313,668,673] and that GnRH actually causes a PKC-mediated inhibition of PAC_1-mediated cAMP elevation.[313] Accordingly, if gonadotropes are exposed to stimulatory concentrations of (local or hormonal) PACAP in vivo, GnRH pulses could actually inhibit rather than stimulate cAMP production.[313]

Extracellular Nucleotides

The purines adenosine, ADP, and ATP, and the pyrimidines UDP and UTP (uridine 5′ diphosphate and uridine 5′ triphosphate, respectively), can all act as extracellular signaling molecules. Many cells release them in a regulated manner, to act on target cells via specific cell surface purinergic receptors.[651,689] ATP is well established as an extracellular signaling molecule for skeletal muscle and adrenal chromaffin cells, as well as fibroblasts and endothelial cells. It also acts as a neurotransmitter, co-transmitter, or neuromodulator in the central and peripheral nervous systems.[689] Extracellular ATP is rapidly metabolized to ADP, and then via adenosine monophosphate to adenosine.[651,689] Extracellular ATP can activate G-protein coupled P2Y receptors (P2YRs) or ion-conducting P2X receptors (P2XRs), whereas ADP activates P2YRs (but not P2XRs), and adenosine activates G-protein coupled adenosine receptors (ARs) but not P2XRs or P2YRs. UTP and UDP are also native agonists for P2YRs. Anterior pituitary cells express at least six of the seven cloned P2XRs, two of the eight cloned P2YRs, and all four cloned ARs.[281,651,667,689] At least four of the mammalian P2YRs ($P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$) act via $G_{q/11}$.[281,667] The P2XRs are ATP-gated ion channels, with each subunit having two transmembrane domains, and three subunits forming the functional receptor. They gate Na^+, K^+, and Ca^{2+} primarily, and activation characteristically increases Ca^{2+} entry across the plasma membrane either directly or secondary to depolarization by P2XR-mediated ion currents.[281,667,689]

Pituitary cells store ATP in secretory granules and co-secrete it with hormones during agonist-stimulated exocytosis.[651,689–692] Pituitary ATP receptors were first characterized in gonadotropes and in gonadotrope-derived cell lines.[690,693,694] These were identified as P2YRs that were activated by ATP and UTP and caused a $G_{q/11}$-mediated activation of PLC with consequent mobilization of Ca^{2+} from intracellular stores and activation of PKC.[690,693,694] However, cell enrichment protocols have shown that Ca^{2+}-mobilizing P2YRs are located predominantly in lactotropes and not gonadotropes.[689] The reason for this discrepancy is unknown but may relate to the use of different rat strains (Wistar versus Sprague–Dawley, as discussed in Ref. 689). The cell enrichment study also indicated that P2XR is expressed by all of the anterior pituitary secretory cell types.[689] Multiple P2XR transcripts are expressed by pituitary cells, but functional profiling revealed the presence of P2X2R in gonadotropes (and somatotropes), as opposed to P2X3R, P2X4R, and P2X7R in lactotropes. GnRH can stimulate ATP release from dispersed pituitary cell cultures,[690,691] and ATP can stimulate LH secretion and amplify GnRHR-mediated gonadotropin secretion.[651,689–694] Consequently, although physiological roles are not established, it seems likely that ATP of autocrine or paracrine origin acts via P2XRs and/or P2YRs on gonadotropes to amplify the GnRH effects on Ca^{2+} signaling, as well as the downstream consequences of such signaling.

Natruiretic Peptides, Nitric Oxide, and Guanylyl Cyclases

The major mammalian natriuretic peptides are atrial-, B-type, and C-type natriuretic peptide (ANP, BNP, and CNP, respectively). They act via cell surface receptors with intrinsic guanylyl cyclase activity termed NPR1 (GC-A) and NPR2 (GC-B).[667] These enzymes, also known as particulate guanylyl cyclases, have a single transmembrane domain but are thought to act as homodimers.[667,695] NPR1 is selectively activated by ANP and BNP, whereas NPR2 is selective for CNP, and all mediate elevation of cGMP levels in their target cells. ANP and BNP are well-characterized regulators of cardiac and renal function, but the physiological roles of CNP are less clear. Transgenic deletion of the mouse CNP gene (*Nppc*) causes dwarfism and skeletal abnormalities, revealing a critical role in endochondral ossification.[342] Female mice with this deletion are also infertile, as are mice in which the gene encoding GC-B (*Npr2*) is deleted, and this is consistent with evidence supporting a role for CNP in the hypothalamo–pituitary–gonadal

axis.[695–701] Thus, the anterior pituitary contains the highest tissue concentration of CNP, and immunostaining revealed particularly strong expression in gonadotropes,[702] where it is found within secretory vesicles.[700] Moreover, CNP causes marked increases in cGMP levels in GnRH-expressing neurons, primary cultures of pituitary cells, gonadotrope cell lines, as well as endocrine cells of the testis, ovary, placenta, and uterus.[703–710] The fact that gonadotropes produce and respond to CNP is suggestive of an autocrine function,[702,711] although other anterior pituitary cell types are also CNP responsive, suggesting a possible paracrine role for gonadotrope-derived CNP.[651,700,701] Although CNP does not stimulate LH secretion or modulate GnRH-stimulated LH secretion,[695,698,700] work with gonadotrope-derived cell lines reveals that it can stimulate the human αGSU promoter[700] and that GnRH may stimulate CNP expression (transcriptional regulation of the proximal *Nppc* gene).[700] Nevertheless, the physiological relevance of pituitary CNP–NPR2 signaling in the pituitary remains unknown, largely because so little is known about physiologically relevant cGMP targets in this tissue.

Nitric oxide (NO) is a gaseous signaling molecule that plays important roles in the immune, cardiovascular, and neuronal systems. It is produced from L-arginine by nitric oxide synthase (NOS) and exerts physiological effects by activation of soluble guanylyl cyclases to generate cGMP. There are three forms of NOS; neuronal (nNOS) and endothelial (eNOS), which are expressed constitutively and are activated by Ca^{2+}–calmodulin, and inducible (iNOS), which is Ca^{2+} independent.[251,651] nNOS is expressed in the anterior pituitary, where it is located primarily in gonadotropes and folliculostellate cells. In male rats nNOS is located predominantly in folliculostellate cells, whereas in female rats it is predominantly found in gonadotropes.[651] nNOS expression is also controlled by GnRH, as agonists can increase nNOS expression in gonadotropes.[712,713] Moreover, gonadotrope nNOS expression increases during the afternoon of proestrus and can be increased by gonadectomy, and both of these effects are prevented by GnRH antagonists.[712,713] Such effects may reflect the presence of the AF2 transactivation domain of NR5A1 (SF1) in the nNOS promoter[714] as a likely target for GnRH. In addition to effects on expression, GnRH would be predicted to acutely increase nNOS activity by activation of Ca^{2+}–calmodulin. In accord with this, GnRH has been shown to acutely increase cGMP concentrations in pituitary cells in vitro, and this effect was blocked by NOS inhibition.[712]

In vitro studies have shown that NO can stimulate basal LH and FSH secretion and inhibit GnRH-stimulated LH and FSH secretion.[715–717] Thus, GnRH-stimulated gonadotropin secretion can be inhibited with an NO donor and enhanced with an nNOS inhibitor. Similarly, in vivo treatment with an NOS inhibitor has been shown to reduce LH and FSH responses to GnRH in humans.[718] However, effects of NO on gonadotropin secretion remain rather controversial (see Denef 2008 for a discussion of stimulatory and inhibitory effects[651]), and as with the natriuretic peptide studies discussed here, understanding the physiological role of nNOS in this system is hampered by a lack of information on NO–cGMP targets in pituitary cells. Here, it is important to recognize that NO activates a number of target proteins, including cycloxygenase enzymes and ion channels as well as the soluble guanylyl cyclases. At physiological concentrations its effects are largely mediated by soluble guanylyl cyclases, but at higher concentrations it also has pronounced cytotoxic effects, reacting for example with superoxide to produce peroxynitrite that further decomposes to generate cytotoxic oxygen radicals. Such effects presumably underlie the ability of NO donors to stimulate LH and FSH secretion, as these responses were shown to be cGMP independent,[716] and no such effect is seen when cGMP is elevated with CNP (as discussed here).

The fact that gonadotropes appear to use regulators of multiple guanylyl cyclases for autocrine or paracrine regulation raises the obvious question of whether the source of cGMP (i.e., generation by particulate or soluble guanylyl cyclases) is relevant for function. GnRH-stimulated gonadotropin secretion is modulated by NO donors or NOS inhibitors (in a cGMP-dependent manner) but not by CNP (as discussed here), which implies that effects of soluble and particulate guanylyl cyclases are not identical. In terms of potential cross-talk, it is intriguing that GnRH causes a rapid PKC-mediated inhibition of CNP-stimulated cGMP accumulation[711] and a Ca^{2+}–calmodulin -mediated activation of nNOS, so it has the potential to rapidly shift the balance of signaling from particulate to soluble guanylyl cyclases. Further potential for cross-talk arises as activation of cGMP-dependent phosphodiesterase activity provides the potential for CNP to modulate the effects of NO or PACAP (on cGMP and cAMP, respectively), whereas PACAP has been shown to increase nNOS expression and potentiate the GnRH-stimulated increase in cGMP in gonadotropes.[719]

CONCLUSION

The past few decades have seen massive advances in our understanding of gonadotropes, with increasingly detailed models published for gonadotrope development, the GnRHR signaling network, the transcriptional regulation of gonadotrope signature genes, and the local control of gonadotrope function. In spite of the increasingly complex nature of such models, they can all be assumed to be vast oversimplifications, not least because most have little or no information on kinetics and compartmentalization. The importance of kinetics is self-evident in a system where the CNS provides a frequency-encoded

signal to gonadotropes, yet surprisingly little is known about mechanisms of GnRH pulse frequency decoding. We know a great deal about how different Ca^{2+} responses are created and are beginning to understand how MAPK responses are shaped, but we know very little about how they are interpreted by gonadotropes. Similarly, we are just beginning to understand the dynamic nature of the transcriptome, but we know remarkably little about the dynamics of autocrine or paracrine regulatory systems. Compartmentalization of signaling proteins is equally important, as highlighted by recent work showing that the majority of hGnRHRs are actually intracellular and that GnRHR signaling is dependent upon its location within the plane of the plasma membrane. Here, we know very little about what controls the proportion of GnRHRs reaching the cell surface and how the membrane raft partitioning of GnRHRs and their effectors are controlled. Accordingly, we believe that a major challenge for future work is to overlay space and time on existing schemas for gonadotrope biological control mechanisms. Adding spatiotemporal features to already complex biological models will inevitably challenge existing approaches, so it is likely that mathematically rigorous system approaches will be increasingly important, irrespective of whether the system studied is the GnRHR conformation ensemble, the gonadotrope signature gene transcriptome, the GnRHR signaling network, the anterior pituitary, or the individual. Here, an obvious caveat is that mathematical modeling can predict likely regulatory mechanisms extremely efficiently, but their biological relevance can only be established by testing in biological systems with increased use of genetically tractable systems such as mouse models. Mathematical modeling can also enable system simplification by highlighting parameters likely to have little influence on system behavior. However, an alternative approach to simplification would be to focus on parameters known to influence gonadotrope function in vivo. Here, it is telling that only a handful of gonadotrope proteins are known to be essential for normal reproductive function in vivo (GnRHR, αGSU, LHβ, FSHβ, and ERKs) and that pharmacological manipulation of gonadotrope function still directly targets only GnRHRs. Perhaps the greatest challenge for the future lies in translating the large amount of mechanistic information available from cell lines and mouse models into genuine therapeutic benefits to control pituitary-dependent reproductive outcomes.

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CHAPTER

11

Physiology of the Adult Gonadotropin-Releasing Hormone Neuronal Network

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INTRODUCTION

This chapter aims to provide an overview of knowledge regarding the properties and operating features of the gonadotropin-releasing hormone (GnRH) neuronal network as it exists in the normal adult mammal. The GnRH neuronal network is defined as the GnRH neurons and their associated assembly of neuronal and glial cells responsible for controlling GnRH secretion into the pituitary portal circulation. Multiple internal homeostatic and external factors influence the activity of GnRH neurons to achieve levels of fertility appropriate for the animal. This chapter does not extend to a review of the mechanisms through which stress, aging, lactation, seasonality, or adverse metabolism impact upon GnRH neurons. These are all topics of separate chapters in this volume. The fascinating developmental biology of GnRH neurons has been reviewed recently[1,2] and is not touched on here. In addition, the mechanisms underlying the activation of GnRH neurons at puberty in rodent, sheep, and primates can be found in separate chapters of this volume, alongside species-specific chapters on the ovarian cycles of mammals.

The last version of this chapter devoted considerable attention to other forms of the GnRH decapeptide, in particular GnRH-II.[3] Due to the limited evidence for a role of GnRH-II in reproductive control, and the relatively few advances made in this area over recent times, the reader is referred to the 2006 chapter[3] and a recent review[4] for information on this subject.

For this review, "GnRH-I" is represented as "GnRH". It is pertinent to note that the 10–amino acid peptide was originally named "luteinizing hormone-releasing hormone" (LHRH), being isolated and established as a potent stimulator of luteinizing hormone (LH) secretion in the early 1970s by the Schally and Guillemin laboratories.[5,6] Although LHRH was recognized to stimulate the secretion of both LH and FSH, the possibility that a selective FSH-releasing factor may exist[7] resulted in the persistence of the LHRH nomenclature for many years. Today, LHRH is almost invariably termed GnRH.

Since writing the 2006 chapter, it is readily apparent that the last few years have been dominated by kisspeptin. The discovery of a role for kisspeptin in fertility control has sparked huge renewed enthusiasm in investigating the neurobiology of the GnRH neuron and been an incredible boon for scientists in the field in many ways. The author is embarrassed to note that kisspeptin got "just another neuropeptide" coverage in the last version of this chapter. This is now corrected. While undoubtedly representing the most important neuropeptidergic input to GnRH neurons presently known, it remains that the network requires more than just kisspeptin and GnRH neurons to function. Importantly, other fields of enquiry have continued to make important discoveries.

As noted in 2004, progress in the field of GnRH neurobiology is almost totally dependent upon technological advances in neuroscience, and these continuously revise our understanding of the GnRH neuron. For example, the 1990s were the years of the GT1 cell and GnRH mRNA in situ hybridization, and while observations from those studies were no doubt correct, their impact as techniques upon our current understanding of GnRH neuron physiology has lessened. The past decade has been dominated technically by the use of transgenic and knockout mouse models allowing a plethora of brain slice electrophysiological and in vivo reproductive

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profiling studies to be undertaken. As new neuroscience techniques are brought to the GnRH neuron field, it will be fascinating to observe how results from the current mouse models will stand the test of time.

THE GnRH NEURON

Neuroanatomy

Julien Barry and colleagues working in Lille, France, were the first to generate antisera directed against GnRH that could be used for immunocytochemistry.[8] During the 1970s this group dominated the neuroanatomical mapping of GnRH neuron cell bodies and fibers in the brains of a wide variety of vertebrate species including humans (for review see Ref. 9). These studies were complemented in the late 1970s by neuroanatomical investigations by Ann-Judith Silverman[10] and Gloria Hoffman[11] in the USA and Bela Flerko[12] in Hungary. After this there was a worldwide explosion of interest in mapping GnRH immunoreactivity in multiple species at different developmental time points and in relation to varying endocrine states. Major landmarks in GnRH neuron neuroanatomy have also come from ultrastructural studies undertaken by the Silverman, Witkin, Jennes, Beauvillian, Liposits, and Lehman laboratories (for review see Ref. 13) in addition to experiments identifying the hypophysiotropic population of GnRH neurons that project to the median eminence.[14–16] Functionally, the discovery by Hoffman and colleagues that the immediate early gene cFos could be used to indicate activity patterns of GnRH neurons, at least in relation to the GnRH surge,[17,18] has had an enduring influence on experimental GnRH neurobiology.

Distribution of GnRH Neuron Cell Bodies

As a consequence of the unique developmental origins of GnRH neurons within the nose, the GnRH cell bodies come to reside as a scattered "continuum" along their migratory pathway within the mammalian forebrain (Figure 11.1). The GnRH neuron cell bodies can be found to reside anywhere along an axis extending from the olfactory bulbs to the medial septal nuclei and preoptic area through to the ventral aspects of the anterior hypothalamic area (AHA) and mediobasal hypothalamus (MBH). This distribution is sometimes referred to as the "inverted Y" pattern with the rostral-most midline GnRH somata in the medial septum (MS) splitting at the beginning of the third ventricle within the rostral preoptic area (rPOA) to form the two arms of the inverted "Y" that extend back into the ventral AHA and MBH (Figure 11.1). Although this continuum can be found in all mammals, there are species differences as to where the majority of GnRH neurons come to reside along this pathway.[13] In the sheep, rat, and mouse the great majority of GnRH somata are located near the junction of the inverted "Y" in and around the rPOA. Whereas small numbers of GnRH neurons do exist further caudal in the sheep and mouse, it is debatable whether any GnRH perikarya exist in the MBH of the rat. In contrast, a more even distribution of GnRH cell bodies throughout this rostro-caudal continuum is evident in guinea pigs, monkeys, and humans.[19–23]

Whereas the olfactory-to-hypothalamic continuum accounts for nearly all GnRH neurons in mammals, individual cell bodies can occasionally be found outside this distribution in regions such as the bed nucleus of the stria terminalis, subfornical organ, and even the corpus callosum and cingulate cortex.[15,24] The locations of these "aberrant" GnRH neurons may result from individual cells encountering abnormal migratory cues during development.[25]

It is worth noting that GnRH is also expressed in multiple extrahypothalamic cell populations during development in mice and primates.[26,27] The expression of GnRH mRNA and GnRH peptide within these other cell types declines soon after birth in the mouse.[26] This may not be the case in the primate where widespread GnRH-1 mRNA-expressing cells remain evident in the adult human.[28] However, whether these cells utilize GnRH-1 as a neuropeptide is doubtful as these extrahypothalamic neurons appear to express only fragments of the decapeptide such as GnRH$^{1\text{-}5}$.[29]

GnRH Neuron Morphology

Studies undertaken over the last decade have substantially modified how we view the GnRH neuron. In particular, the ability to fill individual living GnRH neurons in situ with small molecular weight dyes has enabled the whole of the GnRH neuron to be visualized rather than just those parts of the cell that contained GnRH peptide.[30] This has revealed the full extent of the GnRH neuron dendrite and revolutionized our understanding of how GnRH neurons must function.

In most species, the GnRH neuron is comprised of a cell body and two simple dendrites giving it a bipolar morphology (Figure 11.2(A) and (B)). The orientation of the bipolar GnRH neuron depends upon its location in the brain; the rostral GnRH neurons in the MS exhibit a vertical orientation and this changes gradually along the continuum to a near horizontal orientation for the caudal-most GnRH neurons in the MBH. This changing orientation may reflect the pathway of GnRH neurons as they migrate through the brain to reside in these different locations (Figure 11.1). However, it should be noted that not all GnRH neurons exhibit this simple bipolar morphology. In sheep, for example, some GnRH neurons can display complex multipolar morphologies

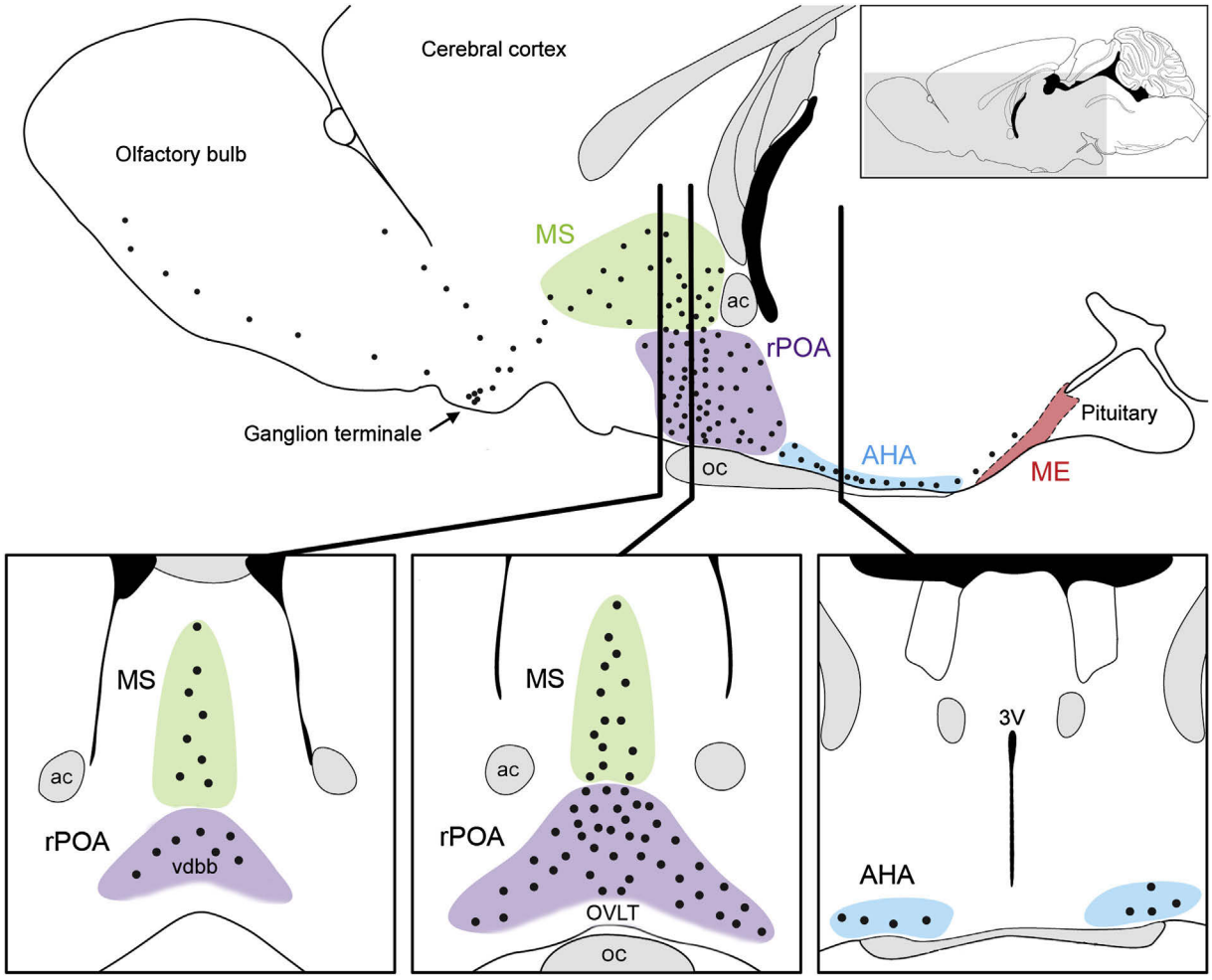

FIGURE 11.1 Distribution of GnRH neurons in the mouse brain. Schematic diagrams showing the distribution of GnRH neurons (black dots) in the sagittal (top) and coronal (bottom) planes. The GnRH neuron continuum extends from the olfactory bulb through to the median eminence (ME), with the great majority of GnRH neurons residing within three arbitrarily defined anatomical brain regions: the medial septal (MS) population, the rostral preoptic area (rPOA) population, and the caudal-most anterior hypothalamic area (AHA) neurons. Note the projection zone for GnRH neurons in the median eminence. Coronal views at the three locations indicated on the sagittal plane are shown below where the concept of the "inverted Y" distribution is more clearly observed. Abbreviations: ac, anterior commissure; oc, optic chiasm; vdbb, vertical limb of the diagonal band of Broca; OVLT, organum vasculosum of the lamina terminals; 3V, third ventricle. *Source: Diagram courtesy of Dr Michel Herde.*

(Figure 11.2(C))[31] as can GnRH neurons located near the organum vasculosum of the lamina terminalis (OVLT) in mice.[32] There is also growing appreciation that the dendritic morphology of a GnRH neuron is modifiable; a notable rearrangement of dendritic structure occurs in the mouse around the time of puberty,[33,34] and seasonal differences have also been observed in the sheep.[35]

Cell filling experiments in transgenic GnRH-green fluorescent protein (GFP) mice have revealed that GnRH neurons exhibit remarkably long dendrites that can extend for over 2000 μm[36] (Figure 11.2(D)). These dendrites are usually oriented parallel to each other and, remarkably, can bundle together being held in direct contact by specialized zona adherens.[37] Shared synapses, where one input synapses on two adjacent GnRH neuron dendrites, are found at these bundling sites, providing one possible location for synchronized control of GnRH neurons by afferent inputs.[37] In addition to vertical dendritic bundling, a small number of GnRH neurons extend dendrites in a manner perpendicular to the vertical GnRH neuron dendrite streams.[37] These new observations have emphasized that, despite the scattered distribution of their somata, the GnRH neurons may form a connected syncytium through their dendritic projections. However, beyond these shared synapses, and rare cytoplasmic bridges,[38] precisely how these dendritic alignments might help synchronize the GnRH neuron population remains unknown.

Another major observation originating from cell filling experiments has been that GnRH neurons in the mouse exhibit numerous spines on their somata and dendrites.[34,36] Prior to this, electron microscopic

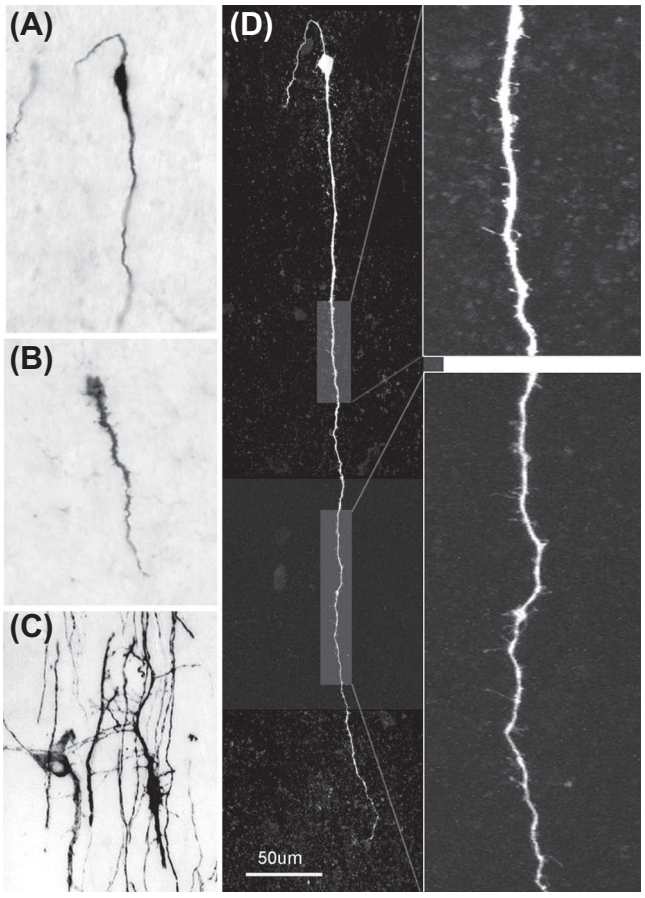

FIGURE 11.2 GnRH neuron morphology in the mouse and sheep. The typical morphology of GnRH neurons in the mouse is shown by the cells in (A) and (B), with (B) exhibiting a clear "spiny" morphology following GnRH immunocytochemistry. The two cells in (C) exhibit the more complex and variable morphology of GnRH cell bodies in the sheep. (D), a montage showing the soma and dendritic structure of an adult mouse GnRH neuron filled with biocytin in situ. Note the length of the primary dendrite (over 500 μm) and the high density of spines throughout the length of the dendrite (two highlighted insets).

investigations had found very little evidence for synapses on GnRH neuron somata[39–42] and, thus, generated the once widely held concept that GnRH neurons receive few synaptic inputs. The observation of numerous spines decorating the long GnRH neuron dendrites (Figure 11.2(D)) provided clear evidence that GnRH neurons receive substantial synaptic input.[36] Thus, while it may be true that synapse density on GnRH neuron somata is relatively low compared with neighboring unidentified cells, this is more than compensated for by the numbers of spines and synaptic inputs along the considerable length of the GnRH neuron dendrites. Although almost all GnRH neurons exhibit numerous dendritic spines, there is heterogeneity in the numbers of spines[36] and this correlates with early work reporting the presence of "spiny" and "smooth" contour GnRH neurons using GnRH immunocytochemistry (Figure 11.2(A) and (B)).[39,42–44] The neurochemical identities of inputs to spines on GnRH neurons are not yet established. However, spine density has been shown to be plastic with alterations observed during pubertal development[34,45] and following changes in circulating gonadal steroid concentrations in the adult.[46–48] Remarkably, it is only the cFos-activated GnRH neurons of the rPOA that exhibit spine changes leading up to the GnRH surge.[46] Studies are just beginning to understand the intracellular mechanisms regulating spine density in GnRH neurons in vivo.[49]

A notable feature of GnRH neurons at the ultrastructural level has been that of substantial glial cell contact or "wrapping" of the cell body.[42,50,51] In the monkey and sheep, a wide range (0–90%) of glial ensheathment of individual GnRH neurons has been observed,[50,51] and recent studies have also reported astrocytic interactions with GnRH neuron cell bodies in humans.[52] Interestingly, glial cell invaginations can be seen to separate presynaptic terminals from postsynaptic densities on the GnRH neuron in rats and sheep.[42,50] Such observations emphasize the potential importance of glia in modulating the efficiency of afferent inputs to GnRH neurons.

GnRH Neuron Projections to the Median Eminence (the Dendron)

The principal projection site of the GnRH neuron is the external zone of the median eminence where GnRH is released into the pituitary portal circulation. Retrograde tracing studies have revealed that between 50% and 70% of all GnRH neurons project to the median eminence.[14,16,23] In the rat, mouse, and sheep, the somata of the GnRH neurons innervating the median eminence are scattered throughout the GnRH continuum.[14,16,53,54] A similar situation exists in the monkey, although the percentage of GnRH neurons projecting to the median eminence is highest within the preoptic area and MBH, with none of the rostral-most GnRH neurons in the MS and diagonal band of Broca being hypophysiotropic.[23]

One of the more remarkable recent findings in the GnRH neuron field has been that the great majority of GnRH neurons do not have axons but send atypical dendritic projections to the median eminence.[55] Using mouse brain slices that maintain GnRH neuron projections to the median eminence, coupled with cell filling and electrophysiology, it was discovered that the GnRH neuron projection to the median eminence is actually a dendrite that receives synaptic inputs along its complete length while also propagating action potentials.[55] This blend of both dendritic and axonal properties has led to the process being termed the "dendron".[55] Once arriving at the median eminence, the GnRH neuron dendron splits into many individual axon-like processes that project to blood vessels and ramify widely throughout the median eminence. Detailed reconstructions of GnRH

neurons also show that the dorsal-orientated dendrite, which initially projects away from the median eminence, often does a hairpin loop turn and itself also eventually projects to the median eminence. Alongside evidence for the initiation of action potentials in the proximal dendron,[56,57] this morphology marks the GnRH neuron as being unique within the central nervous system.

In essence, the GnRH neuron could now be looked upon as one very long information processing cable with three key specializations: (1) a cell body that is responsible for biosynthetic regulation and patterning of electrical activity;[58] (2) an action potential initiation zone in the proximal part of the most ventral dendron[57] (see following); and (3) terminal modifications of the dendron into axon-like nerve terminals for secretion of GnRH into the portal circulation.[55] This new morphology of the GnRH neuron may provide an explanation for curious past experimental results, not the least being the initial puzzling over why GnRH neurons should have such long dendrites. It also predicts important features of GnRH neuron activity and regulation. First, synaptic inputs along the full length of the dendron will have a role in regulating its activity at the median eminence. Those near the spike initiation site will be especially important in determining the overall level of cell excitability, those at the cell body for regulating gene expression, and those at the distal dendron should be able to modulate directly GnRH secretion within the median eminence. The past practice of examining synaptic inputs/appositions on the GnRH neuron cell body alone may be of little relevance in terms of understanding the regulation of GnRH secretion. The presence of synaptic inputs on the distal dendron just before it splits into axons in the median eminence may account for the curious but long-standing lack of evidence for presynaptic synapses on GnRH nerve terminals themselves.[59,60] Equally, neurotransmitter inputs to the distal dendron may explain why so many neurotransmitters and neuropeptides were able to modulate GnRH secretion from "median eminence" preparations in vitro that had no GnRH neuron cell bodies.

Within the median eminence itself, GnRH axons form close associations with tanycytes, the specialized ependymal cells of the third ventricle, so that GnRH neurovascular terminals are often encapsulated by tanycytic endfeet.[61–63] In addition, the GnRH terminals do not sit directly adjacent to the endothelial basal lamina ready to release GnRH into the pericapillary space of the portal system.[50,64–66] Instead, the tanycyte-encapsulated GnRH terminals are located several microns from the basal lamina,[64] and astrocytic processes may exist as a further barrier between the terminal and portal vasculature.[60] This arrangement suggests that access of the decapeptide to the portal system upon release from the GnRH terminal is not straightforward and that ultrastructural variations may represent an additional mechanism for regulating portal GnRH profiles.[61,65,67] Several molecules derived from nonneuronal cells in the median eminence may regulate this process in a steroid-dependent manner (see the section Glial Elements of the GnRH Network).

GnRH Neuron Projections Elsewhere in the Brain

There are abundant GnRH neuron projections in the brain outside the median eminence. For example, in the rat, GnRH-immunoreactive fibers are found in all of the circumventricular organs, medial and cortical amygdaloid complex, stria terminalis, habenula, hippocampus, interpeduncular nucleus, neocortex, and periaqueductal gray.[24,68] There is also a significant number of GnRH neurons located in olfactory brain regions that may provide GnRH neuron inputs into these networks (for review see Ref. 13). Whether GnRH neurons project axons or dendrites to these extrahypothalamic sites remains unclear. Although the great majority of GnRH neurons only have dendrites,[55] a small number have been found to elaborate an axon in addition to their dendrites (Herde and Herbison, unpublished).

One surprising recent observation has been that many of the GnRH neurons in the medial aspects of the rPOA extend one of their dendrites into the OVLT.[32] Such dendrites begin as a normal projection from the cell body, but once they get within approximately 100 μm of the OVLT they begin to branch substantially and ramify throughout the OVLT. As assessed by the presence of spines and electrophysiological responses to excitatory neurotransmitters such as glutamate and kisspeptin, these processes maintain dendritic properties within the OVLT although, ultrastructurally, both axonal and dendritic GnRH-immunoreactive profiles were observed in the mouse OVLT.[32] As these dendrites, and indeed GnRH neuron somata located within 100 μm of the OVLT, are outside the blood–brain barrier, they are well placed to monitor blood-borne molecules. Approximately 85% of GnRH neurons with dendritic projections in the OVLT are thought to be involved in the GnRH surge mechanism,[32] and it is speculated that this ability to sample blood constituents beyond the blood–brain barrier may be of particular importance in regulating the preovulatory surge. However, it remains that the function of the dense OVLT GnRH-immunoreactive fiber innervation has been debated for many years without any clear resolution.[69]

It is likely that most GnRH neuron projections to extrahypothalamic brain regions represent locations where GnRH is released to act as a neuropeptide transmitter. These non-hypophysiotropic GnRH neurons are rarely investigated despite the likelihood that they comprise up to 30% of all GnRH neurons.[16] One experimental difficulty in characterizing this subpopulation is the present inability to actually identify which GnRH neurons

are hypophysiotropic in brain slice preparations. This no doubt contributes to the frequently encountered issue of GnRH neuron heterogeneity. Indeed, despite substantial evidence showing neuromodulatory effects of GnRH throughout the brain,[70,71] the physiological roles of GnRH in these brain areas are essentially unknown. One exception has been the GnRH neuron projections to the midbrain periaqueductal gray that are believed to be involved in regulating female reproductive behavior.[72,73] A role for GnRH in sexual behavior raises the possibility that a mechanism may exist to coordinate the release of GnRH at the median eminence and the midbrain periaqueductal gray on proestrus. One possibility would be for GnRH neurons with dendrons projecting to the median eminence to also send axons to the periaqueductal gray. Another, for which there is initial evidence, is that GnRH overflows into the cerebrospinal fluid from the arcuate-median eminence complex at the time of the surge to coordinate the activity of distant reproductive neural networks such as the periaqueductal gray.[69,74] Interestingly, another likely site of action for GnRH in the cerebrospinal fluid is the hippocampus where it may modulate spine density in a cyclic manner.[75] At present, very little is known about the functions of non-hypophysiotropic GnRH neurons. One recent interesting speculation is that GnRH may mediate antiaging effects throughout the brain.[76]

GnRH Neuron Electrophysiology

GnRH Neuron Firing Patterns

Direct electrical recordings of adult GnRH neurons were first achieved in acute brain slices obtained from guinea pigs,[77] followed by mice,[78–80] and, subsequently, rats.[81] Whereas the initial studies were extremely laborious, requiring post hoc identification of GnRH neurons,[77,82] the development of three transgenic GnRH-GFP mouse lines[78,79,83] and a GnRH-GFP rat[84] has greatly facilitated investigations into the electrical properties of adult GnRH neurons (for recent review see Ref. 85).

To date, GnRH neurons have been recorded in the acute brain slice preparation using three different modes of electrophysiology: (1) on-cell recordings in which an electrode is placed against the cell membrane to measure action potential firing; (2) whole-cell recordings in which the patch electrode "breaks into" the cell to measure ionic currents and subthreshold membrane potential changes; and (3) perforated-patch recordings in which a patch electrode is placed on the outside of the cell but where molecules within the electrode "drill" small holes in the membrane to enable sufficient electrical communication to measure ion channels and membrane potential. Unlike whole-cell recordings, the latter method has the advantage of leaving the internal milieu of the GnRH neuron relatively undisturbed. The choice of recording mode depends upon the question asked and the expertise available.

While acute brain slices from GnRH-GFP mice have been critical in making GnRH neurons accessible for electrophysiological experiments, it is important to be aware of the caveats of this approach. Foremost, it is an in vitro experimental preparation in which both the GnRH neurons and their afferent inputs are damaged. The extent of this deafferentation is difficult to assess, but, considering results from other neuronal cell types,[86,87] there is almost certainly a reduction in synaptic input to GnRH neurons compared with in vivo. For example, magnocellular vasopressin neurons behave quite differently in the brain slice compared to in vivo.[88] Equally, damage to the long GnRH neuron dendrites is unavoidable during the brain slicing procedure and can have a significant impact upon the observations of electrophysiological experiments.[89] Some of these concerns have, indeed, been recently highlighted by the first report of GnRH neuron firing patterns in vivo in the anesthetized mouse.[90] While this study showed that, as in the brain slice, GnRH neurons exhibit heterogenous firing patterns, the patterns of firing in vivo were different to those found in vitro. For example, whereas ~65% of GnRH neurons in vitro exhibit burst firing, only ~15% of GnRH neurons in vivo exhibit this behavior, with the great majority showing relatively high levels of unpatterned firing.[90]

Experimenters have employed different slice preparations to try to alleviate some of these issues, including conventional coronal and sagittal slices, although, in the end, these have had rather little impact on the properties or firing characteristics of GnRH neurons recorded.[89,91] Unconventional slicing procedures have, nevertheless, been able to preserve specific inputs to GnRH neurons such as that from neurons in the rostral periventricular region of the third ventricle (RP3V) using a precisely angled, horizontal brain slice preparation.[92] More recently, a thick hypothalamic "chunk" preparation has been developed in which AHA GnRH neurons and their projections to the median eminence are preserved.[89]

Investigators have found individual adult GnRH neurons in the rat and mouse to exhibit a range of different spontaneous firing patterns. Although seldom detailed fully in papers, this includes GnRH neurons that are silent, continuously active, or irregularly active, including what are commonly called bursting cells (Figure 11.3). As this variability in firing behavior also exists in vivo,[90] it likely represents true heterogeneity within the GnRH neuron population. In this regard, it is interesting to note that cultured (i.e., undamaged) embryonic GnRH neurons also exhibit marked heterogeneity in their firing characteristics,[93,94] as do adult GnRH neurons stripped of their dendrites.[95] Disappointingly, no GnRH neuron electrophysiological "finger-print" has emerged, and it

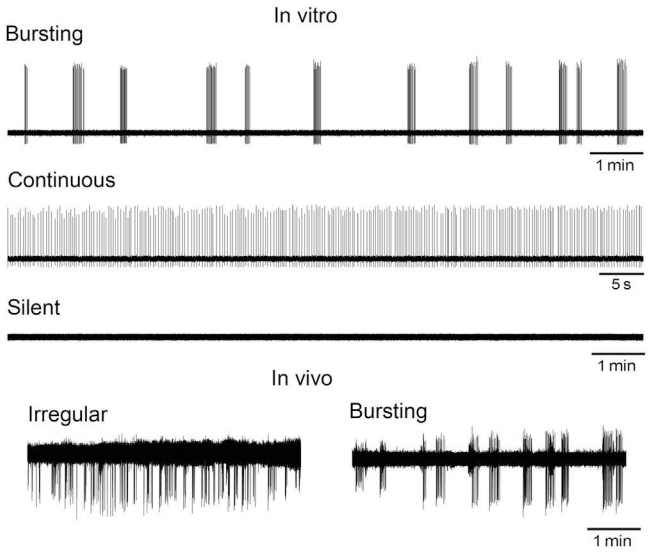

FIGURE 11.3 Typical firing patterns of adult rodent GnRH neurons in vitro (top) and in vivo (bottom). The three top traces show the typical bursting, continuous, and silent types of firing exhibited by adult GnRH neurons in the acute brain slice preparation using cell-attached recording mode. The bottom two recordings show cell-attached firing patterns from GnRH neurons in vivo in the anesthetized adult mouse. Each deflection up or down represents a single action potential.

has turned out to be extremely difficult to correlate these heterogenous firing patterns with suspected pulsatile or surge patterns of GnRH/LH secretion.[96–98]

The heterogeneity in firing, alongside the different methods of recording, characterizing, and documenting GnRH neuron activity by the various laboratories, makes it challenging to be conclusive about what "on average" a GnRH neuron population looks like in terms of its spontaneous electrical firing. Nevertheless, guided by data from minimally invasive cell-attached recordings,[58,89,96,97,99,100] it seems reasonable to state that the majority of adult GnRH neurons in the acute brain slice exhibit burst firing, while another population is silent, and a further smaller group exhibits continuous activity (Figure 11.3). These patterns are observed in gonadectomized as well as intact male and female mice throughout the estrous cycle. Studies from the Herbison laboratory indicate this split to be ~65% bursting, ~30% silent, and ~5% continuously firing GnRH neurons in various brain slice preparations made from male and female mice.[58,89] In contrast, in vivo recordings of GnRH neurons show this split to be ~15% bursting, ~15% silent, with the remainder exhibiting ongoing irregular-continuous firing[90] (Figure 11.3).

GnRH Neuron Burst Firing

Much attention has focused upon burst firing in GnRH neurons on the supposition that, if similar to magnocellular oxytocin and vasopressin neurons,[101] this represents that most effective release pattern for generating pulses of secretion from neuroendocrine nerve terminals. It is important to note that this supposition has not yet been tested for GnRH neurons, and whether burst firing is especially important for pulsatile GnRH secretion is unknown.[102] Nevertheless, it is very likely to be useful to establish the mechanisms underlying burst firing even if only for the fact that it occurs in the majority of GnRH neurons in vitro and may represent a "default" pattern of firing for a denervated GnRH neuron in vivo. Information from several studies of adult and embryonic GnRH neurons indicates that most burst firing in GnRH neurons can be characterized as consisting of 2–5 action potentials occurring over ~2 s, with intraburst frequencies of up to 10 Hz and the interburst interval being either every ~20 s or longer.[58,85,89,93,94,96]

To understand the etiology of burst firing, the first challenge has been to establish whether it represents an intrinsic property of a GnRH neuron or if it requires extrinsic inputs. This issue has still not been resolved satisfactorily. One early piece of evidence for a completely intrinsic "burst generator" has come from the observation that acutely disassociated adult GnRH neurons are still able to exhibit episodic firing.[95] However, the very erratic firing exhibited by those individual disassociated cells is not typical of the relatively evenly spaced burst firing observed in individual GnRH neurons in the slice preparation (Figure 11.3).[85] Further, those cells did not have dendrites, and it is now established that most action potentials are initiated in the GnRH neuron dendrite.[56,57]

Another piece of evidence widely used to support the presence of an intrinsic burst generator in GnRH neurons is the observation that the immortalized GnRH-secreting GT1 cell lines exhibit episodic calcium events, burst firing, and pulsatile GnRH secretion.[103,104] However, it is becoming increasing apparent that GT1 cells are not an appropriate model of adult GnRH neurons in vivo. For example, episodic activity in GT1 cells depends upon complex calcium signaling and gap junctional intercommunication, and yet no evidence has been found for gap junctional coupling between adult GnRH neurons in situ.[105] Furthermore, GT1 cells utilize calcium differently than native GnRH neurons.[106]

Although the case for an intrinsic pulse generator underlying burst firing is not strong, it remains that evidence for an important role of extrinsic inputs is similarly weak. For example, a study has recently demonstrated that GnRH neuron burst firing is independent of all fast ionotropic GABA and glutamate signaling.[107] Thus, the impact and balance of intrinsic versus extrinsic influences upon GnRH neuron burst firing remains unresolved. Recent progress in deciphering some of the ionic mechanisms operating within a GnRH neuron to control burst firing dynamics (see following) indicates that GnRH neurons are, at least, able to modulate their patterns of bursting. However, the key unresolved

issue is whether each burst is triggered by an intrinsic or extrinsic mechanism or, indeed, by both processes. Depressingly, studies in magnocelluar neurons have highlighted that the mechanisms underlying burst firing in vitro are likely to be different from those encountered in vivo.[88]

Ion Channels Expressed by GnRH Neurons

Investigations over recent years have described several distinct ion channels and conductances in adult rodent GnRH neurons (Table 11.1). These studies show that GnRH neurons use typical combinations of voltage-dependent sodium channels and A-type potassium, large-conductance calcium-activated potassium (BK) channels, as well as delayed-rectifier potassium channels to generate action potentials[79,82,109,110] (Table 11.1). Following the action potential, however, a relatively unusual cluster of ion channels is used by GnRH neurons to generate the after depolarization potential (ADP) and after hyperpolarization potential (AHP) that summate to determine post-spike excitability (Figure 11.4(A)).

TABLE 11.1 Ion Channels Expressed by Adult GnRH Neurons in the Rat and Mouse

Channel	Role in GnRH Neurons	References
Volatge-gated Na^+	Action potential initiation	79
Slow TTX-sensitive Na^+	After-depolarizing potential (ADP), burst firing dynamics	108
Delayed rectifyer K^+	Repolarization of membrane after action potential	82,109,110
A-type K^+	Suppression of action potential initiation, repolarization of membrane after action potential	82,109
BK calcium-activated K^+	Repolarization after action potential	110
SK calcium-activated K^+	After-hyperpolarizing potential (AHP), general excitability and burst firing dynamics	58,111–113
UCL calcium-activated K^+	Slow AHP, burst firing dynamics	58
ATP-sensitive K^+	Metabolic regulation of excitability	114
Hyperpolarization-activated cation	Depolarizing influence on membrane potential, possible burst firing dynamics in some GnRH neurons	114,115
Voltage-gated T-type Ca^{2+}	Low threshold voltage activation of calcium entry	81,116,117
Voltage-gated L-type Ca^{2+}	Depolarization-evoked calcium entry into soma and dendrite	57,81,118,119

The ADP in GnRH neurons is activated ~200 ms after the action potential and can be rather slow, lasting for up to 1–2 s.[108,120] An unusual TTX-sensitive sodium current, possibly located within the soma of the GnRH neuron, underlies this ADP.[108] As the ADP elevates membrane potential soon after the action potential, it provides a depolarized plateau facilitating repetitive firing that would contribute to a burst (Figure 11.4(A)).

The AHP acts in the opposite manner to the ADP by generating a period of hyperpolarization following the action potential (Figure 11.4(A)). The AHP in GnRH neurons is driven predominantly by calcium-activated potassium conductances; the small-conductance calcium-activated potassium channels (SK) and "UCL-sensitive channels".[58,111–113] Although encoded by the normal SK1-3 transcripts, the SK channel is unique in GnRH neurons in that, once activated, its current lasts for several seconds, compared with ~200 ms in other neurons (Figure 11.4(A)). As a hyperpolarizing influence on the cell, the SK current counteracts the ADP and suppresses firing immediately after the first action potential. Thus, the SK channel plays a role in terminating burst firing by controlling the duration of the burst and the spacing of action potentials within it. In terms of burst patterning, activated and spontaneous bursts in GnRH neurons often exhibit spike frequency adaptation whereby the interval between individual spikes in the burst becomes progressively longer; this phenomenon is attributed to the SK channel in GnRH neurons.[58,112]

The other channel contributing to the AHP in GnRH neurons is an unusual, molecularly uncharacterized, calcium-activated potassium channel that is antagonized by UCL2077 (Figure 11.4). This "UCL channel" (named after University College London where the antagonist was discovered) is extremely slow, activating over ~10 s and lasting for a further ~20 s. Being active outside the time of the ~2 s burst itself, the channel does not impact on intraburst dynamics but, rather, has a role in determining the interburst interval in GnRH neurons.[58]

Intracellular calcium within the GnRH neuron soma acts to coordinate several of the ion channels involved in bursting.[58] Although it had been appreciated for some time that embryonic GnRH neurons exhibit spontaneous increments in intracellular calcium concentration,[121,122] it was only recently that this was also found to occur in adult GnRH neurons[123] (Figure 11.4(B)). Subsequent studies in adult GnRH neurons have shown that these ~10 s long calcium transients are generated by small amounts of calcium entering through voltage-gated L-type calcium channels that evoke massive calcium-induced calcium release from inositol-1,4,5-trisphosphate receptor-dependent stores.[58] Once generated, one of the roles of the calcium transient is to coordinately

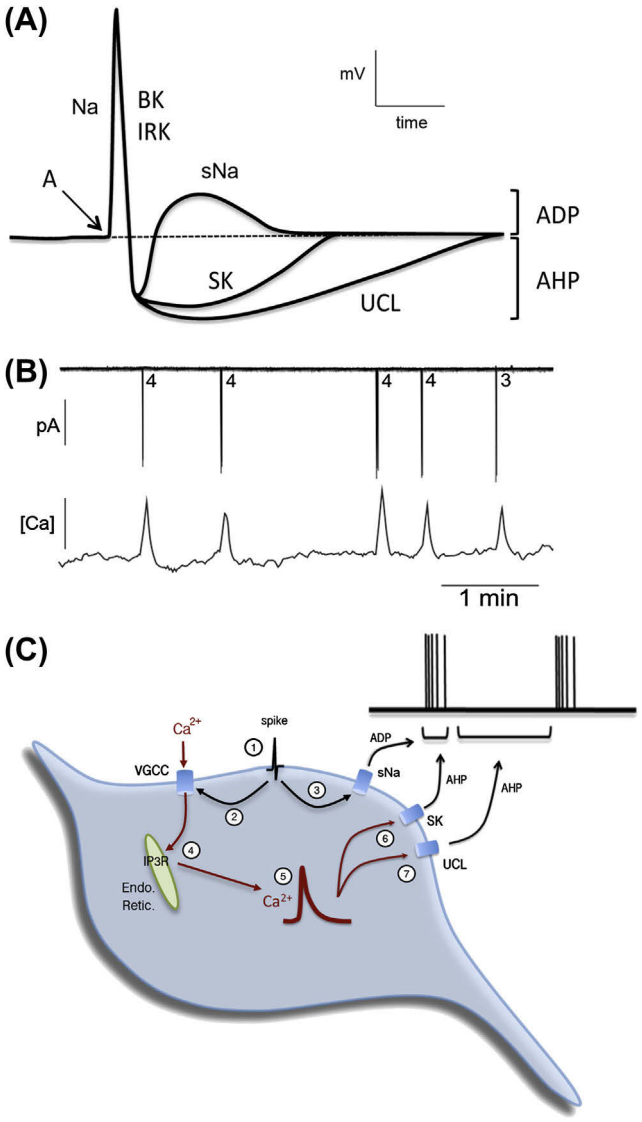

FIGURE 11.4 Electrical properties of adult rodent GnRH neurons. (A) Schematic (not to scale) diagram of the action potential and after potentials in GnRH neurons and the underlying currents. The action potential is generated in the normal manner by activation of voltage-gated sodium channels (Na) with repolarization occurring through inward rectifier (IRK) and large-conductance calcium-activated potassium (BK) channels. The A-type potassium current (A) is indicated to suppress the initiation of the action potential. Following the action potential, several different voltage- and calcium-dependent channels are activated to create the after depolarization potential (ADP) and after hyperpolarization potential (AHP). These include the activation of a slow voltage-gated sodium channel (sNa) to generate the ADP and a slow small-conductance calcium-activated potassium channel (SK) and even slower calcium-activated potassium channel (UCL) that generates the AHP. (B) Experimental trace showing simultaneous recording of bursts of action potentials (top, each number shows the number of action potentials in the burst) and intracellular calcium transients (bottom) occurring in adult GnRH neurons. (C) Schematic diagram indicating known channels involved in generating burst firing in GnRH neurons. The sequence starts with a spike (1) that (2) initiates calcium entry through voltage-gated calcium channels (VGCC) and (3) activates the slow sodium channel (sNa) that, in turn, drives the ADP promoting burst firing. The small calcium influx through VGCC is then amplified (4) through an IP3 receptor-dependent mechanism at the endoplasmic reticulum calcium stores to generate the ~10 s calcium transient (5). The transient activates SK channels (6) to generate an AHP that acts to suppress burst firing and also a slower calcium-dependent UCL channel (7) that prolongs the AHP and to help time when the next burst may occur.

activate the SK and UCL channels to control burst firing dynamics (Figure 11.4(C)). While calcium does not appear to directly regulate the sodium channel underlying the ADP, it does modulate its magnitude by controlling the AHP.[108,112] Blocking or modifying calcium dynamics at different points in this cascade alters burst firing in GnRH neurons.[58,118]

Together these studies suggest that a simple "core model" for burst firing in GnRH neurons could result from: (1) initiation of an action potential; (2) activation of the ADP to start the burst; (3) activation of the SK component of the AHP to terminate the burst; and (4) activation of the UCL component of the AHP to determine when the next burst will occur (Figure 11.4(C)). Indeed mathematical simulations with these basic elements are sufficient to generate GnRH neuron bursting behavior.[58,124] The mechanism underlying the initiation of the first action potential is unknown and forms the center of the debate regarding the intrinsic versus extrinsic origin of burst firing in GnRH neurons.

Although controversial,[116,117] two channels associated with pacemaking activity elsewhere in the brain can be found in some GnRH neurons and can evoke rebound depolarization and spiking. These are the fast-acting (~20 ms) T-type calcium channel and slower (~100 ms) hyperpolarization-activated cyclic nucleotide-gated (HCN) channel.[115–117] If either or both channels were activated by the slow AHP in GnRH neurons, they would provide a range of depolarizing sources that might then contribute to the ongoing burst or initiation of the first action potential required for the next burst. Although undoubtedly simplistic, each of (2–4) in the model outlined above are likely to be regulated by extrinsic inputs and, as such, would give the spectrum of firing observed in GnRH neurons. For example, upregulated SK channel activation, via altered intracellular calcium dynamics, would enhance the AHP and abolish burst firing, while downregulated UCL channels, through neurotransmitter modulation, would increase interburst frequency.

GnRH Neuron Dendrite Electrophysiology

As noted before, the discovery of the blended axon and dendrite (termed the *dendron*) requires a considerable revision of how the GnRH neuron functions to generate secretion in the median eminence. Unfortunately, recording the electrical activity and characteristics of

the GnRH neuron dendron is technically demanding.[56] Nevertheless, using cell-attached recordings and both sodium and calcium imaging, it has been possible to show that the great majority of action potentials are initiated from a spike initiation zone located 100–150 μm from the cell body down the dendron.[57] This has been further verified through visualization of the location of Ankyrin G, the scaffolding protein clustering together the channels required for spike initiation, at the same proximal dendritic location in GnRH neurons.[55]

Thus action potentials are generated in the proximal dendron and propagate to reach the median eminence to initiate GnRH secretion. Although action potentials traveling down the dendron can initiate calcium transients, these dendronic calcium events are very different from the long-duration calcium transients observed in the GnRH neuron cell body[57] and would not be sufficient to pattern the required calcium-activated potassium channels (Figure 11.4(C)). This, alongside evidence for key "burst firing channels" being located in the GnRH neuron soma (see previous discussion), indicates that the actual patterning of GnRH neuron firing probably occurs at the GnRH neuron soma. In this scenario, the spike initiated in the dendron would reach the soma to activate "burst channels" to (Figure 11.4(C)) generate subthreshold potentials that quickly propagate back to the spike initiation zone to generate the patterned firing of the cell.

The identity and roles of synaptic inputs along the GnRH neuron dendron are not established. This information is difficult to attain but, nevertheless, there is emerging evidence for both gradients and microdomains of $GABA_A$ and $GABA_B$ receptors on the GnRH neuron dendron.[89,118] How these and other neurotransmitter inputs function to control excitability, action potential conduction down the dendron, and subthreshold potentials in the GnRH nerve terminals within the median eminence, remains to be established.

GnRH Biosynthesis

GnRH-1 Gene

The *GnRH-1* gene was cloned in the human and rat by the Seeburg laboratory in the mid-1980s.[125,126] The *GnRH-1* coding sequence is found on chromosome 8 in the human and chromosome 14 in the mouse.[127] The gene is comprised of four short exons and three long introns spanning approximately 4.5 kb (Figure 11.5). The GnRH decapeptide is ultimately derived from exon 2, whereas GnRH-associated peptide (GAP) is transcribed from exons 2, 3, and 4 (Figure 11.5).[125,126] Transcription of the *GnRH* gene begins at multiple sites; for example, in the human, transcription starts at +1 for GnRH neurons and upstream at −579 for reproductive tissues such as the placenta (Figure 11.5).[128,129]

Much work has been undertaken examining the proximal promoter and enhancer elements within the *GnRH* gene 5′ sequence responsible for the regulation and neuron-specific expression of GnRH (for recent review see Ref. 130). The rat *GnRH* gene has received the most attention with the identification of a minimal 173 bp promoter (−173/+1) and three critical enhancer regions conferring neuron-specific *GnRH* expression located at −1863/−1571, −3135/−2631 and −4199/−3895[131–133] (Figure 11.5). Transgenic mice in which reporter constructs comprised of the 173 bp promoter and first 300 bp enhancer target the majority of GnRH neurons in vivo.[134] Substantial progress has been made in defining many of the transcription factors and their binding sites within the *GnRH* gene promoter and enhancers. These include POU (Oct1 and Brn2) and other homeodomain proteins (Msx, Dlx, Six6), as well as Otx2 and GATA transcription factors, that bind to motifs located throughout the *GnRH* promoter and enhancers.[135–139] In addition, important coactivators (Prep1/Pbx1) and corepressors (Goucho-related-gene proteins) active at the Oct1 binding site have been identified.[140,141] A recent study has shown that there are also elements within intronic regions of the *GnRH* gene that contribute to its regulation.[142] As the individual regulatory elements become apparent, an important challenge for this field will be to understand the mechanisms coordinating the expression of the various transcription factors to regulate *GnRH* gene expression during development and in the adult. For example, it is proposed that protein kinase C (PKC) phosphorylates or modulates no less than five different transcription factors to suppress *GnRH* gene expression.[130]

Less is known about the cell-specific and critical enhancer regions of the human GnRH gene. Initial studies using fragments of human *GnRH* 5′ sequence in murine GT1 cells indicated that important *GnRH* neuron-specific elements were located in the first 500 bp.[143] However, subsequent investigations using human *GnRH* sequences as transgenes in mice revealed that elements between −795 bp and −992 bp were critical for hypothalamic expression in vivo.[144] This is a similar region to that shown to be important for enhancing transcription at the upstream/placental transcription start site of the human *GnRH* gene.[128] Although caution is required when interpreting results obtained using human sequence in a mouse transcriptional environment, these data suggest that different regions of the rat and human GnRH promoter are used to confer GnRH neuron specificity (Figure 11.5). Interestingly, a CpG-rich island has recently been identified in the distal 5′ (−2126 to −1863) region of the rhesus monkey *GnRH* gene and this was found to be demethylated with GnRH neuron maturation in culture.[145] While the relationship of this demethylation to GnRH

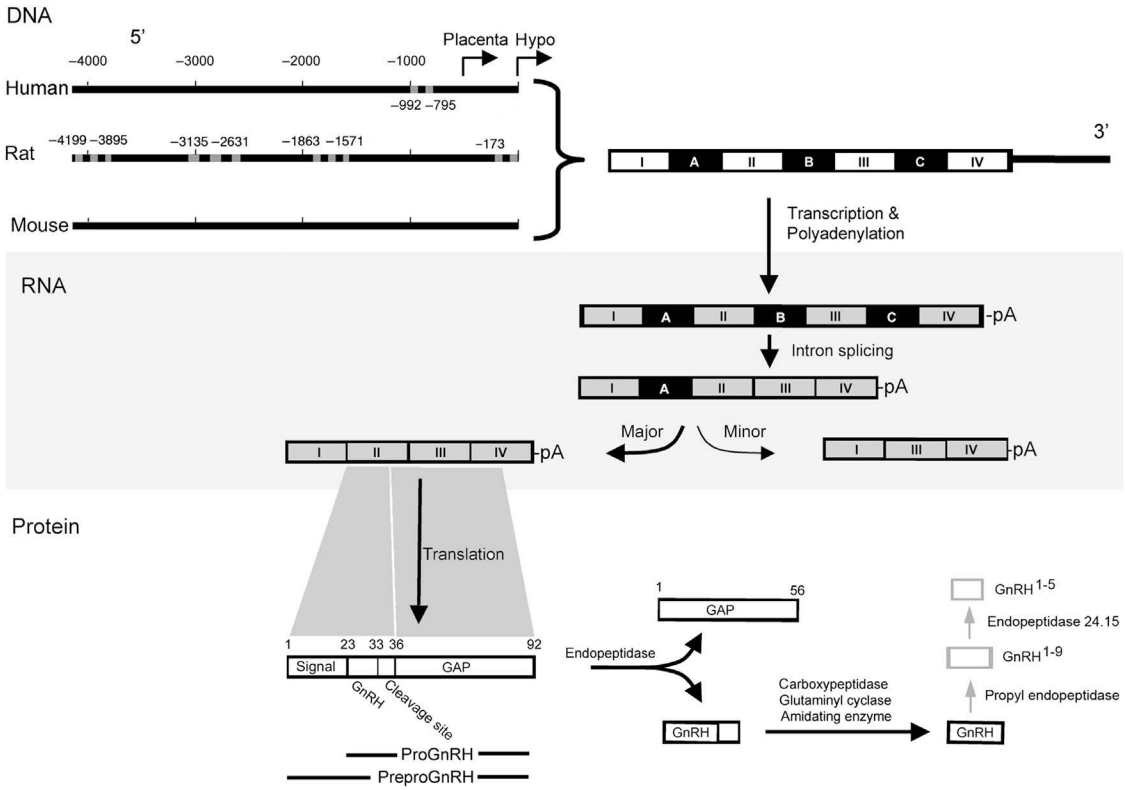

FIGURE 11.5 **Biosynthesis of GnRH and GAP.** Top panel depicts the structure of the GnRH gene with the 5′ regulatory sequence of human, rat, and mouse overlaid and specific regions of known function highlighted (see text). Middle panel depicts GnRH RNA and various splicing and processing intermediates. Bottom panel shows translation and posttranslational modifications that result in the final GnRH decapeptide.

biosynthesis is not yet clear, this study demonstrates that the *GnRH* gene may be subject to epigenetic remodeling.

The functional organization of the mouse *GnRH* gene has been examined in vivo utilizing transgenic approaches in mice. Transgenes comprised of different parts of mouse *GnRH* sequence coupled to the *lacZ* reporter have demonstrated that nearly all GnRH neurons are targeted using 5.2–3.5 kb of 5′ flanking sequence and that transgene expression levels in GnRH neurons decrease with shorter elements of 5′ flanking sequence.[146–148] These studies have not yet defined the role of any smaller regions of flanking sequence and the importance of the relatively conserved rat-mouse 173 bp promoter and first 300 bp enhancer of the rat.[130,149] Studies in the mouse have, however, provided some insight into the importance of 3′ flanking sequence as, although the first 3.5 kb of 3′ flanking sequence does not contribute substantially to the targeting of expression to the GnRH neurons, it does appear to contain sequences that suppress GnRH expression in non-GnRH neurons.[146]

Interestingly, there is evidence that GnRH gene transcription occurs in a fluctuating manner in GT1-7 cells. This is regulated by calcium and involves Oct1 transcriptional actions within the first enhancer site of the rat *GnRH* gene.[150,151] This spontaneous oscillation of GnRH transcription has recently been confirmed in vivo in transgenic mice where it exhibits a 10 h periodicity.[152] Whereas studies in cell lines have indicated that many different hormones and peptides can alter *GnRH* gene transcription in vitro (for recent review see Ref. 130), alterations in *GnRH* gene transcription in vivo have seldom been examined. Perhaps the best-characterized example of regulated *GnRH* gene transcription in vivo is that associated with estrogen positive feedback in the rat where a ~40% increase in GnRH gene transcription has been demonstrated 2–4 h prior to the onset of the GnRH surge.[153,154] Whether this increase is required for the generation of new peptide that will be released a few hours later, or whether it may be used to replenish stores for the next GnRH surge four days away, remains unknown. Changes in GnRH gene transcription rates have now also been detected in relation to estrogen negative feedback in the male mouse.[147]

GnRH-1 mRNA

Transcription of the *GnRH* gene produces a polyadenylated primary transcript of ~4200 bases that is processed by splicing to a mature transcript of 560 bases[125] (Figure 11.5). Maurer and Wray[155] estimated that single GnRH neurons contain >1000 GnRH mRNAs, making

them a high copy number transcript with levels comparable to that of housekeeping genes. One unusual feature of the GnRH neuron is that 30–40% of all GnRH transcripts are located in the nucleus at any one time,[156,157] and it has been suggested that this acts as a large steady-state pool of mRNA available for translocation into the cytoplasm when required.[158]

The processing of the primary transcript appears to be rapid, with a half-life in the order of 18 min for the primary transcript and 10 min for the mature transcript.[156,159] Studies in the rat and mouse indicate that intron B is rapidly excised from the primary transcript followed by intron C, with intron A being spliced out more slowly.[157,160] The removal of intron A is critical to enable efficient translation of the mature GnRH mRNA.[161] Whereas introns B and C contain appropriate consensus splice sites, intron A exhibits a suboptimal 3′ splice site that does not allow efficient splicing by the spliceosome complex.[160] Intron A can only be spliced out following the formation of a splice enhancer complex comprised of the serine/arginine-rich RNA-binding protein Tra2a and associated interacting proteins.[162] This complex must interact with exon splicing enhancers located in exons 3 and 4 to achieve the splicing of intron A.[162] Thus, it is postulated that introns B and C are removed in the normal manner, thereby bringing exons 3 and 4 physically closer to intron A to allow the recruitment of the splice enhancer complex that facilitates splicing of the suboptimal 3′ splice site in intron A.[161] The relative difficulty in excision of intron A explains why intron A-containing transcripts are found alongside the mature mRNA in the brain[157] and also provides an explanation for the presence of low levels of a further GnRH RNA splice variant missing exon 2 (Figure 11.5).[160,163] In this case, it is likely that splicing occurs between the normal 5′ splice site of intron A and the 3′ site of intron B, thus excising the whole of exon 2.[161]

The functional relevance of intron A splicing was revealed by demonstrating that the translation of intron A-containing transcripts is very poor compared with the mature RNA.[164] These observations regarding intron A provide a nice explanation for the failure of the *hpg* mouse to produce GnRH decapeptide.[165] The *hpg* mouse is infertile as a result of a spontaneous ~30 kb deletion in chromosome 14 that involves exons 3 and 4 of the GnRH gene.[165] It was unclear why RNA transcribed from exon 2, that is present in these mice and encodes GnRH, did not produce GnRH peptide. It now seems likely that the absence of exons 3 and 4 in these mice results in truncated RNAs unable to recruit the splice enhancer complex required to splice out intron A and thus results in the presence of an intron A-containing RNA that cannot be translated efficiently.[166] Together, these studies indicate that pre-RNA processing and the excision of intron A, in particular, is critical for the biosynthesis of GnRH decapeptide.

The mechanisms underlying the degradation of cytoplasmic GnRH mRNA are not established, but the half-life of transcript decay in primary cultured neurons has an initial rapid phase of 5–13 min followed by a much slower phase lasting around 30 h.[159] A primary role of poly(A+) tail shortening in transcript decay is suspected,[158] and the 3′ UTR of the mature mRNA is known to exhibit two highly conserved motifs that may bind factors regulating degradation.[159] As GnRH mRNA content has been shown to change in the presence of unaltered primary transcript during puberty,[167] and in response to neurotransmitters like NMDA,[168] the regulation of GnRH mRNA degradation or stability appears to be a major form of biosynthetic regulation in vivo.

GnRH-1 Peptide

Translation of the mature GnRH transcript results in the production of a preprohormone consisting of 92 amino acids (Figure 11.5). The first 23 N-terminal amino acids comprise a signal sequence with the remainder representing the prohormone in which GnRH is located at the N-terminal end and separated from GAP by a three–amino acid cleavage site (Figure 11.5). As PKC activation results in a significant decrease in the numbers of ribosomes associated with GnRH mRNA,[169] it is possible that the translation of preproGnRH is itself regulated. At least four different enzymatic steps are involved in the processing of the prohormone.[170] The initial step involves cleavage of the prohormone to create a GnRH intermediate and mature GAP (Figure 11.5). This is achieved by one or more endopeptidases and both prohormone convertase 1/3 and 2 are implicated in this process.[171–173] The GnRH intermediate then undergoes cleavage of its C-terminal basic residues by carboxypeptidase E.[170,171] The final steps in generating the biologically active decapeptide require the conversion of the N-terminal glutamine (Gln^1-GnRH) to pyroglutamate ($pGlu^1$-GnRH) by glutaminyl cyclase and the amidation of the C-terminal glycine by peptidylglycine alpha-amidating monooxygenase.[174] Immunocytochemical studies suggest that the processing of the GnRH prohormone occurs within vesicles during its transport down the dendron and within the nerve terminals.[175–177] It remains unclear whether the enzymatic processing of GnRH represents an opportunity for regulating GnRH biosynthesis in a physiological context.

One further posttranslational modification of GnRH is that of hydroxylation of the proline residue to create hydroxyproline (Hyp^9) GnRH. Gautron and colleagues have shown that as much as 10% of GnRH-like peptide in the hypothalamus exists as (Hyp^9) GnRH in adult rats.[178] The physiological significance of (Hyp^9) GnRH remains unknown.

Multiple enzymes appear to be involved in the degradation of the GnRH decapeptide with the most

important being zinc metalloendopeptidase EC 3.4.24.15 (EP24.15) and propyl endopeptidase (PEP). It is thought that PEP cleaves the carboxy-terminal glycine to generate $GnRH^{1–9}$ that is then processed by EP24.15 at the Tyr^5-Gly^6 bond to create the $GnRH^{1–5}$ and $GnRH^{6–9}$ fragments (Figure 11.5) (for recent review see Ref. 179). Studies examining the distribution of EP24.15 have shown that it is located in the perivascular space around the GnRH nerve terminals in the median eminence and that it can be secreted into the portal circulation.[180] Investigations have further shown that a selective antagonist of EP24.15 reduces GnRH degradation and enhances LH secretion in vivo.[180–182] Thus, it seems likely that endopeptidases represent a further factor capable of determining the profile or amounts of GnRH reaching the gonadotrophs. The degradation of GnRH may also be regulated physiologically as a small reduction in PEP levels was observed at the time of the LH surge in the sheep, suggesting that a decrease in peptidase activity may contribute to enhanced GnRH release at that time.[183] In contrast, levels of EP24.15 were not found to fluctuate over the estrous cycle of rats[180] or in sheep.[183] It is intriguing to note that $GnRH,^{1–5}$ the product of EP24.15, may itself be biologically active and not simply a degradation product.[179] The $GnRH^{1–5}$ fragment has been shown to function as an NMDA receptor antagonist and also modulate female reproductive behaviour.[184]

GnRH-Associated Peptide

The 56-amino acid GAP is generated from the propeptide following endopeptidase processing (Figure 11.5) and is present in GnRH nerve terminals and released into the portal system.[185,186] Soon after cloning the GnRH/GAP gene, it was reported that human recombinant GAP stimulated LH and FSH release in a GnRH receptor–independent manner and also inhibited prolactin secretion from rat pituitary cultures.[187,188] This sparked substantial investigation into the role of GAP in the regulation of prolactin and gonadotropin secretion in the late 1980s. In essence, the effects of GAP on prolactin secretion were not found to be repeatable by other laboratories or, at best, only very weak, and a similar fate awaited the role of GAP in the regulation of LH and FSH secretion (for review see Ref. 3). Activity in this field died in the early 1990s, and, perhaps surprisingly, the functional significance of GAP remains unknown.

Regulation of Biosynthesis

It is clear from the previous description that, in addition to the electrical activity of a GnRH neuron, multiple biosynthetic and catabolic processes are responsible for determining the final levels of GnRH entering the portal circulation. Whereas the various biosynthetic steps must be operational to enable GnRH secretion from the nerve terminal, the physiological impact of regulating their efficiency at any one point is much less clear. For example, a variety of different stimuli have been shown to alter GnRH mRNA levels by 20–30%[158,189] indicating that transcription and mRNA processing can be regulated. However, it remains to be resolved whether these relatively modest changes in mRNA levels have any functional impact upon the long-term functioning of a cell type that maintains "housekeeping gene" levels of GnRH mRNA content.

As GnRH neurons maintain a very high level of GnRH decapeptide (approximately 1 pg or 10^9 molecules/cell)[155] it might be argued that a high constitutive level of GnRH biosynthesis is sufficient without the need for complex regulation. If biosynthesis were to be regulated, however, it would seem sensible to have biosynthesis coupled to secretion in a neuroendocrine neuron. Whereas evidence for this exists prior to the GnRH surge in the rat,[154,158,190] it is not typically the case. During development in vitro, GnRH mRNA expression lags significantly behind GnRH secretion in the monkey,[145] and large increases in GnRH secretion can even occur in the face of decreasing GnRH mRNA expression.[158,191,192] Thus, it would appear that a tight relationship does not necessarily exist between secretion and biosynthesis in GnRH neurons, with the level of coupling likely to depend more upon the precise location (soma versus dendron) and type of stimulus presented to the GnRH neuron.

Neurotransmitters Co-Expressed by GnRH Neurons

Electron microscopic investigations have identified the presence of both dense core and clear synaptic vesicles within GnRH neurons.[42,50] Many dense core vesicles in GnRH neurons label for GnRH as well as GAP, demonstrating that, as expected, they contain the peptides encoded by *GnRH*. However, the clear vesicles in GnRH neurons, along with some dense core vesicles, are not labeled by GnRH,[42] suggesting that other neurotransmitters may be synthesized and released by GnRH neurons.

Over the years many neuropeptides have been reported to be expressed in GnRH neurons including pro-opiomelanocortin-related peptides,[193,194] delta sleep-inducing peptide,[195] galanin[196,197] cholecystokinin,[198] neurotensin,[198] and orphanin FQ.[199] In most cases these observations of co-expression have come from a laboratory working in a single species and their generalization to all mammalian GnRH neurons is unclear. The exception is galanin that has been well documented to be co-expressed by subpopulations of GnRH neurons in multiple species, although its role remains unknown. In relation to the clear vesicles identified in GnRH neurons, it is interesting to note evidence that GnRH neurons may use glutamate as a neurotransmitter (see following).

Galanin

The neuropeptide galanin was first identified to be expressed by GnRH neurons in the rat.[196,197] Subsequent studies showed that galanin was synthesized in a highly sexually dimorphic manner being present in ~70% of GnRH neuron soma and terminals in the adult female but only around 20% in the male rat.[200,201] Whereas a similar pattern of sexual dimorphic expression is seen in the mouse,[202] this does not exist in the sheep where all GnRH neurons express galanin regardless of sex.[203] In contrast, no galanin was reported in GnRH neurons of the macaque monkey.[204] Although the GnRH neurons expressing galanin are found throughout the GnRH neuron continuum, there is a slight overrepresentation of co-localization in the preoptic subpopulation activated at the time of the GnRH/LH surge.[205]

The biosynthesis of galanin in GnRH neurons is regulated in a gonadal steroid- and/or activity-dependent manner with galanin gene expression in GnRH neurons peaking at the time of the GnRH/LH surge.[206–208] With evidence that galanin facilitates GnRH-stimulated LH secretion directly from gonadotrophs,[196,209,210] as well as GnRH release from the median eminence,[197,211] it is proposed that co-released galanin aids the generation of the LH surge by acting at both brain and pituitary sites.[212] As some GnRH neurons express galanin receptor mRNA,[213,214] it is conceivable that galanin acts directly back upon its own co-secreting nerve terminal in the median eminence to facilitate GnRH secretion.

Galanin is likely to be used as a signaling molecule by multiple elements within the GnRH neuronal network, and it is important to distinguish between roles of galanin secreted by GnRH neurons and galanin released by many other neurons within the network. For example, GnRH neurons are themselves excited directly by galanin.[215] Unfortunately, it is usually impossible to distinguish between these two origins of galanin release in physiological experiments. Furthermore, as noted by Shine[216] remarking on the similarities between galanin and a politician "It (galanin) has not been found to be primarily responsible for any important activity, but rather to modulate many and thus take credit for a wide range of outcomes". This may be the situation for galanin within the GnRH neuronal network. Whereas a plausible modulatory role can be suggested, evidence for a critical role of co-expressed galanin in GnRH neurons is lacking. The galanin knockout mouse is fertile and exhibits no reproductive phenotype attributable to abnormal GnRH function.[217,218] Equally, mice with a functional deletion of the GalR1 gene are reported to breed normally.[219] Nevertheless, the LH surge can be abolished by i.c.v. galantide, the galanin receptor antagonist in the rat.[211] Whether galantide is acting to block effects of galanin secreted by GnRH neurons, galanin on GnRH neurons, or galanin released by other cells within the network is unknown. Nevertheless, it seems reasonable to suggest that galanin co-released from GnRH neurons plays a role in facilitating GnRH and LH secretion, particularly at the time of the surge.

Glutamate

The first indication that glutamate may be synthesized by GnRH neurons for use as a neurotransmitter came from the observation that essentially all GnRH neurons in the male rat expressed vesicular glutamate transporter 2 (VGlut2) mRNA.[220] Although not in agreement with previous and subsequent studies that found no evidence for vGlut2 protein in GnRH neurons,[35,221] another investigation found that a vGlut2 promoter construct directed expression to a subpopulation of GnRH neurons located in the medial septum of the mouse.[222] These interesting observations suggested that GnRH neurons may release glutamate from their nerve terminals in the median eminence. However, roles for glutamate at the pituitary in regulating gonadotropin secretion are unclear. It turns out that almost all hypophysiotropic neuroendocrine cells express VGlut2 mRNA,[223] so it is difficult to envisage how any specificity might be achieved between the various neuroendocrine axes all secreting glutamate into the pituitary portal system.

There is, nevertheless, evidence for recurrent-collateral innervation of GnRH neurons,[224,225] so it remains possible that glutamate might be involved in some form of auto-excitation. Repetitive depolarization of a GnRH neuron can decrease the frequency of GABAergic synaptic events at its cell body. As this phenomenon is blocked by metabotropic glutamate receptor (mGluR) antagonists, it is possible that "self" release of glutamate may activate mGluR on GABA inputs to GnRH neurons to control their excitability.[226] At present, the functional significance of VGlut2 mRNA expression by rat GnRH neurons, and the role of glutamate in auto-feedback regulation of the GnRH neuron, require further exploration. Notably, recent studies have failed to detect VGlut2 within the GnRH nerve terminals of mice or humans.[227]

COMPONENTS OF THE GnRH NEURONAL NETWORK

The GnRH neuronal network is defined here as the GnRH neurons and the associated assembly of brain cells responsible for controlling GnRH release into the pituitary portal circulation. Broadly these "brain cells" can be defined as either neurons or glial cells and will be dealt with separately. For clarity with regard to GnRH neuron dendritic processes, the word *dendron* will only be used in reference to a dendritic projection that innervates the median eminence. In all other cases the

structure will be referred to as a *dendrite* even though the investigated process may well end up innervating the median eminence and, in fact, be a dendron.

Neuronal Elements

If one were to make a conservative assumption that an individual GnRH neuron received inputs from 1000 cells[36] and that each of these interneurons, in turn, received ~5000 inputs,[228] the activity of any one GnRH neuron would be influenced directly or indirectly by approximately 5 million first- and second-order neurons. If third-order neuronal connections were considered, then the numbers of potential neurons involved becomes substantial. As the 5 million primary and secondary afferent inputs to a single GnRH neuron are likely to utilize a wide range of different neurotransmitters, it is not surprising to find that essentially any neurochemical administered into the ventricular system of an animal alters LH secretion (Tables 11.2 and 11.3). As such, it would seem that the challenge is not to catalog every possible neurotransmitter involved in the network but rather to define the functional hierarchy of these inputs.

The definition of those neural inputs that represent the primary afferents to the GnRH neurons will be of most interest, as these should have the greatest influence upon the behavior of the GnRH neuron. Ideally, the definition of a neurotransmitter used by a functionally significant primary afferent within this network would require: (1) evidence that it alters LH and/or FSH secretion when administered into the brain in vivo; (2) demonstration that it can alter GnRH secretion either in vivo or in vitro; (3) anatomical evidence for direct neuronal inputs to GnRH neurons, particularly if this was corroborated with evidence for the expression of an appropriate receptor by the GnRH neurons; and (4) electrophysiological evidence for a direct effect of the particular neurotransmitter on the electrical excitability of a GnRH neuron. A review of the existing literature using these criteria (Tables 11.2 and 11.3) shows that there are several neurotransmitter candidates that have fulfilled many of the criteria listed herein.

Having defined these "primary afferent neurotransmitters" on this basis it is critical to establish the location of their cell bodies, their physiological roles, and relative contribution to the final output of the GnRH neuron. Some primary inputs to GnRH neurons might be expected to have only subtle modulatory or permissive effects, whereas others would represent powerful, tonically active drivers of their excitability. This is a formidable challenge and partial sets of data exist for only a small number of neurotransmitters. The following review focuses on the amino acid transmitters GABA and glutamate, the classical neurotransmitter norepinephrine (NE), and several neuropeptides including GnRH, kisspeptin, neurokinin B (NKB), neuropeptide Y (NPY), and RFamide-related peptide-3 (RFRP-3).

TABLE 11.2 Catalog of Known Classical and Non-Neuropeptidergic Neurotransmitter Actions upon LH Secretion and GnRH Neurons in Adult Mammals

	ICV LH/FSH Release	In Vitro GnRH Release	ICC Fiber Apposition	EM Synapses	Receptors on GnRH Neurons	Direct Effects on GnRH Neurons
GABA	See text					
Glutamate	See text					
Norepinephrine	See text					
Epinephrine	**r** see in Ref. 229,230		**r**[230]			
Dopamine	**r** see in Ref. 229 **s** see in Ref. 231,232	**r**[233–236] **s**[237]	**r**[238] **s**[239,240] **m**[241]	**r**[242–244] **s**[237]	**m**[245]	**m**[245]
Serotonin	**r** see in Ref. 229,246 **s**[247]	**r**[248]	**m**[249]	**r**[250]		
Histamine	**r**[251–253]	**r**[254]	**r, p**[251]			
Acetylcholine	**r**[255–260]	**r**[261,262]	**r**[263]			
Cannabinoids	**r**[264–266]	**r**[267]				**m** (indirect)[268]

The definition of a neurotransmitter that is used by a primary afferent input to the GnRH neurons requires six criteria to be filled. This includes evidence that the neurotransmitter (1) alters gonadotropin secretion when injected intracerebrally (IC) or intracerebroventricularly (ICV), (2) can alter GnRH secretion, (3) is present in fibers observed by immunocytochemistry (ICC) to be in the vicinity of the GnRH cell bodies, dendrites, or terminals, (4) is present in terminals observed by electron microscopy (EM) to synapse upon GnRH neurons, (5) has cognate receptor protein or mRNA expressed by GnRH neurons, and (6) evokes direct actions upon GnRH neurons to alter their electrical excitability. The prefix indicates the species in which the evidence exists (**r** = rat, **m** = mouse, **s** = sheep, **p** = primate, **gp** = guinea pig), and the appropriate reference is given in superscript.

TABLE 11.3 Catalog of Known Neuropeptide Actions upon LH Secretion and GnRH Neurons in Adult Mammals

	ICV LH/FSH Release	In Vitro GnRH Release	ICC Fiber Apposition	EM Synapses	Receptors on GnRH Neurons	Direct Effects on GnRH Neurons
α-MSH	**r**[269]	**r**[269]				m[270,271]
Angiotensin	see in Ref. 272	**r** [273]				
β-endorphin	**r**[274,275]	**r**[276]	**r**[277] **s**[278] **p**[279]	**r**[224,280] **s**[281] p[282]		**gp**[77] **m** no actions[270]
Bombesin	**r**[283]				**m**[214]	**m**[214]
Bradykinin	**r**[284]	**r**[284]				
CART		**r**[285,286]	**r**[287] **hmst**[288]			**m**[270]
CGRP	**r**[289]					
CRF	**r**[290–293] **s**[294]	**r**[291,293,295]	**p**[296]	**r**[297]	CRH-R1 m[298]	
Cholecystokinin	**r**[299]				**m**[300]	
Dynorphin	**r**[275,301,302]		**r**[277] **s**[281]	**s**[281]		
Endothelin		**r**[303]			ET_B **r**[304]	
Enkephalin	**r**[275,305]	**r**[306]	**r**[277] **p**[307]			
galanin	see in Ref. 212,272	**r**[211,308]	**r**[197] **m**[309] **p**[310]		**r**[213] **m**[214]	**m**[214]
GALP	**r**[311] **m**[312,313] **p**[314]	**r**[315]	**r**[316,317]			
Ghrelin	**r**[318,319]	**r**[320]				
GnRH	see text					
Kisspeptin	see text					
Melanin-concentrating hormone	**r**[321,322]		**m**[323] **r**[324]		MCHR1 **r**[324]	**m**[323]
Natriuretic peptides	**r**[325–327]	**r**[325]				
Neurokinin B	see in Ref. 272					
Neuropeptide Y	see text					
Neurotensin	see in Ref. 272,328		**m**[329]		NT1 **r**[330] NT2 **m**[331]	
Orexin	**r**[332–336]		**r**[332,337] **s**[338]		OXR1 **r**[337]	OXR1 **m**[339]
Orphanin FQ		**r**[340]				
Oxytocin	**r**[341]	**r**[342,343]				
RFRP-3	see text					
Somatostatin	**r**[344,345] **s**[346]	**r**[347]	**m**[348] **r**[349]		**m**[214,348] **r**[349]	**m**[214,348] **r**[349]

TABLE 11.3 Catalog of Known Neuropeptide Actions upon LH Secretion and GnRH Neurons in Adult Mammals—cont'd

Vasopressin	**r**[350–352] **p**[353]	**r**[354]		**p**[355]	R1b **m**[298]	
VIP	**r**[356–358]	**r**[359,360]	**r**[361–363]		VIP2 **r**[362]	**m**[364]

The definition of a functionally relevant neuropeptide that is used by a primary afferent input to the GnRH neurons requires six criteria to be filled. This includes evidence that the neuropeptide (1) alters gonadotropin secretion when injected intracerebrally (IC) or intracerebroventricularly (ICV), (2) can alter GnRH secretion, (3) is present in fibers observed by immunocytochemistry (ICC) to be in the vicinity of the GnRH cell bodies or terminals, (4) is present in terminals observed by electron microscopy (EM) to synapse upon GnRH neurons, (5) has cognate receptor protein or mRNA expressed by GnRH neurons, and (6) evokes direct actions upon GnRH neurons to alter their electrical excitability. The prefix indicates the species in which the evidence exists (**r** = rat, **m** = mouse, **s** = sheep, **p** = primate, **gp** = guinea pig, **hmst** = hamster), and the appropriate reference is given in superscript.

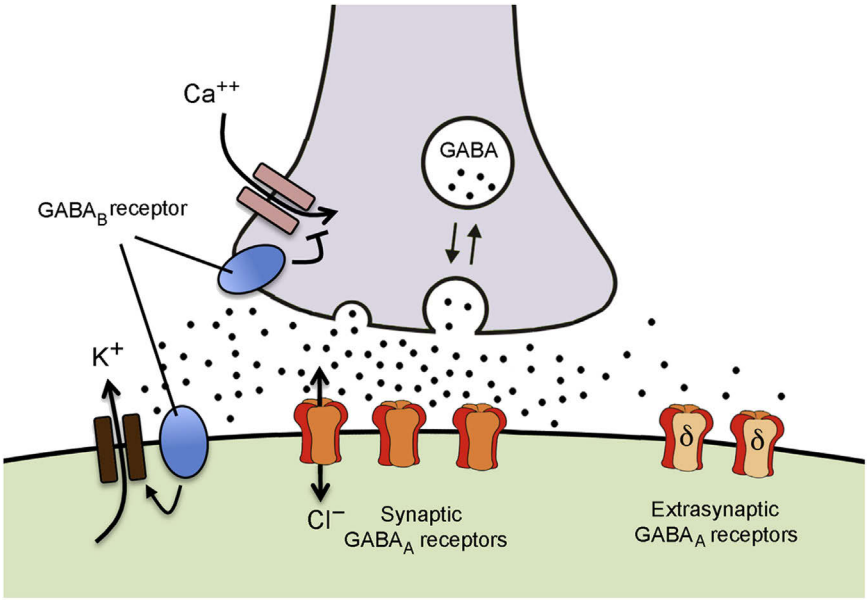

FIGURE 11.6 Modes of GABA action upon GnRH neurons. Presynaptic GABA terminals from the RP3V and other brain sites release GABA into the synaptic space where it activates $GABA_A$ receptors to initiate phasic transmission and $GABA_B$ receptors to suppress excitability. GABA spilling outside of the synapse activates extrasynaptic $GABA_A$ receptors, comprised of delta $GABA_A$ receptor subunits that generate a tonic membrane hyperpolarization. GABA diffusing back to the nerve terminal can activate presynaptic $GABA_B$ receptors that act to inhibit calcium entry into the terminal and suppress presynaptic vesicular release.

GABA

There is little doubt that GABA directly regulates the activity of all GnRH neurons in mammalian species. The most comprehensive assessment of GABA actions to date has been undertaken in the rodent where electrophysiological and gene-profiling studies show that all GnRH neurons express postsynaptic ionotropic $GABA_A$ receptors (for review see Ref. 365) and the vast majority (~90%) are influenced by pre- or postsynaptic metabotropic $GABA_B$ receptors[89,366,367] (Figure 11.6). Electrophysiological studies have also found that almost all GnRH-GFP neurons in the acute brain slice preparation are under a constant barrage of GABA release recorded as fast postsynaptic currents (PSCs) resulting from activation of $GABA_A$ receptors on the membrane of GnRH neurons.[80,368] In the acute brain slice, GABA release on GnRH neurons is far greater than that of glutamate as the rate of $GABA_A$-mediated events is typically 5- to 10-fold greater than glutamate events.[91,369–372]

The sources of GABA inputs to GnRH neurons remain poorly defined. Recent electrophysiological studies have indicated that GABA neurons located in the anteroventral periventricular area (AVPV),[92] lateral preoptic area,[373] and, possibly, the suprachiasmatic nucleus (SCN)[91] provide direct GABAergic inputs to GnRH neurons. These inputs are likely to co-express a variety of other neuropeptides, and a recent investigation has demonstrated the frequency-dependent coding of GABA versus neuropeptide release from such afferents to GnRH neurons.[92]

It seems likely that GABA neurons target the GnRH neuron cell body as well as its dendrites and dendrons projecting to the ME. Electron microscopic studies provided initial evidence that rat GnRH neuron cell bodies receive GABAergic inputs,[374,375] and this was extended by immunohistochemistry experiments showing that GABAergic terminals are apposed to the GnRH neuron cell body as well as the proximal 50 µm of dendrite in mice[34] and sheep.[35] More recent electrophysiological studies have shown that functional $GABA_A$ receptors exist at least up to 200 µm along the proximal dendrite[118] as well as on the dendron as it enters the ME.[55] It is possible that $GABA_A$ receptor

density is highest in the very proximal dendrite and then falls at the level of the cell body as well as more distally along the GnRH neuron dendron in mice. Investigations in human males have also shown the presence of GABA appositions on the cell body and proximal dendrites of GnRH neurons although, in this species, the cell body appears to have the highest density.[227]

The $GABA_A$ receptor on GnRH neurons signals in two different ways, termed *phasic* and *tonic*. Phasic refers to the normal fast activation of $GABA_A$ receptors within the synapse generating the PSCs. Tonic represents the activation of extrasynaptic $GABA_A$ receptors by low ambient concentrations of GABA in the extracellular space[376] and generates a persistent current that helps determine resting membrane potential. Whereas phasic $GABA_A$ receptor signaling has long been recognized in GnRH neurons, it is only recently that tonic $GABA_A$ signaling has been identified in these neurons.[373,377] Mediated by δ subunit-containing $GABA_A$ receptors and partly dependent upon the activity of glial and neuronal GABA transporters, tonic $GABA_A$ receptor signaling was found to hyperpolarize the membrane by ~5 mV in mouse GnRH neurons.[377] Whether this tonic inhibitory current can be modulated is presently unknown, but it may represent an important means for determining the excitability of GnRH neurons.

Another characteristic of $GABA_A$ receptor signaling in GnRH neurons is that, in the acute brain slice preparation, PSCs are not dependent to any large extent upon electrical activity in the presynaptic GABA neurons.[91,378,379] This type of activity-independent or "spontaneous" GABA release is also observed when recording from other neurons, and it remains unclear whether this simply reflects chaotic vesicular release at the presynaptic terminal membrane or is a regulated, purposeful form of neurotransmission.[380] Nevertheless, investigators have suggested that rates of spontaneous $GABA_A$ PSCs may reflect the degree of GABAergic input to a cell[381] and be involved in multiple functions including stabilizing synaptic networks.[380,382] Certainly, the frequency of $GABA_A$ receptor PSCs has been found to change in response to a variety of different experimental manipulations (for review see Ref. 85), and these may reflect changes in the density of GABA inputs to GnRH neuron cell bodies. Finally, with respect to the characteristics of $GABA_A$ receptor signaling in GnRH neurons, it is worth noting that all recordings to date have been made from GnRH neurons in vitro that will have had most of their inputs severed in the brain slicing. Recordings from other neuronal cell types in vivo have revealed that levels of synaptic activity are up to 50-times higher in vivo compared with the acute brain slice preparation and that this has important consequences for the behavior of those cells.[383,384] Establishing the actual rate and pattern of GnRH neuron $GABA_A$ PSCs in a more intact preparation will be an important future goal.

For the majority of neurons in the mammalian forebrain, GABA acts through $GABA_A$ receptors to stimulate their excitability during development, before switching to its well-established inhibitory role in the adult nervous system.[385,386] While there is agreement that $GABA_A$ receptor activation can exert an excitatory influence upon rodent GnRH neurons up until the time of puberty, its role in the adult has been controversial (for review see Ref. 365). Much of this controversy arose from initial studies reporting either inhibitory[387] or excitatory[388] effects of exogenous $GABA_A$ receptor activation upon GnRH neuron firing. Although there is now a consensus that exogenous $GABA_A$ receptor activation is at least transiently excitatory in adult GnRH neurons,[89,365,389] it remains that the role and direction of endogenous $GABA_A$ receptor activation is unclear. Investigations in the acute brain slice preparation have demonstrated inhibitory, excitatory, or, indeed, no effects of a range of different $GABA_A$ receptor antagonists on GnRH neuron firing.[83,92,99,390,391] Although only small numbers of GnRH neurons have been tested as yet in vivo, there is a consistent suppressive action of $GABA_A$ receptor antagonists on GnRH neurons firing.[90] This indicates that, in vivo, $GABA_A$ receptor signaling is essential for GnRH neurons to exhibit their normal firing patterns.

The membrane chloride ion gradient is the principal determinant of whether $GABA_A$ receptors depolarize or hyperpolarize a cell. As such, an understanding of the regulation of transporters and channels responsible for determining chloride ion equilibrium within GnRH neurons remains critical. The potassium-chloride co-transporter 2 (KCC2) is thought to be the key protein maintaining a low intracellular chloride concentration compatible with hyperpolarizing effects of GABA in adult neurons.[392] Although KCC2 mRNA was initially not detected in adult GnRH neurons,[388] a subsequent study has demonstrated KCC2 immunoreactivity in approximately one-third of adult mouse GnRH neurons.[393]

Given that all neurons express $GABA_A$ receptors, it has not been surprising to find evidence for roles of GABA in multiple aspects of reproduction including puberty onset gonadal steroid modulation of LH secretion (see following), seasonality and the metabolic control of reproduction (see other chapters in this volume). However, the physiological roles of GABAergic inputs to GnRH neurons themselves have not yet been established with certainty. Although striking correlations can be found between the frequency of $GABA_A$ receptor events in GnRH neurons and a wide range of physiological and pathophysiological scenarios (for review see Ref. 85), this does not necessarily mean that they are causally related. Furthermore, these events are almost always action potential–independent, and the real patterns of $GABA_A$

receptor activation are likely to be substantially different in vivo. A further difficulty in ascribing an important role to $GABA_A$ receptor activation comes from a recent study in genetically manipulated mice reporting that a selective 70–90% knockdown of $GABA_A$ receptor signaling at the GnRH neuron itself had no major impact upon LH secretion or fertility.[394] This indicates that either GnRH neurons can maintain normal function with only minimal $GABA_A$ receptor signaling or that there is sufficient network compensation to overcome the loss of this receptor. It is also notable that acute $GABA_A$ receptor inhibition does not alter burst firing in GnRH neurons,[390] although, again, this may be an artifact of the brain slice preparation.[88]

Thus, despite the seemingly large amount of data, it is difficult to pin down a specific physiological role for $GABA_A$ receptor–mediated events at the GnRH neuron in regulating their activity. For this, we may still have to fall back on in vivo whole-animal investigations that have shown that $GABA_A$ receptor signaling in the vicinity of the GnRH cell bodies in the preoptic area is necessary for both pulsatile and surge modes of LH secretion to occur normally.[395–398] Whether those effects arise from the direct modulation of $GABA_A$ receptor signaling at the GnRH neuron or indirect actions on neuronal afferents to GnRH neurons, remains unknown.

As first recognized in guinea pigs,[77] the great majority of GnRH neurons are also regulated by $GABA_B$ receptors (Figure 11.6). Receptor profiling studies have since shown the presence of both $GABA_B$-R1 and $GABA_B$-R2 subunits in most mouse and sheep GnRH neurons,[367,399] and electrophysiological experiments have demonstrated that 80–90% of mouse GnRH neurons express functional $GABA_B$ receptors.[366,367] There is agreement that both exogenous and endogenous $GABA_B$ receptor activation inhibits the electrical excitability of GnRH neurons most likely through activation of an outward potassium current.[77,89,366,367] There is initial evidence that $GABA_A$ receptor signaling may predominate at the GnRH neuron soma while $GABA_B$ signaling dominates at the dendrite.[89] Studies have also shown that $GABA_B$ receptors act presynaptically to suppress the activity of GABA and glutamate inputs to GnRH neurons.[366]

The emerging importance of $GABA_B$ receptors in modulating GnRH neuron excitability is paralleled by studies in vivo where $GABA_B$ receptor activation has been found to suppress LH secretion in rats and sheep.[400–405] Furthermore, alongside several other defects, GnRH secretion and fertility are abnormal in global $GABA_B$ receptor knockout mice.[406,407]

While much attention has focused upon GABA actions at the GnRH neuron cell body, it is important to bear in mind that GABA is also likely to modulate distal elements of the GnRH neuron near the median eminence. Both $GABA_A$ and $GABA_B$ receptor activation have been shown to alter GnRH secretion at this level,[408,409] and the GnRH neuron dendron receives GABAergic inputs that signal through the $GABA_A$ receptor.[55]

Glutamate

Substantial evidence implicates glutamatergic neurons as being an important class of primary afferents to the GnRH neurons in mammalian species (for recent review see Ref. 410). Adult GnRH neurons in the mouse respond to exogenous glutamate, AMPA, NMDA, and kainite,[79,95,389,411] and the activation of glutamatergic inputs from the AVPV to GnRH neurons has revealed the presence of substantial endogenous AMPA and NMDA receptor–mediated transmission in these cells.[92] Whereas AMPA and NMDA receptor activation both occur quickly, the AMPA current is short lived compared with the longer-duration NMDA current.[92] Although only able to examine low frequency (~0.1 Hz) action potential–independent release, the majority of GnRH neurons in the acute brain slice exhibit ongoing AMPA-mediated transmission with lesser amounts of NMDA-mediated synaptic activity.[370] Overall, these electrophysiological studies have shown that most (80–100%) GnRH neurons exhibit functional AMPA receptors, whereas only a small subpopulation (~20%) have detectable NMDA receptors.

In general, the previous functional observations fit with the many immunohistochemical and in situ hybridization studies that have been undertaken over past years examining the expression of NR1, 2A and 2B (NMDA), GluR1-4 (AMPA), GluK1 and 5 (kainate) receptor subunits in rodent GnRH neurons (for review see Ref. 410). Also, electron microscopic evidence for glutamatergic synapses on GnRH neuron cell bodies has been found in both the rat and monkey.[221,412]

Less attention has been paid to the presence and role of mGluRs on GnRH neurons. Nevertheless, there is evidence for mGluR subunit mRNAs in GnRH neurons,[214] and a subgroup of GnRH neurons in the MS are excited by Group I mGluR agonists.[222] There is also evidence that Group II/III agonists are indirectly active by modulating GABA inputs to GnRH neurons.[226]

Data also suggest a potential role for glutamate actions at the level of the GnRH nerve terminal to regulate secretion. In vitro preparations of GnRH nerve terminals that are devoid of GnRH cell bodies but maintain the GnRH neuron dendron have been found to release GnRH in response to glutamatergic agonists.[409,413] Whether this represents a direct effect of glutamate upon the GnRH nerve terminal or dendron is not known, but immunocytochemical evidence exists for the expression of both NMDA and kainate receptors by GnRH nerve terminals,[59] and the distal dendron responds to glutamate.[55] Remarkably, the NMDA receptor has been localized to the dense core vesicles actually containing GnRH.[414] There are abundant glutamatergic fibers within the

median eminence and some lie directly adjacent to the GnRH nerve fibers, but, as is the case generally, there have been no demonstrations of conventional glutamatergic synapses upon GnRH nerve terminals.[59,412]

Although it is very likely that GnRH neurons receive glutamatergic inputs from many different brain regions, at present, the only documented input is from the AVPV.[92] This lack of detail underscores the difficulty in ascribing a role of primary afferent glutamatergic inputs to GnRH neurons and is further compounded by the genetic deletion of GluR2 or all NMDA receptors from GnRH neurons having no fertility phenotype.[415] This suggests that NMDA and GluR2-containing AMPA receptors on GnRH neurons are either not essential for fertility or can be compensated for in the mutant mouse. Nevertheless, multiple acute antagonist studies in vivo have documented the necessity of AMPA/kainate and NMDA signaling for enabling both pulsatile and surge modes of LH secretion.[416–419]

GnRH

Electron microscopic studies show that GnRH-immunoreactive terminals comprise up to 10% of all synapses detected on GnRH cell bodies in the rat[225,420,421] and monkey.[282] Further, investigations in the mouse have shown that GnRH neurons express GnRH-1 receptor (GnRH-R1) transcripts[214,422,423] and that their electrical activity is regulated directly by GnRH.[424] Together these data demonstrate that GnRH neurons receive direct inputs from GnRH neurons. Whether this represents recurrent collateral innervation or connections between different GnRH neurons is unknown.

The precise effects of GnRH on the electrical excitability of GnRH neurons are contentious. In the immortalized GT1 cells, GnRH-R1 signals through at least three different G-proteins (Gs, Gq, and Gi) in a concentration-dependent manner. Whereas nM GnRH activates a Gs-cAMP pathway, μM GnRH activates an opposing Gi-cAMP cascade while μM or nM GnRH stimulates the Gq-$[Ca^{2+}]$i pathway. Evidence that a dose-dependent G-protein cascade may also exist in adult GnRH neurons was presented by Xu and coworkers[422] who showed that 20 nM GnRH decreased the firing rate of GnRH neurons from gonadectomized male mice, whereas 200 nM and 2 μM increased firing rate. However, these same responses were not identified in a subsequent study examining the effects of GnRH on GnRH neurons in intact male and female mice where the predominant response was one of modest depolarization of ~50% of GnRH neurons that was independent of GnRH concentration.[424] Also, GnRH was found to activate GnRH neurons in embryonic rats.[425] It is possible that the initial nM inhibitory effects arose from indirect actions[371] or are a property of gonadectomized mice. The subcellular mechanisms involved in GnRH-R1 signaling in adult GnRH neurons are not fully established. One study has shown that GnRH decreases intracellular calcium levels in GnRH neurons,[424] while another has implicated the M-current in GnRH actions.[423]

The GnRH modulation of GnRH neurons provides an example of a neurotransmitter or neuropeptide where its direct actions on GnRH neuron electrical excitability do not fit well with its reported effects on LH secretion in vivo. Numerous studies in vivo with nonprimate species have shown that i.v. or i.c.v. GnRH inhibits portal GnRH or plasma LH secretion[426–429] suggesting a potential short-loop negative feedback role for GnRH within the GnRH neuronal network. This has also been observed in vitro where GnRH decreases, and GnRH antagonists increase, the frequency of GnRH pulses from adult hypothalamic cell cultures.[430] Thus, the suppressive influence of GnRH administered into the brain upon GnRH/LH secretion is not mirrored by its stimulatory effects on GnRH neuron excitability. One explanation may lie in the observation that GnRH inhibits GnRH release from tissue fragments containing only GnRH axons and terminals,[428,431,432] whereas it stimulates LH secretion when applied directly to the region of the GnRH cell bodies in the preoptic area.[433] Thus, as for other neurotransmitters, it is possible that when given i.c.v., GnRH acts at more than one site to regulate GnRH secretion with possible inhibitory actions at the terminals and stimulatory effects at the cell bodies. At present, the mechanisms of GnRH action upon the GnRH terminals are unknown.

What might be the function of a stimulatory GnRH ultrashort-loop feedback at the level of the GnRH soma? This depends very much on whether the GnRH inputs to GnRH neurons are from the same cell (recurrent collateral autofeedback) or from other GnRH neurons. One possibility, related to the latter scenario, would be that GnRH neuron–GnRH neuron signalling enables the coordination of the anatomically dispersed GnRH cell bodies. For example, the LH surge is abolished following intracerebral GnRH-R1 antisense treatment,[434] and it is possible that even a modest depolarizing influence of GnRH could build to a generalized activation of the 50% of preoptic GnRH neurons recruited at the time of the GnRH surge.[18] Intriguingly, however, when this hypothesis was tested in brain slices, it was found that some GnRH neurons switched from being depolarized to hyperpolarized by GnRH specifically during the proestrous phase of the estrous cycle.[424] While some recent progress has been made in defining the effects of GnRH on GnRH neuron excitability, it remains that the physiological role of GnRH-R1 signaling within the adult GnRH neuron is unclear.

Kisspeptin

The kisspeptin projections to GnRH neurons represent an important "high hierarchy" component

of the neuronal network that plays a critical role in many facets of GnRH neuron functioning. This section outlines the neuroanatomy of the kisspeptin system in rodents, sheep, and primates and then briefly considers the roles of kisspeptin neurons in regulating GnRH neurons in rodents. Readers are referred to the species-specific chapters in this volume on reproductive control for detailed reviews of kisspeptin action in sheep and primates. Kisspeptin also features strongly in other chapters on puberty and metabolic control.

ANATOMY

In virtually every mammalian species examined to date, neurons synthesizing kisspeptin are clustered in two brain regions: the preoptic area and mediobasal hypothalamus (Figure 11.7). Within the rat and mouse, the preoptic area kisspeptin neurons lie very close to the third ventricle located within the AVPV and periventricular nucleus of the preoptic area (PVpo)[435,439–441] (Figure 11.7). This distribution lies within the RP3V, a brain region previously defined to contain the estrogen receptor–expressing neurons that innervate GnRH neurons in the mouse.[442] The RP3V kisspeptin neurons in the mouse elaborate a relatively simple multipolar dendritic tree that runs parallel with the surface of the third ventricle.[443] Within the MBH of the rodent, a large population of kisspeptin neurons is found throughout the rostro-caudal extent of the arcuate nucleus (ARN)[439,440] (Figure 11.7). Due to the dense kisspeptin fiber innervation within the ARN that essentially outlines the borders of the nucleus, the identification of ARN kisspeptin cell bodies by immunocytochemistry has often been challenging in both rodent species.[435,444,445] In addition to these two major populations of kisspeptin neurons, small numbers of cells synthesizing authentic Kiss1 mRNA and/or kisspeptin are also found in the dorsosmedial

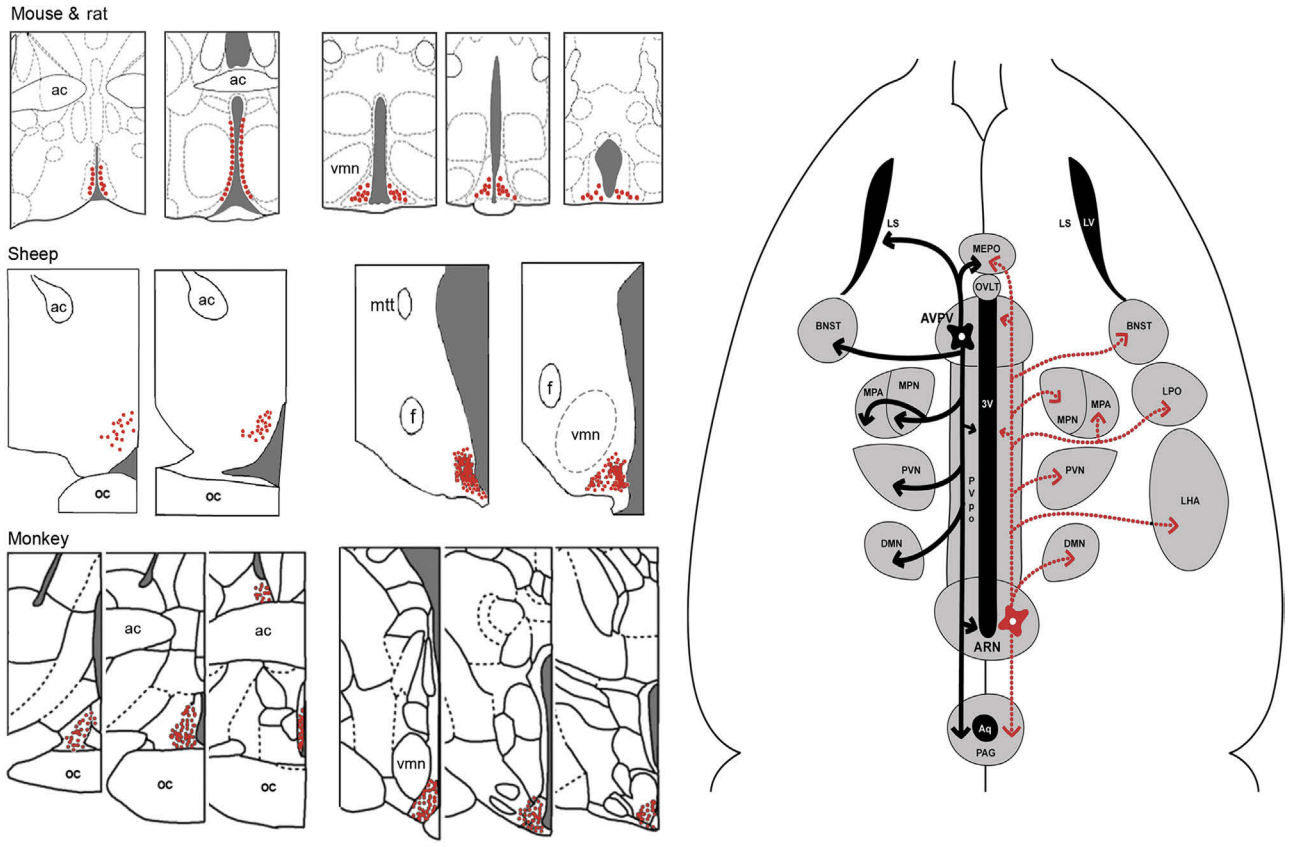

FIGURE 11.7 Sites of kisspeptin neurons in the hypothalamus and their projections in the mouse brain. Left: The locations of kisspeptin cells bodies (dots) in the hypothalamus of the rodent, sheep, and monkey are shown. *Source: These schematic diagrams are based on data reported in Ref. 435–437.* Right: the projections of AVPV (solid lines) and ARN (dotted lines) kisspeptin neurons are shown on a schematized horizontal flat-map of the mouse brain. Abbreviations: Aq, cerebral aqueduct; ARN, arcuate nucleus; AVPV, anteroventral periventricular nucleus; ac, anterior commissure; BNST, bed nucleus of the stria terminalis; DMN, dorsomedial nucleus; LS, lateral septum; LHA, lateral hypothalamic area; LPO, lateral preoptic area; LV, lateral ventricle; MEPO, median preoptic nucleus; MPA, medial preoptic area; MPN, medial preoptic nucleus; mtt, mammilothalamic tract; oc, optic chiasm; OVLT, organum vasculosum of the lamina terminalis; PAG, periaqueductal gray; PVN, paraventricular nucleus; PVPO, periventricular nucleus; vmn, ventromedial nucleus. *Source: Published with permission from Yeo and Herbison[438].*

hypothalamus[435] and amygdala[446] of normal adult rodents.

The distribution of kisspeptin cells in the sheep is very similar to that of the rodent with the exception that the relatively small number of preoptic cells do not reside close to the wall of the third ventricle and are scattered more medially within the preoptic area[436,447,448] (Figure 11.7). The ARN population is again found throughout the rostro-caudal aspects of the ARN.[436,447,448] Mapping studies have also shown the existence of the two major kisspeptin populations in higher primates.[437,449,450] The monkey exhibits a preoptic kisspeptin population that is scattered within the preoptic area and also located in periventricular nuclei[437] (Figure 11.7). The other population of kisspeptin cells is found in the infundibular nucleus, analogous to the ARN in other species (Figure 11.7). A further population of kisspeptin neurons is observed in the anterior division of the bed nucleus of the stria terminalis of the monkey;[437] this has not yet been observed in other species.

It is evident from the wide distribution of kisspeptin fibers within the hypothalamus and other limbic brain regions that the preoptic and ARN kisspeptin neurons project to many different brain nuclei.[435,445] Tract tracing studies have been undertaken in rodents to characterize the kisspeptin neuron projections from the preoptic area and ARN;[438,451,452] this information is summarized in part in Figure 11.7. In essence, both populations are found to innervate many different hypothalamic brain areas with, in general, the preoptic population projecting to more medially located nuclei compared with ARN projections (Figure 11.7). These observations serve to reinforce the notion that the kisspeptin neurons are involved in regulating a wide range of limbic brain functions in addition to the control of gonadotropin secretion.

One interesting feature of kisspeptin neurons is that they co-express numerous classical and neuropeptidergic neurotransmitters. Studies in rodents have shown that subpopulations of RP3V kisspeptin neurons also synthesize GABA, dopamine, met-enkephalin, and galanin. It is reported that ~70% of RP3V kisspeptin neurons express glutamic acid decarboxylase-1 mRNA[453] and that 50% and 80% synthesize tyrosine hydroxylase protein and mRNA, respectively, in adult mice.[241,454] Interestingly, this differs from rats where <20% of RP3V kisspeptin neurons express TH mRNA.[440] In terms of neuropeptides, ~30% of RP3V kisspeptin neurons have recently been found to co-express met-enkephalin and up to 80% express galanin.[455,456] To date, no similar studies have been reported for the preoptic kisspeptin neurons of other species.

Within the ARN, a high degree of neuropeptide co-expression is found between kisspeptin, NKB, and dynorphin, and this has led to these cells being termed the KNDy (Kisspeptin, NKB, Dynorphin) neurons.[457] Initially discovered in the sheep,[458] this pattern of co-expression has now been identified in rodents, primates, and several other species.[457] However, the degree of co-expression between these three neuropeptides appears to vary according to sex, gonadal steroid status, and species. For example, only approximately 50% of NKB neurons in the ARN of the male mouse express kisspeptin,[459] and a recent study did not find high levels of co-expression between any of the three neuropeptides in the infundibular nucleus of human males.[460] In addition to NKB and dynorphin, galanin has also been detected in ARN kisspeptin neurons in the adult mouse.[455,456] Similar to the RP3V, there is evidence that some ARN kisspeptin neurons synthesize classical amino acid transmitters GABA and glutamate.[453,461]

PHYSIOLOGY

In keeping with the early evidence that kisspeptin was an extremely potent activator of LH secretion in several species,[449,462,463] it has been no surprise to find that kisspeptin is the most potent activator of GnRH neuron excitability yet discovered.[464] Different technical approaches all indicate that the great majority (~85%) of GnRH neurons express the kisspeptin receptor (GPR54 or KISS1R) in adult rodents.[464–466] Furthermore, GnRH neurons are likely to synthesize GPR54 before birth[466] and may even do so from early in embryonic development.[467,468] While there is abundant evidence for potent direct actions of kisspeptin on GnRH neurons, there may also be indirect pathways through which kisspeptin can modulate the activity of GnRH neurons. Kisspeptin is reported to stimulate nitric oxide (NO) release around the OVLT[469] and also modulate amino acid transmission to GnRH neurons.[470]

It is becoming increasingly evident that kisspeptin inputs to GnRH neurons are critical for these cells to exhibit their normal patterns of activity in response to a variety of stimuli. For example, GPR54 knockout mice are unable to exhibit high frequency, high amplitude LH secretion following gonadectomy,[471] and neurotransmitters known to stimulate LH secretion are barely effective in the GPR54 knockout mice.[472] While often interpreted as demonstrating a serial relationship (i.e., neurotransmitter X acts on kisspeptin neurons to activate GnRH neurons), it is much more likely this reflects a "dependent parallel" relationship whereby neurotransmitter X is no longer able to activate GnRH neurons if the kisspeptin input is not present. The opposite is not true, however, as many mouse models and experiments that result in suppressed reproductive function or hormone secretion are "rescued" by kisspeptin treatment. Again, these studies are sometimes wrongly interpreted as showing that the defect is in kisspeptin; it is more likely that kisspeptin is such a potent activator of GnRH neurons that it can overcome many different insults to these cells.

Several laboratories[222,470,473,474] have investigated the intracellular signaling mechanisms underlying the remarkably potent actions of kisspeptin on native GnRH neurons. Overall, there is good agreement that GPR54 activation results in the modulation of at least two different ion channels: closure of a tonically active inward rectifying potassium channel and opening of a nonselective cation channel, very likely to be a member of the transient receptor potential-like channel family. Precisely how activation of the Gq-coupled GPR54 receptor modulates these channels is unclear as, although phospholipase C is involved, the roles of the expected downstream signaling molecules such as diacylglycerol, inositol trisphosphate, and intracellular calcium remain contentious.[473,474] Another interesting, but as yet unexplained,[473] feature of kisspeptin action is the mechanism that enables brief kisspeptin exposure to generate very prolonged activation of the GnRH neurons in vitro.

RP3V KISSPEPTIN NEURONS

The RP3V kisspeptin neurons are thought to have a key role in activating GnRH neurons to generate the preovulatory GnRH/LH surge in female rodents. This population of cells exhibits all of the key features expected for a neuronal input to GnRH neurons to be involved in the surge mechanism. The kisspeptin neurons: (1) are located in the RP3V, the key brain region involved in the generation of the GnRH/LH surge in rodents;[442] (2) sexually dimorphic, with 10- to 20-fold more kisspeptin neurons in the RP3V of females compared with males;[440,475] (3) express ER alpha,[476] the key receptor isoform involved in the surge mechanism;[477] (4) exhibit estradiol-regulated ion channels and electrical activity;[478,479] (5) are activated coincidentally with GnRH neurons at the time of the surge, as assessed by cFos immunohistochemistry;[480,481] (6) project to and directly activate GnRH neurons;[92] and finally (7) are essential alongside GPR54 for the generation of the GnRH/LH surge in mice and rats.[441,481,482] There is also evidence that circadian inputs may impact upon RP3V kisspeptin neurons to time the onset of the GnRH surge in rodents.[483–485] Although sexually dimorphic populations of kisspeptin neurons are also located in the preoptic area of the sheep[486] and primate,[450] their role in the GnRH surge mechanism is unknown. Recent studies have, however, shown that sheep preoptic kisspeptin neurons express cFos at the time of the surge in this species.[436,487] It remains unknown whether all the kisspeptin neurons in the RP3V are involved in the surge mechanism or whether there may be functional subpopulations. Certainly, the surge mechanism can occur with less than 50% of the normal number of RP3V kisspeptin neurons.[488]

Studies also indicate that the RP3V kisspeptin neurons are involved in puberty onset in female rodents. Intriguingly, RP3V kisspeptin-immunoreactive cell bodies and kisspeptin fibers around GnRH neurons appear just at the time of puberty onset in male and female rodents.[475,489] As this increase in kisspeptin biosynthesis is dependent upon estradiol, it is likely that the RP3V kisspeptin neurons represent a population of neurons brought into play in late puberty to enable the final development of the positive feedback mechanism in female rodents.[490] Mice in which ERα is deleted from all kisspeptin neurons showed markedly disordered puberty.[482] The role of the small RP3V kisspeptin population in male puberty onset is unclear despite having the same gonadal steroid-dependent pattern of postnatal expression.[491]

ARCUATE KISSPEPTIN NEURONS

The arcuate kisspeptin neurons have been suggested to have roles in the estrogen negative feedback mechanism, puberty onset, and GnRH pulse generation. Unlike the RP3V population, these neurons are found to express kisspeptin from mid-embryogenesis with a peak in expression in both sexes just before birth.[492] It remains controversial whether sex differences exist in the numbers of arcuate neurons that synthesize kisspeptin in rodents.[440,489,493]

Surprisingly, the pathway(s) through which arcuate kisspeptin neurons regulate GnRH neurons are not yet established. Although arcuate kisspeptin neurons send projections to the preoptic area,[438] there is presently little evidence for direct innervation of the GnRH neuron cell bodies.[451,455] Furthermore, it seems unlikely that kisspeptin fibers directly innervate GnRH nerve terminals in the external zone of the median eminence as: (1) very few kisspeptin fibers are found in this area;[435] (2) no ultrastructural evidence has been found for direct presynaptic kisspeptin inputs on GnRH nerve terminals;[494] and (3) kisspeptin neurons do not take up Fluorogold injected peripherally in rats or mice.[438,494] Thus, the anatomical connection between arcuate kisspeptin neurons and GnRH neurons remains unknown and is an important point that requires clarification.

One intriguing property of arcuate kisspeptin neurons is that the biosynthesis of kisspeptin is regulated by estradiol in a manner opposite to that of the RP3V kisspeptin cells. Thus, gonadectomy enhances, and estradiol suppresses, Kiss1 mRNA and protein levels in the ARN. This has led to the hypothesis that estradiol reduces a stimulatory kisspeptin input to GnRH neurons to suppress GnRH secretion and thereby bring about estrogen negative feedback. This hypothesis was supported by investigations showing that the postovariectomy increase in LH secretion was blunted in GPR54 knockout mice[471] as well as in rats with arcuate nucleus-specific, saporin-deleted, kisspeptin neurons.[495] However, these studies could also be interpreted as showing that arcuate kisspeptin neurons are simply required for GnRH neurons to drive pulsatile LH secretion without any role

in mediating estradiol actions. Recent electrophysiological studies examining arcuate kisspeptin neuron firing in acute brain slices have also questioned the estrogen negative feedback model as gonadectomy resulted in even lower firing rates than the already low levels found in intact mice—a result opposite to that predicted by the model.[496] Thus, the estrogen negative feedback role for arcuate kisspeptin neurons in the rodent presently rests on biosynthesis data with definitive functional evidence yet to be presented.

Arcuate kisspeptin neurons may also have a role in puberty onset. Some investigators have observed transient or sustained increases in arcuate kisspeptin levels across postnatal development.[489,493,497] Furthermore, genetic deletion of ER alpha from kisspeptin neurons results in the early onset of puberty in female mice, and this may be attributable to the arcuate kisspeptin population.[482] As for the negative feedback theory, the case for arcuate kisspeptin neurons being involved in puberty onset requires further support.

Studies with GPR54 antagonists demonstrate that kisspeptin-GPR54 signaling is required for normal pulsatile GnRH and LH secretion in a range of experimental animals.[498] Whether this function derives from the RP3V and/or arcuate kisspeptin neuron populations is unclear at present. Mice with ARN diphtheria toxin–deleted kisspeptin neurons fail to exhibit regular estrous cycles,[499] suggesting a possible role of arcuate cells in pulse generation. Also, infusion of GPR54 antagonists directly into the ARN is found to suppress pulsatile LH secretion,[500] indicating an important role for kisspeptin-GPR54 signaling within this area for pulse generation. Most importantly, like NE and NPY, the release of kisspeptin occurs in an episodic manner within the MBH of the monkey, and this coincides with pulsatile GnRH release.[501] Another influential push for a role of arcuate kisspeptin neurons in pulse generation comes from a theoretical pulse generator model involving interconnected KNDy neurons within the ARN.[457] Morphological and electrophysiological studies have provided evidence that kisspeptin neurons may be reciprocally connected as NKB can excite kisspeptin neurons in the mouse.[459] The model proposes that different dynamics of dynorphin and NKB signaling within this pathway would generate oscillatory activity in the arcuate kisspeptin neurons that would then entrain GnRH neurons to generate pulses. Much has yet to be done to explore this interesting model, and further evidence for a role of arcuate kisspeptin neurons in pulse generation remains to be gathered.

Neurokinin B

Neurons expressing NKB are located throughout the brain with several populations found within the hypothalamus including the ARN, ventromedial nucleus, lateral hypothalamic area, and extending into the zona incerta.[502,503] Studies in the early 1990s by the Rance laboratory initiated interest in NKB by showing an increase in tachykinin family gene expression in the infundibular nucleus of postmenopausal women.[504] This was followed by studies suggesting a role for arcuate NKB neurons in the control of GnRH neurons in the rat[505] and experiments in the sheep showing that almost all arcuate NKB neurons expressed estrogen receptors and were sexually dimorphic.[506,507] The recent identification of infertility in humans with *NKB* (known as *TAC3* in humans and *Tac2* in rodents) and the NKB receptor *NK3R* (known as *TAC3R/Tacr3*) mutations[508] has sparked substantial new interest in this neuropeptide. As noted before in the Kisspeptin section, the observation that a subpopulation of NKB neurons in the ARN express kisspeptin in rodents,[451,459,509] sheep,[458] goats,[510] and primates[460,511] has further focused investigators' attention on NKB.

A substantial number of recent studies have examined the effects of ICV NKB upon LH secretion in several species. In general, investigations in rodents have not provided any clear consensus of NKB action, with NKB being reported to inhibit, stimulate, or have no effect upon LH secretion.[512–516] If anything, the very familiar "neuropeptide pattern" of: (a) LH inhibition in gonadectomized rodents; versus (b) LH stimulation in intact or steroid-supplemented gonadectomized rodents appears to exist for NKB, as it does for many other neuropeptides administered ICV.[272] Given the existence of several distinct NKB cell populations projecting throughout the brain[451,452,503] and the widespread activation of neurons following ICV NKB,[516] it is perhaps not surprising that little consensus has been achieved with this approach. Studies in the sheep have shown that ICV NKB stimulates LH secretion in the follicular phase but has no effect in luteal ewes.[517] An investigation in agonadal prepubertal male monkeys revealed a stimulatory effect of intravenous NKB on LH secretion, suggesting that NKB may even act outside the blood–brain barrier to achieve its actions.[511]

At present, relatively little is known about how any of the NKB populations in the brain interact with GnRH neurons to modulate fertility. Studies in the female mouse and rat have indicated that GnRH neurons express mRNA and protein for NK3R,[215,518] although others have failed to detect the receptor in GnRH neurons in the male mouse and ewe.[459,519] The relative absence of NKB fibers around GnRH neuron cell bodies in the rodent,[451,455,518] and lack of any effect of NKB on GnRH neuron excitability in the mouse[459] indicate that the GnRH soma and proximal dendron may not express the appropriate receptors for NKB. In contrast, early immunohistochemical studies reported that NK3R was present on GnRH nerve terminals in the median eminence[518] and abundant NKB fibers have been detected

in nearby GnRH neuron elements within and around the median eminence in all species examined.[451,460,511,518] Thus, it has been proposed that direct effects of NKB on GnRH neurons may occur at the level of the median eminence,[505] presumably on the GnRH neuron dendron. This would be consistent with intravenous NKB being able to modulate LH secretion.[511] As there is substantial, but not complete, co-expression of kisspeptin with NKB fibers in the median eminence,[451,452] it is thought that the relevant NKB population regulating GnRH neurons is that located in the ARN.

The precise roles of NKB within the GnRH neuronal network remain to be established. As NKB is expressed in arcuate KNDy neurons, it shares many of the putative roles of that cell population. For example, as NKB stimulates the electrical activity of arcuate kisspeptin neurons, it has been proposed to be part of the KNDy pulse generator mechanism.[459] However, in general, when examined in the same assays or protocols, the effects of NKB are overshadowed greatly by kisspeptin. For example, in contrast to the complete infertility of kisspeptin and GPR54 knockout mice, NK3R knockout mice are only subfertile.[520] Whereas the effects of kisspeptin on the reproductive axis are very consistent across the sexes, reproductive states, and animal species, this is not the case for NKB,[505] where, in addition, complex interactions exist with other neurotransmitter systems.[521] Furthermore, recent studies have examined the potential for NKB to be involved in puberty onset. Again unlike kisspeptin, there is no evidence for a significant role of NKB in puberty with, if anything, only a modest modulatory influence observed.[502,522]

Similar to kisspeptin biosynthesis, NKB mRNA in the rodent arcuate nucleus is suppressed by estradiol in an ERα-dependent manner,[512,523,524] and this has led to the suggestion that the coordinated suppression of both kisspeptin and NKB in KNDy neurons may be involved in the estrogen negative feedback mechanism.[459] Also, NKB and kisspeptin expression is increased, and dynorphin reduced, in the infundibular neurons of postmenopausal women.[505] While these studies clearly show that NKB gene expression is modulated potently by gonadal steroids, a role for arcuate NKB neurons in mediating estrogen negative feedback has yet to be established.

Neuropeptide Y

Neuroanatomical investigations in several species indicated the likelihood that NPY neurons located in both the arcuate nucleus and brainstem provide direct inputs to GnRH neuron cells.[3] Over recent years, the major advance in understanding NPY actions in relation to fertility has come from electrophysiological studies examining the effects of NPY on GnRH neuron excitability. In the ovariectomized (OVX) rat, NPY was found to directly hyperpolarize GnRH neurons through the NPY Y5 receptor.[525] In adult female mice, the same direct hyperpolarizing action of NPY was identified, although it occurred through the Y1 receptor.[270] Unexpectedly, direct stimulatory actions of NPY were also observed acting through the Y4 receptor that has low affinity for NPY and is likely to be the natural receptor for pancreatic polypeptide.[270] Finally, studies in embryonic GnRH neurons also found a likely direct inhibitory action of NPY acting through Y1 receptors.[526] Thus, it would appear that NPY exerts a predominant direct inhibitory influence upon GnRH neuron excitability and that this is mediated by Y5 receptors in rats and Y1 receptors in embryonic and adult intact female mice. Whether a true species difference exists for the NPY receptor subtype expressed by GnRH neurons remains unclear. Receptor labeling studies in the rat have indicated that GnRH nerve terminals exhibit Y1 receptor immunoreactivity, while GnRH cell soma are immunoreactive for the Y5 receptor.[527,528]

It is difficult at present to reconcile these direct inhibitory actions of NPY on the GnRH neuron excitability with the reported effects of NPY on rodent LH secretion in vivo. This is most probably due to NPY acting at multiple levels within the GnRH neuron network and pituitary gland to control gonadotropin secretion. For example, there is an array of different NPY receptors reported to be involved in the activation of GnRH secretion within the median eminence,[529,530] in addition to direct Y1 receptor-mediated facilitatory effects upon the gonadotroph itself.[531–535] Thus, ICV NPY is likely to act at multiple different sites to modulate LH secretion and, indeed, exhibits the typical "neuropeptide pattern" of inhibiting LH in gonadectomized animals and stimulating LH in intact or estradiol-treated rats.[272] Studies in NPY knockout mice have not advanced the understanding of NPY to any great extent, as LH levels in OVX and OVX estradiol-replaced mice are normal, although the magnitude of the LH surge is halved in mutant mice compared with wild-type mice.[533,536] Thus, at present, it would seem that the GnRH neuron can be modulated by NPY directly and indirectly at multiple locations, including the cell body and terminals, with none of these actions being essential for their normal behavior, at least in mice.

It is possible that NPY plays a more influential, or at least easily decipherable, role in regulating GnRH neuron secretion in the monkey. In this species, the infusion of NPY into the median eminence of females stimulates GnRH secretion irrespective of its gonadal status.[537–539] Furthermore, NPY levels fluctuate in synchrony with GnRH release within the monkey median eminence, and pharmacological reductions of NPY activity suppress GnRH pulsatility.[540,541] It is interesting to note that NPY release fluctuates in the same manner as NE within the median eminence of the monkey,[542] suggesting that it

may be the brainstem neurons co-expressing NPY and NE that provide this input. Together, these observations indicate that NPY acting in the vicinity of the MBH may have an important physiological role in enabling pulsatile GnRH release in the female monkey and that NPY is most likely to act at the level of the GnRH dendron and nerve terminals to achieve this regulation.[543] In contrast, NPY may exert very different actions in the male primate as i.c.v. NPY inhibits pulsatile LH secretion in gonadectomized male monkeys.[544]

Brought together, it seems likely that NPY is used by a variety of different inputs to GnRH neurons subserving different roles. One of these may be to facilitate GnRH neuron pulse generation at the level of the GnRH dendron/nerve terminals, but other functions related to nutrition and metabolism, for example, almost certainly exist.[545] Establishing the roles of each specific NPY input to GnRH neurons will be a very significant challenge. Aside from the likely different physiological roles of ARN and brainstem NPY inputs to the GnRH neurons, there remains the issue of establishing the significance of co-released agouti-related peptide (Agrp), GABA and NE. Looking at NPY actions independently of Agrp, GABA and NE may be misleading when investigating a neuropeptide that is an established neuromodulator of other neurotransmitter actions.[546]

Norepinephrine

Relatively little new information has accrued on NE since the last chapter published in 2006.[3] A series of experiments in the mouse have, nevertheless, demonstrated that A2 and A6 NE inputs to GnRH neurons exert a direct suppressive influence on GnRH neuron excitability.[249,547] Prior studies on this topic had relied upon confocal appositions[548] or tract-tracing studies that could not be certain whether the labeled NE cell bodies innervated GnRH neurons or other cells in the neighborhood.[549–553] Using conditional pseudorabies virus in GnRH-Cre mice, it was possible to undertake GnRH neuron-specific retrograde tracing, and this showed that NE neurons located in the brainstem A2 and A6 cell groups innervated GnRH neurons directly.[249] Subsequent electrophysiological studies using GnRH-GFP transgenic mice showed that NE acts through both α1 and β-adrenergic receptors to exert direct hyperpolarizing actions on the great majority (80–90%) of adult GnRH neurons.[547] These studies agreed well with prior GnRH neuron gene-profiling work demonstrating the presence of α1 and β-adrenergic receptors transcripts in mouse GnRH neurons.[215] Thus, it would appear that direct NE inputs from the A2 and A6 cell groups act to suppress GnRH neuron excitability in the mouse.

In relation to the new neuroanatomical information, it is interesting to note that species differences appear to exist in the location of ascending NE inputs to GnRH neurons. Although not established with the same confidence allowed in the mouse, it seems that A1, A2, and A6 cells are important in the rat[549,550,554] while only A1 and A6 cells are involved in the network in sheep.[552] Furthermore, it is possible that NE inputs also influence GnRH secretion indirectly within the median eminence[554–557] and an input to the AVPV has also been reported.[558]

A case has been made for a role of NE in the generation of pulsatile GnRH/LH secretion.[559] Experiments in the monkey have shown the importance of α-adrenergic receptors in enabling pulsatile LH secretion[560] and revealed a striking correlation between NE levels in the MBH and pulsatile LH secretion.[542] Early studies in rats also showed that NE inputs near GnRH neurons are critical for pulsatile LH secretion; infusions of *either* adrenergic agonists or antagonists into the vicinity of the GnRH cell bodies in rats and monkeys were found to have the *same* effect of reducing pulsatile GnRH and LH secretion.[542,561–564] These results suggested that NE exerts a permissive influence within the GnRH neuronal network by enabling network function rather than driving it in any particular direction.[559] This idea is further supported by evidence for the reemergence of pulsatile LH release several weeks after complete destruction of the ascending NE pathways.[565,566] Elsewhere in the brain, and particularly in sensory neural networks, NE is well established to act as a permissive neuromodulator that does not carry any specific information but modulates the responses of target neurons to their inputs.[567] This can occur through NE altering signal-to-noise ratios and/or altering the threshold for activation of neurons to specific stimuli; for example, NE reduces the "noisy" spontaneous firing of thalamic neurons to make them more receptive to the effects of key afferent inputs.[568]

Perhaps the clearest indicator of NE's permissive role within the GnRH neuronal network is in relation to the preovulatory GnRH/LH surge. There is evidence for the activation of brainstem NE neurons at this time,[558,569] and studies in rats have shown that preoptic NE concentrations in the vicinity of the GnRH neuron cell bodies begin to increase on the morning of the surge.[558,570–572] This activation of NE neurons on the morning of proestrus appears to depend upon the rising levels of estradiol at this time.[573] As NE levels are elevated hours before the abrupt activation of GnRH neurons on proestrous afternoon, this suggests that they are facilitators rather than the trigger. A similar conclusion has been reached in the sheep,[574] and brainstem NE neurons have also been implicated in the mechanism underlying induced-ovulation in the rabbit.[575–577] Interestingly, the progressive failure of the LH surge mechanism in older rats is associated with reduced or absent increases in preoptic area NE levels.[571]

Precisely how NE acts within the network to promote the presumed high-output state of GnRH neurons

required for individual pulses or the surge is unknown. The recent electrophysiological studies clearly show that NE exerts a potent suppressive influence upon the spontaneous firing of about 50% of GnRH neurons on proestrus.[547] One possibility is that, as in other brain areas,[567] NE reduces spontaneous firing to make the effects of specific inputs more apparent; in this case spontaneous firing could be reduced to increase the overall impact of potentially time-locked RP3V inputs that initiate GnRH neuron activation.

Together, these studies indicate an important permissive role for NE within the GnRH neuronal network. Whether this permissive influence occurs through primary NE afferents to GnRH soma or terminals, or involves parallel, indirect actions of brainstem NE neurons is unknown. Equally, the influence of co-released molecules such as NPY and, possibly ATP,[578] on NE actions remain to be established.

RFRP-3

In 2000 a family of novel peptides with a C-terminal LPXRFamide (X = L or Q) motif was discovered and termed the RFamide-related peptides (RFRPs).[579] Remarkably, an avian homolog of RFRP was found to inhibit gonadotropin secretion directly at the pituitary gland in the quail,[580] raising the possibility that a gonadotropin release-inhibiting hormone had been found. As such, this quail RFRP was termed gonadotropin-inhibitory hormone (GnIH).[580] Subsequent studies in many different bird species have shown that the GnIH peptide is synthesized predominantly by hypophysiotropic parvocellular neurons located in the paraventricular nucleus.[580,581] Receptors for GnIH are present in the avian pituitary,[582,583] and GnIH inhibits both the synthesis and release of LH in all avian species examined to date (for review see Ref. 584). In addition to an action at the pituitary, avian GnRH neurons express Gpr147, the receptor for GnIH,[585] and GnIH-immunoreactive fibers exist close to the cell bodies of GnRH neurons in birds.[585,586] Hence, avian GnIH neurons exert a dual influence upon gonadotropin secretion by directly regulating both pituitary gonadotrophs and GnRH neurons. The widespread distribution of GnIH and its receptor within the avian brain and periphery suggest that there may also be indirect actions of GnIH on the reproductive axis, in addition to roles for GnIH in a range of other systems.[584]

The GnIH peptide itself does not exist in mammals with the mammalian gene orthologous to avian GnIH encoding an precursor that produces two similar LPXRFamide neuropeptides termed RFRP-1 and RFRP-3.[579,587] In situ hybridization and immunocytochemistry studies have shown that cell bodies expressing RFRP precursor mRNA or RFRP-1 and -3 protein are located within and around the dorsomedial hypothalamus of rodents and that these RFRP neurons project widely within the brain.[588–592] Sheep and monkeys have their RFRP neurons located primarily within the dorsomedial and paraventricular nuclei of the hypothalamus.[437,593,594]

The functional role of RFRPs in regulating reproduction in mammals has been more difficult to ascertain and appears to be species, and possibly even sex, dependent. Whereas there is evidence for a direct, albeit modest, role of RFRP-3 in regulating pituitary gonadotropin release in vivo in the sheep,[595,596] this is much less certain in other species. RFRP-immunoreactive fibers have been detected within the median eminence of hamsters, but not primates, although Gpr147 is expressed by gonadotrophs of both species.[437,593,597,598] Studies examining the effects of peripheral RFRP-3 administration on LH secretion have been inconclusive, with inhibitory, stimulatory, or no effects observed.[591,597,599] The hamster is one species where effects of RFRP-1 have also been detected on LH secretion.[591] In the case of the rat, no evidence has been found that RFRP neurons project to the median eminence,[600] and peripheral RFRP-3 has been found to have highly variable inhibitory or no effects upon LH secretion in vivo or in vitro.[589,600–603]

If RFRP does act directly at the pituitary in mammals it remains unclear how this might occur. A recent portal bleeding study in the sheep found that, while detectable in portal blood, RFRP does not fluctuate in relationship to LH pulses.[596] This demonstrates that RFRP is unlikely to have a role in generating or shaping pulsatile GnRH and LH secretion, although it remains possible that it may have more general actions on gonadotroph activity during different seasons in the ewe.[596]

Although the evidence for RFRP being a true hypophysiotropic releasing factor in mammals is slim, it remains that intracerebral administration of RFRP-3 can modulate LH secretion in rats, sheep, and hamsters. This most likely reflects central actions of RFRP within the GnRH neuronal network itself. There is evidence for RFRP fibers around GnRH neuron cell bodies in all mammalian species examined to date[437,589,594,597,598,604] and subpopulations of GnRH neurons express Gpr147.[591,592,604] Most importantly, RFRP-3 is found to exert direct, predominantly suppressive, actions upon the excitability of a subpopulation of GnRH neurons in the mouse.[605,606] Also, administration of RFRP-3 can suppress the cFos activation of GnRH neurons at the time of the LH surge in the rat.[603]

Together, these data demonstrate that, as in birds,[584] the GnRH neuronal network includes a direct RFRP neuron projection from the dorsomedial/paraventricular nucleus to a subpopulation of GnRH neurons in mammals. The physiological role of this projection is uncertain with studies suggesting possible roles in mediating the effects of season[591,594,596,599,607] and stress[608,609] upon the reproductive axis. Given the widespread projections of RFRP neurons throughout the brain, it has not been surprising to find that this neuropeptide is also implicated

in regulating the release of multiple pituitary hormones, as well as a variety of other central functions.[584,610]

Glial Elements of the GnRH Network

As noted in the neuroanatomy section of this chapter, there is substantial evidence for a close physical relationship between glial cells and GnRH neurons at the level of their cell bodies as well as their terminals in the median eminence. Led by the Prevot and Ojeda laboratories, much recent work has been undertaken examining astrocyte-GnRH soma/dendrite interactions and the complex endothelial-tanycyte-GnRH nerve terminal dynamics within the median eminence (for reviews see Ref. 611,612). Together, these studies indicate potent roles for glial cells within the GnRH neuronal network.

Neuronal-glial Interactions at the GnRH Neuron Soma

Astrocytes modulate the activity of GnRH neurons through both direct physical means as well as through signaling molecules. In relation to the former, individual GnRH neurons have up to 90% of their cell soma in direct apposition to glial cell elements depending on the gonadal steroid environment.[51] Specifically, fine protrusions from glial cells can be seen to interdigitate between presynaptic terminals and the postsynaptic membrane of the GnRH neuron.[42,50] Thus, it seems possible that plasticity in glial cell wrapping could modulate the afferent input to a GnRH neuron. Firm evidence for this is lacking, however. A consistent correlation does not exist between levels of glial cell ensheathment and the predicted activity levels of GnRH neurons.[278,613] To date, there is no information on glial cell wrapping of the GnRH neuron dendrites and dendrons.

It is now evident that astrocytes in the vicinity of the GnRH neuron cell bodies secrete prostaglandins, specifically PGE2, to modulate the electrical excitability of GnRH neurons.[614,615] The secretion of PGE2 from astrocytes is regulated by activation of the ErbB receptor family that, in turn, can be controlled by other glial factors such as transforming growth factor alpha (TGFα) and neuregulin[616] as well as neuronal inputs involving activation of glutamatergic metabotropic and ionotropic receptors.[617] All GnRH neurons are reported to be activated by PGE2 and, remarkably, in the acute brain slice, show dependency on tonic PGE2 release to maintain their normal levels of electrical activity.[615] These investigations indicate a powerful role for local glial cells in determining GnRH neuron excitability; further studies are now needed to identify precisely which aspects of GnRH neuron physiology are modulated by this pathway. One study has shown that preoptic levels of ErbB receptors are maximal at the time of the GnRH surge and that knockdown of erbB signaling in this area results in acyclicity.[618] In addition, it seems likely that other glial-derived signaling factors such as TGFβ may be involved.[619] As the tools are developed, this area of GnRH neuron biology will likely become increasingly complex.

Endothelial-Tanycyte-GnRH Nerve Terminal Interactions

Electron- and light-microscopic studies have shown that the GnRH nerve terminals within the median eminence are typically enveloped by tanycytic end-feet and sit away from the basal lamina of the endothelial cell wall.[61,63,67,620] Remarkably, at the time of the preovulatory GnRH surge, substantial structural remodeling occurs involving tanycytic end-feet retraction, sprouting of GnRH nerve terminals to reach the hypophysial blood vessels, and increased permeability of the blood vessels themselves.[64,65,621] These changes, although allowing only approximately 10% of GnRH nerve terminals to be abutting portal vessels,[65] are thought to play a role in enhancing GnRH secretion into the portal system at the time of the surge.[611] How critical this process is to successful fertility is not yet established.

Some progress has been made in understanding the mechanisms underlying the dynamic changes in tanycyte morphology at proestrus. Again, this has been found to involve the erbB receptor family and PGE2; in this case, TGFα released by tanycytes acts back on erbB receptors to promote PGE2 synthesis by tanycytes that, in turn, secretes TGFβ to retract tanycyte processes.[622] Some clarity was brought to understanding how this pathway works by showing that estradiol upregulates the TGFα converting enzyme within the median eminence,[623] indicating that the proestrous rise in estradiol would facilitate tanycyte retraction by promoting TGFα secretion.

Prevot and colleagues have also addressed the important question of whether the vascular endothelial cell is itself involved in this tanycyte retraction mechanism. It is proposed that the endothelial cells produce NO in response to the elevated levels of estradiol on proestrus and that this has two major effects; first, it directly activates GnRH secretion from the nerve terminal, and second, NO activates both soluble guanylyl cyclase (to enhance cGMP levels) and cyclooxygenase (to generate PGE2 secretion) to facilitate tanycyte end-feet retraction.[624–626] Together with the TGF studies outlined before, it would seem that there is a very sophisticated, multidimensional mechanism for regulating tanycytic morphology within the median eminence.

SYNCHRONIZATION AND PULSATILITY

Generation of a GnRH Pulse

Portal bleeding and microdialysis approaches in a variety of species have documented the pulsatile nature of GnRH release and its typically tight correlation with

pulsatile LH secretion.[542,627–636] The intervals between GnRH pulses in vivo range from 40 to 60 min in the gonadectomized monkey, sheep, and rat, with longer intervals reported in intact animals.[542,632–634,637–640] Although GnRH secretion has not been measured in the mouse in vivo, inference from pulsatile LH measurements suggests that GnRH pulses also occur approximately every 60 min in gonadectomized female mice[641,642] and every 3 h in intact males.[643] Detailed analysis of the dynamics of a GnRH pulse has been undertaken in the sheep and revealed that each pulse begins abruptly, plateaus for ~5 min, and then falls precipitously.[631,637] As a general concept, Goodman and Karsch coined the term *hypothalamic pulse generator* in 1981 to describe the neural mechanism responsible for this pulsatility.[644] However, 30 years later the question of what makes up the pulse generator, and how the dispersed GnRH neurons generate a sharp ~5 min pulse of GnRH into the portal system every hour, remains one of the most important unanswered questions in GnRH neurobiology.

While it is self-evident that GnRH neurons ultimately have a role in pulse generation, precisely which GnRH neurons are involved in generating a GnRH pulse remains unknown. Studies involving the transplantation of GnRH neurons into the ventricular system of the *hpg* mouse (that have a genetic mutation resulting in no GnRH peptide expression) indicate that as few as 20 GnRH neurons are sufficient to generate pulsatile LH secretion.[645] Further, a mouse line with only ~80 GnRH neurons can support normal reproductive activity in males, implying their ability to release LH in a pulsatile manner.[646] Thus, under pathological conditions, it would appear that <10% of GnRH neurons are sufficient for pulsatile GnRH secretion to occur. Exactly which of the ~1000 GnRH neurons are involved in generating pulses is unclear. Are they located in a specific area or intermingled with other GnRH neurons? Do the same GnRH neurons generate each pulse, or are they the same GnRH neurons as those generating the surge? The answers to these important questions remain to be determined. There is, however, some evidence that the caudal-most GnRH neurons may be specifically involved in pulse generation. Studies using cFos have reported that a small number of ~100 GnRH neurons located in the MBH of the sheep are activated prior to an LH pulse.[647] Similarly, the most caudal GnRH neurons in the rat express cFos following naloxone treatment that evokes a pulse of LH.[648]

Another pressing question is that of defining whether the GnRH neurons act independently or together with other neurons and glial cells within the network to generate GnRH pulses. It was expected that the ability to make electrical recordings from adult GnRH neurons in situ would be of considerable benefit in answering this question. Based on the observations from GnRH portal bleeding investigations and evidence that 10 Hz activation of GnRH terminals is required to release GnRH in the median eminence,[649] there was great expectation that at least some GnRH neurons would exhibit ~10 Hz firing for 5 min periods once every hour in gonadectomized rodents. As noted in the section GnRH Neuron Electrophysiology, electrical recordings from individual adult GnRH neurons in the rodent brain slice have not found such a correlate or resolved the issue of GnRH pulse generation. Although most GnRH neurons in vitro fire in a bursting mode, this occurs as ~2 s bursts most often every 20 s, whole orders of magnitude out of phase with 5 min LH pulses that occur once every hour. Furthermore, on rare occasions where it has been possible to record the burst firing of multiple GnRH neurons in the same slice preparation, there has been no evidence found for the synchronization of bursting between cells.[650] This complete failure to provide any temporal correlation between episodic GnRH neuron firing patterns and pulsatile GnRH secretion suggests that either: (1) GnRH neuron burst firing measured at the cell body is not driving the patterning of secretion at the median eminence, or (2) that the acute brain slice procedure is inappropriate for examining this issue. The recent demonstration that only ~15% of GnRH neurons exhibit burst firing in vivo[90] may support the second possibility.

The inability to detect GnRH neurons displaying the expected "electrophysiological pulse profile" has greatly hindered progress in identifying how GnRH pulses occur. As the importance of GnRH neuron burst firing to pulsatile LH secretion remains dubious, whether or not burst firing originates from intrinsic and/or extrinsic mechanisms within the GnRH neuron (see the section GnRH Neuron Electrophysiology) is not especially helpful in deciphering the mechanism generating LH pulses. Arguably, most progress in understanding this mechanism has come from experiments involving embryonic GnRH neurons. After two to three weeks in culture, embryonic nasal explant cultures begin to exhibit pulsatile GnRH release with a frequency of ~50 min in the monkey[651] and sheep,[652] ~30 min in the rat,[653] and ~25 min in the mouse.[654]

Terasawa and colleagues have examined the cellular mechanisms behind this pulsatile GnRH secretion in the monkey olfactory placode and shown that GnRH neurons exhibit calcium oscillations with intervals of ~8 min and that these become synchronized every 50 min.[121,655] A similar scenario was reported by the Wray laboratory for GnRH neurons from the mouse olfactory placode where calcium peaks synchronized every ~20 min and that, when >30% of GnRH neurons were involved in a synchronized event, this was associated with a GnRH pulse.[122,654] As in adult GnRH neurons,[58] the calcium peaks in cultured embryonic cells reflect episodes of bursting electrical activity.[656] As such, these data suggest

that synchronization mechanisms exist between embryonic GnRH neurons that are important for generating pulsatile GnRH secretion. Although enriched in GnRH neurons, these preparations also contain other neuronal and nonneuronal cell types, and it remains to be determined whether GnRH neurons interact with these other cells to generate a pulsatile pattern of release.[657]

Another experimental model that has been used to examine GnRH pulsatility has been the GnRH-secreting GT1 cell line. As these cultures contain only GnRH-secreting cells, their ability to generate pulsatile GnRH secretion with an interpulse interval of 20–40 min[103] has been taken to infer that GnRH neurons can generate pulses on their own. However, the relevance of GT1 cells to adult GnRH neurons in vivo has increasingly been questioned. With regard to pulsatility, episodic GnRH secretion from GT1 cells is known to depend upon extensive gap junctional coupling.[658,659] However, adult GnRH neurons in vivo do not utilize gap junctions for communication.[105] Thus, while it remains interesting that cultured GT1 cells can organize themselves into an episodic secretory population, the mechanism being used does not resemble that employed by GnRH neurons in vivo.

Other avenues of investigation into the nature of GnRH pulsatility have focused upon the roles of cAMP and circadian clock genes in GnRH neurons. In terms of the former, using a rat transgenic strategy, Weiner and colleagues reported that the depletion of cAMP levels within GnRH neurons reduces pulsatile LH secretion in vivo.[660] Whether this depletion in cAMP acts specifically on the pulse-generating mechanism or more generally to alter electrical excitability of GnRH neurons is not known. The appeal of circadian genes as modulators of episodic activity in GnRH neurons has been substantial[661] despite the difficulty of aligning a 24 h process with the 30–60 min pulsatility required for GnRH neurons. Nevertheless, molecular clocks with periodicity as low as 2 h have been reported,[662] and, overall, their importance in pulse generation remains to be determined.

GnRH Neuron Synchronization

At least a subpopulation of GnRH neurons must be synchronized to achieve episodic GnRH secretion at the median eminence. How this occurs remains unknown and leaves room for speculation about the possible mechanisms. Two possible models for GnRH neuron synchronization are noted below (Figure 11.8).

Intrinsic Pulse Generation

This model is centered around GnRH neurons themselves having sufficient interconnectedness to achieve a synchronous output (Figure 11.8). This is termed *intrinsic* in that it involves only GnRH neurons. Precisely how individual GnRH neurons may communicate with one another to achieve this synchronization is unclear. Classical neurotransmission between GnRH neurons using GnRH or one of its co-expressed transmitters is one possibility. Studies indicate that GnRH has modest stimulatory effects on the excitability of about 50% of GnRH neurons (see the section Neuronal Elements). Alternatively, as some GnRH neurons may co-express glutamate (see the section Neurotransmitters Co-expressed by GnRH Neurons), this may provide a more potent synchronizing stimulus. To date, however, limited numbers of dual recordings from GnRH neurons in the brain slice have failed to show any classical form of reciprocal neurotransmission.[105] Humoral forms of intercommunication between GnRH neurons have also been proposed. Intriguingly, acutely disassociated adult hypothalami secrete GnRH in a pulsatile manner that is not different to that of intact hypothalami maintained in vitro.[430,663] These data suggest that diffusible molecules may be involved in the synchronization of GnRH release in vitro. Roles for NO,[469,664] endocannabinoids[268,614] and ATP[655] have been suggested, although, in these cases, the source of synchronizing influence would not be "intrinsic".

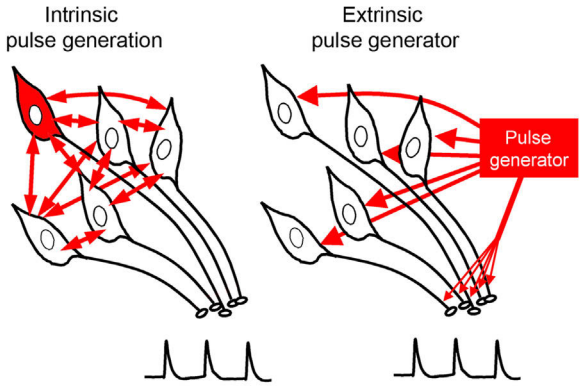

FIGURE 11.8 GnRH neuron synchronization. Schematic diagram showing two potential models for GnRH neuron synchronization. In the Intrinsic Model, the GnRH neurons alone are able to form reciprocal communication with one another thus allowing a buildup of network excitability. The episodic nature of this activity might be driven by one subset of GnRH neurons (shaded) that determine the pace of the interconnectedness. In the Extrinsic Model, the synchronization of the GnRH neurons is generated and controlled by a remote set of neurons with pulse-generating properties.

If the GnRH neurons are able to influence each other's activity, the source of the periodicity needs to be ascertained. It may be that a small subset of GnRH neurons has pulse generator capabilities and is able to drive the activity of all the others. This possibility takes inference from investigations in magnocellular oxytocin neurons where prior to the beginning of each oxytocin burst, "leader" oxytocin neurons become activated and then drive or recruit the activity of the rest of the oxytocin neurons

to generate a period of synchronous high frequency firing.[665] Based on cFos expression studies, "surge GnRH neurons" do not all suddenly fire together at the onset of the surge but, rather, appear to be progressively recruited over the ascending phase of the surge.[666] Interestingly, a very small number of burst-firing GnRH neurons (~1%) exhibit large "spontaneous" membrane oscillations that might represent a strong driving force within the GnRH neuron population.[100] It may, of course, also be the case that there is no "leader" group of cells and that excitability gradually gains momentum both spatially and temporally within the appropriate GnRH neuron population until it reaches a level upon which coordinated bursting activity of large numbers of GnRH neurons occurs.

Extrinsic Pulse Generation

This model is based around the possibility that GnRH neurons have no powerful direct interactions with one another and that the synchronizing influence is generated by an entirely separate group of neurons within the GnRH neuronal network (Figure 11.8). There are, in fact, several blends of this model that extend from GnRH neurons being complete slaves to an extrinsic pulse generator, through to them having a modest degree of episodic activity that is enabled or further entrained by network interactions. For example, in the deafferentated acute brain slice, the short 2 s burst behavior of GnRH neurons may represent something akin to an idling engine awaiting extrinsic acceleration.

Where the extrinsic pulse generator(s) may reside is difficult to assess and controversial. Studies in the rat have suggested that neurons located in the MBH may comprise a critical element of the pulse-generating network. Deafferentation experiments in which the preoptic area is disconnected from the MBH were found to be compatible with pulsatile LH secretion.[667–669] However, as there is an almost complete absence of GnRH neuron cell bodies in the MBH of the rat, it is difficult to understand how the dendron or terminals alone could maintain GnRH secretion for anything more than a few hours. Importantly, subchiasmatic projections from preoptic GnRH neurons to the median eminence were discovered, and it is possible that these were left intact in these deafferentation studies.[670] Those investigations may have revealed, instead, that a small number of preoptic GnRH neurons using a subchiasmatic axonal pathway are sufficient to enable pulsatile GnRH secretion.[670] Nevertheless, Weick and colleagues[668] found that, when combined with anterior hypothalamic deafferentation, highly localized lesions of the rostral-most pole of the ARN completely abolished pulsatile LH release. A similar effect was found when deafferentation procedures involved the rostral ARN[671] suggesting that a group of cells in this area, or fibers passing through it, were critical for pulsatile LH release. Interestingly, electrolytic or chemical lesions of the main body of the ARN itself have no effect upon pulsatile LH secretion with or without anterior hypothalamic deafferentation,[668,672] suggesting that it may be cells or fibers passing through the rostral pole of the arcuate nucleus that are critical.

As monkeys and sheep have GnRH neuron cell bodies spread throughout the preoptic area and MBH, similar types of lesion studies may not be as revealing in terms of defining the location of neurons involved in the pulse generator. Nevertheless, in both species, anterior hypothalamic deafferentation studies indicate that GnRH neurons within the MBH are sufficient to maintain pulsatile GnRH secretion,[673–675] although they do not exclude a similar role for preoptic GnRH neurons under normal circumstances. However, relatively selective lesions of the ARN in two monkeys were shown to suppress LH secretion, indicating a clear role for this brain region in the regulation of LH secretion.[676] Electrical multiunit recording studies have further shown an excellent correlation between the occurrence of action potential volleys in the MBH and pulsatile LH secretion in several species.[677–680] However, despite speculation that it may be the kisspeptin neurons,[510] the identity of the cell types generating these multiunit recordings has not been determined to date.

A pulse generator may regulate the GnRH neuron cell bodies, dendrons, and/or terminals to synchronize and drive episodic activity (Figure 11.8). A recent study found that the long dendrites of GnRH neurons often bundle together and receive shared synapses where one axon innervates two adjacent bundling dendrites.[37] Although the identity of the axons participating in the shared synapses is not known, this observation provides key information that synchronization may occur through afferent input to the dendrite/dendron. Indeed, the clustering of GnRH neuron dendrons and terminals in the vicinity of the median eminence makes them very attractive targets for any external synchronizing influence. Curiously, episodic GnRH release is reported to continue for several hours in vitro in the absence of the GnRH cell bodies[409,681,682] suggesting that some degree of synchronization exists at the GnRH neuron distal dendron and terminal. The nature of this synchronizing influence is unknown and may derive from synaptic inputs from a pulse generator located in the adjacent ARN, for example, or as a humoral form of communication, for example, by NO.[626,683]

As noted, these models are speculative and represent theoretical models for experimental testing. It seems most likely to the author that GnRH pulse generation occurs from a blend of intrinsic interconnectedness coupled with an external pulse generator.

Generation of a GnRH Surge

It is well established that the preovulatory LH surge in mammalian females is generated by a relatively

abrupt and prolonged increase in GnRH release within the portal vasculature[631,632,634,638,684–692] (Figure 11.9). Detailed analyses of portal GnRH secretion in the ewe have shown that GnRH release changes from a strictly episodic pattern to one of significant nonepisodic GnRH release intermingled with high-frequency pulsatile GnRH secretion prior to the surge.[690,693,694] Ultimately, the GnRH surge itself appears to be composed of high-frequency pulsatile events superimposed upon a high constant level of GnRH outpouring (Figure 11.9). This massive increase in GnRH secretion continues for a period of >24 h, considerably beyond the duration of the LH surge it induces, before returning to a strictly episodic pattern of release.[684,689]

The profile of the GnRH surge is thought to be similar in other species although, because of technical constraints, less detailed information is available. In the monkey, both median eminence and third ventricular monitoring of GnRH concentrations have shown that a marked increase in GnRH release occurs at the time of the LH surge.[687,688] The precise profile of GnRH release has not been studied on a minute-by-minute basis but seems likely to be comprised of pulsatile events superimposed upon high basal outpourings of GnRH.[687] Like the sheep, the GnRH surge in the monkey long outlasts the LH surge.[687] Investigations of this kind in the rat have used push–pull or microdialysis approaches to sample GnRH in the median eminence. Hampered by a lack of good temporal resolution, these studies have, nevertheless, reported a clear increase in GnRH secretion correlated with the LH surge.[630,685,686] The dynamics of the GnRH surge in the rat appears to take the form of a marked increase in GnRH pulse amplitude with more minor variations in GnRH pulse frequency and interpulse secretion.[630,639,695,696] Whether the proestrous increase in GnRH secretion in the rat persists for several hours after the end of the LH surge, as it does in the sheep and monkey, is not clear.

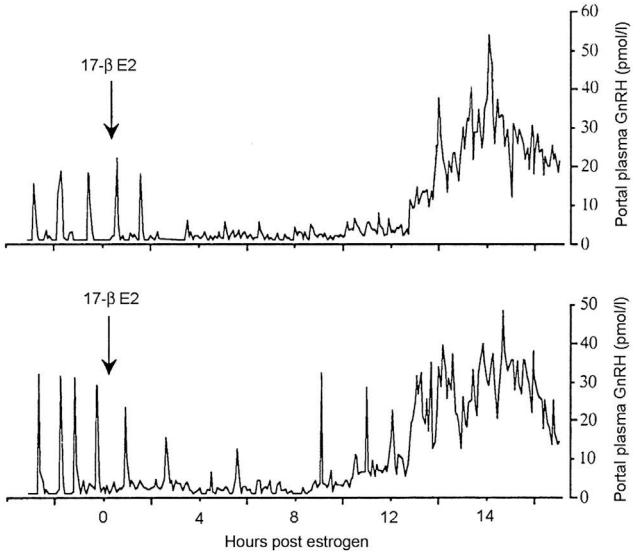

FIGURE 11.9 Effects of estradiol on GnRH secretion in vivo. Portal plasma GnRH concentrations in two OVX ewes showing their response to parenteral 17β-estradiol administration. Note that GnRH secretion is clearly pulsatile with sharp episodes of GnRH secretion. Following estradiol there is a relatively rapid phase of negative feedback with reduced amplitude and frequency of GnRH pulses, which is then followed approximately 12 h later by an increase in pulsatile and baseline GnRH resulting in the GnRH surge. *Source: Adapted with permission from Caraty et al. (1989).*[632]

It seems highly likely that the GnRH neurons responsible for the generation of the LH surge represent a specific subpopulation. It is clear in the rat that ~50% of preoptic area GnRH neurons clustered around the OVLT express cFos at time of the surge,[17] and it seems reasonable to assume that these cells are activated to release GnRH at this time.[18] A similar situation exists in the mouse where ~50% of GnRH neurons around the OVLT and extending back into the AHA express cFos at the time of the surge.[89,477] Interestingly, the surge-activated GnRH neurons preferentially co-express galanin[205] and exhibit highly selective changes in spine density at the time of the surge.[46] Whereas similar studies in the sheep have also found that only a subpopulation of GnRH neurons express cFos at the time of the surge, no preferential topography of the cFos-expressing GnRH neurons was detected.[697] Intriguingly, no increase in the numbers of GnRH neurons expressing cFos was detected at the time of the surge in the monkey.[698]

Given that around half of GnRH neurons in the preoptic area are involved in the GnRH surge mechanism, it seemed likely that targeted electrical recordings of GnRH neurons in the acute brain slice would provide important information about their patterns of electrical activity at the time of the surge. Profiling of the surge in sheep indicated hugely elevated basal and episodic GnRH secretion into the portal vasculature (Figure 11.9). However, to date, only one group has reported any positive data on this subject. Using a model in which ovariectomized, estradiol-treated mice exhibit daily LH surges, mean GnRH neuron firing rates were found to be elevated by ~2-fold on the afternoon of the surge compared with ovariectomized, vehicle-treated mice.[98] The pattern of GnRH neurons firing was highly episodic in both surging and ovariectomized mice with no difference in instantaneous firing frequency. This was a surprisingly modest change in GnRH neuron firing considering the presumed substantial change in GnRH secretion that occurs ate this time in vivo.

As for the GnRH pulse, this lack of a substantial correlation between GnRH neuron firing in the brain slice and the GnRH/LH surge may arise as a consequence of the brain slicing or, indeed, represent the true situation at the level of the cell body. As noted in the section Glial Elements of the GnRH Network, there is substantial

morphological rearrangement of GnRH nerve terminals in relation to their proximity to the vasculature at the time of the surge, although this is unlikely on its own to drive sufficient GnRH secretion to account for the surge. Equally, it is possible that the surge is generated by alterations in synchronization at the level of the distal dendron. How this issue will be resolved remains uncertain, but it may, ultimately, require in vivo recordings of multiple GnRH neurons at the time of the surge.

HETEROGENEITY WITHIN THE GnRH NEURONAL PHENOTYPE

Aside from intrinsic properties common to all neurons, it would appear that the only features shared by all GnRH neurons are that of GnRH biosynthesis and the presence of functional GABA and glutamatergic inputs. Virtually everything else that has been described so far in GnRH neurons has been detected in only subgroups of these cells. As highlighted throughout the chapter, this heterogeneity exists across multiple parameters including GnRH neuron projection patterns, light and electron microscopic appearance, co-expression of neuropeptides, firing patterns, rates of GnRH biosynthesis, as well as ion channel and neurotransmitter receptor expression. Of these parameters, only two have functional correlates as yet. The first is whether GnRH neurons project to the median eminence or not—those that do being hypophysiotropic GnRH neurons and those that don't being involved in other ill-defined actions of GnRH within the brain. The second is their classification as to whether or not they express cFos at the time of the GnRH surge, where it is presumed that those that do are involved in GnRH surge generation.

The relationship of the various heterogenous features to each other has been difficult to ascertain. The one exception has been with regard to the GnRH neurons that express c-Fos at the time of the surge. This subpopulation of GnRH neurons is more likely to project outside the blood–brain barrier;[699] receive more galanin,[309] glutamate,[700] and vasoactive intestinal polypeptide (VIP)[701] inputs than other GnRH neurons; exhibit spine plasticity at the time of the surge;[46] be more transcriptionally active in terms of GnRH mRNA;[702] and are more likely to co-express galanin.[205] In the rat and mouse, these cFos-expressing GnRH neurons are found predominantly, but not exclusively, in the vicinity of the OVLT,[17,481] where gonadal steroids exert their most profound effects upon GnRH biosynthesis.[703–705] Together, these observations suggest that a functionally distinct subpopulation within the GnRH population is located preferentially around the OVLT and is involved in the generation of the GnRH surge.

Another potential functional subpopulation, but with much less supporting evidence, concerns GnRH neurons located in the MBH in appropriate species. Lesion studies in the monkey[673] and guinea pig[19] have indicated that GnRH neurons located in the mediobasal hypothalamus may be of particular relevance to episodic gonadotropin secretion in those species. Also, a study in the sheep indicated that MBH GnRH neurons expressed cFos preferentially in relation to LH pulses.[647] This subpopulation of GnRH neurons may also have a different pattern of innervation compared with GnRH neurons in the preoptic area in monkeys.[51]

Other heterogenous properties of GnRH neurons can also be distributed in a topographic manner. For example, the rostral-most GnRH neurons in the MS of the monkey do not project to the median eminence,[23] while only MS GnRH neurons in the rodent express the GalR1 receptor[213] as well as specific $GABA_A$ receptor subunit mRNAs.[706] However, not all MS GnRH neurons are alike as distinct populations of kisspeptin or metabotropic glutamate receptor-sensitive GnRH neurons are found in the mouse MS.[222] Viewed from a different neuroanatomical perspective, it has also been reported that the GnRH neurons located closest to the third ventricle express NMDA receptors whereas those placed more laterally do not.[707] Nevertheless, as noted before, many of the heterogenous characteristics of GnRH neurons occur in a manner independent of the location of the cell body. This is perhaps best highlighted by the observation that the hypophysiotropic GnRH neurons are scattered throughout the GnRH neuron continuum of rodents and sheep[14,16,53] and the preoptic area and MBH of monkeys.[23]

What is clear is that it is not appropriate to consider the GnRH neuron population as a homogeneous group of cells with a unitary function. A major challenge for future research will be to establish the functional significance of this extensive level of heterogeneity within the GnRH neuron population. With new techniques allowing better cellular and molecular definition of the GnRH neurons, it will likely become increasingly important to refine investigations to particular subsets of GnRH neurons.

ESTROUS CYCLE PLASTICITY

Whereas pulsatile GnRH secretion occurs in a regular and continuous manner in males, spontaneously ovulating females exhibit cycles of episodic GnRH secretion over the course of the ovarian cycle. This section concentrates upon the mechanisms responsible for this remarkable plasticity in the GnRH neuronal network of females. While taking insight from the sheep and monkey, the section focuses upon rodent studies. Readers are also

referred to other chapters in this book devoted to control of the ovarian cycle in the sheep and monkey. While dedicated to the understanding of GnRH neurons, it is appreciated that significant feedback actions also occur at the pituitary gonadotrope to help shape gonadotropin secretion (see Chapters 10,26,27,28 and 33 for a detailed review of this subject).

In female rodents there are clear interactions between a circadian signal and the fluctuating gonadal steroid concentrations that drive GnRH neuron network plasticity.[708–710] It is most convenient, therefore, to consider circadian and then estradiol and progesterone mechanisms separately before providing an integrated model for estrous cyclicity.

Circadian Inputs

In rodents, lesions involving the SCN result in a complete abolition of the preovulatory LH surge, indicating the critical role of this structure in generating a circadian signal within the GnRH network of these species.[711–715] Mutations of the core circadian clock gene *clock* also result in failed cyclicity and surges.[716] How the SCN relays this information to the GnRH neurons is under investigation with both direct and indirect pathways likely. The involvement of diffusible molecules originating from the SCN, as has been found for wheel-running rhythms,[717] seems unlikely to be relevant for GnRH neuron activation.[712,718]

Anterograde tracing studies have shown that SCN neurons project directly to the GnRH neurons in rodents[719,720] and that this principally involves VIP inputs from the ventrolateral SCN.[361,719] Experiments in the rat have shown that ~40% of GnRH neurons express the VIP receptor VPAC2[362] and that ~30% of GnRH neurons in the mouse are surrounded by VIP-immunoreactive fibers.[721] Furthermore, VIP inputs selectively target GnRH neurons that express cFos at the time of the surge.[701] Importantly, electrophysiological studies have recently shown that VIP exerts a time-of-day- and estradiol-dependent excitatory action upon a subpopulation of GnRH neurons; VIP increases the mean firing rates of approximately half of GnRH neurons from ovariectomized, estradiol-treated female mice but only when recorded between 1400 and 1600 h.[364]

In addition to direct projections to GnRH neurons, SCN inputs also innervate the AVPV where they likely regulate ER-expressing cells.[720,722] A circadian rhythm in cAMP levels exists within cells of the AVPV of the rat.[723] Recent studies have demonstrated that kisspeptin neurons in the AVPV exhibit circadian patterns of cFos, circadian transcription factors, and *Kiss1* gene expression, and that this likely originates from a dorsomedial SCN vasopressin input.[483–485,724,725] Interestingly, neonatal cocultures of the SCN and preoptic area that enable a circadian pattern of GnRH release to occur correlate better with vasopressin secretion rather than that of VIP.[653] Furthermore, the inability of *clock* mutant mice to exhibit an LH surge can be reversed to some extent by treatment with vasopressin acting through the vasopressin 1a receptor.[716] However, the precise effects of vasopressin on AVPV neurons are not as yet known.

Thus, the SCN appears to be able to deliver a circadian input to the GnRH neuronal network through at least two different pathways: one involving a direct likely VIP input to the GnRH neurons and another indirect vasopressin input to the AVPV neurons that then relay the signal to the GnRH neurons.[484] There is evidence that the effectiveness of both inputs is critically dependent upon the prevailing estradiol concentrations.[364,485] Similarly, circadian differences in GABA and glutamate inputs to GnRH neurons are only present in the presence of estradiol.[91,726] While there is a clear anatomical substrate for the interaction between vasopressin and estradiol within the ER-expressing cells of the AVPV, it is less certain how estradiol may modulate the direct VIP input to GnRH neurons.

Although the SCN undoubtedly provides circadian information to the GnRH neuronal network, exactly when this occurs is unclear. Classic studies using pentobarbital to anesthetize rats for 1–2 h identified a "critical period" of 5–7 h prior to "lights off," during which neural activity was essential for the LH surge to occur.[709] A similar time point of ~5 h prior to "lights off" has been demonstrated in the mouse.[726] It is possible that this critical period represents a time at which a key circadian signal is delivered to the network, although the importance of other neural processes mediating estradiol actions at this time cannot be excluded. Interestingly, a mouse model that clamps estradiol levels to examine circadian influences finds differences between GnRH neuron firing rates in the morning and afternoon in vitro,[98] suggesting that the circadian signal is "embedded" in the local circuitry even after brain slicing.

Estradiol Inputs to GnRH Neurons

In spontaneously ovulating species, the rising plasma estradiol levels of the mid-to-late follicular phase of the cycle evoke a positive feedback action upon the GnRH network to induce the gonadotropin surge. At other times of the cycle estradiol is responsible for exerting a suppressive negative feedback effect upon gonadotropin secretion. At least three fundamentally different modes of estradiol action work together to influence the output of the GnRH neuronal network, and these will be reviewed briefly before considering the negative and positive feedback mechanisms.

Direct Estradiol Actions on GnRH Neurons

Although initially controversial,[727] it is now well accepted that GnRH neurons express only the ERβ isoform of the ER in all mammalian species examined to date, including mice,[728] rats,[729–731] sheep,[732] and humans.[733] However, the classical transcriptional or other roles for ERβ in GnRH neurons remain unclear. The receptor is not required for the regulation of GnRH mRNA expression by estradiol[734] but may possibly have a role in steroid regulation of galanin synthesis in GnRH neurons.[735] There is also evidence that ERβ in GnRH neurons may mediate rapid effects of estradiol on GnRH neurons (see following).

One of the principal difficulties in assigning a physiological role for ERβ in GnRH neurons has been the highly variable reproductive phenotype of the different ERβKO mouse lines. The initial mouse line[736] was reported to have only modestly impaired fertility as female knockout mice had fewer litters and fewer pups. The next ERβKO line to be produced exhibited a more variable reproductive phenotype with female mice displaying normal fertility through to infertility.[737] As some of this variation may have resulted from the presence of ERβ splice variants missing exon 3, a complete ERβKO null mouse was generated in which ERβ and all of its known splice variants were deleted.[738] These mutant male and female mice were uniformly infertile with females displaying disordered estrous cyclicity. While much of the reproductive phenotype of these mice is attributed to abnormalities in the gonads,[737,739] it remains possible that some of the phenotype derives from ERβ expressed by GnRH neurons.

There has been substantial interest over the last decade in the possibility that estradiol may exert direct, nonclassical actions upon adult GnRH neurons (for recent reviews see Refs 740–742). This idea was spawned by an early electrophysiological study demonstrating a rapid hyperpolarizing influence of estradiol upon GnRH neurons in the guinea pig.[77] Subsequent investigations have shown that estradiol rapidly phosphorylates cAMP response element binding protein (CREB),[743] modulates potassium-ATP channels,[744] elevates intracellular calcium concentrations,[745] and also alters the firing rate[745–747] of adult mouse GnRH neurons. Estradiol has also been found to rapidly modulate calcium levels in embryonic GnRH neurons from the mouse and monkey.[748,749]

In relation to the acute effects of estradiol on GnRH neuron firing rate in adult mice, there has been little consensus between laboratories with inhibitory or excitatory effects reported to depend upon the dose of estradiol tested[746] or the basal level of GnRH neuron activity prior to testing with estradiol.[747] Some of this inconsistency may come from the use of the sudden bath application of widely varying low pM to high nM concentrations of estradiol in the face of almost no understanding of the actual profile and levels of estradiol a GnRH neuron would normally be exposed to during the estrous cycle.[741] Furthermore, the precise identity and location of the receptors mediating these variable rapid effects of estradiol on adult GnRH neurons are only poorly defined, with little evidence that they are, in fact, direct actions on GnRH neurons. Indeed, the only consistent finding from adult studies so far has been that low pM, presumably physiological, concentrations of estradiol act indirectly via ERα to modulate GABA inputs to GnRH neurons.[745,746] To date, CREB phosphorylation is the only rapid effect of estradiol on adult GnRH neurons that has been proven to depend upon ERβ expressed by these cells.[750] Whereas the rapid effects of estradiol on calcium peaks in embryonic GnRH neurons are also dependent upon ERβ in the mouse,[749] the receptors mediating these actions in the monkey are, curiously, different with both an STX-sensitive receptor and GPR30 implicated.[751]

In summary, while there is good evidence for the expression of ERβ in GnRH neurons, and therefore direct actions of estradiol on these cells, the transcriptional targets of ERβ remain unknown. Similarly, while there are now a plethora of nonclassical rapid effects of estradiol reported for GnRH neurons, deciphering which, if any, of these actions are physiologically relevant remains an important goal.

Indirect Transsynaptic Pathways of Estradiol Action

Given the predominant role of ERα in mediating estrogen feedback actions upon GnRH neurons (see following) and absence of ERα in GnRH neurons,[727] it is apparent that estradiol must exert significant indirect actions on GnRH neurons. In this light, it has been a major challenge to ascertain the locations of estrogen-receptive afferent inputs to GnRH neurons. This is no trivial matter and has been frustrated by conventional tract-tracing methodologies not being able to generate any definitive description of the identity and locations of ER-expressing neurons that project to the GnRH neurons. For example, it has been possible to define the locations of ER-expressing neurons that project to the general vicinity of the GnRH cell bodies,[550] but it has not been possible to confirm that these cells make direct synaptic contacts with GnRH neurons.

The combination of transgenic strategies with viral tract tracing has recently made it possible to undertake GnRH neuron-specific retrograde tract tracing in the mouse brain.[752] Using this approach, the primary afferents to preoptic GnRH neurons have now been determined in the mouse.[249,477,753] Dual labeling for ERα in these experiments demonstrated that the estrogen-receptive primary afferents to GnRH neurons are highly

concentrated within three contiguous hypothalamic nuclei: the AVPV, PVpo, and median preoptic nucleus, subsequently defined as the RP3V.[442] A smaller number of ERα-expressing afferents were also found within the arcuate nucleus.[477] While these brain areas were not entirely unexpected, this tracing strategy provided the essential neuroanatomical framework for understanding the direct transsynaptic regulation of GnRH neurons by estradiol.

Key questions remain as to what the neurochemical phenotypes of these ER-expressing cells may be. Likely cell types within the RP3V include neurons utilizing GABA,[754,755] glutamate,[756] or both amino acid transmitters,[757] as well as neuropeptides such as neurotensin[758] and kisspeptin.[441,480,481] The development of a new angled brain slice preparation has enabled the functional effects of AVPV afferents to GnRH neurons to be examined in situ. This approach has provided functional evidence that AVPV neurons expressing GABA, glutamate, and kisspeptin all project to and regulate the excitability of GnRH neurons.[92]

Indirect Non-Neuronal Sites of Estradiol Action

As noted in the section Glial Elements of the GnRH Network, glial cells form close structural and signaling relationships with GnRH neuron cell bodies, dendrites, and terminals. As astrocytes and ependymal cells express both classical and membrane ERs,[759–761] they may serve as indirect targets for estradiol to alter the activity of GnRH neurons. Importantly, estradiol-dependent alterations in glial cell morphology are not necessarily slow, and significant changes may be observed within 5 h in vitro.[762]

In terms of direct structural modifications, an ultrastructural study in the monkey reported that ovariectomy resulted in a significant increase in the apposition of glial processes to the GnRH neuron soma, with a concomitant decrease in synaptic input, and that this was partly reversed by estradiol treatment.[51] This effect was not observed in the female rat[763] but, nevertheless, suggests the possibility that glial cell "wrapping" of GnRH neurons may be regulated by estradiol and that this may impact on the amount of synaptic innervation a cell receives.

In contrast to the cell body, there is more substantial evidence that the GnRH nerve terminals are subjected to estradiol-dependent glial cell wrapping. In this case, estradiol modifies the tanycyte envelopment of the GnRH nerve terminals so that, at the time of proestrus, the tanycytic end-feet are retracted, allowing increased numbers of GnRH terminals within the pericapillary space.[64,65,764] Recent investigations have identified that the high levels of estradiol on proestrus act through at least two mechanisms to retract the tanycytic end-feet: one involving the promotion of TGFα levels within the median eminence and the other enhancing NO release from endothelial cells[623,625] (see the section Glial Elements of the GnRH Network).

The other potential mechanism through which glial cells could contribute to the plasticity of the network would be through the estradiol-dependent secretion of molecules that regulate the activity of GnRH neurons. For example, an important role for prostaglandins secreted by astrocytes neighboring GnRH neurons was recently revealed in controlling the electrical excitability of GnRH neurons.[615] However, it remains unclear whether this signaling pathway is modulated by gonadal steroids. Although estradiol is reported to increase PGE2 receptor expression in GnRH neurons,[765] the impact of PGE2 on GnRH neuron excitability was found to be equivalent throughout the estrous cycle.[615] Other molecules secreted by glial cells, such as TGFβ,[766] are known to be estradiol sensitive, but their role in driving estrous cycle plasticity in vivo remains unclear at this time.[767]

Estrogen Positive Feedback

It is first important to define what the positive feedback effects of estradiol are on GnRH neurons. In terms of GnRH secretion, some of the most useful data have come from portal bleeding experiments in sheep.[632,640,693,694,768] Those studies demonstrate that the first change from the strictly episodic negative feedback pattern of GnRH secretion begins about 4 h before the onset of the GnRH surge when significant interpulse GnRH secretion is observed. This is followed by an increase in GnRH pulse frequency and amplitude and further enhanced by elevated interpulse secretion over the next 2–4 h immediately prior to the abrupt onset of the GnRH surge comprised of a massive, continuous release of GnRH with superimposed pulses. Hence, in the ewe, estradiol's positive feedback effects are to evoke a gradual increase in episodic GnRH secretion alongside the initiation of a continuous pattern of release that ultimately "explodes" into the prolonged GnRH surge (Figure 11.9). Portal bleeding studies have not been possible in the rat, but there is, nevertheless, evidence of increased irregular GnRH secretion prior to the surge in this species as well.[696] The activation of preoptic GnRH neurons, as evidenced by cFos expression, suggests that while the first cells are activated rather abruptly, the remaining "surge" neurons are progressively recruited as the GnRH/LH surge advances.[666]

How does estradiol, in association with a circadian input in rodents, bring about these changes in GnRH neuron secretion? One clue lies in the dynamics of the estradiol signal that are interpreted by the GnRH network to evoke the surge. In all mammalian species, the LH/GnRH surge is only induced by an estradiol signal of sufficiently high or increasing levels that lasts for several hours.[632,687,689,708,769–772] Attempts to refine the

exact period of estradiol exposure required in the ewe have suggested that a 7–14 h period is the absolute minimum.[773] Critically, as found earlier for the LH surge in the rat,[708] studies in the sheep also reported that estradiol did not need to be present at the actual time of surge initiation for a normal GnRH surge to occur.[773] Together, these observations indicate that the GnRH network "reads" a prolonged, high circulating estradiol concentration as the appropriate signal to initiate positive feedback. The length of the estradiol exposure and the fact that estradiol need not be present at the time of surge onset itself is compatible with the idea that estradiol acts in a classical genomic manner to alter gene expression profiles within the network to induce the surge. This concept has been supported by a study showing that mice expressing a mutated ERα unable to bind to DNA estrogen response elements are unable to generate positive feedback.[774]

Which cell types within the network might be regulated by estradiol in this manner to induce the changing patterns of GnRH release at the time of the surge? Several lines of evidence strongly suggest that estradiol uses an ERα-expressing RP3V transsynaptic input to drive positive feedback in rodents (for review, see Ref. 442) (Figure 11.10).

Lesion, Implant and Neurochemical Studies

The great majority of lesioning and estradiol implant studies addressing the sites of action of estradiol in regulating LH secretion have been undertaken in the rat. Lesions of both of the principal RP3V nuclei, the AVPV and PVpo, were found to result in persistent estrous and the abolition of the estradiol- or estradiol-plus-progesterone-induced LH surge.[711,775,776] Furthermore, estradiol placed in the medial preoptic area was able to generate an LH surge in the rat,[777,778] while similarly positioned implants of antiestrogens inhibited the estradiol-induced LH surge.[779,780] In contrast, implants of estradiol into the MBH do not elicit a surge-like alteration in LH secretion in the rat.[777,778]

There is abundant ERα and ERβ expression within neurons of the RP3V,[758,781,782] and a subpopulation of ERα-expressing neurons in this region provides direct inputs to GnRH neurons in the mouse.[477] This, coupled with cFos evidence for the activation of RP3V neurons at the time of the LH surge in rats and mice,[481,783] strongly suggests that estradiol regulates the activity of RP3V neurons to activate GnRH neurons.

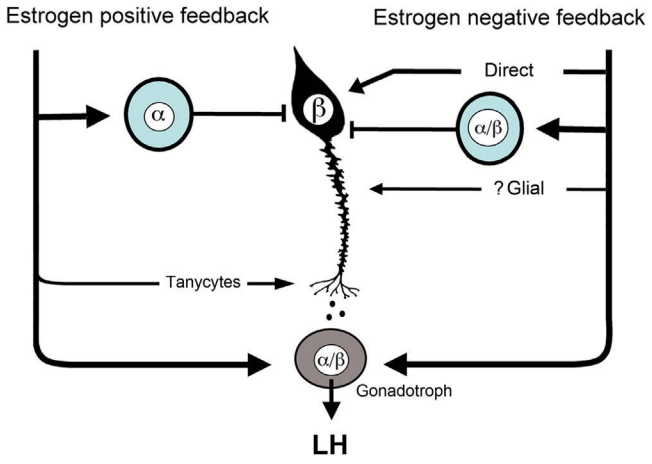

FIGURE 11.10 Model of estradiol feedback actions. Schematic diagram showing proposed modes of estradiol action within the GnRH neuron network for positive and negative feedback. α and β indicate location of ERα and ERβ, respectively.

Transgenic and Mutant Mouse Studies

The generation of mouse models with ER deletions and mutations has been important in determining the ER isoform involved in positive feedback. The global ERαKO mouse lines are infertile with severely abnormal gonadotropin hormone levels whereas, as noted above, ERβKO mutants show a milder hormonal phenotype and varying degrees of infertility.[734,736,737,739,784] Investigations employing global ERαKO and ERβKO mice have shown that the LH surge is absent in ERαKO mice but present in ERβKO.[477] Furthermore, studies with ERα- and ERβ-selective agonists demonstrate that only ERα activation can evoke the LH surge in wild-type mice.[477] Using cell-specific transgenic strategies, it was also shown that it is neurons expressing ERα rather than other cell types that are essential for the LH surge in mice.[477] Together, these studies identified that ERα-expressing neurons are both necessary and sufficient for the estrogen positive feedback mechanism in mice. This does not mean that ERβ or nonneuronal ERα-expressing cells are not involved in the surge mechanism, rather that they play a replaceable or modulatory function within the network. For example, the infusion of an ERβ antisense oligonucleotide into the third ventricle of rats prolongs estrous cycle duration but does not stop it.[782]

GnRH Neuron Electrophysiology Studies

Using a daily surge mouse model, investigators have shown that mean GnRH neuron firing rates are elevated at the time of the surge compared with recordings made earlier in the morning.[98] As this model clamps estradiol at a constant high level, it is unclear what the impact of estradiol is other than enabling a strong circadian imprint on the network. Nevertheless, this important facilitatory role of estradiol is known to be dependent upon classical ERα signaling[785] and correlated with changes in $GABA_A$ receptor signaling.[91] Other studies using standard ovariectomized, estradiol-treated mouse models have indicated that the rising levels of estradiol required to generate the LH surge are correlated

with increased expression and function of T-type calcium channels in GnRH neurons.[116] These channels are involved in rebound excitation and have been suggested to contribute to increased excitability of GnRH neurons on proestrus.[116]

Specific ERα–RP3V Afferents to GnRH Neurons

The previous studies provide good evidence that an ERα-expressing population of cells located in the RP3V relays the estrogen positive feedback signal to GnRH neurons in rodents. A variety of classical neurotransmitters and neuropeptides are synthesized by RP3V neurons, and several of these are known to express ERα including glutamate,[756] GABA,[755] dynorphin,[786] enkephalin,[786,787] galanin,[788] substance P,[789] calcitonin gene-related peptide,[790] kisspeptin,[441,480,481] and neurotensin.[758] At present, a case for the involvement of RP3V glutamate, GABA, and kisspeptin neurons can be made in the positive feedback mechanism, with a role for neurotensin recently being excluded in the mouse.[331]

Glutamate. Glutamatergic neurons in the AVPV provide direct inputs to GnRH neurons and act to enhance GnRH neuron excitability.[92] Furthermore, "surge GnRH neurons" exhibit a substantial increase in dendritic spine density at the time of the surge, and it is possible that spine density reflects glutamatergic input to these cells.[46] Glutamate levels are reported to increase within the vicinity of the GnRH neuron cell bodies at the time of the surge in the rat,[791,792] although it is not certain whether this glutamate arises only from RP3V afferents. Recordings of glutamate-mediated postsynaptic events in adult GnRH neurons in the brain slice have failed, however, to detect any change at the time of positive feedback, although it needs to be borne in mind that this study was only able to measure action potential–independent glutamate activity.[370] Finally, although not addressing the role of RP3V glutamatergic cells specifically, antagonists of glutamatergic receptors administered into the ventricular system of rats block the occurrence of the LH surge.[417,418,793]

GABA. There is a consistent set of data across species showing that GABA levels within the vicinity of the GnRH cell bodies rise in the morning and then fall precipitously in the afternoon just before the onset of the LH surge.[791,794–797] Pharmacological studies with $GABA_A$ receptor antagonists have further indicated that this fall in GABA release is necessary for the GnRH surge to proceed.[397,798,799] Whether this fluctuation in GABA levels results from changes in circulating estradiol concentrations and/or circadian influences remains unclear.[573,796] The origin of GABAergic inputs to GnRH neurons is unclear as, in addition to a substantial input from the RP3V,[92] GABA neurons in the lateral preoptic area,[373] as well as many other possible locations, innervate GnRH cells.

Perhaps more problematic is determining what effects these changes in GABA levels may have on GnRH neuron excitability. A study examining $GABA_A$ receptor–mediated synaptic events on GnRH neurons in the brain slice found that their frequency and amplitude increases in about one-third of GnRH neurons at the predicted time of the onset of the GnRH surge.[91] This temporal profile of changing $GABA_A$ receptor activity does not, however, fit with the in vivo microdialysis and push–pull analyses noted previously, and indicates that either: (1) the GABA release data are not an appropriate representation of GABA concentrations at the $GABA_A$ receptors expressed by GnRH neurons; or (2) that the brain slice is not providing a true reflection of GABA inputs in vivo. It is very likely that both of these possibilities are in play. Although the great majority of in vivo i.c.v. experiments indicate that $GABA_A$ receptor activation is inhibitory to LH secretion,[3] it needs to be borne in mind that i.c.v. drug administration is a very blunt tool for probing neuronal circuitry in the brain. Equally, the GnRH neuron in the brain slice suffers from substantial deafferentation, with all distant GABAergic inputs to GnRH neurons being absent. The final solution to this issue seems likely to require better experimental models of the GnRH neuronal network. For example, the use of thick hypothalamic "chunk" rather than a thin brain slice has shown that the effects of GABA on GnRH neurons are dependent upon how much of the GnRH neuron soma and dendron exist in the preparation.[89] Ultimately, however, this issue may only be resolved in vivo. Interestingly, a mouse model in which 70–90% of $GABA_A$ receptor signaling was deleted selectively from GnRH neurons exhibited normal positive feedback.[394]

The GnRH neurons also express $GABA_B$ receptors,[366,367] and GABA release activated from the RP3V can, at higher stimulation frequencies, inhibit GnRH neuron activity through the $GABA_B$ receptor.[92] Thus, there may be a fine balance between the amount of GABA released and whether it activates stimulatory $GABA_A$ receptors and/or inhibitory $GABA_B$ receptors. In addition, it is now recognized that $GABA_B$ receptors exert a potent inhibitory presynaptic influence on RP3V terminals synapsing on GnRH neurons.[366] Intriguingly, the potency of these $GABA_B$ receptors declines substantially at proestrus, thereby maximizing the influence of RP3V inputs on GnRH neurons at this time.[366]

Kisspeptin. The RP3V kisspeptin neurons have all the characteristics required of a neuronal phenotype involved in the positive feedback mechanism. These cells project to GnRH neurons and, when activated at >5 Hz stimulation frequencies, release kisspeptin to initiate prolonged GnRH neuron firing.[92] The RP3V kisspeptin neurons are highly sexually dimorphic, being 20-fold more plentiful in females,[440,475] express ERα,[441,480,481] and exhibit estradiol-regulated currents determining their intrinsic

excitability.[478] On the basis of cFos expression, RP3V kisspeptin neurons are activated at the time of surge onset in parallel with that of the GnRH neurons.[441,480,481] It is noteworthy, however, that recent electrophysiological studies of RP3V kisspeptin neurons did not find a significant change in kisspeptin neuron firing rate at the time of the surge in the acute brain slice.[496] Again, this may be due to the deafferentation of the kisspeptin neurons in that preparation. Finally, although not selective for the RP3V, mice with global mutations of kisspeptin or GPR54 fail to exhibit positive feedback,[481] as do mice with ERα deleted from all kisspeptin neurons.[482]

In summary, a variety of investigations now provide solid evidence that estrogen positive feedback in rodents originates primarily from classic transcriptional activity of ERα within RP3V neurons. While an important role for kisspeptin neurons has been delineated in transmitting this excitatory stimulus to GnRH neurons, it is likely that other transsynaptic inputs are also involved. As yet, there is a relatively poor correlation between the firing rates of GnRH neurons measured in the acute brain slice with the presumed massive increase in GnRH secretion within the median eminence of the mouse. The ERα-expressing cells of the RP3V provide a convenient locus for the integration of estradiol and circadian stimuli within the network, but precisely how this may occur remains a mystery. Outside of direct transsynaptic modes of action for positive feedback, there is also compelling evidence for estradiol-dependent morphological rearrangements involving the GnRH nerve terminals, although their relative importance to the generation of the GnRH surge remains to be established.

Estrogen Negative Feedback

Compared with the positive feedback mechanism, where there has been significant progress over the last decade, the mechanisms underlying estrogen negative feedback remain unclear. Indeed, the potent negative feedback actions of estradiol at the pituitary gland have made it difficult to establish whether there is a significant role for the GnRH neurons in this mechanism. Nevertheless, by measuring GnRH secretion directly in a variety of species, it has been possible to show that bolus estradiol administration to gonadectomized animals results in a relatively rapid (1–2 h) suppression of GnRH secretion[632,770,800] (Figure 11.9). These changes principally involve a decrease in GnRH pulse amplitude although changes in frequency have also been observed.[637,800,801] In rats, the opposite strategy of examining the effects of gonadectomy on GnRH secretion has also been assessed. The results of these studies indicate that the time following gonadectomy is critical as male rats gonadectomized for over 1 month showed no difference in GnRH secretion[630] whereas rats gonadectomized for 1 or 4 days exhibited significant increases in GnRH pulse frequency and/or amplitude.[633,802]

Brain Sites and ER Isoform Studies

The brain sites at which estrogen negative feedback occur have been difficult to elucidate. In rats, estradiol implants within the vicinity of the GnRH cell bodies in the preoptic area[803] as well as within the MBH[804–806] have been shown to suppress LH secretion. Although some of the MBH results may have been confounded by diffusion of estradiol to the pituitary,[807] the suppressive effects of estradiol within the preoptic area do not agree with observations that antiestrogen implants or lesions of this area do not prevent negative feedback.[711,776,779,780] Thus, it remains unclear where within the brain estradiol acts to suppress GnRH release in rodents.

Investigations employing mouse genetic models have been used to determine the ER isoform involved in estrogen negative feedback in the mouse. As for positive feedback, ERα appears to be the major receptor involved. Basal LH levels in intact ERαKO mice are similar to those of OVX wild-type mice and do not suppress following estradiol treatment.[739,808] This clearly suggests a major role for ERα in negative feedback somewhere within the pituitary and/or GnRH network. Both the pituitary- and neuron-specific ERαKO mouse lines can exhibit normal basal LH levels, indicating that one system may compensate for the deletion of the other.[477,809] The modest suppressive influence of gonadal steroids upon GnRH mRNA expression is also absent in ERαKO mice,[734] indicating that ERα-dependent elements are also involved in the suppression of GnRH biosynthesis.

Importantly, it appears that ERα acts in both a classical and nonclassical manner to suppress LH secretion. The generation of a mouse line in which ERα is no longer able to bind to consensus DNA estradiol response elements revealed that approximately 50% of estradiol-induced LH suppression resulted from classical ER signaling while the other half utilized a different mechanism.[774] In contrast to ERα, the ERβKO mouse exhibits normal[739] or only modestly impaired[734] LH levels and normal GnRH mRNA negative feedback.[734] Thus, at present, the role of ERβ, and ERβ expressed by GnRH neurons, in estrogen negative feedback cannot be excluded, but appears to be relatively minor compared with ERα.[810]

GnRH Neuron Electrophysiological Studies

Several GnRH neuron electrophysiological studies have been undertaken by the Moenter group examining the firing rates of GnRH neurons at the time of negative feedback. Initially this group examined GnRH neurons in a standard ovariectomized, estradiol-treated mouse model where recordings were undertaken in the afternoon 7 days after ovariectomy and estradiol/oil replacement. Although finding that overall mean GnRH

neuron firing rates were not different between ovariectomized and estrogen negative feedback mice, a significant difference was detected in the patterning of firing with estradiol-treated mice having longer and more frequent silent periods.[99] A further study in this model revealed that estradiol regulated the expression of two different potassium channels, although in such a way that it would contribute to increased, rather than decreased, GnRH neuron excitability at a time of negative feedback.[109] It has later been suggested by the authors that the timing of this model may in fact reflect estrogen positive feedback mechanisms.[98]

Subsequent studies by the Moenter laboratory have used an estradiol-treated, ovariectomized mouse model that uses constant high estradiol levels to generate an LH surge every day. Caution needs to be taken in interpreting the results of these investigations as circadian factors are the key determinant of dissimilarities between morning and afternoon recordings, rather than the normal differences in circulating estradiol levels found across the 4–5 day estrous cycle. Under these circumstances, "morning recordings" show GnRH neurons to have a significantly lower mean firing rate than "afternoon recordings," and this is dependent upon amino acid transmission and classical ERα signaling.[98,726,785] However, comparisons between mean firing rates in the morning from ovariectomized oil- and estradiol-treated animals revealed no significant differences, indicating little correlation between GnRH neuron firing and the known high and low LH levels at these times.[98] Interestingly, despite having similar mean firing rates, the frequency of ionotropic glutamate receptor-mediated events in GnRH neurons was markedly reduced in morning recordings of estradiol-treated mice compared with ovariectomized controls.[370] This suggests that glutamatergic inputs are modulated by estradiol-dependent circadian factors in this mouse model.

Direct GnRH Neuron and Rapid Estradiol Actions

The mechanisms through which estradiol restrains GnRH pulse frequency and amplitude to help achieve negative feedback may well incorporate all three modes of estradiol action: direct, indirect transsynaptic, and indirect nonneuronal. In relation to direct actions of estradiol, the genes regulated by ERβ in GnRH neurons are unidentified and, as such, the direct classical actions of estradiol on these cells are unknown. The only mechanism established to date to occur directly at the GnRH neuron involves the ERβ-dependent phosphorylation of CREB.[49,743,750] A series of studies have shown that estradiol acts through ERK1/2 and other upstream kinases to increase CREB phosphorylation within 15 min in GnRH neurons in vitro and in vivo.[750] This effect was abolished in both the GnRH neuron-specific ERβKO mouse in vivo and by synaptic transmission blockers in vitro, indicating that estradiol may act through two dependent parallel pathways involving ERα/ERβ synaptic input as well as ERβ in GnRH neurons to phosphorylate CREB.[750] It was recently reported that intact GnRH neuron-specific CREB knockout mice exhibit normal basal levels of LH alongside moderately disordered estrous cycles and an inability of estradiol to acutely suppress LH secretion in OVX animals.[49] This suggests that direct estradiol-dependent CREB phosphorylation in GnRH neurons may be involved in aspects of negative feedback that underlie the acute suppression of LH secretion following bolus administration of estradiol. This rapid, nonclassical mechanism of estradiol action may, nevertheless, occur persistently throughout the estrous cycle.[743,750]

Other rapid actions of estradiol on GnRH neurons have been reported, but it remains unknown whether they are direct actions and/or what their role may be in the nonclassical[774] aspects of estrogen negative feedback. Acute treatment with physiological low pM concentrations of estradiol was not found to have any effects on the intrinsic properties of GnRH neurons but did impact upon GABA and glutamate signaling to these cells in an ERα- and ERβ-dependent manner.[746] Another study demonstrated that high nM estradiol concentrations activated an atypical membrane ER that utilized PKC and PKA signaling to ultimately enhance potassium ATP channel activity in GnRH neurons.[744] A further report has shown that low nM estradiol modulates L-type and R-type voltage-gated calcium channels in GnRH neurons through unknown mechanisms involving ERβ and GPR30, respectively.[117]

Several reports from embryonic GnRH neurons demonstrate that estradiol enhances the frequency of calcium transients, suggesting that acute estradiol enhances GnRH neuron burst firing in these preparations.[748,749] It is unclear how this might correlate with estrogen negative feedback. Recent studies have also found that acute estradiol can also enhance calcium transients in GnRH neurons from adult mice.[745] However, in this case, it is interesting to find that the acute effects of estradiol in adult GnRH neurons were state-dependent such that silent cells were activated and spontaneously active neurons were inhibited.[747] This indicated an overall tendency for acute estradiol to "even out" activity patterns within the GnRH neuron population. What these different GnRH neuron populations may represent functionally is unknown.

Finally, it is important to note that other signaling cascades may also be involved in the acute negative feedback network action. P21-activated kinase in the preoptic area is rapidly phosphorylated by estradiol in a nonclassical mechanism, and acute estradiol suppression of LH is blocked by a p21-activated kinase antagonist given ICV.[811]

Indirect Transsynaptic Actions

Evidence that negative feedback can be initiated remote to the GnRH neurons and is dependent upon ERα suggests the presence of indirect transsynaptic modes of estrogen action. As noted, the ERα-expressing primary

afferent inputs to GnRH neurons are located predominantly in the RP3V and arcuate nucleus while those for ERβ are unknown. The absence of a marker (like cFos for positive feedback) indicating those neuronal populations that have altered activity at the time of negative feedback has greatly hindered progress in identifying the relevant neuronal populations within the network. Over the years, many different neurotransmitter systems have been implicated in the negative feedback mechanism.[229] Nevertheless, two neurochemical phenotypes have more evidence than most as being potentially important: preoptic GABAergic neurons and arcuate kisspeptin cells.

The GABAergic neurons of the rodent preoptic area are sensitive to fluctuations in circulating estradiol levels,[755] and GABA neurons in the RP3V and lateral preoptic area project directly to GnRH neurons.[92,373] Preoptic GABAergic neurons express ERα, and estradiol has been shown to increase GABA release, GABA reuptake mechanisms, and $GABA_A$ receptor subunit expression in the preoptic area (for review, see Ref. 755). Thus, in the presence of estradiol, GABAergic neurotransmission within the preoptic area is increased, and, accordingly, it has been hypothesized that estradiol acts to enhance the activity of GABAergic primary afferents to the GnRH neurons to suppress their electrical firing. This concept is supported by: (1) evidence for an inverse correlation between endogenous GABA levels within the vicinity of the GnRH cell bodies and circulating LH levels;[812–814] (2) activation of preoptic $GABA_A$ receptors resulting in the suppression pulsatile LH secretion in OVX animals;[395,396,398] and (3) the pharmacological blockade of $GABA_A$ receptors elevating LH levels in OVX estradiol-treated rats.[815] However, mouse electrophysiological studies have found that $GABA_A$ receptor activation exerts predominant excitatory, and not inhibitory, actions on GnRH neurons.[365] This discord between "inhibitory rat in vivo" and "excitatory mouse in vitro" might be explained, in part, by GABA actions at $GABA_B$ receptors, in addition to the likelihood that excessive $GABA_A$ receptor activation in vivo may well depolarize GnRH neurons into a state where they are unable to fire action potentials and thus become inhibited.[365]

One curious result has come from mice in which 70–90% of $GABA_A$ receptor signaling was deleted selectively from GnRH neurons. These animals show normal fertility but have a markedly enhanced rise in LH following ovariectomy and increased sensitivity to acute estradiol negative feedback.[394] How this apparent increase in estrogen negative feedback sensitivity comes about is not known. It does, however, suggest that the network may compensate for the $GABA_A$ receptor loss by generating a different type of negative feedback mechanism. While such observations provide evidence that a $GABA_A$ input to GnRH neurons is probably involved in the normal negative feedback mechanism in mice, precisely what these GABA afferents do is unknown.

There is presently substantial enthusiasm for the possibility that kisspeptin neurons in the rodent ARN are a site of estrogen negative feedback. Ovariectomy increases arcuate Kiss1 mRNA expression, and estradiol treatment returns transcript levels to normal in mice and rats.[476,480] As such, it was hypothesized that estradiol suppresses an excitatory arcuate kisspeptin drive to the GnRH neurons.[816] In other words, ovariectomy allows the emergence of the arcuate kisspeptin cells to drive GnRH neurons to generate high LH secretion. It is established that almost all arcuate kisspeptin neurons express ERα and that estradiol utilizes ERα in a nonclassical manner to regulate Kiss1 mRNA levels in these cells.[476,817] This hypothesis has received support from studies showing that the postovariectomy increase in LH secretion was reduced in GPR54 knockout mice[471] as well as in rats with arcuate nucleus-specific, saporin-deleted, kisspeptin neurons.[495] However, as noted in the Kisspeptin section Neuronal Elements, estradiol was still able to suppress LH secretion in both of these experimental models. This may indicate that arcuate kisspeptin neurons are more important for GnRH neurons to exhibit the pattern of activity required to drive the high amplitude, high frequency LH secretion of ovariectomy. Recent electrophysiological studies examining arcuate kisspeptin neuron firing in the acute brain slice have also questioned the kisspeptin estrogen negative feedback hypothesis as gonadectomy resulted in even lower firing rates than the already low levels found in intact mice—a result opposite to that predicted by the model.[496] Thus, at present, the estrogen negative feedback role for arcuate kisspeptin neurons in the rodent requires further evidence.

In summary, investigations to date have emphasized the role of ERα in the estrogen negative feedback mechanism, but the brain sites and neurochemical identity of the cells involved in this process remain obscure. It seems likely that negative feedback is multimodal in nature with estradiol exerting both classical and nonclassical actions over different temporal time frames to directly and indirectly suppress GnRH pulse frequency and amplitude. There is evidence for a modest rearrangement of GnRH neuron firing pattern, rather than firing rate, associated with negative feedback, although better mouse models need to be developed to address this further. Whereas some rapid, nonclassical estradiol actions may occur directly at the GnRH neuron and be partly responsible for acute estradiol suppression, the physiological significance of most remain unclear. It seems highly likely that ERα-expressing transsynaptic inputs are involved in sensing and transmitting information on circulating estradiol levels to GnRH neurons, although there is actually rather little compelling evidence supporting a role for any specific neuronal phenotype in negative feedback. Finally, glial cell structural rearrangements involving GnRH neurons could be envisaged to

provide a relatively stable form of negative feedback, but, again, evidence for this remains to be gathered.

Progesterone

Progesterone exerts an important regulatory influence on the activity of GnRH neurons at several different time points. Foremost, the elevated levels of circulating progesterone during the luteal phase exhibit a potent suppressive influence upon GnRH and LH secretion in spontaneously ovulating species including humans.[818–820] The principal role of this luteal-phase increase in progesterone may be to prevent GnRH surges on the days subsequent to ovulation.[821] In addition to this inhibitory action, it is also evident that progesterone can enhance the time of onset and amplitude of the GnRH/LH surge in rodent and primate species.[822–824] There has, however, been some debate as to precisely when circulating progesterone levels increase in relation to the onset of the LH surge.[825–827] It has been argued that this positive feedback effect of progesterone may, instead, originate from production by astrocytes within the brain.[828]

Modes of Progesterone Action

Recent discoveries have greatly expanded the potential modes of progesterone action on cells. Classic genomic regulation, as well as rapid effects, arise through the progesterone receptor (PR) isoforms PR_A and PR_B, while rapid actions of progesterone can also occur through at least two different classes of progesterone-binding receptors: progestin/adipoQ (PAQR) receptors (sometimes called *membrane PRs*) and progesterone receptor membrane component 1 (PgRMC1).[829] In addition to these receptors, progesterone is also rapidly metabolized in the brain to neuroactive steroids that allosterically modulate the activity of a variety of neurotransmitter receptors.[830,831]

In terms of direct actions on GnRH neurons, it seems that these cells do not express appreciable amounts of the classic PRs. Immunocytochemistry studies have failed to detect PR in the sheep[832] and monkey,[833] although a small population of medially located GnRH neurons were observed to express PR immunoreactivity in the guinea pig.[834] A recent study of cultured nasal placode GnRH neurons has, however, demonstrated that all embryonic GnRH neurons express mRNA and protein for PgRMC1 but not PR or PAQRs.[835] That study also showed that a subpopulation of adult GnRH neurons in the mouse expressed PgRMC1 immunoreactivity.[835] In terms of neurosteroids, the 5α-pregnan-3α-ol-20-one (allopregnanolone) metabolite is known to directly modulate the activity of $GABA_A$ receptors expressed by GnRH neurons,[836,837] implicating a role for glial cell neurosteroid synthesis in transmitting progesterone feedback to GnRH neurons.

In addition to direct actions, it seems likely that progesterone modulates GnRH neuron activity through transsynaptic inputs. Progesterone receptors have been detected in many hypothalamic populations including glutamatergic,[838] GABAergic,[833,838,839] dopaminergic,[840,841] and neuropeptidergic[842–844] cells, as well as brainstem NE neurons.[845,846] However, as yet, the PR-expressing neurons that project directly to the GnRH neurons are not known.

Progesterone Negative Feedback

Evidence to date suggests that progesterone utilizes a combination of genomic and rapid actions to suppress LH pulsatility during the luteal phase. Investigations by Skinner and colleagues in the ewe have shown that progesterone can act relatively quickly (~50 min) to suppress GnRH pulse frequency and that this requires the presence of estradiol-induced PRs.[847] Investigations in the mouse suggest a mixture of modes of progesterone action as intact PR knockout mice exhibit elevated LH levels despite maintaining rapid suppressive effects of progesterone.[848,849] The evidence for PgRMC1 in adult GnRH neurons and demonstration that 10 nM progesterone rapidly suppresses calcium peaks in about 50% of embryonic GnRH neurons[835] provides one mechanism for rapid, direct suppression of GnRH neuron activity. It is of note, however, that high 1 μM concentrations of progesterone were not reported to alter the excitability of adult mouse GnRH neurons.[836]

Classic PR signaling may suppress GnRH pulse frequency through any one of a number of different transsynaptic inputs. Investigators have shown a role for endogenous opioid peptides in progesterone negative feedback,[637,850–852] and work in the sheep has also highlighted the potential importance of PR-expressing dynorphin (i.e., KNDy) neurons within the arcuate nucleus within the GnRH network in this species.[281,842,853] There is also evidence to suggest that opioid peptides are involved in the progesterone-induced suppression of LH secretion in humans.[854]

The direct and rapid modulation of GnRH neurons by progesterone metabolites derived from nearby glial cells may also play a role in suppressing pulsatile LH secretion during the luteal phase of the cycle. Allopregnanolone has been reported to suppress GnRH secretion,[855] LH release,[856] and ovulation[857] in the rat, but has no effects on LH in the mouse[849] or sheep.[847] Further confusion about the precise roles of allopregnanolone comes from the observation that the rapid allosteric modulation of $GABA_A$ receptors would be expected to enhance, rather than suppress, GnRH neuron excitability in the mouse.[365]

Thus, as for estrogen negative feedback, progesterone negative feedback during the luteal phase seems likely to result from a combination of direct and indirect

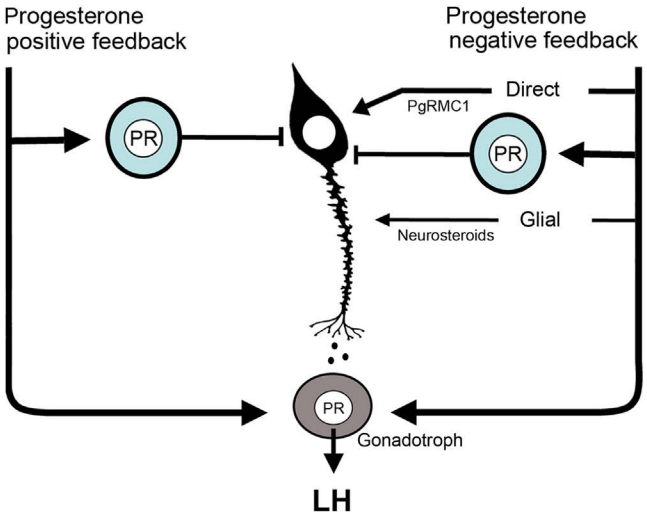

FIGURE 11.11 Model of progesterone feedback actions. Schematic diagram showing proposed modes of progesterone action within the GnRH neuron network for positive and negative feedback. Abbreviations: PR, progesterone receptor; PgRMC1, progesterone receptor membrane component 1.

transsynaptic and glial mechanisms involving both classical transcriptional and nonclassical modes of action (Figure 11.11).

Progesterone Positive Feedback

Progesterone can advance the time of onset of the GnRH/LH surge and also greatly magnify its amplitude in rodents and primates.[822–824,858] Both of these effects have a critical requirement for estradiol-induced PRs[848,859–861] suggesting that classical mechanisms of estradiol-dependent gene transcription are involved. Further, PR antagonists given before the onset of the proestrous or estradiol-induced LH surge result in its marked attenuation.[860,862,863] While the previous evidence indicates a predominant classical PR mode for progesterone positive feedback, it is noteworthy that progesterone can exert rapid stimulatory effects on GnRH secretion,[864] and the direct allopregnanolone actions on GnRH neurons may be excitatory (see previous discussion).

However, a major difficulty in understanding the physiological significance of these stimulatory effects of progesterone has revolved around the lack of evidence for significant circulating progesterone secretion before the onset of the endogenous GnRH/LH surge.[272,825–827] Two solutions to this issue have been proposed. The first involves the proposition that follicular-phase estradiol acts in a nonclassical manner to induce astrocytes to secrete progesterone de novo within the brain (astrocrine hypothesis) and thereby provides sufficient presurge progesterone exposure within the GnRH network in the absence of significant circulating levels of progesterone.[828,865] The second solution hypothesizes that ligand-independent PR activation may underlie the stimulatory roles of PRs in the surge generation.[696] In that model, the rise in follicular-phase estrogens initiates or enhances PR expression in RP3V cells that then enables them to be transactivated by stimuli other than progesterone to help initiate the surge.

The neurochemical identity of neurons within the GnRH neuron network involved directly in the transsynaptic activation of the GnRH neurons by progesterone is not clear. Again, however, endogenous opioid peptides have been favored as having a key role. There is abundant evidence that progesterone can facilitate the estradiol/circadian-induced decline in both β-endorphin levels and opioid receptor expression prior to the onset of the LH surge in the rat, thereby enhancing the disinhibition of the GnRH neurons.[866–873] It has also been suggested that the role of progesterone to suppress the occurrence of daily LH surges after ovulation results from the activation of opioid peptide signaling within the GnRH network.[858] One difficulty with this proposed pathway is that GnRH neurons in the rat do not express the appropriate opioid receptors[874] and rarely respond directly to β-endorphin or μ opiate receptor agonists in mice.[270]

Another progesterone-modulated transsynaptic input that has received recent attention is the NE input from the locus coeruleus.[558] In a recent study, both the preovulatory LH surge and rise in preoptic NE concentrations were greatly enhanced by progesterone treatment, and this was shown to originate from the locus coeruleus NE cells known to express PR.[558,846] Also, it is evident that the RP3V kisspeptin neurons implicated in estrogen positive feedback mechanism express PRs.[481] This indicates that progesterone facilitation of the GnRH/LH surge may well occur through the same kisspeptin pathway driving estrogen positive feedback.

Together, these investigations indicate the importance of classical estradiol-induced progesterone actions in the facilitation of the GnRH/LH surge. The source of progesterone and its targets within the GnRH neuronal network are currently being established. However, it is possible that progesterone simply utilizes the existing ERα positive feedback pathway to enhance activation of the GnRH neurons at the time of the surge.

An Integrated Model of Estrous Cycle Plasticity

Figure 11.12 provides an overview of a model bringing together estradiol, progesterone, and circadian influences within the network to generate estrous cycle plasticity in GnRH secretion.

It is apparent that the potent circadian input within the network occurs through at least two transsynaptic mechanisms: one direct to the GnRH neurons and one indirect pathway that regulates steroid-receptive inputs to GnRH neurons. The relative importance of these two

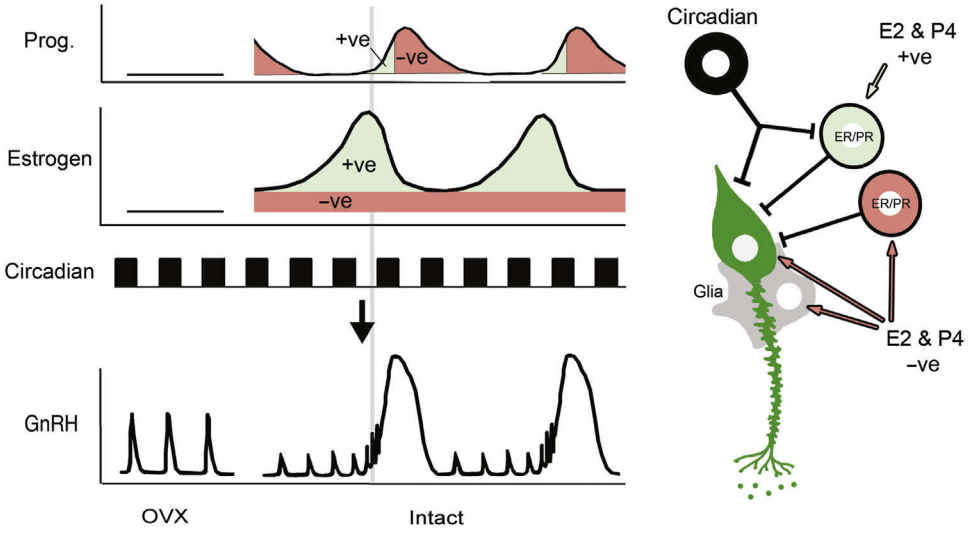

FIGURE 11.12 Model of integrated circadian, estradiol, and progesterone influences within the GnRH neuronal network. Left: Schematized profiles of circulating progesterone (prog.), estradiol, and the daily circadian signal are given above the profile of GnRH secretion in ovariectomized (OVX) and intact rats. In OVX rats, the secretion of estradiol and progesterone is minimal, and this reveals "free-running" high amplitude, high frequency GnRH pulsatility that is not strongly entrained by the circadian input in adults. In intact female rats, significant progesterone secretion is only observed coincident with the onset of the GnRH surge (light gray vertical line). Whereas the initial period of progesterone secretion exerts a stimulatory feedback effect (marked as +ve), further boosting estrogen positive feedback, the subsequent progesterone secretion exerts negative feedback actions (marked as –ve). Estradiol feedback is proposed to exist as two separate feedback mechanisms that run simultaneously; the positive feedback pathway is entrained by the rising estradiol levels of the follicular phase and "breaks through" the relatively constant negative feedback pathway (marked as –ve) once every cycle to generate the GnRH surge. The precise timing of the surge is entrained by circadian inputs (black boxes) to the positive feedback pathway. Right: Schematized model showing the nature of the different pathways within the GnRH neuronal network. Circadian inputs regulate GnRH neurons through direct and indirect pathways. Both estradiol (E2) and progesterone (P4) positive feedback act primarily through an ERα/PR-expressing transsynaptic mode. In contrast, the negative feedback actions of both estradiol and progesterone appear to be multimodal utilizing direct, transsynaptic and glial cell modalities to suppress the activity of GnRH neurons.

pathways is not yet clear. However, there is abundant evidence that circadian influences within the network are only clearly observed in the presence of estradiol, suggesting that the indirect pathway is prominent.

Estradiol is the principal driver of plasticity across the cycle, and it is proposed that the negative and positive feedback actions of estrogens are driven by separate neuronal/glial ensembles. Not only are the temporal dynamics of negative and positive feedback different but there is evidence that one can occur without the presence of the other.[875–877] Thus estradiol is not modeled as a negative–positive feedback "switch," as is commonly assumed, but as two separate pathways that are active in parallel. In essence, estrogen negative feedback is always present and relatively constant in impact, while the strength of the estrogen positive feedback pathway is totally dependent upon the threshold level of circulating estradiol. This means that the efficacy of positive feedback is directly related to circulating estradiol levels and, as such, it "breaks through" the negative feedback influence once every cycle driven by high estradiol levels of the late follicular phase. Thus, the ultimate impact on the GnRH neuron is the sum of the two pathways. There is abundant evidence that estrogen positive feedback represents a classical concentration-dependent, transcriptional mode of action, and that this likely resides within RP3V cells projecting to GnRH neurons.

The estrogen negative feedback pathway is proposed here to result from direct, transsynaptic, and glial cell modes of action. The multimodal nature of this pathway likely underlies the present difficulty in deciphering its components and precise characteristics. With several components using multiple different modes of communication and mechanisms of estradiol action, it may also explain the robustness of negative feedback from a physiological perspective. While positive feedback can be perturbed rather easily, defects in the suppressive influence of estradiol on LH secretion are rarely encountered.

Present evidence indicates that the mechanism and mode of progesterone positive feedback within the network looks much like that of estrogen positive feedback. In many ways it makes sense that progesterone should "piggyback" on the estrogen positive feedback transsynaptic pathway as, during the first few hours of the surge, it is required to generate the same stimulatory action on GnRH secretion. A subpopulation of the ERα-expressing neuronal afferents to GnRH neurons will likely be the same cells expressing estradiol-dependent PRs. Again, similar to estradiol, the negative feedback pathways appear to be complex

and involve multiple different signaling mechanisms and cell types. Whether there is actually any overlap between the estradiol and progesterone negative feedback pathways is unclear, but given the different temporal domains that they are working within, this may be less likely.

Brought together, this simplistic model of circadian, estradiol, and progesterone mechanics can relatively easily explain the marked plasticity of the network across the cycle. Studies over the coming years will no doubt substantiate or invalidate parts of the model.

CONCLUSION

This review has documented the very substantial progress that has been made in the field of GnRH neurobiology over the last decade and how this has built upon our existing knowledge. With respect to the GnRH neurons themselves, the ability to now examine the electrophysiological properties of native cells with relative ease in vitro has provided a whole new dimension of detailed information as to what makes the GnRH neuron active. Perhaps surprisingly given the decades of prior research, the morphology of the GnRH neuron has also evolved further in recent years, again made possible by developing new ways of looking at these cells. In contrast, our understanding of the biosynthesis of GnRH is relatively mature and has not advanced to the same extent in recent times.

While there is every reason to be optimistic about making substantial future advances in understanding the GnRH neuronal phenotype, the larger and more daunting challenge will be to make headway in deciphering the functioning of the GnRH neuronal network, in particular, how the network functions together to generate a pulse of GnRH and how the gonadal steroid negative feedback mechanism works. It is likely that little further will be gained by measuring changes in LH following third ventricular administration of yet another neuropeptide or transmitter. Rather, the challenge is to develop technologies that can interrogate different components of the GnRH neuronal network in a selective manner. This will mean developing new techniques tailored to address the actual key questions rather than simply reusing old, familiar approaches to generate data. The neuroendocrine field has an excellent pedigree in driving technical innovation, and new methodologies may come from within the community or by adapting advances elsewhere in neuroscience. The considerable resources, risks, and creativity required to develop these new approaches will likely require collaborative efforts between the various GnRH neurobiology laboratories to facilitate any rapid progress.

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CHAPTER

12

Hypothalamic Control of Prolactin Secretion, and the Multiple Reproductive Functions of Prolactin

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INTRODUCTION

Prolactin is an anterior pituitary hormone that is essential for the process of lactation, and it is this function from which its name is derived. In addition to this critical role in reproduction, however, prolactin is also known to have many other actions in the body. A seminal review from Charles Nicoll published in 1974[1] listed 85 different actions of prolactin; this list was updated and expanded by Paul Kelly and colleagues in 1998,[2] and no doubt could be expanded further. It has now become almost a cliché to describe prolactin as having over 300 distinct biological actions. The breadth of potential functions is conceptually difficult to comprehend. In both the original and updated lists of prolactin actions, the various functions were subdivided into a number of different categories, of which reproduction was but one. Other functions included water and electrolyte balance, growth and development, endocrinology and metabolism, brain and behavior, and immunoregulation. The experimental evidence for each action is quite clear, but, when examined in isolation, many of the reported actions of prolactin do not appear to be essential. Hence, prolactin has been thought of as a pleiotropic adaptive hormone, having multiple distinct roles in the homeostatic regulation of many different processes, but not essential or irreplaceable for any of them (apart from lactation).

For the purposes of this chapter, we have taken a different approach. Rather than look at reproduction as one category out of many prolactin-responsive functions, we have considered how the multiple different actions of prolactin might, in fact, contribute to the one primary outcome of successful reproduction. In essence, rather than contemplate over 300 different actions of prolactin, we have asked the question "What are 300 things that need to change in a mammal to enable pregnancy and lactation?" When faced with a novel action of prolactin, for example in bone or the pancreas, we tried to think about why this tissue might need to change its function during pregnancy or lactation. In this context, an action on bone might be necessary to mobilize calcium for milk production, and metabolic effects of prolactin might balance glucose supply for support of fetal growth and maternal survival or conditions for lactation. While this might not completely account for all of the known actions of prolactin, it provides a useful contextual framework to think about the roles of prolactin in the physiology of reproduction. An important concept in considering the roles of prolactin in reproduction is that these functions are in fact mediated by multiple ligands (pituitary prolactin and the placental lactogens), acting through a common receptor.

The breadth of prolactin's functions likely originates from the evolutionary history of this molecule. While

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in mammals prolactin provides the critical signal that induces mammary gland development and milk production, the hormone predates mammals in evolutionary time. Thus, prolactin is present in fish, reptiles, amphibia, and birds, where it clearly has functions unrelated to lactation. It appears to have arisen as a duplication of an ancestral gene in early vertebrates, resulting in the formation of the prolactin and growth hormone genes.[3] These two paralogs have subsequently evolved quite distinct functions in modern vertebrates. In non-mammalian vertebrates, prolactin is involved in osmoregulation and fluid balance, regulating ion and water flux across epithelial layers in a variety of tissues.[4] It is also involved in regulation of the skin and integument. At least some of these actions are targeted toward the reproductive process. For example, in some birds, prolactin induces a nutritious secretion from the epithelial cells of the crop sac, adding components to food that is then regurgitated to feed their offspring. This is highly analogous to lactation, where mammals provide nutrition to their offspring through secretions from epithelial glands located in the skin. Prolactin also influences a number of reproduction-related behaviors, including migration in amphibia and nest building in birds. In mammals, the roles of prolactin appear to have specialized further, but may have a similar basis. Lactation generates a demand on fluid balance and a demand on metabolic processes for energy, and it requires behavioral changes in the mothers. It seems logical that the same signal that was co-opted by evolution to stimulate milk secretion would also be used to induce modifications in other processes to facilitate this key reproductive process.

In this chapter, we have focused on the broader role of prolactin in reproductive physiology. We have reviewed the hypothalamic control of prolactin secretion and its regulation at the level of the anterior pituitary gland. The different patterns of prolactin secretion are discussed, including the important role that placental lactogenic hormones play during pregnancy and the overall role of prolactin in reproduction. We have then reviewed the major reproductive functions of prolactin, both its actions in peripheral tissues and its actions in the brain to influence important reproductive behaviors. Within this section, there is a focus on the distribution of prolactin receptors, and identification of the cell types responding to prolactin. Finally, we have looked at emerging evidence for the involvement of prolactin in a range of reproductive disorders, ranging from infertility to neurological and mood conditions and a number of reproductive cancers. Because we have taken quite a different approach, we consider that the present chapter complements, rather than replaces, the authoritative chapters published in earlier editions of this book (e.g., Refs 5–7).

THE PROLACTIN RECEPTOR AND SIGNAL TRANSDUCTION

The prolactin receptor is a member of the cytokine family of receptors,[2] and its expression is widespread in the body, consistent with the pleiotropic roles of prolactin in reproduction and other functions. Before discussing the ligands in detail, we think it is useful to have a basic understanding of the signal transduction properties of this receptor. Later in this chapter, we will discuss tissue-specific receptor distribution when describing functions of prolactin. In this section, however, we will examine prolactin signal transduction pathways (with a summary diagram illustrating the major pathways shown in Figure 12.1).

The prolactin receptor is a single transmembrane-spanning protein without an intrinsic kinase domain, and signaling occurs via activation of receptor dimers. A number of different receptor isoforms has been identified, and each is formed by posttranscriptional processing of the prolactin receptor transcript.[8–11] Although multiple promoters and first exons have been reported,[12,13] the extracellular domain (coding sequence begins at exon 3) is common to all receptor subtypes and is well conserved among species.[14] The different first exons and associated promoter regions are involved in the tissue-specific expression of the receptor, enabling differential control of receptor expression in different tissues.[15] The transmembrane domain is also shared by all subtypes, apart from circulating "soluble receptors" that may be formed by alternative splicing of the primary transcript[11,16] or by proteolytic cleavage of the extracellular domain.[17] The transmembrane domain may be critical in the formation of ligand-free receptor dimers,[14] and this is potentially important in signal transduction mechanisms (discussed further in this chapter). There are multiple different intracellular domains that are formed by alternative splicing of the primary transcript.[8–11] Full signal transduction requires the "long form" of the receptor,[2] while a number of short forms have been described in different species, typically involving replacement of exon 10 of the gene with an alternative and different last exon. These short forms lack the tyrosine phosphorylation sites that form docking sites for Src homology 2 (SH2) proteins (discussed in this section), and therefore cannot activate many of the signaling pathways that are activated by the long form of the receptor. However, the short forms do retain some signal transduction capacity, and specific functions are beginning to be ascribed to these isoforms. There is some evidence that the short forms may act as regulatory molecules by forming heterodimers with the long form of the receptor and thereby blocking signal transduction,[18–21] but recent studies looking specifically at

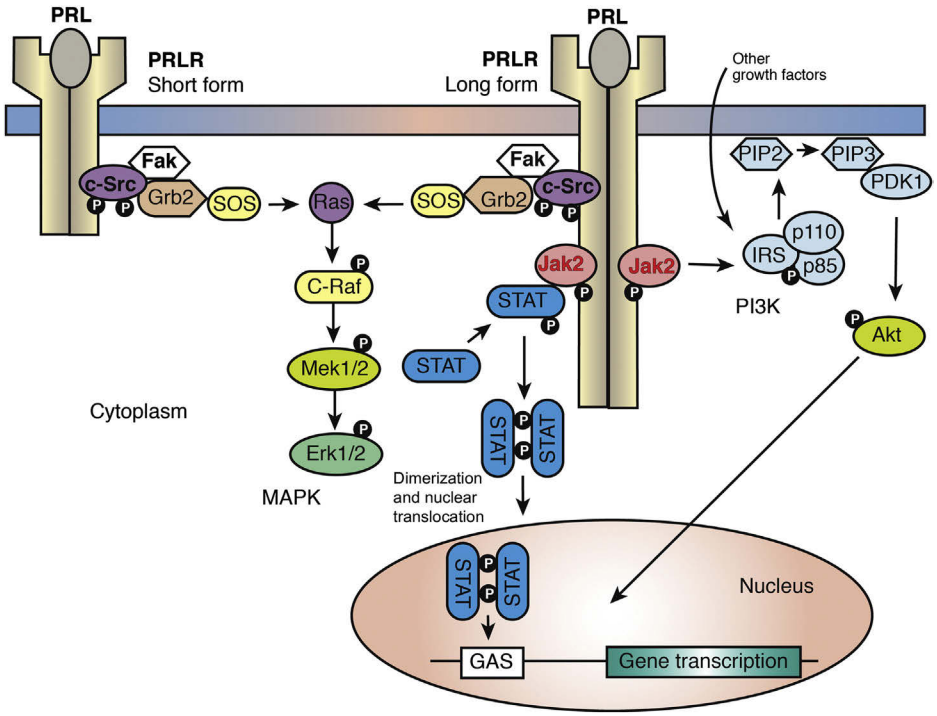

FIGURE 12.1 Diagrammatic representation of the multiple signal transduction pathways mediating prolactin action in different tissues (see the text for definitions of abbreviations). The major pathway mediating prolactin action is the JAK–STAT pathway, which is activated by the long form of the prolactin receptor. The short form of the receptor has a truncated intracellular domain that lacks the STAT binding residues but can activate the MAPK pathway in a manner that can be independent of JAK2. The long form may also activate the PI3K pathway or at least amplify responses through this pathway that are induced by other growth factors, such as insulin, IGF, and leptin.

the functions of the short forms have begun to identify roles for these isoforms that are distinct from those of the long form.[22–24]

Prolactin binds to two prolactin receptor molecules to form an active heterotrimeric receptor–ligand–receptor complex. Brooks recently reviewed the evidence suggesting that this complex is formed by a ligand-induced dimerization of two receptor molecules.[14] His group has demonstrated that binding sites on the prolactin molecule interact in sequence, with a receptor binding at "site 1" on the ligand inducing a conformational change that allows binding of a second receptor molecule to "site 2" on the ligand,[25] followed by a dimerization of the receptor molecules through interface of the two extracellular S2 subdomains.[14] An alternative view is that the receptors are present in the membrane as ligand-independent dimers.[26] Sequential binding of one receptor to site 1 and then the second receptor to site 2 induces a conformation change resulting in activation of the complex.[27] Regardless of whether the initial hormone binding is to monomers or dimers of the receptor, it is clear that binding must occur in this sequence[28,29] and that activation of signaling requires the full heterotrimer of one ligand plus two receptors[a]. Ligand binding induces a conformation change in the hormone-binding site that is communicated via allosteric interactions through the transmembrane domain to induce structural changes in the internal domain,[29] allowing autophosphorylation and activation of the receptor-associated Janus kinase 2 (Jak2). Jak2 phosphorylates the receptor, establishing docking sites of SH2 domain proteins (Figure 12.1). The major substrates for these sites are the signal transducer and activator of transcription (Stat) molecules, Stat5a and Stat5b.[2] Upon binding to the receptor, Stat5 is phosphorylated by Jak2, triggering the formation of transcriptionally active Stat dimers. Either homodimers or heterodimers of Stat5a/b then translocate to the nucleus, binding to specific sequences of the DNA to activate the transcription of target genes. Other SH2 domain–containing signaling proteins, such as Src, Fyn, and Tec, may also bind to this Jak2-activated site and participate in prolactin signaling. There are 10 tyrosine residues on the long form of the prolactin receptor, and the unique amino acid composition of these 10 sites within the

[a] Modeling of the ligand-induced structural changes underlying prolactin receptor signaling has informed the design of prolactin antagonists[30,31] and provided insight into the mechanisms underlying how some variants can act as both antagonist and agonist.[32] Such antagonists could be used as therapy for conditions such as breast or prostate cancer by counteracting the effects of activating mutations of the prolactin receptor or competing with locally produced prolactin (which is unresponsive to classic prolactin-lowering drugs such as bromocriptine).[33,34] Animal studies with these antagonists have been limited by their short half-life in vivo, but transgenic models with ubiquitous antagonist expression have been developed to demonstrate their ability to prevent prolactin-induced tumorigenesis.[35]

prolactin receptor will likely result in a unique subset of the 110-member SH2 domain family of proteins[36] to be activated in a particular pattern, generating the specific functional consequence of prolactin signal transduction in a cell.[14]

While extensive evidence demonstrates that Jak2 is critical to many of the functions of prolactin, particularly those involving activation of Stat signaling, prolactin has also been shown to activate multiple other signaling pathways, both dependent on and independent of Jak2 activation (Figure 12.1). For example, prolactin acting through Jak2 can induce phosphorylation of insulin receptor substrate 1 and 2 (IRS1 and IRS2, respectively),[37,38] providing docking sites for the p85 subunit of phosphatidylinositol-3-kinase (PI3K), resulting in activation of PI3K and its downstream biological effects. More recently, it was suggested that Jak2 does not initiate activation of Akt (a downstream target of PI3K) but greatly intensifies this through signaling cross-talk with other growth factor receptors.[39] Prolactin also activates the mitogen-activated protein kinases (MAPK) such as extracellular-regulated kinase (ERK) 1 and 2,[40,41] and this can occur in cells lacking functional Jak2,[39] possibly through activation of c-Src kinase independently of Jak2.[39,42] Thus, prolactin-induced signal transduction is complex, and different combinations of signal transduction pathways may be activated in different cell types. Future studies will be required to characterize the unique complex of downstream mediators activated in each tissue of interest.

Signaling through the JAK–STAT pathway is attenuated by members of a family of cytokine-inducible suppressors of cytokine signaling (SOCS) proteins.[43–45] These proteins are activated by cytokines, including prolactin, and then act as intracellular feedback inhibitors of signaling.[46,47] There are eight members of the SOCS family (Cis and SOCS1–7), and each has a different mechanism of action to suppress JAK–STAT signaling. For example, SOCS1 binds directly to Jak2 and inhibits its catalytic activity, while SOCS3 binds to specific regions in the intracellular domain of the cytokine receptor, preventing STAT binding.[48] The analysis of transgenic mice lacking SOCS genes has begun to shed light on their in vivo roles. Although *Socs1*$^{-/-}$ mice die perinatally due to immune dysregulation, the *Socs1*$^{-/-}$ mice can be rescued if bred on an interferon γ–deficient background.[49] These mice displayed enhanced mammary lobuloaveolar development during pregnancy and increased milk production. Moreover, deletion of a single copy of *Socs1* rescued lactational defects in mice that were heterozygous for a knockout of the prolactin receptor.[49] These data suggest an important physiological role for SOCS1 in limiting prolactin signaling. SOCS2 also functions as a physiological inhibitor of prolactin signaling. It is transcriptionally regulated by prolactin, and deletion of *Socs2* within the mammary gland rescues lactation in heterozygous prolactin receptor knockout mice.[50] SOCS3 is closely related to SOCS1 and is upregulated by prolactin in some tissues, but it appears to preferentially target STAT3 and hence may not be a major regulator of prolactin signaling.[48] In addition to the SOCS molecules, a number of other regulators of the JAK–STAT pathway have been identified, including the protein inhibitors of activated STATs (PIAS) and SH2-containing phosphatases.[51] While the full extent of the physiological roles of these proteins in prolactin signaling remains to be determined, it is clear that endogenous regulation of the JAK–STAT pathway is an important controlling mechanism that is likely to play a significant role in the reproductive functions of prolactin.

NEUROENDOCRINE CONTROL OF PROLACTIN SECRETION

Prolactin Secretion is Inhibited by Dopamine from the Hypothalamus

Unique among pituitary hormones, prolactin is predominantly inhibited by the hypothalamus and will be spontaneously secreted at high levels should hypothalamic inhibition be interrupted or if pituitary cells are grown in culture.[52,53] This was originally demonstrated when Everett observed that the rat pituitary could maintain the corpus luteum even after the pituitary stalk had been severed or after the transplantation of a pituitary gland under the kidney capsule.[54] As prolactin was known to be a luteotrophic hormone in rodents (as discussed here), these observations suggested the spontaneous secretion of prolactin from lactotroph cells of the pituitary gland without a requirement for input from the hypothalamus. Subsequently, it was shown that ectopic pituitary grafts could maintain lactation, again suggesting high levels of prolactin secretion without hypothalamic stimulation.[55] Repeating the classic experiments of Geoffrey Harris and Dora Jacobsohn that first proved humoral regulation of the pituitary gland by the hypothalamus,[56] Nikitovitch-Winer and Everett conducted a series of experiments in hypophysectomized rats, transplanting a pituitary gland either under the kidney capsule or back under the median eminence of the hypothalamus. They showed that while pituitaries located away from the hypothalamus would induce pseudopregnancy, a marker of elevated prolactin secretion in rodents (also discussed here), normal cycles would result when pituitaries were revascularized by portal vessels under the median eminence.[57] The use of ectopic pituitary glands, relieved of the hypothalamic inhibition of prolactin secretion, subsequently became a standard model of hyperprolactinemia.[58] With the

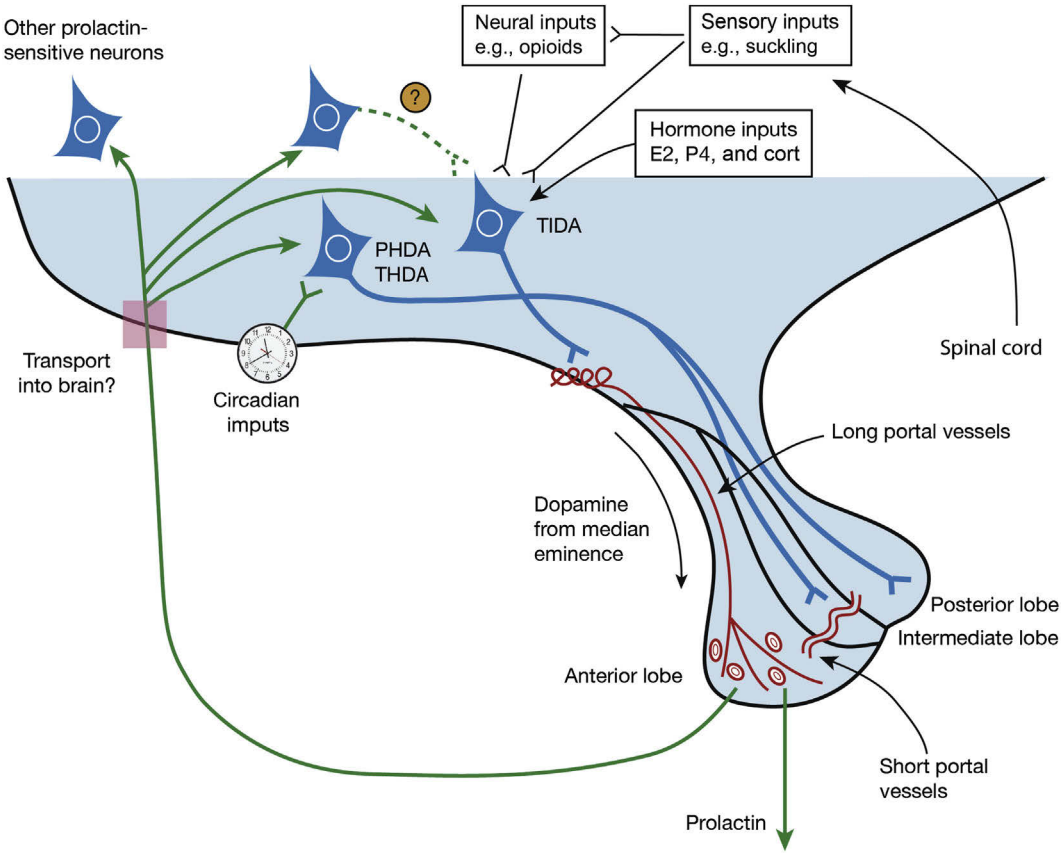

FIGURE 12.2 Neuroendocrine dopamine (NEDA) neurons in the hypothalamus, consisting of the tuberoinfundibular dopamine (TIDA) neurons projecting to the median eminence, and the tuberohypophyseal (THDA) and periventricular hypophyseal (PHDA) dopamine neurons projecting to the posterior lobe and intermediate lobe, respectively. These neurons regulate prolactin secretion, with dopamine reaching the anterior pituitary from the TIDA neurons via the long portal vessels, and from the THDA and PHDA neurons via the short portal vessels. Prolactin directly stimulates these three populations of neurons, resulting in short-loop negative-feedback regulation of its own secretion, as well as activating a number of other prolactin-responsive neurons in the brain. Multiple factors regulate prolactin secretion indirectly through altering activity of the NEDA neurons, including circadian, sensory, and neural inputs, as well as other hormones such as estradiol (E2), progesterone (P4), and corticosterone–cortisol (Cort).

development of prolactin radioimmunoassays in the early 1970s, it was confirmed that median eminence lesions[59] or severing the pituitary stalk[60] resulted in elevated prolactin secretion, consistent with the concept of a prolactin-inhibitory factor coming from the hypothalamus (Figure 12.2).

Two groups independently demonstrated that the hypothalamus released a prolactin-inhibitory factor that could prevent prolactin secretion from anterior pituitary cells in vitro.[61,62] The identification of this prolactin-inhibitory factor as dopamine, a catecholamine neurotransmitter, was controversial because all other hypothalamic hormones identified had been peptide hormones. Observations that drugs such as reserpine, which depletes endogenous dopamine, induced pseudopregnancy in rats[63] had provided the first clue, but it was assumed that the catecholamines were working as neurotransmitters in the brain by altering the release of a hypothalamic hormone.[64] However, about this time, Fuxe identified high levels of dopaminergic nerve terminals in the median eminence[65] consistent with the possibility of neurosecretion into the portal blood. MacLeod championed the view that dopamine was, in fact, a hypothalamic hormone, demonstrating that dopamine inhibited prolactin secretion from isolated pituitary glands and that dopaminergic agonists were also effective at suppressing prolactin secretion in vivo.[66] Dopamine was subsequently identified in the pituitary portal blood,[67] and an elegant series of studies showed that changing levels of dopamine in the portal blood could account for much of the variation in prolactin secretion from the anterior pituitary gland.[68–71] Dopamine receptors have subsequently been identified on lactotroph cells in the pituitary, with inhibition of prolactin mediated through the dopamine 2 (D2) receptor (D2R) subtype[72] (see the section Dopamine Inhibition of Lactotroph Function and Proliferation via the D2 Receptor on pituitary regulation). Mice lacking D2R are hyperprolactinemic.[73,74]

In the original studies of hypothalamic neurosecretion of dopamine, there was some suggestion that levels of dopamine detected in the portal blood were insufficient to account for the full inhibition of prolactin secretion. These studies involved depleting endogenous catecholamines with an inhibitor of tyrosine hydroxylase (TH, the rate-limiting enzyme involved in dopamine synthesis) and then infusing exogenous dopamine at the concentrations detected in the portal blood. Prolactin was suppressed by only about 60% with these "physiological" levels of dopamine, suggesting that there might be other prolactin-inhibitory factors.[71,75] However, it is now clear that these early experiments did not take into account an important additional source of dopamine reaching the anterior pituitary from the posterior pituitary gland by means of short portal vessels.[76] Evidence now suggests that prolactin secretion is regulated by three populations of neuroendocrine dopaminergic neurons[77]: the tuberoinfundibular (TIDA), tuberohypophyseal (THDA), and periventricular hypophyseal (PHDA) dopaminergic neurons. TIDA neurons arise from the dorsomedial arcuate nucleus and project to the external zone of the median eminence[78] (Figure 12.2). Dopamine diffuses into the capillaries of the pituitary portal blood vessels and is transported to the anterior pituitary. THDA neurons originate in the rostral arcuate nucleus and project in the hypothalamo-hypophyseal tract to the intermediate and neural lobes of the pituitary gland.[79,80] PHDA neurons arise more rostrally in the periventricular nucleus, with axons terminating solely in the intermediate lobe of the pituitary gland.[81] Dopamine from THDA and PHDA neurons contributes to basal regulation of prolactin secretion after transport to the anterior pituitary gland through short portal vessels from the neurohypophysis.[76] Together, these hypothalamic sources of dopamine are sufficient to account for all of the inhibition of prolactin from the pituitary gland. While they are anatomically distinct populations, most evidence suggests that they are functionally similar in terms of their regulation and pattern of dopamine release. Moreover, there is considerable overlap in their dendritic fields.[82] Hence, we support using the terminology introduced by Freeman et al.[83] to refer to these neurons involved in prolactin secretion collectively as the neuroendocrine dopamine (NEDA) neurons (Figure 12.2).

Characteristics of Neuroendocrine Dopamine Neurons

The NEDA neurons are typically described as being small parvocellular neurons approximately 20 μm in diameter, and they have 3–5 primary dendrites generating a relatively large dendritic field extending several hundreds of microns from the cell bodies.[82,84] Dendrites can be identified in both the coronal and sagittal planes, with more rostral and dorsal dendrites typically arranged in a dorsoventral orientation, while the more ventral dendrites tend to be oriented in parallel with the rostrocaudal plane. The axon principally originates off one of the main dendritic shafts and projects ventrally toward the median eminence, although some axons projecting elsewhere have also been described.[84] Within the median eminence, there are many large TH-immunoreactive nerve terminals (approximately 1 μm in diameter) in the vicinity of the primary capillary plexus of the portal blood system.[82] Recent electrophysiological studies of TIDA neurons, either in rats with post hoc identification[85] or in transgenic mice expressing green fluorescent protein (GFP) under the control of the TH promoter (TH–GFP)[86] (see Figure 12.3), have offered the opportunity to fill cells with a marker dye, revealing the full extent of the dendritic arborization. The dendrites can be seen to branch throughout the arcuate nucleus, with spiny processes visible that are indicative of extensive innervation, in contrast to the fine axonal processes arising off the primary dendrite and projecting ventrally to the median eminence.

Because of the scattered nature and mixed phenotype of hypothalamic neurons, electrophysiological studies of NEDA neurons have been difficult to conduct. There have been a few studies of unidentified cells in the arcuate nucleus that have been interpreted as "putative" TIDA neurons based on a variety of characteristics.[87–89] A more specific approach is to label the cells being recorded and then identify them by immunohistochemistry post hoc (e.g., Ref. 90). This is an accurate method, but it can be extremely time-consuming as many cells need to be examined and only a relatively small proportion will be the cells of interest. A recent study using this approach in prepubertal rats was able to identify a particular pattern of electrical oscillations that was unique to the TIDA neurons in the dorsomedial arcuate nucleus, allowing a thorough investigation of electrophysiological properties of these neurons.[85] This study described TIDA neurons exhibiting a robust oscillation between hyperpolarized and depolarized states, with periodicity of about 20 s and a spontaneous firing rate of approximately 4 Hz during the depolarized "upstate". Remarkably, the TIDA neurons were found to show a synchronous pattern of firing suggestive of an interconnected network, dependent on the functional expression of gap junctions.[85] It was proposed that this synchronous firing might provide optimal population output from the neurons to generate sufficient dopamine to inhibit prolactin secretion at the level of the lactotroph. An alternative approach to study TIDA neurons in mice has been to take advantage of transgenic technologies to label these neurons with a fluorescent marker. For example, TH–GFP mice[91] or mice expressing yellow fluorescent protein in dopamine transporter (DAT)-positive

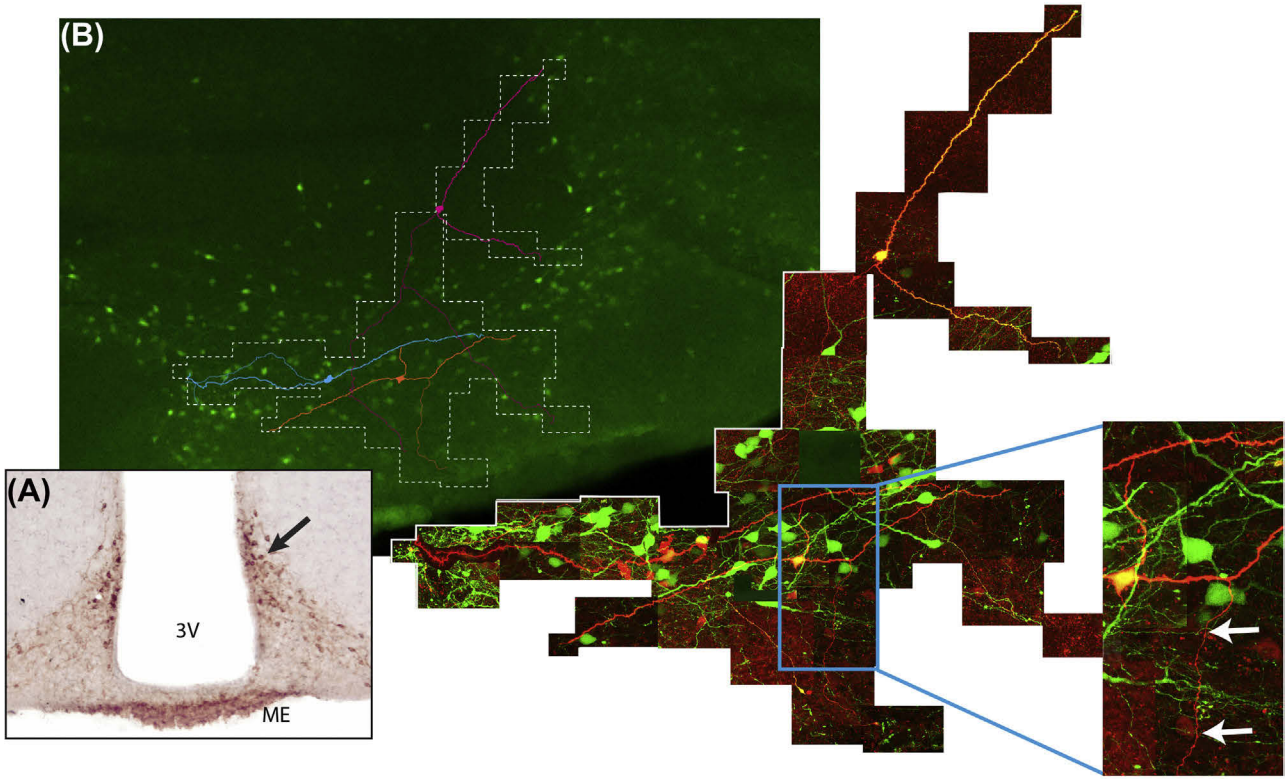

FIGURE 12.3 Neuroanatomy of the TIDA neurons. This figure is reproduced in color in the color plate section. Panel A shows a coronal section through the mouse hypothalamus, with TIDA neurons identified by tyrosine hydroxylase (TH) immunoreactivity (3V: third ventricle; ME: median eminence). Note the cell bodies in the dorsomedial arcuate nucleus (arrow), as well as strong staining in the nerve terminals in the external zone of the median eminence. Panel B shows a low-power sagittal view of the hypothalamus from a mouse in which green fluorescent protein is expressed under the control of the TH promoter (green). These neurons were visualized in a brain slice, and then three individual TH-positive neurons were filled with neurobiotin (red; but pseudocolored pink, blue and orange in the low power view), allowing visualization of the full extent of the nerve processes. The montage (right) is constructed from multiple high-power views obtained using a laser confocal microscope, illustrating the dentritic tree of three filled cells extending a long distance throughout the nucleus. In the enlargement (right), note the major dendrites coming off the cell body, identified by a wide diameter and multiple spiny processes. The thin, smooth axon (arrow) arises off a primary dendrite and projects down toward the median eminence in the base of the hypothalamus. *Source: Images provided by Drs Michel Herde and Allan Herbison, based on work published in Ref. 86.*

neurons[92] allow identification of NEDA neurons in a brain slice, and the firing patterns can be monitored in these models. The DAT model is potentially more specific as TH is expressed in all catecholamine neurons, whereas DAT is specific to dopaminergic neurons. In the arcuate nucleus, however, the only cells labeled in each case are dopaminergic neurons, the majority of which are NEDA.[84] In practice, studies using these approaches in mice have observed a similar pattern of firing to that seen in the rat, although only a small proportion of TIDA neurons showed the phasic oscillations in this model. It is possible that this approach allowed identification of additional NEDA neurons not detected in the rat study, whereas the oscillating cells are only a subset. Nevertheless, a major additional finding from the mouse work was the demonstration that patterns of firing of an individual TIDA neuron were reflected in the pattern of dopamine release from the population, as measured using in vivo amperometry in the median eminence (measuring dopamine by electrochemical detection of its oxidation induced at a specific voltage via a carbon fiber electrode).[92] These data support the concept postulated by Broberger and colleagues[85] that the neurons act as a synchronous network to release dopamine in a pulsatile or phasic fashion.

Short-Loop Negative Feedback

Concurrent with studies establishing the role of dopamine as a physiological prolactin-inhibiting factor, it was found that prolactin stimulated hypothalamic dopamine synthesis[93] and turnover,[94,95] increased metabolism in the median eminence,[96] and promoted dopamine secretion into the pituitary portal blood.[97] These observations identified the afferent limb of a classic negative-feedback mechanism regulating prolactin secretion (see Figure 12.2). Multiple studies in the subsequent two decades have confirmed the reciprocal relationship between prolactin and hypothalamic dopamine secretion. By comparison with other pituitary hormones that act on endocrine

tissue in the body, and for which feedback is provided through the secretions of those endocrine organs (e.g., gonadal steroids for gonadotropic hormones, or glucocorticoids for adrenocorticotrophic hormone), this has been termed "short-loop" feedback, because the pituitary hormone itself provides the feedback signal.

Prolactin acts directly on NEDA neurons, which express prolactin receptors.[98–100] Prolactin stimulates gene expression of TH in NEDA neurons[101] and also modulates the phosphorylation of TH, resulting in increased dopamine synthesis.[102] Dopamine synthesis and release are increased in response to acute or chronic increases in prolactin levels in blood,[103,104] while hyperprolactinemia results in suppression of dopamine secretion,[101] thus fulfilling the criteria to be a homeostatic regulatory system. Activation of NEDA neurons by prolactin requires activation of the JAK2–STAT5b signal transduction pathway.[105–110] As only the long form of the prolactin receptor can activate the JAK–STAT pathway, involvement of STAT5b in the prolactin regulation of NEDA neurons strongly implicates this isoform as the critical protein-mediating prolactin action, and this isoform has been specifically identified on TIDA neurons.[100] In addition to activating STAT5b, prolactin activates a range of other signaling pathways in TIDA neurons, including MAPK, and these pathways are also involved in regulation of dopamine synthesis through modulating the phosphorylation of TH, resulting in increased activity of this enzyme.[102] While the short form of the receptor cannot activate the JAK–STAT pathway, it can mediate some actions of prolactin through MAPK pathways.[111] The short form of the receptor is expressed in the arcuate nucleus of the hypothalamus[112,113] and could possibly influence TH phosphorylation. As described in this chapter, the short form of the receptor may act as a competitive inhibitor of prolactin action by forming inactive heterodimer receptor complexes with the long form,[18–21] thereby preventing activation of the JAK–STAT pathway. Interestingly, short-form mRNA levels in the arcuate nucleus increase during late pregnancy and lactation,[114] raising the possibility that an increasing ratio of short- to long-form prolactin receptors may contribute to a change in the responsiveness of NEDA neurons to prolactin at this time (see the section Prolactin Secretion during Pregnancy in Rodents on pregnancy and lactation).

Using biochemical indices of activity of TIDA neurons, a complex time course of prolactin action has been described, with a "rapid" component of increased activity observed 2–4 h after prolactin treatment[115] and a delayed component seen approximately 12 h after prolactin treatment.[104,116] The former component likely involves rapid, prolactin-induced serine phosphorylation of TH,[102] resulting in increased enzyme activity. These effects of prolactin are presumably mediated acutely by a range of other signaling pathways, including protein kinase C and MAP kinase.[102] The latter component requires protein synthesis,[117] consistent with a role for transcriptional signaling through the JAK–STAT5 pathway. Recent data from three groups have demonstrated very rapid actions of prolactin on the electrical activity of TIDA neurons in transgenic mice expressing fluorescent markers in dopamine neurons[90,91] or in prepubertal male rats, where TIDA neurons were identified by their characteristic phasic firing pattern.[118] These studies show that prolactin induced a fourfold increase in the firing rate within minutes of application, reversibly changing the firing pattern from a basal phasic pattern to a tonically active pattern. The prolactin-induced increase in firing was directly associated with an increased release of dopamine from the median eminence, measured by in vivo amperometry.[92] It seems unlikely that these rapid actions involve the JAK–STAT pathway, as this pathway is involved in transcriptional regulation (see Figure 12.1). Hence this indicates a physiological role for other pathways in mediating prolactin action in these cells. The rapid action of prolactin appears to involve two components: a low-voltage component from transient receptor potential (TRP)-like currents and a high-voltage component from inhibition of Ca^{2+}-dependent, BK-type K^+ currents. These components are mediated through distinct pathways, with the high-voltage component being wortmannin sensitive, suggesting an involvement of the PI3K pathway, whereas the low-voltage component is not.[118]

Interestingly, prolactin action through TRP channels has been previously reported to increase excitability in trigeminal sensory neurons[119] (specifically through TRP-V1 receptors). Prolactin-induced hyperalgesia may be mediated through local production of prolactin in response to tissue inflammation,[120] but this raises an interesting question about whether there might be a role for circulating prolactin in regulating sensory nociception during lactation.

Combined with earlier data on prolactin feedback, it is now apparent that multiple levels of signaling occur in the NEDA neurons (see Figure 12.4). It seems likely that the JAK–STAT pathway mediates transcriptional responses to prolactin, including an increase in mRNA expression for TH.[101,102] In addition to this transcriptional regulation, however, prolactin also increases the phosphorylation of TH through a pathway involving intracellular protein kinases,[102,121] leading to enhanced activity of the enzyme[122] and increased synthesis of dopamine. The data on electrical activity provide evidence for a third level of control in that prolactin also rapidly increases the firing rate of these neurons, resulting in increased dopamine secretion into the portal blood. Thus,

it would appear that TIDA neurons are regulated by prolactin through rapid and transient changes in electrical activity, slower but more prolonged changes in protein phosphorylation, and transcriptional events maintaining changes in gene transcription to regulate neuronal function over a much longer time course.

The complex, multilevel regulation of TIDA neurons by prolactin likely reflects the importance of maintaining tight control over the function of these cells. In terms of the transcriptional response, the TIDA neuronal population is highly sensitive to prolactin, such that a high proportion of neurons show phosphorylated STAT in their nucleus, when exposed to only basal levels of serum prolactin.[123] This is consistent with observations from neurochemistry studies showing that these neurons are tonically activated by basal levels of prolactin, with bromocriptine-induced suppression of prolactin leading to decreased activity.[101] Despite the fact that low levels of basal prolactin are sufficient to maintain a transcriptional response in TIDA neurons, the neurons remain responsive and are still able to show an acute change in firing rate following exposure to additional prolactin. The ability to rapidly respond to changes in prolactin levels and remain responsive throughout a range of prolactin concentrations may be critical to allow TIDA neurons to tightly regulate prolactin secretion through negative feedback.

Effect of Prolactin on Development of the TIDA Neuronal System

Prolactin plays a critical role in the development of the NEDA neurons. Mice that exhibit mutations of the transcription factors *Prop1* or *Pou1f1* (e.g., Ames dwarf or Snell dwarf mice, respectively) lack prolactin (along with growth hormone and thyroid-stimulating hormone (TSH)) and have markedly fewer NEDA neurons in their hypothalamus, as measured by TH immunoreactivity.[124,125] This deficiency develops postnatally, as the number of NEDA neurons present soon after birth is normal.[126] Treatment of these mice with prolactin during the neonatal period and up to puberty,[127] but not in adulthood,[128] rescues the NEDA neurons. NEDA neurons are present with functional connections from immediately after birth,[129] and thus these data suggest that prolactin or growth hormone is required to maintain NEDA neurons postnatally, and that without exposure to prolactin these neurons are lost. Interestingly, transgenic mice that specifically lack prolactin do not exhibit the same loss of NEDA neurons,[130] although they do show a loss of dopamine production,[131] suggesting that some sort of compensation during development is able to make up for the lack of prolactin. It is likely that the presence of growth hormone in these models can account for this compensation during development.[132] While treatment with prolactin in the adult was initially reported as ineffective at rescuing the NEDA neurons, subsequent studies using

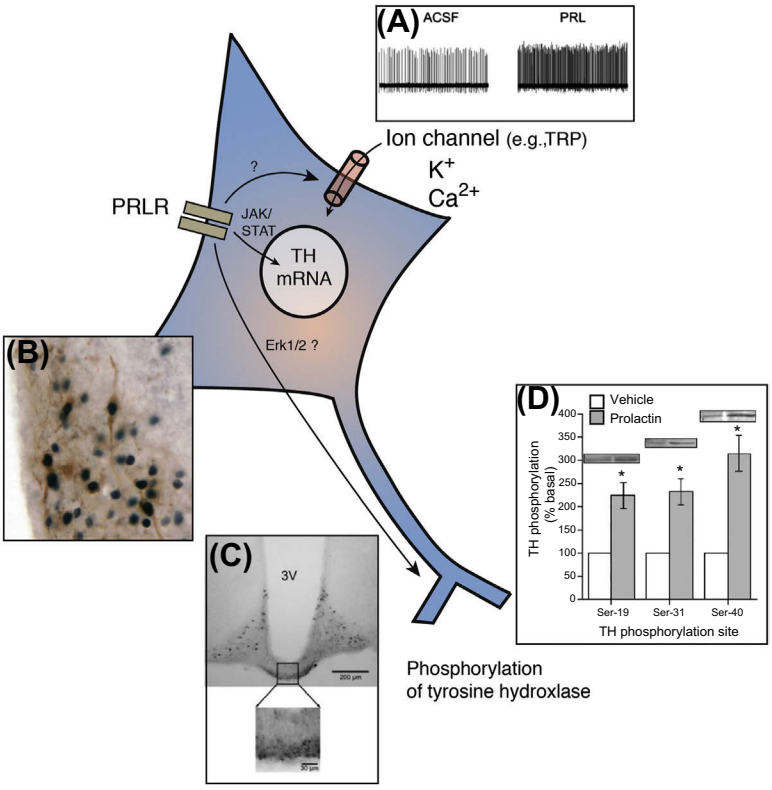

FIGURE 12.4 Diagrammatic representation of the multiple levels of prolactin feedback on NEDA neurons. This figure is reproduced in color in the color plate section. Prolactin binds to prolactin receptors on NEDA neurons and then acutely (within 5 min) increases the firing rate of those neurons. Inset (A) shows prolactin-induced increase in the firing rate of a TIDA neuron.[92] Prolactin also increases the phosphorylation of STAT5 in TIDA neurons (approximately 15–20 min), inducing a transcriptional response. Inset (B) shows prolactin-induced STAT5 immunoreactivity (black) co-localized with immunoreactivity for tyrosine hydroxylase (TH) (brown), demonstrating direct prolactin action in these cells.[91] Finally, prolactin induces the phosphorylation of TH (approximately 15–30 min) both within the cell body and, more particularly, in the nerve terminals within the median eminence. Inset (C) shows prolactin-induced serine 40 phospho-TH immunoreactivity in the arcuate nucleus and median eminence (unpublished data); inset (D) shows prolactin-induced phosphorylation of TH at serines 19, 31, and 40 in mediobasal hypothalamic cultures.[102] ? indicates an uncertain mechanism; $^*P < 0.05$.

homologous hormone from pituitary grafts and longer periods of prolactin exposure in the adult were able to increase the numbers of NEDA neurons present in the hypothalamus.[133] This observation suggests that plasticity is possible in the mature hypothalamus, possibly involving transdifferentiation of other neuronal phenotypes into dopamine cells with sufficient stimulation.

Transport of Prolactin across the Blood–Brain Barrier

The clear role for prolactin as a feedback regulator of hypothalamic dopamine neurons suggests that there must be a mechanism for prolactin to enter the brain. Being a relatively large polypeptide hormone (197–199 amino acids, 21 kDa), prolactin would be expected to be excluded from nervous tissue by the specialized tight junctions between vascular endothelial cells in the brain that form the so-called blood–brain barrier".[134] While it is possible that some prolactin-responsive regions, such as the arcuate nucleus–median eminence region, may have an incomplete blood–brain barrier such that hormones may have more direct access to neurons in this area,[135,136] many prolactin-responsive neurons are found well away from such regions. There is clear evidence, however, that systemic prolactin gains access to the cerebrospinal fluid (CSF). Prolactin levels in the CSF parallel changes in prolactin in the peripheral circulation.[137–139] Moreover, circulating prolactin was found to enter the brain through a saturable, carrier-mediated transport system.[140] By administering radiolabeled prolactin in rodents, high levels of binding were observed in the choroid plexus.[141,142] Consistent with these observations, the choroid plexus contains the highest level of prolactin receptor mRNA and protein of anywhere in the brain,[113,114,143] and levels of both the short and long forms increase in response to estrogen,[144] during pregnancy and lactation,[114,145] and in response to stress.[146] It has been widely assumed that the prolactin receptor might mediate prolactin entry into the CSF, but evidence for this is only circumstantial. Because the ependymal cells lining the cerebral ventricles lack tight junctions and do not limit diffusion into the neural parenchyma, prolactin entering the CSF would have ready access to neurons. Neurons expressing prolactin receptors in the brain are concentrated in periventricular regions, particularly in the hypothalamus.[143,147] Using prolactin-induced phosphorylation of STAT5 as a marker, it can be shown that peripheral administration of prolactin rapidly (within 30 min) activates these receptors in the brain.[123,147] This pattern of activation is essentially identical to that achieved by administration directly into the cerebral ventricles. Taken together, these data are all consistent with the concept that the choroid plexus–CSF route is the principal mechanism by which prolactin enters the brain, and it is possible that prolactin receptors in the choroid plexus may mediate this transport.

Interestingly, the short form of the prolactin receptor is highly expressed in the choroid plexus of the rat,[113] and this pattern of expression is homologous with another cytokine-type receptor, the leptin receptor. Like prolactin, leptin is synthesized and secreted in the periphery, and is transported into the brain. While there is evidence for leptin transport in the cerebral microvessels of the blood–brain barrier,[148,149] it is also clear that this hormone is transported into the CSF at the choroid plexus. The nonsignaling, short form of the leptin receptor is highly expressed in the choroid plexus[150] and is widely thought to act as a transporter.[151] By analogy, it has also been assumed that the short form of the prolactin receptor may be the key transport molecule for prolactin entry into the brain.[152] This assumption has not been tested, and the precise mechanism that translocates prolactin across this epithelial layer into the CSF remains to be determined. Prolactin and prolactin receptor internalization is well documented,[153] although this is typically associated with receptor degradation.[154] Receptor-mediated transport across an epithelial layer remains to be proven.

Regulation of NEDA Neurons

Many of the factors that regulate prolactin secretion do so by influencing the activity of the NEDA neurons in the hypothalamus. There is a vast literature on the range of different factors that can influence NEDA neurons, and this is well reviewed by Freeman and colleagues.[52] For the purposes of this chapter, we will focus on a few key factors that influence the reproductive functions of prolactin through an action mediated by changes in the activity of NEDA neurons: circadian rhythms, gonadal steroids, and opioid peptides.

Circadian rhythms: There is considerable evidence that dopamine secretion from the hypothalamus varies across the light–dark cycle,[89,155–157] and this is a major contributor to the circadian pattern of prolactin secretion in different states, including the preovulatory surge of prolactin[158] and the daily surges of prolactin[155,159] that are critical for the establishment of pregnancy in rodents. The daily rhythm of activity has been identified through neurochemical measures of dopamine synthesis,[160,161] expression of immediate early genes in the NEDA neurons,[162] and changes in the electrophysiological activity of presumed TIDA neurons (dopamine-inhibited neurons in the dorsomedial arcuate nucleus).[89] The dopaminergic tone is reduced at certain times during the day, such that prolactin secretion tends to be linked to specific times of day. For example, the proestrous prolactin surge in the rat peaks around the time of lights out, while

there are twice-daily surges of prolactin at specific times in the late afternoon and early morning during early pregnancy, which are associated with decreases in dopamine secretion from the median eminence. Superimposed on the inhibitory tone provided by hypothalamic NEDA neurons, there may also be a stimulatory tone that becomes apparent only when dopamine secretion is lowered.[163,164] Such circadian rhythms are typically coordinated by the suprachiasmatic nucleus, and lesions of the suprachiasmatic nucleus abolish rhythms in NEDA function.[156] It seems likely that vasoactive-intestinal peptide from the suprachiasmatic nucleus regulates the activity of NEDA neurons, entraining their activity to the circadian clock.[165] It is also clear, however, that specific clock genes are expressed within NEDA neurons, and expression of these genes shows a circadian rhythm.[166] Thus, these neurons maintain an independent oscillator, allowing a "free-running" rhythm, even in constant light or in the absence of inputs from the suprachiasmatic nucleus. Transient inhibition of clock genes by antisense oligonucleotides did not alter patterns of prolactin secretion,[166] however, making it difficult to interpret their physiological role.

Gonadal steroid hormones: Gonadal steroid hormones exert a marked influence on prolactin secretion, and this is particularly important to integrate the reproductive functions of prolactin with other parts of the hypothalamic–pituitary–gonadal axis. Gonadal steroids influence NEDA function in both males and females.[167–171] The NEDA neurons express receptors for both estrogen (estrogen receptor alpha (ERα)) and progesterone,[172–176] although they may not express androgen receptor.[177] Estradiol appears to render the NEDA neurons less sensitive to the stimulatory effects of prolactin.[178,179] The mechanism of this effect is not clear, as estradiol increases prolactin receptor expression on NEDA neurons,[98] and hence it is possible that chronic estradiol may affect prolactin responses downstream of the receptor through upregulating endogenous inhibitors of the JAK–STAT pathway, such as the SOCS proteins.[180] The effect of estradiol on NEDA neurons is further complicated by the fact that it activates prolactin synthesis and secretion from the anterior pituitary gland (see the section Lactotroph Function on pituitary). Thus, acute increases in estradiol are associated with activation of TIDA neurons, mediated by the estrogen-induced increase in prolactin from the pituitary.[181] In contrast, the predominant direct action of estradiol on NEDA neurons is one of inhibition,[171,178,179,182] suppressing TH expression[183,184] and activity[185] and reducing secretion of dopamine into the portal blood,[186] thereby facilitating prolactin secretion. Testosterone action in NEDA neurons in males appears to be similar to that of estradiol in females, decreasing the activity of TIDA neurons to facilitate prolactin secretion,[170,187] but it does not alter their responses to prolactin.[170]

The effects of progesterone on NEDA activity are equally complex. Progesterone treatment acutely decreases TH activity in these neurons,[188,189] but chronic elevations in progesterone result in increased TH activity[171,190] and increased secretion of dopamine into the portal blood.[191] Progesterone can also antagonize the inhibitory effects of estradiol on TH mRNA levels.[168] The acute inhibitory actions appear to involve a rapid decrease in the phosphorylation of TH, inactivating the enzyme and reducing dopamine synthesis,[189,192] and hence do not seem to involve the classical steroid hormone action via gene transcription. Effects of progesterone on NEDA neurons are dependent on the presence of estradiol,[184,189,193] likely because expression of the progesterone receptor in the brain is dependent on estradiol.[194–197] Hence, it is difficult to evaluate the effects of progesterone on NEDA neurons in isolation from the effects of estradiol.

Endogenous changes in ovarian steroids and the consequent changes in activity of NEDA neurons play a physiological role in the control of prolactin secretion during reproduction (see the section Patterns of Prolactin Secretion). For example, the endogenous circadian rhythm of NEDA neurons is dependent on ovarian steroids.[157] The estradiol-induced proestrous prolactin surge is associated with a steroid-dependent decline in TIDA activity,[188,192,198,199] with a prominent role for progesterone suppressing dopamine release during the plateau phase of the surge.[169,193] Similarly, ovarian steroids play a critical role in controlling the twice-daily prolactin surges required to sustain pregnancy in rodents.[200,201] During late pregnancy, a stimulatory role of progesterone predominates to maintain NEDA activity at high levels and suppress pituitary prolactin secretion. Progesterone withdrawal following luteolysis leads to the suppression of dopamine secretion and the induction of prolactin secretion.[202]

Opioid peptides: Endogenous opioid peptides have a critical role in regulating activity of TIDA neurons, thereby affecting pituitary prolactin secretion in several reproductive states. Opioids inhibit TIDA neurons, contributing to the suppression of dopamine secretion during the prolactin surges of early pregnancy[203,204] and during the antepartum period,[204] as well as during the prolactin secretory response to suckling.[205–209] These effects may be mediated by dynorphin neurons within the arcuate nucleus, as dynorphin terminals are found in close proximity to TIDA neurons.[210] Similarly, β-endorphin neurons have been observed to contact TIDA neurons,[211] but these make up a relatively small proportion of opioid inputs to these neurons. In addition, the TIDA neurons themselves express met-enkephalin,[212] and levels of enkephalin expression increase markedly during late pregnancy and lactation.[212,213] It is possible that increased local release of enkephalin might

contribute to the suppression of dopamine synthesis and release during these times, acting in an autocrine fashion on the TIDA neurons. Pharmacological targeting using subtype-specific receptor antagonists suggests that mu and kappa receptor subtypes, but not the delta receptor, are critical to endogenous opioid peptide regulation of prolactin secretion during these times.[204,208,214,215] These observations are consistent with the possible effect of dynorphin and endorphins, which act predominantly at the kappa and mu receptor subtypes, respectively.[216] Enkephalins are most potent at the delta receptor, which does not appear to be involved in the control of prolactin secretion, but they could also act via mu receptors to suppress dopamine secretion.[216] The opioid-like receptor (ORL1), which does not bind any opioid peptide with high affinity,[217] and the selective ligand orphanin FQ (OFQ) may also regulate prolactin secretion. Intracerebroventricular (ICV) injections of OFQ significantly increased prolactin secretion in males and females.[218,219] Like other opioid peptides, OFQ effects on prolactin seem to be mediated by an action on TIDA neuronal activity. ORL1 expression is found in the arcuate nucleus,[220,221] and OFQ treatment rapidly decreased activity of TIDA neurons, preceding the rise in prolactin secretion.[219]

Interest in the arcuate nucleus dynorphin neurons has recently been elevated by the observation that these neurons also express kisspeptin.[222] This peptide was discovered 10 years ago as being essential for fertility and reproduction,[223,224] and kisspeptin neurons have emerged as critical afferent connections in the neuronal circuit regulating gonadotropin-releasing hormone (GnRH) neurons.[225] The arcuate nucleus population of kisspeptin-producing neurons has recently been found to coexpress dynorphin and neurokinin-B[222,226] and may project widely in the hypothalamus[227] (the extent of these projections appears to have marked species differences[228]), and thus may have a range of functions other than simply stimulation of GnRH neurons. Such functions include appetite and food intake[229] and temperature regulation.[230] The original description of a dynorphin input to TIDA neurons[210] has recently been complemented by the description of kisspeptin–neurokinin B innervation of these neurons.[231,232] Kisspeptin injection into the lateral ventricle stimulates prolactin secretion,[233] consistent with the hypothesis that these neurons have a complex role in the regulation of TIDA neurons, potentially involving multiple bioactive peptides.

Prolactin-Releasing Factors

From the time of discovery of the hypothalamic hormones, it has been assumed that all pituitary hormones would be controlled by a hypophysiotrophic prolactin-releasing factor. The evidence outlined in this chapter, showing that prolactin was predominantly inhibited by the hypothalamus, did not detract from the search for a prolactin-releasing factor to complement dopaminergic inhibition.[234–236] In particular, there was evidence that removal of dopaminergic inhibition was not sufficient to account for the secretion of prolactin induced by stress[237] or suckling.[75] Early studies identified thyrotrophin-releasing hormone (TRH) as a potent stimulator of prolactin secretion, but TRH release from the median eminence did not increase in response to suckling[238,239] and there is no concurrent suckling-induced release of TSH,[240,241] suggesting that it is unlikely that TRH is a physiological regulator of prolactin secretion during suckling. Similarly, both positive and negative evidence exists for vasopressin[242] and oxytocin[243,244] as prolactin-releasing factors. To date, the evidence for a prolactin-releasing factor remains inconclusive, and no one factor convincingly meets the criteria originally assigned by Harris for a hypothalamic hypophysiotrophic factor (these criteria are as follows: (1) present in the median eminence, (2) present in the portal blood at concentrations greater than in the general circulation, and (3) levels fluctuate in the portal blood with electrical or reflex stimulation of the hypothalamus, and this correlates with pituitary hormone secretion).[245] Hence, interest remains high in the identification of hypophysiotropic prolactin regulatory factors, and some high-profile reports have produced a number of false dawns! For example, a novel prolactin-inhibitory factor was discovered within the precursor protein of GnRH,[246] but a physiological role for this "GnRH-associated peptide" in regulating prolactin secretion has not been forthcoming. Ben-Jonathan and colleagues identified a prolactin-releasing activity in the neurointermediate lobe,[247] but despite many subsequent studies, the identity and physiological role of this factor remain elusive. There was much excitement in the field when a novel neuropeptide was identified and named "prolactin releasing peptide", based on in vitro actions.[248] Unfortunately, based on a range of subsequent anatomical and physiological studies, this peptide is almost certainly not a physiological prolactin-releasing factor.[249,250] One interesting new observation is that the dopamine derivative salsolinol may act as a prolactin-releasing factor.[251] This metabolite would essentially originate from the NEDA neurons, particularly the PHDA and THDA populations terminating in the neurointermediate lobe, but exert an opposite effect to that of dopamine, consistent with the concept of a switch from negative to positive feedback. There is some evidence that salsolinol may be involved in both stress- and suckling-induced prolactin secretion,[252] but again, the physiological importance of these observations remains to be confirmed. At this stage, the jury is still out as to whether one or more prolactin-releasing factors are involved in the stimulation of prolactin release, and if so, their identity and function remain to be determined.

LACTOTROPH FUNCTION

The anterior pituitary gland is the main source of circulating prolactin, which is secreted from lactotrophs, one of the five specialized endocrine cell types in this organ. The gland develops from a region of oral ectoderm that forms Rathke's pouch around embryonic day 9 (E9.0) in the mouse.[253] The cells of Rathke's pouch proliferate to generate undifferentiated progenitors that initiate commitment to the various specialized hormonal cell types after exit from the cell cycle[254,255] and terminally differentiate as a result of expression of a cascade of transcription factors and signaling molecules.[253] The full repertoire of transcriptional activators and repressors that specify lactotrophs has not yet been defined, but the cells derive from the pituitary lineage expressing the transcription factor *Pou1f1* (also known as *Pit1*), as do somatotrophs and thyrotrophs, cells secreting growth hormone and TSH, respectively. Loss of functional POU1F1 protein in mice and humans leads to a hypoplastic pituitary gland and a failure of normal lactotroph specification and proliferation, with an absence of postnatal pituitary prolactin gene expression.[253] Recently, late-embryonic pituitary prolactin in the absence of POU1F1 has been described in mice with altered pituitary development,[256] possibly analogous with the *Pou1f1*-independent thyrotroph population that is found in the embryonic pituitary.[257]

Prolactin gene expression is first detected in the embryonic pituitary at E17.5 in the mouse, but only a few cells are detectable by immunohistochemistry before birth.[258] After birth, there is a rapid expansion of the number of lactotroph cells, which in mice increase exponentially from birth to 5 weeks of age but then remain static from 8 weeks of age in the absence of physiological change or tumors.[259] In rodents, the number of lactotrophs in adult females is higher than in males, a sexual dimorphism that begins around puberty and continues until adulthood, when the proportion of lactotrophs is approximately 35% of all pituitary cells in females but 20% in males.[260] The limited studies in humans suggest that there is no sexual dimorphism in lactotroph cell number in men compared with nulliparous women, although there is an increase in multiparous women.[261] Lactotrophs may not be a homogeneous population of cells, as subpopulations have been identified, based on morphology,[262] response to stimulation,[263] or spontaneous secretory activity.[264]

Stimulation of Lactotroph Proliferation

The factors controlling postnatal proliferation of lactotrophs have been only partially characterized, and it is apparent that a complex interaction of peripheral, hypothalamic, and intrapituitary signals determines the control of this cell population. There is an increase in lactotroph cell number in adult $Prl^{-/-}$ and $Prlr^{-/-}$ mice, indicating that there is a homeostatic regulation of the cell population under normal conditions.[265,266] A role for insulin-like growth factor 1 (IGF1) in the early postnatal expansion of lactotrophs has been shown in studies of IGF1 knockout mice, where there is a reduced number of pituitary lactotrophs by postnatal day 20.[267] This study also demonstrated the ability of estradiol and epidermal growth factor to cause lactotroph hyperplasia in mice before puberty, but it suggested that IGF1 may be permissive for these actions. In the adult, interaction of estradiol with growth factors modulates lactotroph proliferation, which is higher in females than males. The transient estradiol-induced increase in lactotroph mitotic activity on the afternoon of estrus[268] is likely mediated by ERα, since loss of this receptor leads to lactotroph hypoplasia.[269] A direct effect on lactotrophs has been suggested by in vitro studies where antagonists of this steroid receptor reduce lactotroph proliferation,[270] an effect that is dependent on antagonist-mediated degradation of the estrogen receptor.[271] Other studies, however, have suggested that in vivo the effects of estradiol may be indirect, since the increased lactotroph proliferation at estrus is blocked by anesthesia on the afternoon of proestrus, indicating that hypothalamic factors mediate at least some of the actions of estradiol.[272] The proliferative response of lactotrophs to estradiol is only transient,[273] and recently it has been shown that it also has a role in apoptosis of lactotrophs (reviewed in Ref. 274), demonstrating an important role for the steroid in maintaining tissue homeostasis.

A wide range of other factors has been described as causing proliferation of lactotrophs, several of which are paracrine factors released by other pituitary cell types or autocrine factors released by the lactotrophs themselves (reviewed in Ref. 275). The most convincing evidence for a role of these factors in driving lactotroph proliferation is from transgenic and knockout mouse studies, with hyperplasia in older adult females resulting from transgene overproduction of pituitary galanin,[276] nerve growth factor,[277] and transforming growth factor alpha,[278] whilst loss of galanin leads to a reduction in lactotroph proliferation in response to estradiol.[279] It has been suggested that prolactin itself may have a role in lactotroph proliferation, since prolactin receptors have been found in the pituitary and the rate of induction of pituitary tumors in mice correlates with the level of prolactin secretion.[74] Studies of both $Prlr^{-/-}$ and $Prl^{-/-}$ mice, however, suggest that this is unlikely, since the absence of prolactin signaling leads to even more dramatic lactotroph hyperplasia than that found in $D2r^{-/-}$ mice (see the section Dopamine Inhibition of Lactotroph Function and Proliferation via the D2 Receptor).[265,280]

Dopamine Inhibition of Lactotroph Function and Proliferation via the D2 Receptor

Unlike other pituitary hormone cell types, and indeed most other endocrine cells, lactotrophs secrete prolactin spontaneously in the absence of dopamine inhibition (as discussed in this chapter). Lactotrophs display spontaneous electrical activity, and Ca^{2+} influx through voltage-gated Ca^{2+}channels (VGCCs) leads to prolactin secretion (described in more detail in Ref. 6). Inhibition of calcium influx by dopamine results in reduced prolactin secretion from storage granules by altering the activity of VGCCs[281] and membrane hyperpolarization.[282,283] Of the five dopamine receptors, the two members of the D2-like receptor family, D2R and D4R, are found in the pituitary gland,[284,285] and it is through these D2-like receptors, which are coupled to a pertussis toxin–sensitive G protein ($G_{i/o}$),[286–288] that dopamine acts to inhibit lactotroph cell function and proliferation.[53,72] The importance of the role of D2R specifically in lactotroph regulation is apparent from the hyperprolactinemia observed in knockout mice with a loss of this receptor subtype.[73,74] Although there have been reports of dopamine stimulation of prolactin secretion at low concentrations,[289,290] this may be a rebound effect of dopamine removal,[291] which has been shown in perfused pituitary slice culture.[292] In addition to its effect on secretion, dopamine-induced suppression of adenylate cyclase leads to a reduction in prolactin gene expression,[293–295] which may also involve activation of protein kinase C[296] and ERK1/2[297] signaling pathways. The transcription factor POU1F1 has been shown to have a role in mediating the effects of dopamine on prolactin gene expression. This occurs at two levels, since POU1F1 has been shown to directly mediate dopaminergic effects on the prolactin promoter,[298] and the expression of POU1F1 is also downregulated by reduced adenylate cyclase activity,[293] leading to reduced prolactin gene expression.

It is well established that dopamine inhibits lactotroph proliferation, as this effect has been demonstrated in cultures of pituitary cells[295] as well as in vivo by suppression of estradiol-induced proliferation.[299] The effects of loss of D2R,[73,74] leading to abrogation of dopamine inhibition of the lactotroph, and of loss of the DAT,[300] leading to reduced reuptake of dopamine in median eminence nerve terminals with a consequent increase in dopamine reaching the pituitary, best define the in vivo role of dopamine in regulating lactotroph proliferation. In young adult animals, loss of dopamine inhibition has only a mild effect[74] or no effect[73] on lactotroph cell number, but with increasing age, there is a dramatic lactotroph hyperplasia, leading to prolactinoma formation in females and much later in males.[74,301] Increased dopamine from loss of DAT leads to a severe postnatal reduction in lactotroph proliferation, which leads to a dramatic reduction in the number of lactotrophs by 8 weeks of age.[300] The similarity of phenotypes between D2R knockout mice and those with a specific reduction of hypothalamic dopamine[302] suggests that D4R, while present in the pituitary, has a minor role in control of lactotroph proliferation. Interestingly, mice with a loss of prolactin receptor and D2R have a more advanced and severe hyperplasia and tumor formation than those with loss of either receptor alone,[280] demonstrating that dopamine inhibition is not the sole regulator of lactotroph proliferation and the existence of other homeostatic mechanisms controlling their cell number. Taken together, these models suggest that early lactotroph proliferation is principally regulated by nondopaminergic mechanisms, although cells remain responsive to high levels of dopamine from birth. The factors regulating this are undefined but may include IGF1. In adult animals, there is a complex interaction between dopamine and a number of other factors in the regulation of lactotroph proliferation, with a role for nondopaminergic factors that respond to a loss of prolactin signaling. In older animals, there is a physiological change that permits increased lactotroph hyperplasia despite hyperprolactinemia, suggesting a breakdown of regulatory homeostatic mechanisms, which can lead to tumor formation.

Of note in all of the models of lactotroph hyperplasia and tumorigenesis, both those resulting from overexpression of growth factors and those with reduced dopamine inhibition, is a dramatic effect of sex. A bias toward lactotroph hyperplasia and more rapid generation of pituitary tumors in females could be expected from the actions of estradiol, but this may not be the sole factor leading to the increased female hyperplasia. In *D2r*$^{-/-}$ mice with hyperplasia resulting from loss of dopamine inhibition, it was noted that males and females had similar serum estradiol levels.[74] The role of sex steroids in lactotroph hyperplasia in *D2r*$^{-/-}$ mice was further characterized by Low and colleagues, who found that gonadectomy reduced the lactotroph hyperplasia and tumor formation but that this could not be fully rescued by estradiol replacement, suggesting that ovarian factors other than estrogen are required for the proliferation of lactotrophs.[266]

Other Factors Regulating Prolactin Secretion in the Pituitary

In addition to dopamine, a number of other factors have been described as regulating secretion of prolactin from the lactotroph, although the physiological significance of these is not yet established. Hypothalamic somatostatin inhibits prolactin secretion through both cyclic adenosine monophosphate (cAMP)-dependent and -independent mechanisms,[303] and calcitonin, which may be of hypothalamic origin, has also been shown

to inhibit secretion.[304] In addition, local production of endothelin-1 and transforming growth factor beta may be autocrine or paracrine inhibitors of prolactin secretion.[305,306] Whilst estradiol is a major stimulator of prolactin secretion in mammals, this is principally through increased prolactin gene expression,[307] by increasing the number of lactotrophs (as discussed in this chapter), and by modifying lactotroph responsiveness to other regulators,[308–310] although a rapid nongenomic action of estradiol-stimulating prolactin secretion has been described.[263] A large number of other factors have been suggested as directly stimulating prolactin secretion, although a clear physiological role for many of these has not yet been demonstrated (reviewed in Ref. 52). These include the following:

- *Vasointestinal peptide (VIP)*: This is produced in the hypothalamus[311] and in lactotrophs themselves.[312] As well as in vitro and in vivo demonstrations of effects on prolactin secretion, passive immunization against VIP leads to reduced prolactin secretion.[313] It is possible that VIP was the ancestral regulator of prolactin secretion, since it is the primary prolactin-releasing factor in nonmammalian vertebrates.[314,315]
- *TRH*: This is released from the hypothalamus, is a regulator of thyrotrophs, and stimulates prolactin synthesis and secretion from lactotrophs,[316] which express TRH receptors.[317] However, no effect on prolactin secretion was found after stimulation of cultures of mouse pituitary slices,[292] and nonpregnant TRH-deficient mice have normal pituitary and serum prolactin,[318] although this could suggest species-specific effects of TRH. However, pituitary and serum prolactin were reduced in lactating TRH-deficient animals, even after thyrotrophin replacement, suggesting a role for TRH in stimulation of prolactin gene expression in lactating mice. The role of TRH may be further complicated by interaction with other regulatory factors, since an interaction between estradiol and TRH in stimulation of prolactin secretion has been described.[319]
- *Oxytocin*: This can reach the anterior pituitary from both peripheral circulation and short portal vessels linking the posterior and anterior lobes of the pituitary. Treatment of mouse pituitary slices with oxytocin stimulates prolactin release,[292] and antagonism of oxytocin leads to reduced prolactin secretion.[244,320] Oxytocin may have a complex interaction with dopamine in the control of prolactin secretion.[321]
- *Galanin*: Galanin is synthesized in both the hypothalamus and pituitary, has been shown to stimulate lactotroph function, and may be one factor mediating the effects of estradiol on lactotrophs.[276,279,322]

A number of other autocrine and paracrine factors have been shown to have prolactin-releasing activity (reviewed in Ref. 52).

Changes in Pituitary Function during Pregnancy and Lactation

Studies in rats and humans have shown an increase in lactotroph cell numbers in lactation, which in humans (but not rats) begins early in pregnancy,[323,324] with rapid regression back to prepregnant numbers after cessation of lactation.[325] Lactotroph hyperplasia was not found in lactating mice,[326,327] although this does not preclude increased lactotroph turnover resulting in altered function. This does suggest, however, that there may be species-specific differences in meeting the increased physiological demand for prolactin during lactation. There are several mechanisms that may lead to changes in the number of lactotrophs during pregnancy and lactation, including proliferation of lactotrophs, transdifferentiation between hormone cell types, apoptosis, and proliferation and differentiation of progenitor or stem cells:

Lactotroph proliferation: The sexual dimorphism in cell numbers in rodents is accompanied by a difference in the mitotic rate of lactotrophs, which is lower in males than females and begins to decline before puberty, whereas in females the mitotic index of lactotrophs peaks at 60 days of age.[268] Increased mitosis of lactotrophs has been found in late-pregnant rats[328] and in pregnant women.[323,329] There is a transient increase in lactotroph mitotic activity on the afternoon of estrus, which is dependent on estradiol.[268]

Transdifferentiation: The presence of some bihormonal GH–prolactin cells, known as mammosomatotrophs, in the pituitary, together with the timing of hormone expression and shared transcription factor requirements, have led to the suggestion that lactotrophs develop by transdifferentiation from somatotrophs. This was proposed to account for increased lactotrophs in pregnancy and lactation in rats[324] as well as in humans.[329] Studies using lineage tracing in mice, however, have suggested that, at least in this species, only a small proportion of lactotrophs is derived from somatotrophs.[330] The proportion of mammosomatotrophs in mice is much lower than in rats,[326] so it is possible that the proportion of lactotrophs arising from transdifferentiation may be higher in rats, the species where this has been studied in the most detail. Lineage studies have also demonstrated that lactotrophs do not transdifferentiate into any other pituitary cell type.[326]

Apoptosis: The increased lactotroph mitosis that occurs at estrus is accompanied by an increase in apoptosis

at the subsequent proestrus when pregnancy does not occur,[331] as well as at the end of lactation.[332] An interaction of estrogen and dopamine has been implicated in regulating this apoptosis.[333–335]

Progenitor cells: Recently, a population of cells in the pituitary with the characteristics of stem cells has been described.[336–338] These cells are potentially capable of differentiation into all pituitary hormone cell types. In the absence of lineage-tracing tools, the contribution of these cells to the lactotroph population in the normal pituitary is unclear; however, cell ablation studies suggest that they contribute to regeneration of lactotrophs in adult mice.[339]

Thus, a combination of processes results in altered function during pregnancy and lactation, optimizing prolactin production for lactation. The precise mechanism of these changes is not known and seems likely to involve some or all of the above processes, but the relative importance of each process may be different in different species.

Organization of Lactotrophs into a Network Affects Function

In recent years, the importance of cell organization in the function of pituitary cells has become apparent (reviewed in Ref. 340). An indication that lactotroph function is affected by contact between cells is suggested by in vitro studies showing that increasing the density of dispersed lactotrophs in culture augmented the inhibitory effects of dopamine.[341] The organization of lactotrophs in the female mouse pituitary has been studied using multiphoton imaging of pituitary slices with lactotrophs identified by the transgenic expression of DsRed fluorescent protein.[292] Lactotrophs are distributed throughout the anterior pituitary, with a higher density in the ventral region, and are organized into honeycomb-like structures.[327] This organization is distinct from that described for other pituitary cell types, for example somatotrophs,[342] suggesting that the pattern of organization may be related to the different secretory patterns of the hormones. In lactation, lactotroph organization is modified so that the honeycomb structure becomes more apparent and there is an increase in contacts between lactotrophs (see Figure 12.5) This increased structural organization is a response to the high demand for prolactin in lactation, rather than simply the result of physiological changes associated with pregnancy, because restricting prolactin demand by reducing the number of suckled pups reduces the extent of augmented network organization.[327] The change in organization of lactotrophs is accompanied by an increase in the synchronization of lactotroph secretory activity, monitored by calcium imaging, and is mediated by gap junctions. This suggests that the network is important for the increased secretory output of lactotrophs in lactation by coordinating the activity of subpopulations of cells. Surprisingly, after weaning, the augmented organization of lactotrophs persists, even after 3 months, and leads to a change in coordinated cell behavior and a further increase in prolactin output, showing that lactotrophs demonstrate experience-related structural and functional plasticity.[327]

PATTERNS OF PROLACTIN SECRETION

The pattern of prolactin secretion varies between species in a manner that is specific to the reproductive strategy of that species. Common to all mammals is the prolactin stimulus provided by suckling of the nipple by the young, which produces a signal in afferent somatosensory neuronal pathways that is relayed to the hypothalamus and then the pituitary, inducing prolactin secretion. This is a classical neuroendocrine reflex, where the amount of prolactin released depends on the intensity and duration

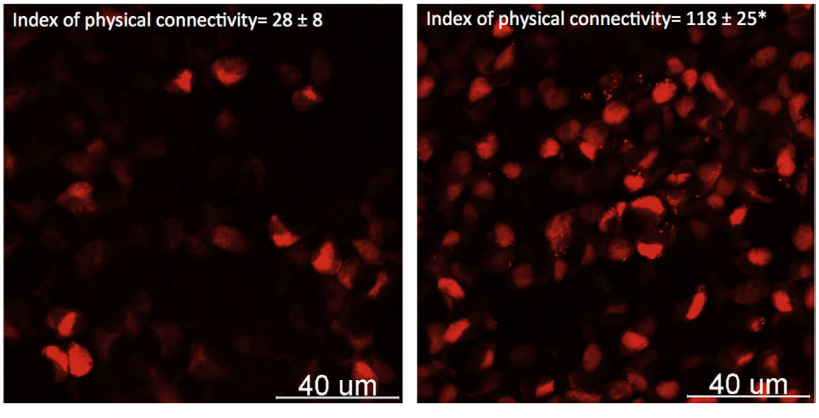

FIGURE 12.5 Biphoton imaging of pituitaries from virgin (left panel) and lactating (right panel) mice with transgenic expression of DsRedExpress fluorescent protein identifying lactotroph cells. This figure is reproduced in color in the color plate section. In the virgin pituitary, lactotrophs are organized into a network of honeycomb-like structures. In the lactating pituitary, these honeycomb structures become more pronounced, and there is an overall increase in the proportion of lactotrophs with homotypic contacts. The "index of physical connectivity" is generated by three-dimensional reconstructions of the fluorescent label and reflects the degree of structural connections of lactotrophs with other lactotrophs. *P <0.05. *Source: Image courtesy of Chrystel Lafont, Taoufik El Yandouzi, and Patrice Mollard, Montpellier, France, based on work published in Refs 327,340.*

of suckling.[5] Apart from suckling, the most physiologically relevant stimulus to prolactin secretion is estradiol, which stimulates prolactin secretion both through actions on the pituitary gland and through hypothalamic mechanisms. This action is particularly important during the reproductive cycle and during pregnancy. Interestingly, unlike the suckling-induced release of prolactin that is tightly linked to the stimulus itself, estradiol-induced prolactin secretion tends to be a global change in secretion that is superimposed on patterns generated by other factors, such as the light–dark cycle.[158] For example, estradiol induces a single afternoon prolactin surge observed during the afternoon of proestrus,[343] or in ovariectomized animals, chronic elevation in estradiol induces a daily afternoon prolactin surge.[344] During late pregnancy, there is another estrogen-dependent surge of prolactin secretion, again timed to the light–dark cycle.[180,345,346]

Another interesting prolactin stimulus is stress, which induces an acute increase in prolactin secretion in many species.[347] Stress-induced prolactin secretion is thought to be involved in modulation of the immune system, or as a counterregulatory mechanism to limit the hypothalamic–pituitary–adrenal axis response to stress. Prolactin can also act in the brain[348] to prevent the stress-induced formation of gastric ulcers.[349,350] This effect is associated with increased prolactin receptor expression in the choroid plexus[146] and in the paraventricular nucleus (PVN) of the hypothalamus.[348] Antisense-induced inhibition of prolactin receptor expression in the PVN prevents the ability of prolactin to block stress-induced changes in plasma calcium and gastric ulceration.[351] Thus, stress-induced changes in prolactin secretion might have protective actions in the body as well as reducing anxiety associated with the stressor. While some have argued that this release of prolactin is unlikely to have a major reproductive function,[352] the influence on the stress axis has been raised as potentially important in birds for maintaining reproductive function in the face of stress.[353] This latter context may be similar to a role of elevated prolactin in suppressing the stress response in mothers during pregnancy and lactation in mammals (discussed further in this chapter). For the purposes of this chapter, we will describe the patterns of prolactin secretion during the full reproductive cycle in rodents (as key experimental species) and contrast this with what is known about humans.

Prolactin Secretion during Pregnancy in Rodents

Prolactin secretion is relatively low during the estrous cycle in rats, resulting in concentrations in blood of <20 ng/ml, except for an estradiol-induced surge (peaking around 200–300 ng/ml) during the afternoon of proestrus,[354] which is largely coincident with the preovulatory luteinizing hormone (LH) surge (Figure 12.6). Some labs report a secondary surge during the afternoon of estrus,[355] which is likely dependent on how long estradiol levels stay elevated. The vaginocervical stimulation that female rats experience during mating provides the afferent input of a unique neuroendocrine reflex, resulting in the initiation of twice-daily surges of prolactin that are of similar magnitude to the proestrous surge.[200,356] These surges are not typical of a normal neuroendocrine reflex, as the stimulus does not need to be repeated for another surge to occur. Instead, the prolactin surges are timed by the light–dark cycle, with daily diurnal and nocturnal prolactin surges, the first in the afternoon at approximately the same time as the proestrous surge, and the second peaking approximately 2 h before the end of the dark phase. These surges provide luteotropic support for corpus luteum production of progesterone[343,357] and thus are critical for the maintenance of pregnancy. Prolactin surges persist for about 10 days in pregnant rats. A similar pattern of secretion has been reported in mice.[358,359]

The timing of the prolactin surges may be generated by the characteristics of the feedback mechanisms controlling prolactin secretion (Figure 12.6). Hence, estrogen- or mating-induced increases in prolactin activate hypothalamic dopamine neurons by short-loop feedback. Notwithstanding the rapid feedback effects described in this chapter, there is a 4–8 h delay in the overall neurochemical activation of the NEDA neurons,[104,116] likely involving changes in gene expression and new protein synthesis, before tonic dopamine secretion rises sufficiently to bring prolactin back to presurge levels. Falling prolactin then relieves the stimulatory drive to NEDA neurons, and dopamine release falls, potentially allowing a further prolactin surge to occur. Thus, NEDA neurons also exhibit a semicircadian pattern of activity during early pregnancy, with reduced dopamine release observed during the prolactin surges and elevated release between surges.[155,159,201] It is hypothesized that once initiated by the mating stimulus, the mating-induced prolactin surges are driven by a reciprocal interaction of an endogenous rhythm of oxytocin neurons stimulating prolactin release, followed by an inhibitory dopamine response with an intrinsic delay. This reciprocal fluctuation is entrained to the light–dark cycles by innervation of oxytocin neurons by vasoactive-intestinal peptide projections from the suprachiasmatic nucleus.[165,360,361]

The prolactin surges of early pregnancy are terminated in response to the increased secretion of placental lactogens from the developing conceptus.[362,363] The placental lactogens are structurally similar to prolactin and act in an identical manner, crossing into the brain and activating prolactin receptors on NEDA neurons to cause a sustained increase in dopamine release[159] and thereby inhibit the secretion of prolactin from the maternal pituitary.[362,364,365] Two forms of placental lactogen have been

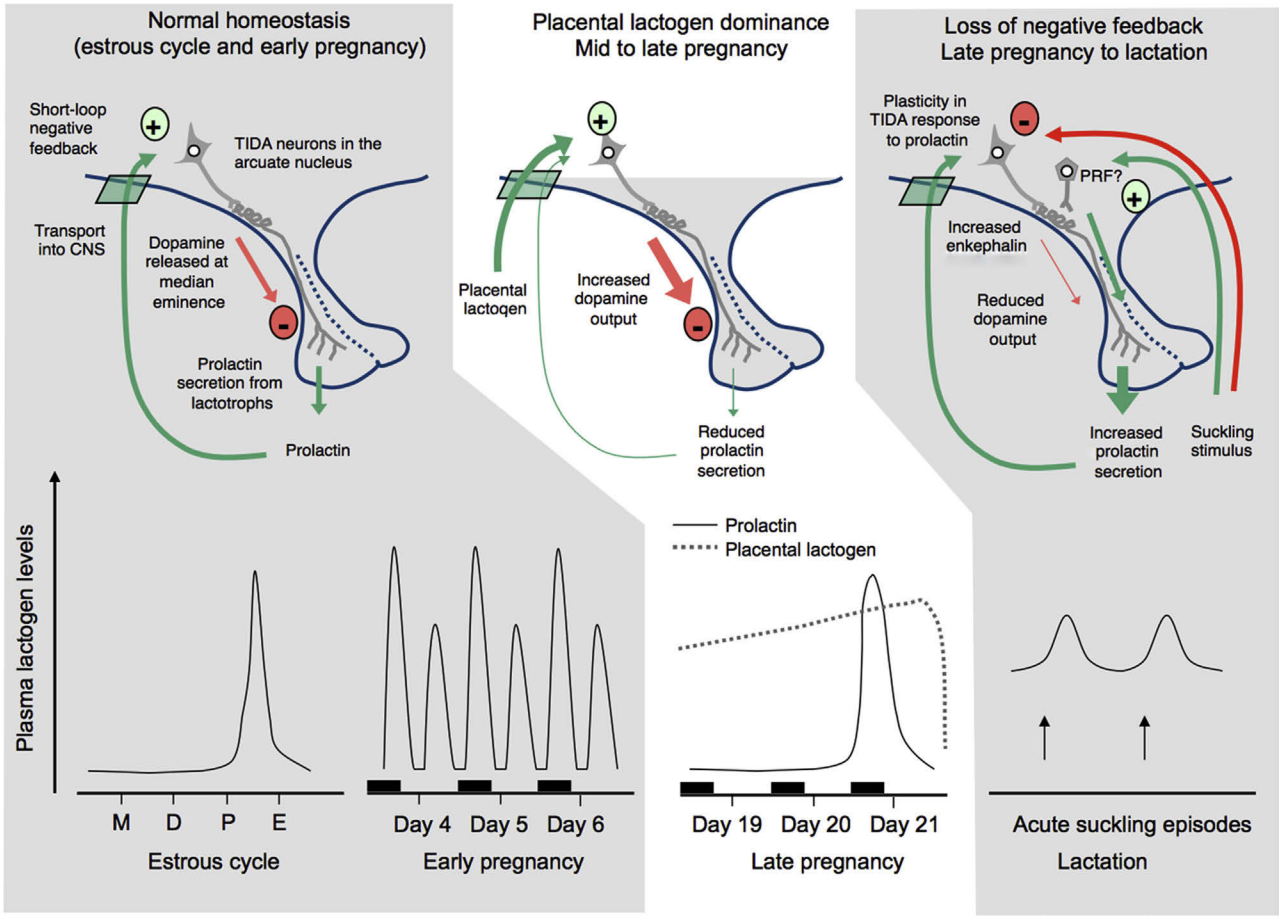

FIGURE 12.6 Diagrammatic representation of the major patterns of lactogen secretion in the rodent (prolactin: solid line; placental lactogen: dotted line), together with a cartoon depicting the changes in the hypothalamic control of prolactin secretion in the different physiological states. See the text for details. ? indicates an uncertain mechanism; *P <0.05.

identified in rats, placental lactogen-I (rPL-I) and -II (rPL-II).[366] rPL-I is present from day 8 of pregnancy and peaks in the circulation around day 13,[367] while rPL-II is first detected around day 10 of pregnancy, peaking near parturition.[368] Both variants bind to the prolactin receptor[367] and thus activate the short-loop feedback system inhibiting prolactin secretion.[364] As rPL-I is present earlier, it is most likely the signal that terminates early-pregnancy prolactin surges,[369] while rPL-II maintains this inhibition of prolactin secretion during the remainder of pregnancy. Thus, from the time of cessation of the prolactin surges until very near term, NEDA neuronal activity is high and maternal prolactin secretion is suppressed.[345] Because placental hormone secretion is not under the inhibitory control of the hypothalamus, this placental lactogen secretion essentially bypasses the maternal regulatory feedback pathways to provide high levels of prolactin receptor activation throughout the second half of pregnancy. Thus, at day 10 of pregnancy, there is a change in the pattern of prolactin receptor activation, from a phasic pattern of activation provided by twice-daily prolactin surges during early pregnancy to the chronic stimulation induced by placental lactogen (Figure 12.6). This chronic secretion of placental lactogen continues until term.

There is a distinct change in the mechanisms regulating prolactin secretion from late pregnancy and into lactation.[370] Up until this time, feedback regulation of prolactin secretion dominates. Despite the continued presence of placental lactogens, however, there is a decrease in NEDA neuronal activity during late pregnancy[345] associated with a nocturnal surge in pituitary prolactin secretion (300–500 ng/ml) immediately before parturition.[346,371] The timing of this event appears to be controlled by the changes in ovarian steroid hormones associated with this time, in that prolonging pregnancy with progesterone treatment delays the prolactin surge, while the surge can be advanced by estradiol treatment following progesterone withdrawal.[346] NEDA neurons no longer release dopamine in response to prolactin or placental lactogen at this time, rendering the short-loop negative-feedback system functionally inactive.[371] This adaptation persists into lactation, and NEDA neuronal activity remains low even during periods of elevated prolactin secretion.[68,372] Furthermore, treatment with

prolactin during late pregnancy[373,374] or lactation[372,375] does not affect NEDA activity, such that the high levels of pituitary prolactin secretion that are required for lactation and maternal behavior can be maintained throughout late pregnancy and lactation unencumbered by an inhibitory feedback system. There is no evidence that this loss of sensitivity is mediated by a downregulation of prolactin receptors on the TIDA neurons,[100] and indeed, recent evidence suggests that the rapid increase in electrical activity seen in response to prolactin persists in lactation.[92] Downstream of the prolactin receptor, however, there is a change in the cellular response. TH phosphorylation (serine 40) in the median eminence is decreased, and because phosphorylation of TH at this site is required for enzyme activity,[376] this means that the activity of TH is reduced, and the neurons no longer release dopamine in response to a change in firing rate.[92] Prolactin-induced activation of the transcription factor, STAT5b, which is essential for prolactin-mediated stimulation of dopamine synthesis and release,[106] is suppressed (but not abolished) during lactation, likely mediated by an upregulation of a family of endogenous inhibitors of STAT signaling, the SOCS proteins.[108,109,180] There is an increase in enkephalin expression in the NEDA neurons,[212,377,378] and it seems likely that elevated prolactin drives this enkephalin expression.[379] The function of enkephalin expression in NEDA neurons is not clear, but as described here, extensive data show that opioids inhibit the activity of NEDA neurons. It is therefore possible that prolactin-induced enkephalin release from these neurons will contribute to the suppression of dopamine secretion, effectively changing the short-loop feedback from a negative-feedback to a positive-feedback system, facilitating prolactin secretion (Figure 12.6).

After parturition, when placental lactogen is lost, prolactin secretion from the maternal pituitary again becomes the major source of lactogenic hormones in the maternal circulation. Prolactin secretion is now closely linked to the suckling stimulus from the young, which directly induces maternal prolactin release and thus ensures continued production of milk. Essentially, the production of prolactin during a feeding bout stimulates milk production to replace what was consumed, and the longer the suckling bout, the longer prolactin is elevated, matching milk production to demand. This highly coordinated release of prolactin is caused by a suckling-induced decrease of activity of TIDA neurons,[372,380,381] resulting in profound suppression of TH mRNA levels,[382] dephosphorylation of TH protein in the median eminence,[383] and reduced secretion of dopamine into the portal blood.[68] The specific pathway mediating the suckling-induced suppression of NEDA neurons is not clear. A direct neuronal pathway is involved, transmitting the somatosensory afferent information from the nipple via the spinal cord to the hypothalamus.[384] Recent evidence suggests that there is a direct pathway from the subparafascicular nucleus–posterior thalamus to the ventrolateral arcuate nucleus, possibly connecting with the dynorphin neurons located in this region.[385] Neurons in this pathway express the tuberoinfundibular peptide of 39 residues (TIP39), and this peptide may be a critical regulator of prolactin secretion in response to suckling,[386,387] acting through the parathyroid hormone 2 receptor[388] to suppress activity of NEDA neurons.

There is much debate about whether disinhibition per se is sufficient to account for the full suckling-induced release of prolactin. Certainly, administration of a dopamine D2R antagonist can result in a release of prolactin of similar magnitude to that seen during suckling.[389] However, whether such a total withdrawal of dopamine ever occurs in vivo is unlikely.[390] Where measured, the fall in portal blood dopamine may not account for the full release of prolactin.[75] Hence, there have been many attempts to identify prolactin-releasing factors that might be involved in stimulating prolactin secretion, particularly in response to suckling. Lesions of the PVN markedly impair the suckling-induced release of prolactin,[391,392] suggesting that this nucleus plays a key role in stimulating prolactin secretion. Similarly, removal of the posterior lobe of the pituitary blocks the suckling-induced release of prolactin.[247] A range of putative candidates from the PVN and/or posterior pituitary has now been examined, but the evidence for a physiological prolactin-releasing factor remains inconclusive (as discussed in this chapter).

Patterns of Prolactin Secretion in Pregnant and Lactating Women

Unlike rodents, in women no clear rise in prolactin secretion during the menstrual cycle has been documented that is associated with ovulation (reviewed in Ref. 393). Some studies show no change in prolactin at this time,[394] while others report a small rise.[395] In an excellent overview of multiple studies looking at hormonal changes during the menstrual cycle, Rubinow and colleagues concluded that there likely was a transient surge in prolactin associated with ovulation, but that many studies have missed this due to timing of sampling or suboptimal experimental conditions.[396] Even if this is correct, it is clearly not as prevalent as that seen in other species. It is possible that this is due to the relative lack of sensitivity of the human prolactin promoter to estradiol,[397] resulting in pituitary prolactin secretion being much less sensitive to changing levels of estradiol than is seen in rodents.[393]

While many studies have measured prolactin levels during pregnancy in women, they have been the focus of systematic investigation in relatively few. Both prolactin[398,399] and human placental lactogen (hPL; also known as chorionic somatomammotropin)[400] are present in the blood during pregnancy, in increasing amounts

with advancing gestation. There is also growth of the pituitary gland (reviewed in Ref. 401), with increased lactotroph size and number,[329] as discussed in this chapter. The concurrent rises in both prolactin and placental lactogen suggest that placental lactogen is ineffective at activating the short-loop feedback system to suppress prolactin secretion. This is interesting because unlike rodent placental lactogens, which are closely related to prolactin, hPL is in fact much more similar to human growth hormone (sharing an 85% homology, as opposed to a 22% homology with prolactin).[14] As growth hormone can activate the human prolactin receptor and is therefore considered lactogenic[b], the closely related placental lactogen can also activate the prolactin receptor. However, each ligand will bind to the receptor in a unique manner, potentially generating subtly different responses. It is possible that, as happens in late pregnancy in rodents, the NEDA neurons alter their response to prolactin (and placental lactogen), but that this happens relatively early in human gestation to allow the concurrent rise in prolactin and placental lactogen, although this has not been studied. As in other species, suckling induces a rapid increase in plasma prolactin, which is proportional to the duration and intensity of the suckling episode.[403]

Thus, in humans and other mammalian species, there are at least three adaptations to ensure high levels of lactogenic hormone activity during pregnancy and lactation. Firstly, the production of placental lactogen bypasses the inhibitory regulation of the maternal pituitary gland, providing constantly elevated levels of lactogenic hormones throughout pregnancy. Secondly, the maternal NEDA neurons change the manner in which they respond to prolactin (or placental lactogen) and cease to release dopamine, causing dopamine to remain low during late pregnancy and lactation despite elevated prolactin. Finally, maternal behavior and the introduction of the suckling stimulus provide the most powerful stimulus to prolactin secretion that is known in mammals. While these elevated levels of lactogenic hormones are clearly required for development of the mammary gland during pregnancy and for milk production during lactation, prolactin and placental lactogen are also able to act in a wide variety of other tissues in the body to facilitate the adaptive responses that are required for successful transition through these different reproductive states.

Extrapituitary Prolactin

The patterns of prolactin secretion described in this chapter all originate from secretion from the anterior pituitary gland, where the prolactin gene is expressed under the control of the pituitary-specific *Pou1f1* promoter. In humans and other primates, however, the prolactin gene is also expressed at extrapituitary sites, including immune tissues, adipose tissue, skin, and decidualized endometrium.[393] This extrapituitary expression is driven by an alternative promoter located 5.8 kb upstream of the pituitary transcription start site within a primate-specific long terminal repeat transposable element[c]. Of the various sites of extrapituitary prolactin production, decidual prolactin would appear to have an important function in reproduction. Decidual prolactin is identical to human pituitary prolactin and is present in amniotic fluid by week 9 of pregnancy, increasing to peak levels around midgestation. While decidual prolactin does not get into the maternal circulation in significant concentrations,[405] and hence its contribution to lactogenic effects in the mother is minimal, it is dramatically upregulated during pregnancy,[406] and its presence at the maternal–fetal interface is thought to have important paracrine roles in implantation,[407] controlling endometrial growth, as well as the volume and composition of amniotic fluid.[408] Local production of prolactin from adipocytes in the mammary gland may also play an important role in lactation[409] and certainly influences mammary carcinoma[410] (see the section Prolactin and Reproductive Disorders).

As rodents do not have this "primate-specific" distal promoter, extrapituitary prolactin would not be expected.[393] Nevertheless, numerous examples of extrapituitary

[b] The lactogenic activity of growth hormone was largely responsible for the delay in the discovery of human prolactin until the early 1970s. Prior to that time, many endocrinologists doubted that human prolactin existed. Although extracts of human pituitary glands could be shown to have prolactin-like effects, attempts to purify it identified only growth hormone.[402]

[c] The human prolactin gene locus is markedly different from that of the rodent prolactin gene. In humans, there is a single prolactin gene on the short arm of chromosome 6, surrounded by approximately a megabase of noncoding DNA. By contrast, the rodent prolactin gene exists as a large family of up to 26 closely related paralogs arising from multiple gene duplication events.[3] These genes are clustered on chromosomes 13 and 17 in the mouse and rat, respectively. It has been proposed that the use of an alternative promoter to drive human prolactin secretion in extrapituitary sites is an alternative strategy to compensate for the lack of expansion of the prolactin gene in primates.[404] No mutations of human prolactin have been described, and one interpretation of this is that such mutations might be lethal due to the lack of redundancy in the human prolactin gene. Another observation is that primates have evolved an expansion of the closely related growth hormone locus, with five different growth hormone–related genes providing the plasticity to produce pregnancy-related ligands, such as human chorionic somatomammotropin (human placental lactogen) and placental growth hormone variant, while rodents have a single growth hormone gene. The common theme is that many members of these gene families are expressed at the maternal–fetal interface, either in the decidual tissue or in the trophoblast cells of the placenta, suggesting an important role in pregnancy.

expression have been reported in rats and mice. For example, autocrine prolactin from mammary gland appears to be required for lactation initiation in mice.[411] "Pituitary" prolactin (as well as multiple prolactin-related genes) is also expressed in decidual tissue in rats[412] and mice.[413] The prolactin receptor is also expressed in these reproductive tissues in rats[414] and mice,[415] providing the substrate for autocrine mechanisms controlling cellular function. A potential explanation for this extrapituitary prolactin in rodents has recently been provided by the identification of a novel alternative promoter region distinct from that observed in humans.[416] These authors found that decidual prolactin is not a characteristic of all placental mammals, but has evolved at least three separate times in humans, rodents, and elephants, each with a different distal promoter. The regulation of expression is unlikely to be conserved, but the convergent evolution of prolactin expression at the maternal–fetal interface must convey an evolutionary advantage to these species. A Pouf1 variant has also recently been described in mice, potentially driving prolactin expression in mouse germ cells.[417]

REPRODUCTIVE FUNCTIONS OF PROLACTIN

Prolactin was identified and named for its critical role in the physiology of lactation, and this remains its best characterized reproductive function. As outlined in the introduction, however, in this chapter we have taken the approach of considering whether many of the multiple other pleiotrophic functions of prolactin are also functioning to prepare the mother for lactation. Thus, prolactin could be promoting adaptive responses in the mother, throughout pregnancy and lactation, to optimize her system for the metabolic and emotional demands of the lactating state. In this section, therefore, we will examine direct reproductive functions of prolactin in mammary gland, ovary, and brain, and then review a range of actions of prolactin in different tissues and examine how prolactin actions in these tissues might contribute to preparation for or support of lactation. In addition, however, it has to be acknowledged that some actions of prolactin do not fit clearly within this hypothesis. For example, approximately 25% of the male pituitary gland consists of lactotrophs, suggesting prolactin also has some function in males! Thus, we will also examine other reproductive roles of prolactin that may be unrelated to pregnancy or lactation. Importantly, while many of the functions of prolactin have been elucidated by the generation of prolactin[418] and prolactin receptor[419] knockout mice, it must be recognized that reproduction is controlled differently in different species, and some reported functions of prolactin may not be modeled well in mice. In addition, it is also important to recognize that if a particular function is not altered in a prolactin ($Prl^{-/-}$) or prolactin receptor ($Prlr^{-/-}$) knockout mouse, this is not absolute evidence that prolactin is not normally involved in that function. Developmental plasticity can sometimes compensate for deficient signaling pathways to maintain critical functions[d]. Hence, while we have drawn heavily on the transgenic mouse literature for the following sections, we have also tried to draw attention to key studies in other species, even when those observations are not necessarily present in the mouse.

Mammary Gland (See Also Chapter 46)

Hormones are not required for the embryonic and prepubertal development of the mammary gland, and the elongation of the ductal network that occurs during puberty is dependent on estrogen and growth hormone, but not prolactin.[422] Although both long and short forms of prolactin receptor are expressed in the fetal and neonatal mammary gland,[423] prolactin has not been shown to be critical for the early development of the mammary gland (mammogenesis). Indeed, $Prlr^{-/-}$ mice have normal embryonic postnatal development before puberty, showing that prolactin signaling is not required at these stages.[424] However, prolactin has a role in all of the processes of mammary development in the adult, including those leading to lactation and its maintenance[425]:

1. *Ductal side branching and formation of alveolar buds*: At puberty, ductal side branches and alveolar buds develop and increase in density at each subsequent estrous cycle, and both of these morphological changes fail to occur in both $Prl^{-/-}$ and $Prlr^{-/-}$ mice. By replacing progesterone in $Prlr^{-/-}$ animals, the effect of prolactin on ductal branching has been shown to be indirect, through its stimulation of progesterone synthesis.[426] Alveolar buds failed to develop after progesterone replacement, however, demonstrating a direct requirement for prolactin at this stage of mammary development.
2. *Lobuloaveolar development*: In pregnancy, there are dramatic changes in the structure and function of the mammary gland, with alveolar buds developing into lobuloalveoli that are capable of milk production (lactogenesis). The role of prolactin

[d] A classic "neuroendocrine" example is the oxytocin knockout mouse, which has a deficit in milk ejection but is able to give birth relatively normally.[420] This does not disprove the wealth of data showing that oxytocin is involved in parturition, but rather demonstrates that there are other processes that can compensate for this essential function in the absence of oxytocin.[421]

and its receptor in these processes has been dissected by transplantation of mammary epithelial cells from *Prlr*$^{-/-}$ animals into the cleared mammary glands of wild-type animals. Despite a completely normal endocrine environment in the host animals, lobuloalveolar development fails to occur in the transplanted tissue, demonstrating the absolute requirement for prolactin or placental lactogen in this process.[426] This role for prolactin signaling in lobuloalveolar development is confirmed by overexpression of a dominant negative form of the receptor in mammary epithelium[427] and is consistent with the impaired lobuloalveolar development and lactational failure seen in pregnant mice that are heterozygotes for the prolactin receptor deletion (*Prlr*$^{+/-}$).[428] Prolactin also plays a key role in polarization of the mammary epithelium and establishment of tight junctions during lactogenesis, establishing the cytological structure within the gland to enable milk secretion.[425]

3. *Lactation*: Prolactin is also required for the maintenance of lactation (galactopoiesis), although the specific requirements for prolactin vary across species (reviewed in Refs 425,429). Prolactin affects the production of milk by increasing the production of its constituents, in particular β-casein, whey acidic protein, and α-lactalbumin, lactose, and lipids.[430] A role for prolactin in increasing the production of β-casein and whey acidic protein is confirmed by their reduced expression in mice with targeted expression of dominant negative prolactin receptor to mammary epithelial cells.[427] Prolactin may also act as a lactating mammary cell survival factor by inhibiting the apoptosis and remodeling associated with involution.[431]

The mechanisms of prolactin's actions on the mammary gland are complex, with potential interactions with other hormones and their receptors, as well as paracrine factors that may mediate and modify prolactin signaling. In particular, the gonadal steroids estradiol and progesterone are critical co-regulators, as are glucocorticoids and insulin.[425] Whilst both long and short forms of the receptor are found in the gland from embryonic development to adulthood, there is considerable variation in total receptor expression as well as the relative abundance of different isoforms and heterogeneous distribution within the gland.[423] In lactating rats, there is a dramatic increase in expression of the long form of the prolactin receptor, with a less significant change in the expression of the short form, compared with either virgin or multiparous animals, although interestingly this is not reflected at the protein level.[432] The dynamics of prolactin receptor expression and distribution suggest differential actions of prolactin at different stages of gland development and function. Effects of prolactin appear to be mediated predominantly by the long form of the prolactin receptor, through activation of Stat5a, as there is an absence of ductal branching in mice lacking this transcription factor.[433] A role for the short form of the receptor at critical stages of gland development, however, is indicated by the rescue of lactation in *Prlr*$^{+/-}$ mice overexpressing this specific isoform.[434] Although the prolactin receptor is expressed in both epithelial and stromal compartments of the gland, the epithelial expression is sufficient for lobuloalveolar development, as demonstrated by transplant of stromal *Prlr*$^{-/-}$ cells and wild-type epithelium into cleared mammary fat pads, resulting in normal gland development.[426] A large number of factors downstream of prolactin receptor signaling in the mammary gland have been identified, including members of the Wnt pathway, epidermal growth factor receptor ligands, the TNF family member receptor activator of nuclear factor κB ligand (RANKL), and IGF2 (reviewed in Ref. 422). The complexity of prolactin signaling in the gland is further complicated by the emerging evidence for cross-talk between the prolactin and progesterone receptors.[435]

Ovary

One of the major roles of prolactin in nonprimate mammals is the maintenance of pregnancy, demonstrated by the infertility of both *Prl*$^{-/-}$ and *Prlr*$^{-/-}$ mice[418,419] and caused by a failure in the maintenance of the corpus luteum. The midcycle LH surge drives ovulation and initiates luteinization of the postovulatory follicle into a corpus luteum. Unlike primates, which have a long luteal phase allowing progesterone secretion to support a fertilized embryo for a sufficient period to allow implantation and initiation of chorionic gonadotropin secretion to enable maternal recognition of pregnancy (see also Chapters 23 and 28), rodents have a particularly short luteal phase. The mating stimulus is required to rescue the corpus luteum and maintain progesterone secretion[e] to allow implantation and the

[e] Because the prolactin surges of early pregnancy are induced by the physical vaginocervical stimulation provided by the act of mating, they do not depend on a fertile mating and can be induced by mating with a sterile male or by an appropriate artificial stimulation.[436] The prolactin surges induced in the absence of fertilized ova result in a state called pseudopregnancy, with hormonal changes that are essentially identical to those of early pregnancy.[356] The surges persist for approximately 12 days without further reinforcement, and their termination is thought to involve an inhibitory factor from the uterus.[437] The prolonged prolactin secretion maintains progesterone production, resulting in a sequence of diestrous-like vaginal smears. This characteristic of prolactin-induced acyclicity was used as a biomarker for hyperprolactinemia in early studies characterizing the control of prolactin secretion,[57] and it continues to be a useful marker in present biomedical experiments.

establishment of pregnancy, and in the absence of mating, the corpus luteum regresses within hours of ovulation. The twice-daily surges of prolactin resulting from cervical stimulation (as discussed here) are responsible for the increased life span of the corpus luteum and its secretion of progesterone early in pregnancy. In concert with the prolactin surges, there is an LH-driven upregulation of ovarian prolactin receptor expression, with short-form expression increased relatively more than the long form.[438] Maintenance of the corpus luteum is achieved through two actions of prolactin: increased corpus luteum expression of the luteinizing hormone receptor,[439,440] leading to increased expression of the steroidogenic enzymes required for progesterone synthesis,[441] and repression of 20α-hydroxysteroid dehydrogenase,[442] the enzyme responsible for progesterone metabolism in the ovary.[443] Prolactin also has an important role in corpus luteum estradiol biosynthesis via increased expression and activity of aromatase P450 and 17 beta-hydroxysteroid dehydrogenase (17βHSD) (reviewed in Ref. 444). Interestingly, part of this stimulation may occur specifically through interaction of 17βHSD with the short form of the prolactin receptor.[445] In addition to increasing estradiol synthesis, prolactin increases the signaling of this steroid as estrogen receptor expression in the corpus luteum is stimulated by prolactin, through Stat5 response elements present within the promoters of both ERα and ERβ.[444]

Differential effects of the two isoforms of prolactin receptor in ovarian function have been described in studies with animals in which one of the short forms of the prolactin receptor (PRLR-S1) is overexpressed in *Prlr*$^{+/-}$ mice. These studies have confirmed an essential role for the long isoform of the receptor in maintenance of the corpus luteum, because the short form could not rescue the corpus luteum following ovulation,[446] but they have also identified specific roles for the short form. Mice overexpressing PRLR-S1 show premature follicular development, followed by follicular atresia, such that by 5 months of age the ovarian follicles are completely depleted.[446] There were also a number of changes in the expression of specific transcription factors and gene targets.[23] Prolactin action through PRLR-S1 resulted in inhibition of MAPK signaling through activation of a MAPK-specific phosphatase identified as deoxyuridine diphosphate 1.[22] As MAPK signaling is critical to normal follicular function,[447] this inhibitory effect likely mediates the detrimental consequence of overexpression of PRLR-S1.

Clearly, the luteotrophic function of prolactin fits the hypothesis of a role in the maternal adaptation to pregnancy. However, in addition to this role in the maintenance and function of the corpus luteum, there is also evidence in rats that prolactin may exert a luteolytic action, with the preovulatory prolactin surge contributing to the regression of the corpus luteum from the previous ovulation.[448,449] Luteolysis fails to occur normally if the proestrous surge of prolactin is blocked.[450] Several studies have suggested that this action of prolactin may not be a direct action on cells of the corpus luteum but rather via stimulation of macrophage and lymphocyte cells of the immune system.[451,452] It is also possible that prolactin has a direct effect on the corpus luteum in mediating this immune cell–mediated luteolysis, since intercellular adhesion molecule-1, which plays a role in facilitating recruitment and binding of macrophages, is upregulated by prolactin in regressing corpora lutea.[453]

The rodent pattern of secretion and function of prolactin in ovarian function is not conserved in humans or other primates (see Ref. 393 for a review). While there is no dramatic change in prolactin secretion during the menstrual cycle (discussed in this chapter), it is possible that ovarian production of prolactin[454,455] may mediate some of the roles that are fulfilled by pituitary prolactin in rodents. Importantly, prolactin is not required for corpus luteum maintenance in humans. LH promotes the original development of the corpus luteum, and then human chorionic gonadotropin fulfills the role of rescue of the corpus luteum and the establishment of pregnancy.[456] However, the prolactin receptor is expressed in luteinized human granulosa cells,[454,457] and prolactin acts as a survival factor in human granulosa cells in vitro,[458] suggesting that it is possible that local or circulating prolactin may have a physiological function in this tissue.

Reproductive Tract

In addition to expression in the ovary, there is good evidence for expression of the prolactin receptor in the female reproductive tract. In mice, prolactin receptors are present in the uterus[415] and fallopian tube,[459] and levels of receptor expression are regulated by ovarian steroids. Similarly, in women, prolactin receptors are found in both the nonpregnant[460,461] and pregnant[406] uterus, as well as the fallopian tube.[459] Deletion of the prolactin receptor in *Prlr*$^{-/-}$ mice causes the uteri to be refractory to implantation,[462] suggesting that prolactin may be involved in the establishment and maintenance of pregnancy.

Fetal Development

Prolactin receptors are present in the preimplantation embryo[463] and are widely expressed during embryogenesis in both rodents[464–467] and humans,[468] particularly in bone and cartilage, brain, and pituitary. In rodents, much of fetal development occurs during the second half of pregnancy, when prolactin secretion is low, and while prolactin mRNA can be detected in the fetal pituitary at approximately embryonic day 15.5[469] or 17.5,[470]

prolactin protein is not present until much later in gestation,[258] and prolactin is not detectable in the serum until soon after birth.[471] Hence, the placental lactogens are likely to be the major ligands for fetal prolactin receptors during gestation. PL-II is present in fetal serum during the latter half of gestation[472] and presumably acts on prolactin receptors present at this time. Prolactin is also present in amniotic fluid in women, and this may also influence fetal prolactin receptors.[405,473] Disruption of the prolactin receptor does not result in fetal lethality but does induce a range of developmental defects.[419] This observation is supported by recent work using a zebrafish model, where temporary disruption of prolactin production during embryonic development caused multiple morphological defects, consistent with a role for prolactin in organogenesis.[474]

The observation that prolactin receptors were particularly abundant in the olfactory system in the fetus, and in the olfactory bulb specifically,[475] was very interesting because there is little or no expression detected in this region in the adult.[10] As well as being expressed in the olfactory epithelium and sensory neurons of the olfactory bulb, prolactin receptors are found in the fetal trigeminal ganglion, tongue, whisker follicles, and facial musculature. These organs are essential for odor detection, facial sensation, taste, and the neonatal suckling reflex. One tantalizing possibility is that since prolactin is secreted into milk[476] and can be absorbed intact across the neonatal intestine,[477,478] prolactin from the mother might access the offspring's olfactory system during suckling, providing a novel mechanism to signal from the mother to her infant, fostering appropriate behavioral interactions.

Reproductive Behaviors

Lordosis and female reproductive behavior: Because of the increase in prolactin associated with ovulation and the onset of estrous behavior, there has been some interest in a possible role for prolactin in regulating reproductive behavior, particularly in the female. The data, however, do not provide a clear picture. High levels of prolactin can inhibit lordosis in female rats,[479] although this may be due to its antigonatorophic effect (discussed further in this chapter). In contrast, bromocriptine-induced inhibition of prolactin secretion during the proestrous prolactin surge inhibits female receptivity,[480] and acute administration of prolactin directly into the midbrain stimulates lordosis,[481] consistent with a stimulatory role for prolactin in female reproductive behavior. In women, while the evidence of a preovulatory surge of prolactin is marginal (described in this chapter), there is a coitus-induced release of prolactin associated with orgasm.[482] This increase following orgasm is also seen in men[483,484] and has been proposed to be involved in the feedback suppression of sexual drive[485] contributing to the latency period following orgasm. More recently, it has been proposed that the long-lasting elevation of prolactin in women following coitus may be involved in the initiation of pregnancy, facilitating decidualization and implantation.[482]

Maternal care: The behavior of female rodents toward pups (either their own or those of another animal) changes dramatically at parturition, and new mothers display a range of pup-directed behaviors that include nursing, licking, and grooming as well as retrieval of the pups to a nest site.[486] This maternal care can be induced in virgin female rats with repeated exposure to pups, albeit with a latency period of several days.[487] There is a strong body of findings that support a role for prolactin in the central stimulation of the onset of maternal behavior. The initial observations utilized a rat preparation in which gonadectomized virgin rats were treated sequentially with a systemically administered steroid regimen that consisted of 10 days of progesterone exposure followed by exposure to estradiol. This steroid treatment elevated circulating prolactin concentrations[488] and stimulated a rapid onset of maternal care toward foster young.[489] Moreover, when steroid-treated females were concurrently treated with bromocriptine to suppress endogenous prolactin secretion, the onset of maternal behavior toward foster young was delayed.[488] These behavioral effects of prolactin can be mimicked by placental lactogen.[490] Prolactin actions on maternal behavior are mediated in the central nervous system, demonstrated by the observation that ICV infusion of prolactin or placental lactogen will induce maternal behavior, whereas the same dose administered systemically has no effect.[490,491] The medial preoptic area has previously been identified as an important region for maternal care.[492–494] Using localized injections into this region, Bridges and colleagues have identified the medial preoptic area as a critical site for prolactin action to stimulate maternal behavior.[491] Prolactin receptors are expressed throughout this region,[112,143,495,496] and levels are increased during lactation.[496,497]

Earlier work in mice has also implicated the hypothalamus as a possible site of prolactin action in stimulating maternal behavior and nest building in this species.[498] The critical role of prolactin in maternal care was confirmed in the prolactin receptor knockout model, with a deficiency in pup-induced maternal care in both virgin and primiparous $Prlr^{+/-}$ mice, and an even more severe deficit in virgin $Prlr^{-/-}$ animals[499] (full knockouts are infertile, precluding the study of maternal behavior in parous animals). Interestingly, $Prl^{-/-}$ animals exhibit some maternal behavior,[418] possibly as a result of embryonic or neonatal exposure to prolactin or placental lactogen or of the actions of other ligands on the prolactin receptor.[500] Prolactin actions on maternal behavior in

mice are not limited to the medial preoptic area. Prolactin-induced neurogenesis in the subventricular zone (SVZ) during early pregnancy[501] is also critical for the normal expression of maternal behavior postpartum.[359,502]

PROLACTIN-INDUCED ADAPTIVE RESPONSES CONTRIBUTING TO SUCCESSFUL REPRODUCTION

In addition to the direct roles of prolactin in reproductive tissues and on reproductive behaviors, as described in this chapter, prolactin has a wide range of other functions in the body. While at first look, a number of these seem quite unrelated to reproduction, we consider that many of these actions result in adaptive functional changes that will assist the mother in coping with the physiological, metabolic, and behavioral challenges of pregnancy and lactation. This is particularly true of the multiple actions of prolactin exerted in the brain. In this section, we will review a variety of prolactin actions from this context: adaptive changes in physiology to meet the novel demands of the transition through pregnancy and into lactation.

Metabolic Effects

There are numerous studies linking prolactin to the regulation of metabolism, and prolactin seems to be acting both in the central nervous system and in peripheral tissues to mediate these effects. While many of these studies show effects in males and nonpregnant females, prolactin is likely to be high in such individuals only under pathological conditions. From a physiological perspective, many of these metabolic effects of prolactin make more sense if considered as part of the metabolic adaptation to pregnancy and lactation.[503] During these states, there is a dramatic increase in maternal metabolic demand to support fetal growth, parturition, and milk production.[504] To meet this increased demand, a number of physiological changes occur in mothers, including a reduction in energy expenditure, increased food intake, and an increase in nutrient absorption from the gut. There is also a change in energy partitioning to ensure adequate nutrient supply for fetal growth, as well as increased white adipose tissue mass during pregnancy and subsequent mobilization of these fat stores in lactation. Here we will examine a wide range of metabolic responses to prolactin, and consider how this might contribute to the metabolic adaptations of pregnancy and lactation:

1. *Nutrient uptake*: Prolactin receptors are expressed in the digestive tract,[505,506] and prolactin has been reported to affect ion and nutrient absorption, epithelial cell proliferation, and gastrointestinal motility. Elevated prolactin alters the expression of a number of genes related to fluid and electrolyte homeostasis, especially for Ca^{2+} (which may be critical for adaptive changes in bone metabolism during pregnancy, as discussed in the section Bone and Calcium Homeostasis), Na^+, K^+, and Cl^- transport[507] and cell proliferation in the duodenum.[508] These data suggest that prolactin contributes to the hyperplasia of the intestinal epithelium and the increased expression of transporters that occurs during pregnancy and lactation.[509,510] Prolactin also upregulates hepatic bile salt transporters in rats, which may increase the absorption of lipids and fat-soluble vitamins.[511] In addition, administration of exogenous prolactin to cycling female rats results in the downregulation of liver enzymes that control amino acid oxidation and urea synthesis,[512] consistent with the hypothesis that prolactin may contribute to the alterations in protein metabolism seen in pregnant and lactating rats.
2. *Energy partitioning*: The prolactin receptor is expressed in adipose tissue in mice,[513] rats,[514] and humans,[515] and recent evidence indicates a role in adipogenesis and in the regulation of leptin secretion from adipocytes. *Prlr*$^{-/-}$ mice show a reduced adipose tissue weight, without overall reduction in body weight or food intake.[516] The effects on leptin secretion are complex, with prolactin apparently exerting negative effects on leptin secretion by adipose cells from mice[517] and rats[514] in vitro, but circulating leptin levels are elevated in hyperprolactinemia in vivo.[518] In contrast, chronic hyperprolactinemia results in hypoglycemia and decreased adiposity in mice.[513,519] An increase in brown-like adipocytes in white fat depots occurs in *Prlr*$^{-/-}$ mice,[520] promoting energy expenditure. The increased adiposity resulting from overexpression of the long form of prolactin receptor in the absence of the short form suggests that a balance in receptor signaling might be important for the regulation of adipose tissue.[521] Again, these effects or the mechanisms underlying them may be species specific, exemplified by different effects of prolactin on adipose tissue lipolysis in rats and mice.[522] Taken together, these data from nonpregnant animals are consistent with a role for prolactin in regulating energy partitioning at the level of the adipose tissue. Prolactin appears to predominantly promote adipogenesis and

energy storage. In the context of pregnancy, the rising levels of prolactin are therefore likely to favor energy storage, contributing to the anabolic state that characterizes early pregnancy. Prolactin may also exert other effects on metabolism through actions in the liver. High levels of prolactin receptors are expressed in the liver, and this is an interesting organ because it is one of the few where the short form of the receptor is predominant.[523] There are dramatic increases in liver expression of the long form of the receptor during pregnancy in mice, which revert to virgin levels in lactation.[524] In rats, there are significant changes in the ratio of isoforms expressed in the liver during lactation.[432] The specific function of prolactin receptors in the liver, however, is unknown.

3. *Body weight*: Hyperprolactinemia is associated with weight gain in a number of species, although these effects may be species specific. Chronic elevation of prolactin in humans is frequently associated with increased weight gain, which is reduced after normalization of prolactin,[525–527] and a similar effect is found in rats.[528–530] Evidence from genetically modified mice, however, is less clear, with either excess or loss of prolactin having little effect on weight gain.[513,522] Loss of prolactin receptors in knockout mouse studies has also produced inconsistent data,[531,532] although there may be a small effect of reduced body weight in female animals. Effects on body weight represent the composite of food intake and nutrient absorption, together with energy expenditure, and provide a crude insight into the potential metabolic functions of prolactin. Data on acute effects of prolactin on appetite are more consistent and are reviewed next.
4. *Appetite regulation*: Systemic prolactin administration increases food intake in a variety of species.[528,533–535] In rats, this treatment not only increases food intake but also causes a suppression of cyclic estradiol release and increases in plasma progesterone concentrations, effectively inducing a pseudopregnancy.[534,536] The anorectic effects of estrogens are well established.[537,538] Estradiol suppresses food intake, specifically reducing meal size[539,540] and increasing energy utilization.[541] Thus, low estradiol during pregnancy might enhance food intake and suppress leptin responsiveness.[542] Conversely, progesterone treatment stimulates weight gain and fat deposition as well as food intake.[543,544] The increase in food intake in female rats following prolactin treatment, therefore, could be mediated through effects on gonadal steroids. ICV administration of prolactin increases food intake without influencing estrous cyclicity, however, whether given by bidaily injections or via chronic infusions using an osmotic minipump.[534,545,546] Moreover, ICV administration of prolactin also increases food intake in ovariectomized rats.[546] Together, these data argue for the ability of central prolactin receptor activation to increase food intake independent of steroid hormone levels. Thus, the phasic prolactin secretion of early pregnancy is likely to contribute to the rapid increase in food intake seen in pregnant rodents,[547–549] both indirectly (by promoting progesterone secretion) and directly (through actions in the hypothalamus). Prolactin receptors are found in many of the nuclei involved in the homeostatic regulation of food intake, including the arcuate, ventromedial, and paraventricular nuclei,[143,147,495] and hence these nuclei form likely targets for prolactin action during pregnancy and lactation. However, prolactin receptors do not appear to be expressed in the arcuate neuropeptide Y (NPY) and proopiomelanocortin (POMC) neurons[100,550,551] that regulate appetite. Hence, it seems likely that prolactin acts downstream of the arcuate neurons, such as at the PVN. Consistent with this hypothesis, localized injections of prolactin directly into the PVN stimulate food intake in a dose-dependent manner in female rats.[552] Pregnancy is an interesting state with respect to leptin action, in that the hyperphagia in pregnancy persists despite the increase in serum leptin resulting from the increased fat mass.[553,554] These data have been interpreted to indicate a condition of leptin resistance during pregnancy. Whilst it is possible that the available leptin in the brain is blunted as a result of increased leptin-binding protein,[555,556] saturation of a transporter mechanism,[557] or reduced transporter expression,[558] there is a reduced response to leptin infusion directly into the brain in pregnancy,[548,559] suggesting that hypothalamic leptin resistance is an important factor in the continued hyperphagia throughout pregnancy (reviewed in Refs 560–563). To test the hypothesis that elevated prolactin mediates the pregnancy-induced leptin resistance, two groups have examined leptin responses in hyperprolactinemic animals. Woodside and colleagues used chronic ICV infusion of prolactin in nonpregnant female rats, and found that elevated prolactin prevented the reduction in body weight or food intake normally seen in response to a central injection of leptin.[545] This prolactin-induced leptin resistance occurs whether the leptin is administered to satiated rats or after a 24h fast.[545] The lack of a behavioral response to leptin was accompanied by a reduction in the ability of leptin to induce Fos expression and phosphorylation of STAT3 in both the paraventricular and ventromedial hypothalamic nuclei.[545] Consistent with these data, Augustine and colleagues have shown that ICV prolactin infusions induce leptin resistance in pseudopregnant rats.[564]

Interestingly, the pseudopregnant rats, which are hyperphagic in response to early-pregnancy-like changes in prolactin, estrogen, and progesterone, remain fully responsive to leptin. It was only after chronic prolactin infusion, mimicking the chronic elevation in placental lactogen secretion that is characteristic of midpregnancy, that they became leptin resistant like pregnant animals. Taken together, these data suggest that the prolactin surges during early pregnancy stimulate an initial orexigenic response, and the resultant hyperphagia is then maintained, in the face of rising leptin levels and a positive energy balance, by placental lactogen-induced leptin resistance. The mechanism of prolactin action on leptin responses is not clear. Prolactin receptors are widespread in the hypothalamus,[143,147,495,565] including the major nuclei involved in body weight regulation: the arcuate, ventromedial hypothalamic, and paraventricular nuclei (discussed in this chapter). In neurons, prolactin activates STAT5,[105,107,566] and in particular STAT5b,[106,110] whereas leptin predominantly acts though STAT3.[567] As both hormones are acting through the JAK–STAT pathway, there is potential for the endogenous regulatory proteins, such as SOCS proteins, to cross-talk between the two signaling pathways. In ovariectomized rats, prolactin induces SOCS1, SOCS3, and CIS but not SOCS2 within the arcuate nucleus.[108] This suggests that, as in response to other cytokines, these proteins are induced as a part of the prolactin signal transduction response. During late pregnancy and lactation, levels of SOCS1, SOCS3, and CIS mRNA were all markedly elevated in the arcuate nucleus,[108] and it seems likely that these proteins mediate the loss of prolactin-induced activation of STAT5b in TIDA neurons during lactation.[108,109,180,370] Conceivably, prolactin-induced SOCS proteins might also impact other cytokine signal transduction pathways, such as leptin. While the prolactin and leptin receptor are localized in the same nuclei, however, there is little evidence of co-localization.[563] As discussed in this section, leptin receptors are expressed on POMC and NPY neurons in the arcuate nucleus. Prolactin receptors are strongly expressed in the arcuate nucleus, but these are predominantly on dopamine neurons[100] and not on POMC or NPY neurons.[100,550,551] Similarly, in the ventromedial hypothalamic nucleus, leptin receptors are predominantly in the dorsomedial aspect of that nucleus, whereas prolactin receptors are exclusively in the ventrolateral part. Furthermore, even when expressed in the same cell type, the two pathways may not inhibit each other.[568] Hence, it seems unlikely that prolactin might act directly on the first-order leptin-responsive neurons to cause leptin resistance, and if prolactin is mediating the pregnancy-induced changes in these nuclei, it must be through an indirect pathway. At least part of the resistance to leptin during pregnancy, however, is caused by a downstream loss of responsiveness to αMSH,[569] presumably in the PVN.[570] Oxytocin neurons may function as a major downstream target of αMSH from leptin-responsive POMC neurons, and αMSH stimulates oxytocin release that then has anorectic actions.[571,572] These neurons also express leptin receptors,[573] and hence form a potential site for integration of reproductive and metabolic signals to regulate food intake. Prolactin receptors are expressed on oxytocin neurons in the PVN,[574] and prolactin acutely reduces the firing rate of oxytocin neurons. Hence, these data suggest that elevated prolactin during pregnancy might act to functionally antagonize αMSH action in the PVN, resulting in secondary leptin resistance, and are consistent with the hypothesis presented here that prolactin might act to stimulate appetite in these downstream pathways.[552] There is also evidence that prolactin, acting on central prolactin receptors in the hypothalamus, contributes to the hyperphagia of lactation (reviewed in Refs 575,576). Prolactin has been implicated in stimulating changes in NPY expression in lactating rats,[551] with prolactin receptors identified on NPY neurons in the dorsomedial hypothalamus (but not arcuate nucleus) during lactation.[550] Prolactin-induced stimulation of NPY in the dorsomedial hypothalamus[551] may augment the orexigenic effects of other NPY inputs to the PVN. As described in this chapter, prolactin is the major lactogenic hormone in the rat, and thus investigations of appetite and food intake by manipulating prolactin levels in postpartum females can be confounded by effects on milk production, since the energetic demands of milk production may cause a change in appetite, independent of any direct effects of prolactin. To distinguish the energetic demands of milk production from the sensory and hormonal responses to the suckling stimulus, elegant experiments have been completed in lactating rats following transection of the galactophores (the tube that delivers milk from the mammary gland to the nipple). In these animals, maternal behavior and suckling are normal, but the inability to deliver milk leads to rapid involution of the mammary gland and the consequent loss of the metabolic energy demands of milk production. These studies have shown that the increased food intake induced by suckling is maintained even in the absence of milk production.[577] Because galactophore-cut postpartum rats have similar hormonal profiles to those of intact lactating rats, it has been possible to use this model to study the specific contributions of

suckling-induced prolactin to hyperphagia. When prolactin levels are suppressed in galactophore-cut rats using the dopamine D2 agonist bromocriptine, food intake and body weight are decreased. Replacement of prolactin by chronic ICV infusion restores food intake, suggesting that the orexigenic effects of prolactin acting on prolactin receptors within the brain make a major contribution to the hyperphagia of galactophore-cut, suckling rats.[575,578]

5. *Glucose homeostasis*: Prolactin receptors are expressed in pancreatic β-cells.[579,580] Exposure of cultures of human, mouse, and rat β-cells to prolactin or placental lactogens in vitro leads to increased insulin secretion, upregulation of glucose-stimulated insulin secretion, increased insulin expression, and β-cell proliferation.[581–583] Prolactin also stimulates expression of glucose transporter 2[584] and promotes glucose entry into the β-cells, and as a consequence it enhances the activity of glucose-sensitive enzymes such as glucokinase.[585] Rats with chronically high prolactin have enhanced insulin secretion,[586] and targeted overexpression of placental lactogens in pancreatic β-cells increases β-cell mass and insulin production.[587] Conversely, mice deficient in prolactin receptors show reduced β-cell mass as early as 3 weeks of age, reduced insulin expression, and glucose-stimulated insulin secretion.[588] These data suggest that prolactin may play an important role in development of the β-cells, and in particular may promote the surge of maternal β-cell replication that occurs in the perinatal period. Consistent with the overall hypothesis in this chapter, much recent work has focused on the role of prolactin and placental lactogen in stimulating β-cell function during pregnancy.[589] As described here, there are marked changes in maternal energy balance during pregnancy, from anabolic conditions in early pregnancy leading to accumulation of fat stores to a catabolic state later in pregnancy promoting mobilization of energy stores and increased partitioning of nutrients to the conceptus to meet the high demand resulting from rapid fetal growth. Maternal tissues develop insulin resistance to meet the very high demands of the fetal–placental unit for glucose, which can consume 50% of total maternal glucose utilized by late pregnancy,[590] leading to an increased demand for maternal insulin secretion. A failure of pancreatic islets to meet this demand leads to gestational diabetes, affecting 135,000 pregnancies annually in the United States[591] and leading to a 70% increase in the risk of developing type 2 diabetes.[592] The pancreatic islet adapts to this altered demand by increasing β-cell mass, principally by increased cell proliferation,[593] enhanced insulin synthesis,[594] a decreased glucose-stimulated insulin threshold,[595] enhanced glucose and cAMP metabolism,[596] and increased gap junction coupling between β-cells.[597] There is good evidence that these changes in β-cell biology are a result of the chronic increase in prolactin and placental lactogen acting on the prolactin receptor.[598] As described in this chapter, pregnancy is associated with high levels of prolactin signaling, both due to prolactin itself and due to high levels of placental lactogen. There is a dramatic increase in prolactin receptor levels in the pancreas during pregnancy,[580,589] and hence many of the documented effects of prolactin on islet function, including expansion of β-cell fraction, enhanced insulin expression, and increased sensitivity to glucose-stimulated insulin secretion, are most physiologically relevant at this time. In $Prlr^{+/-}$ mice that have a 50% reduction in β-cell expression of prolactin receptors, there are reduced β-cell mass and proliferation, decreased glucose-stimulated insulin release, and impaired glucose tolerance during pregnancy.[599] These data demonstrate that prolactin and placental lactogen play a key role in the adaptations of the β-cells during pregnancy. The mechanism by which prolactin and placental lactogen increase the proliferation of β-cells has been studied extensively in recent years, as a result of the interest in increasing β-cell proliferation for therapeutic purposes in diabetes. The main pathway activated by prolactin in β-cells is STAT5,[583,600,601] although prolactin induction of insulin expression may be independent of that pathway.[602] Prolactin can also activate the PI3K and MAPK pathways in β-cells,[38] and reduce expression of the transcription factor FoxO1,[601,603] an inhibitor of β-cell proliferation.[604] The D cyclins have been shown to be essential for β-cell proliferation,[605] and transcription of cyclin D2 has been shown to be induced in β-cells by prolactin-induced activation of STAT5, leading to increased proliferation.[606] A role for menin, previously characterized as an endocrine tumor suppressor, in the regulation of the cell cycle inhibitors in β-cell has been described, as menin and its associated cell cycle inhibitors are downregulated in islet cells in pregnancy.[607] This response was also dependent on Stat5,[607] implicating signaling from the prolactin receptor, and has also been shown to involve induction of the transcription factor FoxM.[608] Recent studies have implicated pancreatic serotonin as an important factor that is induced by placental lactogens in islet cells of mice and can increase β-cell proliferation.[609,610] The serotonin-synthesizing enzymes Tph1 and Tph2 are induced by placental lactogen in primary pancreatic cultures

from nonpregnant animals, and their expression is increased in islets of pregnant mice, peaking at the 12th day of gestation, when there is also an increase in islet serotonin. It is possible that there is also an increase in serotonin receptor expression in islets in pregnancy, and loss of the 5HTr2b serotonin receptor was found to lead to a reduction in β-cell proliferation in pregnancy.[610] There is evidence for this important role for placental lactogen in β-cell adaption in humans, although given its pattern of secretion, prolactin may also have a role. Patients with prolactinomas have increased insulin secretion in response to glucose and decreased glucose tolerance similar to that found in pregnancy,[586,611,612] and the increased insulin is reduced after bromocriptine treatment to normalize prolactin secretion.[613] hPL has been found to increase β-cell survival and increase insulin secretion in vitro,[614] and prolactin has been shown to protect human β-cells from apoptosis.[615] The protective effects of prolactin and placental lactogen have been suggested to be of importance in countering the effects of a number of factors in pregnancy that have detrimental effects on β-cell survival and function, such as high glucocorticoids.[598,616]

Bone and Calcium Homeostasis

Prolactin receptors have been identified in multiple sites involved in calcium metabolism, including gastrointestinal tract, kidney, and bone, implicating a direct effect in these areas.[505,523,617–619] The *Prlr*$^{-/-}$ mouse is characterized by delayed bone formation, despite no apparent reduction in the number of osteoclasts.[618] Long- and short-form prolactin receptors have been localized specifically to chondrocytes[620] and osteoblasts,[618,621] but not osteoclasts.[618] Studies predominantly involving cultured cells have begun investigating direct prolactin-stimulated effects on bone at both basal and high prolactin levels.[622,623] Prolactin has been implicated in affecting osteoblast function rather than differentiation as it can decrease alkaline phosphatase activity and osteocalcin mRNA expression, two markers of osteoblast activity.[624] Furthermore, prolactin can alter the ratio of osteoblast-derived factors, RANKL and its decoy receptor osteoprotegerin (OPG), in a dose- and age-dependent manner.[624,625] In adult rats, prolactin stimulates an increase in RANKL expression and a simultaneous decrease in OPG levels, resulting in elevated bone resorption.[625] Pathological hyperprolactinemia leads to osteopenia and osteoporosis,[626,627] although this might also be secondary to the prolactin-induced decrease in estradiol levels (discussed further here).

Consistent with the overall hypothesis of this chapter, it seems likely that prolactin actions on bone and calcium homeostasis relate to the marked changes that occur during pregnancy and lactation. During pregnancy, women lose approximately 200–300 mg/day of calcium in the third trimester for mineralization of the developing fetal skeleton. This increases to 300–1000 mg/day for lactogenesis.[628] Consequently, adaptations in calcium metabolism are necessary during these reproductive states to safeguard the maternal skeleton against increased demand for calcium and other minerals from the growing fetus or neonate. Prolactin, which is elevated during pregnancy and lactation, has been described as the principal calcitropic hormone,[510] in terms of its action both on calcium absorption, described here, and on regulating bone turnover. During pregnancy, there is a net gain in bone density,[621] but during lactation, bone resorption is a major source of calcium for milk production.[628] The bone resorption occurring during lactation is dependent on prolactin,[621] but cannot solely be explained by a shift in the RANKL:OPG ratio.[625] In addition, prolactin has been shown to induce mRNA of other osteoblast-derived factors involved in osteoclastogenesis.[623] These include monocyte chemoattractant protein (MCP1), cyclooxygenase 2 (Cox2) at a prolactin concentration of 300 ng/ml, and tumor necrosis factor alpha (TNFα) and interleukin 1 (IL1) at a prolactin concentration of 500 ng/ml.[624] This indicates that increasing prolactin levels during pregnancy and especially lactation may act to switch on certain genes in a time-dependent manner. Furthermore, although prolactin has the ability to directly affect osteoblast function, it also acts as an indirect modulator of osteoclast production and activity.

The direct effects of prolactin on bone metabolism are well established, but it is possible that prolactin also acts indirectly to influence calcium homeostasis via the central nervous system. It is known that the hypothalamus exerts a regulatory control of peripheral calcium homeostasis.[629] Recent evidence has identified a role for the brain in mediating the actions of leptin on bone function[630,631] via the sympathetic nervous system. While there is no evidence as to whether prolactin might influence bone in the same manner, the stimulation of prolactin receptors in the hypothalamic PVN of the rat has been shown to be protective against stress-induced hypocalcemia,[348] and knockdown of prolactin receptor expression in this nucleus influenced circulating plasma calcium levels in nonstressed and stressed states,[351] thereby implicating a central prolactin mechanism in regulating circulating calcium levels.[351] It seems possible that such a mechanism could also play a role in the adaptive changes in calcium homeostasis during pregnancy and lactation.

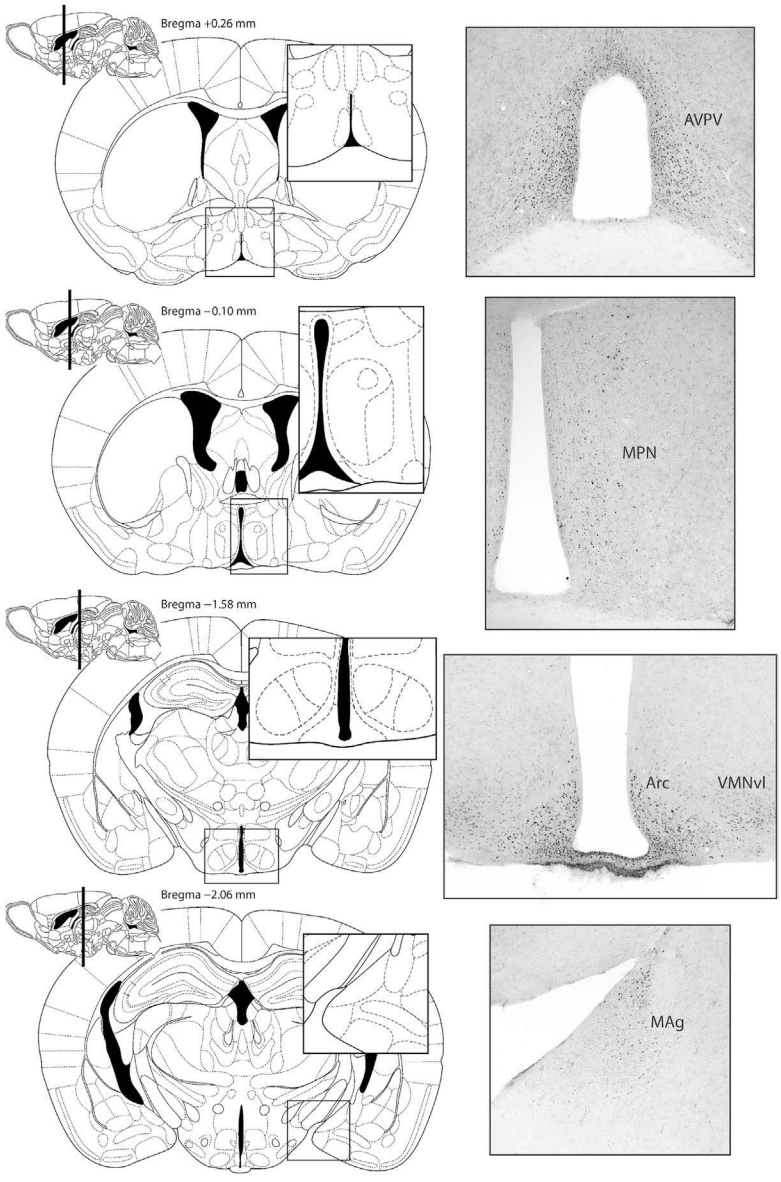

FIGURE 12.7 Map of prolactin-responsive neurons in the mouse brain (based on Refs 147,638). Diagrams on the left show the major structures of the mouse brain *(Source: Adapted from the Paxinos and Franklin brain atlas[639].)*. Micrographs on the right show prolactin-induced pSTAT5 (black dots represent nuclear staining of individual neurons) in different brain regions at that particular level. Major areas showing positive labeling of prolactin-responsive neurons are labeled (AVPV, anteroventral periventricular nucleus; MPN, medial preoptic nucleus; Arc, arcuate nucleus; VMNvl, ventromedial hypothalamic nucleus, ventrolateral subdivision; MAg, medial amygdala; MCG, midbrain central gray matter; AP, area postrema; NTS, nucleus of the solitary tract).

Brain Actions of Prolactin

In addition to the specific effects on reproductive behaviors, the central effects on appetite and metabolism, and the effects on NEDA neurons to influence its own secretion by short-loop negative feedback, outlined in this chapter, prolactin also exerts many other actions in the brain[632] that can be seen as facilitating reproductive processes.

Expression of prolactin receptors in the brain: Prolactin receptors have been identified in the brain, particularly in the rat, using in vitro binding assays and autoradiography,[633–636] immunohistochemistry,[143,496,637] and in situ hybridization.[112,495,565] More recently, prolactin-induced phosphorylation of STAT5 has been used to investigate prolactin-responsive neurons in the mouse brain.[147,638] All of these studies have provided a consistent picture of the distribution of prolactin receptors in the brain (see Figure 12.7). Expression is widespread, particularly in medial hypothalamic nuclei from the preoptic region to the caudal arcuate nucleus, but also in a number of extrahypothalamic regions. There are also

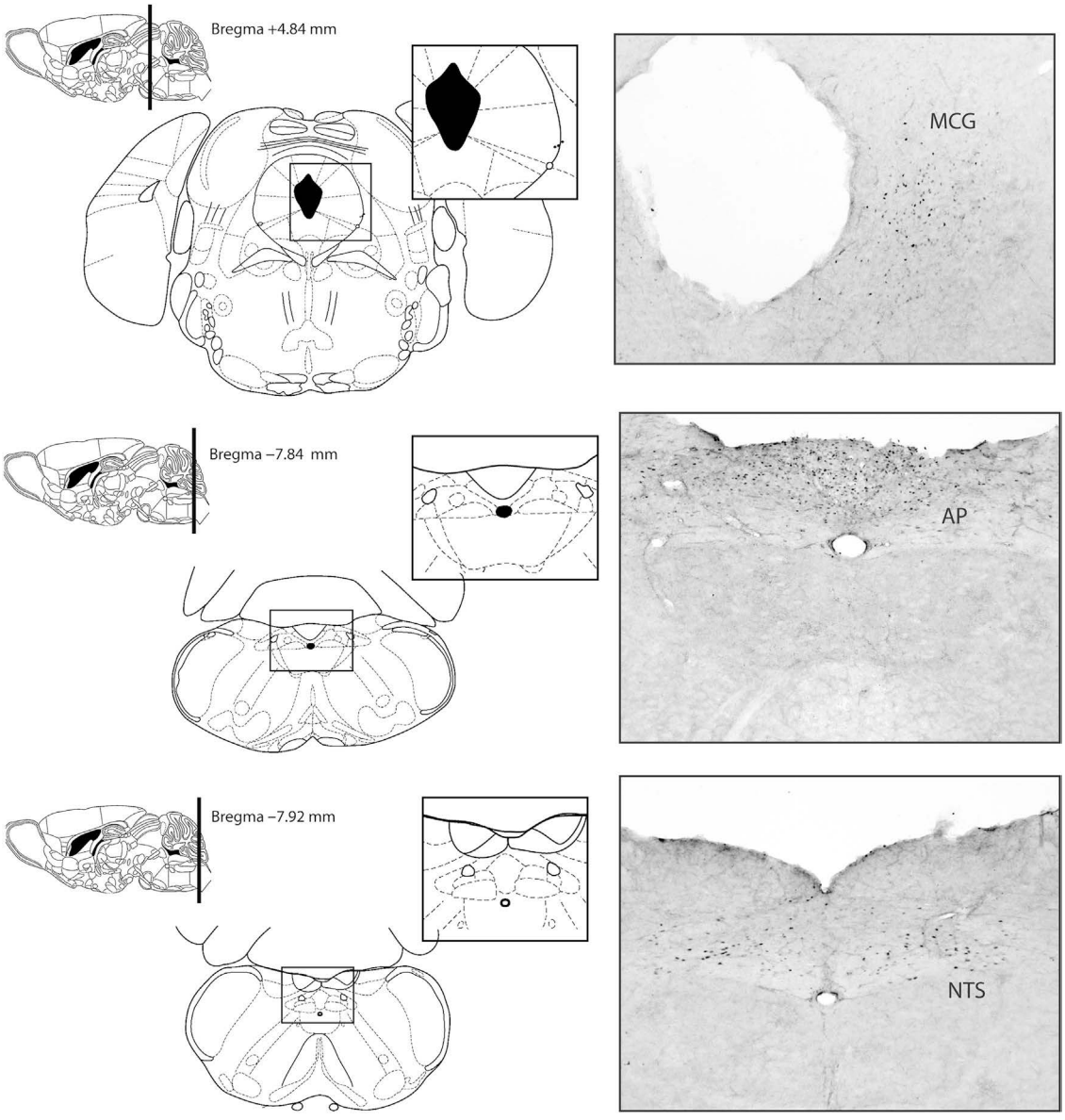

FIGURE 12.7 Cont'd

exceptionally high levels in the choroid plexus, and it has recently been reported that multiple variants of exon 1 of the prolactin receptor drive expression in this tissue.[640] Using the mouse as a model species, prolactin responsive neurons are observed to be particularly dense around the organum vasculosum of the lamina terminalis and rostral part of the third ventricle, in the anteroventral periventricular nucleus, periventricular preoptic nucleus, and median preoptic nucleus. At this level of the brain, there is also extensive prolactin receptor expression throughout the bed nucleus of the stria terminalis. Moving caudally, prolactin receptors are highly expressed in the medial preoptic nucleus, the supraoptic nucleus, and both the magnocellular and parvicellular subdivisions of the PVN. In the mediobasal hypothalamus, prolactin receptors are expressed in the arcuate nucleus and the ventrolateral subdivision of the ventromedial nucleus. Prolactin receptor expression is also seen in the medial amygdala at this level of the brain. In the midbrain, prolactin receptors are expressed in the parafascicular nucleus and central gray matter, and then in the hindbrain, area postrema, and nucleus of the solitary tract. Thus, there is considerable potential for prolactin to influence brain function.

The overall distribution of prolactin receptor–containing neurons strongly resembles the distribution of ERα in the mammalian brain.[641] There is significant evidence that prolactin receptor expression is directly influenced by estradiol[98,635,642,643] and progesterone,[644] and some of

the biological actions of prolactin in the brain are dependent on estradiol.[645] Prolactin receptor expression in the brain is also regulated during pregnancy[114,495,497,646] and lactation.[496,497,647–649] The mechanisms mediating increased expression of prolactin receptors in the brain of pregnant and/or lactating rats are not known. Prolactin itself may mediate an upregulation of its receptors,[650,651] as occurs in some other tissues. It is probable that hormonal changes of pregnancy, such as the high progesterone levels, prolonged elevations of placental and then pituitary lactogens, and the increasing estrogen–progesterone ratio prior to parturition, all play a role in stimulating the expression of prolactin receptors during pregnancy and lactation. The elevated prolactin receptor expression during lactation appears to result from the combined stimulatory effects of suckling-induced hyperprolactinemia, and the neural input from the suckling stimulus per se.[652]

The neurochemical phenotype of prolactin-responsive neurons in various regions is gradually being described. As well as being expressed on TIDA neurons in the arcuate nucleus,[98,100] prolactin receptors are also expressed in enkephalin neurons,[653] kisspeptin neurons,[654] as well as some as yet unidentified neurons in this nucleus. Despite evidence that prolactin might regulate β-endorphin production,[655–657] only a few POMC neurons express the long form of the prolactin receptor.[100] In the supraoptic and paraventricular nuclei, oxytocin neurons express the long form of the prolactin receptor.[574] In contrast, vasopressin neurons do not express the long form of the prolactin receptor but may express the short form of the receptor, as prolactin receptor-like immunoreactivity has been identified on these cells.[658] Prolactin has been reported to activate MAPK in corticotrophin-releasing hormone neurons in the PVN,[659] although this could be direct or indirect. In the dorsomedial hypothalamic nucleus, but not the arcuate nucleus, prolactin receptors are expressed on NPY-containing neurons.[550] In the ventromedial hypothalamic nucleus, prolactin receptors are localized on enkephalin neurons.[653] In the anteroventral periventricular nucleus, most kisspeptin neurons express the prolactin receptor, as well as many GABAergic neurons,[654] while very few GnRH neurons express prolactin receptors.[660] Hence, consistent with the pleiotropic role of prolactin, there are a wide variety of prolactin-sensitive neurons in the hypothalamus.

Adult neurogenesis: The changes in behavior associated with motherhood, as described in this chapter, are associated with neuronal plasticity leading to remodeling of neural circuits (reviewed in Ref. 661), some of which may be mediated by adult-born neurons in the hippocampus and olfactory bulb that are associated with learning.[662] Although recent reports have suggested a role for prolactin in neural stem cell proliferation[663] and increased hippocampal neurogenesis in both virgin female and male mice,[664] there is no evidence for either increased hippocampal neurogenesis in early or late gestation in rodents[359,501,665] or decreased postpartum proliferation during lactation.[666–669] There is increasing evidence, however, for increased neurogenesis in the SVZ of the forebrain during mate choice, early pregnancy, and lactation in rodents,[359,501,670–672] although the timing of this may differ between species.[665] A direct role for prolactin in this generation of new neurons has been shown by a similar increase in SVZ neurogenesis after infusion of prolactin either peripherally or intracerebroventrically in ovariectomized mice,[501] and reduced SVZ neurogenesis either in pregnant $PRLR^{+/-}$ mice[501] or after a suppression of prolactin in early pregnancy.[359] The newly generated neurons migrate to the olfactory bulb, where they are likely to have a role in the learning of novel odorants,[673] which is required for offspring recognition.[674] Prolactin has also been shown to have a role in the proliferation of both astrocyte[675] and oligodendrocyte[676] populations, which potentially leads to recovery of the brain after injury.[677] Intriguingly, this may be a mechanism underlying the remyelination of neurons associated with the remission of multiple sclerosis in the third trimester of pregnancy.[678] Taken together, these data indicate a key role for prolactin in promoting plasticity within the central nervous system, a function that is likely to be particularly prominent during pregnancy and lactation when prolactin levels are high. More work is required to determine the physiological relevance of these processes, although impaired maternal behavior has been observed when prolactin-induced neurogenesis is blocked during pregnancy in mice.[359,502]

Anxiety and stress responses: In pregnancy and lactation, there is a marked attenuation of anxiety as well as the hypothalamic–pituitary–adrenal axis response to stress[679–681] (see also Chapter 44). This is thought to help protect the fetus from adverse programming effects of elevated glucocorticoids during early development. Prolactin acts as an endogenous anxiolytic agent, able to induce dose-dependent suppression of anxiety behaviors[682] as well as the acute stress response.[683,684] Both of these effects could be prevented by central administration of antisense oligonucleotides directed against the prolactin receptor.[351,684] Chronic prolactin treatment blocked the stress-induced increase in corticotrophin-releasing hormone mRNA in the PVN, and reduced neuronal activation in response to stress, as measured by *c-Fos* mRNA.[682] In addition, stress-induced prolactin secretion has been associated with increased prolactin levels detected in the PVN.[685] Thus, it seems possible that prolactin may have a role in the suppression of the stress response

during pregnancy.[686] As with induction of maternal care, prolactin may have acute anxiolytic effects during late pregnancy or lactation, but it can also influence postpartum anxiety through its effect on neurogenesis early in pregnancy.[359]

Regulation of oxytocin neurons: Oxytocin neurons play a critical role in parturition, milk ejection, and maternal behavior. Prolactin receptors are expressed in oxytocin neurons,[574] and prolactin increases phosphorylation of STAT5,[687] induces expression of the transcription factor Egr1,[688] and acutely suppresses the firing rate of these neurons in nonpregnant females.[574,689,690] In contrast, prolactin appears to increase the secretion of oxytocin in males[691] and ovariectomized females,[692] likely through activation of neuronal nitric oxide synthesis.[691] As discussed here for TIDA neurons, it seems unlikely that the JAK–STAT response is mediating the acute effects on electrical activity; it is more likely to be inducing a change in gene transcription in these neurons. It has been proposed that the acute inhibitory effect on firing might reflect a feedback role,[693] regulating a putative prolactin-releasing role of oxytocin.[321,694] In lactating animals, prolactin stimulates oxytocin secretion[695,696] and expression of oxytocin mRNA,[697,698] likely involving the JAK–STAT pathway to activate gene transcription. Moreover, ICV administration of a prolactin receptor antagonist impairs parturition,[699] consistent with a role for prolactin in stimulating the normal firing pattern of oxytocin neurons at this time. Hence, prolactin has a complex role in the regulation of oxytocin neurons, and specific effects may be dependent on the physiological state of these neurons.

Suppression of fertility: Hyperprolactinemia is an established cause of infertility. Prolactin suppresses both the frequency and amplitude of LH pulses[700–702] and reduces GnRH secretion into the portal blood.[703–705] Prolactin can also act in the pituitary gland to suppress LH secretion.[706–708] In humans, pulsatile GnRH replacement can reverse the infertility induced by hyperprolactinemia,[709–711] suggesting that a prolactin-induced suppression of GnRH release is the primary cause of infertility. Such an effect might be mediated directly on GnRH neurons,[712] but since the majority of GnRH neurons do not express the prolactin receptor,[660] it is more likely that prolactin acts indirectly through prolactin-sensitive afferent neurons, such as kisspeptin or GABA neurons.[654]

During pregnancy and lactation, suppression of fertility may be a physiological function of hyperprolactinemia. Lactation is associated with a period of physiological suppression of fertility in most mammalian females, including women.[713] This is an important adaptive response, allowing the mother to focus her energy on feeding her new offspring before investing resources in a further pregnancy.[714] In humans, this function has served as a critical regulator of population growth, spacing the timing of births to allow the mother to ration her metabolic investment in reproduction across sequential pregnancies.[715] Other species, such as rodents, have evolved mechanisms to bypass this limit on fecundity, with postpartum ovulation and mating immediately after birth allowing a condition of concurrent pregnancy and lactation—despite anovulation throughout the duration of the lactation.[716] Even here, a delayed implantation ensures that the new litter is not born until the appropriate time of weaning of the original pups.[717,718] Despite the extensive impact on mammalian reproductive physiology, our understanding of the mechanisms mediating lactational infertility remains incomplete.[713,714] It is clear that the suckling stimulus is the critical inhibitory signal,[719] and the proximal cause of infertility is an almost-complete suppression of GnRH secretion from the hypothalamus and the consequent loss of pituitary gonadotropin secretion.[720] It seems likely that prolactin might be involved in the lactation-induced suppression of pulsatile secretion of GnRH, but earlier studies looking at this have been equivocal. Bromocriptine-induced suppression of prolactin secretion was not sufficient to fully reverse the effects of lactation in the presence of an ongoing suckling stimulus in rats,[719,721–724] although suppression of prolactin by itself was sufficient to increase pulse generator function in a subset of lactating monkeys.[725] The timing of these experiments might be important, as some studies have revealed changes in the relative roles of prolactin and/or suckling across the duration of pregnancy.[719] Studies have recently shown a loss of kisspeptin expression during lactation,[726–729] and given the prolactin receptor on these neurons,[654] this is again consistent with a possible role for prolactin in the lactation-induced suppression of GnRH.

Brain prolactin: While there is extensive evidence of systemic prolactin being transported into the brain, the possibility that central prolactin receptors are influenced by prolactin of brain origin should also be considered. The role of brain prolactin remains controversial, however, despite numerous reports of both the protein and its mRNA in brain tissue. As discussed in this chapter, it seems unlikely that the pituitary-specific promoter would drive prolactin expression in the brain in the rodent, but it remains possible that a novel promoter could induce expression in nervous system cells. The original observation of immunoreactive prolactin in the hypothalamus of rats[730] has been independently replicated by several groups,[731–736] although one study showed cross-reactivity of prolactin antiserum with POMC.[737] Importantly, prolactin-like immunoreactivity was not depleted by hypophysectomy, suggesting that it was unlikely to

be of pituitary origin. Moreover, de novo prolactin synthesis and prolactin release could be detected from hypothalamic fragments in vitro,[733,738,739] suggesting local production of prolactin in neurons or glial cells. Subsequently, prolactin mRNA was detected in brain tissue by real-time polymerase chain reaction[738,740,741] and by in situ hybridization in fetal sheep brains.[742] Prolactin expression in the brain has been colocalized with estrogen receptors[731,736] and appears to be stimulated by gonadal steroids.[731,738,740,743,744] Prolactin mRNA in the brain is also increased by stress and during lactation,[685,745] although there have not yet been any studies demonstrating prolactin mRNA in the adult brain by in situ hybridization. Although there is not universal agreement, most studies report prolactin or its mRNA in the medial preoptic, paraventricular, periventricular, and arcuate nuclei, where there are high levels of prolactin receptor, and thus it seems possible that some actions of prolactin in the brain might be mediated by prolactin acting as a neuropeptide as well as a neuroendocrine hormone.[746] It is not clear what levels of prolactin might be released into the central nervous system, relative to levels entering the brain from the systemic circulation. When pituitary prolactin is blocked by bromocriptine treatment, however, there is a complete absence of phosphorylated STAT5 in the brain,[147] suggesting that if brain prolactin is present, levels are insufficient to activate this pathway under basal conditions in the adult. It remains possible, though, that brain prolactin may play a role under specific conditions, such as during brain development[664] or in response to brain injury.[4,327,376,677]

REPRODUCTIVE ACTIONS OF PROLACTIN IN THE MALE

While our focus in this chapter has been on the broad roles of prolactin in helping the mother adapt to pregnancy and lactation, it is important to recognize that prolactin also has reproductive functions in the male. These functions have not been as widely studied as in the female, probably because the *complete* infertility found in knockout models is restricted to that sex.[418,419] Nevertheless, prolactin exerts physiologically significant functions in the male reproductive system, and we will summarize available data here.

Fertility

The male infertility and loss of libido associated with acute hyperprolactinemia[747] are most likely the results of inhibition of GnRH secretion (as discussed in this chapter). However, prolactin may also influence the male reproductive system directly. Prior to the generation of prolactin and prolactin receptor knockout models, roles for prolactin in testis function had been suggested by the observation that prolactin receptors are expressed in the testis,[748] and studies showing the effects of prolactin on Leydig cell response to LH[749,750] and on the Sertoli cell response to FSH.[751] The physiological importance of these roles was questioned, however, by reports that male *Prlr*$^{-/-}$ and *Prl*$^{-/-}$ mice were able to reproduce and have normal plasma testosterone concentrations, indicating normal testicular response to LH.[131,752] This has led to widespread assumption that prolactin is not involved in the normal regulation of fertility in the male. As discussed here, however, it is important to recognize that compensation is common in knockout models. The lack of overt phenotype in *Prlr*$^{-/-}$ mice does not necessary rule out a physiological function of prolactin in male fertility. In fact, the original description of *Prlr*$^{-/-}$ male mice reported reduced fertility in 33% of animals, and a further 20% were completely infertile,[419] which was confirmed in one subsequent study[753] but not another using the same model.[752] In *Prl*$^{-/-}$ mice, there was no reduced male fertility,[131] but these mice would have some prolactin and placental lactogen exposure from the mother and placenta. The lack of an infertility phenotype in *Prl*$^{-/-}$ males, as well as the inconsistent findings with *Prlr*$^{-/-}$ mice (which may be a result of different genetic background),[752] would be consistent with compensation by other factors or pathways. In studies of *Prlr*$^{-/-}$ mice where reduced fertility was found, mating behavior, intromission, ejaculation, testes weight, and spermatogenesis were all normal, and the cause of the infertility is unclear. Interestingly, in one study reporting *Prlr*$^{-/-}$ male mice with reduced fertility, those animals that were fertile had a prolonged latency to the first pregnancy, but subsequent mating with the same female was normal (postpartum mating).[753] These data implied that an abnormal male ejaculate–female reproductive tract interaction may be responsible for the reduced fertility in *Prlr*$^{-/-}$ mice. A role for the female immune response to semen has been suggested in preeclampsia, with tolerance in subsequent pregnancies with the same father.[754]

There are significant quantities of prolactin in human seminal plasma[755] and even higher concentrations in epididymal fluid,[756] and studies have suggested possible roles in sperm capacitation.[757,758] Prolactin receptors are strongly expressed in the midregions of human spermatozoa,[756] and prolactin acts as a pro-survival factor for these cells by suppressing entry into a programmed cell death pathway. While there is clear evidence for this pro-survival pathway, it is likely a redundant mechanism, with a number of other growth factors able to exert the same function.

Prostate Gland (See Also Chapter 18)

The prolactin receptor is expressed in the prostate gland,[759,760] and prolactin acts as a survival factor for

prostate cells in culture.[761] As seen in the testis, knockout of prolactin or its receptor did not reveal a key role for prolactin signaling in mouse prostate physiology,[752] but there is plenty of evidence for prolactin regulation of normal prostate function.[762] Analysis of changes in prostate gene expression resulting from loss of prolactin receptors implicates prolactin in the regulation of genes with roles in sperm–oocyte interaction, copulatory plug formation, and protection of sperm from female immune response.[753]

Paternal Behavior

The role and extent that the mammalian father plays in parental care of offspring vary amongst species, principally being dependent on ecological conditions that provide an advantage from biparental care for reproductive success.[763,764] Whilst numerous studies in a range of species, including humans,[765,766] have found an association between prolactin and paternal care,[767] the role that prolactin plays in the induction of paternal behavior is unclear and controversial, even in rodents.[768,769] This is, in part, a result of the differences in paternal behaviors between species. Nevertheless, paternal recognition of offspring to prevent infanticide and attacks on adult sons and daughters is consistent amongst most species. Pup contact by male rats can lead to some forms of parental care behavior, and this is associated with an increase in serum prolactin, as well as increased expression of the long form of the prolactin receptor.[770] Further evidence for a direct role for prolactin in paternal recognition of offspring has been shown by studies of *Prlr*$^{-/-}$ fathers, who fail to distinguish adult offspring from nonoffpring, possibly as a result of failure of prolactin-induced neurogenesis in the SVZ and the dentate gyrus.[771]

PROLACTIN AND REPRODUCTIVE DISORDERS

While the focus of this chapter has been on the role of prolactin in the physiology of reproduction, it is important to acknowledge that inappropriate prolactin levels, either too little or too much, are associated with a range of pathological conditions. As might be expected from its primary roles, prolactin is principally associated with disorders of the reproductive system, with the major pathologies being fertility disorders and cancers of the breast and prostate. However, in accordance with its potential roles in a modulation of a wide range of physiological systems, prolactin has been implicated in diseases as diverse as autoimmune disorders,[772] endometrial carcinoma,[773] peripartum cardiomyopathy,[774] chronic renal failure, hepatic insufficiency, and polycystic ovarian syndrome.[775]

Hyperprolactinemia

In addition to the high levels of prolactin secretion associated with a variety of normal physiological states, hyperprolactinemia can result from a number of pathological conditions and pharmacological treatments. Hyperprolactinemia is the most common endocrine disorder,[776] occurring in 1% of women,[777] and is recognized as a major cause of reproductive dysfunction. Delayed puberty is observed in both males and females.[778] Women with chronic hyperprolactinemia present with infertility, galactorrhea, amenorrhea, reduced libido, and even habitual abortion. Up to 40% of women presenting with secondary amenorrhea have increased serum prolactin levels.[779,780] Men may similarly present with infertility and galactorrhea, in addition to decreased libido. In men, hyperprolactinemia is present in approximately 16% of patients with erectile dysfunction and 11% of men with oligospermia.[747] Hyperprolactinemia is also a serious side effect of neuroleptic drugs, resulting in infertility and loss of libido in up to 70% of patients, representing one of the major causes of noncompliance with antipsychotic medication.[780] Most of the symptoms can be accounted for by an altered pattern of GnRH secretion, with a marked reduction in both the frequency and amplitude of LH pulses[709] (a direct result of GnRH pulses), and the suppression of LH pulsatility can be reversed by reducing serum prolactin concentrations to normal.[781] Pulsatile GnRH replacement can reverse the infertility induced by hyperprolactinemia,[709,710] suggesting that a prolactin-induced suppression of GnRH release is the primary cause of infertility.

The two principal pathologies leading to hyperprolactinemia are prolactin-secreting tumors known as prolactinomas (discussed in this section) and other sellar and parasellar lesions that lead to a functional disconnection of the normal dopaminergic control of lactotrophs. A functional disconnection of dopaminergic control can also occur after traumatic brain injury (TBI), which can result in pituitary stalk damage or posttraumatic deficits of dopamine. Hyperprolactinemia has been described in 52% of individuals during the acute period post TBI, with levels correlating with the severity of injury.[782] Pharmacological treatments that antagonize dopamine D2R or lead to reduced dopamine secretion are also a major cause of hyperproactinemia.

Prolactinomas: Whilst asymptomatic pituitary tumors are found in 14–22% of individuals at autopsy, symptomatic tumors are much rarer, occurring with a frequency of 1:1000 to 1:1300 in the general population.[783] These tumors are generally benign, and metastases occur in less than 0.5% of symptomatic pituitary tumors.[784] Prolactinomas are the most common, accounting for 30–35% of pituitary tumors, and are classified according to their size as microprolactinomas (<10 mm) or macroprolactinomas

(>10 mm). Prolactinomas have been reported to occur more frequently in females than males, most commonly between 20 and 50 years of age, although the more obvious symptoms in females may account for part of the apparent higher frequency. Prolactinomas are commonly successfully treated with dopamine agonists, such as cabergoline, which result in normalization of prolactin levels and reduction in tumor size.[785] Prolactinomas have a variable response to dopamine agonists, however, and in some tumors there is a complete resistance to these drugs.[786] There are multiple potential mechanisms underlying this dopamine agonist resistance (reviewed in Ref. 787), including reduced receptor density, disruptions of autocrine growth factor–signaling pathways, and potential alterations in dopamine receptor isoform expression.

The molecular alterations leading to prolactinoma formation are unclear, but the monoclonal origin of the tumors indicates a mutation occurring in a single cell that confers a growth advantage from activation of a proto-oncogene or inactivation of a tumor suppressor gene.[788] Mutations leading to the reduced dopamine sensitivity of lactotrophs are excellent candidates, as demonstrated by the tumor occurrence in D2R-deficient and dopamine-deficient mice.[302] However, it is likely that there are several different mechanisms underlying prolactinoma formation, with the low incidence of progression from microprolactinoma to macroadenoma, which occurs in only 4–7% of cases, suggesting that the two types of tumor have distinct origins.[789]

Breast Cancer

Both prolactin and its receptor have been shown by immunohistochemistry to be expressed in human breast carcinoma,[790] and the role that the hormone plays in normal breast development and function suggests that it is likely to have a role in mammary tumorigenesis, although this remains controversial.[791,792] Epidemiological data linking prolactin with the development of breast tumors are limited,[793] but an increasing number of studies are demonstrating a correlation between high circulating prolactin concentration and risk of subsequent development of breast cancer in women with a range of ethnic and genetic origins (e.g., Refs 794,795). There are potential complicating factors in demonstrating a link between high prolactin and risk of breast cancer. For example, patients with prolactinomas and extremely high levels of circulating prolactin frequently have hypogonadism,[796] and the resulting reduced estradiol may influence tumor development or even be protective if the breast tumor is estrogen dependent.[792] Both in vitro analyses of human breast cancer cell lines and in vivo studies of animal models have provided strong evidence for a role of prolactin in the etiology of breast cancer (reviewed in Refs 34,792). The effects of antibody and antagonist treatment indicate that local production of prolactin by breast tissue itself may have a role in mammary tumorigenesis[34,410] and it is possible that mammary gland prolactin may have a different effect than pituitary prolactin on the tissue, either from post-translational modification or by intracrine actions.[797] More recent studies have shown that the microvasculature of human breast tumors expresses the prolactin receptor and that prolactin may have a direct effect on endothelial cell migration,[798] suggesting a potential key role for the hormone in tumor angiogenesis.

Prolactin receptor signaling may also be an important variable in the development of breast cancer, with studies showing increased receptor concentration in malignant tissue[799] as well as differences in expression of different isoforms.[800] More recently, activating mutations in the prolactin receptor have been described that are associated with the development of breast fibroadenomas.[801] Prolactin effects on mammary carcinogenesis are likely to be mediated by Stat5 signaling, since reduced Stat5 decreases the incidence of mouse mammary tumors.[802] Other prolactin receptor–signaling pathways may have a role, however, for example the recent description of nuclear prolactin receptor signaling leading to increased ERα and progesterone receptor expression in breast cancer cell lines.[803]

A role for prolactin in mammary tumorigenesis has also been demonstrated in vivo in animal models with increased or decreased exposure of mammary tissue to prolactin. Transgenic mice with ubiquitous overexpression of rat prolactin have a 100% incidence of mammary carcinomas by 11–15 months of age,[804] whilst those with expression restricted to mammary epithelial cells have an increased incidence of mammary tumors from 5% to 65–80% in mice older than 19 months.[805] Conversely, loss of prolactin[806] or the prolactin receptor[807] leads to a retardation of mammary tumor formation in mice.

Prostate Cancer (See Also Chapter 18)

Although prostate and breast cancer have very different apparent pathologies, an association between these cancers is suggested by several similarities such as their biochemistry, hormone dependence, incidence, and risk factors.[808] Although in the case of prostate cancer there is no evidence for an increased risk from high circulating prolactin,[809] there is increasing evidence for a role of locally produced prolactin acting on prolactin receptors within the tissue in the etiology of these tumors.[760] Prolactin activation of the Jak2–Stat5a/b pathway has been demonstrated in ex vivo cultures of human prostate cancers, and there is a correlation between activated Stat5 and the histological grade of tumors.[810] As with in vitro studies

of breast cancer cell lines, blocking prolactin signaling with a receptor antagonist leads to decreased cell viability through induction of apoptosis,[811] whilst prolactin acts as a mitogen in primary cultures of rat prostate cells and in vivo.[761,812] Animal studies also implicate prolactin in the etiology of prostate cancer, with transgenic overexpression of prolactin in mice causing enlargement of the prostate gland[813,814] and a reduction in tumor incidence in prolactin receptor knockout mice[753] or following prostate-specific overexpression of a prolactin receptor antagonist.[35]

CONCLUSION

The textbook definitions of prolactin action focus on its essential role in stimulating milk production in lactation. It is presented as a simple hormone with a single function. While this function is important, arguably, it is not irreplaceable (at least in humans), and diseases of prolactin signaling are typically not life threatening. Moreover, when issues with prolactin secretion do arise, such as a pituitary adenoma causing hyperprolactinemia, these can be treated in the clinic using dopaminergic drugs such as bromocriptine or carbergoline. Hence, it can be tempting to consider that we know all we need to know about this hormone. However, we do not think this is the case! In this chapter, we have attempted to expand the definition of prolactin function by acknowledging the fact that prolactin influences numerous cell and tissue systems, and by emphasizing how this breadth of function might maximize the reproductive success of an individual. While knockout animals are viable and have failed to display major deficits consistent with all of the hormone's "300 biological actions", this does not mean that prolactin functions are not important. They might not be critical to the individual, but they may have a more general role in optimizing the survival of a species. As the hormone responsible for lactation, prolactin has clearly played a critical role in the evolution of mammals, as this function defines this class of animals. In this context, distinct roles for the hormone in different species, as well as in genetically distinct groups within the same species, and diverse mechanisms resulting in analogous functions are not unexpected.

There remains much for us to understand about the direct and indirect roles of prolactin in reproduction. This issue is complicated by the interaction of prolactin with many other biological systems, and by the many other neural and endocrine inputs that influence those same biological systems. Looking forward, it is likely that the specific roles of any one hormone within this complex milieu will be clarified by new research tools, such as the use of genetically modified mice with alterations of prolactin signaling in specific tissues and cell types, and at specific times during development. The mechanisms underlying the plasticity of the neuroendocrine control of prolactin during pregnancy and lactation also remain important questions to be answered. Similarly, the potential of prolactin in fetal programming and transgenerational modification of function is another potentially important question, supported by the broad range of adaptive changes promoted in the mother during pregnancy, the high levels of prolactin present during development, and the high levels of prolactin receptor expression in the fetus. Again, it seems likely that a mixture of genetic and epigenetic analyses and new technologies will be required to fully understand these questions. In addition, there is increasing evidence for local prolactin production altering tissue function, and this may be a more important factor than circulating prolactin, particularly when looking at prolactin-related diseases such as cancers. This has been largely overlooked until recently and will continue to be a fruitful area for research, as will the development of antagonists as potential therapies for a range of disorders and cancers.

It is without question that elevated prolactin is present in all mammals during pregnancy and lactation. The hypothalamic control of prolactin secretion exhibits profound plasticity during these states, changing from a predominantly inhibitory feedback system to one that facilitates a state of physiological hyperprolactinemia. The elevated prolactin is clearly essential for synthesizing and secreting milk from the mammary gland. Mammalian reproduction is far more complex than this relatively simple function of milk secretion, however, and we have highlighted how prolactin regulates and facilitates multiple parts of the full complex process of reproduction, culminating in lactation (see Figure 12.8). Thus, we have described how prolactin is important in the reproductive cycle of the female and enhances the fertility of the male. After conception, prolactin is involved in implantation and the establishment and maintenance of pregnancy. It acts on a wide variety of tissues, such as bone, pancreas, gut, liver, adipose tissue, and brain, to induce maternal systems to change their function to adapt to the new reproductive state. And it contributes to the behavioral changes that are an essential part of enabling milk delivery to the offspring. Disorders of prolactin function within these processes, by themselves, may not irretrievably compromise reproduction. Nevertheless, it would seem that understanding prolactin function within these processes will help us optimize reproductive events and help us intervene appropriately when these processes are impaired in individuals.

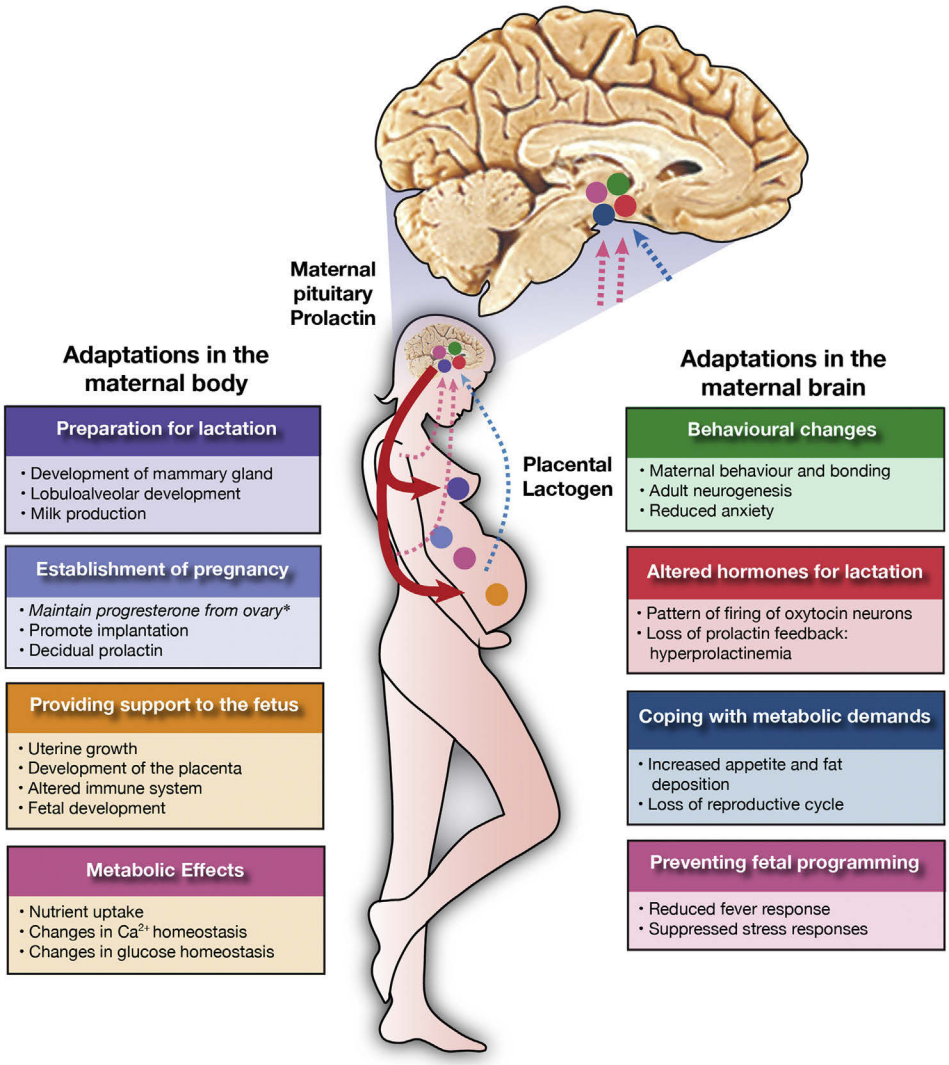

FIGURE 12.8 Summary of adaptive responses in the pregnant female that may be induced or regulated by prolactin and/or placental lactogen. This figure is reproduced in color in the color plate section. Note that while this figure shows a human pregnancy, most of the data are based on experimental data from laboratory animals, and some have not yet been confirmed in women. *Those shown in italics are known to occur only in rodents.

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CHAPTER

13

Central Nervous System Control of Oxytocin Secretion during Lactation

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INTRODUCTION

For mammals, providing milk during lactation is critical to infant growth and survival. Successful lactation depends on many factors, but critically on sufficient milk production, instigated largely by the circulating adenohypophysial hormone prolactin, on the morphological development of mammary glands for milk storage and ejection, and on the secretion of the hormone oxytocin (OT) in order to expel the milk from alveolar storage to mammary ducts (or cisterns, or sinuses) for its retrieval by the nursing young. This chapter is concerned with this latter aspect, specifically focusing on OT neurons of the hypothalamus and their axonal projection to the neural lobe of the neurohypophysis: how their electrical activity relates to hormone release and milk ejection, how this activity is formed through intrinsic and synaptic properties, and how critical events during pregnancy may shape this system for optimal neuronal activity, and thus efficient secretion, during parturition (where OT promotes labor by uterine contractions) and lactation (where it contracts the myoepithelium surrounding alveoli to eject milk).

Although it is over a century since posterior lobe (a.k.a. neural lobe, or pars nervosa) extracts were first demonstrated to have powerful pressor, antidiuretic, uterotonic, and galactagogic activity[1–3] and now several decades since the neuronal character of OT neurons was established fully, historically there was slow acceptance that neural lobe secretions had a neuronal origin. Many investigators suggested an anatomical relationship between the neurohypophysis (which consists of the median eminence, pituitary stalk, and the neural lobe) and the hypothalamus based on a variety of methods. From the monograph of Geoffrey Harris summarizing this literature,[4] we know that histologists as early as 1860 had described an innervation of the neurohypophysis—Cajal[5] himself described unmyelinated fibers connecting the supraoptic nucleus (SON; his tangential nucleus in rat) to this region, but considered that the pituitary was a sensory organ, communicating afferent stimuli to the hypothalamus. In later studies the efferent nature of connections from hypothalamus, especially from the SON, was surmised from axonal degeneration after hypothalamic lesions and from the retrograde atrophy of hypothalamic neurons following tract section or hypophysectomy.[6–8] But it was not until the definitive histological work of Ernst Scharrer, Wolfgang Bargmann, and Bargmann's student Walter Hild that the origin of the active posterior lobe principles was shown to be hypothalamic neurons.[9] In mammals, fish, and amphibians, the Gomori-positive (i.e., cysteine-rich material stained with chrome-alum hematoxylin) axons with associated varicosities observed in the neurohypophysis could be traced from the gland back to large cells in the hypothalamus in the SON and paraventricular nucleus (PVN; or their homologs in lower vertebrates). Experimentally, the accumulation of transported material in proximal axons after pituitary stalk section, and its subsequent disappearance in the neurohypophysis, was consistent with the notion of anterograde axonal transport demonstrated in peripheral nerve.[10] Soon the physiologically active extracts from the posterior lobe of mammals were purified and their amino acid composition and sequence characterized as the closely related nonapeptides OT[11] and vasopressin (VP).[12]

In spite of what today seems the obvious force of the histological arguments, a continued hurdle lay in relating how the hypothalamus exerted this control. Electrical stimulation of the pituitary or the supraoptico-neurohypophysial tract had been known since the late 1940s to produce pressor, antidiuretic, and oxytocic actions, demonstrated in a classic series of experiments by Harris in rabbits.[4] Cross and Harris[13] even directly

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observed the ejection of milk from a cannulated rabbit's teat when stimulating this tract. Other studies showing that damage to this tract often led to pronounced diuresis from the loss of VP[6] and a disruption of milk ejection[14] reinforced the primacy of hypothalamic control over the pituitary. Roughly coincident with Harris's 1951 monograph,[4] for example, was the aforementioned article by Bargmann and Scharrer[9] summarizing years of work using Gomori-positive stains and crystallizing the concept of neurosecretion. While believing, and in large part himself demonstrating, that hypothalamic electrical activity controlled neurohypophysial and adenohypophysial secretions, Harris[4] nevertheless espoused a view common at the time about the source of the hormone, writing: "the active substance present in the posterior extracts is formed in the tissues of the neurohypophysis, though the particular cells involved are unknown." However many histologists even outside the German school had commented on the fact that the cellular components of the neural lobe were not Gomori-positive, or at least, not richly so like hypothalamic neurons or the secretory cells of the anterior lobe and pancreas.[8] In 1958, Harris[15] acknowledged the now accepted view that it was the SON and PVN neurons that also synthesized the active pituitary principles, transporting them to the neural lobe for secretion from axon terminals.

Once it was established that the PVN and SON were the source of the active posterior lobe principles, and given that it had been known for many years that these hormones could be released by electrical stimulation either of the lobe itself, the hypothalamo-neurohypophysial tract, or from within the hypothalamus, it remained to be determined whether OT (or VP) cells were fully neuronal, that is, capable of firing regenerative action potentials, receiving synaptic input, etc. It's easy to understand why these functions were not intuitively assumed. The Gomori-positive axons were decorated with large swellings (Herring bodies) ending near capillaries (and not making synapses with neurons) in the neural lobe, and with the exception of their elongated axon (dendrites not extensively visible), their appearance was not unlike other gland cells, such as those in the adenohypophysis. This issue was directly addressed by Kandel's[16] landmark study of goldfish neurosecretory neurons in the magnocellular preoptic nucleus. These neurons make hormones related to VP and OT, isotocin or vasotocin, which are also secreted from the posterior lobe. Kandel[16] found that these neurons fired action potentials either spontaneously, or antidromically from pituitary stimulation, and also showed synaptic potentials in response to olfactory tract stimulation. Although osmotically responsive extracellular action potentials had been recorded from in or near the SON of rabbits[17] and cats,[18] it was not until antidromic identification was applied that it became certain that rodent neurosecretory neurons were similar to their lower vertebrate counterparts, verified first in the SON by Yagi et al.[19] The technique was later applied to the PVN of rabbits,[20,21] and similar studies were accomplished in the SON or PVN of cats,[22,23] dogs,[24] and monkeys.[25] Most studies found a relatively slow estimated conduction velocity (<1 m/s) consistent with the histological evidence of small diameter, unmyelinated axons in the neural stalk and lobe. Together with the anatomical work discussed earlier, and the classic experiments on stimulus-secretion coupling in the neurohypophysis from Douglas and Poisner,[26] these seminal electrophysiological forays form the scaffold for this chapter's exploration of how the central nervous system (CNS) controls OT release during lactation. Although there has been substantial work on the release of OT in many mammalian species, particularly cats, rabbits, pigs, primates, and guinea pigs, it is primarily in rat (but see below) that the behavior of OT neurons has been directly observed during secretion, and for which an overwhelming amount of experimental work on the neural control of OT neurons exists. In focusing on these neural aspects, it should be mentioned that there are many species differences in milk storage and ejection, and in mammary gland morphology, that have received excellent and extensive treatment in prior reviews and need not be revisited here.[27]

Long before the firm establishment of the hypothalamo-neurohypophysial system as described earlier, it was commonly thought that the brain influenced OT release. But this was not based initially on what later become known as the "suckling reflex"—where sensory information from the suckled nipples reached the brain to initiate milk ejection—it appears rather to be largely anecdotal evidence. Schäfer[28] commented in his classic text that while it is a "common experience that the quantity and quality of the milk is in women materially influenced by the condition of the nervous system," the results from animal experiments were "either entirely negative, or at most of a somewhat conflicting nature." The anecdotal "experience" is the knowledge that emotional factors influenced the ability of women to lactate—both positively (e.g., hearing a baby cry) and negatively (stress). That these experiences would be more apt to signal nervous system involvement than the response to suckling itself is not difficult to understand; suckling was often viewed as a way to locally remove milk, but not necessarily viewed as something needing the CNS. Thus it is the stimulation from other sensations, including emotional distress, that would be assumed to engage the nervous system at many levels. Initially, knowledge that cattle could be conditioned to increase milk let down by stimuli accompanying milking, as well as frightened or given adrenaline to "hold up" their milk,[29] prompted investigation of the autonomic nervous system. Early experiments led Bacq[30] to conclude that denervation of most sympathetic nerves

had little effect on lactation in rats; a similar conclusion was reached by Ely and Petersen[29] in cattle. The interest that adrenaline's ability to inhibit milk ejection might be the basis for emotional interference did not wane however,[31] and eventually it was determined that adrenaline can exert direct inhibitory effects on mammary tissue,[31,32] most likely due to an action on the myoepithelial cells mediated by β-adrenergic receptors.[33] Adrenaline's local actions on the pituitary could not account for all inhibition of milk ejection to stressful or even distracting visual, olfactory, and auditory stimuli, as peripheral OT injections could often overcome this stress-induced inhibition.[34,35] The brain-mediated stress-induced inhibition was convincingly, if by today's standards grotesquely, shown by Taleisnik and Deis,[36] who found that inhibition of OT release to leg fracture in rats was completely abolished during cortical spreading depression. This study not only suggested that cortical processing of painful stimuli was involved in this suppression but importantly suggested that cortical activity in rats is somehow permissive, and that sensory activation of the cortex is *not necessary* for suckling-induced OT release, a point that will be made again later in this chapter. The ability of electrical stimulation of the cortex to inhibit lactation was later confirmed by Ausebrook and Holland,[37] a finding confirming Cross's[32] original notion that the hypothalamus possessed inhibitory as well as excitatory pituitary influences.

The known ability of some anesthetics/analgesics to either block milk ejection outright, like ketamine or neuroleptics, or others to block or severely delay lactation at higher doses, *without* interfering with OT's ability to eject milk after intravenous injection, is further indication of central nervous involvement in the reflex.[38,39] Interestingly, experimentally and in the rat, especially, anesthetics are necessary for prolonged examination of the reflex even if they may delay its onset after the young attach, because the dam is easily distracted by environmental stimuli and will stop nursing.[38] In this regard even as our best animal model in terms of experimental access and an overwhelming history of reference, the rat is somewhat unusual among mammals in that cortical slow-wave sleep always accompanies lactation.[40,41] Not only is slow-wave sleep *not* a requirement among other experimental mammals studied,[42,43] many animals appear vigilant when nursing,[39] whereas suckling is soporific in conscious rats.[41]

MILK EJECTION IN RESPONSE TO SUCKLING IS AN ATYPICAL NEURONAL REFLEX

There is great variety in the nursing patterns of mammals, which for those studied experimentally, ranges from the once-a-day, short feedings of rabbits, to the prolonged nursing bouts of rats.[39] While in all mammals suckling stimulates OT release from the neural lobe, which in turn contracts the alveolar myoepithelium of the gland to force milk into ducts (or analogous chambers) where the young can access it, ruminants represent a specialized case since milk ejection at nursing, while present, is not immediately required due to the storage capacity of udders. In addition, the reflexive release is variable within and across animals: the start of the OT secretion can be relatively immediate, or delayed for minutes. This is especially true in animals under anesthesia, as indicated previously.

The best early suggestion of a centrally, neuronally mediated, reflexive release of OT in response to the sensory stimulus of suckling was probably provided by Ingelbrecht,[44] who found that in rats receiving lower spinal cord section (between last thoracic and first lumbar segments), thus removing ascending somatosensory pathways from the posterior teats, pups given only posterior teats to suckle died, whereas those given only anterior teats survived. Importantly, when pups together suckled the anterior teats *and* posterior teats in this preparation, *all suckling pups survived!* Thus once the sensory limb of the reflex successfully activated the efferent component, *all* mammary glands reacted. This study was thus not only critical in addressing both the necessity of spinal pathways to transmit the suckling stimulus but in determining the manner of the efferent limb of milk ejection. Having already ruled out the relative importance of the autonomic nervous system for lactation,[30] the latter result was best explained through a humoral factor, independent of motor innervation of the glands. A similar recognition of sensory nerve involvement in mammary gland function (although through prolactin release), came from Selye and coworkers[45,46] who noted the ability of suckling in intact teats to maintain mammary gland engorgement in others with nipple excision (and thus, not suckled), the ability of newborn pups to maintain gland development after weaning the original litter, and the ability of suckling to maintain the gland even when milk retrieval is prevented. This sensory/humoral link was again demonstrated in mice by Hooker and Williams,[47] who found that even without nursing, stimulating even a few nipples with turpentine (a known skin irritant) was sufficient to retard involution in all the glands, and that nipple excision did not prevent a particular gland from engorgement, so long as other intact nipples were suckled.

While varying concepts of a milk "let down" reflex, in the vein of spinal cord mediated reflexes, were proffered as early as 1915 by Gaines,[48] and summarized by Hammond[49] in his extensive review, Ely and Petersen[29] are credited with first suggesting that the efferent limb involved OT release and not nerves innervating the gland. In their classic experiment, the sympathetic motor nerves of one half of a cow's udder were sectioned

without altering gland development or milk yield during milking from that half. The sensory innervation remained intact throughout their experiments, which led to the stimulated release of OT from the hypothalamus that would reach both intact and denervated areas. These authors also suggested the reflex could be classically conditioned and that sensory information from the teat was just one source of afferent stimuli that could evoke OT release. Finally, the similarity of the increase in intramammary pressure recorded during milking to that recorded with the oxytocic extract Pitocin convinced these workers that the efferent limb was humoral. Folley[50] crystallized this view and clearly separated the phenomena of milk secretion (which includes milk synthesis and its release into the alveolar lumen) from the discharge of milk occurring during lactation, convincingly arguing a change from the "make-shift term letdown" to something that reflected the active process of the contracting alveoli forcing milk into ducts, cisterns, or sinuses for retrieval upon the suckling stimulus. The term "milk ejection", with its more active connotation, was officially proposed by Cowie et al. in 1951[51] and has been thereafter adopted by most workers in the field.

Thus the milk ejection reflex is atypical in at least three ways from classic spinal reflexes. Firstly, the latency to the response is relatively slow and variable. In anesthetized rats, for example, milk ejection may be delayed several minutes, even tens of minutes, after suckling onset. Secondly, as just discussed, the efferent limb has a humoral component following neuronal activation that requires OT transport in circulating blood. And thirdly, milk ejection is *pulsatile* in the face of a continuous suckling stimulus in virtually all species studied,[39,52] including humans[53–56] (Figure 13.1).

Interestingly, the young typically stimulate the nipples much more strongly *after* OT has reached the mammary gland and altered intramammary pressure. This pressure change, detected in all species studied both during milk ejection and in response to intravenous OT injection, is sensed by the nursing young and excites them to suckle more vigorously and withdraw milk. In animals with repetitive, periodic milk ejections, like the rat, nipple attachment for the minutes between ejections is critical to instigate and to maintain the reflex, but intense suckling is only present after intramammary pressure increases.[38] Often, pups detach from the nipple after withdrawing the milk, and then avidly move to reattach (Figure 13.1).

Although many of the earliest studies focused on rabbits and ruminants,[15] in no species has CNS involvement in milk ejection reflex been studied as intensively as it has in rats. This resulted not only from their general cost efficiency but also more importantly from the finding that the reflex was present for long periods under various anesthetic agents in this species[38,52] (Figure 13.1).

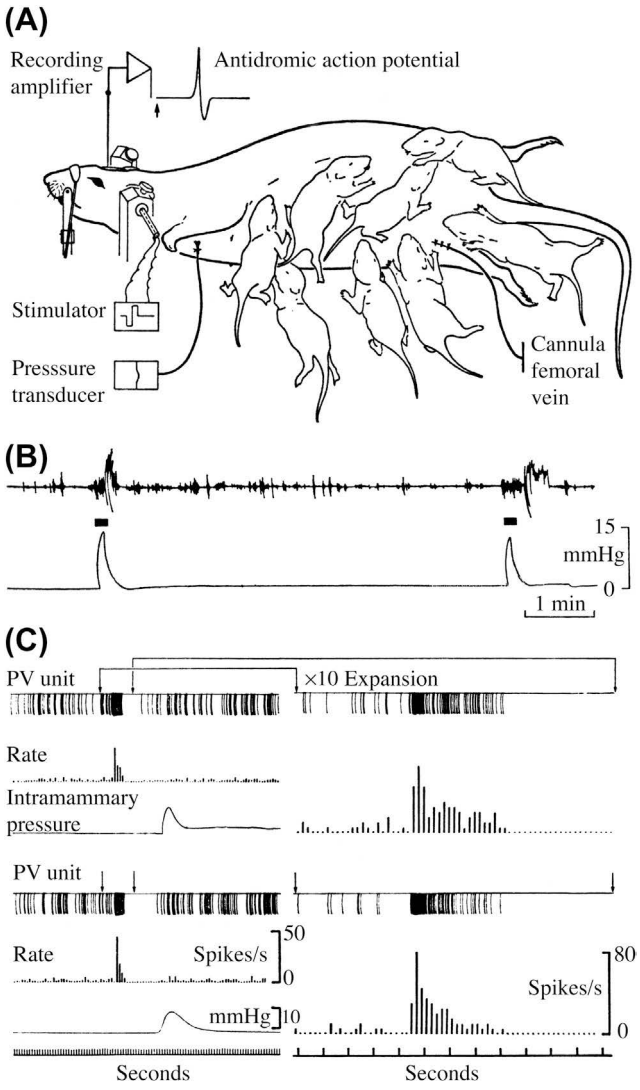

FIGURE 13.1 Features of the milk ejection reflex in anesthetized lactating rat. (A) Schematic showing simultaneous recording of antidromically identified action potentials in the PVN and intramammary pressure during suckling. The stimulating electrode was placed in the neurohypophysis transaurally through the hollow ear bar of the stereotaxic apparatus. The femoral vein is cannulated so oxytocin can be administered to calibrate the change in intramammary pressure. An antidromic spike is shown from stimulation (arrow) of neurohypophysis. *(Source: Redrawn from Ref. 57 with permission.)* (B) Activity record of pup movement (top trace) recorded simultaneously with intramammary pressure during suckling, showing two milk ejections. The pup's stretch response upon sensing the intramammary pressure increase is indicated by the bar, and is followed by even larger movements when detached pups search for a nipple, which often occurs after withdrawing milk. *(Source: Redrawn from Ref. 38 with permission.)* (C) Recordings from two antidromically identified PVN neurons during the milk ejection response. There are three traces for each unit. Arrows point to the burst of action potentials in the pen record (top trace) and the integrated rate (middle trace). Lower trace shows the intramammary pressure change signaling arrival of oxytocin at the gland. At the right for each neuron are expansion of the pen recording and rate meter. Note the: (1) abrupt onset and exponential decay of the spike discharge; (2) the peak frequency >50 Hz; (3) the period of after-inhibition; (4) the latency between burst discharge and milk ejection; and (5) the similarity in the response of the two neurons. *Source: Redrawn from Ref. 57 with permission.*

Even in mice, the milk ejection reflex has not been well described, and there are no apparent studies on the behavior of OT neurons during release. One exception is rabbits,[58–60] to be discussed below.

THE BEHAVIOR OF OT NEURONS DURING MILK EJECTION—THE RAT MODEL

The Milk Ejection Burst Established in PVN Neurons

The demonstration that neurosecretory neurons could be antidromically identified in vivo opened the gates for a flood of research utilizing this technique to study their activity during hormone release. Although nipple stimulation had been shown to alter neuronal activity in the region of the PVN in lactating cats, and to evoke release,[61,62] it was Wakerley and Lincoln[57] who first combined antidromic identification with a fully functioning and periodic milk ejection reflex, which they previously had demonstrated possible in anesthetized rats[38,52] (Figure 13.1). About 30 min after applying the pups to the dam milk ejections, as monitored by abrupt changes in intramammary pressure recorded from two cannulated teats, began to occur at regular intervals from 10 to 20 min. Coincident with the transient increase in intramammary pressure signaling milk ejection, the pups became more active and exhibited a stretch response, wherein they suckled vigorously with outstretched limbs and an arched back, as described in detail by Lincoln et al.[57] It is during this period that the pups withdrew milk—so vigorously that they often detached themselves and attempted to find another nipple. Within 30 s, this activity had subsided and the pups were again somnolent. The increase in intramammary pressure indicative of OT release was preceded by a burst of activity for 2–4 s in more than half the identified neurons (Figure 13.1). Peak firing rates in the burst (measured over 0.5 s intervals) ranged from 24 to 84 spikes/s and were characterized by a range of increase over background firing levels of 338–11,000%! The transient form of the increase in intramammary pressure was mimicked by a bolus injection of 1 milliunit (1/1000 international unit) of OT, and by electrical stimulation of the neurohypophysis (4 s, 40 Hz). The latency to the increase in intramammary pressure from IV injection was ~10 s, and that to electrical stimulation (~16 s) was similar to the average delay from milk ejection bursts (~18 s). Importantly, other nearby hypothalamic neurons appeared unrelated to the reflex, as did the remaining antidromically identified PVN neurons. In the latter category were several phasically bursting neurons previously speculated as possible OT neurons.[63] However, the phasic bursts of these neurons were not only temporally unrelated to milk ejections, they were much longer (10–30 s), had a faster periodicity (10–30 s) and were asynchronous. These neurons, classified initially as "non-milk ejection" neurons, were soon identified as VP secreting owing to their response to other stimuli.[64,65] Indeed, OT appears to be selectively released over VP during lactation in rabbits[4,66] and in rats.[67] The seminal result of Wakerley and Lincoln[57] was later achieved in awake, lactating rats with chronically implanted recording and stimulating electrodes,[68] and has been since verified in a number of studies.[65,69]

The association of antidromically identified PVN neurons and milk ejection has also been studied in awake and freely moving rabbits, apparently the only other species beside rats studied in this regard.[58–60] Although the nursing pattern is dramatically different in the two species—rabbits typically nurse once or twice a day, and for only a few minutes—putative OT neurons in the PVN burst just prior to the increased sucking of the young (mammary glands were not cannulated for obvious reasons, but the activity of the pups was monitored to record the reflex). These bursts are similar in duration to those in rat (1–4 s), but are more frequent: bursting 5–9 times during the short session (~5 min). Interestingly, the dams often groom their nipples following a nursing session, and this stimulation also slightly raised neuronal activity in the same neurons that burst during nursing, reaffirming the sensory transmission to the hypothalamus observed by others,[61,70] seen as well with direct mammary nerve stimulation.[71]

The SON Also Contains Milk Ejection Neurons

The initial electrophysiological studies during lactation focused first on the PVN, undoubtedly due to the lingering supposition that OT may derive largely from there, rather than the neuroendocrine counterpart, the SON. This supposition seems today based primarily on studies wherein large lesions of the PVN strongly reduced the oxytocic activity of the neural lobe, with little effect on antidiuretic activity.[72–74] However, while electrical stimulation of the PVN promoted OT release in goats[75] and rabbits,[32] the same stimulus also promoted antidiuresis.[75] And as noted by Lincoln and Wakerley,[76] oxytocic and antidiuretic activity had been found in extracts of both the PVN and SON, and lesions of either nucleus seemed to deplete both peptides from the neural lobe. Deafferentation of the PVN also failed to block milk ejection.[77]

Using the method described previously in rats, Lincoln and Wakerley[76] found milk ejection bursts in about half of the SON neurons, which were indistinguishable from those in the PVN; given its larger size, this suggested an importance of the SON equal if not greater than the PVN in importance for OT release. The localization

of both hormones in both nuclei in the rat was confirmed the following year with the seminal utilization of specific antibodies for VP and OT in immunohistochemical studies,[78–80] and has since been confirmed in many species, albeit with varying estimated proportions. Cell counts by Swaab et al.[78] and Rhodes et al.[81] each revealed that the SON contained more OT neurons than did the PVN, with Swaab et al.[78] reporting an average of 2019 ± 71 OT neurons in the SON compared to 842 ± 48 in the PVN, and Rhodes et al.[81] reporting 1440 SON OT neurons and 1174 PVN OT neurons. Whereas both authors found about the same numbers of VP as OT neurons in the PVN (~1000), the SON contained 1.5–2x as many VP as OT neurons. Thus numerically the SON is more important than the PVN for OT, but still even more important for VP. A final caveat for this quantitative consideration relates to the accessory neurosecretory nuclei, which can add >1000 more neurons to the system, and probably more OT than VP neurons.[81] This is certainly true of the anterior commissural nucleus, which is overwhelmingly OT containing (another 616 OT, but only 15 VP neurons reported in this nucleus[81]). Like other accessory nuclei, the anterior commissural nucleus also projects to the neural lobe.[82] It is unknown to what extent its neurons, separated from the main portion of the PVN by 300–400 μm, have been sampled in any of the electrophysiological studies of the PVN considered herein. Finally, while in this chapter the assumption will be made that both nuclei contribute OT during milk ejection in most mammals, this is based overwhelmingly on work in the rat.

Phasic-bursting "non-milk ejection" neurons (see previous discussion) were again recognized in the SON (76) and were, for the most part, not correlated with milk ejection. However, a few of these neurons did have an "on" phase correlated with milk ejection, something that would occasionally be observed in future studies by these and other investigators.[83] Still, these studies, coupled with immunocytochemical investigation, gave credence to the one hormone-one cell hypothesis. While we now understand the genetic capability of most magnocellular neurons to possess mRNA for both peptides,[84] there are only small amounts of peptide co-expression reported in unchallenged animals. Based on the selective OT release during lactation, however, co-secretion of VP during milk ejection in significant amounts seems unlikely, even during this upregulated state when co-expression may reach 15–20%.[85] Thus the importance of OT-VP expression remains largely undetermined.

Milk Ejection Bursts are Synchronous and Highly Periodic

Lincoln and Wakerley[76] not only confirmed the similar behavior of SON and PVN milk ejection neurons and the resulting probable numerical importance of the former over the PVN regarding OT release but they first reported, from one double recording and multiunit recordings, synchronous bursting in neighboring neurons. This synchrony was later confirmed from a larger number of double recordings between the two nuclei (and from between the right and left halves of the same nucleus) from separate electrodes[86,87] (Figure 13.2). Thus some 13–16 s following these 2- to 4-s bursts in both nuclei, a milk ejection response in the mammary gland can be recorded, the lag reflecting diffusion within the neural lobe to fenestrated capillaries and vascular circulation. Burst onset between paired recordings can vary from 0 to 680 ms, but the average lag time between cells is relatively short (~200 ms), and the lag-to-peak firing rate even shorter (~122 ms).[87] Although paired recordings revealed units that may even switch regarding which of the two neurons fired first, in the great majority of cases, whichever cell fired first did so at a higher frequency than the second cell in the pair (also see Ref. 86). This intimated some type of communication among OT neurons, something that had been suggested from this and earlier studies showing that central OT facilitated, whereas its antagonism suppressed, bursting activity.[88,89] The role of locally released OT will be discussed in detail below.

Several aspects of the pulsatile milk ejection reflex suggest a central gating mechanism. Lincoln and Wakerley[70] first examined this in detail by varying the number of pups whilst simultaneously recording the bursts and intramammary pressure. In the urethane-anesthetized rat, a threshold of ~5 pups was needed to generate the reflex. While increasing the number of pups increased the amplitude of bursts and the resulting intramammary pressure, it had little effect on periodicity. Even when milk ejection bursts were precipitated earlier than expected based on the ongoing periodicity by stimulating an individual gland, the

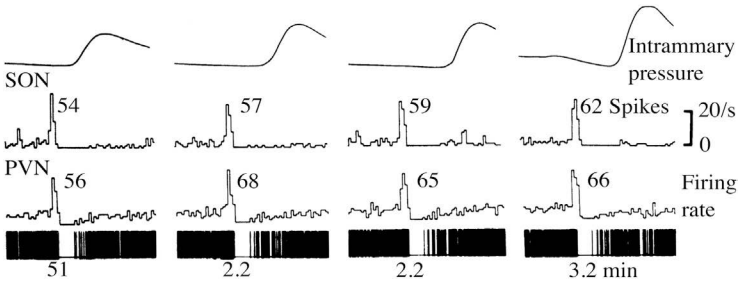

FIGURE 13.2 Simultaneous single unit recordings from SON and PVN neurons during suckling. Note the synchronization of bursts in the two units, each of which was recruited at the onset of milk ejections, 51 min after pups were applied. The time between bursts thereafter is noted beneath the spike record for the PVN neuron (bottom trace). Note also the progressive increase in burst amplitude (numbers of spikes in each burst) in both neurons, and the progressive increase in intramammary pressure. *Source: Redrawn from Ref. 87 with permission.*

session was reset and the prior periodicity was resumed. Thus afferent summation controlled a cell's response, and the amount of OT released per burst, but not necessarily the periodicity. In later experiments weak, but constant, electrical stimulation of nipples also resulted in periodic milk ejections.[90] Whereas transient stimulation of dorsal spinal cord sensory pathways can also elicit a single milk ejection, continuous stimulation of the cord was not accompanied by periodic release,[91] suggesting that gating occurs more rostral in the CNS. Wang et al.[92] found that increasing pup numbers also increased burst amplitude, especially pups contralateral to the recorded OT neuron. However, these authors also noted that rats suckled bilaterally had shorter interburst intervals, suggesting afferent summation may have some influence over periodicity.

THE AFFERENT PATHWAY MEDIATING MILK EJECTION

It should be stated at the outset that the precise sensory pathway from mammary gland to OT neurons underlying the milk ejection reflex is still unknown. There may even be multiple ways by which mammary sensory information could reach OT neurons. This subject may benefit from newer tracing methods allowing for more robust transynaptic transport from the nipple, but as of this writing this has not been done. Intense work in the past, summarized following, has relied heavily on deficits following lesions that varied in size and location, sometimes coupled with axonal tracing methods.

Spinal Cord and Brainstem

The innervation of the mammary gland is much denser than the adjacent skin and has been examined in many species, including human.[27] The nerve supply is from both smaller myelinated and unmyelinated axons and a small percentage of larger myelinated axons that largely terminate in free nerve endings and are most numerous in the tip of the nipple. These sometimes occur in bundles and may be associated with an enveloping Schwann cell. Electrical recordings from either the mammary nerve or spinal cord indicate a range of conduction velocities from <1 m/s–60 m/s, with the fastest myelinated fibers being very sensitive to both rapid cheek movements and the slower myelinated fibers to slower jaw movements associated with sucking.[93–95] Recordings from the dorsal spinal cord indicate the sensory fibers may adapt fairly rapidly.[95,96] The slowest, unmyelinated fibers appear to give rise to a late response to nipple stimulation or nerve stimulation, with a higher threshold. It is unknown to what extent different fiber types play in activating pathways essential for OT release, however, in nursing rats, unmyelinated fibers were larger diameter than in virgin animals.[94]

Within the spinal cord, lesion studies coupled with suckling tests in many different animals indicate a predominately ipsilateral pathway.[97–100] Application of the sensitive tracer, wheat germ agglutinin conjugated to horseradish peroxidase to the mammary nerve or nipple directly appears to give different results regarding the precise terminus in rats. Haller[93] reported abdominal (T11-12) mammary nerve projections primarily to Clarke's column (typically regarded as spinocerebellar) and to the lateral spinal nucleus, a source of spinothalamic, spinohypothalamic, and spinomesencephalic fibers.[101,102] Tasker et al.[103] reported segmental projections from nipples on one side directly to the dorsal horn, primarily superficially to the substantia gelatinosa but also to lamina II and deeper. Haller[93] reported a small amount of contralateral labeling in the cord, whereas Tasker et al.[103] found none. Although neither study reported labeling in the dorsal columns, Tasker et al.[103] did find some sparse labeling in dorsal column nuclei.

Lesion studies uniformly find that the dorsal column–medical lemniscal somatosensory pathway is not critical to the milk ejection reflex.[97–100,104] Even in sheep, in which lesions of the dorsal columns in the thoracic regions do impair the reflex, rostral cord lesions of the columns do not.[99] The afferent pathway appears to travel in the dorso- or ventrolateral funiculus[97,98,100,104,105] and could originate from the dorsal horn, as well from the lateral spinal and lateral cervical nuclei. The latter two nuclei may get direct dorsal root ganglion projections, but it is likely that most sensory projections are routed first through the dorsal horn.

Somatosensory projections from the spinal cord to the thalamus are probably not involved in the milk ejection reflex. Large lesions of the ventrobasal-ventral posterolateral thalamus had no effect on milk ejection in sheep[99] or rat.[104] Similarly, section of the compact portion of the medial lemniscus failed to block milk ejection.[99,106] These results should not be interpreted to mean that sensory information from the nipple does not reach the thalamus, or for that matter, pass through the dorsal columns at some point—only that the dorsal-column pathway is not critical for milk ejection. Indeed mammary afferents may reach dorsal column nuclei,[103] and nipple stimulation can evoke c-Fos expression (the protein resultant from the proto-oncogene c-*fos*, thought to indicate neuronal activation) activity in both the lateral and medial cuneate in sheep, in addition to the dorsal horn and lateral cervical nucleus.[107]

The target of the spinal cord neurons conveying suckling information necessary for milk ejection neurons is not well understood. What is agreed upon is that the information is transmitted largely contralaterally through the brainstem; the precise point of crossing is unknown. The pathway is largely discerned from lesion studies as hinted before. In contrast to ineffective

thalamic lesions, even relatively small bilateral lesions of the midbrain lateral tegmentum significantly slowed or blocked the reflex,[104–106] and unilateral lesions impaired the suckling stimulus from the contralateral side of the animal[106,108] (Figure 13.3). Stimulation of mesencephalic lateral tegmentum has been shown to facilitate OT release in guinea pig and rabbits,[109,110] and a study of lactating rats revealed that stimulating this region excited milk ejection neurons when in the presence of central OT.[111] Thus a contralateral sensory pathway conveys the suckling stimulus to the hypothalamus, which does not involve the thalamus and does not require cortical activity.[36] Spinal cord projections from the region of the lateral cervical nucleus and including the dorsal horn, as well as other spinal afferents from fibers of passage in the lateral funiculus, converge in the external inferior colliculus, the intercollicular zone, and the deep layers of the superior colliculus, near the region of effective blockade,[104,105] and these are all targets of somatosensory projections (e.g., Ref. 112) (Figure 13.4). Importantly, Hansen and Kohler[113] directly addressed the issue of fibers of passage versus cell group importance by injecting the neurotoxin ibotenic acid in the lateral midbrain tegmentum in approximately the same regions highlighted by the effective lesions. These more specific "cellular" lesions blocked milk ejections as measured by decreased pup weight during 2 h of nursing in these mothers (as well as sexual behavior in both male and female rats), whereas control injections in overlying structures had no effect. There was little difference between the effects of large (0.5 µl) versus small (0.2 µl) injections in the effective region. Repetition of these experiments while recording the periodic release of OT using mammary cannulation, and/or OT milk ejection bursts, could be most revealing.

Tracer injections into effective lesion sites in the midbrain tegmentum retrogradely labeled cuneate-gracile, dorsal horn, and lateral cervical neurons contralaterally, as well as many other brainstem regions, and also labeled sparse anterograde axonal projections to the lateral hypothalamus, zona incerta, and Fields of Forel, but failed to label the SON and PVN.[104] However, in sheep, injections into the PVN directly labeled the lateral cervical nucleus, which in turn received dorsal horn projections.[114]

Thus regardless of species differences, it appears that a contralateral spinomesencephalic pathway is involved in transferring suckling information to the SON and PVN, but the number of connections between the cord and these nuclei is unknown. There is even the possibility of a direct spinal pathway to the PVN in sheep and rat.[115] However, spinal projections that were followed to the hypothalamus clearly pass through mesencephalic lateral tegmentum near the effective lesion placements discussed before[115] and could make synapses there as well.

Although the midbrain lateral tegmentum appears critical for transmitting the contralateral suckling stimulus to the hypothalamus, the point of bilateral sensory integration, necessary to activate both sides of the hypothalamus, is unclear. The midbrain lateral tegmentum may have some bilateral hypothalamic projections.[116]

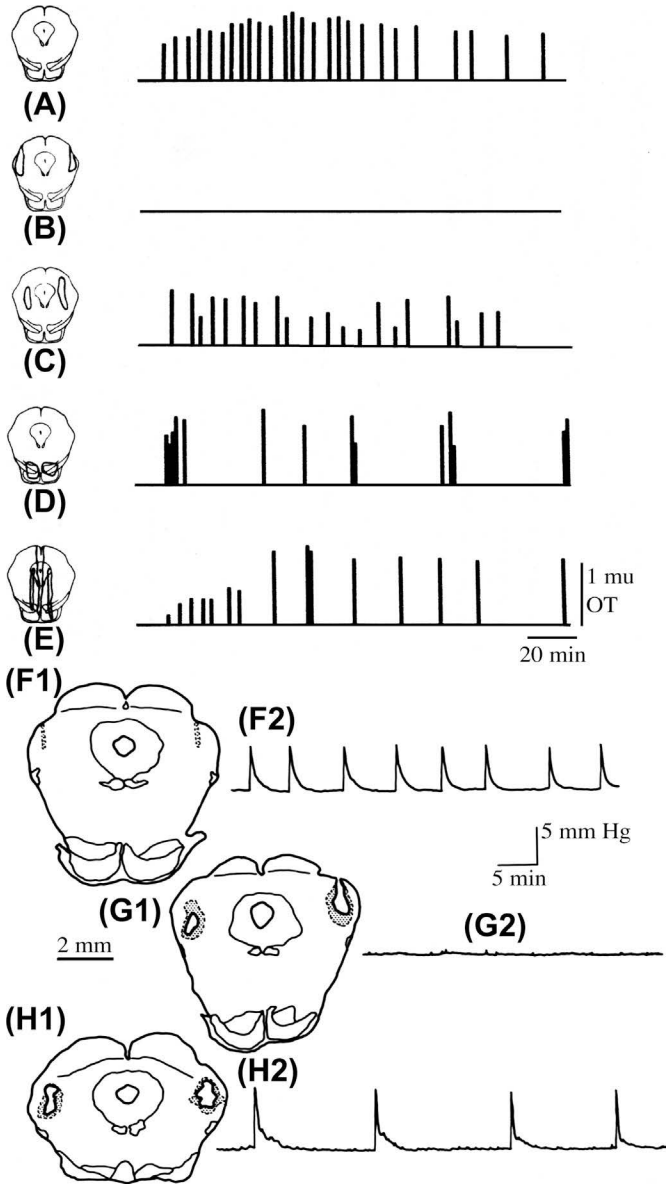

FIGURE 13.3 Lesions in the intercollicular zone of the midbrain lateral tegmentum block the milk ejection reflex. (A–E) Drawings from lesion sites (left) and associated patterns and amplitudes of intramammary pressure change (right) in four different experimental animals (B–E) and a control rat (A). *(Source: Redrawn from Juss and Wakerely Ref. 106 with permission.)* Note that some lesions produced changes in the reflex pattern, but only that in (B) blocked the reflex. (F–H) Results from a similar study by Dubois-Dauphin et al.[105] (F1) Small, ineffective bilateral lesions in the intercollicular zone. (F2) Tracings from intramammary pressure recordings during lactation show frequent milk ejections. (G1) Lesions in the intercollicular zone that completely blocked milk ejections (G2). Note the similarity in location to the lesions blocking milk ejections in the Juss and Wakerley[106] study shown in (B) above. (H1) Lesions involving the posterior aspect of the medial geniculate nucleus, just rostral to those shown in (G1), which failed to block milk ejections (H2). *Source: Modified from Ref. 105 with permission.*

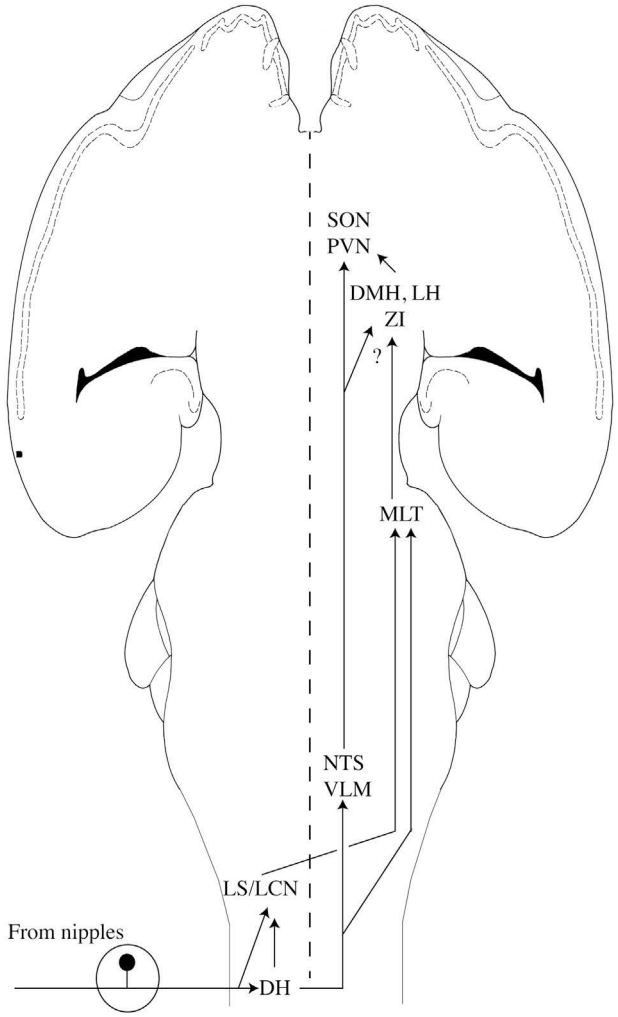

FIGURE 13.4 Schematic diagram illustrating some possible routes of suckling-associated sensory stimulation to the hypothalamus. Spinal (mammary) nerve inputs terminate in dorsal horn (DH), lateral spinal (LS), and lateral cervical (LCN) nuclei. The LS and LCN may also receive inputs ipsilaterally from DH. Axons from the LS, LCN, and DH project contralaterally to the midbrain lateral tegmentum (MLT), and to the nucleus of the solitary tract (NTS) and ventrolateral medulla (VLM). The NTS and VLM are known to project directly to magnocellular SON and PVN neurons, and may also project to nearby regions of the hypothalamus (perhaps through the MLT), such as the dorsomedial nucleus (DMH), lateral hypothalamus (LH), mammillary nuclei (MAM, not shown) and zona incerta (ZI). These areas may then project to OT neurons in SON and PVN, or perhaps immediately adjacent areas such as the PNZ of the SON or the parvocellular PVN (not shown). The MLT has no known direct projections to the SON and PVN, but projects to LH, ZI, and DMH. The MLT includes several areas, such as the intercollicular zone, the external nucleus of the inferior colliculus, and the paralemniscal nucleus, which could be damaged with the MLT lesions that block the milk ejection reflex (Figure 13.3). While pathways such as those from the NTS and VLM to the hypothalamus may have some bilateral components, they are primarily ipsilateral.

However, unilateral midbrain transections block afferent stimulation from the contralateral side but do not block the synchronized milk ejection activities of the SON bilaterally when pups are allowed to suckle ipsilateral to the lesion.[108] In intact rats allowed to suckle only on one side, sufficient to activate SON and PVN bilaterally, the milk ejection activity of the OT neurons in the contralateral SON was stronger than in the ipsilateral SON.[92] Thus while it's clear that a crossed pathway from the nipples to as far the midbrain is critical, what happens rostral to the midbrain is at this point unknown.

Some other brainstem areas receiving somatosensory input deserving exploration regarding the milk ejection reflex include the A2 noradrenergic cell group in the nucleus of the solitary tract (NTS) and the A1 group in ventrolateral medulla, both of which project directly to the SON and PVN (see Ref. 117,118 for review) (Figure 13.4). These projections are strongly ipsilateral with a weaker but present contralateral input—thus, if important to the reflex, it would be obvious that the relevant spinal inputs will have crossed by the lower medulla. These cell groups, and perhaps even the locus coeruleus (A6 cell group),[118,119] may be of particular importance given the suggested role of noradrenaline in mediating OT cell activation during the reflex. The NTS does receive somatosensory spinal cord afferents from the dorsal horn, and the pattern of dorsal horn labeling after NTS tracer injections is similar to that of spinomesencephalic projections.[120] The NTS-A2 projection may even favor OT neurons.[121–123] The NTS-A2 pathway may be important in mediating the specific OT release that occurs in rats with peripheral cholecystokinin (CCK) injection.[124,125] Synaptic adrenergic (or noradrenergic) profiles making contact with OT neurons have been observed.[126] Another NTS potential peptide transmitter, activin, favors innervation of OT regions and has been implicated in OT release.[127]

While it is unclear whether the CCK data suggesting a more viscerally related yet apparently specific involvement of the NTS in OT release is relevant to milk ejection, this area remains important for further study. A large number of NTS catecholaminergic neurons projecting to the PVN or SON expressed c-Fos following peripheral CCK injection,[125,128] and the noradrenergic transmission during CCK activation is critical for magnocellular activation.[125,129] Although noradrenaline is also released in the PVN during suckling and blocking adrenergic receptors within the nucleus suppress the OT release,[130] it is unknown which of the catecholaminergic cell groups are responsible for this release. Li et al.[131] found that suckling-induced c-Fos expression was prominent in the A1 and A6 cell groups but not in the NTS, whereas Moos et al.[132] reported activation of both A1 and A2 cell groups in the medulla and found numerous noradrenergic neurons could be labeled that projected to the SON. However, Moos et al.[132] further indicated that the only medullary cell group that projected bilaterally to the SON was a noncatecholaminergic group in the ventromedial region between A1 and the raphé obscurus. This region appears to have strong ipsilateral

projections and some contralateral hypothalamic connections, the latter of which cross within the optic chiasm to approach the SON. Although the evidence is strong that the noradrenergic innervation of the PVN (or SON) may be important for the milk ejection reflex (see below), it may not be these areas that provide afferents through the midbrain lateral tegmentum. Hansen and Kohler[113] found that injection of the catecholamine toxin 6-HODA into the midbrain lateral tegmentum had no effect on the amount of milk intake in a 2-h nursing test (see also previous discussion). However, these authors did not verify whether the A1 or A2 cell groups were depleted.

Caution should be exerted in interpreting c-Fos or other early intermediate gene products used to indicate neuronal activity. In the magnocellular system for example, electrical activation of PVN or SON neurons alone (with antidromic stimulation) is insufficient to increase c-Fos expression, whereas direct transmitter receptor activation does,[133] and stimuli involving synaptic activation such as suckling,[131] parturition, or osmotic stimulation (e.g., Ref. 134) or peripheral CCK administration[135] are effective in this regard. The absence of a c-Fos response may be an issue of sensitivity rather than lack of involvement. And while c-Fos may indicate a structure's eventual involvement after stimulation, the sequence of events is unknown due to its slow temporal response. Increased electrical activity may not be assumed, even if generally correlated with its expression.

With that caveat, suckling induces c-Fos expression in a number of other brainstem regions, including the parabrachial nucleus (which also projects to the PVN but not the SON), periaqueductal gray, and midbrain lateral tegmentum nucleus.[131,136] Although the precise location of midbrain neurons was not identified by Lin et al.,[136] the paralemniscal nucleus identified by Li et al.[131] is near, but more ventral to and more posterior to, the regions of the mesencephalic lateral tegmentum that effectively block or impair milk ejection when lesioned[105,106] (Figure 13.3). The most dorsal and anterior extent of paralemniscal nucleus, or its rostral projections, may have been damaged by lesions of the midbrain that disrupted milk ejection patterning but that failed to block the reflex.[106] Although most often associated with the auditory system, the paralemniscal region probably has diverse functions and even provides descending inputs to the spinal cord via the dorsolateral funiculus.[137] It is possible that midbrain tegmental lesions could interfere with the sensory gating apparent in producing the milk ejection reflex rather than simply blocking the afferent pathway. In this regard, the PVN is known to project to the spinal cord, including the dorsal horn, and some of these projections are OTergic.[138] Even a brief period of suckling increases OT release in the spinal cord, and OT-positive fibers are found in spinal lamina near c-Fos-activated neurons after the suckling bout.[139] This raises the possibility that central gating of afferent input could come from the PVN. In all likelihood, these OT axons would not be collaterals of neurohypophysial axons but probably a separate parvocellular system also involved in preautonomic innervation[82,140] or limbic system projections.[141]

Hypothalamic Circuitry

Some extrahypothalamic regions aside from those in the brainstem or spinal cord could be involved in the milk ejection reflex and will be considered next. Here it is pertinent to discuss whether other hypothalamic structures, aside from the PVN and SON, participate in and perhaps may even be necessary for the reflex. The structure for which the most evidence exists is the dorsomedial hypothalamic nucleus (DMH) (Figures 13.5 and 13.6). Although more often implicated in feeding behavior, the DMH is strongly activated by suckling as indicated by c-Fos expression[131,143] and by upregulation of neuropeptide Y (NPY).[144,145] This activation has supported the suggested involvement of the DMH in lactation-associated hyperphagia, consistent with its putative role in obesity.[143] The DMH is densely interconnected with many other hypothalamic nuclei, most notably

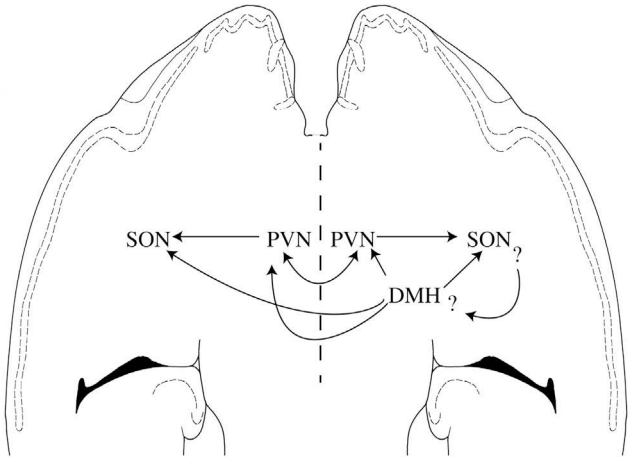

FIGURE 13.5 Schematic diagram illustrating two possible intrahypothalamic pathways that could contribute bilateral connectivity to OT neurons. The dorsomedial hypothalamic nucleus (DMH) is thought to project to both the SON and the PVN, bilaterally, is critical to the reflex, and receives brainstem inputs from regions also thought to be involved in the reflex (see Figure 13.4). The two PVN are thought to be connected, and the PVN may connect to the SON. The outputs from PVN to the SON are likely from parvocellular neurons, but this is not completely determined. The DMH is also thought to connect with other hypothalamic nuclei, such as the LH, that receive brainstem inputs (Figure 13.4). OT injections into the SON, or its electrical stimulation, are known to affect contralateral OT neurons. However, these effects may be due to stimulation of nearby neurons, which may then connect with a region such as the DMH that is known to project bilaterally. The vagueness of this SON intrahypothalamic connectivity is illustrated by question marks for source and target.

with the parvocellular regions of the PVN,[146–148] connections that dwarf the diverse yet sparser extrahypothalamic connections. Projections from the DMH to the SON have also been reported,[148,149] and although ipsilateral connections are strongest, the DMH also projects contralaterally to the SON with fibers passing beneath the third ventricle, and to the PVN, using fibers dorsal to the ventricle.[148] It is noteworthy that the DMH receives ascending projections from the brainstem, in particular the A1-A2 catecholaminergic regions that travel in the midbrain lateral tegmentum,[148] and also directly from the spinal cord.[115] A significant number of the PVN afferents from the DMH are glutamatergic, and an even larger number come from the nearby lateral hypothalamus,[150] which is the recipient of some suckling afferents (see previous discussion).

The suckling-induced increased c-Fos expression in the DMH may be directly related to a change in the activity of DMH neurons observed during the milk ejection reflex. Takano et al.[151] recorded hypothalamic neurons near the PVN during the reflex, all of which could be antidromically identified from ipsilateral SON stimulation but did not project to the neurohypophysis. About a third of these neurons recorded during the milk ejection reflex exhibited a bursting pattern similar to OT neurons, and some of these were in the DMH, as further documented by Honda and Higuchi[142] (Figure 13.6). Electrical stimulation of the DMH facilitates OT neuronal activity in the SON.[152,153] Even though the antidromic DMH projections to the region of the SON are more strongly ipsilateral,[142] DMH stimulation can activate the SON bilaterally.[152] Unilateral electrolytic lesions of the DMH completely block suckling to contralateral (but not ipsilateral) suckling.[154] However, the importance of the DMH per se is still clouded by the possibility that fibers of passage are responsible for both orthodromic and antidromic responses to SON stimulation, as well as lesion effects. Furthermore, when targeting the DMH specifically, Honda and Higuchi[142] reported that although a few DMH neurons showed bursting activity during milk ejections, the overwhelming majority did not. Finally, the DMH is just one of many other areas immediately surrounding the PVN that produce excitatory and inhibitory inputs to magnocellular (and parvocellular) neurons when stimulated with locally applied glutamate.[155,156]

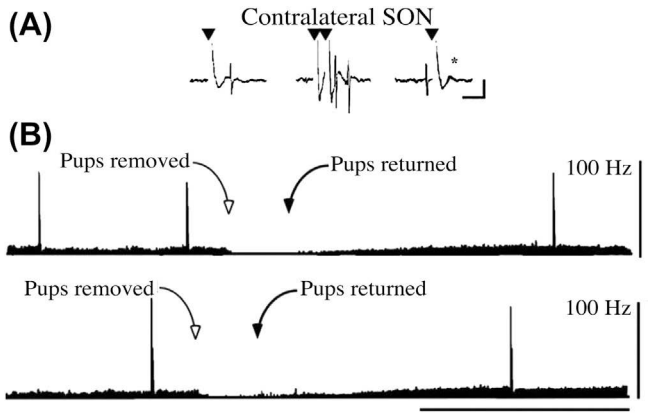

FIGURE 13.6 A dorsomedial hypothalamic neuron projecting to the contralateral SON and exhibiting bursts prior to milk ejections. (A) Oscilloscope traces showing the antidromic response criteria of constant latency (left), high frequency following (middle), and collision with orthodromic spike (right) following SON stimulation (arrowheads). * Indicates expected location of antidromic spike following collision with orthodromic spike from the dorsomedial nucleus. Scale, 1 mV, 1 s. (B) Bursts (large vertical deflections on ratemeter), and their dependence on pups suckling (arrows). Horizontal scale, 10 min. *Source: Redrawn from Ref. 142 with permission.*

An additional hypothalamic area proffered to mediate activation of the SON and PVN during suckling is an area just dorsomedial to the optic chiasm. Neurons in this region project both to the SON and PVN.[157] However, these projections appear largely ipsilateral, and there is no other indication of their importance to milk ejection. This region is near what is commonly referred to as the SON perinuclear zone (PNZ), which also has documented projections to the SON (see Refs. 117,158 for review). In addition, while potentially projecting to both nuclei, the lack of contralateral projections from this region still leaves the question of how the four nuclei communicate. The afferent intrahypothalamic connections of the PVN and SON have been difficult to document due to the nuclei's small size—nevertheless, hypothalamic connections with the PVN, including the lateral hypothalamus, which does receive projections from the midbrain lateral tegmentum, are thought to be extensive,[147,159] whereas afferent connections to the SON are sparse but do include the DMH projecting to OT-rich parts of the nucleus.[147]

Regardless, interhypothalamic communication is critical for full expression of bursting.[132,160,161] For example, hypothalamic midline knife cuts prevent most OT neurons on the side ipsilateral to the nursing pups from expressing milk ejection bursts, whereas bilateral suckling, or placing pups on the contralateral side to nurse, produces relatively normal bursting, although burst amplitudes are reduced.[161] Moos et al.[132] however, found that lactating rats suckling bilaterally, but with extensive hypothalamic hemisection, failed to show OT burst synchronization, and the bursting that was observed was dramatically reduced in amplitude compared to controls. Partial cuts allowed some synchronous bursting but with reduced burst amplitudes, similar to the results of Wang et al.[161]

Recently, the mammillary region of the hypothalamus has been suggested to mediate the burst synchronization.[162] The tuberomammillary nucleus and related nuclei have been shown to project bilaterally to the SON[163]—these are largely histaminergic neurons. Lesions involving the mammillary complex (tuberomammillary and supramammillary nuclei, mammillary body) produced

gross desynchronization of pair-recorded OT-bursting neurons during lactation, as did caudal hypothalamic hemisection.[162] Rostral hemisections similar to those performed by Moos et al.[132] produced some impairment of paired bursting. Since the mammillary lesions produced electrolytic damage that would destroy fibers of passage, it's not certain whether damage to these nuclei per se, or an ascending tract, is responsible for this effect. In addition, the extent of damage is difficult to assess from schematic, rather than histological, representation, but there was no reported damage to the DMH or the arcuate nucleus. The effects of these more discrete lesions suggest that a more compact region and/or pathway in the ventromedial hypothalamus (VMH) is critical to bilateral distribution of suckling information.

Another area of potential interest is the arcuate nucleus, which projects to the PVN and SON using peptide transmitters typically thought involved with feeding such as pro-opiomelanocortins and neuropeptide Y (NPY), or agouti-related protein (AGRP) (see Ref. 164 for review), and the innervation of which favors OT neurons.[165] Stimulation of the arcuate nucleus produces biphasic inhibition-excitation of OT neurons,[166] some of which is probably due to co-localization of arcuate neurons with glutamate or gamma amino butyric acid (GABA). Photostimulation of arcuate AGRP axons in the PVN strongly inhibits OT neurons through release of GABA, but it is unknown whether magnocellular and parvocellular OT neurons are both affected—the presumption is that parvocellular neurons projecting extrahypothalamically are the primary target as these neurons are involved in the suppression of feeding.[167] Arcuate dopaminergic neurons (which project axons to the external layer of median eminence) are clearly sensitive to suckling when they are profoundly inhibited and release dopamine's tonic inhibition of prolactin in the adenohypophysis (Ref. 168; also see Chapter 12). However there are no data, positive or negative, to suggest whether the arcuate nucleus is critical for the milk ejection reflex.

Perhaps the densest hypothalamic input to the SON and PVN comes from the region of the organum vasculosum of the lamina terminalis and the median preoptic nucleus (see Ref. 158 for review). While this region seems critical to osmoregulatory and cardiovascular duties of the PVN and SON and provides them both excitatory and inhibitory inputs, extensive lesions in this region to do not impair the milk ejection reflex,[169] compatible with the results of rostral hypothalamic deafferentation discussed earlier.[162]

Interconnections of the PVN and SON

It has often been speculated that the SON and PVN interconnect, either ipsilaterally or between the nucleus on one side and its contralateral counterpart. While there is evidence for polysynaptic communication, especially for OT neurons, direct connections have been difficult to document. Early retrograde tracing experiments reported ipsilateral projections from the SON to the PVN, and between the two PVN,[118,170] but no such interconnections were reported for the two SON.[119,149] Tribollet et al.[118] also reported projections from some accessory neurosecretory nuclei to the PVN, such as the anterior commissural nucleus (which contains a great majority of OT neurons). In later studies, smaller injections of retrograde tracers into the PVN failed to label the SON, and vice versa.[147]

Electrical stimulation of the SON on one side excites neurons in the contralateral SON[171] or produces both inhibitory and excitatory responses in the ipsilateral PVN,[172] but these appear to be through polysynaptic connections and may not involve magnocellular neurons but possibly those in the PNZ or dorsal chiasmatic area near the SON. Dyball and Leng[173] found synaptic responses from neurons near the SON following neurohypophysial stimulation, but their activity was generally unrelated to milk ejections. Interestingly, Takano et al.[171] reported that most contralateral OT neurons recorded in the SON were excited by SON stimulation, whereas a much smaller number of VP neurons were affected, and of these an equal number were inhibited. Some collaterals of magnocellular axons have been observed near the PVN,[174] in the PNZ of the SON,[175] and for OT neurons, at least, in the dorsolateral hypothalamus,[176] but the origin is unknown. OT-positive synapses were reported in the SON by Theodosis[177]—these might likely be intrahypothalamic, as such synapses are present in hypothalamic organotypic explants,[178] but again, the origin is undetermined. Morphologically, processes immunoreactive for neurophysin can be observed crossing the midline from one PVN to the other.[158] While these have the appearance of dendrites, this is not confirmed. In hypothalamic slices, a small number of PVN neurons can be excited by local glutamate or noradrenaline application to the contralateral PVN, or to the ipsilateral caudal SON.[179] The precise origin of these projections from either nucleus is unknown, and they could derive from parvocellular PVN neurons or PNZ neurons near the SON. In this regard, Thellier et al.[157] reported that parvocellular PVN neurons projected to the ipsilateral SON and to the contralateral PVN. Application of OT to one SON or PVN also potentiates OT neuronal activity in their contralateral counterparts during lactation[160,180] (also oxytocin below), and as mentioned earlier, electrical stimulation of the SON affects the contralateral SON. With the paucity of anatomical data showing magnocellular SON projections outside the neurohypophysis, it seems likely that the PNZ or dorsal chiasmatic areas may provide important connections, perhaps through a nucleus known to have bilateral projections and to affect OT neurons, such as the DMH (Figure 13.5). And while there is good evidence for some type of PVN to PVN

connections, the PVN per se appears to not be essential for synchronized bursting in the SON, since lactating rats with large PVN lesions still show milk ejection bursts in the SON,[181] albeit with a slower frequency and smaller amplitude. In addition, intracerebroventricular (ICV) OT was still able to facilitate the reflex with these PVN lesions. OT's role in the reflex will be considered in more detail later. Some possible connections underlying bilateral communication between the SON and PVN are summarized in Figure 13.5.

Using a modern, molecular biological approach involving adenovirus transfection of OT neurons, OT-specific pathways from the PVN to the amygdala have been demonstrated.[141] This approach has powerful ramifications for physiological experiments as well, since transfecting a gene expressing the light-sensitive channel rhodopsin was tied to the OT promoter and used to specifically release OT within the amygdala. A similar approach will undoubtedly prove useful for determining interconnectivity of the SON and PVN and their accessory neurosecretory nuclei.

Forebrain Regions Modulating Milk Ejection Activity

While forebrain regions may modulate milk ejection frequency and amplitude, they are not likely critical for generating the reflex. For example, the septum projects both to the PVN (primarily parvocellular) and to the PNZ of the SON,[147,149,182,183] but its destruction does not influence the milk ejection reflex, even though its stimulation can inhibit OT neurons and delay milk ejection bursts.[184]

The bed nucleus of stria terminalis (BNST) is a complicated area with multiple subdivisions, most of which project particularly strongly to anterior, parvocellular regions of the PVN,[185,186] many of which do contain extrahypothalamically projecting OT (and VP) neurons. The anteromedial BNST projects to PVN regions containing magnocellular OT neurons.[187] While many divisions project to the PNZ of the SON, the anteromedial division appears to have direct, if somewhat sparse, connections to the SON.

Large lesions encompassing the septum and BNST do not block the milk ejection reflex,[184,188] consistent with data showing the rostral separation of the hypothalamus from the forebrain with extensive knife cuts also fails to block the OT neuronal burst synchronization.[162] However, stimulation of the BNST either electrically or with OT injection facilitates the reflex,[188] and this region contains a high density of OT receptors that increase postpartum.[189] BNST lesions do impair, but do not block, the ability of ICV OT to facilitate the reflex (see the following on OT's role in milk ejection), and antagonism of OT receptors within the BNST slows burst frequency.[188] Some OT-positive fibers have been traced into the BNST and OT excites some BNST neurons, especially those stimulated orthodromically by PVN stimulation.[190] Interesting, none of the antidromically activated BNST neurons from PVN stimulation were affected by OT. Some BNST neurons subtly change their level of activity during milk ejection, but there are not strictly timed correlations with OT neuron bursting as has been observed in some DMH neurons.[191] Thus the BNST remains a site of interest as a target for central OT actions facilitating the reflex, but it would appear the output of the OT-excited BNST neurons is probably polysynaptic to OT milk ejection neurons, and the overall importance of this structure to the reflex per se is unclear.

The olfactory bulb has projections to the hypothalamus,[192,193] clearly plays a role in maternal behavior,[194] and shows c-Fos activation at parturition.[195] It has been speculated that mitral cells directly innervate neurosecretory neurons in the SON. While retrograde tracing studies from the SON suggest a bulb and an accessory bulb projection to at least near the SON,[195,196] the evidence for direct synaptic innervation of the SON from the bulb is partial. In slices, stimulation of the cut lateral olfactory tract elicited monosynaptic, glutamatergic excitatory postsynaptic potentials (EPSPs) in SON neurons,[197] but current spread and fibers of passage remain an issue with that result. Anterograde tracing studies indicate that the olfactory bulb and accessory bulb send projections to the ventral and lateral dendritic zone of the SON, which extends toward the periamygdaloid cortex.[196,198] A recent study using transynaptic tracing suggests bulb projections might be specific to VP neurons in both the SON and PVN.[193] To date, electron microscopically verified synapses between bulb efferents and magnocellular neurons have not been documented. Nevertheless, inducting maternal behavior in nulliparous female rats by housing pups nearby is sufficient to induce some of the morphological changes associated with plasticity of the OT system during pregnancy and lactation.[199]

There are other important inputs to the SON and PVN, both from more rostral and more caudal areas, but to date none has been strongly implicated in the milk ejection bursting of OT neurons. Some of these inputs are very dense, such as those from preoptic area (median preoptic and organum vasculosum of the lamina terminalis) and subfornical organ, but appear to relate to the role of both OT and VP in cardiovascular homeostasis. Other brainstem inputs of possible importance are the lateral parabrachial nucleus (projection primarily to the PVN) and serotonergic inputs from the raphé system.[158] There is evidence for some involvement of serotonin in OT bursting, to be reviewed in the following discussion.

Summary of Afferent Pathway

Although the precise sensory pathway from nipple to OT neuron is still unknown, there is agreement that the sensory pathway is largely contralateral, probably

crossing in the lower brainstem or upper spinal cord, that it passes in the lateral columns but not the dorsal columns of the spinal cord, that a synapse in dorsal horn, lateral spinal nucleus, or lateral cervical nucleus is likely, and that processing through conventional sensory centers, such as sensory thalamus and cortex, is unnecessary (Figure 13.4). Indeed, cortical activation in rodents inhibits the reflex. Within the brainstem, it appears likely that the NTS and ventrolateral and ventromedial medulla are involved, but it is unclear whether these nuclei directly transmit suckling information to OT neurons, even though they can make direct projections with them. Once crossed, the pathway in pons is unknown, but in the midbrain there is large agreement that afferents utilize the midbrain lateral tegmentum either as a passageway for fibers or make synapses there. This region is multisensory, does have neurons projecting to hypothalamus (perhaps even directly to the PVN), some of which are activated by suckling. Furthermore, lesions sparing axons of passage in this area do impair OT release. Nevertheless the possibility of many relevant sensory pathways simply passing through this area must be entertained. Although some medullary brainstem neurons project bilaterally and have been suggested to mediate bilateral transmission of nursing, they are typically more strongly ipsilateral, and unilateral lesions in posterior hypothalamus or midbrain lateral tegmentum block OT release and OT neuron bursting from contralateral suckling.

There is little agreement about how, once past the midbrain, sensory afferents eventually transmit bilaterally to OT neurons within the hypothalamus. Bilateral hypothalamic connections are critical to burst synchronization, but from which nuclei? The DMH exhibits some electrical activity correlated with milk ejection bursts, has bilateral projections to both the SON and PVN, impairs the reflex when destroyed, and has many neurons expressing c-Fos during suckling (Figures 13.5 and 13.6). It remains to be seen whether DMH axons directly innervate OT neurons, but this seems a possibility. The intrahypothalamic pathways are likely to be somewhat diffuse (typically large hemisections are required to disrupt the reflex), probably involving both supra- and subventricular crossings, and may involve more than one nucleus. In addition to the DMH, the medial dorsal chiasmatic area, the lateral hypothalamus, and the PVN are likely candidates for making such a distribution, but to date these connections are thought to be largely ipsilateral.

NEUROTRANSMITTERS/ NEUROMODULATORS MEDIATING THE MILK EJECTION REFLEX

Rather than explore in detail all transmitters or neuromodulators known to affect OT or VP neurons, the following discussion will focus on those thought to modulate the milk ejection reflex, especially at the OT neuron itself. The strongest evidence exists for glutamate and noradrenaline for transmitting information to the hypothalamus, and to OT itself for its role in facilitating and coordinating the reflex. These three have been implicated as necessary for the reflex, whereas many other transmitters can clearly modulate the amplitude or frequency of OT bursting.

Glutamate

As in most regions of the CNS, glutamate is a ubiquitous excitatory neurotransmitter in the hypothalamus, including neuroendocrine nuclei.[200] There is abundant evidence that both OT and VP neurons are heavily endowed with various glutamate receptors, most of which are excitatory, and which have been implicated in a variety of physiological processes, including synaptic plasticity during lactation (see "Synaptic Plasticity During Lactation" below). Glutamate receptors further mediate the EPSPs that can be recorded from OT neurons in vitro[201] and which are modulated during lactation.[202,203] However, the best evidence that glutamate is involved in transferring the suckling stimulus rests primarily on the results of the use of antagonists during lactation, and the data are surprisingly sparse. Injection of AMPA glutamate receptor antagonists into the third ventricle blocked suckling-induced OT release.[204] However, the range of diffusion of the antagonist is undetermined, thus it is possible the synaptic blockade was not at the OT neuron, and as indicated before, glutamatergic synapses are ubiquitous. Even though identified OT neurons contain AMPA-mediated EPSPs[201] and AMPA evokes OT release from lactating rats when directly injected into the SON,[204] these data are just further evidence that OT neurons have glutamate receptors and receive glutamatergic synapses, not necessarily for specific involvement in the reflex at the OT neuron. Moos et al.[205] recorded milk ejection bursts from OT neurons and applied the NMDA receptor antagonist, AP-5, locally in the SON. NMDA antagonism reduced background firing and burst amplitude but did not block bursts or reduce their periodicity, consistent with other in vivo work showing the interfering with NMDA receptor activation attenuated suckling induced OT release.[206] Unfortunately, AMPA-antagonists were not tested by Moos et al.[205] NMDA-mediated EPSPs are present on OT neurons[201] and may be involved in the synaptic plasticity occurring during lactation (Ref. 207, also see "Synaptic Plasticity During Lactation" below). According to Parker and Crowley,[206] there is a synergism between NMDA and AMPA-receptor activation controlling OT release.

A second line of evidence comes from the only in vitro system wherein synchronous OT bursts reminiscent of those during milk ejection have been observed.[208] In

this preparation, hypothalamic slices were taken from pups of unidentified sex at 5–7 days of age and organ-cultured for 4–7 weeks. Pairs of neurons were recorded and later identified as OT or VP subtypes. Many pairs of OT, but not VP, neurons were found to synchronously burst in a manner similar to that observed in lactation (Figure 13.7). Bursts were found underlain by synchronous volleys of AMPA-receptor mediated, glutamatergic EPSPs. OT facilitated both bursting and synaptic activities (also see below). This result suggests the circuitry regulating milk ejection bursts is intrahypothalamic.

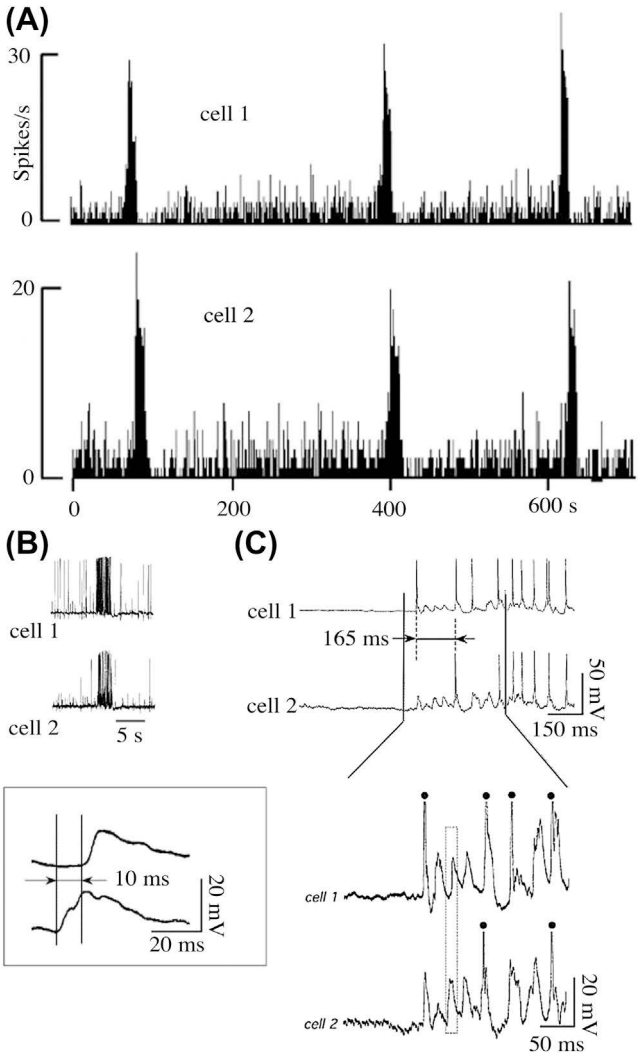

FIGURE 13.7 OT neurons with milk ejection-type bursts in organotypic cultures of hypothalamus, driven by glutamatergic EPSPs. (A) Two neurons exhibiting synchronous burst discharges characteristic of OT neurons in these cultures, similar to those occurring during lactation in vivo. (B) Another pair of neurons with simultaneous bursts. (C) The sweeps are expanded to show that the occurrence of the first action potentials in each is delayed by 165 ms. Below, the traces are further expanded to show the underlying synaptic activity driving the burst. EPSPs are highly synchronized, delayed by ~10 ms (see boxed inset). Dots show truncated action potentials. *Source: Redrawn from Ref. 208 with permission.*

While it should be noted that such cultures are known to make inappropriate connections, it is nevertheless astounding that a pattern like this should emerge. Since the sex of the donors is unknown, it is unlikely to involve steroid preconditioning or other sex-dependent electrophysiological and morphological changes known to occur with OT neurons during pregnancy and lactation (see "Synaptic Plasticity During Lactation" below). The clue to its relationship to milk ejection bursts in vivo may in fact be the massive amount of excitatory synaptic activity present in the organotypic slice. In adult hypothalamic slices, synchronous OT bursting has never been observed, even from lactating rats, although pharmacologically induced bursts of individual OT neurons has been reported, even in male rats.[209,210] However, tonic excitatory synaptic activity in hypothalamic slices is low, is largely from miniature EPSPs and is insufficient to alter OT firing patterns.[211] Perhaps increasing this excitatory activity in adult slices would be a means of further testing the role of glutamate in burst generation.

Where would the local glutamatergic inputs arise? It is useful to recall here that both the SON and PVN do receive excitatory connections from neighboring regions, such as the PNZ of the SON[156,212] and regions surrounding the PVN, including the DMH, VMH, and the lateral hypothalamus.[150,152,155,156] Indeed the number of hypothalamic regions suggested to provide glutamatergic inputs to the SON is large, and in addition to those mentioned earlier, includes mammillary structures, the PVN, and the suprachiasmatic nucleus.[212] Thus there would appear to be ample opportunity in the organotypic culture for local glutamatergic neurons to develop synapses with OT and VP neurons.

Noradrenaline

A case has long been made for noradrenaline as the principal transmitter mediating the milk ejection reflex.[213] As mentioned before, there is adequate evidence for noradrenergic synaptic input, primarily from the NTS (A2) and A1 regions of the medulla, to both nuclei, but NTS input favors OT neurons. Activation (c-Fos expression) of A1, A2, and A6 (locus coeruleus) neurons has been reported in response to suckling.[131,132] Similarly, there is abundant evidence of the presence of various adrenergic receptors on OT neurons.[117,213,214]

Suckling increases turnover of noradrenaline in the SON and PVN, and local destruction of catecholaminergic inputs blocks suckling-induced OT release.[215] Microdialysis studies indicate noradrenaline is released in the PVN in response to suckling, but such release was not detected in the SON.[130] Third ventricular administration of α_1-but not β-adrenergic receptor blockers likewise blocks the milk ejection reflex,[213] but local applications of adrenergic antagonists within the SON or PVN have

not been used to study the response of milk ejection bursts per se. Indeed, these investigations indicate that β-adrenergic receptors appear to tonically inhibit OT release. Local application of either class of antagonists blocks the intranuclear, suckling-induced OT release in the PVN,[130] and noradrenaline, as well as glutamate, may play critical roles in local OT release (see below).

Although noradrenaline appears to excite OT (and VP) neurons via α_1 receptors in vitro,[216,217] direct effects may be no more important than the simultaneous presynaptic excitation of glutamatergic inputs.[218] It is unknown whether direct connections between brainstem catecholamine and OT neurons actually mediate the milk ejection reflex. There are no data demonstrating the electrophysiological responses of brainstem catecholaminergic neurons to suckling, and no data demonstrating that a selective impairment of any one of these cell groups would block suckling-induced OT release—even though such an experiment could prove difficult due to the involvement of these nuclei in other important physiological functions, such as cardiovascular homeostasis. Finally, the milk ejection-like bursting response of the organotypic explant[208] is clearly not dependent on an intact noradrenergic system, even if this model system may be an aberrant representation of the physiological network. While catecholaminergic inputs to OT may be activated during suckling, their precise role in controlling the bursting activity is still debatable. It should be mentioned that other, non-catecholaminergic brainstem neurons project to the SON and PVN, including a group in the ventromedial medulla that have bilateral projections,[132] and the NTS has other neurons with peptides associated with OT activity, such as activin,[127] which has been implicated in suckling-induced OT release.

Noradrenaline, through α_1 receptors, has recently been found to produce milk ejection–type bursts in OT neurons in hypothalamus slices from lactating rats.[219] The significance of this action to OT neurons during lactation is unclear, however, since a similar phenomenon has been observed in male rats.[209,210] The bursts are thought to not be synchronously activated in this preparation, and the studies of Wang and Hatton[209,219] were performed in an abnormal slice medium (low in Ca^{++}), which itself promotes atypical bursting in these neurons.[220]

Oxytocin

Clearly the most exciting work on the mechanisms underlying the milk ejection bursting has come from the demonstration that central OT itself is an important player. ICV injections of OT were found to dose-dependently facilitate the reflex in two important ways[88,89] (Figure 13.8). Firstly, OT increased background firing, the burst amplitude in the PVN (increased number of spikes/burst), the intramammary pressure associated with release, and the frequency of the periodicity. Secondly, neurons that were not correlated with OT release initially could be recruited into the bursting pattern by OT. VP had no effect in this regard, and a later study showed ICV administration of an OT antagonist not only blocked OT's effect but also suppressed the ongoing reflex[89] (Figure 13.8). Freund-Mercier and Richard[89] further found that: (1) quantities of OT as low as 10 pg were effective; (2) injection into the third ventricle was more effective than the lateral ventricle; (3) the effects were dependent on pups suckling; (4) OT could instigate reflex onset in cases where it failed to show within the first hour of suckling; and (5) VP neurons were unaffected. The results of the OT antagonist suggested that the effect of ICV OT was probably not pharmacological, that OT released centrally during suckling was critical to reflex expression. These seminal findings have been repeated several times, are true for both SON and PVN OT neurons, and have led to vigorous research concerning the mechanism and location of action of OT, as well as its source.

Shortly following the seminal studies of Freund-Mercier and Richard,[88,89] it was discovered that OT was released in vitro from tissue containing either the SON or PVN,[221,222] and that OT agonists facilitated this local OT release.[221] Using push–pull cannulae, Moos et al.[223] demonstrated the release of OT in vivo from the SON during suckling, release that was facilitated by neurohypophysial stimulation mimicking OT bursts and by OT agonist injection (Figure 13.9). Intranuclear release of OT during parturition and suckling has since been repeated with finer microdialysis probes in both the SON and PVN, appears to result from release within or very near these nuclei,[130,224] and is first increased at low levels after pup application but *before* milk ejection bursts commence, when it increases further.[223] In addition, local retrodialysis of OT antagonists suppressed the local OT release and the milk ejection reflex.[225] Further evidence that the action of OT is likely within the PVN and SON was suggested by the effectiveness of small amounts of OT in facilitating bursts when injected directly into the SON or PVN, but not in adjacent regions of the SON.[160] Tellingly, these injections facilitated OT neuronal bursting in the *contralateral* nuclei as well, but only with an intact midline, suggesting the importance of hypothalamic intercommunication referred to earlier[160] (Figure 13.8). Complementarily, local injection of an OT antagonist into either the PVN or SON reduced bursts from all neurons tested within the injected nucleus, and the majority of neurons in the *contralateral* PVN or SON, and reduced the amount of OT released.[180] Thus information about OT release within either a single SON or PVN nuclei is somehow communicated ipsi- and contralaterally, but as mentioned previously, the precise connections are unknown. Electrical or osmotic stimulation

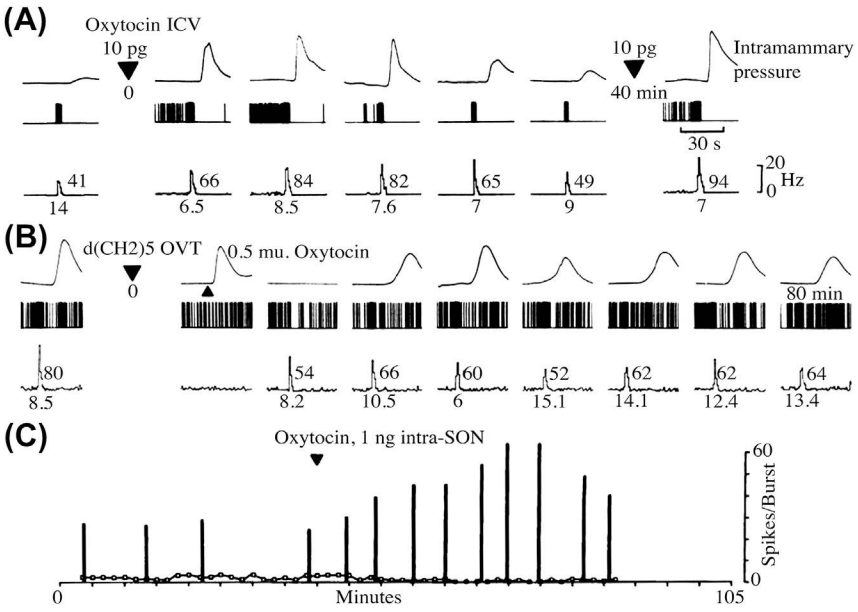

FIGURE 13.8 Oxytocin (OT) facilitates the milk ejection reflex. (A) OT delivered intracerebroventricularly (ICV) increased the burst amplitude and decreased interburst interval of an antidromically identified PVN neuron, and simultaneously increased the amount of OT released during each milk ejection. *Top traces*, intramammary pressure indicating OT release at each milk ejection; *middle traces*, pen recording of single unit activity of the PVN neuron; *bottom traces*, ratemeter histograms of firing rate. The number at the side of each burst represents its amplitude, or spikes/burst. The number below represents the number of minutes between bursts. (B) An OT antagonist, $d(CH_2)_5$ OVT injected ICV (downward arrowhead) blocked the next milk ejection burst in a PVN neuron and the milk ejection reflex. To ensure the mammary gland was responsive, OT was injected intravenously (upward arrowhead). The burst in this neuron returned 8.2 min later, but was reduced in amplitude and was not accompanied by the milk ejection reflex. The reflex returned 10.5 min later. *(Source: (A) and (B) Redrawn from Ref. 89 with permission.)* (C) OT injected directly into the SON facilitated burst of an OT neuron in the *contralateral* SON. Injections near the SON were ineffective (not shown). Note the increase in burst amplitude and the much faster interburst interval following the injection. *Source: Redrawn from Ref. 160 with permission.*

of the SON unilaterally, for example, has no such effect on OT bursts in the other nuclei, suggesting activation of an OT-sensitive circuit that probably does not involve direct PVN-SON connectivity of magnocellular OT neurons. Thus it is possible that neurons near magnocellular neurons, such as PNZ neurons in the SON, the DMH, or parvocellular PVN neurons, receive a suckling-specific OT signal from neurohypophysial projecting neurons, and communicate this among the nuclei.

To date, however, the source and particularly the target of the intranuclear OT-facilitating OT–milk ejection bursting are under debate. The current ongoing hypothesis concerning the source is that OT release is dendritic, from the same magnocellular neurons projecting to the neurohypophysis. This is a parsimonious conclusion and enjoys much evidence.[226] In addition to evidence for local OT release from the SON and PVN, and the effects of locally applied OT agonists and antagonists on milk ejection bursting, magnocellular dendrites have clusters of dense core vesicles (DCVs). Stimulation produces DCV exocytosis,[227] as well as calcium-dependent DCV mobilization and associated actin mobilization.[228,229] Some requisite molecules associated with vesicular release have been found in OT or VP dendrites, such as syntaxin-1 and munc-18.[229,230] Considering more conventional sites of release, axon collaterals of immunoreactive OT and VP axons have been reported near the SON,[175] but collaterals are not commonly observed from intracellularly filled SON neurons.[117] Collateral branching is, however, prevalent among PVN parvocellular neurons,[174] and it is possible that some of these could be OT containing. As previously mentioned, there is some evidence for OT-positive synaptic terminals in the SON,[177] but this is not commonly reported and their source is unknown.

Thus the current wisdom suggests that OT is released from dendrites at nonsynaptic locales during suckling. While diffusion within the third ventricle to remote sites of action is possible, the evidence favors more limited diffusion within the SON, PVN, or to nearby nuclei, for effects. Local OT release is not easily produced by antidromic stimulation, but a large release can be evoked after calcium mobilization with thapsigargin that is thought to prime (i.e., move toward the plasma membrane) the DCVs for release.[226] However, this result may be a question of detection sensitivity. Antidromic stimulation can evoke bursts in OT neurons,[173,231] again dependent on pups suckling, and in some labs, trains of antidromic spikes have been shown to release OT in the SON without priming.[223] The effects of antidromic facilitation on OT neurons are independent of invasion in the recorded neuron, as stimulation adjusted to collide with orthodromic action potentials from the OT neuron still

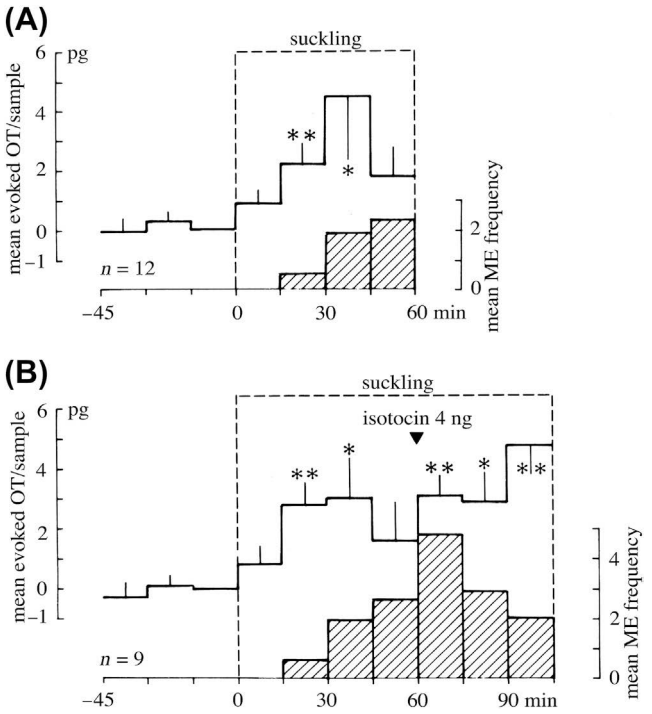

FIGURE 13.9 Evoked oxytocin (OT) release in the SON measured using a push–pull cannula in the nucleus during the milk ejection (ME) reflex. The mean number of ME events was calculated for each 15-min push–pull sampling session. (A) Mean increase in intranuclear release during the ME reflex. Note the increased release during suckling. (B) Mean increase during suckling and following facilitation of the reflex with the OT agonist isotocin delivered intraventricularly. *, $p<0.05$; **, $p<0.01$, Student's paired *t*-test. *N* = number of rats tested. *Source: Redrawn from Ref. 223 with permission.*

evokes bursting[231] (Figure 13.10). In putative but unidentified OT neurons, depolarization during whole cell recording by direct voltage steps[232] or by NMDA application increased somatodendritic exocytosis as measured with capacitance changes—the latter effect of which was upregulated from neurons taken from lactating rats.[233]

It should be noted here that there are multiple groups of OT neurons in the hypothalamus that project to the neural lobe,[158] including many accessory neurons such as the anterior commissural nucleus, implicated in the BNST-OT connection.[188] While the assumption based on their neurohypophysial projection would be the participation in most if not all aspects of the OT functions typically ascribed to SON and PVN neurons, there are no reported in vivo electrophysiological studies of these neurons during lactation, and the difficulties in observing c-Fos activation during lactation compared to parturition in large numbers of OT neurons, even in the SON and PVN,[134,136] make their participation in the reflex difficult to assess.

As for the target of OT's actions, OT neurons have been shown to possess the OT receptor (OTR) based on several lines of evidence, and this would be consistent with local effects, from local release. The increase in intracellular Ca^{++} evoked by OT or agonists in dissociated OT neurons is blocked with a specific OT antagonist.[234] OT applied to hypothalamic slices excited putative OT neurons in the SON, an effect that remained, albeit smaller, in low Ca^{++}-Hi Mg^{++} solution thought to block synaptic transmission.[235] Specific binding of radiolabeled OT antagonists has been observed in the SON during lactation[236] and late gestation.[237] OTR mRNA has been located in both the SON and PVN,[238–241] although there is disagreement as to whether OTR mRNA is upregulated in both the SON and PVN during gestation or at parturition.[239,241]

Despite the strength of the evidence that OT activates OT neurons to facilitate the reflex, aside from the study of Yamashita et al.,[235] there are few studies demonstrating OT's *direct* effects on membrane potential or membrane currents of OT neurons, unlike the extensive results obtained in extrahypothalamic targets possessing OT or VP receptors.[242] Better documented is the effect of OT on synaptic inputs to OT neurons. Originally described by Kombian et al.,[243] OT is thought to *suppress* glutamatergic synaptic inputs. The suspected targets of OT initially were presynaptic OTRs, but of late many of the presynaptic effects of OT on glutamatergic synaptic inputs have been attributed to OT's ability to release cannabinoids from OT neurons, presumably due to OT's ability to increase intracellular Ca^{++}.[244] Cannabinoids so released are then thought to act on CB_1 receptors presynaptic to OT neurons, again, to suppress EPSPs. However, there are no data concerning any differential effects of OT, or cannabinoids for that matter, on EPSPs impinging on OT neurons in lactating animals compared to control animals. Thus it is not clear what role OT's indirect means of suppressing glutamatergic synapses would play in lactation. There are studies showing that glutamatergic transmission may undergo plasticity during lactation, but results are not uniform (see below under "Synaptic Plasticity During Lactation").

Interestingly, ICV-administered OT does unveil excitatory effects on milk ejection burst that occur ~100 ms after stimulating the midbrain lateral tegmentum,[111] an effect observed only when pups are suckling. This effect was not observed when stimulating excitatory inputs from the medial septum, and was not observed with general excitation of OT neuronal background firing (with osmotic stimulation). This is consistent with antidromic stimulation's,[223] or OT's ability to enhance OT bursts and background firing *only* when pups are suckling, and not with generalized excitation.[89] The facilitation observed with midbrain tegmental stimulation followed the time course of the OT-induced bursting facilitation, and was greatest prior to an expected burst and lowest immediately after a burst. A similar phenomenon was observed when stimulating the DMH during

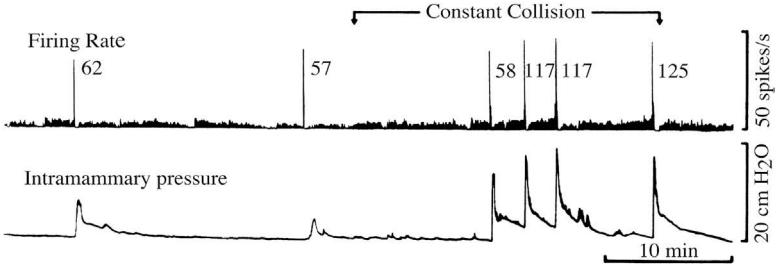

FIGURE 13.10 Antidromic activation of PVN neurons facilitates milk ejection bursts from OT neurons during suckling. *Top trace*, ratemeter from an antidromically identified PVN neuron showing milk ejection bursts before (two at left) and after neurohypophysial stimulation. *Bottom trace*, intramammary pressure indicating milk ejections. When the neurohypophysis was given a single shock after each orthodromic action potential, milk ejection bursts and OT release were facilitated even though the antidromic spike from the recorded neuron did not reach the PVN. Note the increased frequency of bursts and the increase in burst amplitude (spikes/burst) shown beside each burst. Thus the facilitation of the OT release during antidromic stimulation is not dependent on invasion of the recorded neuron but instead mostly likely derives from the back propagation of spikes in other magnocellular neurons. *Source: Redrawn from Ref. 231 with permission.*

lactation.[153] However, a long latency to this effect, similar to that of midbrain lateral tegmentum stimulation, was reported. Indeed, some short latency (~10 ms) excitatory responses to DMH stimulation were not potentiated by OT. Thus the location of OT's action is unknown in regard to both studies, and may not be at the OT neuron or even at presynaptic terminals directly on these neurons, but perhaps elsewhere in the afferent circuitry. Given the multiple locations of OTRs in the brain (see below), it is possible that other sites known to project to the SON or PVN could be involved in the effects of *exogenous* OT administration.

The results from in vitro experiments support a critical role for local OT in regulating the reflex, probably through glutamatergic inputs. In organotypic hypothalamic explants that show synchronous OT neuronal bursting with patterns similar to those observed in vivo during lactation, OT and its agonists facilitate this bursting both in amplitude and periodicity. OT antagonism blocks the effects of endogenous OT and suppresses spontaneous bursting, but primarily by increasing interburst intervals and not burst amplitude.[245] As discussed earlier, the OT bursting is underlain by glutamatergic EPSPs that are highly synchronized when driving bursts[208]—indeed, EPSP synchronization is significantly tighter than that of action potential onset (Figure 13.7). OT's actions on bursting are likewise dependent on glutamatergic synapses. However, in this preparation, OT has no obvious effect on EPSPs (even the presynaptic inhibition reported by others in slices), but instead is suggested to act presynaptically at GABAergic terminals at low concentrations.[246] This enhancement of IPSPs is thought to then contribute to rebound action potentials that are generated from low threshold (T-type) Ca^{++} currents and to the increased background firing of OT neurons that is also characteristic of OT's actions.

A change in background firing, expressed as an increase in rate and an increased irregularity, has been noticed in OT neurons in vivo prior to OT bursts,[247] as has an increase in coordinated activity.[248] This change may reflect an increase in synaptic activity or a change in its pattern from neurons presumptively driving the bursts, but there is no direct evidence of this. In the organotypic culture, basal firing also increases with OT application and also prior to bursts, but there has been no demonstration that GABAergic activity was necessary for OT bursting, or even for OT's ability to facilitate bursting. As for the presence of T-channels in OT neurons, this is inconsistently reported in the literature. An absence of low threshold currents was noted in the PVN magnocellular neurons by Luther and Tasker,[249] but they did find this current characteristic of one class of PVN parvocellular neuron, and this has been likewise observed in enkephalinergic neurons near the PVN.[250,251] In the rat SON, Foehring and Armstrong[252] found no evidence of low threshold Ca^{++} currents, but such currents were reported by Fisher and Bourque,[253] and in guinea pig SON VP, but not OT neurons, by Erickson et al.[254]

In hypothalamic slices, OT has been found to produce brief burst discharges in OT neurons[255] similar to those evoked by noradrenaline discussed earlier. Although these experiments were performed in slices from lactating rats, there was no comparison with virgin rats as to the efficacy of OT in this regard. Surprisingly, OT concentrations in the fM range were effective in this study, which is a couple of orders of magnitude below the reportedly low nM K_d of the historically well-characterized OT receptor.[256,257] The effects of OT involved progressive membrane depolarization and were blocked by AMPA-receptor antagonists, and like noradrenaline, did not promote burst synchronization. More recent studies suggest that OT's direct effect on OT neurons may involve actin mobilization,[258] which could relate to OT's ability to prime its own somatodendritic release.[226,259]

Given that (1) OT's action on the milk ejection neurons is greater for third ventricular compared to lateral

ventricular injections, (2) OTRs have been shown to be autoreceptors on OT neurons, and (3) the reflex is maintained when other important regions with OTRs, such as the BNST, are separated from the hypothalamus, it seems likely that central OT's role in milk ejection minimally requires some action in the magnocellular nuclei. However, in considering OT's central effects, it should be remembered that the OTR has been located in other regions in hypothalamus besides the SON and PVN, some with great density.[260] The densest distribution of OTR is in the VMH, which has no obvious tie to the milk ejection reflex, but the OTRs of which are sensitive to reproductive state and estrogen.[189,261,262] Additional regions of interest include the preoptic area and some members of the mammillary complex, the latter of which have been implicated in controlling the reflex.[162] Thus it is possible that exogenously administered OT may act in multiple regions to modulate the reflex. But if the prime source of released OT during milk ejection is dendritic, then diffusion to more remote regions becomes problematic due to the fast enzymatic degradation of released OT.[243]

Gamma Amino Butyric Acid

Like glutamate, GABA is a ubiquitous transmitter in the CNS, is likely responsible for at least one half of the synapses on magnocellular neurons,[263] and its effects (largely inhibitory) on magnocellular neurons have been well characterized.[117,214] The sources of GABAergic inputs to OT neurons could be intrahypothalamic, even near the SON or PVN, as well as some well-known extrinsic afferents such as the preoptic area, the BNST, the mammillary complex, the subfornical nucleus, and the organ vasculosum of the laminar terminalis.[158] GABAergic synapse numbers increase during lactation and are part of well-documented reproductive plasticity in this system (see Plasticity below).

Moos[264] and Voisin et al.[265] addressed the specific role of GABA in the milk ejection reflex in vivo. Moos[264] found that whereas local GABA or GABA agonist applications to the SON decreased baseline firing as expected, they actually *precipitated* OT milk ejection bursts, whereas the specific $GABA_A$ receptor antagonist GABAzine considerably delayed the occurrence of the next expected burst. In contrast, Voisin et al.[265] reported that both the $GABA_A$ receptor agonist muscimol and the $GABA_A$ antagonist bicuculline inhibited the reflex, whether injected ICV or into the SON. Aside from implying the importance of GABA activity to the reflex, it is difficult to interpret these two studies mechanistically.

Perhaps the role of GABA in the reflex rests on OT's actions on GABAergic synapses, which are largely inhibitory, either postsynaptically[266] or presynaptically.[232] These actions, accompanied by increased Ca^{++} somatodendritic release of OT during lactation,[232] are largely interpreted as OT's ability to retrogradely shape inputs, and permit the increased excitatory input needed for OT release at parturition and lactation.[267] However, OT's presynaptic inhibition of GABA-mediated IPSPs appears almost solely due to OT's action on the OT cell itself, releasing cannabinoids to reach presynaptic CB1 receptors on GABAergic terminals. High-frequency activation of these inputs results in powerful OT neuronal inhibition that is dependent on cannabinoid action.[268] Although not tested in lactating rats, this suggests that the powerful inputs driving each burst, probably from glutamatergic synapses, would release OT sufficient to shut down any competing GABAergic activity both pre- and postsynaptically. However, given the needed time for cannabinoid diffusion following its release for local OT action, it would seem that this inhibition might arrive late to facilitate any burst associated with massive local OT release, at least at burst onset. However, it is possible that OT release from glutamatergic inputs actually *precedes* burst onset, allowing some local OT the time to shape the burst.

Serotonin and Dopamine

Based on light microscopy, the serotonergic innervation from the median and dorsal raphé nuclei to the PVN and SON may favor OT neurons.[269] Iontophoretically applied serotonin (5-hydroxytryptamine, or 5HT) inhibits OT neurons during suckling-induced bursting,[270] and both $5HT_{1A}$[271] and $5HT_{1B}$ receptors[272] have been found in magnocellular neurons of the SON and PVN. Pharmacological data suggest a role of 5HT in modulating milk ejection bursts, but it is unlikely to be the primary neurotransmitter,[273] and there is no evidence to suggest that suckling affects raphé neurons. However in rats, the apparent necessity of sleep-like EEG to allow milk ejections suggests that pharmacological manipulations of 5HT levels could influence the reflex indirectly. The actions of 5HT agonists and antagonists on the reflex were opposite in anesthetized compared to conscious animals, although both expressed the milk ejection reflex.[273] Thus 5HT's role in the reflex remains undetermined.

There are dopaminergic inputs with identified synapses in both the SON and PVN, although the input to the PVN is much denser,[274,275] and a specific projection to OT neurons has not been identified. The source of the dopamine to the PVN is likely the medial zona incerta,[276] but also could derive from parvocellular PVN groups. Dopamine has direct depolarizing actions on SON neurons; mediation through both D_1 and D_2 type receptors has been suggested.[277,278] ICV injection of the dopamine agonist apomorphine facilitates OT milk ejection bursting, although not as powerfully or as immediately as does OT[279,280]—on the other hand, dopamine antagonists inhibited milk ejection bursts[279] and suckling induced OT release.[281]

In addition to its direct depolarizing effects on magnocellular neurons, dopamine also inhibits both IPSPs[282] and EPSPs[283] presynaptically in the SON, effects thought mediated by D_4 receptors. According to Balmoukhametova et al.,[284] OT neurons in normal animals show short-term facilitation of repetitively activated GABAergic synapses, but dopamine did not appear to impact this effect, in contrast to its ability to prevent short-term depression in VP neurons. While neither the synaptic nor the direct depolarizing effects of dopamine have been examined on OT neurons from lactating rats, it is of interest that the dopamine neurons of the medial zona incerta are extremely close to the PVN and adjacent structures thought to modulate bursting, such as the DMH. OT from PVN dendrites could diffuse to the zona incerta and thereby modulate dopaminergic inputs. Furthermore, medial hypothalamic lesions and knife cuts that are thought to affect milk ejection bursts may easily impinge on this region.

Histamine

Another transmitter that is released into the PVN and SON by suckling is histamine.[285] Direct injection of histamine into the PVN releases OT both peripherally and locally,[286] and histamine H_1 receptor antagonists suppress suckling-induced OT release.[287] However, the data are not so clear regarding mechanisms. Oddly, both H_1 and H_2 receptor antagonists blocked the histamine-induced intranuclear release,[285] and the excitatory effect of histamine on both local and peripheral OT was in addition blocked by an α_1 noradrenergic receptor antagonist.[286] Although histamine has direct excitatory effects on VP neurons through H_1 receptors,[288,289] Yang and Hatton[290] found evidence for direct inhibitory actions of histamine on OT neurons that were mediated by H_2-type receptors, carried by monosynaptic inputs from the tuberomammillary nucleus, and histamine has been shown to induce dye coupling in OT neurons.[291] Since CNS histamine is made solely in the tuberomammillary and adjacent tuberal nuclei in the hypothalamus, the recent results from Wang et al.[162] showing that small lesions in this region block suckling-induced OT release may yet herald an important role for this region, and should be repeated with neurotoxic destruction of this region to avoid issues of fibers of passage.

Other Neurotransmitters/Neuromodulators

There is a wide variety of other peptides, neuromodulators, and hormones that might not be critical for, but nevertheless do influence, the milk ejection reflex based on their known actions in the SON and PVN, and these are reviewed elsewhere.[117,210,213,214,292,293] Among these, the steroids estrogen and progesterone, as well as the neurosteroid allopregnanolone, will be dealt with in the section Synaptic Plasticity During Lactation. The normally inhibitory actions of local nitric oxide on OT release appear to be greatly reduced at parturition, suggesting, like that of GABAergic control,[267] a release from inhibition when OT demand is acutely high.[210] While the role of this gaseous transmitter has not been studied specifically within the SON or PVN during the reflex, recent studies show it behaves as a retrograde messenger in these nuclei, capable of facilitating GABAergic inputs to magnocellular neurons.[294–296]

An intriguing peptide of interest is α melanocyte-stimulating hormone (MSH), which is synthesized in the arcuate nucleus, which has well-documented connections to the PVN and suggested projections to the SON. Locally applied α-MSH was found to selectively inhibit the electrical activity of OT (not VP) neurons in female rats in the SON even while promoting an increase in c-Fos expression, an increase in local OT release, and an increase in intracellular Ca^{++}, probably from internal stores.[297] This study shows the difficulty in interpreting results from increased c-Fos expression as excitatory, showing a clear separation of function of somatodendritic depolarization from electrical activity. The most likely scenario is that the increase in dendritic intracellular Ca^{++} promotes a Ca^{++}-dependent afterhyperpolarization in these neurons, similar to what has been observed in substantia nigra by some dendritic inputs.[298] Clearly the role of α-MSH deserves more study in the regulation of OT bursting during lactation.

The peptide neurotensin causes OT neurons to adopt an irregular firing pattern,[299] similar to that which occurs prior to milk ejection bursts.[247,248] However, OT neuron burst initiation was not reported by Johnstone et al.[299]—indeed neurotensin exerted no effects on OT neurons during lactation. Thus firing irregularity, while highly correlated with an impending burst, seems to reflect some other change in the system, but may not itself lead to, or even signal, an impending burst.

ELECTROPHYSIOLOGICAL PROPERTIES OF OT NEURONS AND THEIR PLASTICITY DURING LACTATION

An obvious question regarding the mechanism of OT milk ejection bursts is whether intrinsic membrane properties play a role in this activity. The short answer is that intrinsic properties, even those known to change during lactation, are probably insufficient to underlie milk ejection bursts, but they could certainly shape them to an important degree.

The first comparison of membrane properties of OT and VP neurons identified few differences in input resistance, spike parameters, or afterhyperpolarizing potentials in male rats.[300] Both types were also found to possess

a slow (1–2 s) depolarizing afterpotential (sDAP) thought to underlie the phasic bursts of VP neurons and which was more prominent in VP neurons, especially after treatments blocking an overlapping spike afterhyperpolarization (AHP). However, the amplitude and form of sDAPs in OT neurons was not different from those in VP neurons.

Stern and Armstrong[301] described a sustained outwardly rectifying potassium current (SOR) that was much more prominent in OT neurons and which was increased during lactation (Figure 13.11). The SOR is of interest because it contributes to spike frequency adaption, and also promotes a rebound depolarization, part of which is Ca^{++} dependent, that could contribute to the onset of OT bursts.[302] The studies showing that IPSP-induced hyperpolarizations lead to rebound spikes,[246] combined with the facilitating effect of GABA on OT bursting while also reducing background firing,[264] suggest that strategically placed hyperpolarizations could precipitate bursting, and perhaps the SOR could facilitate this. However, Israel et al.[246] found that bursts were not coincident with OT's actions on rebound firing, but instead found that OT contributed to spike firing irregularity and frequency via this mechanism. Li et al.[211] found that blocking randomly occurring IPSPs in OT neurons regularizes firing, supporting a role for GABAergic synapses in shaping the background firing that changes dramatically during interburst intervals.[247,248] However, since irregularity can be separated from bursting, these changes in background firing observed in vivo may reflect ongoing synaptic activity relevant to the oncoming burst, but do not necessarily lead to burst discharge.

Other changes were noted during lactation by Stern and Armstrong,[301] including an increase in the size of AHPs following spike trains (Figure 13.12), an increased spike width and a reduced spike height. Enhanced AHPs during lactation were specific to OT neurons and have been observed in other studies,[303,304] but the increased spike width and reduced height has also been seen with

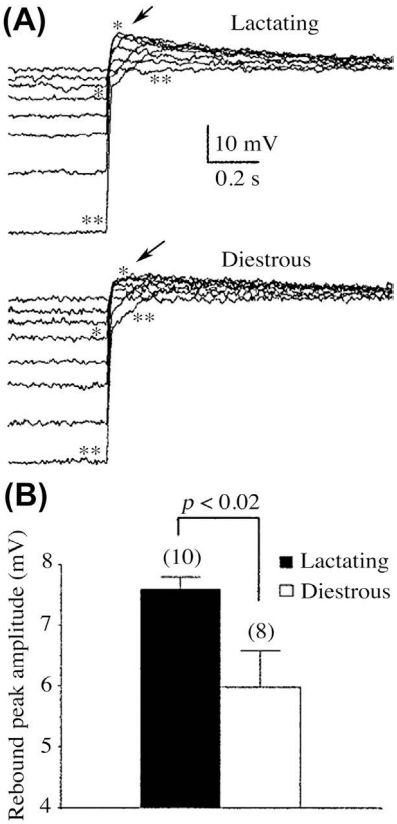

FIGURE 13.11 The peak amplitude of the rebound depolarization observed in OT neurons is enhanced during lactation. (A) The rebound depolarization following deactivation of a sustained outward rectifying potential elicited at the offset of 1.5 s hyperpolarizing pulses in OT neurons is shown for a lactating and a diestrous rat. Arrows point to the peak rebound. All traces were taken in the presence of 0.5 μM tetrodoxin, which blocked fast sodium action potentials and thus allowed appreciation of the rebound's amplitude. Neurons were current clamped at approximately −45 mV. Note that the largest rebounds are associated with relatively weak hyperpolarizations turning off the sustained outward rectification (*), whereas strong hyperpolarizations are associated with the smallest rebounds (**). (B) The rebound peak amplitude is enhanced during lactation ($p < 0.02$; Student's *t*-test). *Source: Redrawn from Ref. 301 with permission.*

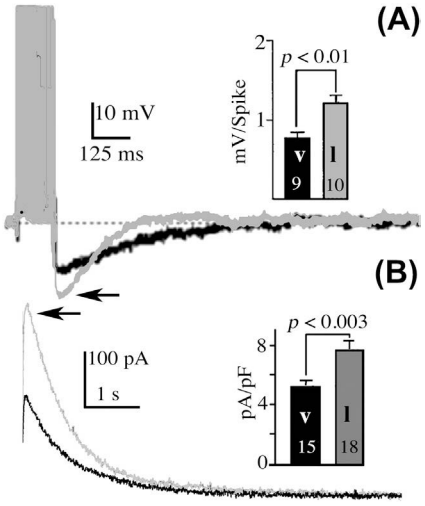

FIGURE 13.12 AHPs are larger in OT neurons during lactation. (A) Example traces from an OT neuron from a virgin (black) and a lactating (gray) rat. Note the larger AHP (arrow) in the neuron from the lactating rat versus that in a virgin rat following a spike train triggered by a 180-ms depolarization. *Inset*, Histogram showing the mean difference between lactating (L) and virgin (V) rats. Amplitude was calculated per spike because trains could have different spike numbers. *(Source: Redrawn from 301 with permission.)* (B) The current underlying the apamin-sensitive AHP in voltage clamp is enhanced in OT neurons during lactation. As with the AHP in (A), the I_{AHP} is larger (arrow) in lactating (gray trace) compared to in virgin rats (black). The I_{AHP} was evoked by a 200 ms depolarizing step to +10 mV from a holding potential of −70 mV. Traces are the averages from all neurons recorded. *Inset*, Histogram showing the mean difference between lactating (L) and virgin (V) rats. The I_{AHP} was calculated as current density to account for larger whole-cell capacitance observed during lactation in OT neurons. *Source: Redrawn from Ref. 303 with permission.*

VP neurons during lactation.[304] However, spike height and rise time are more sensitive to membrane potential in OT than in VP neurons, likely due to the fact that the fast sodium current underlying spikes in OT neurons is much less dense than that in VP neurons, probably reflecting fewer sodium channels.[305] OT spike height is thought to rapidly decrease during milk ejection–type bursts pharmacologically induced in vitro.[219]

The increase in AHPs observed by Stern and Armstrong[301] and Teruyama and Armstrong[303,304] involves two types of potentials following spike trains, one of medium duration (mAHP) (tau 100–200 m) and a slow AHP (tau 1.5–2 s) (sAHP). The mAHP is mediated by an apamin-sensitive, SK3-type potassium channel, and the current density underlying the mAHP was increased during lactation[303] (Figure 13.12). This increase was not accompanied by an increase in intracellular Ca^{++} during spike trains, or in whole cell Ca^{++} current under voltage clamp. Thus these authors could not confirm the increase in Ca^{++} current from unidentified SON neurons reported by De Kock et al.[232] during lactation. Both AHPs appear to first enlarge during late pregnancy,[304,306] suggesting that suckling is not the triggering stimulus.

The increase in AHPs in OT neurons in late pregnancy and lactation suggests that intrinsic inhibition is important to sculpting the burst. OT neurons fire during milk ejection bursts at rates not observed with other forms of activation, such as osmotic stimulation; bursts are typically ~4 s and reach rates from 50 to 80 Hz in the first second.[65] Synchronous bursting releases a bolus of OT that maximally and transiently stimulates mammary myoepithelial cells, avoiding OTR desensitization, and burst termination and the rapid reduction in firing rate needed to rapidly shut off OT release would be aided by these increased AHPs.[301,304] In addition, OT neurons undergo a post-burst inhibition[65,247,258] that is likely a combination of these enhanced AHPs and of OT's abilities, perhaps through cannabinoid release, to transiently suppress excitatory inputs.

What governs this plasticity? A popular theory is that OT neurons and the circuits controlling their activity may be programmed for adaptations needed at parturition and during lactation by the changing sex steroid milieu known to occur in late pregnancy.[307] Thus the rising levels of progesterone and estradiol (E2) during pregnancy, followed by a precipitous fall in progesterone during the last 24–48 h before birth, may trigger several brain events, presumably via central steroid receptors. In steroid replacement models in ovariectomized females, the withdrawal of progesterone in the continued presence of E2 is accompanied by increased maternal behavior,[307] and by upregulation of OTR binding[237] and in OT mRNA[213] in the SON and PVN. These indices, as well that of OTR mRNA,[241] are known to increase naturally in late pregnancy (also see Chapter 43), and increased OTR receptor binding is evident in the SON during lactation.[236] Thus even though local OT release appears to not increase over baseline until parturition[224] and then again during lactation,[223,224] the increased OTR presence could serve to enhance the effect of any constitutive OT release even before the enhanced OT release begins. The precise site of the estrogen and progesterone receptors in the brain mediating these effects is undetermined. While OT neurons have estrogen receptor ß,[308] they do not have estrogen receptor α or progesterone receptors, although these may be in regions connecting to the SON and PVN, such as the medial preoptic area,[309] BNST[310] and PNZ of the SON.[311] In addition, E2 is known to upregulate OTRs.[239,312] There is also the possibility that a novel membrane receptor for estrogen could participate in some manner in OT neuronal behavior.[313]

The steroid replacement model was used by Montagnese et al.[314] to determine the factors involved in the morphological plasticity of the OT system known to occur in lactation, well documented for over 30 years.[315,316] Changes involve hypertrophy of OT neurons, an increase in synaptic density and in the number of so-called shared synapses (terminals that contact more than one postsynaptic element), and an increase in close appositions related to the withdrawal of astrocyte processes. Much of the plasticity begins late in pregnancy, just prior to parturition. Interestingly, OT itself delivered ICV can induce this plasticity, but only in female rats in constant diestrous, or in ovariectomized animals receiving progesterone treatment followed by E2.[314] ICV OT had no effect in animals treated with progesterone or E2 alone. While the role of endogenous OT was not determined, the studies nevertheless implicated the peptide in the restructuring of its own system.

Teruyama et al.[306] investigated the role of central OT in AHP plasticity. Pregnant rats were infused ICV for about a week during late gestation starting on day 13 with the specific OT antagonist desGly-NH2-d(CH2)5[D-Tyr2, Thr4]OVT (OTA) or vehicle. In slices made from these animals sacrificed at days 19–22, the enhanced AHPs of OT neurons in vehicle-treated animals were absent in OTA-treated rats (Figure 13.13). In an in vitro model, Langle et al.[317] found that a 1-h OT (100 nM) treatment was sufficient to induce morphological plasticity when applied to slices from day 19 pregnant rats, an effect enhanced by relatively high doses of E2 (16.5 µM) and blocked by the OTA. GABA synapses were increased by this treatment, demonstrated both morphologically and electrophysiologically.[318] Armstrong et al.[319] found that using this model, OT (10 nM) and E2 (500 pM) i.e., at or near physiological doses, also enhanced sAHPs specifically in OT neurons. These studies suggest that autoregulation of OT neurons in combination with sex steroid changes plays a role in OT neuronal plasticity, beginning

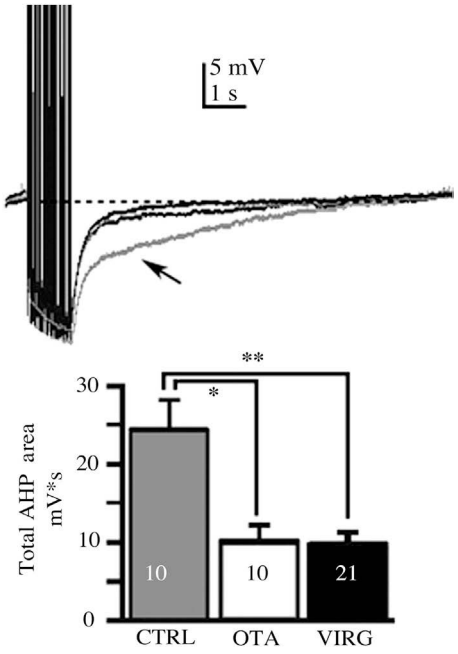

FIGURE 13.13 Enhanced AHPs during late pregnancy were blocked by intraventricular OT receptor antagonist (OTA) administration. The OTA ($d(Ch_2)_5$, $Tyr(Me)^2$, Orn^8-vasotocin) was delivered for 7–9 days at 200 ng/μl at 5 μl/h during late pregnancy via osmotic minipump. OT neurons from pregnant rats (days 19–21) treated with the OTA or artificial cerebrospinal fluid vehicle were compared with virgin rats. *Top*, Averaged traces of AHPs generated from a 20-Hz spike train for 1 s are shown. Note that OTA-treated pregnant animals had AHPs similar to virgin rats, whereas pregnant rats treated with vehicle (gray trace, arrow) were enhanced as shown previously.[304] Spike heights are clipped for convenience. *Bottom*, Histogram showing the mean difference between the three groups in the total area of the AHP. *, $p < 0.0009$; **, $p < 0.0001$. *Source: Redrawn from Ref. 306 with permission.*

in late pregnancy. Thus OT may behave in ways beyond those that underlie acute changes affecting electrophysiological or synaptic properties, actions that would be considered more akin to those exerted by growth factors.[316] This concept is not new and has been put forth to explain other long-lasting effects of OT in peripheral target organs that may involve mitogen-activated protein kinases.[320,321]

SYNAPTIC PLASTICITY DURING LACTATION

As discussed earlier, OT and VP neuronal systems undergo remarkable plasticity when challenged, with changes in the OT system appearing in late pregnancy and extending through lactation.[315,316,322] One of the first changes appreciated was the increased close appositions among magnocellular neurons and their dendrites, both in appositional length as well is in the number of elements involved, and the concomitant withdrawal of astrocyte processes that normally separate these processes. These findings, observed first with dehydrational challenge, led to speculation that appositions might underlie gap junctional or even ephaptic (i.e., electric field) communication among neurons, which might facilitate synchronization.[323] However, there has appeared no evidence for increased *electronic* communication among OT neurons in lactation, despite reported increases in dye coupling (thought to be mediated by gap junctions) among them,[315] and the presence of gap junction proteins.[324] Indeed, the evidence for electrical coupling is weak in the SON[325] and thought to be absent in synchronously bursting OT neurons in vitro.[208] Ephaptic interactions are possible but have not been reported, but could be subtle and also mediated in part by connexin hemichannels, such as occurs in retina.[326]

The change in morphology is accompanied by a significant decrease in astrocyte transmitter buffering near synapses (see following discussion), a reduction in the size and tortuosity of the extracellular space,[327] and in a small amount of extracellular buildup in potassium during synchronous bursting, the latter of which may permit weak depolarizations across immediately neighboring OT neurons.[328] Morphological changes may thus contribute to at least some of the reported physiological differences in synaptic transmission, discussed later.

Glutamate

Given the prominent role of glutamate-regulating milk ejection-related OT release, a natural question is whether glutamatergic transmission is altered during lactation. El Majdoubi et al.[329] first found that axosomatic synapses increased proportionately with somatic membrane of OT neurons, and a later study indicated an increased density of synapses in the neuropil of the SON[330]—in both studies, it is apparent that the number of shared, or double synapses, also increases. This plasticity appears to be permitted by adhesion molecules, primarily located on astrocytic membranes, like polysialated (PS) NCAM (neural cell adhesion molecule). While PS-NCAM does not increase during lactation, inhibiting its activity prevents astrocyte withdrawal and synaptic plasticity.[316]

Since the number of contacts per unit length of membrane appears to be static, it could be argued that the increase in synapse numbers simply compensates for the hypertrophy of OT neurons, an argument made for the increase in GABA synapses in this system.[331] On the other hand, the increase in shared synapses could reflect the closely timed synchronization of excitatory inputs inducing bursts.[208] Stern et al.[202] noted a doubling in the frequency of miniature EPSCs (mEPSCs) impinging on OT neurons in the SON of lactating rats, possibly reflecting this increase in synapse number or perhaps reflecting some constitutive increase in release probability. Using

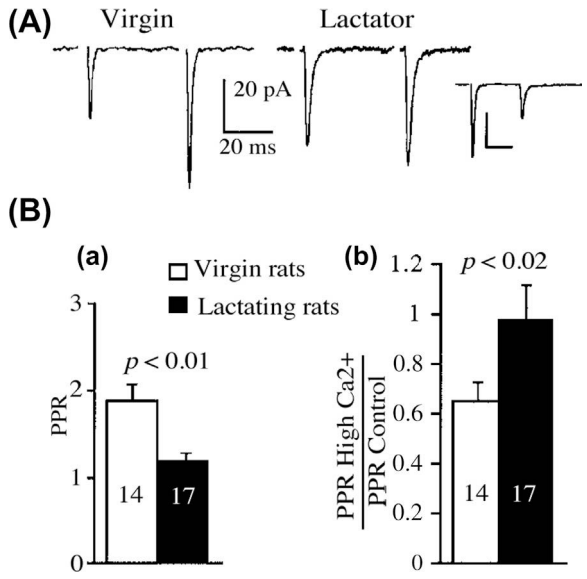

FIGURE 13.14 Paired-pulse facilitation (PPF) is decreased in OT neurons during lactation. PPF indicates an increased amount of transmitter is released to the *second* volley of action potentials produced by two closely spaced stimuli. A decrease in the ratio of the second response (PP2) to the first (PP1) is taken as an increased probability of release to the first stimulus. (A) Representative examples of averaged responses ($n = 100$) to pairs of extracellular stimuli in OT neurons in virgin and lactating rats. Note that the PPR ratio (PP2/PP1) decreases in lactation. *Inset*, In 36% of cases, a switch from PPF to paired-pulse depression was noted during lactation. (Scale: 20 pA, 20 ms). (Ba) PPF was significantly reduced in OT neurons during lactation. (Bb) The PPR in high $[Ca^{++}]_o$ compared to controls is shown for both virgin and lactating rats. Note the decreased sensitivity to increased $[Ca^{++}]_o$ in lactating rats. *Source: Redrawn from Ref. 202 with permission.*

paired-pulse stimulation of glutamatergic synapses (a standard method of evaluating synaptic potentiation or inhibition to repeated axonal stimulation), Stern et al.[202] found that glutamate release probability was likely increased during lactation, as the paired-pulse facilitation of consecutive EPSCs normally observed in virgin rats was decreased specifically in OT neurons during lactation, and a greater number of neurons actually showed paired-pulse depression (Figure 13.14). Increasing extracellular Ca^{++} in virgin rats reduces the paired-pulse ratio consistent with an increase in release probability, but this treatment has little effect in the ratio from lactating rats, whose glutamate release probability is presumably already enhanced.

These results are disparate from a similar study by Oliet and Poulain,[203] who found a *greater* paired-pulse facilitation in unidentified SON neurons during lactation, indicative of a lower probability of release. By pharmacologically manipulating extracellular glutamate uptake, this change was attributed to a reduced buffering of ambient glutamate during lactation due to astrocyte process withdrawal, elevating glutamate enough to increase presynaptic inhibition at glutamatergic terminals through group III metabotropic glutamate receptors (mGluRs). This theory is consistent with the reduced tortuosity and extracellular volume, both of which also would enhance the level of released glutamate.[327] However, the frequency of mEPSCs was not examined by Oliet and Poulain,[203] nor were neurons identified as OT or VP containing. While one might argue that an increased probability of release would maximize the initial EPSPs needed to drive burst onset,[202] in this alternative scenario of reduced glutamate release probability, the initial excitation would be filtered out, and synaptic facilitation would increase release through a spike train. The reduced glial coverage during lactation is also thought to allow potentiation of the NMDA receptor component of EPSPs from repeated EPSP volleys during lactation, through reduced astroglial release of the NMDA co-agonist D-serine,[207] although it is unknown whether this effect is specific to OT neurons. Finally, another consequence of glial withdrawal and the increased levels of ambient glutamate appears to be a switch in the activity of presynaptic glutamate kainate receptors on GABAergic terminals, which normally mediate facilitation of GABAergic transmission, but which during lactation appear to mediate inhibition specifically on OT neurons.[332] This would increase excitability of OT neurons.

Gamma Amino Butyric Acid

Like glutamatergic synapses, GABAergic synapses on OT neurons specifically have been shown to increase in number in the SON during lactation, including an increase in shared synapses.[330] As will be clear when dealing with noradrenergic synapses (discussed below), this suggests a general increase in synaptic numbers that may relate more to the hypertrophy of OT neurons than to any specific role in milk ejection bursts. Nevertheless, GABAergic synaptic transmission undergoes other significant and specific alterations during the reproductive cycle. The biggest documented change appears to be in sensitivity of $GABA_A$ receptors to the allosteric modulator, allopregnanolone, a progesterone metabolite considered a neurosteroid. In virgin animals and in late pregnancy, allopregnanolone prolongs the synaptic decay of mIPSCs on putative OT neurons in the SON (probably by increased $GABA_A$ receptor channel open time) whereas during lactation, it has little effect[267,331,333] (Figure 13.15). IPSC decays were found prolonged (without allopregnanolone) during lactation, returning to normal postlactation.[331] Interestingly, during late pregnancy and continuing through early lactation, mIPSC frequency is doubled from levels in virgins.[331] However, the prolonged increase in mIPSC frequency beginning near parturition and continuing through the first part of lactation suggests that total synaptic impact of GABA per se remains relatively constant, when considered with the hypertrophy of OT neurons.

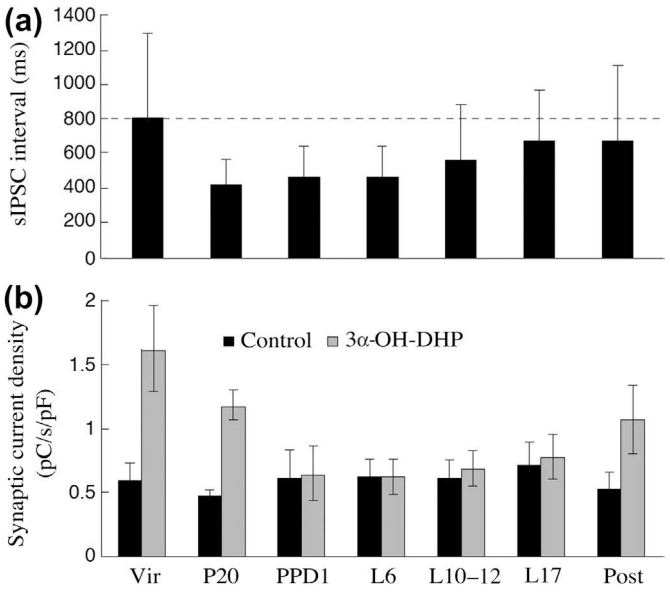

FIGURE 13.15 Histograms of spontaneous inhibitory postsynaptic current (IPSC) intervals and the relative synaptic-current densities of $GABA_A$ receptors on OT neurons during the pregnancy–lactation reproductive cycle. (A) The decreased IPSC interval during pregnancy and early lactation indicates that an increase in the number of GABAergic boutons on oxytocin neurons occurs at this time—consistent with morphological results.[330] (B) The sensitivity of IPSCs to the neurosteroid allopregnanolone (3α-OH-DHP) changes dramatically through the reproductive cycle. In order to estimate the impact of $GABA_A$ receptor-mediated synaptic transmission on OT neurons, the monoquantal IPSC amplitude, frequency, decay, and response to 3α-OH-DHP were combined to provide relative synaptic-current-density indices. This shows that the reduced sIPSC interval at the time of pregnancy has only a small impact on $GABA_A$ receptor efficacy (compare Vir and P20 in absence of 3α-OH-DHP). By contrast, the raised levels of 3α-OH-DHP enhance $GABA_A$ receptor mediated transmission substantially only in late pregnancy (P20). The switch to a 3α-OH-DHP insensitive receptor immediately prior to parturition (PPD1–L17) would provide a powerful mechanism of disinhibition that is involved in the activation of OT neurons at birth and during lactation. Abbreviations: L, lactation day; P, pregnancy day; Post, postlactation; PPD, postparturition day; Vir, virgin. *Source: Redrawn from Ref. 267,331 with permission.*

While initially these changes during lactation were thought correlated with a switch in the ratio of α_1/α_2 $GABA_A$-receptor subunits, favoring more α_2 at parturition and lactation,[267,331,333] more sensitive measures suggest that subunits do not change in amount, but there is an increased clustering of α_2 subunits at many GABA synapses.[334] As for the steroid sensitivity, it seems more likely that local OT itself alters phosphorylation of the $GABA_A$ receptor via protein kinase C inhibition, rendering the receptor steroid insensitive.[335] Thus OT neurons would be maximally inhibited by steroid in late pregnancy, perhaps to avoid premature release of OT, but with the fall of progesterone coupled with increased local OT release at parturition and into lactation, this steroid potentiation of GABAergic effects would be absent.

As a final caveat, however, Armstrong et al.[319] found that the decays of mIPSCs of *both* identified VP and OT neurons were prolonged in early lactation, so specificity of these changes to OT neurons could be questioned. Stern et al.[202] also reported prolonged mEPSCs in *both* cell types during lactation. $GABA_A$ receptors are also subject to heterosynaptic inhibition from glutamate release and the activation of mGluRs, but only during enhanced glutamate release.[327] Thus GABA transmission might be suppressed selectively during strong glutamatergic activation of OT neurons during milk ejection.

An increase in GABA synapses[317] and mIPSC frequency[318] has been induced by OT and potentiated by E2 in an in vitro model using hypothalamic slices from rats taken at day 19 of pregnancy. While these changes are mediated by the OTR, and dependent on Ca^{++} and glutamatergic transmission, they are presynaptic in origin and are not accompanied by changes in mIPSC decay. However, the precise target of OT's action is unknown—it is possible that OTR on astrocytes could promote astrocyte process withdrawal through increased Ca^{++} and consequent cytoskeletal changes, and thus "permit" synaptic increases.[258,336,337] The increased frequency of mIPSCs observed during lactation[333] and in this model is of interest since this tonic activity is much greater on OT compared to VP neurons and contributes to OT firing irregularity.[211] Since OT's ability to enhance GABAergic transmission also was found to contribute to firing regularity,[246] it is tempting to speculate that the increased frequency and increased irregularity of background OT neuron firing that precedes milk ejection bursts[247,248] may herald a dynamic change in synaptic transmission that favors burst onset. Whatever ultimately controls this change is undetermined, but clearly it must relate to continuous stimulation at the nipple provided by suckling pups.

Although its role has not yet been demonstrated in lactation, taurine is a neuroactive amino acid binding to glycine receptors (which are ligand-operated Cl^- channels, as are $GABA_A$ receptors) and is tonically released from astrocytes in the SON, providing a constitutive inhibitory tone.[338,339] Astrocytes are osmotically sensitive, and in response to hyperosmotic challenge, reduce their taurine release to lift this inhibitory tone. However, it seems likely that even without acute, osmotically mediated changes in taurine release, morphological astrocyte process withdrawal during lactation could reduce this inhibitory tone through a spatial change in the taurine concentration gradient.[339]

Noradrenaline

A quantitative microscopy study has indicated that noradrenergic innervation also contributes to synaptic plasticity, increasing specifically on OT neurons in both

the SON and PVN during lactation.[340] Although synapses were estimated from noradrenaline-positive varicosities with light microscopy, when examined these varicosities were typically associated with symmetrical synapses. Although it is tempting to conclude that noradrenergic effects are enhanced during lactation since, the increase in number of noradrenergic varicosities appears to match OT hypertrophy (much like glutamatergic and GABAergic synapses), suggesting again a compensatory innervation to match the increased postsynaptic membrane. These synaptic increases might relate to the increase in noradrenaline turnover[215] and release[130] observed during suckling, but rather than signaling a special role in lactation, a larger number of terminals might be necessary simply to equivalently impact hypertrophied OT neurons.

Conclusion of Synaptic Plasticity

Given that synaptic plasticity occurs during osmotic activation on both VP and OT neurons,[341] and can occur as fast as 30 min following a hyperosmotic stimulus,[342] increased synaptic numbers on OT neurons during lactation should not be viewed as a unique phenomenon. In this regard, while astrocyte process withdrawal facilitates synaptic proliferation, such withdrawal may be instigated by multiple factors, one of which could be OT.[317,318] Thus it seems plausible that OTR on astrocytes may mediate this withdrawal in lactation at least, although this is not certain. It is difficult to attribute the particular change in OT neuronal behavior during lactation to synaptic plasticity, either physiologically with regard to pre- and postsynaptic regulation of transmitter release, or in terms of increased synaptic numbers, although all of these could impact the burst response. It seems most likely that synaptic plasticity in lactation is geared primarily to ensure the now larger OT neurons are adequately impacted by whatever the final relevant stimuli are that drive bursts during suckling. The periodicity assumed in glutamatergic transmission to underlie OT bursts is likely a network phenomenon and may involve synaptic rearrangement at many locations aside from those in the SON and PVN. This seems likely since the *synchronous* bursting response is, as far as has been demonstrated, unique to lactation (although some evidence exists for synchronized bursts during parturition[68]), both of which involve specialized sensory inputs from structures modified by reproductive state.

Neurohypophysial Plasticity

Although the bursting pattern of OT neurons during lactation is a function of interactions within magnocellular nuclei, the terminal environment also undergoes a similar structural rearrangement.[315,316] Normally, pituicyte (modified astrocytes) processes separate neurosecretory terminals from the capillary bed and then withdraw these processes with strong activation as occurs during parturition, dehydration, and lactation. As with the SON and PVN, adhesion molecules on pituitcytes such as PS-NCAM may play a role in this plasticity.[316] It is also possible that OT, via OTRs on pituitcytes, might affect this plasticity through adenosine- and Ca^{++}-dependent changes in stellation[343] similar to that described for VP and $V1_A$ receptors.[344,345] However, to date these factors have not been specifically implicated in changes associated with lactation. Pituicyte withdrawal would give OT quicker diffusional access to fenestrated capillaries, but the limiting step of diffusion for OT's peripheral actions would be 10–20 s needed for vascular transport of the hormone to the mammary gland or uterus—much slower than any diffusion within the neurohypophysis to fenestrated capillaries. The more important consequence of these changes to OT release may relate to the access of terminals to presynaptically neuroactive compounds released from neighboring axons into the perivascular extracellular space, such as co-released dynorphin, CCK, or other peptides and neuromodulators. However, there is thus far little evidence that these factors play a physiological role in the neurohypophysis during lactation.

A MODEL FOR MILK EJECTION

Computational models have increased our understanding of neuronal activity both at the single-cell and network level. Single-neuron models have been developed for SON neurons based on many known properties, with a focus on VP neurons and their phasic bursting activity,[346,347] or on the osmotic response of VP and OT neurons.[348] Recently a very robust network model was developed that successfully produces synchronous bursting with amplitudes and periodicities similar to milk ejection bursts.[349] The model successfully reproduces the background firing of OT neurons as well, including the progressive switch to irregularity prior to bursts. The key features of the model are the priming produced by OT during its slow, progressive release between bursts (producing the long interburst intervals as well), and relatively weak dendritic bundling that allows intercommunication among OT neurons. Synaptic feedback from OT's release of cannabinoids, and AHPs, also play significant roles in shaping post-burst silent periods and the changeable background activity. However, the strong depolarization producing a burst in the model appears primarily due to OT's depolarizing effect on OT neurons and not from glutamate. There is little evidence from recording studies to support such a

powerful, direct ability of OT in this regard. Indeed the OT-induced effects on bursting in slices in vitro involve relatively weak depolarizations and are completely dependent upon glutamatergic transmission underlying the burst.[208,255] Nevertheless, this model is an excellent starting point for interactive studies between computer and experiment that should lead to future refinement and more insights.

CONCLUSION

Although it should be evident from this chapter that great advances have been made in our understanding of the CNS control of milk ejection, there are major gaps in our knowledge. Firstly, at least three neurotransmitters/neuromodulators are deemed essential to the reflex, presumably from their actions within the hypothalamus, if not directly upon OT neurons themselves, but how these interact to influence OT bursting remains a mystery. For example, there is a great deal of evidence to suggest that the suckling stimulus works through noradrenergic afferents—but this system is absent in the only in vitro model that successfully reproduces the synchronous, periodic OT bursting resembling that of the milk ejection reflex. On the other hand, this model may still be a window into the mechanisms of the oxytocin pulse generator, a presumed circuit of neurons that would normally be activated selectively by suckling, but which, in the absence of many normal synaptic inputs, operates due to an increase in excitatory connections that are either aberrant in number or simply lack the normal gating mechanisms that come with connectivity in the adult rat. While these inputs are clearly modulated by somatodendritically released OT, another "essential" neuromodulator for the reflex, we do not understand yet how OT induces, or permits, bursting, despite knowing many of the things that OT does both directly to OT neurons as well as to synaptic inputs.

Related to the importance of neuroactive substances are the locations of critical CNS regions transmitting suckling afferents. While there is general agreement that suckling is carried ipsilaterally in the lateral columns in the spinal cord, and that the pathway crosses to arrive contralaterally in the hypothalamus, the precise players are still not determined. While there are many ascending bilateral inputs to the PVN and SON from the brainstem, most are dominated by ipsilateral connections, suggesting that this crossing likely occurs in the lower medulla or rostral spinal cord. Experiments are still needed to determine, for example, which brainstem noradrenergic populations are *critical* to transferring this information to the SON and PVN, and not simply activated by the stimulus. For example, experiments like those performed in the midbrain lateral tegmentum, showing that neurotoxic lesions sparing fibers of passage reproduced the effects of knife cuts or electrolytic lesions blocking milk ejection, could illuminate the critical role of certain noradrenergic or nearby brainstem cell groups, when comparing the toxins specific to catecholaminergic transmission, for example, with those of ibotenic acid.

Within the hypothalamus, these signals are finally shared bilaterally, but the mechanism of transfer is unknown. The fact that OT injected into the SON recruits contralateral SON OT neurons into the reflex makes it unlikely that bilateral brainstem inputs are critical to bilateral SON and PVN activation. It seems more likely that somatodendritically released OT has access to the hypothalamic pulse generator, which likely connects bilaterally with the SON and PVN. Direct connections among the four SON and PVN nuclei cannot be ruled out, but are not yet well documented. The pulse generator may not be a single entity but may include different neuronal groups that provide interhypothalamic connections through both supra- and subventricular pathways. While some candidates are emerging, including the DMH and adjacent lateral hypothalamus, the PNZ or dorsal chiasmatic area of the SON, much work lies ahead to determine if these regions are essential for OT bursting, or its synchronicity, during the milk ejection reflex.

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CHAPTER

14

Testicular Development

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INTRODUCTION

In 1947 Alfred Jost reported that castration of fetal rabbits prevents masculinization of the fetus thereby demonstrating that the fetal testis determines the phenotypic sex of the individual.[1] Jost later went on to show that the factors involved in this process are testosterone and anti-Mullerian hormone (AMH) secreted by the somatic cells of the developing testis.[2] This chapter describes development of the testis during this period after initial differentiation when it acts to alter the phenotype of the fetus and up until early puberty when spermatogenesis is beginning. This time span covers most of the critical stages of testis growth and functional differentiation with only the completion of meiosis and the process of spermiogenesis occurring later. The major developmental landmarks and circulating hormone levels during testicular development in the mouse (as a representative model) are shown in Figure 14.1. Overall, the emphasis of this chapter will be on the functional differentiation of the somatic cells and how they affect germ cell development.

In most eutherian mammals the gonads differentiate and start to develop during the first quarter of gestation. For example, in the human (gestation 40 weeks) the gonads develop at about six weeks while in the sheep (gestation 145 days) development is around 30–35 days. In rodents, testis development is a little later at just after half way through pregnancy, starting at 11.5 days postcoitum (dpc) in the mouse and 12.5 in the rat.[4] Initially, differentiating pre-Sertoli cells migrate into the developing gonad from the coelomic epithelium where they aggregate with primordial germ cells that have traveled from the yolk sac (see Chapter 7). Interactions between endothelial cells migrating from the mesonephros and interstitial cells lead to the formation of sex cords from the Sertoli/germ cell aggregates, and these cords will go on to form the seminiferous tubules.[5–7] Around 13.0 dpc in the mouse the peritubular myoid cells start to differentiate from interstitial cells, and they go on to form a cellular layer surrounding the developing tubules.[6] At around the same time another major somatic cell type, the Leydig cell, starts to differentiate within the interstitial spaces from cells that originated in either the coelomic epithelium or the mesonephric-gonadal border.[8] This differentiation phase is fairly rapid in the male, and the essential structure of the testis is in place by eight weeks in the human, 35 dpc in the sheep, and about 14 dpc in the mouse. As the testes differentiate morphologically, they start to masculinize the fetus in the process described by Jost through secretion of androgen from the Leydig cells and AMH from the Sertoli cells.

Following initial differentiation, a number of landmark events occur in the testis prior to puberty (Figure 14.1). Fetal testis development is characterized largely by Sertoli cell proliferation and androgen production by the fetal Leydig cells, both of which are essential for masculinization and subsequent fertility. Later in fetal or in early neonatal life, the primordial germ cells migrate to the basement membrane of the seminiferous tubules and give rise to the spermatogonial stem cells (SSC) from which full spermatogenesis will develop after puberty. Shortly after migration of the primordial germ cells, at about postnatal day 7 in the mouse, a second, adult population of Leydig cells develops, which goes on to secrete the high postpubertal levels of testosterone required for fertility and normal male behavior. Finally, in this period, the Sertoli cells stop proliferating as puberty starts and undergo a maturation process that forms the blood–testis barrier and allows spermatogenesis to progress. These events are considered in more detail in the following discussion.

ANATOMICAL, HISTOLOGICAL, AND MORPHOMETRIC DEVELOPMENT

As the testis develops, the sex cords form a flattened, interconnected plexus in the region of the testis next to the mesonephros; this develops into the rete testis. At the

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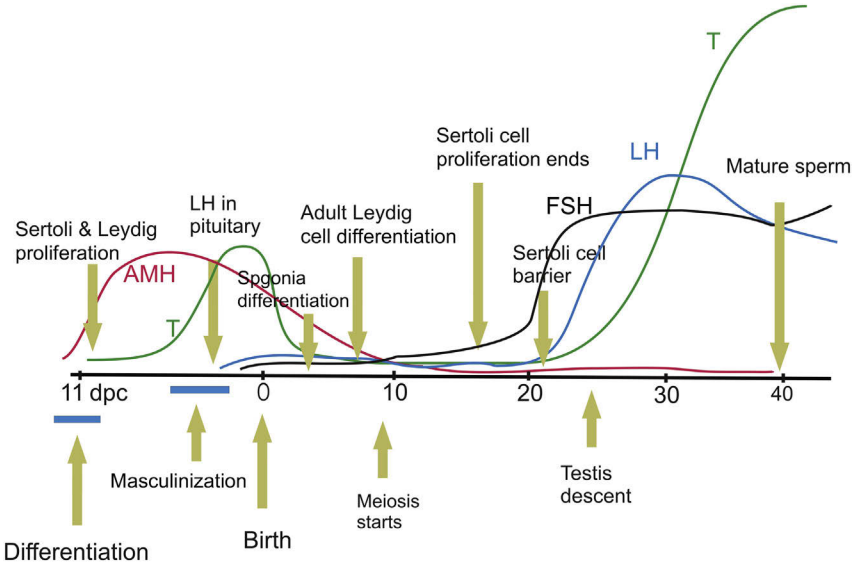

FIGURE 14.1 Major developmental landmarks and relative circulating hormone levels in the mouse during fetal, neonatal, and pubertal growth. This schematic shows the development of the mouse testis from initial differentiation around 11 dpc until puberty. Major changes in the testis (or relevant developmental events in the whole animal) are indicated by arrows. Relative levels of AMH, FSH, LH, and testosterone (T) are shown. *Source: Modified from Ref. 3.*

same time, cords developing away from this region form looped structures originating and returning to the developing rete testis.[9] The 3D structure of these cords is quite complex, with a central group of cords surrounded by an outer group of parallel cords, and fusion and branching occurring throughout (Figure 14.2). In the mouse the total number of these cords is between about 12 and 18.[9] It is the expansion and elongation of these structures, firstly through Sertoli cell proliferation and then as spermatogenesis begins, that causes growth of the testis through fetal and postnatal development. Interestingly, the asymmetric nature of cord development to form the rete testis and precursors to the seminiferous tubules appears to be dependent on G protein-coupled receptor 56 (GPR56), which is present on the Sertoli cells, although the ligands that activate this receptor in the testis remain unknown.[10]

Histological section of the fetal testis shows the presence of the developing seminiferous tubules (Figure 14.3), with the Sertoli cells forming an epithelial lining and the large primordial germ cells lying within the lumen of the tubules. Around the tubules there is a single (rodents, pigs) or multiple (human) layer of peritubular myoid cells lying in contact with the basement membrane that supports the Sertoli cells. The interstitial tissue between the tubules contains the fetal population of Leydig cells, mesenchymal cells, macrophages, and fibroblasts. Also present within the interstitium are blood and lymphatic vessels and nerves. One of the characteristics of the developing testis is the formation of the coelomic blood vessel, which is generated by migrating endothelial cells coming from the mesonephros.[12,13] Branches of this vessel track through the interstitial tissue lying in the space between the developing testis cords. In the ovary, development of this artery is inhibited by Wingless-related MMTV integration site 4 (WNT4).[14,15] Arrangement of the lymphatic vessels in the testis varies between species; in rodents there are large lymphatic spaces present within the interstitium, while in other species the lymphatic vessels are more discreet,[16–18] and in humans they are apparently restricted to the septa dividing the testicular lobes.[19] Testicular nerve types and the structures innervated vary somewhat between species and their function within the testis remains uncertain, although there is evidence that they may regulate testicular blood flow and affect Leydig cell function.[20–22] A detailed and comprehensive review of testicular anatomy can be found in a previous edition of this book.[23]

Morphometric Development

Sertoli Cells

In mammalian species for which we have reliable morphometric data (mouse, human, rat, sheep, monkey), Sertoli cell number increases exponentially during at least part of fetal development. This is followed by a further postnatal increase in numbers, which is either continuous with fetal development or follows on after a period of quiescence.[24] In rodents, Sertoli cell number increases continuously after initial testis differentiation, until early puberty at about day 15 in rats and mice with no further change thereafter.[25–28] Final Sertoli cell numbers in rodents are about 3×10^6 in mice and 38×10^6 in rats. In the ram there is a similar pattern of development with Sertoli cell proliferation extending for between 6 and 12 weeks after birth,[29–31] while in the boar it extends up to 17 weeks[32] and in the bull until about 20 weeks.[33] In these species the germ cells enter meiosis at around the same time as Sertoli cell proliferation ceases, the likely connection being formation of the blood–testis barrier. In rhesus monkeys there is a short proliferative phase after birth, which may or may not be

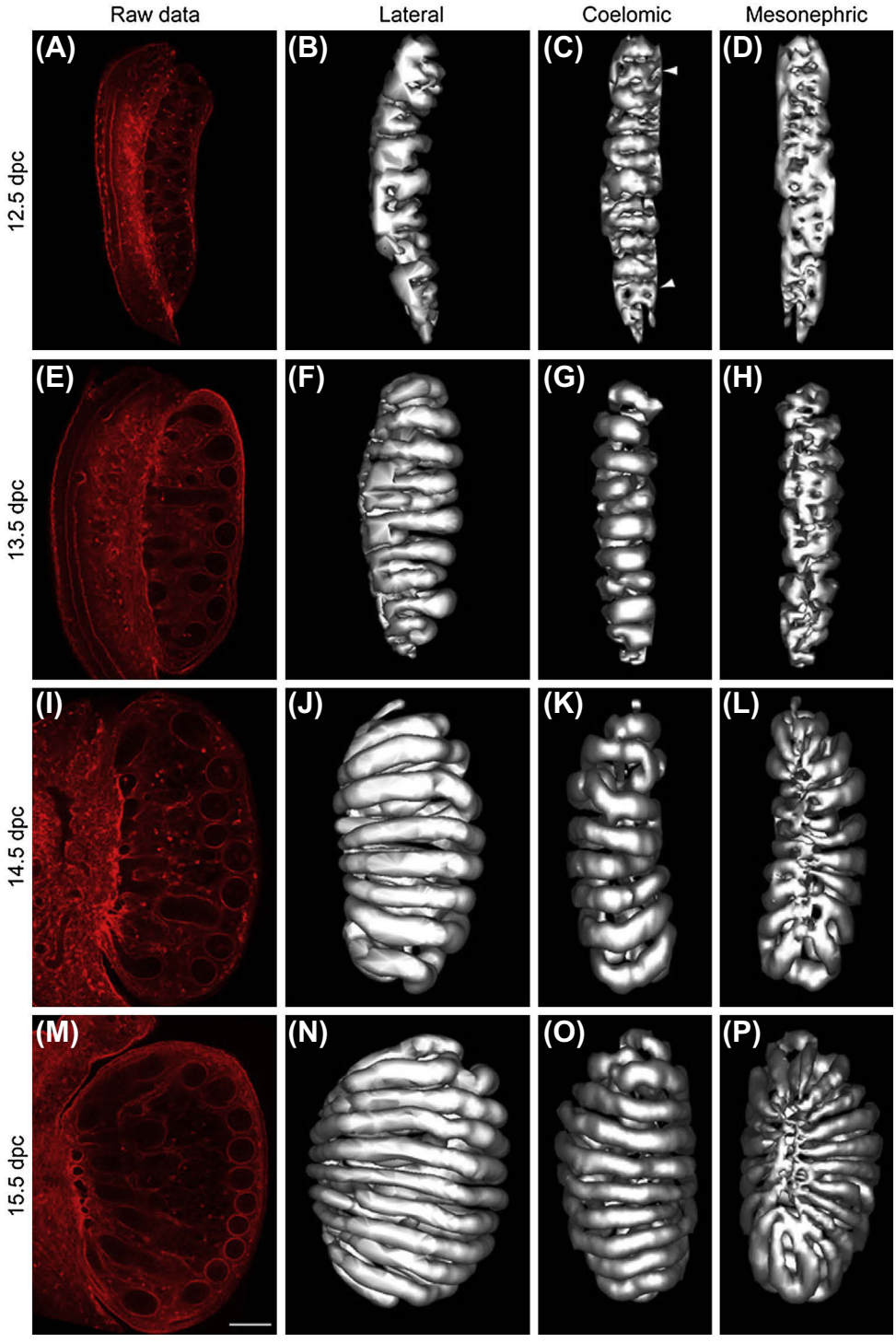

FIGURE 14.2 Modeling of testicular cord development in the mouse. This figure is reproduced in color in the color plate section. This figure is taken from Ref. 9 and shows lateral (B, F, J, N), coelomic (C, G, K, O), and mesonephric (D, H, L, P) views of models generated from testis cord outlines prepared from whole mount testis. The left panel in each row (A, E, I, M) is a representative image from the data set used to compile the models. The results show that cords initially form as a network of irregular cell clusters that are subsequently remodeled to form regular parallel loops, joined by a flattened plexus at the mesonephric side. Branched, fused, and internalized cords are commonly observed. Parts A–D, E–H, I–L and M–P show models of testicular development at 12.5, 13.5, 14.5 and 15.5 dpc, respectively. Scale bar, 100 μm. *Source: Modified from Ref. 9.*

continuous with the fetal phase of development.[34] This is followed by a period of relative quiescence when Sertoli cell number only increases gradually in the prepubertal period.[35] As the animal enters puberty, however, there is renewed proliferation of the cells and a marked increase in Sertoli cell number.[34,36] Data for the human would suggest that Sertoli cell development follows a similar pattern to the rhesus monkey, although the pubertal increase may be less marked. Fetal Sertoli cell number in the human doubles about every two weeks from week 7 of gestation to week 19.[37,38] Thereafter, the rate of proliferation drops, and Sertoli cell number doubles three more times in the remaining 19 weeks of pregnancy to reach about 130×10^6 cells/testis at birth.[37,39] After birth there is a further six-fold increase in cell number that peaks at three months,[39] remains

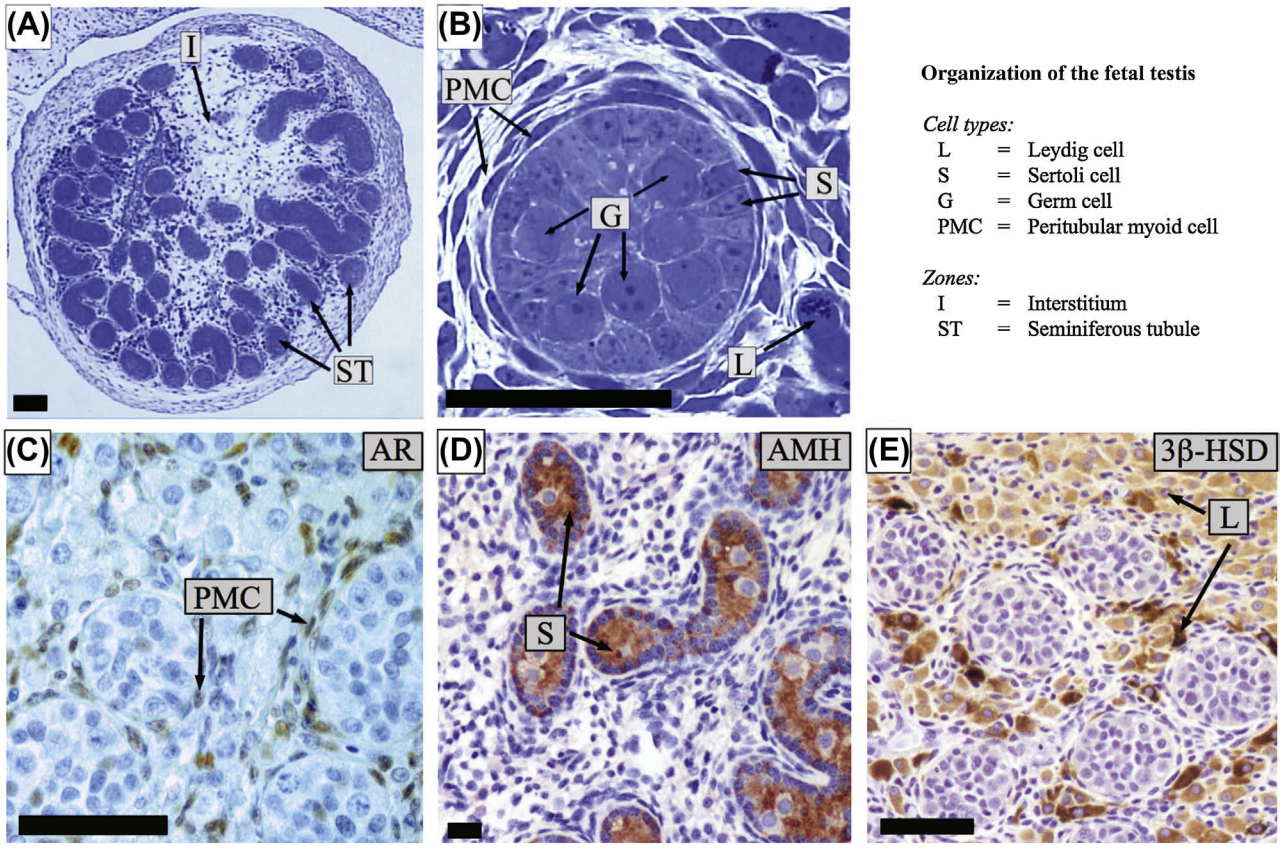

FIGURE 14.3 Histology of the fetal testis. This figure is reproduced in color in the color plate section. (A) Fetal mouse: low magnification of a semithin section showing seminiferous tubules and interstitial tissue (I). (B) Fetal mouse: higher magnification of (A) showing gonocytes (G) in the central part of the sex cord with the Sertoli cells (S) around the periphery. The peritubular myoid cells (PMC) form a concentric layer around the cord, and Leydig cells (L) are present within the interstitium. (C) Fetal human: immunohistochemically labeled for the androgen receptor (AR), which is clearly expressed in PMC and in some interstitial cells. (D) Fetal sheep: immunohistochemically labeled for anti-Mullerian hormone (AMH), which is strongly expressed in fetal Sertoli cells. (E) Fetal human: immunohistochemically labeled for 3β-hydroxysteroid dehydrogenase (HSD3B), which is localized exclusively in the Leydig cells. In all photomicrographs, the bar represents 50 μm. *Source: Reproduced from Ref. 11. © Society for Reproduction and Fertility (2011). Reproduced by permission.*

stable until about 10 years,[39,40] and then doubles again at puberty to reach a final number of about 1800×10^6 cells/testis. In general the rate of Sertoli cell apoptosis is low during the developmental period.[41,42] Thus the increase in cell numbers leading up to puberty will be due largely to changes in cell proliferation rates rather than a change in the balance between proliferation and apoptosis. Interestingly, humans with *ATRX* (alpha-thalassemia, mental retardation, X-linked) syndrome have small testes,[43,44] which appear to be due largely to increased apoptosis in the fetal Sertoli cells,[45] indicating that ATRX is required for Sertoli cell survival. Mutation in the microtubule-associated protein, sperm associated antigen 5 (SPAG5), has also been shown to increase Sertoli cell apoptosis due to disorganization of the spindle apparatus.[46] Control of Sertoli cell proliferation is discussed in the following section.

An interesting question that has recently been raised is whether the adult Sertoli cell represents a terminally differentiated cell (i.e., does not divide, shows no changes in phenotype, and performs a specific function) or an arrested proliferating cell.[47,48] Generally, the Sertoli cell is referred to as terminally differentiated, but data from seasonal breeders show that adult Sertoli cells will undergo proliferative activity in these species.[49,50] In other species, where there is no clear seasonal pattern to spermatogenesis, the blood–testis barrier and presence of tight junctions after maturation may prevent Sertoli cells from reentering the cell cycle,[48] and a balance may exist between cell cycle inhibitors and inducers that in the normal in vivo situation keeps the cells quiescent. If the cell–cell interactions are broken, however, by isolating the cells and placing them in vitro they will show proliferative activity.[48]

Whether or not the adult Sertoli cell is terminally differentiated, in most species postnatal development of Sertoli cells is limited, and the number of Sertoli cells in the adult testis will be critically dependent upon numbers that develop during fetal development. The number of adult Sertoli cells, in turn, will determine adult testis

size and sperm production since each Sertoli cell will only support a limited number of germ cells.[51]

Leydig Cells

There is strong evidence that two distinct populations of Leydig cells arise during development in rodents. The first or "fetal" population of cells develops soon after Sertoli cell differentiation and is present throughout fetal development and into the neonate. The second or "adult" population starts to develop in late neonatal life (about day 7 in mice[52,53]) and persists through the remaining lifespan of the animal. The evidence that these are distinct populations in rodents depends primarily on morphological and functional differences between the cells and the presence of adult Leydig cell stem cells in the adult animals (see the section The Leydig Cell below). While this is largely convincing, until the fate of the fetal Leydig cells can be established through specific labeling of the cells, it remains possible that fetal and adult cells represent different phases of the same population. The presence of distinct Leydig cell populations in other species is largely inferred by reference to the rodent models, but it is certainly the case that two distinct periods of Leydig cell activity, in fetal and adult life, can be seen in other mammals, which would be consistent with this model. There is additional morphological evidence in humans, such as the presence of Reinke crystals, to suggest that adult and fetal cells represent different populations.[54–56] In humans, other primates, and the boar it has also been postulated that there is a third population of Leydig cells that arises during neonatal life,[57–59] but it is not clear whether these are a distinct population or fetal Leydig cells reactivated by a postnatal rise in luteinizing hormone (LH).[60]

In rodents, fetal Leydig cell number increases rapidly after initial differentiation and is then maintained, relatively unchanged, at about 2.5×10^4 in mice and 5.5×10^4 in rats through fetal and neonatal life.[25,26,61] Between postnatal days 10 and 20 in the mouse, there is a marked increase in Leydig cell numbers as the adult Leydig cells differentiate and adult levels (1.5×10^6 in mice and 20×10^6 in rats[25,26,61]) are reached by about day 30. In humans, Leydig cells are first detectable at week 8,[62] and numbers increase exponentially during the first half of the second trimester reaching a maximum number of about 2×10^6 around 18 weeks.[37] Thereafter, Leydig cell numbers decline in the human fetus through a process of dedifferentiation or degeneration.[55,63–66] After birth, Leydig cell numbers increase again in the human at three months (caused by an increase in LH levels due, probably, to altered steroidal feedback at the hypothalamic–pituitary axis after birth) followed by another decline after four months.[65] The cells become very scarce until six to eight years of age when they begin to increase to adult levels, reported as ~800×10^6.[67] Other, nonhuman, primates are reported to show the same fetal, neonatal, and pubertal peaks of testosterone and changes in Leydig cell number during development.[68–75] Leydig cell development in the boar also appears to go through three stages of development (fetal, neonatal, and pubertal),[32,58,59] but evidence from other species is generally lacking. In sheep there is an exponential rise in Leydig cell number from days 40–60 of gestation, and numbers then remain relatively stable until birth.[29] Leydig cell numbers in the neonatal period have not been measured in sheep, but there is no evidence for a neonatal peak in testosterone levels.[76]

Peritubular Myoid Cells

Currently we know little about development of the peritubular myoid cells, although evidence is starting to accumulate suggesting that these cells may play a significant role in normal testis development and function.[77,78] The peritubular myoid cells form shortly after testis differentiation in the mouse (at about 13.5 dpc[79]) and a little later in the human between weeks 9 and 12 pc.[80] There is evidence to suggest that formation of the peritubular myoid cells is dependent on Desert hedgehog (DHH) signaling.[81] In rodents there is normally a single layer of peritubular myoid cells surrounding the seminiferous tubules, although in the human several layers exist.[82] The peritubular myoid cells lie between two layers of basement membrane in rodents,[83] with the Sertoli cells on one side and lymphatic endothelial cells on the other.[16] Cell numbers have been measured in the mouse; they are reported to increase markedly from about postnatal day 18, reaching a maximum number at the end of puberty at about day 30.[26] Whether this increase in cell number is simply a response to expansion of the seminiferous tubules as spermatogenesis develops or whether it plays a more functional role in tubule and testicular development remains to be determined.

Testicular Macrophages

Macrophages are first seen in the testicular interstitium around birth in rodents,[84] and numbers increase slowly until the start of puberty when they show a rapid increase that then declines again after puberty.[26] Interestingly, testicular macrophages are reported to form digitations with Leydig cells in which long, microvillus-like processes from the Leydig cells are inserted within coated pits of the macrophage.[85] These digitations initially form during puberty,[86] at the time when macrophage number is showing a marked increase.

Germ Cells

The primordial germ cells are formed in the caudal yolk sac and migrate to the developing gonad where their fate is determined by the gonadal environment. Primordial germ cells that migrate to an ovary will differentiate into primary oocytes, and in rodents most of them proceed to the diplotene stage of meiosis by around the time

of birth.[87] In contrast, primordial germ cells that migrate to a developing testis do not enter meiosis until after birth.[88] Since germ cells that migrate to non-gonadal tissues will enter meiosis,[89] it has been proposed that the testis produces a meiosis inhibitory factor,[90,91] although it is only in recent years that mechanisms regulating entry into meiosis have started to become clearer. In 2006 two groups reported that retinoic acid, coming from the mesonephros, acts as a meiosis-inducing substance in female gonads but is prevented from acting in the developing testis through degradation by Cytochrome P450 26b1 (CYP26B1) in Sertoli cells.[92,93] CYP26B1 certainly appears to be involved in this process as germ cells will enter meiosis in CYP26B1-deficient mouse testes,[92,94] but the role of retinoic acid (RA) has been challenged.[95,96] A number of other factors have now also been reported to affect germ cell differentiation.[97–102] It has been suggested that germ cell fate in the male is affected through both inhibition of RA signaling and by factors produced by the Sertoli cells.[103]

In humans, germ cell number shows an exponential increase during fetal development from the initiation of testis differentiation until the end of the second trimester.[37,38,104] The rate of increase in germ cell numbers is greatest from 6 until 10 weeks and is then reduced until the end of the second trimester.[105] The reason for the reduced rate of proliferation after 10 weeks is not clear but may be related to the number of Sertoli cells. Proliferation of the Sertoli cells is slower than the germ cells during the second trimester which is seen as a doubling in the germ cell/Sertoli cell ratio from the first to the second trimester.[37] If germ cell number depends on Sertoli cell number at this stage of testis development, as it does in the adult,[51] then germ cells will be limited by Sertoli cell numbers. The undifferentiated fetal germ cells (gonocytes) undergo development in the fetal and neonatal stages toward formation of the SSC and spermatogonia[106–109] in a process that appears to be similar between humans and marmosets.[110] This process is gradual and asynchronous in primates, which means that while spermatogonia form in fetal life, undifferentiated germ cells at different stages of development will still be present through fetal and neonatal development. Studies in the monkey show that there is a marked increase in undifferentiated spermatogonia (presumably including SSC) between birth and puberty, perhaps as a result of an increasing number of Sertoli cell niches.[36] Germ cell numbers in the sheep increase exponentially during fetal development in a process that lasts through most of gestation.[29] In rodents there is marked proliferation of germ cells in the period immediately after testis differentiation, and then proliferation declines sharply by 15 dpc.[111] Germ cell numbers then remain stable through gestation before proliferation starts again after birth as the gonocytes differentiate into spermatogonia.[25]

CONTROL OF TESTIS DEVELOPMENT

The rodent has been the mainstay of research into testicular development, and use of mutant and transgenic mice, in particular, has led to enormous advances in our understanding of the events and mechanisms involved. In terms of the endocrine regulation of fetal testicular development, however, current evidence suggests that the rodent may have limitations as a model for other mammalian species.[11] In the postnatal testis it is well established that LH from the pituitary regulates Leydig cell function, while pituitary follicle-stimulating hormone (FSH) and androgen from the Leydig cells control Sertoli cell activity (see below). During fetal life, however, testicular development and function in rodents does not require pituitary gonadotropins.[112–114] In contrast, in most other species testicular development is dependent upon pituitary hormone stimulation during at least part of fetal life while some, such as the human, have evolved an additional placental LH-like hormone (chorionic gonadotropin; CG), which regulates Leydig cell function early in development.[11] The rodent will, however, fit the general pattern if it is accepted that testicular development in the mouse is a compressed version of that seen in many other species and that it is born "early" in terms of testicular development[112] (Figure 14.4). The description of testicular control that follows rests very heavily on the mouse because of the unprecedented power of the genetic models available, but caution needs to be maintained when extrapolating to other species because of this compressed nature of development, particularly with respect to the endocrine control of development.

The Sertoli Cell

Hormonal Control of Cell Proliferation

Data from rats and mice indicate that proliferation of Sertoli cells during fetal and neonatal development is regulated largely by androgens. For example, studies using the androgen receptor (AR) deficient *Tfm* mouse and the ARKO mouse show that Sertoli cell number at birth is significantly reduced in the absence of androgen action.[27,113,115] Similarly, induced reduction of androgen levels in fetal or neonatal rats significantly reduces Sertoli cells number.[116–118] Interestingly, studies on the Sertoli cell-specific ARKO (SCARKO) mouse show that these effects of androgen are not mediated directly through the Sertoli cells.[27,115,119] This is consistent with AR expression in the rodent during fetal development, which is limited to interstitial cells and peritubular myoid cells, indicating that androgen action on the Sertoli cells is likely to be mediated through these cells in the fetus.[120,121] The role that FSH plays in determining Sertoli cell number during fetal development is less certain. Early studies reported

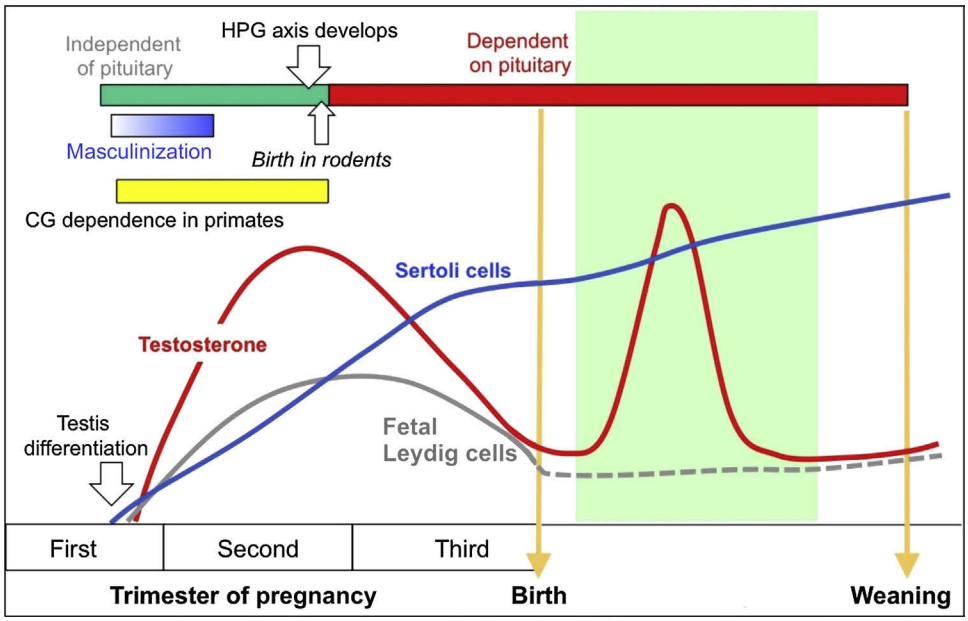

FIGURE 14.4 Schematic representation of the regulation of testicular function during fetal and perinatal development in the mammal. This is a composite diagram of data from several species. It is not meant to represent any single species but is a generalized view of how testicular control develops. Changes in Sertoli cell and fetal Leydig cell numbers and testosterone levels are shown, and dependence on pituitary gonadotropins is indicated along the top. After birth there is a rise in testosterone levels in some species (e.g., primates) caused probably by altered steroid feedback at the hypothalamic-pituitary level. The fate of the fetal Leydig cells after birth is largely unknown, and this is indicated by a dotted line. The arrow indicating birth in rodents suggests that these species follow the same general developmental plan but are born "early" (see text). Similarly, primates follow the same general plan as other species but have additional control from chorionic gonadotropin. *Source: Adapted from Ref. 11. © Society for Reproduction and Fertility (2011). Reproduced by permission.*

that Sertoli cell proliferation in the late fetal rat is regulated by FSH,[122] but subsequent morphometric analysis of Sertoli cell numbers in newborn mice lacking FSH or the FSH receptor (FSHR) failed to see any change in Sertoli cell number.[25,113] Subsequently, an effect of FSHR ablation on fetal Sertoli cell development was shown, but this was only evident when exacerbated by loss of the AR.[27] This led to the view that FSH is relatively unimportant in regulating fetal Sertoli cell numbers in rodents, but the underlying data has recently been challenged[123] and further studies will be required before a consensus is reached. Nothwithstanding this controversy, studies using ARKO, FSHRKO, and compound ARKO.FSHRKO mice indicate that after birth and up to early puberty Sertoli cell number is regulated largely by androgens and hormone-independent mechanisms, with an uncertain contribution coming from FSH.[27] Sertoli cells normally stop proliferating at early puberty in rodents, and numbers remain static thereafter, but in mice lacking FSHR there is a postpubertal decline in Sertoli cell numbers.[27,113] This indicates that FSH is essential for maintaining Sertoli cell numbers postpubertally in mice, either through prevention of apoptosis or through maintenance of cell attachment to the basement membrane.[124] In rats, there is more evidence that FSH may play an important role postnatally in regulating Sertoli cell number. Neonatal treatment of intact rats with recombinant FSH, for example, will increase adult Sertoli cell numbers,[125] while neonatal suppression of FSH by passive immunization will decrease Sertoli cell number without affecting circulating androgen levels.[126]

The relative roles that FSH and androgen play in determining Sertoli cell numbers in other species is more difficult to determine as many of the treatments that affect one hormone (e.g., long-acting gonadotropin releasing hormone agonist [GnRHa] treatment) will also affect the other hormone, and specific genetic models are generally lacking. In sheep and pigs, for example, fetal hypophysectomy, fetal decapitation, or fetal GnRHa treatment[29,58,127–131] will decrease testicular weight in late fetal life or at birth[127,129] with a reduction in Sertoli cell number.[11,29,131] In anencephalic fetal humans, the testes are generally smaller, which is an indication that Sertoli cell number is probably reduced.[132–135] Similarly, treatment of fetal cynomolgus monkeys with GnRHa reduces testis size at birth,[136] indicating that gonadotropins are required for Sertoli cell proliferation in fetal life. AR is not expressed in the Sertoli cells in fetal primates/humans,[137,138] although, as we have seen in the mouse, androgens may still be essential for Sertoli cell development. Generally the data indicate that in all species, apart from rodents, pituitary gonadotropins are essential for fetal Sertoli cell development. It remains uncertain, however, whether FSH or (LH dependent) androgens

are of greater importance during this period. Postnatally, the increase in Sertoli cell number in primates and the subsequent quiescent period correlate with levels of circulating gonadotropins suggesting an obligatory role in Sertoli cell proliferation.[139] Certainly, in the juvenile period of primate development current evidence suggests that both FSH and LH/testosterone will stimulate Sertoli cell proliferation leading up to puberty.[140–142] Evidence from adult humans lacking FSH indicates that there is a reduction in Sertoli cell number[143] in these individuals, though, again, changes in androgen levels may contribute to any reduction in Sertoli cell number.

It is now also clear that thyroid hormone (TH) is involved in the establishment of final Sertoli cell numbers. Sertoli cells express TH receptors,[144–147] and it has been shown that transient neonatal/prepubertal hypothyroidism delays Sertoli cell final maturation, prolongs the period of Sertoli cell proliferation, and leads to an increase in the final Sertoli cell number in the adult testis.[145,148–150] High circulating TH during the same period has an opposite effect, leading to premature Sertoli cell maturation, an end to Sertoli cell proliferation and smaller adult testes.[151,152] The effects of TH on the Sertoli cells are through the α1 isoform of the receptor and appear to be mediated through regulation of the Cyclin-dependent kinase 4/Jun D proto-oncogene/c-myc pathway.[153] Other effects of TH occur through an increase in the levels of connexion 43 (Cx43), the major testicular gap junction protein and a powerful regulator of Sertoli cell maturation.[154,155] Effects of TH on Sertoli cell proliferation have also been seen in other species,[156–158] and in the prepubertal human thyroid failure is associated with testicular enlargement.[159,160] It is clear therefore that TH, and triiodothyronine in particular, acts to regulate Sertoli cell development, although it could be argued that this is a general permissive effect of the hormone—the difference with gonadotropins, for example, being that lack of LH and/or FSH has specific effects on gonadal development while lack of TH affects multiple body systems.

Finally, there are data to suggest that locally produced activins may regulate Sertoli cell proliferation in vitro,[161–163] while local knockout of testicular activin A, or downstream components of the transforming growth factor (TGFβ) signaling pathway, reduces Sertoli cell proliferation.[164]

Sertoli Cell Function during Development

REGULATION OF TESTIS DEVELOPMENT

The question of what Sertoli cells do during testis development is particularly interesting and one to which we do not yet have a clear and complete answer. Initial testis differentiation is a story of Sertoli cell differentiation as described in Chapter 7, and this depends on sex determining region Y (SRY), SRY-box 9 (SOX9), and nuclear receptor subfamily 5A1 (NR5A1).[165,166] Since the Sertoli cell is the only cell type that requires the male-specific sex-determining factor SRY,[167] it is generally assumed that the Sertoli cell then orchestrates subsequent testicular development. For some early events such as sex cord development this appears self-evident, and there is good evidence to suggest that the Sertoli cells are involved in fetal Leydig cell and peritubular myoid cell recruitment through secretion of DHH.[81,168–171] This is unlikely to represent the whole story, however, since it has been shown that the Aristaless related homeobox gene (ARX) is required for fetal Leydig cell differentiation[172] and ARX expression is limited to peritubular myoid cells, endothelial cells, and fibroblast-like cells in the fetal testis. Sertoli cells may also act to ensure that germ cells in the testis follow a male pattern of development and do not enter into meiosis through expression of CYP26B1,[173] although this enzyme is also expressed in the fetal peritubular myoid cells[174,175] (see below).

The role that the Sertoli cell population plays in stimulating the major landmarks of testis development following differentiation, and its importance in maintaining testicular structure up until puberty, is unclear. Evidence for a continued orchestrating role for Sertoli cells beyond initial testis development is patchy, and other somatic cell types of the testis may play important functions in regulating development. For example, it is likely (though it has not been shown) that the Sertoli cells are essential for differentiation of the spermatogonial stem cells, through formation of Sertoli cell niches (see below), and it is clear that they regulate and maintain subsequent spermatogenesis. It remains uncertain, however, whether the Sertoli cells are essential for the activity of the fetal Leydig cells during the pituitary-independent phase of development, whether they trigger development of the adult Leydig cell population, whether they play any role in maintenance and function of the mature Leydig cells, or, indeed, whether they act to limit Leydig cell precursor differentiation.

Recent studies of gonadal differentiation/identity have highlighted the interrelationship between the Sertoli cell and the ovarian granulosa cell and have provided some further clues to the role of the Sertoli cell in testis development. It has been shown that deletion of a single factor, Forkhead box L2 (FOXL2), leads to reprogramming of granulosa cells into Sertoli-like cells,[176] even in adult animals, with some evidence that the reverse reprogramming can also occur following deletion of Doublesex and mab-3-related transcription factor 1 (DMRT1) in Sertoli cells.[177] Interestingly, reprogramming of granulosa cells into Sertoli cells in the adult animal is also associated with an apparent transformation of the theca cells to Leydig cells. This transformation may be a direct consequence of FOXL2 depletion in the theca cells but may also be a consequence of Sertoli cell

differentiation, suggesting that Sertoli/granulosa cells regulate theca/Leydig cell fate. There is also some older evidence to suggest that the adult Sertoli cells can regulate Leydig cell function following differentiation,[178,179] although whether this regulation is of physiological importance in the adult is not clear. Finally, there is one study suggesting that Sertoli cells are required for Leydig cell survival in the longer term.[180] This evidence comes from B-cell CLL/lymphoma 2-like 2 (BCL2L2)-deficient mice, which gradually lose Sertoli cells up to 6–8 months of age and then become depleted of Leydig cells. Since BCL2L2 is not expressed in Leydig cells, this suggests that Sertoli cells may act to maintain the adult Leydig cell population. Evidence exists, therefore, to implicate the Sertoli cells in most aspects of testis development and function, but in all cases this evidence remains far from conclusive.

CHANGING SERTOLI CELL ACTIVITY DURING DEVELOPMENT

During the period of Sertoli cell proliferation, which lasts from initial testis differentiation until near puberty in most species (with or without a quiescent interval), Sertoli cell functional activity is generally low. As the cells stop proliferating, the activity associated with specific differentiated function increases markedly and the cells start to develop the junctional complexes associated with adult Sertoli cells. This can be seen in Figure 14.5 where changing expression of putative Sertoli cell-specific mRNA transcripts in the testis is shown during postnatal development in the mouse. Expression of all transcripts is low until about days 5–7, and as Sertoli cell proliferation comes to an end transcript levels increase markedly. The one notable exception is AMH, which is secreted early in Sertoli cell differentiation and then decreases as the Sertoli cells mature and become androgen sensitive through expression of the AR.[182–184] A critical step in testis development, therefore, is the Sertoli cell switch from a proliferative cell to one showing specific, active, differentiated function. Normal maturation of the Sertoli cells is likely to be androgen dependent as precocious expression of the AR in Sertoli cells appears to accelerate developmental changes, while Sertoli cell-specific knockout of the AR reduces adult transcript levels.[115,185] Another essential factor in the process of Sertoli cell maturation appears to be retinoblastoma protein (Rb), since mice lacking Rb in the Sertoli cells show continued cycling activity and reduced differentiated function.[186] Maturation of Sertoli cells also requires testicular expression of NR5A1[187] while, interestingly, adult cx43-deficient Sertoli cells seem to proliferate while maintaining expression of mature markers.[188]

THE SERTOLI CELL BARRIER

Sertoli cells are typical epithelial cells in that they sit on a basement membrane, attached by hemidesmosome junctions, but they differ in the mature animal by the presence of specialized junctions between adjacent Sertoli cells near to the basement membrane. These junctions form the blood–testis barrier (or Sertoli cell barrier), which acts to restrict movement into the seminiferous tubules and allows the Sertoli cells to regulate the internal environment of the tubules.[189] The structures of these junctional complexes in the Sertoli cells have been covered in a series of excellent recent reviews, particularly from Cheng et al.[189–192] The blood–testis barrier develops in rodents between 15 and 19 days,[193–195] shortly before spermatocytes enter the first meiotic division. Formation of the barrier is an essential step in development of spermatogenesis. Any delay will prevent the onset of meiosis,[195,196] while disruption of the barrier in the adult leads to infertility due to an inability of the spermatogonia to differentiate into spermatocytes.[197,198] The actin filament

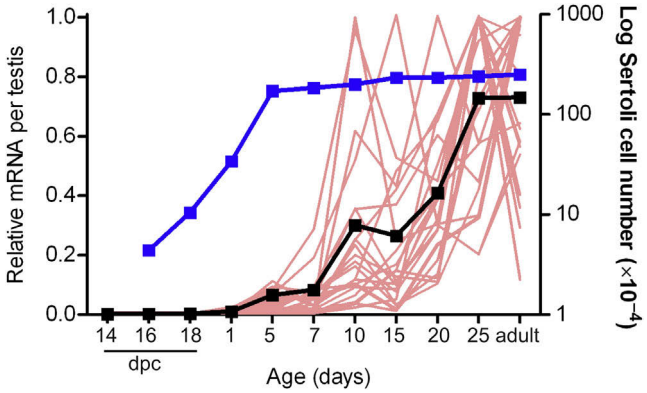

FIGURE 14.5 Changes in Sertoli cell number and transcriptional activity during fetal and pubertal development in the mouse. Data show that Sertoli cell number increases exponentially during fetal development, reaching a maximum before puberty. Sertoli cell activity, in terms of mRNA transcript levels, is shown as thin lines with each line representing a specific Sertoli cell transcript. The thick black line is the mean level of all transcripts combined. Results show that transcript levels start to rise markedly as Sertoli cell proliferation comes to an end. Days postconception (dpc). *Source: Data is taken from Refs 25,26,113,181.*

crosslinker, filamin A, appears to be a critical regulator of blood–testis barrier development through recruitment of adhesion protein complexes (e.g., F11 receptor (tight junction protein 1) and N-cadherin-beta-catenin) and reorganization of actin filaments at the basal ectoplasmic specializations.[199] It is also likely that filamin A works with other actin binding and regulatory proteins to control the assembly of the blood–testis barrier during development and during spermatogenesis.[189,200–203]

Regulation of Peripubertal Sertoli Cell Development and Maturation

ACTIONS OF FSH AND ANDROGEN ON THE SERTOLI CELL

Early studies of the effects of hypophysectomy and hormone replacement identified FSH and testosterone as critical regulators of spermatogenesis.[204–206] Subsequently, in the 1970 and 1980s, and despite occasional reports to the contrary, it was shown that receptors for FSH and androgen are not expressed in the germ cells and that these hormones act through their effects on the Sertoli cells.[120,207–209] More recently, it has also become clear that androgen action through the peritubular myoid cells is an essential requirement for Sertoli cell function and spermatogenesis.[77]

EFFECTS OF FSH With respect to hormonal control, there has been considerable debate about whether FSH or androgen is more important in terms of Sertoli cell development and initiation of spermatogenesis. Over the last decade or so, however, study of mutant and transgenic mice has allowed us to determine the role that each of these hormones plays in these processes, at least in the mouse. At the most basic level, mice lacking the FSHβ subunit or the FSHR are fertile, albeit with a reduced germ cell number, while animals lacking AR, either ubiquitously or specifically in the Sertoli cells are infertile.[27,115,210–216] More-detailed studies of adult animals have shown that FSH increases spermatogonial cell numbers and the entry of these cells into meiosis, while androgens increase the number of germ cells entering meiosis and, critically, enable these cells to complete meiosis.[214,216,217] In the adult, therefore, it is clear that androgens are more important for maintaining spermatogenesis and fertility, but FSH may play a more important role during the initiation of spermatogenesis.[27] In the normal animal, many germ cells undergo apoptosis at the spermatocyte stage during the first wave of spermatogenesis in an event that appears to be essential for subsequent normal adult spermatogenesis.[218] Current evidence suggests that FSH normally acts to limit the extent of the germ cell apoptosis during this first spermatogenic wave.[27,126,219] Whether increased germ cell apoptosis during the first spermatogenic wave in mice lacking FSH explains subsequent defects in adult spermatogenesis remains to be determined. Androgens are also reported to reduce spermatocyte apoptosis during this period,[220] but the effect is probably not mediated primarily through the Sertoli cells.[27]

While both FSH and androgen play essential roles in the control of Sertoli cell and germ cell development, the cellular mechanisms involved remain unclear. Early work showed that FSH would stimulate synthesis of a number of specific proteins such as androgen binding protein, inhibin, AR, transferrin, DMRT1, and inducible cAMP early repressor.[221–227] More recently, FSH has been shown to stimulate nociceptin production,[228] and it has been suggested that FSH regulates protein translation in Sertoli cells.[229] Two studies have used arrays to examine the effects of FSH on testicular transcript levels in the hypogonadal (*hpg*) mouse, which lacks circulating gonadotropins through a mutation in the GnRH gene.[230,231] While results showed that a number of putative Sertoli cell transcripts[232] are FSH sensitive, they did not identify clear mechanisms by which FSH may regulate germ cell development through the Sertoli cells.

One distinct effect of FSH that did emerge from the *hpg* arrays, however, was stimulation of Leydig cell transcript expression within 4 h of hormone treatment.[231] Since FSH receptors are only found on the Sertoli cells[233] and recombinant FSH was used for the studies (i.e., no contamination with other hormones), it must be assumed that FSH stimulation causes Sertoli cells to release a factor or factors that can stimulate Leydig cell development and function. This is a phenomenon that has been described a number of times; early studies with hypophysectomized rats showed that FSH treatment would stimulate androgen production[234–239] and increase Leydig cell numbers,[238,239] while similar effects could be seen in the *hpg* mouse[230,240,241] and in culture.[242] The effects of FSH in these rodent models appear to be largely on the steroidogenic pathway components, increasing enzyme levels and the capacity of the cells to produce androgen but without having a major acute effect on steroidogenic activity.[240] This indirect stimulatory effect of FSH on the Leydig cells is not restricted to rodent models. There is supporting in vitro evidence from rabbit, pig, and human Leydig/Sertoli cell cocultures[243–246] and limited human/primate in vivo evidence.[247–250] The in vivo human evidence is contradictory with two studies in favor[247,248] and one against the hypothesis,[249] but it should be pointed out that in all three human studies, under the same experimental conditions we would expect to see little or no effect in rodent models described herein. In other primates the in vivo evidence supports an indirect effect of FSH on Leydig cell function since FSH will act to increase circulating testosterone in the presence of constant LH.[250] However, while there is strong evidence for an indirect

effect of FSH on Leydig cell function mediated through the Sertoli cells, the developmental and physiological role of such an effect remains unclear and probably will remain so until the pathway is characterized. In terms of developmental relevance the evidence from transgenic mouse models and from human mutations is not particularly illuminating, but there are suggestions that the effects of FSH may be of importance to Leydig cell development. In FSHRKO mice, for example, both Leydig cell number and androgen levels are reduced, although Leydig cell numbers and androgen levels are normal in FSHβKO mice.[179] Of relevance, it has been reported that short-term immunoneutralization of FSH in adult rats will decrease Leydig cell function,[251] suggesting that in FSHβKO mice there may be long-term compensation for the loss of FSH that does not occur in the absence of the receptor. In humans, one adult individual with an FSHβ mutation has been shown to have prepubertal androgen levels despite high LH levels,[252] while in others with an inactivating FSHR mutation testosterone levels were in the low-normal range but with high LH.[253] While the nature of the factor or factors involved in Sertoli–Leydig communication remains unknown, it is likely that they are secreted by the Sertoli cell (there is no direct connection between Sertoli cells and Leydig cells) and, given the speed of the response, it is likely that they act directly in the Leydig cells.

EFFECTS OF ANDROGEN Despite early and abundant evidence that androgen action is required to ensure normal development of the seminiferous tubules, it is only recently that the potential mechanisms by which androgen may act to regulate this process have become more apparent. Early studies showed that androgens can affect Sertoli cell function in culture,[254] but these effects were not marked, and in many studies androgens did not appear to have any direct effect in vitro. More recently, however, a number of array studies have been carried out using a variety of mouse models that have identified potential mechanisms by which androgens may regulate Sertoli cell development and function.[181,255–258] Differences between the mouse models used (i.e., total or Sertoli cell only AR ablation) and the age of the animals studied has meant that there has been a wide variation in the regulated transcripts identified, but some common mechanisms of androgen action have emerged. These include retinoic acid metabolism, protease/protease-inhibitor balance, and development of cytoskeletal and junctional components.[3,259] It is this last action of androgens, on Sertoli cell junctions and blood–testis barrier formation, that has probably been most actively studied. Early work using the (androgen resistant) *Tfm* mouse indicated that the blood–testis barrier was defective in these animals,[260] while more recent studies have indicated that in mice lacking Sertoli cell ARs there is altered expression of junctional proteins leading to failure of an intact barrier to form.[261,262] Another study has also suggested, however, that in these Sertoli cell-specific ARKO mice the disturbances are more subtle, and a functional blood–testis barrier does form but the formation is delayed and partially disturbed.[263] Since androgen treatment of *hpg* mice will initiate tight junction formation[264] and development of a functional blood–testis barrier, it appears likely that androgens act through *both* the Sertoli cells and the peritubular myoid cells to induce normal and timely barrier formation.

One transcript that is consistently altered in transcript arrays of testes from AR-deficient mice is reproductive homeobox 5 (*Rhox5*). This gene is expressed specifically in Sertoli cells, and transcript levels rise markedly as the Sertoli cells come to the end of the proliferative phase.[265,266] Targeted mutation of RHOX5 in male mice leads to subfertility, which is marked by increased germ cell apoptosis, a reduction in sperm number, and reduced sperm motility.[267] More recent studies suggest that RHOX5 serves as an intermediate transcription factor that directs some of the actions of the AR.[268] Activation of RHOX5 by androgens depends on the chromatin-remodeling protein ATRX,[45] which may also have a general role to enhance the expression of androgen-dependent genes through physical interaction with AR. An interesting recent observation that androgens regulate the production of several specific microRNAs by the Sertoli cells[269] may offer an alternative/additional mechanism of androgen action in these cells.

OTHER FACTORS

In addition to FSH and androgen, other hormones and growth factors have been reported to alter Sertoli cell function including, notably, TH and the TGFβ superfamily.[270,271] Most recently, it has been shown that microRNAs are essential for postnatal Sertoli cell development.[272–274] Sertoli cell deletion of Dicer, an enzyme essential for processing microRNAs into their mature form, leads to defective Sertoli cell development and massive apoptosis starting around postnatal day 3.[272,273] This would suggest that miRNAs are essential either for regulating the apoptotic pathway during Sertoli cell development or that dicer, itself, may be required for the establishment of differentiation-dependent heterochromatin marks.

The Leydig Cell

Fetal Leydig Cells

It has been shown that LH will stimulate fetal Leydig cell androgen production in the mouse,[275–277] but *hpg* mice (which lack circulating gonadotropins), LuRKO mice (which lack the LH receptor), and LHβKO

mice (which lack LH) are normally masculinized at birth[114,278–280] with normal intratesticular levels of testosterone.[112,281] This shows that LH is not required during fetal development in mice for normal activity of the fetal Leydig cells. Available evidence would also suggest that fetal Leydig cell development in rats is largely independent of LH stimulation. LH secretion into the circulation, for example, does not start in significant levels until 19.5 dpc, by which time testosterone levels have already reached a peak,[277,282] while fetal decapitation before 19.5 dpc does not significantly affect testosterone levels.[283,284]

Interestingly, in NK2 homeobox 1 (NKX2-1)-null mice (which lack a pituitary gland), intratesticular androgen levels are reduced significantly at birth,[285] suggesting that other pituitary hormones may stimulate fetal Leydig cell function in the absence of LH. ACTH has subsequently been shown to stimulate fetal Leydig cells directly,[276,286] but double knockout mice lacking ACTH and the LH receptor have normal testosterone levels during fetal development.[3] Currently there is no evidence that other pituitary hormones can stimulate fetal Leydig cell function, and it appears more likely that reduced androgen production in NKX2-1-null mice is related to other effects of NKX2-1 depletion.[11] This suggests that androgen production by the fetal Leydig cells in the mouse is either constitutive or is regulated by other endocrine or local trophic factors. A number of factors have been reported that will stimulate fetal Leydig cell activity. These include vasoactive intestinal peptide, adenylate cyclase activating polypeptide 1, and corticotropin releasing hormone,[277,287] though, as we have seen with LH and ACTH, this is no guarantee that they are physiologically essential. An alternative, endocrine factor that may be involved in fetal Leydig cell function is osteocalcin. This bone-derived hormone, secreted by osteoblasts, acts through the G-protein-coupled receptor 6A (GPRC6A) (see below)[288] and has been shown to play a significant role in regulating Leydig cell function in the adult. It is not known whether *Gprc6A* is expressed in fetal Leydig cells or whether osteocalcin affects Leydig cell activity, but it is of interest that in GPRC6A-null mice the anogenital distance (AGD) is reduced in adults.[289] AGD is a measure of androgen exposure during fetal life and is set at that time, so a reduced adult AGD is indicative of reduced fetal androgen levels in mice lacking GPRC6A.[290]

The alternative possibility, that constitutive activity may be of importance during fetal Leydig cell development, has also been strengthened recently by the suggestion that steroidogenesis in these cells is driven by an effective increase in NR5A1 stimulation following downregulation of the competitor Chicken Ovalbumin Upstream Promoter-Transcription Factor II (COUP-TFII), an orphan nuclear receptor of the steroid/thyroid hormone receptor superfamily.[291,292] This study also suggested that factors that reduce fetal testosterone production (such as xenotoxicants) may do so through increasing expression of COUP-TFII in the fetal Leydig cells.[291]

Based on the timing of external masculinization and development of the hypothalamic–pituitary axis, data available from the boar, rabbit, stallion, bull, ram, and dog would suggest that fetal Leydig cell function also develops, at least initially, without a need for LH stimulation in these species.[11] Unlike rodents, however, the available evidence suggests that the fetal Leydig cells become pituitary dependent (probably meaning LH dependent) during the second half of gestation. The evidence for this comes from studies using fetal hypophysectomy, fetal decapitation, and treatment with GnRH analogs.[29,58,129,130,293,294] As a general rule, therefore, it appears that in most mammals the fetal Leydig cells go through an initial pituitary-independent phase and then become pituitary dependent later in gestation (Figure 14.4). In humans, however, it is clear that fetal Leydig cell development is critically dependent upon stimulation through LH receptors for most of fetal development. XY individuals with an inactivating mutation of the LH receptor are pseudohermaphrodites, indicating lack of androgen production in fetal life—particularly during the critical masculinization programming window (MPW).[295–300] This contrasts to individuals with an inactivating mutation in the LHβ gene in which fetal masculinization and testis descent takes place,[301,302] indicating that hCG (which acts through the LH receptor) is the main regulator of fetal Leydig cell function during the first and second trimesters.[301–303] Interestingly, in individuals lacking functional LH receptors, epididymidal remnants remain,[295,298] suggesting that the fetal Leydig cells probably go through an early period in the MPW (which probably starts late in the first trimester in humans[300]) when they can function independently of LH/hCG. This is supported by in vitro studies which have shown that testes from first-trimester human fetuses will show significant testosterone production in the absence of LH.[304] Fetal testosterone levels in vivo peak early in the second trimester in humans, shortly after the peak in hCG,[290,305,306] and then decline slowly over the second trimester.[306] Pituitary LH starts being released into the circulation in significant amounts during the second trimester,[11,290,305–309] and it is likely that the Leydig cells become dependent on this LH as the hCG levels decline. Evidence for LH dependency during this period comes from anencephalic humans in which an apparent loss of fetal Leydig cells occurs during the late second and third trimesters.[310] In addition, the external genitalia are often small in newborn anencephalics,[132–135] which is consistent with reduced testosterone levels in the third trimester, a period essential for androgen-dependent growth of the penis.[290] Evidence from the rhesus monkey has also shown that hypophysectomy in the last third of gestation reduces the overall percentage of Leydig cells at term.[311]

In a number of species, including the human, monkey, and the pig, there is activation of the hypothalamic–pituitary–testicular axis shortly after birth, leading to increased LH and a sharp transient rise in fetal/neonatal Leydig cell activity and circulating androgen levels[312–315] (Figure 14.4). It has been suggested that this surge in androgen may play a role in imprinting sexual orientation and behavior and priming target tissues for subsequent growth and maturation.[316]

It is likely that at least some fetal Leydig cells share a common origin with fetal adrenal cells,[317] and, consistent with this, both cell types show a high degree of overlap in the pattern of transcript expression. This includes expression of the ACTH receptor *Mc2r*[286] and the "adrenal" enzymes cytochrome P450, family 11, subfamily B, polypeptide 1 (*Cyp11b1*) and cytochrome P450, family 21, subfamily A, polypeptide 1 (*Cyp21a1*)[286,317–319] in the fetal Leydig cells. Interestingly, it has also been shown that steroidogenic cells can migrate from the adrenal to the testis during early gonadal development,[320,321] and a population of cells can be identified in the fetal testis, which is more characteristically "adrenocortical" than "Leydig",[321] although the origin of these cells and their relationship to the canonical Leydig cells remains to be determined. In humans with congenital adrenal hyperplasia there is, commonly, development of testicular tumors that resemble adrenocortical tissue.[322] These adrenal rest tumors may arise from the ectopic adrenal cells in the testis through stimulation by the high levels of ACTH that are associated with this condition.[322]

The fate of the fetal Leydig cells in the adult testis remains unclear. In terms of overall cell numbers, fetal Leydig cells present in the neonatal mouse testis are just over 1% of the total number of Leydig cells present in the adult testis,[25] and so the fetal cells may persist, largely unnoticed, in the adult testis.[171,323] Alternatively, they may degenerate as the adult Leydig cells develop,[324,325] but clear evidence either way is currently lacking.

Steroidogenic Pathways in the Developing Testis

In the adult testis the Leydig cells synthesize testosterone through the Δ^4 or Δ^5 pathway shown in Figure 14.6. The particular pathway used is species dependent (e.g., the mouse and rat use Δ^4 predominantly, while dog, pig, rabbit, and human use Δ^5)[326–331] and the Leydig cells are self-sufficient in that they express all of the enzymes necessary for testosterone production. Androgen production in the fetal testis, however, appears to be a more complex process, at least in the mouse and the human.

In the mouse, the final step in testosterone synthesis is reduction of androstenedione catalyzed in the testis largely by the enzyme HSD17B3. Two separate studies have now shown that fetal Leydig cells in the mouse lack HSD17B3 and that these cells are unable to synthesize testosterone.[332,333] Instead, androstenedione produced by the fetal Leydig cells is converted to testosterone by HSD17B3 in the Sertoli cells.[332,333] It is not clear whether this two-cell pathway is specific to the mouse or is more generally utilized, but it is of interest from an evolutionary perspective. It has been proposed, for example, that the Leydig cells evolved relatively late in the vertebrate testis and that prior to this the Sertoli cells were the major steroidogenic cell type.[334,335] Under this scenario, once the Leydig cells evolved (perhaps from modified adrenal cells) the Sertoli cells lost most of their steroidogenic

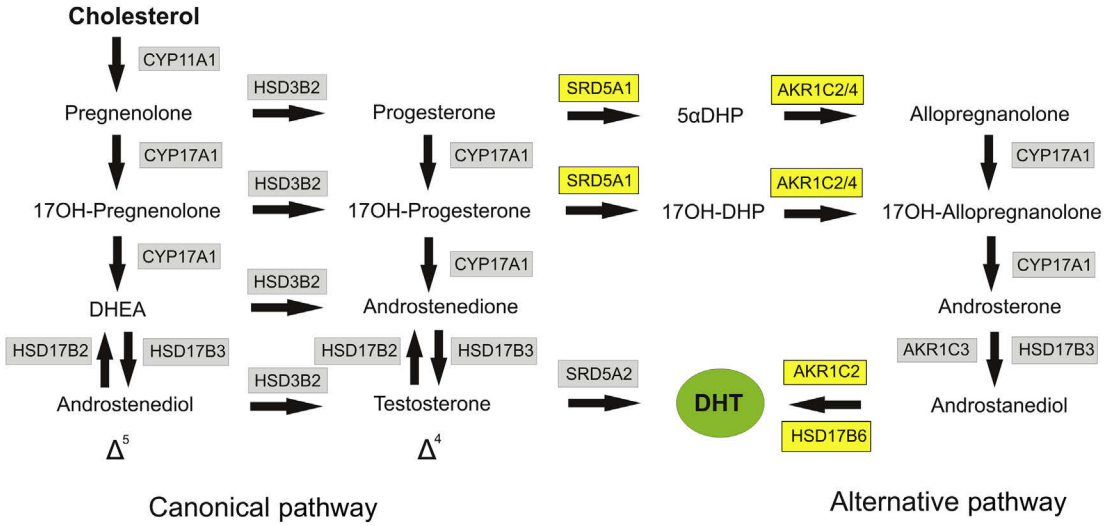

FIGURE 14.6 Potential steroidogenic pathways leading to formation of dihydrotestosterone (DHT) in the testis. The canonical pathway through Δ^4 and Δ^5 steroids is shown on the left with the alternative (backdoor) pathway on the right. Enzymes shaded in gray are required for the canonical pathway (with a number also required for the alternative pathway). Enzymes boxed in black are specific to the alternative pathway.

potential, although in the fetal mouse testis at least, they remain a critical component in the production of bioactive androgen.

Another peculiarity of fetal androgen production is the use of the "backdoor/alternative" pathway by the human fetal testis. In 2003 a novel testicular biosynthetic pathway was described in the tammar wallaby, which generated the potent bioactive androgen dihydrotestosterone without using testosterone as an intermediate[336,337] (Figure 14.6). The relevance of this pathway to human testicular function and fetal masculinization became clear when disordered sexual development in a number of XY individuals was shown to be linked to mutations in *AKR1C2* and *AKR1C4* that encode critical enzymes within the alternative pathway[338] (Figure 14.6). Results from this study indicate that both pathways of testicular androgen biosynthesis are essential for normal human masculinization.[338] Interestingly, the alternative backdoor pathway to androgen synthesis is present in the pubertal mouse testis but not the fetal mouse testis,[333,339] while *AKR1C2* and *AKR1C4* are not expressed in the adult mouse testis.[338] This suggests that the alternative testicular pathway to androgen synthesis is probably widespread in mammals, but the importance of it during fetal development may be species dependent.

Adult Leydig Cells

The development of the adult Leydig cells has been described in some detail in a number of publications.[61,340–344] The cells start to differentiate from stem cells at some point after birth and before puberty, the timing depending on the species. In mice we can time the initial differentiation fairly accurately to around seven days after birth. This comes from studies in which fetal Leydig cell expression of *Insl3* was inhibited by exposure to estradiol in utero.[53] After birth, expression of insulin-like 3 (*Insl3*) can be seen again at seven days, indicating differentiation of the adult Leydig cell population. It could be argued that exposure to estradiol may have altered adult Leydig cell development, but the overall timing fits in with expression of hydroxy-delta-5-steroid dehydrogenase, 3 beta- and steroid delta-isomerase 6 (*Hsd3b6*), a marker of adult Leydig cells, which starts at about day 10.[52] The adult Leydig cell progenitor cells are small, spindle-shaped fibroblast-like cells that develop over the course of pubertal development into easily recognizable polygonal Leydig cells.[342] The origin of these progenitor cells is likely to be the peritubular region, although there may also be some that originate from the perivasculature.[341,345–349] The progenitor cells express some steroidogenic enzyme mRNA transcripts but appear to lack transcripts encoding the LH receptor.[342] As the progenitor cells develop, they enlarge, take on a rounder appearance that is associated with an increase in smooth endoplasmic reticulum, and start to express higher levels of the enzymes required for androgen synthesis.[343,344,350–352] Final maturity of the Leydig cells in rodents occurs during puberty, and these cells do not appear to undergo further cell division,[353] although the adult Leydig cell population will regenerate from stem cells if a significant number of the mature cells are destroyed.[354,355]

Control of adult Leydig cell development remains an area of uncertainty. We are beginning to understand some of the processes involved, but that understanding is very patchy and large areas of uncertainty remain. For example, there is good evidence that platelet-derived growth factors (PDGFs) (and especially PDGFA) are required for adult Leydig cell differentiation,[356–358] but PDGFA is localized to most of the testicular somatic cells[359] and the particular cell–cell interactions involved are not certain. In DHH-null mice, and in rats with a mutation in the DHH gene, there is also failure of adult Leydig cell development although the effect in mice appears to be somewhat variable and strain dependent.[168,171,360] Interpretation of the role that both PDGFs and DHH play in the development of the adult Leydig cell population is also complicated by the effect that knockout of these factors has on early testis development. In both cases there is limited fetal Leydig cell development and/or a reduction in adult Leydig cell stem cells that may be the underlying cause of later defects in adult Leydig cell development. It is of interest, however, that putative Leydig cell stem cells will differentiate down a steroidogenic cell lineage when exposed to PDGF, LH, and other general growth factors.[292] Interesting data has also come from animals in which COUP-TFII has been deleted. If deletion occurs prior to puberty there is a clear and marked failure of adult Leydig cell development,[361] and it is suggested that this factor is involved in both development of Leydig cell progenitors and maturation of the differentiated cell.

In addition to a general uncertainty of the local factors that regulate adult Leydig cell differentiation, we do not know what stimulus drives the whole process—e.g., is there a change in the endocrine environment that initiates progenitor formation from the stem cell? It has been known for many years that LH is essential for adult Leydig cell development, and this can clearly be seen in mice which lack LH (*hpg* and LHβKO) or the LH receptor (LuRKO). In each case androgen levels are very low or undetectable[112,114,278,280,362] and Leydig cell number is markedly reduced.[25] Treatment of *hpg* mice with LH, in turn, has been shown to stimulate steroidogenesis and to induce Leydig cell proliferation.[362] Evidence from *hpg* and LuRKO mice suggests, however, that LH (and FSH) is not required for the initial differentiation of the adult Leydig cells since a number of cells in these animals will start expressing transcripts characteristic of the adult population in the absence of hormone treatment.[281,363]

In addition, the early pattern of adult Leydig cell development suggests that these cells start to show differentiated function before they become LH sensitive.[341] The evidence shows, therefore, that once Leydig cell differentiation has started LH is essential for completion of the process and for activity in the mature cells but that it does not initiate the process. The other major endocrine regulator of Leydig cell function that has been identified recently is bone-derived osteocalcin.[288] To date, published studies have shown that Leydig cell function is reduced in mice lacking osteocalcin, but Leydig cells clearly form in these animals so it is unlikely that osteocalcin is essential for the initial regulation of cell development.[288] Another endocrine factor that may be involved is thyroid hormone as both high and low circulating levels can have marked effects on adult Leydig cell development.[364–366] The nature of these effects suggests, however, that thyroid hormone probably plays a permissive role in normal Leydig cell development, as it does in the Sertoli cells, rather than acting as a trigger for the whole process. There is evidence to suggest that AMH may act as an inhibitory regulator of adult Leydig cell development as Leydig cell differentiation is reported to be reduced in AMH-overexpressing mice.[367] The normal prepubertal decrease in circulating AMH levels may, therefore, be necessary to allow normal Leydig cell development.[184]

Of compelling interest, with respect to Leydig cell development, are the large number of studies carried out using the cytotoxic drug ethane dimethane sulphonate (EDS), which specifically causes Leydig cells in the adult testis to undergo rapid apoptosis.[354,368] Over a three-to-four week period after EDS treatment, the Leydig cell population recovers in a process that appears to mimic normal adult Leydig cell development.[369] This powerful model has allowed us to examine the endocrine requirements for Leydig cell redevelopment more clearly[369] but has, so far, failed to identify what factors are inducing Leydig cell differentiation when the normal population is ablated. It appears likely, however, that this model, or something similar, offers the best current opportunity to understand control of Leydig cell development, particularly as it has been shown recently that some of the effects of EDS can be replicated in vitro.[355] Overall, the weight of available evidence would currently suggest that Leydig cell differentiation is most likely to be initiated by other somatic cells in the testis (the germ cells do not appear to be directly involved as adult Leydig cells develop in germ cell-free testes). Which particular cells are involved and how the cell–cell interactions take place are fundamental questions of testis biology that need to be addressed.

Peritubular Myoid Cells

The peritubular myoid cells have received considerably less research scrutiny than either the Leydig cells or the Sertoli cells (e.g., a PubMed search shows papers concerning myoid cells are about 5% of the total number of papers on testicular somatic cells), but it is likely that this cell type plays a fundamental role in testis development and function. Exactly what those functions are and how they are controlled is now starting to become clearer, largely through study of transgenic mice. We now know, for example, from study of peritubular myoid cell-specific ARKO mice that androgen action through the peritubular myoid cells is essential for normal Sertoli cell and Leydig cell development and for maintenance of spermatogenesis.[77,78] It has also been shown using knockout mice that DHH is required for normal peritubular myoid cell development, and in the absence of DHH there are multiple developmental abnormalities in the testis that may be related to abnormal peritubular myoid cell regulation.[171] Known functions of the cells include contractile activity that is involved in the transport of spermatozoa and testicular fluid through the seminiferous tubules, and a number of factors have been identified that can stimulate or inhibit this activity.[82] In rodents, another recognized function of the peritubular myoid cells is the significant contribution they make to the blood–testis barrier. Studies have shown that in up to 85% of tubules, markers of barrier integrity are blocked at the peritubular myoid cells layer and do not penetrate to the Sertoli cells.[370,371] In primates and probably humans, however, the barrier generated by the peritubular myoid cells appears to be less effective.[372] The presence of a barrier at the level of the peritubular myoid cells in rodents is also of interest as it is likely to inhibit free movement of large molecules between the interstitial compartment (and the blood and lymph) and the Sertoli cells, which may have implications for both endocrine and paracrine regulation in the testis. Another barrier-type role of the peritubular myoid cells may be to regulate movement of retinoic acid into the tubules through expression of CYP26B1. Retinoic acid in the seminiferous tubules can either enter from the interstitium or be synthesized in the Sertoli cells, and it is likely that levels are tightly regulated as both increased and decreased levels of retinoic acid are known to be deleterious to spermatogenesis.[373,374] In the postnatal testis CYP26B1 is localized exclusively in the peritubular myoid cells,[174,175] and the role of this enzyme may, therefore, be to prevent retinoic acid from the interstitium entering the tubules, thereby allowing the Sertoli cells to control intratubular levels. While a number of functions of the peritubular myoid cells are known or suspected, the mechanisms by which the peritubular myoid cells act to regulate Sertoli cell and Leydig cell function remain unknown and will constitute an interesting area of future research.

Testicular Macrophages

Given the intimate connections between macrophages and Leydig cells it is not surprising that macrophages have been postulated to play a role in Leydig cell development. For example, in experimental models with impaired macrophage function it has been reported that there is an absence or delay in Leydig cell development.[375,376] Similarly, depletion of macrophages from the neonatal testis prevents adult Leydig cell development.[375] It has also been reported that adult testosterone production is reduced when macrophages are removed from the testis,[377–380] and it has been suggested that the active factor in this case may be 25-hydroxycholesterol (a potential substrate for testosterone production) secreted by the macrophages.[381] Macrophage production of this sterol is greatest during the prepubertal period, so it may be an active factor in the developmental control of the adult Leydig cell population.[382] It has also been reported that macrophages can inhibit Leydig cell function through the production of nitric oxide, tumor necrosis factor, and/or interleukin-1,[383] although this may be of greater importance under pathological conditions. The characteristics and role of testicular macrophages are discussed further in Chapter 19.

GERM CELLS

Initiation of Spermatogenesis

During fetal development the gonocytes lie within the lumen of the seminiferous tubules, but at some point prior to the start of spermatogenesis they undergo proliferation, extend cytoplasmic processes, and migrate to the basement membrane.[384] This migration appears to be essential for germ cell survival as gonocytes remaining in the lumen of the tubules will undergo degeneration.[385] Once the gonocytes reach the basement membrane they will give rise to the SSCs (A-single spermatogonia; As) in rodents or part of the A-dark (Ad) and/or A-pale (Ap) spermatogonia in primates (see Chapter 15), which are able to undergo either self-renewal to maintain the SSC population or development into spermatogonial cells committed to differentiate into spermatozoa.[386,387] In rodents gonocyte migration/differentiation occurs soon after birth, while in humans it starts toward the end of the second trimester and lasts up to eight months postnatally.[388] The differentiation step from gonocyte to SSC is particularly important as it has been reported that carcinoma in situ cells arise from arrested/dysfunctional gonocytes.[389] The regulatory mechanisms involved in this process remain uncertain, but there is evidence that PDGFs are involved in the migration of the gonocytes,[390] and it has also been suggested that male germ cell miRNAs may act to coordinate the differentiation of gonocytes.[391] Once differentiated, the SSC sit next to the basement membrane in a niche that we assume is contributed to largely by the Sertoli cells, although, interestingly, SSC niches appear to be more frequent in areas of the tubule that border interstitial tissue.[392,393] This may be an indication that Leydig cells contribute to the niche through production of testosterone and possibly other factors,[394] or may simply be a consequence of the presence of blood vessels in the interstitium. Contributions from peritubular myoid cells to the SSC niche are also possible but remain speculative. Once committed, the SSC in rodents gives rise to a pair of a type A spermatogonia (Apr) connected by intercellular bridges, which in turn proliferate to form A-aligned (Aal) spermatogonia. Once spermatogonia become committed to spermatogenesis they undergo proliferation, which is followed by entry into meiosis as primary spermatocytes once the animals start puberty. SSC activity and the process of spermatogenesis are described in greater detail in Chapter 15.

TESTICULAR DEVELOPMENT AND ADULT HEALTH

This chapter started with the seminal studies of Jost who showed that the testes are essential for normal masculinization of the fetus. Studies since then have reinforced the reproductive importance of testis development, but evidence is now also accumulating to suggest that testis development may also have an importance for adult male health and well-being beyond reproductive health. A number of studies have shown, for example, that low adult testosterone is linked to increased incidence of metabolic syndrome (MetS)—a collective group of disorders that increase the likelihood of an individual developing cardiovascular disease, diabetes, and dementia during aging.[395–397] From prospective studies, androgen ablation studies, and testosterone-replacement therapy it is clear that in at least a number of these cases it is the reduction in testosterone that is likely to be causative.[398–402] While it remains to be established, it is highly likely that adult testosterone levels will be dependent on events during pubertal, prepubertal, and even fetal life. For example, it has been shown that AGD in men is significantly correlated with adult testosterone,[403] an effect also seen in rats.[404] This is highly relevant because AGD reflects fetal androgen exposure and is fixed by the time of birth,[299] so a correlation between AGD and adult testosterone levels suggests that adult levels may be dependent on fetal testosterone production. Other studies using sheep, rat, or nonhuman primate models have also shown that reducing Leydig cell function in fetal or neonatal life will reduce adult testosterone levels.[294,405–408] Understanding

of normal testicular development and the control mechanisms involved may, therefore, be essential for understanding processes in later life that can alter male health and well-being.

CONCLUSION

Correlation and integration of data from a number of species on the regulation of somatic cell proliferation and function during fetal and early neonatal life has led to the development of a general model of fetal testis development[11] (Figure 14.4). This model suggests that in all species there is an early period of fetal testicular growth that is independent of pituitary hormone stimulation. This lasts until about half to two-thirds of the way through pregnancy in most species and covers the MPW.[290,299] In the final third of gestation, both Sertoli cells and Leydig cells become pituitary dependent. Within this model rodents and humans have to be treated as special cases, and it is unfortunate that both ourselves, who have to be treated as the primary species of interest, and the model species for mechanistic development fall into this category. In rodents, gestation is short and the pups are born at the end of the pituitary-independent phase, just as the hypothalamic pituitary axis is starting to regulate testicular function. This means that rodents are not good models for the later period of fetal testicular development seen in most other species. Humans and a number of other primate species[409] secrete CG, and following a likely short period of constitutive function, the Leydig cells become highly CG dependent early in testis development. In the last third of gestation, however, CG levels fall markedly, and so the testes probably become LH dependent at this time although the role of FSH remains unclear. Since rodents are not a good model for the pituitary dependent phase of fetal testicular development, what we critically lack at the moment is a clear and detailed understanding of the importance of the fetal pituitary hormones for testicular development in utero and for programming later testicular function and male reproductive health. This chapter has highlighted the enormous progress that has been made in our understanding of testicular cell development and the role of endocrine and local factors in controlling that development. It has also, however, identified some of the more obvious and significant gaps in our knowledge that require to be filled. Outstanding questions include:

- How do the somatic cells interact to regulate testicular development and function?
- What functions do the Sertoli cells have in the period between early fetal and pubertal development?
- Do Sertoli cells regulate Leydig cells in the adult animals and, if so, how?
- What controls adult Leydig cell differentiation?
- What is the overall role of the peritubular myoid cells?
- What regulates gonocyte differentiation?
- How, and to what extent, do changes in fetal or neonatal testicular development alter adult reproductive health and adult well-being?

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CHAPTER

15

Spermatogonial Stem Cells and Spermatogenesis

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INTRODUCTION

Spermatogenesis is a highly organized process that produces millions of sperm each day in postpubertal mammals.[1–3] This productivity is dependent on the activity of spermatogonial stem cells (SSCs)—adult tissue stem cells in the testes that balance self-renewing divisions with differentiating divisions, which maintain the stem cell pool and fuel spermatogenesis, respectively.[4,5] When SSCs differentiate, they give rise to spermatogonia, which undergo a species-specific number of transit amplifying mitotic divisions, followed by two meiotic divisions and spermiogenesis, to produce terminally differentiated sperm (Figure 15.1(A)). SSCs reside in a specialized niche located on the basement membrane of the seminiferous tubules where they are in direct contact with Sertoli cells, which produce some of the paracrine factors required to regulate self-renewal and differentiation fate decisions (Figure 15.1(B) and (C)). Stem, progenitor, and differentiating spermatogonia are all located on the basement membrane of the seminiferous tubules. Differentiating spermatogonia give rise to spermatocytes that initiate meiosis and migrate off the basement membrane, producing spermatids and then sperm (Figure 15.1(C)).

Our current knowledge of the staging of spermatogenesis (discussed in more detail below), including multiple types of spermatogonia and their sequential differentiation, was established in the rat by Leblond and Clermont at McGill University (Montreal, Canada) in the early 1950s.[6,7] In these papers, the authors presented historical perspectives of spermatogenesis research; for example, the stages of the seminiferous epithelium cycle were first reported in 1885.[6] Furthermore, they clearly used the term *stem cells* in their 1952 paper: "These act as stem cells for the next generation. This theory, referred to as 'stem cell renewal,' is described in detail elsewhere."[6] This casual use of the terms *stem cells* and *renewal*, with no references cited, indicates that the concept of stem cells and associated terminology seemed to be relatively familiar to the researchers in the early 1950s.

In a 2007 article, Ramalho-Santos and Willenbring stated that "the early uses of the term stem cell were made in the late nineteenth century in the context of fundamental questions in embryology: the continuity of the germ-plasm and the origin of the blood system".[8] According to these authors, Pappenheim used the term *stem cell* in 1896 for the hematopoietic lineage in the same sense as we use it today.[9] Hematopoietic stem cells are often considered to be the criterion standard of stem

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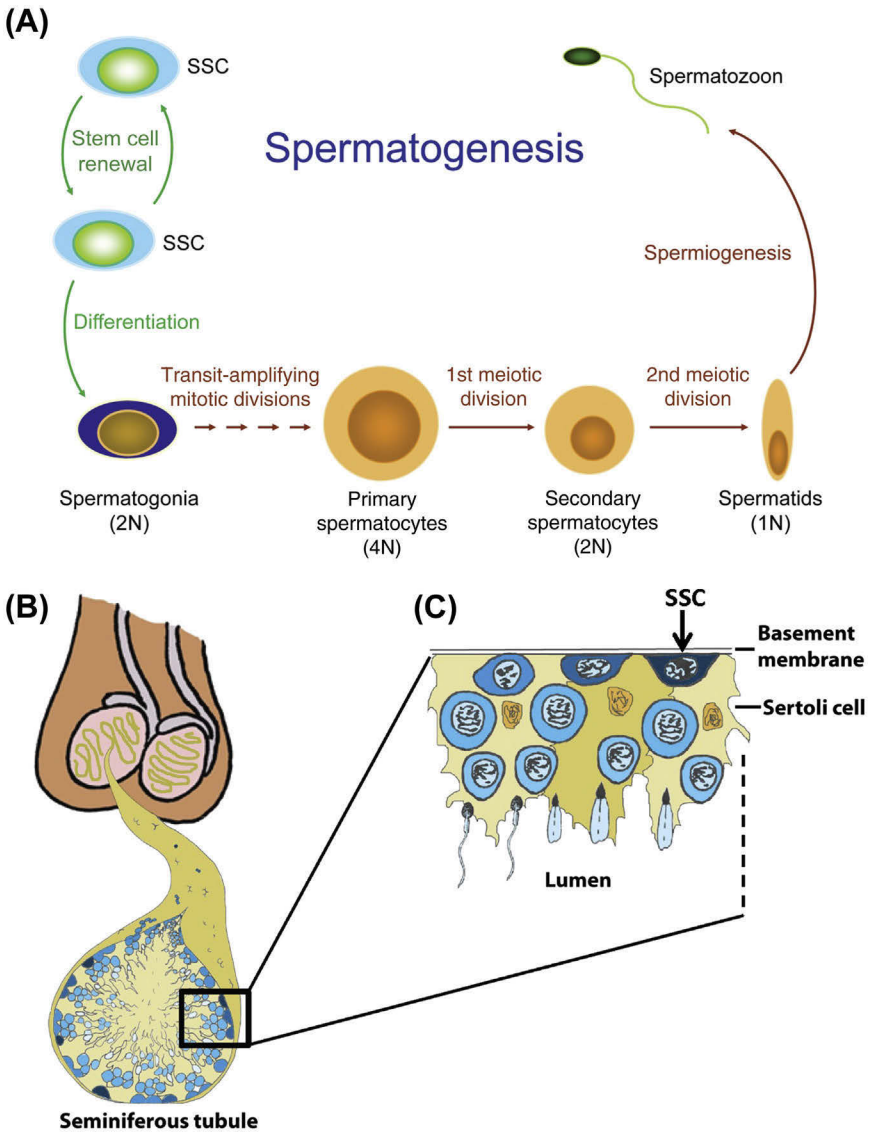

FIGURE 15.1 Mammalian spermatogenesis. (A) The pool of diploid (2N) spermatogonial stem cells (SSCs) balance self-renewing and differentiating divisions to maintain the stem cell pool and also continuously produce sperm. Once committed to differentiate, SSCs give rise to undifferentiated and differentiating spermatogonia (2N), which undergo a variable species-dependent number of transit-amplifying mitotic divisions that can dramatically increase the yield of sperm from a single stem cell. Differentiating spermatogonia give rise to primary spermatocytes (4N), which undergo two meiotic divisions to produce haploid spermatids (1N). The meiotic divisions are followed by spermiogenesis to produce terminally differentiated sperm. (B) Spermatogenesis takes place inside the seminiferous tubules of the testis. (C) Cut out of the seminiferous epithelium. Spermatogonia (including SSCs) located on the basement membrane of seminiferous tubules give rise to primary spermatocytes, which initiate meiosis and migrate off the basement membrane. Two meiotic divisions and spermiogenesis give rise sequentially to secondary spermatocytes, spermatids, and terminally differentiated sperm, which are released into the lumen of the seminiferous tubule. Adapted with permission from Valli et al., Ref 332.

cells, due to pioneering work by Till and McCulloch at the University of Toronto, Canada, which established the concept that stem cells are defined by their function.[10–13] We consider, however, that the concept of stem cells was initially proposed in studies of germ cells. In the 1940s, Leblond initiated autoradiographical investigations into stem cells mainly using spermatogenesis and the intestinal epithelia as a model.[14–17] Thus, we here wish to address when spermatogenesis was recognized as a stem cell system and when the term *spermatogonial stem cell* appeared for the first time in its research history.

Leblond and Clermont stated that "the complete series of successive cellular associations has been named the 'spermatogenic cycle' by von Ebner[18] and Regaud[19]" and that "Regaud in 1901 had already observed spermatogonia of two main types—currently referred to as A and B—the former (type A) being the more primitive which divided to produce the more differentiated type B cells which, in turn, divided to give rise to spermatocytes".[7,20]

When we look at von Ebner's paper from 1888,[18] we find the term *Stammzelle* ("stem cell" in German) but only as a reference and to clarify terminology, and the stem cell itself is not the subject of discussion. The paper he cited was by Biondi from 1885.[21] There, Biondi introduced the term *Stammzelle* for cells at the basal membrane (the first zone) and described how a stem cell divides to produce Mutterzelle (mother cell) in the 'second zone', which in turn produces two daughter cells (*Tochterzelle*) in the third zone, translated as follows: "The spermatozoa often arise, without the appearance of daughter cells, directly from the cells of the first (stem cell) and the second zones (mother cell); then, they protrude as a bundle all from one cell, with their heads closely together."[21] Biondi made these descriptions using rats based on the cell classification that he developed with bull testes and

concluded that spermatozoa are generated by the stem cell. We are, however, uncertain if Biondi identified spermatogonia as stem cells, because he concluded that "the basal nucleus of the Sertoli cell is exactly the same as the stem cell nucleus".[21]

On the other hand, Regaud[19] clearly used the term *stem spermatogonia*' in 1901, in the way we use the term today, as shown in the following translated excerpts from this paper:

"The term *spermatogonia* applies to several distinct generations, at least two. The more primitive—and hence the *origin* of the entire spermatogenic lineage—is formed of cells that possess very little cytoplasm and whose nuclei contain almost no chromatin; I have termed them dusty spermatogonia. Mitotic division of these cells (1st mitotic division) gives rise to a new generation, which I have termed crust spermatogonia, because during the interval between mitotic divisions, the chromatin of the nucleus is arranged like small crusts at its rim, initially safranophilic (stained with saffron), and subsequently hemateiphilic (stained with hemalun)."

Regaud further stated:[19]

"Let us suppose for a moment that, in a seminiferous tubule, the productivity of the seminal epithelium becomes arrested, that the 'developing' spermatogenic lineage completes its evolution (differentiation to spermatozoa) and that in the end, there remains within the syncytial protoplasm between the nuclei of Sertoli nothing save a small number of *stem spermatogonia* that must ensure the future repopulation of the epithelium."

Note that Regaud believed that Sertoli cells formed a syncytial structure and thus preferred calling them "the nurturing syncytium and the nuclei of Sertoli."[19] Interestingly, the cessation of spermatogenesis that Regaud proposes here is not a hypothetical premise, and he takes spermatogenesis in a seasonal breeder as one example. This makes us realize that Regaud considered the functionality of cells—regeneration—to define stem cells in spermatogenesis. Hence, Regaud identified morphological differences among spermatogonia in 1901 and expressed the concept of the cell of origin—the cells that allow for spermatogenic 'regeneration' after depletion of committed germ cells—by calling them *spermatogonies souches*, the exact term that means "stem spermatogonia" in French.

As Leblond and Clermont stated,[6] van Hoof confirmed in 1912 the sequence of spermatogonial differentiation that Regaud identified and also introduced the term *cellules de réserve pour la division suivante*, meaning "reserve cells for the next cell division."[22] This is, to our knowledge, the first introduction of the reserve stem cell concept. Further, van Hoof used the general term *stem cell* (rather than stem spermatogonia) in a phrase where he speculated the following, as translated: "But we do not believe that the stem cell divides only twice to give rise to such a large number of spermatocytes. We therefore think, as does Duesberg[23], that the number of cell divisions is greater than two."[22]

These pioneering papers indicate that researchers in the late 1800s, and at least in 1885 as seen in Biondi,[21] had a grasp of the stem cell concept, and the term *spermatogonial stem cells* was used by 1901 in the way we use it now. It is particularly striking to realize that Regaud's recognition of SSCs was based upon regenerative activity seen in a naturally occurring phenomenon of spermatogenic regeneration in seasonal breeders.

It is also interesting to note that the major descriptive observations of morphological development of spermatogonia presented in these papers survived over time and are very similar to those that we know today. In reading these papers, we further recognize the budding ideas and concepts that later evolved to various theories and models of spermatogonial development and stem cells proposed from the 1960s through early 1980s, most of which were put forth by the pupils of the spermatogenesis study group at McGill University that was founded by Charles Leblond.

In the section Spermatogonial Stem Cells and Spermatogenic Lineage Development of this chapter, we will review the seminal studies that established the models of SSCs and spermatogenic lineage development and provide the framework for our current understanding of mammalian spermatogenesis. We will also review how our current understanding of spermatogenic lineage development in nonhuman primates and humans is similar and how it is different than rodents. In the section Experimental Tools for Studying Spermatogonial Stem Cells, we will describe the experimental tools that are used to study SSCs and spermatogenic lineage development. We will discuss how data generated using those tools should be interpreted as well as the knowledge that has been generated. The section The Spermatogonial Stem Cell Niche will describe factors and cells contributing to the SSC niche and provide examples of how those components regulate SSC self-renewal and differentiation fate decisions. Intrinsic factors that regulate SSC function will be discussed in the section Molecular Mechanisms Governing SSC Developmental Fate to provide a molecular phenotype to the cell-based models of SSCs and spermatogenic lineage development. The section Spermatogonial Stem Cell Aging will discuss the mortality of SSCs and whether aging of SSCs and/or the SSC niche contribute to spermatogenic decline and the paternal age effect. Finally, the sections Transgenesis and Regenerative Potential of Spermatogonial Stem Cells and Translation to the Clinic will discuss ways in which the unique biology of SSCs can be exploited to produce transgenic animals and to treat male infertility.

SPERMATOGONIAL STEM CELLS AND SPERMATOGENIC LINEAGE DEVELOPMENT

Rodents

A_s Model of Spermatogonial Stem Cells

Working independently in the early 1970s, Huckins, Oakberg, and de Rooij proposed a linear model of spermatogenic lineage development for rodents, in which SSCs reside in a population of isolated spermatogonia located on the basement membrane of seminiferous tubules.[24–26] These cells were originally termed $A_{isolated}$ (A_{is}) or A_{stem} (A_s), but the terminology has gradually evolved and most investigators recognize A_{single} (A_s) as the term to describe stem cells in rodent testes. These A_s spermatogonia are rare, comprising 0.03% of all germ cells in the mouse testis;[5] they are evenly distributed along the basement membrane of seminiferous tubules, with a relatively large nuclear to cytoplasmic ratio and diffuse chromatin (lacking heterochromatin). A_s spermatogonia divide to produce A_{paired} (A_{pr}) spermatogonia that either undergo complete cytokinesis to produce two new A_s spermatogonia (self-renewal) or remain connected by an intercytoplasmic bridge and produce a chain of four $A_{aligned}$ (A_{al4}) spermatogonia at the next division. The A_{pr} to A_{al4} transition is considered by many in the field to represent a commitment to differentiate because there is no unequivocal evidence that A_{al} spermatogonia fragment to produce new A_s under steady-state conditions[5,27–32] (Figure 15.2(B)). Successive divisions produce chains of 8, 16, and

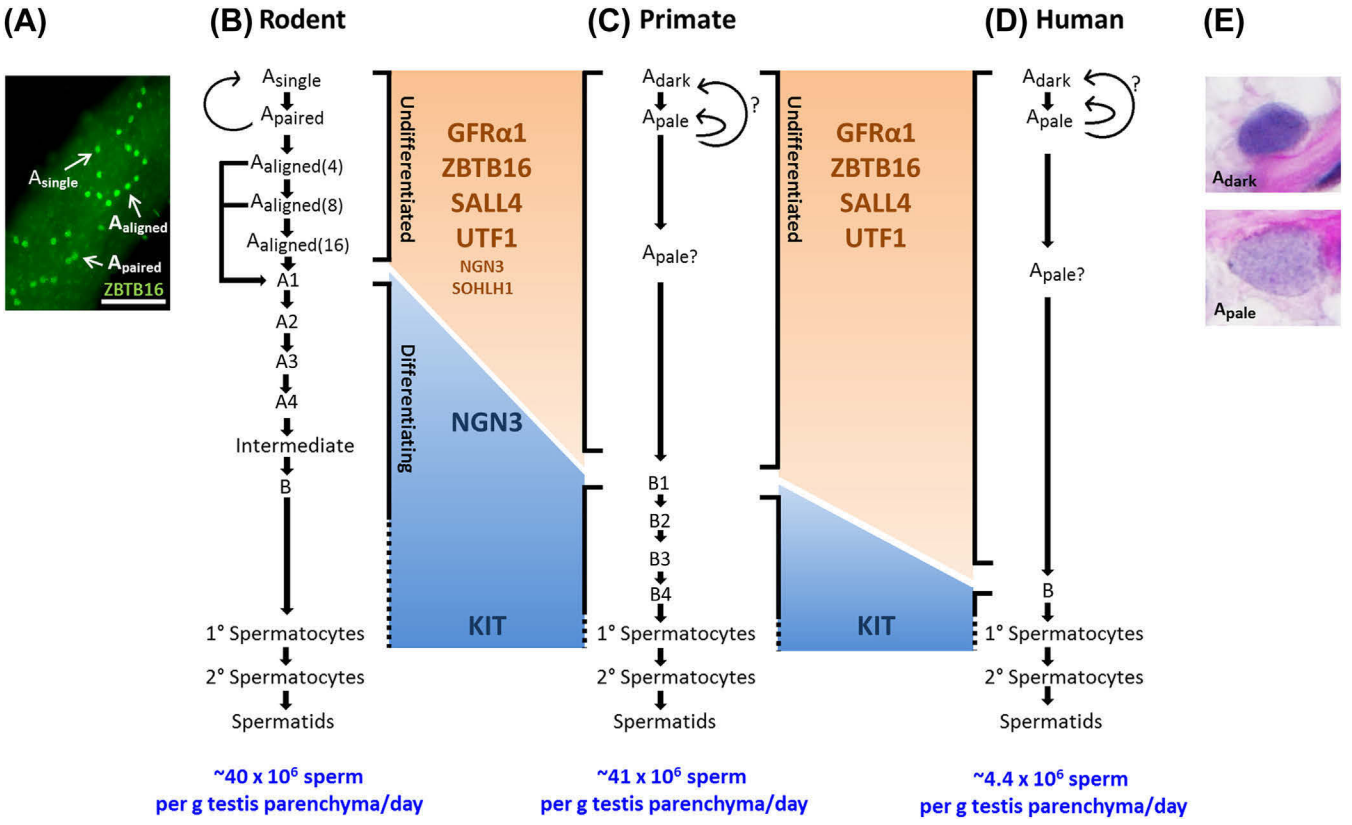

FIGURE 15.2 Current model of rodent, nonhuman primate, and human spermatogenesis. This figure is reproduced in color in the color plate section. (A) Whole-mount immunohistochemistry staining for ZBTB16 in adult mouse seminiferous tubules. ZBTB16$^+$ spermatogonia are identified as A_{single}, A_{paired} or $A_{aligned}$. Scale bar = 100 μm. (B) Rodent undifferentiated spermatogonia, including the SSC pool, is comprised of A_{single} and some A_{paired} spermatogonia and based on whole-mount staining analysis their phenotype is of GFRα1$^+$, ZBTB16$^+$, SALL4$^+$, UTF1$^+$, NGN3$^{+/-}$, and KIT$^-$. Transit amplifying progenitors include some A_{paired} spermatogonia and $A_{aligned}$ spermatogonia (chains of 4–16 cells), with a phenotype of GFRα1$^+$, ZBTB16$^+$, SALL4$^+$, UTF1$^+$, NGN3$^{+/-}$, and KIT$^{+/-}$. The differentiating spermatogonia that are made up of A1–A4, intermediate, and B spermatognia have a phenotype of GFRα1$^-$, ZBTB16$^-$, SALL4$^-$, UTF1$^-$, NGN3$^{+/-}$, and KIT$^+$. (C and D) In nonhuman primate and human testis, the undifferentiated spermatogonia are the type A spermatogonia that are designated A_{dark} and A_{pale} based on nuclear staining intensity with hematoxylin. The B spermatogonia are considered to be the differentiating spermatogonia and in nonhuman primates; they go through four divisions before producing primary spermatocytes, whereas in human there is only one division of B spermatogonia. (E) Sections of human testis stained using periodic acid-Shiff method and counterstained with hematoxylin to show nuclear morphology. ZBTB16, zinc finger and BTB domain containing 16; GFRα1, GDNF family receptor alpha-1; UTF1, undifferentiated embryonic cell transcription factor 1; SALL4, Sal-like 4; NGN3, neurogenin 3; SOHLH1, spermatogenesis and oogenesis specific helix-loop-helix 1. *Adapted from Ref. 75.*

sometimes 32 A_{al} spermatogonia. The synchronized development of spermatogonial clones is facilitated by protein and messenger RNA exchange via the intercytoplasmic bridges that connect the individual cells.[33] Types A_s, A_{pr}, and A_{al} spermatogonia are collectively termed type $A_{undifferentiated}$ (A_{undiff}) spermatogonia (Figure 15.2(B)) and may also appropriately be termed stem and progenitor spermatogonia. Here, we define "stem cells" as cells that have a virtually unlimited capacity for self-renewal and differentiation and "progenitors" as cells that have an undifferentiated appearance but a limited capacity to undergo self-renewing divisions before differentiating.

A precise balance between the self-renewal and differentiation fates among the population of A_{pr} spermatogonia would in principle provide the basis for homeostatic maintenance of the stem cell pool and continuous sperm production. Indeed, Huckins[34] observed after labeling with [^{3}H]-thymidine, "Many of these pairs were subsequently seen to divide again in a second wave of labeled mitoses to form a chain of A_{al} spermatogonia. On the other hand, other daughter cells of A_{is} divisions became separated from one another, and were therefore classified as two new A_{is} spermatogonia". Nakagawa and colleagues recently suggested that the stem cell pool might be expanded to include A_{al4} and possibly larger chains and that these larger clones may fragment to contribute to renewal of A_s spermatogonia and, thus, the stem cell pool. This mode of self-renewal was rarely observed under normal circumstances but occurred more frequently under conditions of regeneration during recovery from cytotoxic damage.[35,36]

Although A_s and A_{pr} spermatogonia are evenly distributed along the basement membrane of the seminiferous epithelium, the differentiation of A_{al} spermatogonia into A1 spermatogonia occurs at specific intervals along the seminiferous tubules and these intervals define the cycle of the seminiferous epithelium.[37,38] One complete cycle of the mouse seminiferous epithelium is depicted in Figure 15.3 and is comprised of 12 stages that are defined by specific associations of spermatogonia, spermatocytes, spermatids, and sperm. Differentiation to type A1 spermatogonia typically occurs from A_{al} chains of 4, 8, or 16 and always occurs at stage VIII of the seminiferous epithelium cycle in mice. This transition initiates a wave of spermatogenesis and is marked by reduced expression of stem and progenitor (A_{undiff}) markers such as GFRα1 (GDNF family receptor alpha-1) and ZBTB16 (zinc finger and BTB domain containing 16, also known as PLZF) and increased expression of differentiation markers such as KIT (also known as cKIT, CD117) (see Table 15.1; spermatogonial markers are discussed in greater detail below). KIT$^+$ A_s and A_{pr} spermatogonia are observed occasionally[35,39] and may indicate that differentiation to A1 spermatogonia can sometimes occur from these small clones, but these events are rare. In Figure 15.3, a clone of A_{al16} spermatogonia in stage VII differentiates (without a mitosis) to a chain of 16 A1 spermatogonia in stage VIII followed by sequential transit amplifying mitotic divisions to produce 32 A2 spermatogonia in stage IX, 64 A3 spermatogonia in stage XI, 128 A4 spermatogonia in stage I of the ensuing cycle of the seminiferous epithelium, 256 intermediate spermatogonia in stage II, 512 B spermatogonia in stage IV, and 1024 preleptotene spermatocytes in stage VI, which enter meiosis and migrate off the basement membrane. Two subsequent meiotic divisions will produce a total of 4096 haploid spermatids, in theory. Note that in the midst of this large clone (colored blue in the online material; open arrow in print), smaller clones underwent SSC self-renewal (black clone; black arrow in stage XI) as well as transit amplifying mitoses (red clone in the online material; arrowhead in print in stages X and I) that will fuel clonal expansion in the next cycle of the seminiferous epithelium. For example, the clone of 8 cells in stages I to VI (red clone in the online material; arrowhead in print) will divide to produce chains of 16 cells, 32 cells, and so on, in the next cycle of the seminiferous epithelium. The clone of two cells (black; black arrow) will either undergo cytokinesis to produce two new A_s spermatogonia or remain connected and produce a chain of four A_{al} spermatogonia at the next division.

Depending on whether the transition from A_{al} to differentiating A1 spermatogonia occurs from A_{al} chains of 4, 8, 16, or 32 spermatogonia, a clone generated from a single SSC can theoretically give rise to between 1024 and 8192 sperm.[40,41] Therefore, a single clone can fill a substantial portion of the seminiferous tubule and the series of successive clones fill in the layers of the seminiferous epithelium and collectively help to define the boundaries of individual stages (see cross-section and longitudinal section views in Figure 15.3). The actual yield of sperm is 50–80% less than what would be predicted by the clonal amplification scheme described above, due to apoptosis that occurs primarily in the A2–A4 spermatogonia in rodents.[26,42–44] The regulation of germ cell loss in differentiating spermatogonia may be "density dependent" and mediated by p53, BCL-2 (B cell leukemia/lymphoma 2) family proteins, and FAS[45–48], as reviewed in Refs 49 and 50.

Each cycle of the seminiferous epithelium in mice takes 8.6 days, and four cycles are required for the differentiation from A1 spermatogonia to mature sperm. Thus, approximately 35 days are needed to produce sperm from the pool of undifferentiated stem and progenitor spermatogonia. This concept is beautifully illustrated in a publications by Huckins and Oakberg[42] and Klein and coworkers in 2010.[51]

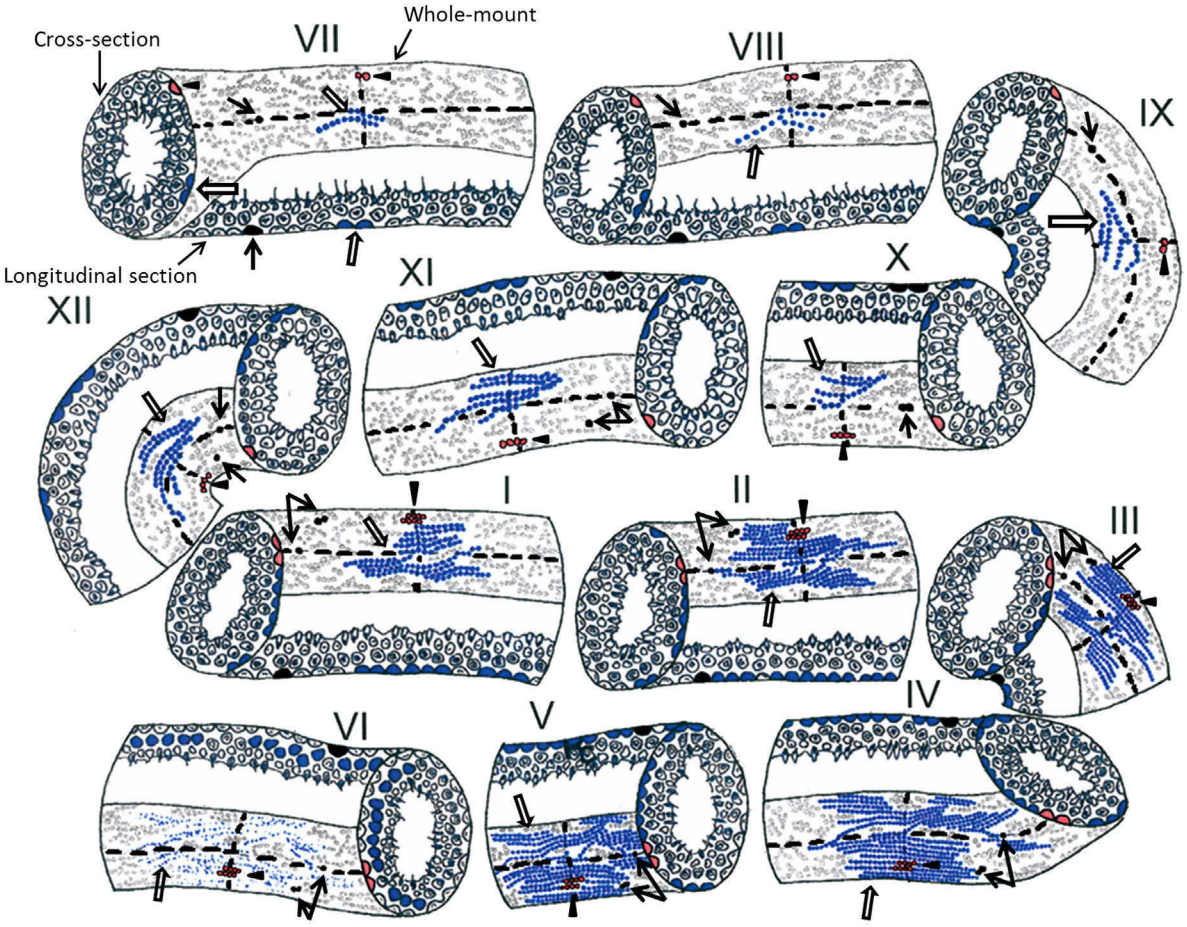

FIGURE 15.3 Schematic of mouse spermatogenic clone development by stage. This figure is reproduced in color in the color plate section. The seminiferous epithelial cycle of the mouse contains 12 stages (I-XII). Each stage is shown in cross-sectional, longitudinal, and whole-mount perspectives (labeled in stage VII). Three putative spermatogonial clones are highlighted in blue (open arrow), red (arrowhead), and black (black arrow). These clones are only representative examples and do not imply A_{single} spermatogonia divide only in stages X and I. A_{single} and A_{paired} spermatogonia are present in all stages of the seminiferous epithelium and tend to divide more frequently in early and late stages than middle stages. The dotted lines in the whole-mount perspective indicate planes of the cross-section and longitudinal section views. Cells that lie on the focal plane in the whole mount appear in the corresponding cross-section and longitudinal section views; this is indicated by matching color or arrows (arrows shown for stage VII only). For example, in stage VII, the red cell (arrowhead) is in the vertical line and therefore appears in the cross-sectional view. A black cell (black arrow) is in the horizontal line, so it is observed in the longitudinal section view. The development of three putative clones (blue/open arrow, red/arrowhead and black/black arrow) through one cycle of the seminiferous epithelium is shown. Stage VII: $A_{al\text{-}16}$ (blue/open arrow); A_{pair} (red/arrowhead); A_{single} (black/black arrow); stage VIII: A1 (clone of 16) (blue/open arrow); A_{pair} (red/arrowhead); A_{single} (black/black arrow); stage IX: A2 (clone of 32) (blue/open arrow); A_{pair} (red/arrowhead); A_{single} (black/black arrow); stage X: A2 (clone of 32) (blue/open arrow); $A_{al\text{-}4}$ (red/arrowhead); A_{pair} (black/black arrow); stage XI: A3 (clone of 64) (blue/open arrow); $A_{al\text{-}4}$ (red/arrowhead); A_{single} (x2) (black/black arrow); stage XII: A3 (clone of 64) (blue/open arrow); $A_{al\text{-}4}$ (red/arrowhead); A_{single} (x2) (black/black arrow); stage I: A4 (clone of 128) (blue/open arrow); $A_{al\text{-}8}$ (red/arrowhead); A_{single} and A_{pair} (black/black arrow); stage II: intermediate spermatogonia (clone of 256) (blue/open arrow); $A_{al\text{-}8}$ (red/arrowhead); A_{single} and A_{pair} (black/black arrow); stage III: intermediate spermatogonia (clone of 256) (blue/open arrow); $A_{al\text{-}8}$ (red/arrowhead); A_{single} and A_{pair} (black/black arrow); stage IV: Type B spermatogonia (clone of 512) (blue/open arrow); $A_{al\text{-}8}$ (red/arrowhead); A_{single} and A_{pair} (black/black arrow); stage V: Type B spermatogonia (clone of 512) (blue/open arrow); $A_{al\text{-}8}$ (red/arrowhead); A_{single} and A_{pair} (black/black arrow); stage VI: primary spermatocytes (lifting off the basement membrane) (blue/open arrow); $A_{al\text{-}8}$ (red/arrowhead); A_{single} and A_{pair} (black/black arrow). *Reproduced with permission from Ref. 28.*

While 12 stages of the seminiferous epithelium have been described for mice,[38,52] 14 have been described for rats,[6,20] 12 for rhesus macaques,[53,54] and 6 for humans.[55–57] The identification of specific stages may be somewhat arbitrary and investigator dependent. Using a modified staining approach, Muciaccia and colleagues[58] recently were able to identify 12 distinct stages of the human seminiferous epithelium as defined by acrosomal development based on acrosin staining. These authors suggested that staging should be normalized among species to facilitate interspecies comparisons.

A0/A1 Model of Spermatogonial Stem Cells

It is important to recognize that there is an alternative model to describe stem cells and spermatogenic lineage development in rodents that predated the A_s model and

TABLE 15.1 Germ Cell Markers in Rodents, Nonhuman Primates, and Humans

	Rodents			Nonhuman Primates			Humans				
	RT-PCR	IHC ICC	TR	RT-PCR	IHC ICC	TR	RT-PCR	IHC ICC	TR	Comments	References
GERM CELL[a]											
ID4	X	IHC-XS IHC-WM								A_s	185
EXOSC10							X	IHC-XS		A_d	376
OCT2								IHC-XS		A_d	377
FGFR3							X	IHC-XS		A_d, A_p	378
EGR3		IHC-WM								A_s, A_{pr}	379
NANOS2	X	IHC-XS IHC-WM	X					IHC-XS		A_s, A_{pr}, A_d, A_p, B-RS	183,187,188,311,380
GFRa1		IHC-XS IHC-WM	X		IHC-XS			IHC-XS		A_s-A_{al4}, A_d, A_p, B1, B2	68,86,156,159,162,166,207,381
UTF1	X	IHC-XS					X	IHC-XS		A_s-A_{al4}, A_d, A_p, BM	186,382
ZBTB16		IHC-XS IHC-WM	X		IHC-XS		X	IHC-XS		A_s-A_{al}, A_d, A_p, B1, B2, BM	39,68,86,91,153,160,206,207,296
FOXO1		IHC-XS IHC-WM								A_s-A_{al}	290
SALL4		IHC-XS IHC-WM			IHC-XS			IHC-XS		A_s-A_{al}, A_d, A_p, B	39,87,206,296
POU5F1 (OCT4)		IHC-XS IHC-WM	X	X	IHC-XS					A_s-A_{al}, BM	383–385
POU3F1		IHC-XS		X						Spermatogonia on the BM	285
RET		IHC-XS								A_s-A_{al}	386
BCLB6		IHC-XS		X						Rare cells on the BM. RS in stage 7	197,281
LHX1		IHC-XS		X						Rare cells on the BM. RS in stage 7	185
ETV5		IHC-XS		X						On the BM	197
NANOS3	X	IHC-XS IHC-WM								A_s-A1	183,187,311,387
SOX3		IHC-XS								A_s-A_{al}	388

(Continued)

TABLE 15.1 Germ Cell Markers in Rodents, Nonhuman Primates, and Humans—cont'd

	Rodents			Nonhuman Primates			Humans				
	RT-PCR	IHC ICC	TR	RT-PCR	IHC ICC	TR	RT-PCR	IHC ICC	TR	Comments	References
GPR125		IHC-XS IHC-WM	X		IHC-XS		X	IHC-XS ICC		A_s-A_{al}, rare cells on the BM	153,206,207,389,390
LIN28 (TEX17)		IHC-XS IHC-WM			IHC-XS			IHC-XS		A_s-A_{al}, rare cells on the BM	184,391
UCHL1 (PGP9.5)		IHC-XS			IHC-XS		X	IHC-XS		Spermatogonia, cells on the BM	153,206,207,392
NGN3		IHC-XS IHC-WM	X		IHC-XS					A_s-A_{al}, A_p, B1–4, PS	68,93,388,393
SOHLH1	X	IHC-XS IHC-WM								A_{pr}-RS	293,294
SOHLH2	X	IHC-XS IHC-WM								A_{pr}-RS	286,287,293,294
CBL							X	IHC-XS		BM	394
DSG2							X	IHC-XS		BM	394
SAGE1								IHC-XS		B	377
TRA-1-81					IHC-XS					Rare cells on the BM	206,395
MAGEA4					IHC-XS			IHC-XS		BM, some spc	156,206,207,220,383
SNAP91							X	IHC-XS		BM, some spc	394
RBM	X	IHC-XS						IHC-XS		A_s-A4 A_d, A_p, B-RS	396–400
RARγ	X	IHC-XS IHC-WM								A_{al}-A1	401
KIT	X	IHC-XS IHC-WM	X	X	IHC-XS		X	IHC-XS		A_{al}-RS A_p, B1–4, PS, BM	39,68,94,95,157,159,161,163,165, 207,402, unpublished data
STRA8	X	IHC-XS IHC-WM	X							A_s-RS	403–405
TAF4b		IHC-XS		X						A_s-Spc	291

DAZL	X	IHC-XS			IHC-XS		X	IHC-XS		A_s-Spc	152,153,406,407
VASA		IHC-XS			IHC-XS		X			A_s-RS	153,159,206,408,409
mRNA-221/222		IHC-XS	X							On the BM	198
mRNA-146	X									Undifferentiated spermatogonia	302
mRNA-20	X	IHC-XS	X							Undifferentiated spermatogonia	303
mRNA-106a	X	IHC-XS	X							Undifferentiated spermatogonia	303
miRlet7		IHC-XS								On the BM	305
CELL SURFACE[b]											
GFRa1		IHC-XS IHC-WM	X		IHC-XS			IHC-XS		A_s-A_{al4}, A_d, A_p, B1, B2	68,86,156,159,162,166,207,381
KIT	X	IHC-XS IHC-WM	X	X	IHC-XS		X	IHC-XS		A_{al}-RS A_p, B1–4, PS, BM	39,68,94,95,157,159,161,163,165, 207,402
THY-1 (CD90)			X			X		IHC-XS	X		68,139,157,159,171,207,410 unpublished data
CDH1 (CD324)		IHC-XS IHC-WM	X							A_s-A_{al}	90,411
CD9		IHC-XS	X					IHC-XS	X	BM	138,141,156,179
CD29 (β1-integrin)			X				X	IHC-XS		BM	170,229,402
CD49f (α6-integrin)			X		IHC-XS		X	IHC-XS ICC	X	BM	157,159,163,170,207,209,402,410 unpublished data
EPCAM		IHC-XS	X				X		X		139,151,153,179,412 unpublished data
SSEA4					IHC-XS	X		IHC-XS	X		157,159,206,210,395
GPR125		IHC-XS IHC-WM	X		IHC-XS		X	IHC-XS ICC		A_s-A_{al}, rare cells on the BM	153,206,207,389,390

X denotes that the technique was used.

IHC-XS, immunohistochemistry cross-section; IHC-WM, immunohistochemistry whole-mount; ICC, immunocytochemistry; TR, transplant; A_s, A single spermatogonia; A_{pr}, A paired spermatogonia; A_{al}, A aligned spermatogonia; A_d, A dark spermatogonia; A_p, A pale spermatogonia; B, type B spermatogonia; Spc, spermatocytes; RS, round spermatids; PS, pachytene spermatocytes; BM, basement membrane.

[a]*We attempted to sort the germ cell markers in the order of their expression from undifferentiated to differentiating spermatogonia.*

[b]*Examined with flow cytometry or FACS experiments. FGFR3 and TRA-1-81 are also cell surface markers but not included here because the have not been studied in the context of flow cytometry or FACS.*

continues to influence our understanding of spermatogenesis in higher primates today (discussed below). The A0/A1 model was described by Clermont and Bustos-Obregon in 1968 and identified five types of A spermatogonia (Figure 15.4).[59] A1 spermatogonia were found in stages II–VIII and were arranged in clusters of 4, 8, and sometimes 16 cells (similar to A_{al} spermatogonia in the A_s system). The A1 cells divided sequentially to produce A2 cells in stage IX, A3 cells in stage XII, and A4 cells in stage XIV of the cycle of the rat seminiferous epithelium. A4 cells divided at stage I of the ensuing cycle, and the authors proposed that they yielded two types of spermatogonia: intermediate and new type A1. Thus, the pool of A1–A4 spermatogonia collectively exhibited the requisite self-renewal and differentiation functions that define stem cells. The authors also recognized a fifth type of spermatogonia (representing 20% of all type A spermatogonia), termed A0, that were arranged as isolated or paired cells, had a darkly stained fine granular chromatin, and rarely divided. Because A0 did not appear to actively self-renew, they were considered to be "reserve stem cells" that could "rebuild the spermatogonial population" after noxious insult. This notion was supported by observations of Dym and Clermont that A0 cells were resistant to radiation-induced damage and could repopulate the seminiferous tubules with A1 spermatogonia that were depleted by radiation treatment.[60] Correlations between the A0/A1 model and A_s model can be inferred because the clonal arrangement of cells in both models has been clearly described (Figure 15.4).

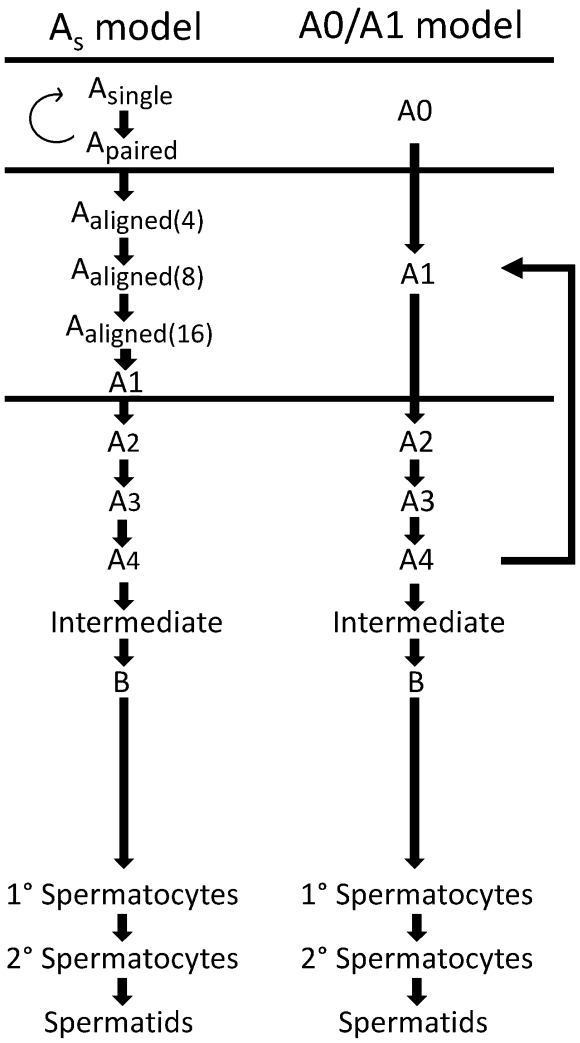

FIGURE 15.4 Comparison of the A_{single} (A_s) model and the A0/A1 model. In the A_s model, the stem cell activity is thought to reside in the A_s population. The A_s spermatogonia divide to produce A_{paired} (A_{pr}) spermatogonia that either undergo complete cytokinesis to produce two new A_s spermatogonia (self-renewal) or remain connected by an intercytoplasmic bridge and produce a chain of four $A_{aligned}$ (A_{al4}) spermatogonia at the next division. In the A0/A1 model, by clone size, these cells would correlate to the A0 spermatogonia. The A0 are thought to be the reserve stem cells that can regenerate spermatogenesis after noxious insult. In the A0/A1 model, the stem cell activity is thought to reside in the A1–A4 spermatogonia pool. The A4 spermatogonia can either produce intermediate or new A1 spermatogonia. The A1 pool correlates to A_{al4}-A1 spermatogonia in the A_s model. When the A_{al4} divide, they give rise to A_{al8} and A_{al16}. The chain of A_{al16} then differentiates into A1 spermatogonia, which will go through several meiotic divisions to eventually produce sperm.

Some differences between the A_s model and A0/A1 model appear to be semantic. A0 spermatogonia appear to correspond to A_s and A_{pr} spermatogonia and the A1 spermatogonia in the A0/A1 system appear to correspond to chains of 4, 8, and 16 $A_{aligned}$ spermatogonia in stages II–VII of the A_s system as well as the differentiating A1 at stage VIII. The critical difference in these models that is not semantic is the identity of the stem cell pool, A_s spermatogonia versus A1–A4 spermatogonia.

Regarding the A0/A1 reserve stem cell model, Oakberg[25] argued that (1) "it is difficult to visualize the selective value of a system which relegates 20% of the type A spermatogonia" to an inactive role; (2) derivation of A1 spermatogonia from A4 spermatogonia that have heterochromatic granules associated with the nuclear membrane would "involve dedifferentiation of cells well-advanced" in the developmental sequence; and noted that (3) the mechanisms that gave rise to chains of A1 spermatogonia were not demonstrated in the study of Clermont and Bustos-Obregon.[59] In this regard, the most compelling evidence supporting the A_s model are the following:

(1) A0 are mitotically active. All undifferentiated A spermatogonia, including A_s and A_{pr}, labeled after repeated administration of [^{3}H]-thymidine. Some isolated stem cells retained label for long periods, but eventually became mitotically active, giving rise to labeled A_{pr} and A_{al}, leading to a gradual disappearance of label.[61]

(2) There is a 10-fold reduction in the population of undifferentiated A_s, A_{pr}, and A_{al} spermatogonia coincident with the appearance of A1 spermatogonia after stage VIII. This population rebuilds until stage VIII of

the ensuing cycle, suggesting that A1 arise from A_{undiff} spermatogonia.[24–26]

(3) The number of intermediate spermatogonia in stage IV is twice the number of A4 in stage II, suggesting that all A4 undergo mitosis to produce intermediate spermatogonia.[24,26]

Nonhuman Primates and Humans

A_{dark}/A_{pale} Model of Spermatogonial Stem Cells

Primate testes contain two morphologically distinct types of undifferentiated (Type A) spermatogonia, which were initially identified as A1 and A2 but are now more commonly classified as A_{dark} and A_{pale}, based on differences in nuclear architecture and staining with hematoxylin in histological sections (Figure 15.2(E)).[37,53,55,62,63] A_{dark} and A_{pale} are both found on the basement membrane of primate seminiferous tubules. However, in contrast to rodents (Figure 15.2(A) and (B)), there are limited data describing their molecular characteristics or clonal arrangement[53,55,64] and conflicting views on whether one or both populations function as active stem cells in steady-state spermatogenesis.[40,55,65–68]

Clermont initially proposed for nonhuman primates[53] and humans[55] that A1 (A_{dark}) were SSCs that undergo self-renewing divisions to maintain the stem cell pool and give rise to A2 (A_{pale}) that subsequently generate differentiated type B spermatogonia. He further proposed that A_{pale} and B spermatogonia were the progressively "more differentiated elements giving rise to preleptotene spermatocytes."[55] However, the same group subsequently revised this A_{dark} stem cell–A_{pale} progenitor model based on observations in the vervet monkey that A_{dark} failed to label with [^{3}H]-thymidine.[65] Since A_{dark} did not appear to self-renew under steady-state conditions, he proposed that A_{dark} and A_{pale} represented reserve and active stem cells, respectively.[63,65] In this reserve stem cell model (Figure 15.2(C)), spermatogenesis is maintained by the "active" pool of A_{pale} SSCs that self-renew to maintain the A_{pale} pool and differentiate to produce type B spermatogonia.[66,69] It is not known whether A_{pale} undergo exactly one self-renewing division or whether they undergo additional division(s) before differentiating into B1 spermatogonia (Figure 15.2(C)). Similar to the A0/A1 model in rodents, Clermont proposed that the reserve A_{dark} SSCs are quiescent and are only activated when the A_{pale} pool is depleted by noxious insult.[65] This notion is supported by observations of de Rooij and colleagues, who noted that X-irradiation caused a rapid decline in A_{pale} spermatogonia. A_{dark} exhibited a delayed decline in numbers that correlated with a resurgence in the numbers of A_{pale} and B spermatogonia.[70] A similar progression of spermatogonial depletion and recovery after X-irradiation has been described in humans.[71–73]

Since Clermont's 1969 study,[65] six additional studies have been performed to quantify the mitotic activity of type A spermatogonia in monkeys. While all reported labeling ([^{3}H]-thymidine, BrdU (bromodeoxyuridine), PCNA (proliferating cell nuclear antigen) staining) in A_{pale} spermatogonia, only four reported labeling in A_{dark} spermatogonia, ranging from 0.6% to 18%.[63,74–77] Consistent with the results of Clermont,[65] two studies failed to observe labeling in the A_{dark} fraction.[78,79] Therefore, the question of whether A_{dark} divide in the adult testis and actively participate in spermatogenesis under normal conditions can still be debated. It is important to note that all of these previous studies were conducted with a single administration of ^{3}H-thymidine or BrdU and most evaluated only a few stages in the cycle of the seminiferous epithelium. Thus, studies similar to those that Huckins performed in rats,[61] with repetitive or chronic administration of the mitotic label to adult monkeys followed by a period of washout are needed to identify slow cycling cells that retain label for an extended period of time—a characteristic that is suggestive of stem cell activity.[80–82] This is a study that could be performed in nonhuman primates as a model for stem cell activity in man.

There is limited information about how dark and pale descriptions of nuclear morphology correlate with molecular phenotype or clone size. A_{dark} and A_{pale} appear to be present in equal numbers in the testes of monkeys and men.[53,55,75,78,83] While A_{dark} are equally distributed along the length of the seminiferous epithelium (similar to rodent A_s and A_{pr}), A_{pale} exhibit a more active, stage-dependent proliferation that results in peak numbers at mid-cycle (stages VII–IX, similar to rodent A_{al}) that precedes the appearance of differentiating B1 spermatogonia in stage X.[74,78] Using three-dimensional reconstruction of serial cross-sections[53] and camera lucida drawings of whole-mount seminiferous tubules,[65] Clermont and colleagues concluded that A_{dark} and A_{pale} existed only in even-numbered clusters, with pairs and groups of four being the most frequent. The arrangement of even-numbered groups suggests a clonal development scheme similar to that described for rodent spermatogonia. The observation of groups of four indicates that there can be at least two transit amplifying divisions that precede differentiation to B1 spermatogonia in primates. Van Alphen and colleagues described the dynamics of spermatogonial depletion and regeneration following low-dose radiation treatment in rhesus macaques.[54,70,84] In the regenerating seminiferous epithelium, the authors observed that both A_{dark} and A_{pale} could be observed as clones of 1, 2, 4, 8, and ≥16.[84] These observations may suggest that undifferentiated spermatogonia in primates can fluctuate between dark and pale phenotypes during clonal expansion, because the only way for an A_{dark} spermatogonia (which do not divide) to attain a chain size of 2, 4, 8, or 16 would be through an A_{pale} intermediate

that does divide. If that is true, is it reasonable to assign different "stemness" identities to these spermatogonial types? Alternatively, it is possible that A_{dark} spermatogonia do divide in the germ cell depleted testes, as has been previously reported for juvenile monkey testes,[85] but that has not been formally demonstrated.

In 2009, we conducted a study to begin unraveling the molecular phenotype of A_{dark} and A_{pale} spermatogonia in rhesus macaques with the objective to gain additional insights about the identity of the stem cell pool.[68] We found that A_{dark} and A_{pale} spermatogonia share many characteristics with A_s, A_{pr} and A_{al} spermatogonia in rodents (Table 15.1). Almost all A_{dark} and A_{pale} expressed the consensus stem and progenitor markers, GFRα1 and ZBTB16. NGN3 (neurogenin 3), which appears to be a transition marker in rodents,[35] is absent in A_{darks}, but expressed by 50% of A_{pales}. Similarly, KIT, the marker of spermatogonial differentiation in rodents, is absent in A_{darks} and expressed in only 25% of A_{pale} spermatogonia. Based on these results, all A_{dark} and 75% of A_{pale} spermatogonia in nonhuman primates share the phenotype GFRα1$^+$/ZBTB16$^+$/NGN3$^{+/-}$/KIT$^-$, which is comparable to the phenotype of most A_s and A_{pr} spermatogonia in rodents (Figure 15.5). GFRα1 and SALL4 (Sal-like 4) appear also to be conserved in A_{dark} and A_{pale} spermatogonia of human testes.[86,87] Based in part on their phenotypic similarities, we have speculated that A_{dark} and A_{pale} spermatogonia may be the same cell at different stages of the cell cycle (i.e., G0 versus G1/S/G2/M).[68] If this is true, it would help to explain why A_{dark} rarely label with [^{3}H]-thymidine or BrdU in the adult testis. In contrast to the adult, A_{dark} do appear to undergo mitosis in the developing testes of juvenile monkeys.[85]

If we accept that (1) rodent SSCs reside primarily in the pool of A_s and A_{pr} spermatogonia, (2) that molecular phenotype predicts function, and (3) that phenotype/function relationships are evolutionarily conserved, then it may be reasonable to suppose that most A_{dark} and A_{pale} spermatogonia in primate testes are stem cells because they are phenotypically similar to rodent A_s and A_{pr} spermatogonia. If that is true, the stem cell pool in rhesus macaques, where A_{dark} and A_{pale} comprise 4% of germ cells in the testis,[68,83,88,89] is substantially larger than in rodents, where A_s and A_{pr} comprise 0.09% of germ cells in the testes.[5,68] Using the same logic, the pool of transit-amplifying progenitor spermatogonia in primates is substantially smaller than rodents. Cells with the phenotype GFRα1$^+$/ZBTB16$^+$/NGN$^+$/KIT$^{+/-}$ comprise 89%

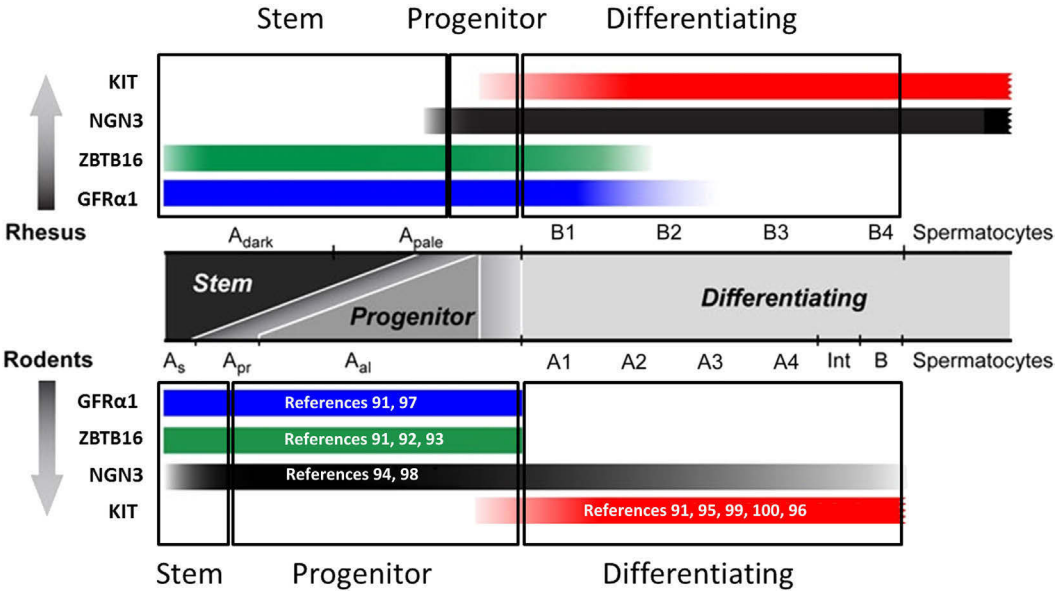

FIGURE 15.5 Correlation between established molecular markers of rodent SSCs (GFRα1, ZBTB16, NGN3, KIT) and morphologically classified spermatogonia (A_{dark}, A_{pale}, B) in the rhesus testis. Sections of adult rhesus testes were evaluated by immunohistochemistry for GFRα1, ZBTB16, NGN3, and KIT.[68] Subsequently, sections were counterstained by the PAS-Hematoxylin to reveal nuclear morphology and identify A_{dark} and A_{pale} spermatogonia, as well as differentiating B spermatogonia. Bars (GFRα1, ZBTB16, NGN3, KIT) indicate the extent of marker expression in the adult spermatogenic lineage for the rhesus monkey (top) rodent (bottom).[95] Stem, Progenitor, and Differentiating functional descriptors, based on rodent data, are indicated in the middle gray bar and may identify rhesus spermatogonia with corresponding phenotype and function. The transitions from stem to progenitor or progenitor to differentiating are noted by gradient shading between these functional categories. Citations for rodent marker expression profiles are indicated within each bar and are as follows.[90–94,96–99] The following abbreviations are used for rodents: A_s, A_{single}; A_{pr}, A_{paired}; A_{al}, $A_{aligned}$. Reproduced by permission of Oxford University Press based on data from Hermann et al.[68] (rhesus, top) or review of the literature (rodents, bottom). Citations for rodent marker expression profiles are indicated within each bar and are as follows.[90–99] Abbreviations: ZBTB16, zinc finger and BTB domain containing 16; GFRα1, GDNF family receptor alpha-1; NGN3, neurogenin 3.

of undifferentiated A_s, A_{pr} and A_{al} spermatogonia in rodents[5,90–99] compared to only 25% of undifferentiated A_{dark} and A_{pale} in primates (Figure 15.5; reviewed in Refs 68 and 75). Despite these differences, adult mice and adult monkeys produce the same number of sperm per gram of testis parenchyma per day (~40 million/gram/day).[1–3] Thus, it appears that pools of stem and progenitor spermatogonia in rodents and primates employ different strategies to meet similar biological demands for sperm production. While rodents have a relatively small stem cell pool with many transit amplifying divisions that precede meiosis, primates have a large stem cell pool with relatively fewer transit amplifying divisions (compare Figure 15.2(C) and (D)).

Based on the onset of KIT expression (Figure 15.2) differentiating A1–A4, intermediate and B spermatogonia in rodents appear to be roughly equivalent to B1–B4 spermatogonia in monkeys. Depending on whether there are zero, one, or two transit amplifying divisions that precede the transition of A_{pale} spermatogonia to B1 in nonhuman primates,[63,78] a single stem cell can theoretically give rise to between 64 and 256 terminally differentiated sperm. In contrast to the four generations of differentiating B spermatogonia in primates, humans have only one generation of B spermatogonia[37,55,57] and this may explain why sperm production in the human testis is approximately eightfold lower than in primates (Figure 15.2(D)). Using the same clonal development logic as we applied to nonhuman primate spermatogenic lineage development above, a single human stem cell can theoretically give rise to between 8 and 32 sperm. The small clone sizes in humans[40] also explain why stages of the seminiferous epithelium in humans are described with a patchy or spiral organization along the length of the seminiferous epithelium.[57] The individual clones are too small to fill an entire segment of seminiferous tubules. Therefore, each human seminiferous tubule cross-section can contain two to three different stages of the seminiferous epithelium.

EXPERIMENTAL TOOLS FOR STUDYING SPERMATOGONIAL STEM CELLS

In rodents, SSCs are identified by the following characteristics, in increasing order of experimental stringency: (1) SSCs are undifferentiated cells with a large nuclear to cytoplasmic ratio and diffuse chromatin located on the basement membrane of seminiferous tubules; (2) SSCs are arranged as individual cells or possibly small chains of interconnected cells; and (3) SSCs have the capacity to regenerate spermatogenesis after toxic insult (e.g., chemotherapy or radiation) or when transplanted into the testes of infertile recipients. Prior to the 1990s, undifferentiated spermatogonia, including SSCs, were identified in histological sections and whole-mount preparations of seminiferous tubules by their morphological characteristics and topographical arrangement along the seminiferous tubule basement membrane. Using these criteria and without the benefit of modern molecular tools, researchers established the models for stem cell maintenance and spermatogenic lineage development that have withstood the tests of time and experimental inquiry. Unfortunately, classic morphologic assessments are becoming a lost art, which is due in part to the fact that they are tedious and time consuming and in part because modern experimental tools (as described below) are now available. Insights about the molecular features of stem and progenitor spermatogonia began to emerge in the 1990s from investigations that employed SSC transplantation as the criterion standard stem cell assay combined with germ cell sorting techniques and genetic or experimental mouse models of male infertility.

Spermatogonial Stem Cell Transplantation

The SSC transplantation technique was developed by Brinster and colleagues in 1994.[100,101] In those pioneering studies, germ cells were isolated from the testes of donor mice and then transplanted into the seminiferous tubules of infertile recipient mice. Examination of recipient testes several months after transplantation revealed normal spermatogenesis and functional sperm arising from donor germ cells. Using transgenic donor cells that express reporter genes (e.g., *lac*Z or *Gfp*) allows the investigator to unequivocally identify colonizing events that arise from the donor cells (see result from *lacZ* donor in Figure 15.6(A) and Figure 15.7(A)–(C)). Infertility in the recipient mouse can be due to a genetic defect (e.g., the *Dominant white spotting* (*W*) mice with a mutation in the KIT receptor tyrosine kinase[102]), or it can be induced experimentally (e.g., Busulfan chemotherapy or radiation treatment[101,103]). SSC transplantation can be used to evaluate the spermatogenic potential of any experimental cell population[104] (e.g., testis cell suspensions, cultured cells, germ cells derived from induced pluripotent stem cells). By definition, only a stem cell can produce and maintain a colony of spermatogenesis and each colony arises from the clonogenic proliferation and differentiation of a single SSC.[105–107] The one-SSC/one-colony relationship was clearly demonstrated by Zhang and colleagues,[106] who mixed testis cells from two different transgenic donor mouse lines in a 1:1 ratio before transplantation. Genotyping of donor-derived colonies from recipient mice indicated that colonies were from one transgenic donor or the other, but not of mixed origin, providing that cells were transplanted at a low enough concentration to prevent colony merging. Kanatsu-Shinohara and coworkers[107] marked donor SSCs with retroviral vectors that integrate randomly into the target

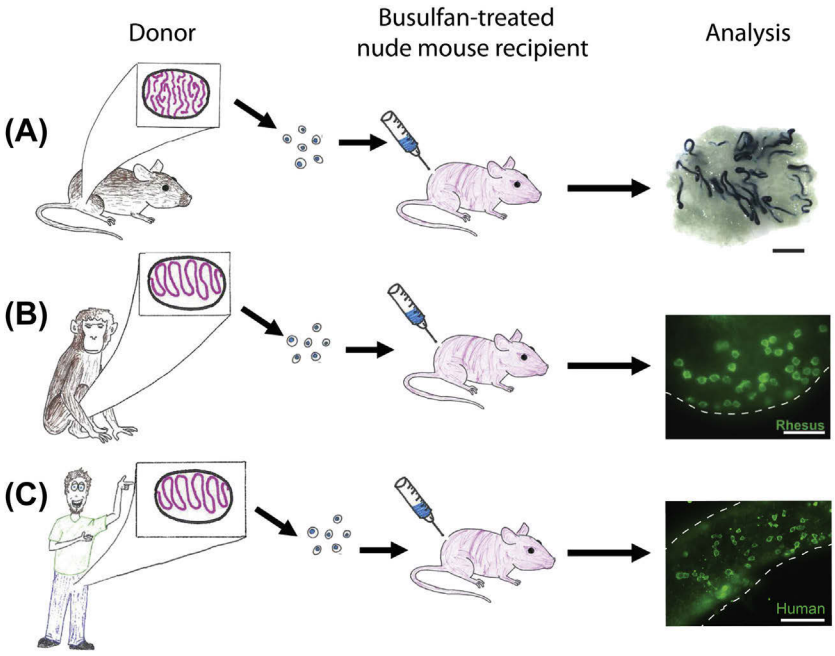

FIGURE 15.6 Spermatogonial stem cell (SSC) transplantation assay. This figure is reproduced in color in the color plate section. The only way to definitively identify SSCs is to analyze SSC function. (A) In this case, a testicular cell suspension is produced from a *lacZ* donor mouse. The cells are injected into the testis of an infertile recipient mouse and the results are analyzed two to three months after transplantation for *lacZ* expressing donor spermatogenesis (dark colonies). Scale bar = 2 μm. (B, C) Xenotransplantation of nonhuman primate (B) and human (C) testicular cells into busulfan treated nude mouse can be used as an assay to identify SSCs by their ability to initiate the early steps of spermatogenesis in the mouse testis. The colonies of spermatogonia are identified by staining with anti-primate antibody that only recognizes primate cells. The colonies of SSCs are identified by the following criteria—they are located on the basement membrane of the seminiferous tubules, they have four or more cells, and the cells are within 100 μm of each other. Scale bars = 100 μm. In the B and C analysis panel, broken lines indicate the borders of seminiferous tubules.

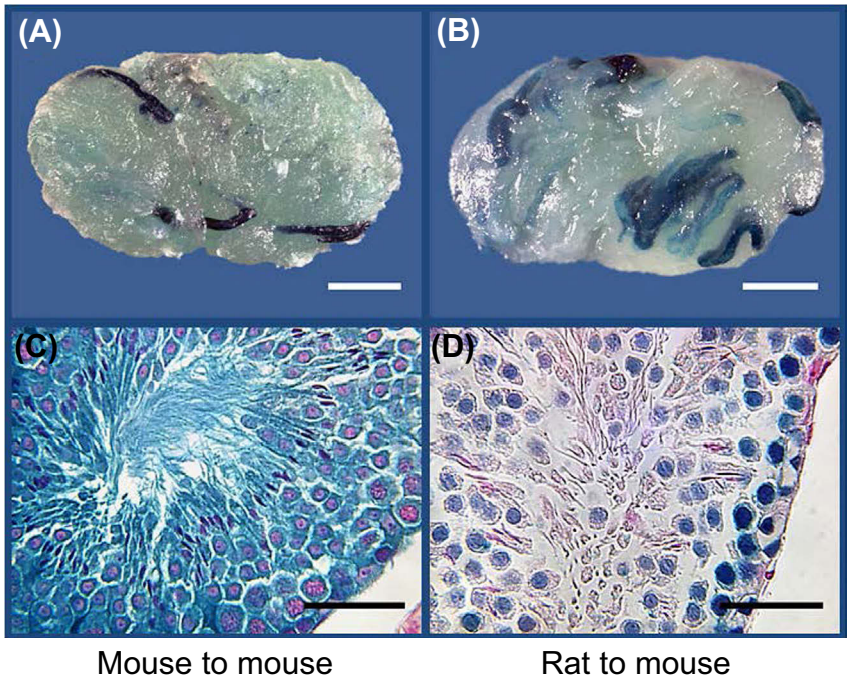

FIGURE 15.7 Macroscopic and histologic appearance of recipient testes transplanted with mouse and rat adult donor testis cells expressing *lacZ*. This figure is reproduced in color in the color plate section. NCr nude (spontaneous mutant T cell deficient mice) recipient mouse testes were stained with X-gal 3 months after transplantation to visualize mouse (A and C) and rat (B and D) donor-derived spermatogenic colonies. Both mouse and rat donor testis cells generated complete spermatogenesis, characterized by multiple germ cell layers and mature spermatozoa, in recipient testes. Note that the lacZ stain is localized in the cytoplasm of ROSA26 mouse testis cells (C) and the nucleus of MT-lacZ rat testis cells (D). Nuclear fast red counterstain (C and D). Bar = 1 mm (A and B), 40 μm (C and D). *Reproduced with permission from Ref. 134.*

cell genome. Haploid germ cells were collected from independent colonies and used to fertilize eggs and create offspring. Offspring generated from the germ cells in the same colony had a common viral integration pattern, while offspring from different colonies had different integration patterns. Therefore, the SSC transplantation provides a quantitative functional assay to characterize SSC activity in any donor cell population.[104]

Mouse-to-mouse SSC transplantation has become routine and, as mentioned above, is the criterion standard for assaying spermatogonial stem cells.

Rat-to-rat transplantation has also been performed to quantify stem cell activity using recipients that were rendered infertile by chemotherapy,[108–111] genetic mutation,[112] or a combination of chemotherapy and genetic manipulation.[113] However, recipient preparation in rats

is more complicated than mice because rats are more sensitive to the systemic toxicity of chemotherapy[109] and radiation treatment appears to damage the somatic environment in rat testes.[114] In total, homologous species SSC transplantations have now been reported in mice, rats, pigs, goats, bulls, sheep, dogs, and monkeys, including the production donor-derived embryos or offspring in mice, rats, goats, sheep, and monkeys.[102,115–123]

For SSC transplant in rodents, the testes are accessed via a mid-ventral abdominal incision. The donor cells are injected via the efferent ducts into the rete testis space, which can be visualized on the surface of the testis and is contiguous with all seminiferous tubules (Figure 15.8(A)–(C)).[124] Testis anatomy in large animals is different than in rodents, with the rete testis being more centrally localized and therefore more difficult to visualize and access. To address this challenge, Schlatt and colleagues pioneered the use of ultrasound-guided rete testis injection into rat, bovine, monkey, and human testes, ex vivo.[125] This technique has now been applied for in situ SSC transplantation in several large animal species, including goats, pigs, sheep, bulls, and monkeys (an example from monkeys is shown in Figure 15.8(D)–(F)).[118–123,126–129] In a recent ex vivo study, Faes and coworkers[130] demonstrated that this approach should be feasible in humans. In contrast to the method used in rodents, ultrasound-guided injection does not require surgery; the injection needle is simply inserted through the scrotal skin and testicular parenchyma into the rete testis space.

Spermatogonial Stem Cell Xenotransplantation

Homologous species transplantation as a routine bioassay for SSCs is only established in rodents. Animal and housing costs, difficulty in preparing infertile recipients, and animal-to-animal variability in outbred large animal species preclude the use of homologous transplantation in those species for quantitative assessment of stem cell activity. To address these challenges, xenotransplantation of cells from various species into the testes of infertile, immune-deficient recipient mice has emerged as an experimentally tractable and cost-effective alternative SSC assay.

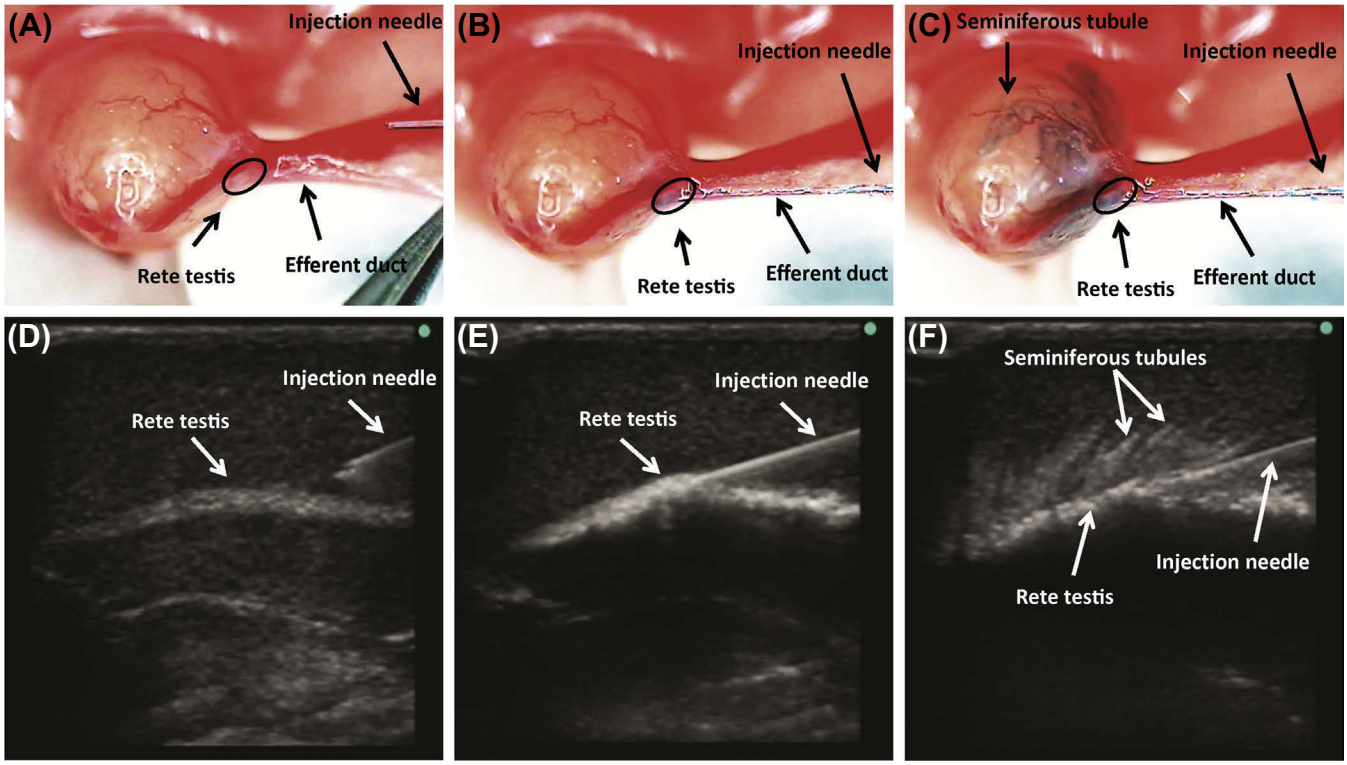

FIGURE 15.8 Testicular cell transplantation. This figure is reproduced in color in the color plate section. (A–C) In rodents, the testicular cells are injected via the efferent ducts into the rete testis space, which can be visualized on the surface of the testis and is contiguous with all seminiferous tubules. (C) Trypan blue is injected with the testicular cells to visualize the filling of the seminiferous tubules. (D–F) Testis anatomy in large animals is different than rodents, with the rete testis being more centrally localized and therefore more difficult to visualize and access. Therefore, ultrasound is used to guide injections. (D) Rete testis (echo-dense structure) is visible on ultrasound. The injection needle is inserted under ultrasound guidance through the scrotal skin into the rete testis space, which is continuous with the seminiferous tubules. (E) Positive pressure is applied to the needle, so the cells are slowly injected into the rete testis and seminiferous tubules. (F) The filling of the seminiferous tubules is observed using microbubbles.

Transplanted mouse SSCs produce chains and networks of spermatogonia on the basement membrane of recipient mouse seminiferous tubules by 2 weeks after transplantation, which subsequently differentiate to produce multiple layers of donor-derived germ cells and sperm within a few months after transplantation (Figure 15.9(A) and (B)).[104] In a similar fashion, rat SSCs can colonize the seminiferous tubules of infertile, immune-deficient recipient mice, giving rise to complete spermatogenesis (Figure 15.7(B) and (D)) and functional rat sperm, despite over 11 million years of evolutionary divergence between these rodent species.[131–134] Interestingly, spermatogenesis from transplanted rat SSCs proceeded according to the cycle of the rat seminiferous epithelium (a new population of A1 spermatogonia produced every 12.9 days) rather than the mouse (8.6 days per cycle, see above).[135] These results indicate that it is the germ cells and not the somatic environment that control the timing of spermatogonial differentiation. Furthermore, rat sperm produced in mouse seminiferous tubules were competent to fertilize rat oocytes by intracytoplasmic sperm injection (ICSI), giving rise to healthy offspring.[132] Thus, rat-to-mouse xenotransplantation can be used as a routine assay for rat SSCs,[108,134,136–142] which reduces animal costs and circumvents the challenges of preparing infertile recipient rats for transplantation. The reciprocal experiment, mouse SSCs transplanted into infertile, immune-deficient rats, also results in the production of donor-derived mouse sperm.[109] SSCs from hamsters, which diverged from mice over 16 million years ago,[143,144] can also colonize the testes of infertile, immune-deficient recipient mice and produce spermatogenesis. However, the hamster spermatids and sperm produced in recipient mouse seminiferous tubules were morphologically abnormal.[143]

Dobrinski and coworkers[145] investigated the effect of increasing phylogenetic distance between donor and recipient by transplanting testis cells from rabbits and dogs into the seminiferous tubules of infertile immune-compromised recipient mice. Donor rabbit and dog cells, which diverged from mice approximately 60 million years ago,[145,146] produced chains and networks of spermatogonia connected by intracellular bridges on the basement membrane of mouse seminiferous tubules. These colonizing events persisted for periods exceeding 1 year, but did not progress to produce complete

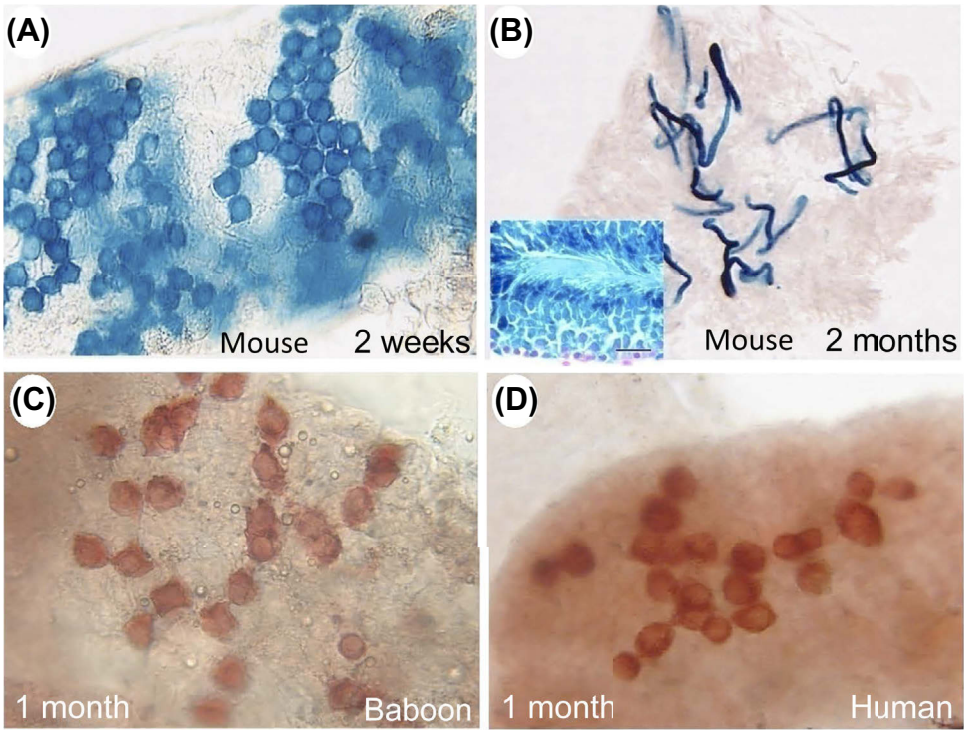

FIGURE 15.9 Colonization of recipient testis. This figure is reproduced in color in the color plate section. (A) Two weeks after transplantation of transgenic mouse donor cells (stained blue) into a recipient mouse seminiferous tubules, small groups of cells can be seen at low magnification. (B) Two months after the transplantation of mouse donor cells, the blue areas are dark in the center, indicating multiple layers of germ cells and development of spermatogenesis. (B inset) Cross-section of a transplanted mouse seminiferous tubule 2 months after transplant. Scale bar = 30 μm. (C and D) Detection of baboon and human germ cells transplanted into the seminiferous tubules of recipient mouse, using an antibody raised against baboon testis cells. One month after the transplant, clusters of baboon (C) and human (D) germ cells are located on the basement membrane of mouse tubules and exhibit characteristics of undifferentiated spermatogonia. The clusters are similar to the ones seen in mouse 2 weeks after the transplant. *Modified with permission from Refs 104 and 149.*

spermatogenesis, as meiotic cells did not emerge. Similar colonizing events have been reported after transplanting porcine, bovine, equine, primate, and human germ cells (Figure 15.9) into recipient mouse seminiferous tubules.[147–150] It is noteworthy that the colonizing events by these nonrodent cells appear very similar to early mouse colonies 2 weeks after transplantation (compare Figure 15.9(A)–(C) and (D)). Although donor germ cells from distantly related mammals do not produce complete spermatogenesis in mouse seminiferous tubules, they do exhibit several characteristics that are consistent with SSC activity: (1) they migrate to the basement membrane of recipient seminiferous tubules, (2) they proliferate to produce characteristic chains and networks of spermatogonia, and (3) they persist for a periods of time ranging from several months to more than 1 year. Based on these criteria, xenotransplantation to mouse seminiferous tubules is becoming a routine bioassay for nonhuman primate and human SSCs[68,148,149,151–157] and should have similar application for other species.

In contrast to mouse and rat donors, testis cells from most species will not have transgenic reporter genes (e.g., *lacZ* or *GFP*) to facilitate identification of donor-derived colonizing events. Therefore, it is necessary to generate species-specific antibodies[145,147,148,152] or preload the donor cells with a fluorescent marker (e.g., PKH26 or CFDA) prior to transplant.[158,159] Using these tools, SSC xenotransplantation can proceed in a manner very similar to mouse-to-mouse transplantation. Testicular tissue from the donor species is digested to produce a cell suspension that is then transplanted into the seminiferous tubules of infertile, immune-compromised recipient mice. Testes of recipient mice are analyzed between a few weeks and a few months after transplantation for colonies of spermatogenesis (Figures 15.7(A) and (B); 9(B)) or colonies of spermatogonia (Figures 15.6(B) and (C); 9(C) and (D)). When the fluorescent dye loading approach is used, earlier analysis (2–3 weeks) is required to minimize the dilution of dye through cell divisions.[158,159]

Molecular Characteristics of SSCs Learned from Mouse Genetic Models

Several mouse models exhibit male infertility with histology that indicates the absence of spermatogenesis in many or all tubules, a phenotype that may suggest defects in the spermatogonial compartment. Three examples are the spontaneous (luxoid mutant) or targeted deletion of the *Zbtb16* (*Plzf*) locus;[91,160] the *Dominant white spotting* (*W*) mice with a mutation in the KIT receptor tyrosine kinase;[94,161] and mice with overexpression or underexpression of the glial cell line derived neurotrophic factor, *Gdnf*.[162]

ZBTB16 encodes a zinc finger transcription factor that is expressed by a subpopulation of spermatogonia (including single, paired, and aligned) located on the basement membrane of seminiferous tubules.[91,160] Most seminiferous tubules in these mice had a Sertoli cell only phenotype (no germ cells) or had a severely disorganized seminiferous epithelium; SSC transplantation indicated that these defects were associated with a depletion of the SSC pool.[91,160] ZBTB16 is a widely accepted marker of SSCs and appears to be conserved in rodents, nonhuman primates, and humans (Figure 15.10(A)–(C)).

Similar to *Zbtb16* mutant mice, adult *W* (*Kit* mutant) mice are infertile with small testes and seminiferous tubules that lack spermatogenesis. This phenotype is due in large part to a failure of primordial germ cells to survive and proliferate during embryonic development. *Kit* mRNA and protein are also expressed by spermatogonia in postnatal testes,[94,161] but unlike ZBTB16, KIT is not expressed in SSCs. Systemic administration of an anti-KIT monoclonal antibody to block KIT signaling in adult mice resulted in rapid depletion of differentiating spermatogonia, while undifferentiated spermatogonia were unaffected.[161] Furthermore, by combining fluorescence-activated cell sorting with SSC transplantation, Shinohara and coworkers demonstrated that the majority of SSC activity resides in the KIT negative fraction of mouse testes.[163] KIT is now widely recognized as a marker of spermatogonial differentiation[95,161] and it is conserved in rodents, nonhuman primates, and humans.[68,157,161,164,165] Based on these studies, mouse SSCs can be partially defined by the phenotype ZBTB16$^+$/KIT$^-$.

GDNF is a distant member of the transforming growth factor-β (TGFβ) family that is secreted by Sertoli cells and signals through a receptor complex that includes the GDNF family receptor α1 (GFRα1) and the RET receptor tyrosine kinase that are expressed by spermatogonia.[162] *Gdnf*, *Gfrα1*, and *Ret* null mice are embryonic lethal, but Meng and coworkers demonstrated that mice with one *Gdnf*-null allele exhibited disrupted spermatogenesis, including some tubules with a Sertoli cell-only phenotype and apparent absence of spermatogonia. In contrast, overexpression of *Gdnf* led gradually to atrophy of the seminiferous tubules and accumulation of undifferentiated (KIT$^-$) spermatogonia.[162] Subsequent sorting and transplant studies revealed that some SSC activity is recovered in the GFRα1$^+$ fraction of rodent testis cell suspensions, confirming that GFRα1 is a marker of SSCs. However, SSC activity is also consistently recovered in the GFRα1$^-$ fraction, which suggests that the stem cell pool may be heterogeneous with respect to GFRα1 expression.[86,166,167] GDNF is the critical growth factor required to maintain rodent SSCs in culture,[110,140,168,169] as will be discussed in more detail below.

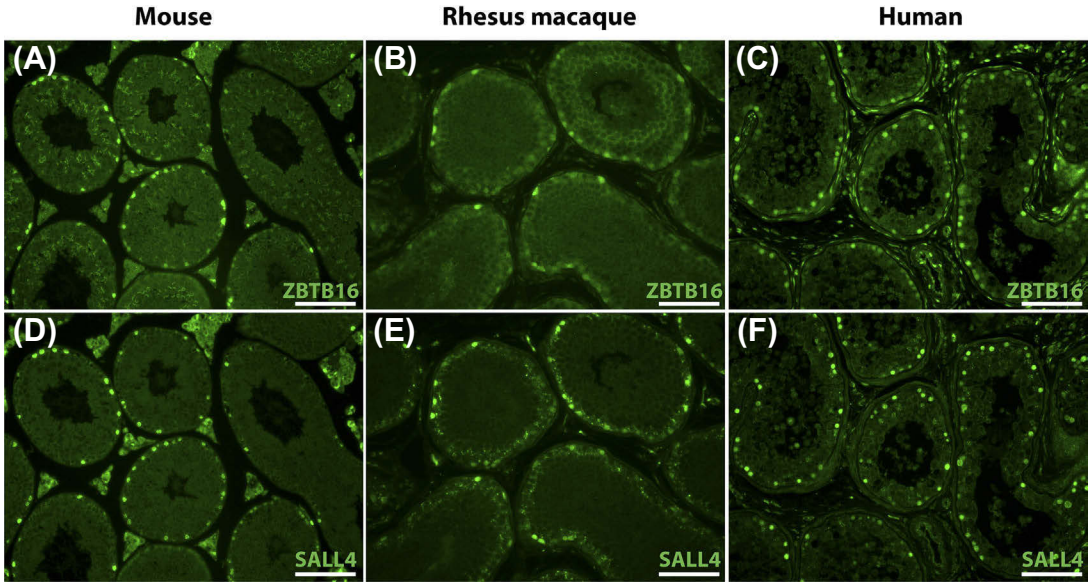

FIGURE 15.10 Conservation of stem and progenitor markers ZBTB16 and SALL4 from mouse to human. This figure is reproduced in color in the color plate section. ZBTB16 (A–C) and SALL4 (D–F) staining in cross-sections of seminiferous tubules from mouse, rhesus macaque, and human testes shows expression of these transcription factors on the basement membrane of the tubule. Both ZBTB16 and SALL4 are conserved markers from mouse to rhesus macaque to human. Scale bar = 100 μm. ZBTB16, zinc finger, and BTB domain containing 16; SALL4, Sal-like 4.

Molecular Characteristics of SSCs Learned from Sorting and Transplantation

As indicated in the KIT and GFRα1 examples above, the SSC transplantation technique is especially powerful for dissecting the molecular phenotype of functional SSCs when it is combined with fluorescence-activated cell sorting (FACS) or magnetic activated cell sorting (MACS) (Figure 15.11(A)). Briefly, a heterogeneous cell suspension is labeled with a vital dye (e.g., Rho123, Hoechst) or with a fluorescent (for FACS)- or paramagnetic microbead (for MACS)-tagged antibody raised against a cell surface antigen. The population is then fractionated based on the level of labeling using a FACS machine or magnetic field. To assess relative stem cell activity, each fraction is transplanted into infertile recipient mice and colonies of donor-derived spermatogenesis are typically quantified 2 months later (Figure 15.11(A)). Based on several studies using this approach, mouse SSCs are now characterized as a subpopulation of testis cells defined by the phenotype ITGB1 (β1-integrin, CD29)$^{+}$, ITGA1 (α6-integrin, CD49f)$^{+}$, THY-1 (CD90)$^{+}$, CD9^{+}, GFRα1^{+}, CDH1 (cadherin 1)$^{+}$, MCAM (melanoma cell adhesion molecule)$^{+}$, FZD3 (frizzled family receptor 3)$^{+}$, Hoechst side population^{+}, Rhodamine 123 (Rho123)^{low}, ITGAC (αv-integrin, CD51)$^{-}$, KIT (CD117)$^{-}$, MHC-I (major histocompatibility complex class I)$^{-}$, EPCAM (epithelial cell adhesion molecule)$^{low/-}$, CD45^{-}, ALDH (aldehyde dehydrogenase) activity^{-}[90,138,163,166,170–180] (Table 15.1). In nonhuman primates, the xenotransplantation technique was utilized to demonstrate that enhanced colonizing activity resides in the SSEA-4 (stage-specific embryonic antigen 4)$^{+}$, THY-1^{+}, CD45^{-} subpopulations of rhesus macaque testes.[68,159,181] Similarly, FACS and MACS sorting in conjunction with human-to-nude mouse xenotransplantation have revealed that human SSCs can be defined in part by the cell surface phenotype, CD9^{+}, EPCAM^{+}, SSEA-4^{+}, HLA-ABC^{-}, CD49e^{-}.[151,156,157] The approach using sorting and transplantation to define the molecular phenotype of functional SSCs is limited primarily to cell surface markers. Although it is feasible to sort a fixed cell suspension based on the expression of intracellular antigens, this method is not compatible with transplantation to evaluate stem cell function because the cells are not viable after fixation. Three studies have shown that it is also possible to isolate and enrich SSCs based on their cellular function (e.g., dye efflux, mitochondrial membrane potential, and aldehyde dehydrogenase)[139,174,177]

Immunohistochemistry to Validate SSC Markers

For candidate SSC markers that are not expressed on the cell surface, immunohistochemistry can be used to determine whether the marker has an expression pattern that is consistent with stem and progenitor spermatogonia. For example, if a candidate marker is expressed by cells located on the basement membrane of seminiferous tubules and co-expressed in cells with established SSC markers (e.g., ZBTB16, GFRα1, CDH1) that have been validated by transplantation,[90,91,160,166,177] then this expression pattern is consistent with SSCs. SALL4 (Sal-like 4) is a zinc finger transcription factor

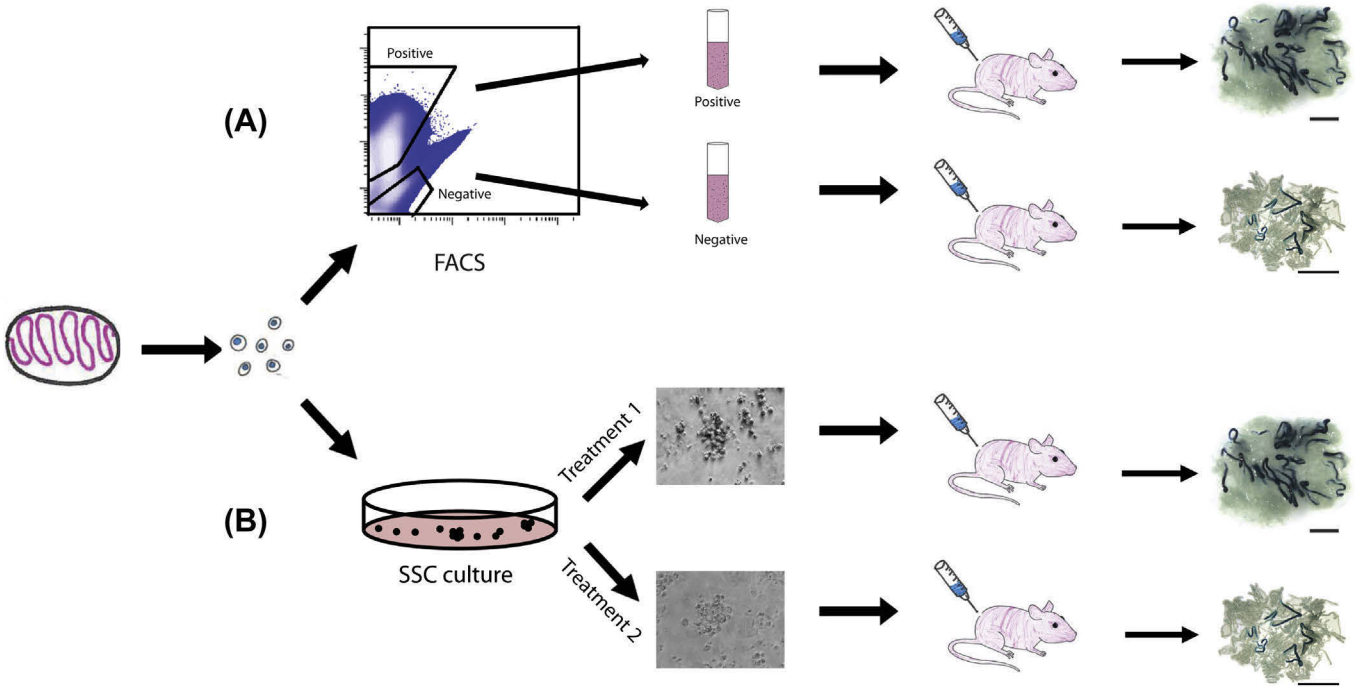

FIGURE 15.11 Experimental designs to study seprmatogonial stem cells. This figure is reproduced in color in the color plate section. SSC transplantation is used to analyze the effectivness of (A) fluorecsence-activated cell sorting (FACS) for a cell-surface protein or (B) a treatment of SSC culture. (A) FACS is used to fractionate the testicular cell population stained with an antibody against a cell-surface protein, based on marker$^+$ and marker$^-$ status. The sorted fractions can then be transplanted into infertile nude mice to determine which fraction contains the SSCs. (B) SSC cultures are used to identify factors important for SSC self-renewal and maintenance or for SSC differentiation. For example, GDNF can be removed from the culture media to determine whether or not it has an effect on SSC self-renewal and/or maintenance. The cells cultured with or without GDNF are then transplanted into testis of infertile recipient mice and, 2 months later, the testis are analyzed for colony formation. FACS, fluorescence activated cell sorting; SSC, spermatogonial stem cell; GDNF, glial cell-derived neurotrophic factor.

that is expressed by cells on the basement membrane of mouse, monkey, and human seminiferous tubules (Figure 15.10(D)–(F)) and exhibits nearly complete overlap of expression with ZBTB16 (Figure 15.10(A)–(C)). Based on these criteria, several markers of stem and/or progenitor spermatogonia have been identified, including ZBTB16, SALL4, GFRα1, CDH1, NANOS2 (nanos homolog 2), NANOS3, UTF1 (undifferentiated embryonic cell transcription factor 1), ID4 (inhibitor of DNA binding 4), LIN28 (TEX17), NGN3, GPR125 (G protein-coupled receptor 125), SOHLH1 (spermatogenesis and oogenesis specific helix-loop-helix 1), SOHLH2 (spermatogenesis and oogenesis specific helix-loop-helix 2), and UCHL1 (ubiquitin carboxyl-terminal esterase L1, also known as PGP9.5) (see references and other markers in Table 15.1). It is important to note that no precise molecular definition of the SSC pool has been determined; this may be a difficult task to achieve because there appears to be some molecular plasticity or heterogeneity in the stem cell pool,[39,90,182–184] which will be discussed in more detail below. In some circumstances, stem cell activity is even observed in cells expressing KIT,[182] which is an established marker of spermatogonial differentiation.[95,161] In histological cross-section, it is not possible to correlate molecular markers with clone size (i.e., A_s, A_{pr} or A_{al}) because observation is restricted to a single plane of view.

Whole Mount Immunohistochemistry to Define Molecular Characteristics of Undifferentiated A_s, A_{pr}, and A_{al} Spermatogonia

Immunohistochemistry analysis in whole-mount preparations of seminiferous tubules allows the investigator to correlate molecular marker expression with clone size (i.e., A_s, A_{pr}, A_{al}). An example of this approach is shown in Figure 15.12, where mouse tubules are co-stained with SALL4 and KIT (Figure 15.12(A), (D) and (G)) while monkey and human tubules are co-stained with UTF1 and KIT (Figure 15.12(B), (C), (E), (F), (H) and (I)). In Figure 15.12(G), smaller clones of undifferentiated spermatogonia exhibit a SALL4$^+$/KIT$^-$ phenotype, while larger clones of transit amplifying differentiating spermatogonia are SALL4$^-$/KIT$^+$. A similar progression is observed in monkeys and humans with small clones of spermatogonia exhibiting an undifferentiated phenotype (UTF1$^+$/KIT$^-$) and larger clones exhibiting a more differentiated phenotype (UTF1$^-$/KIT$^+$). To date, no molecular marker has been identified with expression restricted to

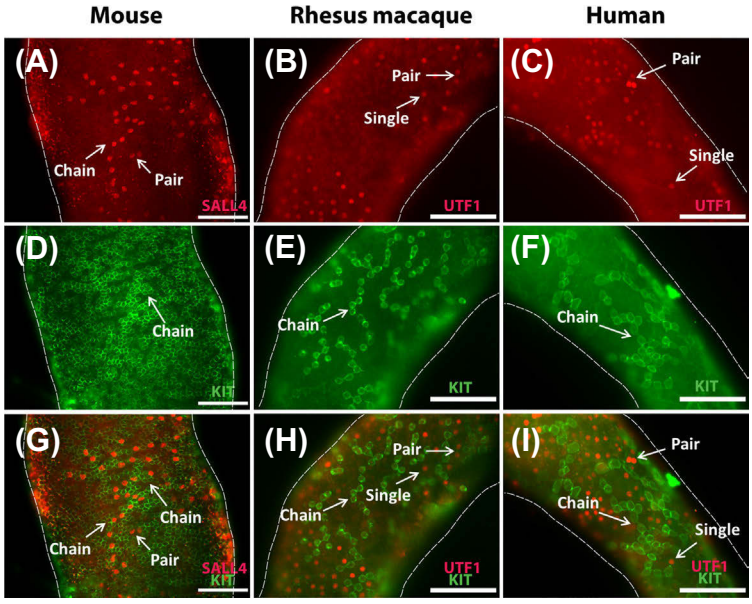

FIGURE 15.12 Whole-mount immunohistochemistry of seminiferous tubules from mouse, rhesus macaque, and human testes. This figure is reproduced in color in the color plate section. Whole-mount immunofluorescence analysis of the undifferentiated spermatogonia marker, SALL4 (A), differentiating spermatogonia marker, KIT (D) and co-staining of SALL4 and KIT (G) in mouse. Whole-mount immunofluorescence analysis of undifferentiated spermatogonia marker UTF1 (B, C), differentiating spermatogonia marker KIT (E, F), and co-staining of UTF1 and KIT (H, I) in the rhesus macaque (B, E, H) and human (C, F, I). Arrows indicate spermatogonia arranged as single cells, pairs, and larger chains for each species. Scale bar = 100 μm. SALL4, Sal-like 4; UTF1, undifferentiated embryonic cell transcription factor 1.

transplantable SSCs. However, whole-mount analyses indicate that the ID4 protein is restricted to A_s spermatogonia[185] and GFRα1, NANOS2, and UTF1are restricted to small clones of A_s, A_{pr} and A_{al4} spermatogonia.[35,39,186–188] Generally, the stem cell pool is considered to reside in the pool of A_s and some of the A_{pr} spermatogonia, whereas larger chains of A_{al} are thought to be committed to differentiation. However, a number of investigations have revealed that clones of rodent spermatogonia exhibit some degree of phenotypic and functional heterogeneity.[90,183,188] GFRα1 is not expressed by all A_s[39,86] and NGN3 is expressed by a subset of transplantable stem cells.[184,189,190] Furthermore, phenotypic heterogeneity has been observed within individual clones of spermatogonia, in which some individual cells within a clone appear different from the rest of the clone.[39,86,90,184,191] The functional significance of the phenotypic heterogeneity is not known, but these observations raise the question of whether clone size alone is adequate to define the stem cell pool. Nakagawa and colleagues suggested that A_{al} clones as large as 16 may be capable of fragmenting and contributing to the stem cell pool.[35]

Spermatogonial Stem Cell Culture

SSC culture has many important applications for human health, animal management, and also basic stem cell research. Culturing SSCs provides new approaches to investigate the molecular mechanisms and cell-signaling pathways that regulate SSC function. It also allows amplification of SSC numbers, which can be beneficial for increasing cell numbers prior to transplantation into testes and may serve as a foundation for experiments to develop a system for in vitro spermatogenesis.

In rodents, SSCs can be maintained in long-term culture with significant expansion in numbers. Cultured SSCs remain competent to produce spermatogenesis and restore fertility upon transplantation.[110,113,140,168,169,192] Several factors were critical to the establishment of long-term SSC cultures. First, methods were needed (e.g., FACS or MACS and/or differential attachment and replating) to enrich the SSCs and remove somatic cells that overproliferate and overwhelm the culture. Second, development of a serum-free, defined medium facilitated the discovery of essential growth factors, specifically the necessity of GDNF to maintain and expand rodent SSCs in culture.[168,192,193] The effects of GDNF in mice and rats is enhanced by the addition of soluble GFRα1 (the receptor for GDNF) and fibroblast growth factor 2 (FGF2).[140,169] Third, STO (SIM mouse embryo-derived thioguanine and ouabain resistant) fibroblast or MEF (mouse embryonic fibroblast) feeder cells are often needed for the survival of rodent SSCs. Although it is possible to maintain mouse SSCs in feeder-free conditions.[194] SSC cultures are often established from mouse pup testes (5–12 days postpartum) because SSCs are enriched at this stage of development due to the absence of differentiating germ cells and because SSCs are predisposed to active cell cycles.[195] However, SSC cultures can be established from all ages (from neonates to adults).[168,169]

Stable SSC culture provides a valuable tool for dissecting mechanisms that regulate SSC renewal and differentiation. For example, through withdrawal and addition of GDNF to mouse SSC cultures in combination with the transplantation assay, it was confirmed that GDNF is an important growth factor for SSC cultures (this experimental design is represented schematically in Figure 15.11(B)) and that GDNF action is mediated by SRC (v-src avian sarcoma)

family kinase/AKT (protein kinase B)-dependent pathways.[196,197] Additionally, the use of a gene specific siRNA enables temporary knockdown of the target gene.[198,199] Thus, through genetic manipulation and transplantation of cultured SSCs, it is possible to identify regulatory pathways required for SSC self-renewal and differentiation. In addition, Yeh and colleagues[200] suggested that SSC culture can be used as a quantitative assay for SSCs based on their observation that there is a linear relationship between the number of germ cell clusters formed in culture and the number of colonies that are produced by transplantation. Elhija et al. reported that testicular cells from 7-day-old mice could be expanded in a three-dimensional soft agar culture system and differentiated to produce post-meiotic germ cells, including morphologically normal sperm.[201] Additional studies are needed to confirm that the resulting sperm are functionally competent.

Several groups have reported extending the SSC culture to large animal species[202–206] and a number of laboratories have even reported culturing human SSCs,[153–155,207–211] including three from the testes of prepubertal patients.[153,155,209] These human culture studies are promising, but they are also challenged by the inability to evaluate cultured cells by homologous species transplantation. To date, each laboratory has used a different approach to culture human SSCs and different methods to assess outcomes. These results need to be replicated in other laboratories and evaluated using robust markers of human spermatogonia and by xenotransplantation to nude mice. Ideally, SSC cultures in humans can serve several purposes. First, similar to rodents, it could be a valuable tool to dissect the molecular-mechanism and cell-signaling pathways that regulate SSC self-renewal and differentiation. Second, it could theoretically be employed to amplify the number of SSCs available for experimentation or clinical application. Third, it could be used to establish a system for in vitro spermatogenesis. The production of spermatozoa in vitro would revolutionize assisted reproductive techniques and would allow the production of spermatozoa without the need for SSC transplantation.

Xenografting

In contrast to SSC transplantation, which involves disaggregation of SSCs from their cognate niches, testicular tissue grafting maintains the integrity of the stem cell/niche unit. Dobrinski, Schlatt, and their colleagues demonstrated that testicular tissues obtained from newborn mice, pigs, and goats could produce complete spermatogenesis when grafted under the skin of nude mice[212] and later reported the production of offspring from sperm obtained after ectopic (under the skin) grafting of mouse testicular tissue.[213] Testis tissue from prepubertal rhesus macaques also produced complete spermatogenesis with fertilization competent sperm after ectopic xenografting into nude mice.[214] Several groups have reported xenografting of human tissue to nude mice, and so far none have observed complete spermatogenesis or haploid gametes.[215–220] However, if the xenograft approach is optimized for humans, it could provide a tool for evaluating the effects of therapies or toxic insults on spermatogenic lineage development in humans or other large animals, using a mouse host. In addition, the xenografting approach may provide an option to preserve and/or restore fertility of boys who are at risk for infertility (e.g., due to cancer treatment). Thus, a testicular tissue that contains only undifferentiated stem and progenitor spermatogonia at the time of biopsy could potentially differentiate to produce fertilization competent haploid human sperm in a mouse host. Survival and spermatogenesis from adult testicular tissue grafts have been less successful to date than immature grafts.[221] Two studies also evaluated autologous testicular grafting in marmoset monkeys,[222,223] and reported that complete spermatogenesis was obtained in orthotopic (in the scrotum), but not in ectopic (under the skin) grafts. Perhaps spermatogenic development from autologous human testicular tissue grafts will be more successful than the human-to-mouse xenografts that have been studied to date.

THE SPERMATOGONIAL STEM CELL NICHE

An SSC niche is a complex entity that is comprised of cells, extracellular matrix, and soluble factors that regulate stem cell dynamics and spermatogenic lineage development. SSCs are in close proximity with elements at the basement membrane of the seminiferous tubules, including Sertoli cells and peritubular myoid cells, which line the inside and outside of the basement membrane, respectively (Figure 15.13).[28,224] These cells secrete the basement membrane components and the adhesion molecules that mediate the interaction of SSCs with their environment.[225] Sertoli cells are polarized columnar epithelial cells that support the SSCs and differentiating germ cells by providing nutrients and mediating external signals to support spermatogenesis.[226] Tight junctions between the Sertoli cells are the anatomical basis of the blood–testis barrier (BTB), which divides the seminiferous epithelium into basal and adluminal compartments and plays an important role in the regulation of germ cell differentiation[227] (Figure 15.13(A)). Sertoli cells must mediate the migration of germ cells from the basal compartment, across the blood–testis barrier to the adluminal compartment without disturbing the integrity of the BTB. This process, described in detail by Smith and Braun,[228] involves the establishment of a new blood–testis barrier beneath a clone of differentiating germ cells before the old BTB degenerates.

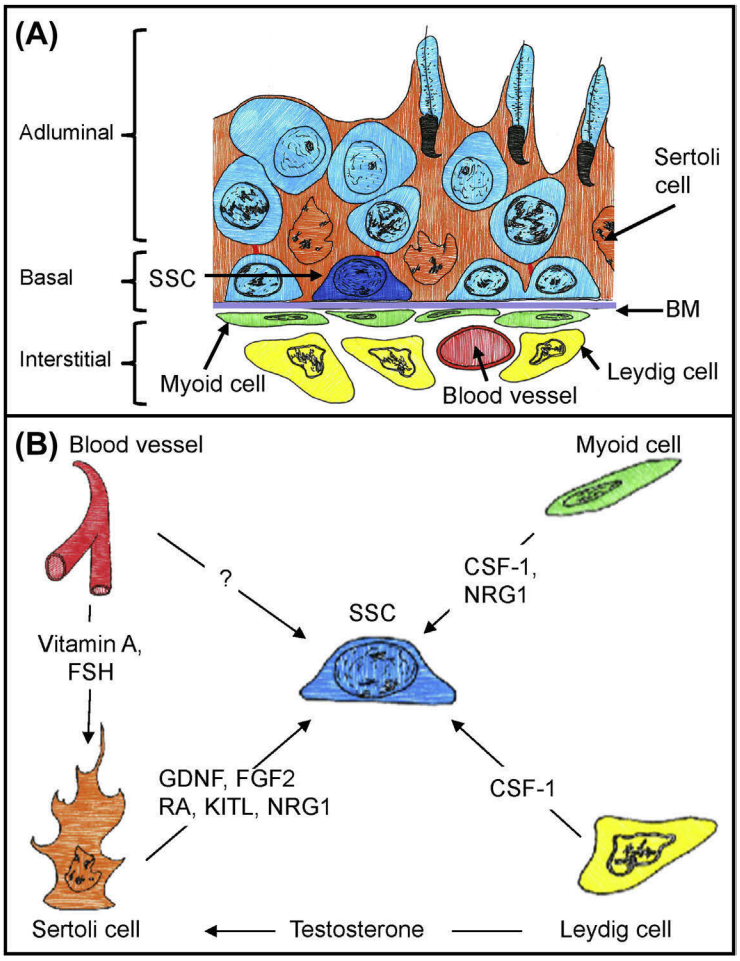

FIGURE 15.13 Spermatogonioal stem cell (SSC) niche. The SSC is diagrammed in its physical niche (A) surrounded by Sertoli cells and differentiating germ cells within the seminiferous tubule. Niche components outside the tubule itself include myoid cells, blood vessels, and Leydig cells. The components of the niche and some factors known to be provided by each are shown in (B). Although some factors are known to act directly on the SSC, such as GDNF, others, such as testosterone, are important for spermatogenesis but act indirectly on the SSC (see Chapter 16). SSC, spermatogonial stem cell; BM, basement membrane; FSH, follicle-stimulating hormone; GDNF, glial cell-derived neurotrophic factor; FGF2, fibroblast growth factor 2; RA, retinoic acid; KITL, KIT ligand; NRG1, neuregulin 1; CSF-1, colony stimulating factor 1. *Modified with permission from Ref. 28.*

In the SSC transplant paradigm, SSCs must migrate from the seminiferous tubule lumen to reach a niche on the basement membrane of seminiferous tubules. In an elegant series of experiments, Shinohara's laboratory used the transplant assay to identify factors important for migration, homing, and attachment to the SSC niche. Their results indicate that ITGB1 expression, both by SSCs and by Sertoli cells, is essential for homing to the seminiferous tubule basement membrane, in vivo. In addition, ITGB1 is required for attachment to laminin, a principle basement membrane component, in vitro.[229] The combination of ITGA6 and ITGB1 comprise a laminin receptor and both molecules are expressed by SSCs.[163] While ITGB1 is essential for attachment to the basement membrane, a separate study from the Shinohara group indicated that the small G-protein, RAC1 (RAS-related C3 botulinum substrate 1), is required for migration across the BTB.[230] The authors reported that passage through the BTB is the key factor that limits homing efficiency, which may explain why transplant efficiency in pup recipient testes that do not have a BTB is significantly greater than in adult testes.[115] *Rac1*-deficient SSCs did not colonize adult recipient testes but were able to engraft and regenerate spermatogenesis in pup recipient testes that lack a BTB.[230] Finally, the Shinohara group identified CXCL12 (chemokine (C-X-C motif) ligand 12) as a chemokine that attracts SSCs to the niche.[231] Overexpression of *Cxcl12* in the Sertoli cells of recipient mice leads to enhanced colonization from transplanted SSCs. The authors also identified CXCR4 (chemokine (C-X-C motif) receptor 4) as a chemokine receptor that is induced by GDNF and expressed more frequently by A_s and A_{pr} than A_{al} spermatogonia. Inhibition or conditional knockout of *Cxcr4* in donor SSCs significantly reduced colonizing efficiency in recipient testes.[232] Targeted disruption of the Ets variant gene 5 (*Etv5*) shows an apparently normal spermatogenesis at the initiation of puberty but eventually results in the loss of germ cells in the older animals.[233] ETV5 is a transcription factor that is expressed in Sertoli cells and loss of ETV5 seems to impair the ability of Sertoli cells to support spermatogonia. Decrease in CLDN5 (claudin-5) in mutant mice indicates that the defect is possibly due to disrupted BTB function.[234] In Sertoli cells, ETV5 is upregulated by FGF2 in vitro, which is important for SSC renewal in culture.[168,169,235]

Around the periphery of seminiferous tubules, undifferentiated spermatogonia (including SSCs) tend to be located in regions adjacent to the interstitial space, rather than regions that are adjacent to neighboring tubules.[97,236,237] Furthermore, using three-dimensional reconstruction of serial cross-sections and real-time in vivo imaging, Yoshida and coworkers demonstrated that undifferentiated spermatogonia were most frequently observed near blood vessels and migrated away from these sites during clone expansion.[97] These observations probably indicate that factors in the general circulation are required for maintaining the pool of stem and progenitor spermatogonia. Factors from the interstitium or blood may have direct effects on spermatogonia or indirect effects that are mediated by other cells, including Sertoli or myoid cells.

Niche Factors Supporting SSC Maintenance and Renewal

Sertoli cells are in direct contact with all SSCs and are the primary target in the testis of follicle stimulating hormone (FSH; see Chapter 16).[238] FSH stimulates the production of GDNF by Sertoli cells,[239,240] and this occurs in a stage-dependent manner with highest levels of FSH receptor expression,[241,242] FSH binding,[243] and GDNF production[244] between stages XIII and I of the rat seminiferous epithelium. These stage-specific increases in FSH receptor expression and GDNF production proceed the maximal proliferation of type A_s spermatogonia in stages I and II.[34] As indicated above GDNF is essential for maintenance and renewal of SSCs in vitro[110,140,153,168,169,192,245] and in vivo.[162,246] Therefore, FSH signaling via the Sertoli cells provides a mechanism for regulating the dosage of GDNF. GDNF signaling is thought to be mediated by both the PI3K (Phosphatidylinositol-4,5-bisphosphate 3-kinase)/AKT (also known as protein kinase B) pathway and the SRC and RAS (rat sarcoma)/ERK1/2 (extracellular signal-regulated kinases) pathway.[196,197,247,248]

WNT5A (wingless-related MMTV integration site 5A) was recently identified as a paracrine cell-extrinsic factor that participates in the control of SSC maintenance.[180] In vitro, WNT5A supports SSC maintenance at least in part by promoting cell survival. Although intracellular signaling mechanisms downstream of WNT5A are not well understood, the JNK (c-Jun N-terminal kinase) pathway was activated upon WNT5A stimulation in cultured spermatogonia, while blocking this signaling pathway negated the cell survival effect of WNT5A. This growth factor is expressed by Sertoli cells in vivo as well as in Sertoli cell lines, but not by germ cells. The WNT5A receptor is expressed by spermatogonia.[180] Interestingly, O'Shaughnessy and coworkers reported that WNT5A expression by Sertoli cells in vivo is transiently augmented within 5 days after a single administration of busulfan, which selectively kills spermatogonia.[249] GDNF transcripts are similarly upregulated after busulfan injection.[250,251] Because busulfan is known to kill SSCs, it is tempting to speculate that the loss of spermatogonia, including SSCs, may be sensed by Sertoli cells, which in turn secrete GDNF and WNT5A to protect and sustain the pool of undifferentiated spermatogonia.

The peritubular myoid cells play a role in spermatogonia maintenance by secreting CSF1 (colony stimulating factor 1). Gene expression profiling showed high expression of the receptor for CSF1 (CSF1R) in a $THY1^+$ spermatogonia population, whereas the CSF1 ligand is expressed in Leydig cells and a subset of myoid cells.[252] Subsequent experiments showed that the addition of CSF1 to SSC culture media significantly enhances the self-renewal of mouse SSCs.[252] Another study by Kokkinaki et al.[253] demonstrated that a $GFR\alpha1^+$ cell fraction isolated from prepubertal mouse testis had higher expression of CSF1R compared to other testis cells. It was suggested that CSF1 may cooperate with GDNF in supporting the maintenance of SSCs.

Niche Factors Promoting SSC Differentiation

In addition to SSC maintenance and renewal factors, Sertoli cells produce KIT ligand (KITL)[254,255] and retinoic acid (RA),[256,257] which are both required for spermatogonial differentiation. Similar to the regulation of GDNF expression in Sertoli cells, FSH regulates the expression of KITL by Sertoli cells[258,259] and KITL levels are highest in stages II-VI that precede the differentiation of A_{al} to A1 spermatogonia in stages VII and VIII[260] in rats. KITL stimulates spermatogonial proliferation[261] and prevents apoptosis.[45] Mice and rats that are maintained on a diet deficient in vitamin A (the dietary precursor of RA) are infertile with seminiferous tubules that contain only undifferentiated KIT^- spermatogonia.[95,256,257,262–264] Treatment of vitamin A deficient (VAD) animals with retinol or all *trans*-RA causes the upregulation of KIT protein expression[95] and restoration of spermatogenesis.[256,257,264] Vitamin A circulates primarily as retinol bound to RBP (retinol binding protein) and is taken up by Sertoli cells in a STRA6-dependent manner.[265] Interestingly, STRA6 is upregulated in later stages of the seminiferous epithelium (stage VII onward in mice) coinciding with the differentiation of A_{al} into A1 spermatogonia.[266] Thus, the coordinated expression of self-renewal (GDNF) and differentiation (KITL and RA) niche factors by Sertoli cells helps to determine the developmental fate of individual stem and progenitor spermatogonia.

Neuregulin 1 (NRG1), which is produced by peritubular myoid cells in rats[267] and Sertoli cells in mice,[268] is a member of the epidermal growth factor (EGF) family that is essential for spermatogonial proliferation and

meiotic initiation. Conditional inactivation of NRG1 in Sertoli cells of 14-day-old mice caused degeneration of the seminiferous tubules, reduced incorporation of BrdU in spermatogonia and spermatocytes, and increased apoptosis. Degenerated tubules contained only Sertoli cells and undifferentiated spermatogonia. In addition, FSH and RA induced proliferation of spermatogonia and initiation of meiosis was blocked in spermatogonia/Sertoli cell aggregation cultures from *Nrg1*$^{Ser-/-}$ mice.[268] Hamra and colleagues[269] demonstrated that rat germ cells express the NRG1 receptor ERRB3 and its signal transducer, ERRB2, and that NRG1 in combination with GDNF are required for formation of longer chains of spermatogonia ($A_{al16-32}$) in cultures of rat spermatogonia.

MOLECULAR MECHANISMS GOVERNING SSC DEVELOPMENTAL FATE

Signal Transduction

GDNF, acting through its cognate co-receptor complex GFRα1/RET on undifferentiated spermatogonia, is critical for SSC maintenance and/or self-renewal. Treatment of cultured SSCs with GDNF leads to rapid phosphorylation of AKT[197,248] and SRC family kinases.[196] GDNF-induced AKT phosphorylation is blocked by treatment with the PI3K inhibitor LY294002 or with SRC family kinase inhibitor SU6656.[197] Oatley and colleagues demonstrated that treatment with LY294002 resulted in loss of germ cell cluster formation in culture and increased apoptosis, suggesting that PI3K/AKT signaling is important for survival. In contrast, SU6656 caused reduced cluster size but no increase in apoptosis, suggesting that SRC family kinase signaling is important for self-renewal. Treatment with either inhibitor reduced the number of transplantable stem cells in culture.[197] Furthermore, Lee and coworkers demonstrated that conditional activation of AKT (myristoylated AKT) can replace GDNF and support long-term maintenance of functionally normal SSCs in culture.[248]

Lee and colleagues reported that the mitogen-activated protein kinase pathway (MEK/ERK) is also activated by GDNF treatment of cultured SSCs, but inhibition of this pathway using the MEK inhibitor PD098059 did not impact the amplification of germ cell clusters in culture.[248] In contrast, using the same inhibitor, He and coworkers[247] reported that PD098059 inhibited GDNF-induced ERK phosphorylation, BrdU incorporation (proliferation), and activation of *c-Fos* expression in the immortalized C18-4 SSC line.[270] Neither study reported the effects of MEK inhibition on transplantable stem cell activity. In vivo, Hasegawa and colleagues[271] observed a stage-dependent activation of ERK1/2 in GFRα1 positive spermatogonia, with higher ERK1/2 phophorylation in stages I–III and lower phosphorylation in stages VII–VIII of the seminiferous epithelium. These investigators injected the MEK inhibitor PD0325901 into the seminiferous tubules of adult mice and observed decreased expression of RET in ZBTB16 positive spermatogonia 6 h later. However, the study did not clearly demonstrate whether this reflected a direct effect on spermatogonia or was mediated indirectly by inhibition of MEK/ERK signaling in Sertoli cells. Therefore, the importance of MEK/ERK signaling for SSC function has not been clearly established.

KIT is expressed by differentiating spermatogonia and is required for the proliferation and survival of these cells.[45,261,272] Interestingly, KIT activates both the PI3K and MEK pathways,[261,273–275] similar to GDNF. Inhibition of either pathway using chemical inhibitors blocks the KIT-mediated proliferative response in differentiating spermatogonia. However, inhibition of MEK and PI3K pathways did not affect the anti-apoptotic effect of KIT signaling in these cells, indicating that the proliferative and anti-apoptotic effects are mediated by different pathways.[261] How is it possible that that self-renewal (GDNF) and differentiation (KITL) factors from the SSC niche signal through the same pathways in spermatogonia to cause different developmental outcomes? One possibility is that the PI3K and MEK pathways mediate proliferation in both undifferentiated and differentiating spermatogonia, but not necessarily "stemness." Future studies will likely identify co-regulatory molecules that modulate signaling through these and other pathways to tip spermatogonial developmental fate toward self-renewing divisions versus differentiating divisions.

Cell Cycle Regulation

Cell cycle regulation is clearly important for both self-renewing and transit-amplifying divisions. Lee and coworkers[276] discovered that GDNF stimulates RAS in an SRC family kinase-dependent manner and upregulates the expression of the cell cycle regulator, cyclin D2. Therefore, these investigators hypothesized that cyclin D2 may mediate the effects of GDNF on SSC self-renewal. However, Beumer and colleagues[277] had previously demonstrated that cyclin D2 was expressed specifically in spermatogonia at stage VIII of the seminiferous epithelium when A_{al} transition to A1 spermatogonia, suggesting the cyclin D2 may be involved in spermatogonial differentiation. Furthermore, cyclin D2 was absent in the undifferentiated spermatogonia in the testes of VAD mice, but was induced by administration of RA. Nonetheless, Lee and colleagues demonstrated that constitutive activation of h-RAS or expression of cyclin D2 + cyclin E1 could stimulate SSC proliferation in culture, even in the absence of cytokines (GDNF, bFGF, EGF). Neither cyclin D1 + E1 nor cyclin D3 + E1 could

mimic this effect, but both combinations could compensate for GDNF when cells were grown in the presence of bFGF and EGF. Cells with activated RAS or cyclin D2E1 transfectants (but not the cyclin D1E1or D3E1 transfectants) colonized recipient mouse testes and indicated a significant amplification of SSC numbers in culture. However, spermatogonial differentiation was disrupted and colonies eventually gave rise to tumors. The authors concluded that RAS and cyclin D2 are important and sufficient for SSC self-renewal, but speculated expression levels relative to other molecules is important to regulate the balance between SSC maintenance and tumorigenesis.[276] These activities may be balanced in part by co-regulation of p21 and p27 cyclin-dependent kinase inhibitors, which compromised proliferation of cultured SSCs.[278]

Cyclins are also important for proliferation of differentiating spermatogonia. Treatment of isolated spermatogonia with KITL results in activation of the PI3K/AKT and MEK/ERK pathways, and this is followed by migration of cyclin D3 to the nucleus and phosphorylation of the retinoblastoma (RB) protein. RB is a pocket protein that is expressed by proliferating spermatogonia in the testis and binds E2F family transcriptional regulators that are involved in transcriptional control and cell cycle progression.[272] These events were associated with cell cycle progression of KIT$^+$ spermatogonia and are abolished by treatment with the PI3K or MEK inhibitors.[261,275]

Two studies recently demonstrated that the retinoblastoma tumor suppressor gene (RB1) is required for maintaining the SSC pool[279,280]. Conditional inactivation of RB1 with VASA-Cre did not affect the establishment of the initial SSC pool or proliferation of GFRα1 positive A_s spermatogonia. However, the balance between self-renewing and differentiating divisions was disrupted, with all divisions giving rise to transit amplifying progenitors rather than new SSCs. In addition, RB1 depletion led to an overproliferation of ZBTB16$^+$, LIN28$^+$, and KIT$^+$ transit amplifying spermatogonia. However, this overproliferation was balanced by increased "density-dependent" apoptosis in RB1-deficient spermatogonia, so the number of spermatocytes was similar to controls. There was no block to terminal differentiation to sperm, so RB1-deficient mice were initially fertile, but gradual depletion of the SSC pool led to infertility by 2 months of age. In contrast, conditional inactivation of RB1 in transit-amplifying spermatogonia using NGN3-Cre or STRA8-Cre disrupted spermatogenic lineage development, leading to tumorigenesis.[280]

Transcriptional Regulation of SSCs

To identify intrinsic factors regulated by GDNF signaling in SSCs, Oatley and colleagues used a microarray approach to identify genes regulated by GDNF withdrawal and replacement in mouse SSC cultures.[281] Three transcription factors showing the greatest level of GDNF regulation included *Bcl6b* (B cell CLL/lymphoma 6, member b), *Lhx1* (Lim homeobox protein 1), and *Etv5* (Ets variant gene 5). *Bcl6b* encodes a BTB/POZ domain transcriptional repressor, similar to ZBTB16. Knockdown of *Bcl6b* expression in SSC cultures using siRNAs impaired SSC maintenance, increased apoptosis, and reduced colonizing activity following transplantation into infertile recipient mice.[197] In vivo, *Bcl6b*$^{-/-}$ mice have smaller testes compared to wild-type mice from the same age and contain seminiferous tubules with degenerating spermatogenesis.[281] *Lhx1* encodes a transcription factor that is important for anatomical morphogenesis in the mouse.[282] Knockdown of *Lhx1* in mouse SSC cultures with siRNA reduced the number of transplantable SSCs, suggesting that *Lhx1* is important for SSC maintenance.[197] ETV5 (also known as ETS related molecular; ERM) is an ETS family transcription factor that is expressed in several tissues, including the testis[283] where it is expressed by both Sertoli cells[233] and undifferentiated spermatogonia.[197] *Etv5* knockout animals produce the first round of spermatogenesis, but this is followed by progressive depletion of germ cells such that most tubules are devoid of germ cells by 10 weeks of age. Furthermore, *Etv5*$^{-/-}$ spermatogonia failed to produce spermatogenesis upon transplantation into infertile recipient mice, which is associated with a decreased level of RET receptor expression per germ cell and decreased proliferation in vitro and in vivo.[284]

In addition to BCL6B, LHX1, and ETV5, undifferentiated spermatogonia express the transcriptional regulators, ID4, UTF1, ZBTB16, POU3F1 (POU class 3 homeobox 1) SALL4, FOXO1 (forkhead box O1), SOHLH1, SOHLH2, NGN3, STAT3, and TAF4b, which may coordinate signals from the niche and activate distinct gene expression networks related to self-renewal and/or differentiation.[39,91,93,160,185,186,285–292] The expression patterns of several of these factors in the pool of undifferentiated (A_s, A_{pr} and A_{al}) spermatogonia are diagrammed in Figure 15.14. ID4 and UTF1 have the most restricted expression patterns, suggesting that they may identify the most undifferentiated spermatogonia with high "stemness" potential.[183,185,186,188] ZBTB16 and POU3F1 are required for the maintenance and/or renewal of the SSC pool.[91,160,285] In contrast SOHLH1, SOHLH2, NGN3, and STAT3 have been implicated in spermatogonial differentiation.[35,93,286–289,292–294] Despite their different functions, many of these factors are expressed simultaneously in undifferentiated spermatogonia, suggesting that these cells may be poised to self-renew or differentiate depending on signals from the local niche. For example, ZBTB16 and SOHLH1 are co-expressed in the majority of undifferentiated spermatogonia, even though they

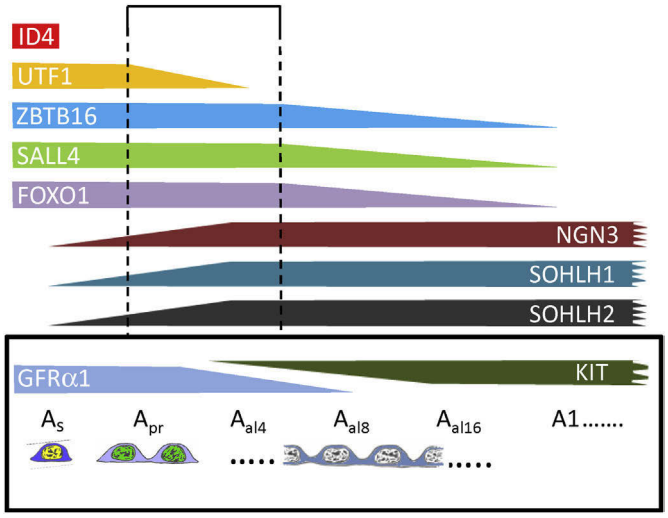

FIGURE 15.14 Summary of transcription factor expression in mouse spermatogonia. Bars indicate the extent of marker expression in the adult mouse spermatogonial lineage based on the data from Table 15.1. ID4, inhibitor of DNA binding 4; UTF1, undifferentiated embryonic cell transcription factor 1; ZBTB16, zinc finger and BTB domain containing 16; SALL4, Sal-like 4; FOXO1, forkhead box O1; NGN3, neurogenin 3; SOHLH1, spermatogenesis and oogenesis specific helix-loop-helix 1; SOHLH2, spermatogenesis and oogenesis specific helix-loop-helix 2; KIT, cKit receptor tyrosine kinase; GFRα1, GDNF family receptor α1. POU3F1, STAT3 and ETV5 are required for SSC maintenance and differentiation, respectively; however, there are no data to correlate the expression of these markers to clone size, so these factors are not shown.

have opposing functions in the regulation of *Kit* expression. ZBTB16 suppresses *Kit* expression and maintains an undifferentiated state, while SOHLH1 activates *Kit* expression and promotes spermatogonial differentiation.[294,295] These cells also express SALL4,[39,87,296] which has been shown to bind ZBTB16. Hobbs and colleagues proposed a model in which SALL4 sequesters ZBTB16 away from the *Kit* promoter, perhaps facilitating access by SOHLH1 to stimulate *Kit* transcription and spermatogonial differentiation.[296] FOXO1 is an effector of PI3K/AKT signaling in undifferentiated spermatogonia and exhibits nearly complete co-expression with ZBTB16. Knockout data suggest that FOXO1 is required for spermatogonial expansion during the first week of life and is also required for the induction of KIT expression, although that regulation may be indirect because there is limited co-expression of FOXO1 with KIT. Direct targets of FOXO1 may include *Sall4*, *Lhx1*, *Ret*, *Egr2*, *Egr4* and *Tex19*. In gonocytes of newborn animals, the FOXO1 protein is located primarily in the cytoplasm but translocates to the nucleus as gonocytes give rise to spermatogonia in the first week of life, which coincides with the onset of *Kit* expression and initiation of the first round of spermatogenesis.[290] A similar cytoplasmic-to-nuclear transition has been reported for SOHLH1 during pubertal development in the monkey, when spermatogonial differentiation is initiated in this primate.[297] The cytoplasmic to nuclear shuttling for these transcription factors appears to be related specifically to the initiation of spermatogenesis because FOXO1 and SOHLH1 are exclusively nuclear in adult spermatogonia.[290,294,297]

Posttranscriptional Regulation of SSCs

Kit mRNA is present in undifferentiated spermatogonia, while the KIT protein is absent.[95,198,281,298] This observation could suggest that SSCs are poised for differentiation and that the production of the KIT protein is initiated by a post-transcriptional mechanism. MicroRNAs (miRNAs) are important posttranscriptional regulators. Two studies demonstrated that inactivation of DICER, the enzyme that catalyzes the processing of miRNA precursors into mature 18–23 bp miRNAs, disrupts spermatogenesis and fertility.[299,300] This activity appears to be independent of the argonaute protein (AGO2) because AGO2 knockout in germ cells does not impact spermatogenesis or fertility, which suggests that DICER may have miRNA independent functions in the testis.[299] Nonetheless, there are dramatic changes in miRNA expression patterns during testicular development that occur in concert with the changing population of germ cells.[301]

Using cultured SSCs, Yang and coworkers[198] demonstrated that disruption of *microRNA 221* and *222* function in undifferentiated spermatogonia causes their transition from a KIT^- to KIT^+ phenotype and loss of potential to regenerate spermatogenesis in recipient mice. They further demonstrated that GDNF, FGF2, and CSF-1 stimulated *miR221/222* expression while treatment with RA reduced *miR221/222* expression, commensurate with increased *Kit* mRNA expression and concomitant increases in KIT protein levels. Overexpression of *miR221/222* inhibited the RA-induced differentiation of undifferentiated spermatogonia. In a similar series of experiments, Huszar and Payne[302] demonstrated that *miR146* inhibits RA-induced spermatogonial differentiation; this is regulated by direct downregulation of mediator complex 1 (*Med1*) expression, which is a co-regulator of retinoid receptors. He and colleagues[303] reported that *miR20* and *miR106a* are also involved in maintenance and renewal of SSCs acting in part by suppression of *Stat3* and *Ccnd1* expression. *MiR21* and the *miR17-92* (*Mirc1*) and *miR106b-25* (*Mirc3*) clusters are overrepresented in the $THY1^+$ fraction of mouse testis cells.[304,305] Knockdown of *miR21* in SSC cultures leads to increased apoptosis and loss of transplantable stem cell activity. ETV5 directly targets the *miR21* promoter and promotes *miR21* expression in SSC cultures.[304] Treatment of $THY1^+$ germ cell cultures

or VAD mice with RA resulted in the downregulation of most members of the *Mirc1* and *Mirc3* clusters. In addition, germ cell specific knockout of the *Mirc1* cluster reduced testis weight, disrupted spermatogenesis and reduced the number of sperm in the epididymis. However, the overall impact on fertility compared to controls was modest. The authors speculated that *Mirc3* family miRNAs may partially compensate for loss of *Mirc1* because *Mirc3* shares several potential genetic targets with *Mirc1* (including *Kit* and *Stat3*) and the *Mirc3* cluster was dramatically upregulated in the *Mirc1* knockouts.[305]

In contrast to *miR221/222*, *MiR20* and *miR106*, *miRlet7* appears to mediate RA-induced differentiation.[305] Tong and colleagues demonstrated that *miRlet7* is expressed by spermatogonia and spermatocytes. RA treatment of VAD mice in vivo and in THY1$^+$ spermatogonia in vitro caused a significant increase in *miRlet7* expression, and this was associated with a significant reduction in LIN28 expression. LIN28 is an evolutionarily conserved small RNA binding protein[306,307] and "stemness" factor[308] that is expressed by undifferentiated spermatogonia and specifically interferes with the DICER-mediated processing of *let7* precursor miRNAs into mature *miRlet7* in cultured SSCs.[184] Tong and colleagues[305] demonstrated that RA regulation is mediated by direct binding of RARα and RARγ to the *Lin28* promoter and also identified *Ccdn1*, *Col1a2*, and *Mycn* as potential targets of *miRlet7* that are known to be involved in spermatogonial development.[196,277,309,310] Similar to LIN28, NANOS2 and NANOS3 (Nanos homolog 3) are RNA binding proteins specific to germ cells in the mouse testis,[311] with expression restricted to A_s, A_{pr} and some A_{al} spermatogonia.[183,188] Immunohistochemical staining in whole mount preparations of seminiferous tubules indicate that NANOS2 may be more prevalent in smaller clones of undifferentiated spermatogonia and NANOS3 may be more prevalent in larger clones. Deletion of *Nanos2* or *Nanos3* caused infertility characterized by a complete absence of germ cells. This is due to the loss of primordial germ cells in the fetal testes and the spermatogenic lineage is never established in the postnatal testis.[311] Sada and coworkers[188] used lineage tracing experiments to demonstrate that NANOS2 is specifically expressed by SSCs in the postnatal testes. Conditional deletion of *Nanos2* at four weeks of age caused a rapid loss of GFRα1$^+$ and ZBTB16$^+$ spermatogonia which led to a complete loss of germ cells by 12 weeks of age. Conditional overexpression of *Nanos2* in the postnatal testis resulted in an accumulation of undifferentiated spermatogonia and a virtual absence of KIT$^+$ differentiating spermatogonia; the authors concluded that this resulted from a block to differentiation rather than increased proliferation or decreased apoptosis of undifferentiated spermatogonia.

SPERMATOGONIAL STEM CELL AGING

In the spermatogenic system, aging is manifested as a decrease in sperm number and quality of sperm in men.[312–315] Aging in the testis is also associated with declines in the number of Leydig and Sertoli cells, thickening of the basement membrane of the seminiferous tubules, and arrested divisions of germ cells.[316] A fundamental question is whether age-related deficits in spermatogenesis are due to deterioration of SSC function, niche function, or both.

Two studies reported a significant decline of stem cell activity (both concentration and total SSCs per testis) by 2 years of age in mice, which as associated with a significant decrease in testicular weight and atrophy of the seminiferous tubules.[250,317] Zhang and colleagues also reported that the length of donor-derived colonies produced in young recipients from 2-year-old donors was significantly reduced, indicating an intrinsic decrease in stem cell activity. In the reciprocal experiment, young SSCs could regenerate spermatogenesis leading to increased testis size in 1-year-old recipients with age-atrophied testes, but could not restore testis size in 2-year-old atrophied testes. These results suggest that there is also an age-related decline in SSC niche function.[317] Consistent with these results, Ryu and colleagues used a serial transplantation approach in which young SSCs were maintained in a perpetually young environment by retransplanting into new young recipients every 3 months. In this context, SSC continued to produce spermatogenesis in recipient testes for more than 3 years with no significant decline in stem cell concentration or colony length. The authors concluded that SSCs are very long-lived and that age-related declines in male fertility can be attributed in large part to a deteriorating niche.[250] The mechanisms of the age-related decline in the SSC niche are not clear, but Ryu and colleagues[250] reported a significant decrease in GDNF expression in aged, atrophied testes, despite the fact that Sertoli cell concentration is relatively increased in the atrophied testis. Additionally, Kokkinaki and coworkers[318] performed microarray analysis for GFRα1$^+$ cells from the testes of young and old mice and observed an age-related increase in genes that are involved in DNA repair (*Ercc1*, *Rcc5* and *Rad18*) or induced by DNA damage (*Gadd45b*) or oxidative stress (*Osgin2*). Whether these changes in gene expression are causes or consequences of SSC aging is not known.

TRANSGENESIS

SSCs are the only stem cells in the postnatal animal that contribute genes to the next generation. Therefore, they are not only crucial for the continuation of the

species, but they are also potentially a vehicle for germline modification and production of transgenic animals. Several groups have attempted to genetically modify SSCs by introducing transgene vectors (plasmid DNA or viral vectors) directly into the testes, similar to the approach used for SSC transplantation. However, in most cases these approaches, which employed viral vectors, lipofection or in vivo electroporation did not lead to efficient genetic modification of SSCs with persistent transgene expression, which indicates that the transgene was not stably integrated into the SSC genome.[319–322] Sertoli cells are likely the major obstacles that prevent transgenic vectors from reaching the target SSCs.

To address this challenge, Nagano and coworkers conducted a series of experiments to determine whether SSCs could be genetically modified when displaced from their cognate niche in the seminiferous tubules.[195,321] The most efficient approach evaluated in those studies involved repeated administration of retroviral or lentiviral vectors carrying a *lacZ* reporter gene to cultured SSCs. Transduction efficiency was greatest using mouse pup donor cells, which was likely due to the fact that spermatogonia are more proliferative at that stage of development. Upon transplantation, the genetically modified SSCs produced colonies of spermatogenesis with *lacZ* expression that persisted for periods ranging from 2 to 6 months. Similar results were obtained in rats.[136] The same group subsequently demonstrated in both mice and rats that recipients of retrovirus or lentivirus treated SSCs could sire transgenic offspring (F0) and that the transgene was passed to the F1 and F2 generations with the expected frequency. Transgenic efficiency in those studies was 4.5% in mice and 6% in rats, calculated as the percent of total offspring produced by the transplant recipient males.[116,323] The advent of SSC culture in 2003[168] expanded the possibilities for germline genetic modification because it allowed for targeted mutagenesis and clonal expansion of modified SSCs.[324,325] Whether germ cell-mediated transgenesis provides advantages relative to the more established pronuclear injection or embryonic stem cell-based approaches in rodents can probably be debated. Nevertheless, these approaches expand the available tools for rodent transgenesis and may be particularly valuable in large animal species where pronuclear injection is inefficient or impossible and ES cell technologies are not available.

Transgenic chicken have been produced by genetic modification (electroporation and *piggyBac* tranposon) and transplantation of germline competent chicken primordial germ cells (PGCs).[326,327] This approach is facilitated by the accessibility of the avian embryo in the egg and by the fact that PGCs can simply be injected into the vasculature of the developing embryo, where they migrate to the embryonic gonad and produce functional eggs and sperm. Zeng and colleagues also reported the production of transgenic pigs using adeno-associated virus and lentivirus constructs to modify SSCs, ex vivo. The transduced SSCs were transplanted into busulfan-treated recipient pigs by ultrasound-guided rete testis injection. Ejaculated sperm from transplant recipients was used to fertilize eggs by in vitro fertilization and gave rise to transgenic embryos.[328] Honaramooz and coworkers also previously demonstrated the feasibility of producing transgenic goats by this approach.[118] In addition to their value for fundamental research, transgenesis in large animals has the potential to improve health and production characteristics of food animals, produce novel bioreactors to generate large quantities of pharmaceutical proteins in eggs and milk and provide tissues and organs for transplantation in humans.

While genetic modification of SSCs by the in vivo approaches is very inefficient (as indicated above), Sertoli cells are efficiently transfected/transduced after injection of transgene vectors into recipient mouse seminiferous tubules,[329–331] and this may be a viable approach for niche therapy treatment of male infertility. Three groups used the rete testis injection approach typically employed for SSC transplantation to introduce adenoviral vectors[331], lentiviral vectors,[330] and plasmid DNA[329] containing *Kitl* cDNA into the *Kitl*-deficient Sertoli cells of infertile *Steel (Sl)* mice. In each case, spermatogenesis was established from latent endogenous SSCs in a portion of the seminiferous tubules in recipient *Sl* mice. Furthermore, sperm collected from the testes of adenovirus and lentivirus-treated recipients were functionally competent to fertilize mouse eggs by intracytoplasmic sperm injection and produced normal offspring.[330] None of the offspring carried the transgene, suggesting that virus-mediated Sertoli cell gene therapy may be a safe treatment for some cases of male infertility. However, the sample size was relatively small (a total of 33 mouse pups were produced in both studies), so additional studies are required before this gene therapy approach can be safely translated to the fertility clinic.

REGENERATIVE POTENTIAL OF SPERMATOGONIAL STEM CELLS AND TRANSLATION TO THE CLINIC

Due to their regenerative potential and the availability of the transplantation method, SSCs may have application for treating some cases of male infertility,[332–335] such as that caused by chemotherapy or radiation treatments for cancer or other conditions. Improved cancer therapies have resulted in a growing population of long-term survivors. Unfortunately, due to its highly prolific nature, the spermatogenic system is an unintended target of chemo- and radiotherapies,[336,337] and some cancer treatments such as high-dose chemotherapy, whole-body

radiation, or radiation to the gonads can cause permanent infertility.[338] The extent that the fertility of a patient is affected depends on the age of the patients as well as the dose, type, and frequency of treatment.[338] There has been significant progress in minimizing the unwanted side effects of the cancer treatment by adjusting and modifying the therapeutic regimens without compromising the efficiency of oncologic treatments.[339] In addition, several rodent studies indicate that suppression of gonadotropins and testosterone using gonadotropin-releasing hormone antagonists stimulated the recovery from endogenous SSCs that survived chemotherapy or radiation treatment.[340–347] However, the results in human clinical trials have been mixed (reviewed in Ref. 348), with only one study reporting successful protection of human spermatogenesis using hormone suppression.[349]

Pubertal and adult males have the option to cryopreserve sperm prior to oncologic treatment. Unfortunately, only a small percentage of men pursue this option prior to initiating treatment.[350] Furthermore, this is not an option for the prepubertal boys, who are not yet making sperm. However, these young patients do have SSCs in their testes that are poised to initiate sperm production at puberty (see Chapter 32). There are several stem cell technologies in the research pipeline that may give rise to new fertility options and allow infertile patients to have their own biological children.

As described above, homologous SSC transplantation has been successfully performed in several animal species, including mice, rats, pigs, goats, bulls, sheep, dogs, and monkeys.[102,113,115–123,126,127,168] Furthermore, Shetty and coworkers[129] recently demonstrated that spermatogenesis from transplanted SSCs was enhanced by hormone suppression with GnRH antagonist in chemotherapy-treated monkeys. This progress suggests that SSC transplantation in humans may be feasible and could provide a new option for patients that have no other alternatives to preserve or restore their fertility. Results in mice, rats, and nonhuman primates indicate that SSCs from donors of all ages, newborn to adult, can regenerate spermatogenesis and produce functional sperm[108,115,123] and SSCs can be cryopreserved and retain their spermatogenic potential upon thawing and transplantation.[68,123,145,147] Wu and colleagues demonstrated that mouse SSCs were competent to regenerate spermatogenesis and produce fertile offspring after 14 years of cryostorage.[351]

Thus, prepubertal boys or adult men should be able to cryopreserve testicular tissue containing SSCs prior to treatment and have these cells reintroduced into their testes at a later date to regenerate spermatogenesis. Several clinics worldwide are already cryopreserving testicular tissue for patients in anticipation that new stem cell therapies will be available in the future. To date testicular tissues or cells have been cryopreserved for over 150 patients that are documented by published reports;[128,154,155,335,352–355] and personal communications (K.E.O.) indicate that the actual number is much higher. Therefore, it is critical for researchers and physicians to responsibly and safely develop new fertility treatments toward clinical application.

In 1999, Radford and colleagues introduced the autologous SSC transplantation technique to the human clinic.[128] Testicular tissue from 12 male non-Hodgkin's lymphoma patients was cryopreserved as a cell suspension prior to initiating chemotherapy. At later dates, seven patients had the cells injected back into their testes.[355,356] To date, there have been no follow-up reports on the fertility status of those patients, so the outcome of the experiment is unknown. Even if the men had fathered children, it would be difficult to demonstrate definitively (in the absence of a unique genetic marker) that those offspring resulted from sperm produced by transplanted stem cells rather than from surviving endogenous stem cells. There have not been other reports of SSC transplantation in humans since 1999, but these initial cases demonstrated that patients are willing to pursue experimental stem cell options to achieve fertility.

Clinical translation of the SSC transplantation technique appears imminent considering the success in several large animal models; however, several questions and challenges remain. First, the small amount of tissue that is obtained from a biopsy from a prepubertal boy may contain a relatively small number of stem cells. Second, in the cancer survivor paradigm, it is essential to eliminate the risk of introducing malignant cells.

In rodents, SSCs can be expanded substantially in culture and maintain competence to produce spermatogenesis and restore fertility upon transplantation.[113,140,168,169] Several groups have reported extending SSC culture to large animal species[202–206] and a number of laboratories have reported progress culturing human SSCs[153–155,207–211] (see the previous section on culture). This approach could theoretically be employed to amplify the small number of stem cells that can be obtained from a biopsy and also to demonstrate the absence of malignant contamination.

Autologous testicular tissue grafting is also an option to restore fertility (see the previous section on Xenografting). Results from monkey studies[222,223] suggest that autologous transplantation may be an option if suitable cryopreservation conditions are developed. Similar to SSC transplantation, autologous grafting will be problematic in cases where malignant contamination of the testicular tissue is suspected. Xenografting of human testicular tissue into animals could circumvent this problem, but it is associated with additional concerns about xenobiotics and has been unsuccessful to date.[215–220]

Additional strategies to remove malignant contamination include FACS sorting the testicular cell suspension

and organ culture. Several groups have demonstrated that it is possible to isolate germ cells and remove malignant contamination from heterogeneous testicular cell suspension using FACS and a combination of germ cell and/or cancer cell markers.[151,172,181,357,358] Despite these promising reports, caution is warranted because even low levels of contamination can lead to cancer and current assays may lack the sensitivity to detect very rare malignant cells.[359,360]

An alternative approach that would eliminate the risk of introducing malignant cells into patients is to produce haploid gametes, in vitro. Sato and coworkers[361] described a method for testicular organ culture in which SSCs are maintained in their niches in the seminiferous tubules. The authors demonstrated that testicular tissue from immature mice could be maintained for several weeks in culture on an agarose platform that was submerged in medium. In this way the testicular tissue was suspended at the air/liquid interface, a method originally described in the 1950s.[362] From immature testicular tissue that initially contained only undifferentiated spermatogonia, haploid round spermatids and sperm were generated by 23 and 42 days in culture, respectively, and used to fertilize mouse oocytes by ICSI or round spermatid injection (ROSI). The fertilized embryos were transferred to recipient females and gave rise to healthy offspring. If these results can be translated to humans, testicular organ culture would circumvent the need to put tissues or cells back into patients and may be a safe option for patients with malignancies that contaminate the testes.

For males who did not preserve sperm or testicular tissue prior to gonadotoxic treatment, generation of transplantable germ cells (primordial germ cells or SSCs) or haploid gametes from embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) has been explored. Several groups have reported the generation of germ cells from nonhuman primate ESCs[363,364] and human ESCs and/or iPSCs,[365–369] including evidence of rare haploid cells in some cases. The safety and feasibility of this system still needs to be evaluated in animal models due to the inability to test the function of the resulting germ cells in human system.

CONCLUSION

Spermatogonial stem cells are at the foundation of spermatogenesis and may have practical applications for producing transgenic animals and treating some cases of male infertility. These adult tissue stem cells can only be definitively identified by their biological potential to produce and maintain spermatogenesis after transplantation or by their ability to regenerate spermatogenesis after toxic treatment. These are retrospective analyses and there is currently no universally accepted method to prospectively identify the entire population of spermatogonial stem cells.

The jargon used to describe SSCs and their progeny is important because it impacts the interpretation of experimental results and, by extension, knowledge in the field. The definition of SSCs is precise and irrefutable. A cell that can produce and maintain spermatogenesis is an SSC. The definitions of progenitor and differentiating spermatogonia may be less obvious. The main distinction between stem and progenitor cells is that stem cells can theoretically replicate indefinitely (or at least for a very long time; see the section on aging), while progenitors have a more limited replicative potential.[370] Progenitor cells are undifferentiated cells that have a *tendency* to differentiate into one or more lineages within a tissue. Note that tendency does not indicate an absolute commitment to differentiate and reflects a populational probability to commitment. For example, most NGN3$^+$, undifferentiated spermatogonia have a tendency to differentiate, but a portion of the NGN3$^+$ cells retain the potential to regenerate spermatogenesis upon transplantation or when spermatogenesis is depleted by toxic treatment.[35,189] SOHLH1, SOHLH2, and NANOS3 have expression patterns very similar to NGN3 and therefore might also be considered markers of progenitors in the spermatogenic lineage. Whether the pools of SOHLH1, SOHLH2, and NANOS3 positive spermatogonia have transplantable stem cell activity remains to be demonstrated, however. When a study is clearly focused on a population of spermatogonia with an undifferentiated phenotype, but it is not possible to determine the population's stem cell activity using the transplantation assay, we suggest that it may be most appropriate to use terms such as "stem and progenitor spermatogonia" or "undifferentiated spermatogonia." Using accurate vocabulary should not compromise the importance of the science and will help to strengthen hypotheses for future studies.

It is also important to understand the biological differences between stem and progenitor spermatogonia to determine which cell type is most appropriate for therapeutic applications. There is relative consensus that differentiating spermatogonia can be identified morphologically by the accumulation of heterochromatin in the nucleus and by expression of specific markers, including STRA8 and KIT. In contrast to progenitor spermatogonia, differentiating spermatogonia have committed to differentiate with no long-term potential to regenerate and maintain spermatogenesis.

There has been significant progress identifying molecules that regulate SSC function; this progress has been fueled in large part by the use of genetically modified animals, the SSC transplantation technique, and SSC culture. However, there remains a very limited understanding of how these individual molecules interact to transduce signals from the SSC niche and coordinate

intrinsic pathways within the SSC that lead to self-renewal versus differentiation developmental fates. Continued discovery of molecules that regulate SSC fate will help to fill the knowledge gaps, and progress will be facilitated by accurate interpretation of results relative to stem, progenitor, and differentiating terminology.

Although spermatogonial clone size (e.g., A_s, A_{pr}, A_{al}) can be indicative of stem cell potential, clone size cannot absolutely define the stem cell pool. The stem cell pool in rodents is generally considered to be comprised of A_s and some A_{pr} spermatogonia (reviewed in Ref. 27). However, several studies have revealed molecular heterogeneity within the pools of A_s and A_{pr} spermatogonia.[35,39,90,183,184] Does molecular heterogeneity suggest functional heterogeneity? It should be possible in future studies to cross different transgenic reporter lines to facilitate isolation of subpopulations of cells with distinct molecular phenotypes within the stem and progenitor pool and determine their relative stem cell activity by transplantation.

A_{dark} and A_{pale} are considered to be the SSCs in nonhuman primate and human testes. In rhesus macaques, all A_{dark} and half of A_{pale} spermatogonia share the undifferentiated phenotype GFRα1$^+$/ZBTB16$^+$/NGN3$^-$/KIT$^-$. Half of A_{pale} also express NGN3 and 25% of A_{pale} express KIT.[68] Does this indicate that the pool of A_{pale} spermatogonia also includes progenitor and early differentiating spermatogonia, such as A_{pr} and A_{al} spermatogonia in rodents? A_{dark} spermatogonia have the potential to regenerate spermatogenesis after noxious treatment with radiation and therefore satisfy the formal definition of SSCs. The potential of A_{pale} to regenerate spermatogenesis in this paradigm has not been demonstrated because A_{pale} are destroyed by radiation.[70,84] In the future, it may be possible to isolate A_{dark} and A_{pale} spermatogonia based on unique molecular phenotypes and then transplant to determine relative stem cell activity. To date, there are no cell surface markers that clearly distinguish A_{dark} and A_{pale} spermatogonia; generation of several transgenic reporter lines in monkeys is possible[371,372] but probably not realistic.

Examination of SSC activity in higher primates is further complicated because it is difficult to test the ability of a test cell population to produce and maintain spermatogenesis after transplantation. Xenotransplantation into nude mouse testes is a valuable tool for studying SSCs in nonhuman primate and human testes,[148,149,151,152] but it is limited because it does not reconstitute complete spermatogenesis. Monkey-to-monkey transplant is feasible and can test the full regenerative potential of a test cell population,[123] but it is too cumbersome and expensive to use as a routine biological assay. Perhaps in the future it will be possible to primatize or humanize mouse testes to support complete spermatogenesis from monkey and human cells. Humanized mice have been valuable for translational studies on human hematopoiesis and immunity.[373] Development of robust functional assays for putative human germ cells will become increasingly important to validate the exciting discoveries that human pluripotent stem cells can be induced to differentiate down spermatogenic lineage.

The SSC field has grown dramatically in the past two decades, due in part to the development of the SSC transplantation technique[100,101] and in part to the explosive development of the stem cell field in general after the discovery of human pluripotent stem cells.[374,375] The SSC transplantation technique has facilitated hundreds of fundamental investigations and opened new opportunities for germline preservation and animal transgenesis as well as pharmaceutical and clinical applications. On the heels of the past discoveries detailed in this chapter, we believe it is reasonable to expect that SSCs or other stem cells will be used to preserve and restore the germlines of valuable domestic species and treat human male infertility in the coming decades.

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CHAPTER

16

Hormone Signaling in the Testis

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INTRODUCTION

The process of spermatogenesis is essential to maintain male fertility. The signals originating from hormones drive intercellular communication and intracellular processes that are required for spermatogenesis. Although there are dissenting opinions,[1] evidence is accumulating that indicates sperm counts are decreasing for men in developed countries. A compilation of human sperm count studies for North American men determined that sperm density decreased by about 40% over the 40 years previous to 1990.[2] For European men, there was a 65% decrease over the same period. If the current trend continues, by 2020–2040 sperm count for the average European man will be 20 million/ml. This level of sperm production is defined as oligospermic and is the level at which decreases in fertility are observed.

It is proposed that environmental influences including endocrine disruptors such as environmental estrogens are major contributors to the decrease in sperm production due to their interference with normal hormone signaling in the testis. These observations highlight the importance of understanding the molecular mechanisms by which hormones support spermatogenesis. Fortunately, transgenic mouse studies have provided better definitions of the physiological and cellular functions of various hormones in specific cell types in the testes. Also, advances have been made in defining the specific genes, proteins, and signaling pathways that are regulated by individual hormones. It is obvious that these advances as well as additional information will be required to counteract the trend of decreasing sperm counts.

We have known for more than 80 years from Greep's pioneering work that two hormones derived from gonadotrophs in the anterior pituitary, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), have separate sites of action in the testis and work in concert to control spermatogenesis.[3] As explained in this chapter, both of these gonadotropin hormones act indirectly to promote the initiation of spermatogenesis, germ cell proliferation, development, and differentiation. FSH acts on the Sertoli cell to activate gene expression and signaling pathways that support the process of sperm production. LH acts on the Leydig cell to promote the production of testosterone that is essential for maintaining spermatogenesis, in addition to its wider role in masculinization and the maintenance of male secondary sexual characteristics. Testosterone acts upon Sertoli cells and other testicular cells to regulate gene expression and the activation of signaling pathways that are required for supporting spermatogenesis. Although Smith first reported in 1930 that testosterone could substitute for the pituitary gland,[4,5] the molecular mechanisms by which testosterone can independently support the development, retention, and release of germ cells is still only partially understood. Additional circulating hormones and testis-derived factors provide needed communication between testicular cells and contribute to maximizing fertility.

In this chapter, we review the intracellular and intercellular signals initiated by the complex milieu of hormonal factors in the testis. The physiological functions of LH and the molecular mechanisms by which LH activates the genes and enzymes required for the production of testosterone are discussed. The multifaceted actions of testosterone in nearly all cells of the testis except

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germ cells and the testosterone-induced communication between testis cells are described from the perspective of transgenic mice lacking the receptor for testosterone in specific cell types. The genes and signaling factors regulated by testosterone are then examined. The multiple signaling pathways regulated by FSH in Sertoli cells are described as well as the differences in FSH signaling that occur in proliferating versus differentiated Sertoli cells. A comparison of signals originating from FSH and testosterone is then made to characterize the synergism shown by the two hormones in the maintenance of spermatogenesis. We also review the signaling pathways by which thyroid hormone regulates the proliferation of Sertoli cells and thus determines the capacity for sperm production.

Interestingly, none of the major endocrine drivers of spermatogenesis (LH, testosterone, and FSH) act directly on germ cells, but instead cause the production of factors that function locally in the testis to enhance the germ cell niche. We discuss the functions of three of these locally acting factors: estrogen, which is derived from testosterone, as well as activin and inhibin, which are regulated by FSH. Finally, a summary is provided of the known contributions of other hormones that fine-tune the testis environment, including osteocalcin, insulin and related factors, glucocorticoids, mineralocorticoids, leptin, and prolactin.

Anatomy of the Testis

Germ cell development and sperm production occur in the seminiferous tubules that feed into the rete testis and then the epididymis (Figure 16.1(A)). Between the tubules in the interstitial space are blood vessels and lymphatic vessels. Leydig cells that produce testosterone are also found in clusters within the interstitial space between the tubules (Figure 16.1(B)), alongside resident macrophages. The seminiferous tubules are surrounded by peritubular myoid (PTM) cells that contract to force released sperm in the tubule toward the rete testis and into the epididymis.

Within the seminiferous tubules, somatic Sertoli cells extend from the basement membrane adjacent to PTM cells to the lumen and surround the developing germ cells (Figure 16.1(C)). Germ cells are present throughout the seminiferous tubule epithelium, with the most immature and undifferentiated cells, including spermatogonial stem cells (SSCs), present on the basement membrane and the most mature germ cells localized to the tubule lumen. At all stages of development, germ cells are in contact with the somatic Sertoli cells that provide niches to support stem cell renewal and differentiation. Sertoli cells also provide growth factors and nourishment for more mature germ cells. During puberty, specialized adhesion junctions are created between each adjacent Sertoli cell that in total form the blood–testis barrier (BTB) near the base of the seminiferous tubules. The BTB divides the seminiferous tubule into the basal and adluminal compartments such that only factors smaller than 1 kDa are able to pass through the barrier. Because of the BTB, Sertoli cells provide a defined, protected microenvironment for the germ cells that are on the luminal side of the BTB. These luminal germ cells do not have access to the outside environment and are dependent upon the Sertoli cell for nourishment, growth factors, as well as other signals required for survival and the development into spermatozoa.[7]

Brief Overview of Spermatogenesis

Details of the process and regulation of spermatogenesis are provided in previous editions of this book. In brief, spermatogenesis is initiated with SSCs located along the basement membrane dividing to produce undifferentiated spermatogonia that are committed to becoming spermatozoa. The undifferentiated spermatogonia undergo a series of mitotic divisions with incomplete cytokinesis to form chains of spermatogonia. The number of spermatogonia divisions is species specific. In rodents, there is extensive amplification of spermatogonia through numerous sequential mitoses. In contrast, there are fewer divisions of spermatogonia in nonhuman primates and humans. A final spermatogonia mitosis gives rise to preleptotene spermatocytes that are programmed to undergo meiosis. During the preleptotene stage of meiosis, spermatocytes detach from the basement membrane and "pass through" the BTB. More precisely, a new series of adhesion sites is formed on the basal side below the preleptotene spermatocyte, after which the BTB on the luminal side of the germ cell is dissolved. Once beyond the BTB, meiosis is completed with the production of haploid spermatids. The resulting spermatids undergo extensive differentiation (spermiogenesis) to become spermatozoa, including condensation of the nucleus, acrosome formation, termination of gene expression, elimination of much of the cytoplasm, cell elongation, and development of a flagellum. Spermatogenesis is completed with the release of spermatozoa into the lumen of the seminiferous tubule (reviewed in Ref. 8).

The seminiferous epithelium is highly organized in the adult testis. Well-defined groups of germ cells appear along the tubule sequentially or at single points on the tubule at specific recurring time points. For example, in rats, within any one region of the seminiferous tubule, the initial transition of undifferentiated spermatogonia to differentiating spermatogonia occurs once every 12.9 days. The length of this cycle is 8.6 days in the mouse and 16 days in humans (reviewed in Ref. 9).

The regular timing of the transition from undifferentiated to differentiated spermatogonia and the

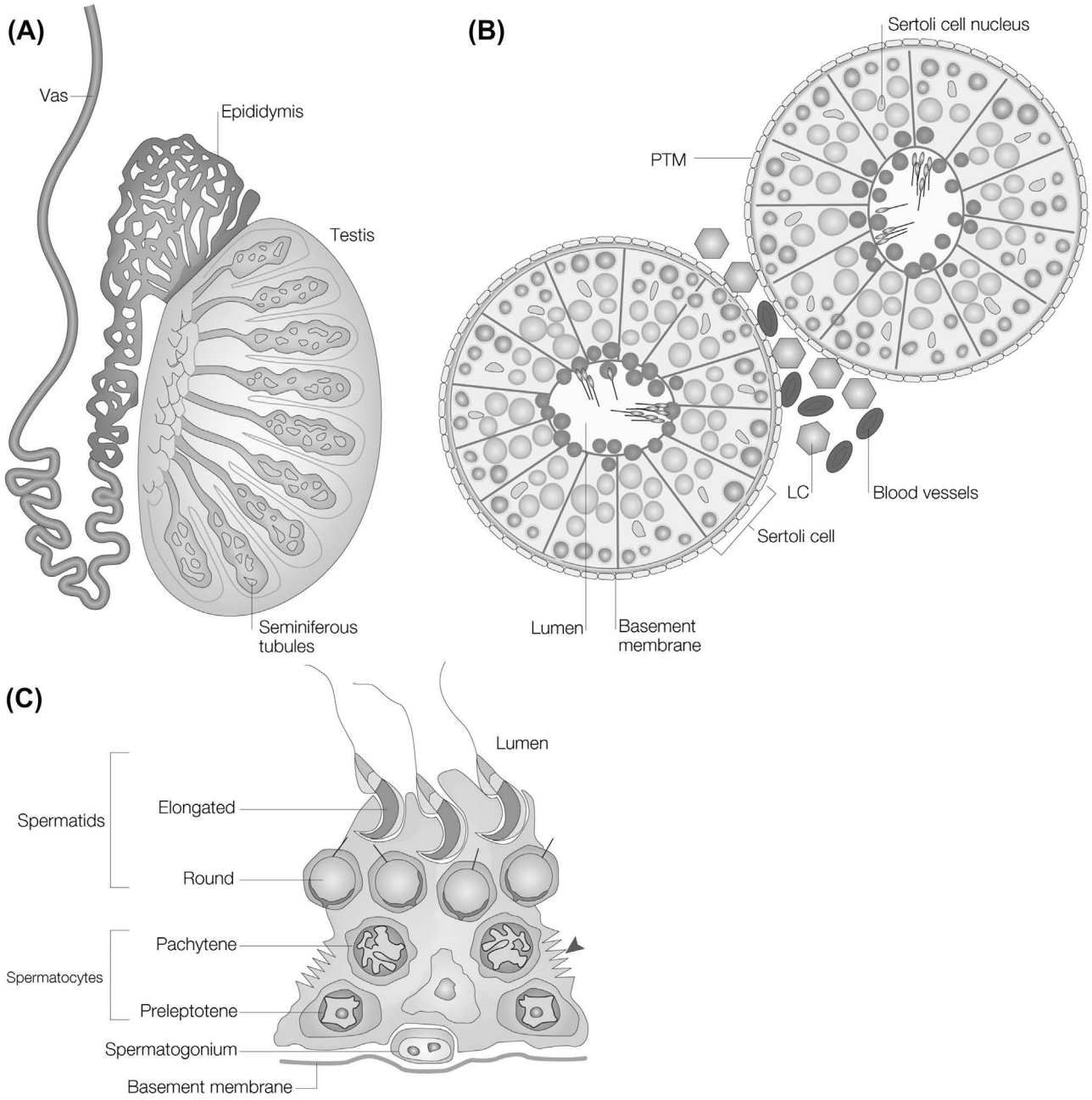

FIGURE 16.1 Schematics of the structure of the testis and seminiferous tubule. (A) The testis consists of lobules that originate from and then return to the rete testes (left side of the testis shown). The rete testis provides a passageway for sperm and fluid to the epididymis. (B) A diagrammatic cross-section through two seminiferous tubules shows germ cells at different stages of development embedded in Sertoli cells. Maturing spermatozoa are shown in the lumen of the tubules. Peritubular myoid (PTM) cells line the outside of the seminiferous tubule. Leydig cells (LC) and blood vessels are located in the interstitial space between seminiferous tubules. (C) A single Sertoli cell and associated germ cells. A region of tight and adherens junctions that forms the blood–testis barrier between Sertoli cells is denoted with an arrowhead. Sertoli cells extend from the basement membrane to the lumen of the seminiferous tubule. The cytoplasm of a Sertoli cell surrounds germ cells during all stages of germ cell development. Spermatogonia lie on the basement membrane of the seminiferous tubule. As germ cells progress through their developmental steps (spermatogonia, preleptotene spermatocytes, pachytene spermatocytes, round spermatids, and elongated spermatids), they move toward the lumen and are released as spermatozoa. *Source: Reproduced with permission from Ref. 6.*

synchronized, clonal pattern of development for each cohort of germ cells result in different generations of germ cells that form associations. Arbitrarily defined associations of the generations of germ cells at various stages of development with defined physiological characteristics are called cell association stages of the cycle of the seminiferous epithelium.[10] There are six stages in humans (I–VI), 12 in mice (I–XII), and 14 (I–XIV) in rats

(Figure 16.2). The cell association stages occur in repeating cycles, with four and one-half cycles of 12.9 days being required in the rat to produce mature sperm from an undifferentiated spermatogonium.[12,13] Along the seminiferous tubule in most rodents and some primates, the stages follow each other in a wavelike fashion[14] (Figure 16.3). In men, the wave occurs as a spiral down the tubule. The results of this cycle of the seminiferous epithelium are that spermatozoa are released at any given point along the tubule once each cycle, and spermatozoa are asynchronously released along the length of the tubule to provide a constant supply of sperm.

Hormone Action

Spermatogenesis is dependent upon hormonal inputs that result in a complex pattern of intratesticular signaling pathways. These hormonal inputs affecting the testis and spermatogenesis can be initiated by the production of hormones at distant tissues, with the transport of the hormones via the circulatory system (endocrine actions). More localized hormonal effects are achieved with the production of hormones by testicular cells and their action on nearby or adjacent cells (paracrine actions) or their secretion from and stimulus of the same cell (autocrine action).

In general, there are two major types of hormones: protein–peptide hormones that act upon receptors at the cell surface, and lipophilic hormones that bind to receptors predominately located in the cytoplasm or the nucleus. Peptide hormones binding to their receptors initiate intracellular signaling pathways that usually activate or inhibit specific kinases, phosphatases, or ion channels, resulting in altering of cellular functions and gene expression (Figure 16.4(A)). Lipophilic hormone binding to their receptors often results in the translocation of the ligand–receptor complex to specific regulatory DNA sites in the genome and the alteration of gene expression patterns (Figure 16.4(B)). However, it is now established that the binding of many lipophilic hormones with their receptors can also cause the activation of kinases within target cells.[15]

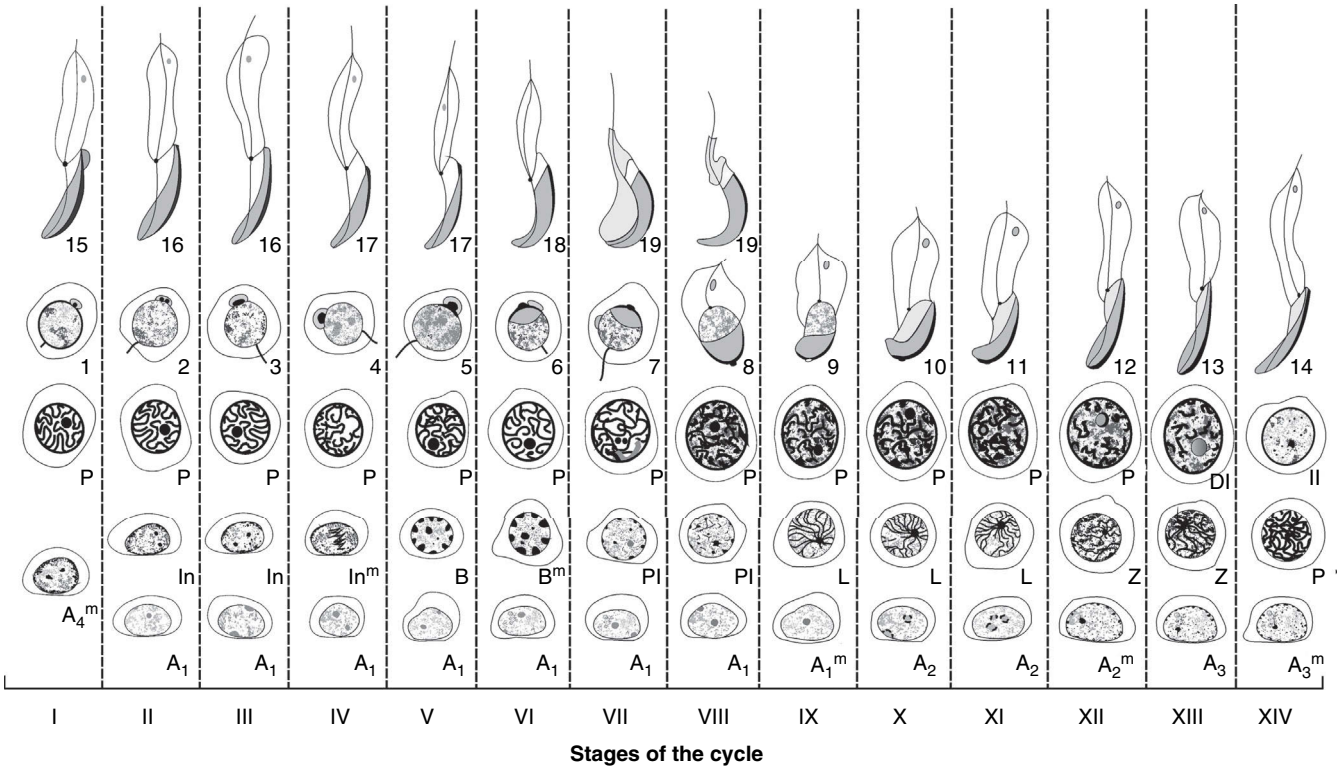

FIGURE 16.2 The cell associations comprising the XIV stages of the cycle of the rat seminiferous epithelium. Each column numbered with a Roman numeral shows the cell types present in one of the cellular associations found in cross-sections of seminiferous tubules. The cellular associations or stages of the cycle succeed one another in time in any given area of the seminiferous epithelium in the rat. Following cellular association XIV, cellular association 1 reappears, so that the sequence starts over again. The stages of the cycle were identified by means of 14 of the 19 steps of spermiogenesis (numbers 1–19). These steps were defined by the changes observed in the nucleus and in the acrosomic structure (the acrosome and head cap are seen applied to the surface of the nucleus). Letters: A_1, A_2, A_3, and A_4 represent four generations of type A spermatogonia; In: intermediate spermatogonia: B: type B spermatogonia; the subscript m next to a spermatogonium indicates occurrence of mitosis; P1: preleptotene spermatocyte; L: leptotene spermatocyte; Z: zygotene spermatocytes; P: pachytene spermatocyte; Di: diakinesis of primary spermatocytes; and 11: secondary spermatocyte. The type A_s, A_{pr}, and A_{al} undifferentiated spermatogonia, present in all stages of the cycle that give rise to type A_1 differentiated spermatogonia, were not included in this drawing. *Source: From Ref. 11 with permission.*

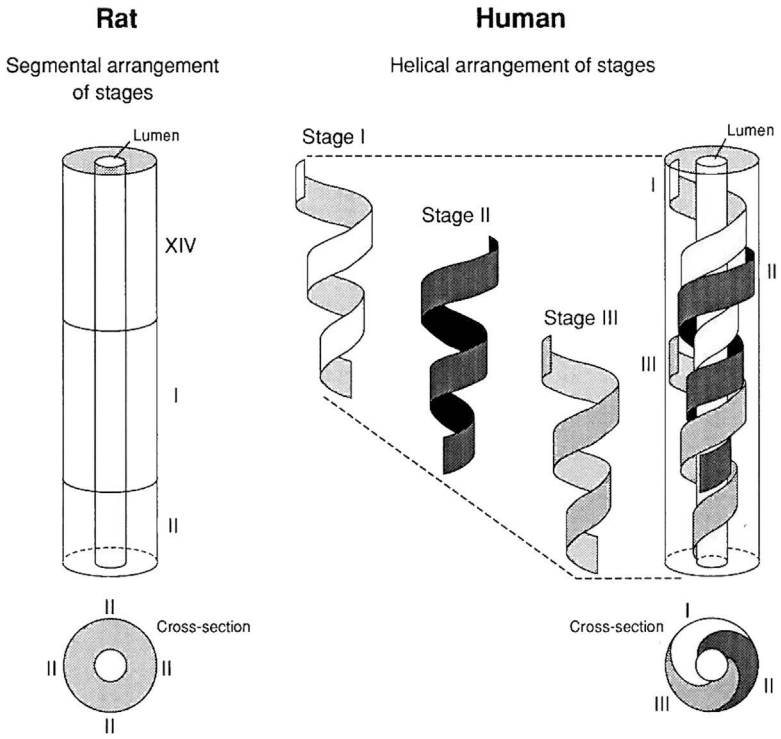

FIGURE 16.3 **Diagram of the progression of germ cell associations (stages of the semiferous epithelial cycle) as a wave along the seminiferous tubule in rat and human.**

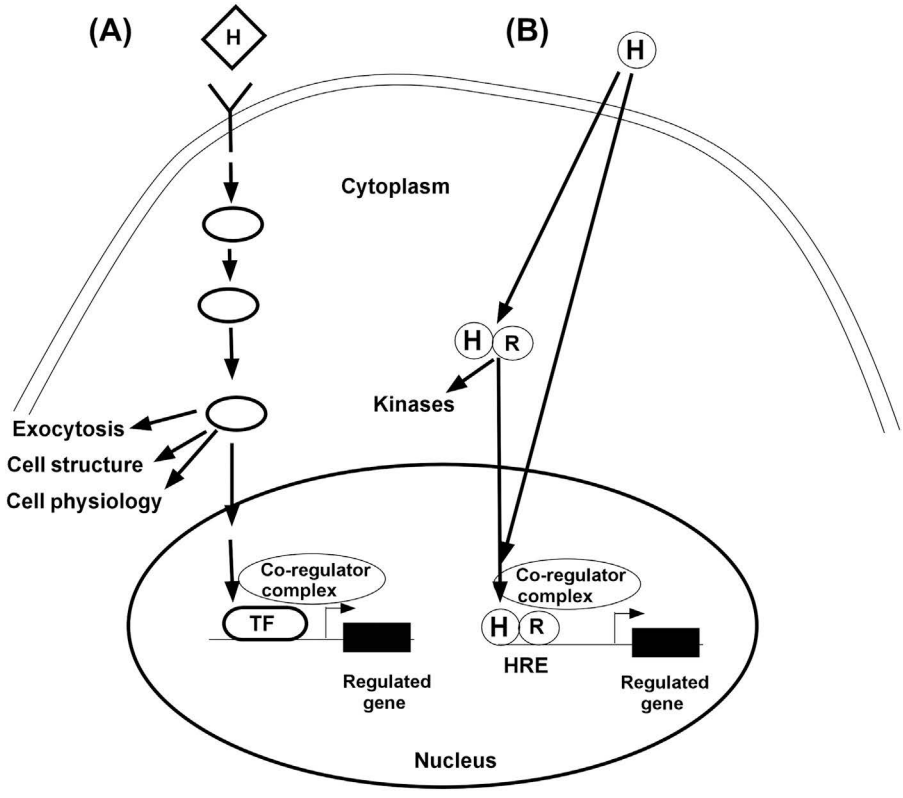

FIGURE 16.4 **Hormone action.** (A) Protein and peptide hormones (H in a diamond) usually bind to membrane-spanning receptors and then alter receptor structure to activate kinase activity, cause dimerization with other receptors, or recruit proteins to the receptor. Often, a series of adaptor proteins are recruited and/or kinases activated that result in changes to cell physiology, cell structure, and/or the exocytosis of regulatory factors. Signals can also be sent to the nucleus to alter transcription factors (TFs) or other members of the gene regulatory machinery to alter gene expression. (B) Lipophilic hormones such as steroids (H in a circle) pass through the plasma membrane to bind receptors (R in a circle) present in the nucleus or in the cytoplasm that then translocate to the nucleus. In the nucleus, the lipophilic hormone–receptor complex acts to alter gene expression. Some lipophilic hormone–receptor complexes are also capable of activating kinases to alter cell function. HRE: hormone response element.

In the testis, LH acting on the Leydig cell and FSH acting on the Sertoli cell are the major endocrine regulators of spermatogenesis. There hormones either directly alter the function of target cells to support spermatogenesis, or result in the production of paracrine and autocrine factors that act on germ cells and other supporting cells to increase sperm production. The hormones regulating spermatogenesis and the

signaling pathways that they initiate are the focus of this chapter.

LUTEINIZING HORMONE

LH and FSH, along with thyroid-stimulating hormone (TSH), are members of the glycoprotein hormone family, each of which shares a common α subunit and a specific β subunit that confers biological specificity. LH is secreted into the bloodstream by the gonadotropes located within the anterior pituitary, upon gonadotropin-releasing hormone (GnRH) stimulation (described in Chapter 28). The primary function of LH in the testis is stimulation of testosterone production by the Leydig cells. This is achieved by binding LH to its cognate receptor, luteinizing hormone–chorionic gonadotropin receptor (LHCGR), on Leydig cells, activating the production of enzymes that is required for the biosynthesis of testosterone from a cholesterol substrate. LHCGR is a member of the seven-transmembrane domain G-protein coupled receptor (GPCR) superfamily (reviewed in Refs 16–18). Treatment of *hpg* mice (which lack gonadotropins) with testosterone restores qualitative spermatogenesis. This effect of testosterone-mediated support of spermatogenesis can also be replicated by exogenous human chorionic gonadotropin (hCG) treatment (which also binds and activates LHCGR) in *hpg* mice, demonstrating that testosterone is the key trophic factor produced by Leydig cells under the control of LHCGR stimulation.[19]

Testosterone release into the bloodstream acts to regulate LH secretion by the pituitary through a negative feedback loop. Elevated circulating testosterone concentrations feed back at the level of the hypothalamus and pituitary to suppress LH secretion by the pituitary, which subsequently reduces LHCGR-mediated stimulation of Leydig cells and thereby reduces testosterone production. Conversely, a fall in circulating testosterone concentrations induces LH secretion by the pituitary and a concomitant increase in testosterone production by the Leydig cells.

LHCGR Localization

Although LHCGR expression has been observed in several tissues in the male, including the seminal vesicles, skin, adrenals, neural retina, neuroendocrine cells, thyroid, and brain, and in certain tumors (reviewed in Refs 16,18,20), the primary site of expression in the normal male is the Leydig cells, both fetal and adult-type Leydig cells. In rodents, LHCGR is expressed in fetal Leydig cells from around embryonic day 16 (e16),[21,22] whereas in humans expression begins within the first trimester. Importantly, germ cells do not express LHCGR at any age, and thus LH control of the seminiferous epithelium is mediated indirectly.

Structure and Function of LHCGR

Expression of LHCGR is controlled by two functional Sp1/Sp3 binding domains identified in both rodents and humans,[23–26] and an upstream imperfect estrogen response element (ERE) direct repeat, with gene expression controlled by a complex balance of repression–de-repression mechanisms, which include suppression by the orphan nuclear receptors EAR2 and EAR3–Coup-TFI, and activation by TR4.[27,28] More recently, epigenetic changes in local chromatin structure have been shown to control gene expression, which works in concert with demethylation of promoter CpG sites to maximize gene expression[29,30] (reviewed in Ref. 18). Several splice variants of LHCGR have been identified across several species (reviewed in Ref. 31). Following translation, the LHCGR protein is shuttled to the surface membrane of Leydig cells, where, like other seven-transmembrane GPCRs, it spans the surface membrane. LHCGR contains an N-terminal extracellular domain that binds LH (and hCG) with high affinity and specificity. This domain contains a number of leucine-rich repeats (LRRs) that are thought to contain successive β-strands and α-helices organized into a horseshoe-shaped structure, with the β-strands forming a parallel β-sheet along the concave surface (reviewed in Refs 16,31). The N-terminal domain is linked to the seven-transmembrane domains that are responsible for signal transduction. These domains are held together by ionic bonds, which are broken upon ligand binding of LHCGR, inducing a conformational change in the receptor. Co-expression of a binding-deficient form of LHCGR and a signaling-deficient form of the receptor was able to rescue the phenotype of an LHCGR-deficient mouse, suggesting that dimerization may perform a functionally important role in LH signaling,[32] as is the case for FSH signaling.[33] However, this interpretation has recently been questioned[34]; thus, the significance and specific function of such dimerization remain to be established, as many GPCRs do not appear to function as dimers (reviewed in Ref. 35).

Role of LH in Adult Leydig Cell Development—Mouse Models

LH signaling in the postnatal testis plays two major roles, controlling both adult-type Leydig cell development and function. In postnatal life, LH expression is upregulated as puberty initiates, while at the same time, the population of adult Leydig cells begins to develop from adult Leydig cell precursor cells (reviewed in Ref. 36). This process of initial differentiation occurs in *hpg* mice that

lack gonadotropin and as such is independent of LH signaling.[37] However, following initial differentiation, these progenitor cells begin to express LHCGR, and LH becomes the most important trophic factor driving adult Leydig cell development. The significance of LH signaling for adult Leydig cell development was firmly established through analysis of several mouse models. In 2001, two groups independently published knockouts of LHCGR: one by targeted deletion of exon 1[38] and one by disruption of exon 11, which encodes the transmembrane and intracellular domains of LHCGR.[39] Both strategies result in models with complete ablation of LHCGR function and with broadly similar phenotypes (reviewed in Refs 40,41). Male mice lacking LHCGR develop normally up to birth, in keeping with the observations that LH signaling is dispensable for prenatal development in rodents.[42–44] The phenotype of the knockout mice remains similar to that of littermate controls up to two weeks of age, but it becomes radically different as the mice enter puberty. Postpubertal LHCGR knockout mice express steroidogenic enzyme genes (including steroidogenic acute regulatory protein (StAR), p450scc, and 17β-HsDIII and Insl3 (insulin-like 3) at very low levels,[45] which is associated with a gross failure of proliferation and maturation of adult Leydig cells and with infertility. The LHCGR phenotype can be partially rescued by testosterone replacement therapy.[46] A similar phenotype is observed in transgenic mice engineered to lack LHβ, demonstrating a clear ligand–receptor interaction.[47] However, because these animals retain a functioning LHCGR, treatment of these animals with exogenous hCG rescues the phenotype.[48] Together with the investigations using *hpg* mice, which demonstrate differentiation of the adult Leydig cell precursors in the absence of LH signaling,[37] these data confirm that LH–LHCGR signaling is essential for the proliferation and function of adult Leydig cells, although it is not required for their initial differentiation.

Overstimulation of LH signaling has also been attempted in various guises (reviewed in Ref. 41). In mouse studies, transgenic overexpression of LH in female mice results in infertility, polycystic ovaries, and ovarian tumors,[49] but male mice were only minimally affected by overstimulation by LH signaling, with mild impairment of adult Leydig cell differentiation.[50,51] Ramping up the system such that transgenic hCG is expressed at 2000-fold the normal elevated circulating LH–hCG bioactivity resulted in focal Leydig cell hypertrophy and increased testicular androgen production, with progressive seminiferous tubule degeneration as the animals aged, but it failed to produce testicular tumors in adult animals (although the animals were infertile, probably due to functional urethral obstruction).[52] Interestingly, overstimulation with hCG during fetal life did result in Leydig cell tumors arising from fetal Leydig cells, but these regressed naturally in line with the normal lifespan of fetal Leydig cells.[53] The most intriguing observation from these overstimulation studies, however, is that chronic overstimulation with hCG failed to advance sexual maturation, despite significantly raised testosterone concentrations.[51,53] The observation that such chronic hCG stimulation interferes with the developmental programming of the hypothalamic–pituitary–gonadal (HPG) axis may provide at least some explanation for this.[54] The situation differs in humans, where activating mutations of LHCGR do cause precocious puberty (if untreated) due to ligand-independent upregulation of testosterone production, reflecting key differences in induction of puberty between rodents and humans.[55–57]

Role of LH in Adult Leydig Cell Development—Humans

In humans, the requirement for LH signaling in fetal life for masculinization makes investigation of the role of postnatal LH signaling complex. In addition to the constitutively activating mutations in LHCGR that drive precocious puberty,[55–57] several loss-of-function alleles and polymorphisms in either LHβ or LHCGR are associated with undermasculinization and aberrant adult Leydig cell development, whereas no inactivating mutations have been identified in hCGβ, presumably due to its essential role in early pregnancy (reviewed in Refs 58,59). Only two reports of men with homozygous missense inactivating mutants in LHβ have been published, and their scarcity is consistent with the fundamental role of LHβ in fertility.[60,61] Complete absence of LHβ results in hypogonadism, associated with hypoplastic seminiferous tubules with greatly reduced spermatogenesis and hypoplastic Leydig cells. However, because hCG remains present to stimulate masculinization, phenotypic effects are limited to postnatal life. Conversely, complete absence of LHCGR (thus preventing both LH and hCG signaling) leads to disorders of sexual development, consistent with the essential role of LHCGR-mediated signaling for masculinization of the human male fetus (reviewed in Refs 58,59).

LH Signaling in Adult-Type Leydig Cell Function

In the adult-type Leydig cell, LH signaling plays an essential role in controlling testosterone production. Briefly, LH binding to the LHCGR leads to stimulation of gene expression of several key steroidogenic enzymes within the Leydig cells, stimulating testosterone production. The intracellular region of LHCGR is associated with heterotrimeric guanonucleotide stimulatory proteins (reviewed in Ref. 16). The best characterized pathway associated with adult Leydig cell function is the Gs–adenylyl cyclase–cyclic adenosine monophosphate

(cAMP)–protein kinase A (PKA) pathway (Figure 16.5). Binding of ligand to LHCGR leads to stimulation of adenylate cyclase, upregulation of cAMP, and subsequent phosphorylation of downstream proteins via PKA (reviewed in Refs 62,63) or protein kinase C (PKC).[64] The cAMP–PKA pathway subsequently induces steroidogenesis in Leydig cells by activating several transcription factors, including cAMP response element binding (CREB) protein, cAMP response element modulator (CREM) protein and GATA4,[65,66] leading to expression of downstream target genes. Production of cAMP also activates a calcium-signaling pathway via increasing concentration of intracellular Ca^{2+} ions,[67,68] a process modulated by PKA and PKC.[69] Although less is known about the molecular cascade triggered by increased intracellular Ca^{2+} and stimulation of the Ca^{2+}–calmodulin (CaM) protein kinase pathway in Leydig cells, recent evidence has demonstrated that expression of StAR is regulated not only by cAMP but also by intracellular Ca^{2+} concentrations. Increasing Ca^{2+} also induces expression of Nur77 (NR4a1), which acts as a mediator of hormone-stimulated StAR expression.[70] Another of the second messenger systems stimulated through LHCGR is PKA-dependent extracellular signal-regulated kinases 1 and 2 (ERK1/2).[71] LH-induced cAMP–PKA signaling transactivates epidermal growth factor receptor (EGFR), which then activates ERK1/2.[72] ERK then promotes expression of StAR[73] and also supports activation of the StAR protein,[74] a process that is modulated by mitogen-activated protein (MAP) kinase phosphatases (MKPs), which act to attenuate ERK1/2 signaling.[75,76] ERK signaling is also important in transducing the proliferative signal that LH–LHCGR binding confers on developing Leydig cells, a role that has been demonstrated both in primary Leydig cell cultures[77] and in mice lacking MEK (MAP kinase kinase, the upstream regulator of ERK1/2) that display Leydig cell hypoplasia.[78] Interestingly, although both LH and hCG ligands activate the same pathways, emerging evidence from in vitro studies suggests that hCG and LH stimulate different intracellular pathways with different efficacies, with hCG weighted toward cAMP, whereas LH favors ERK.[79]

Genes Regulated by LHCGR Signaling

The production of testosterone by Leydig cells requires coordinated expression and function of several transcription factors and steroidogenic enzymes, many under the overarching control of LH. LH achieves this either via posttranslational modification of transcription factor proteins or via de novo induction of transcription factor gene expression (e.g., NR4a1).[80] Regardless of the method of activation, LH stimulation of cAMP-signaling pathways recruits NR5A, GATA, and often CREB–CREM, NR4A, or Sp1 transcription factors to regulatory regions of each gene's promoter to elicit the transcription of genes that is necessary for steroid biosynthesis (reviewed in Refs 81–83).

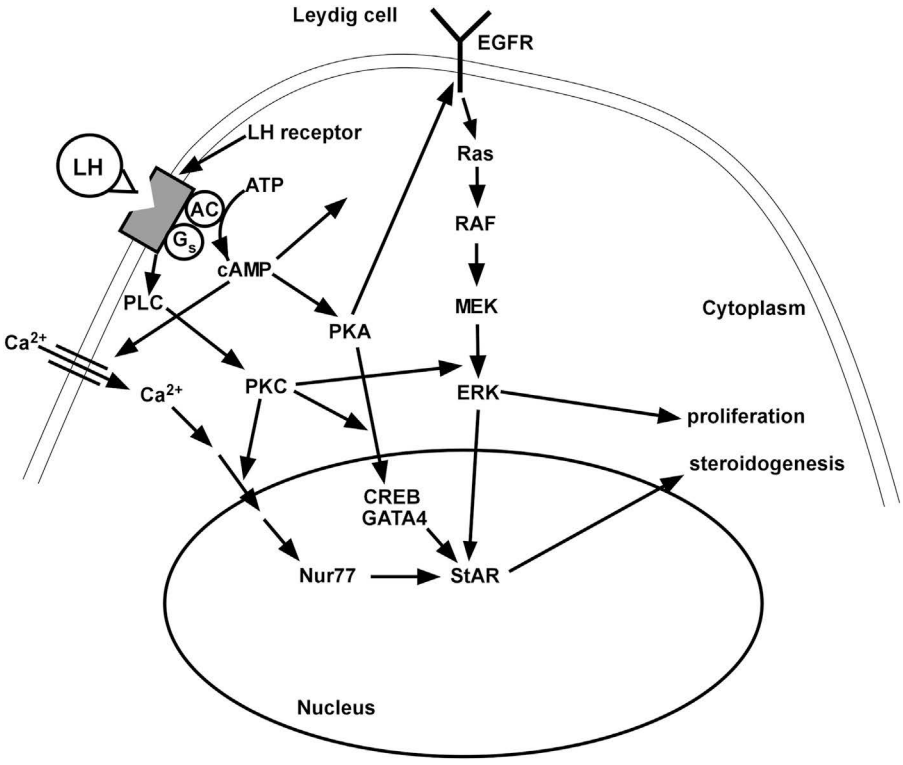

FIGURE 16.5 LH-mediated signaling in a Leydig cell. The major pathway regulated by LH binding to its receptor is the activation of adenylate cyclase (AC) via G_s proteins. The resulting increase in cAMP causes the activation of protein kinase A (PKA) that can directly regulate transcription factors such as CREB or GATA4 to increase the transcription of genes required for steroidogenesis, including *StAR*. LH stimulation can also cause the activation of the MAP kinase pathway (from Ras to ERK), in part via PKA-mediated activation of the epidermal growth factor receptor (EGFR). Activation of ERK increases the expression and phosphorylation of StAR and promotes the proliferation of Leydig cells. Ca^{2+} influx into Leydig cells increases in response to LH and results in the activation of the Nur77 transcription factor that induces *StAR* transcription. LH-mediated increases in phospholipase C (PLC) and then protein kinase C (PKC) activity facilitate the activation of ERK and result in the amplification of ERK, Ca^{2+}, and PKA stimulation of StAR activity.

Several promoters of target genes activated by LH signaling contain cAMP response elements (CREs), an eight-base-pair palindromic sequence (or variant thereof) that can be bound by CREB, CREM, and activating transcription factor 1 (ATF1) to drive gene transcription (reviewed in Ref. 84). The ability of these proteins to activate transcription from CRE-regulated promoters is enhanced upon cAMP–PKA-mediated phosphorylation of the transcription factors. CRE-binding sites have been identified in the promoters of several key genes involved in steroid hormone biosynthesis, and CREB is able to promote transcription of *StAR*, *Cyp11a1*, and *3β-Hsd*.[85] Leydig cell–specific ablation of CREB in transgenic mice leads to downregulation of expression of these three genes in addition to reducing expression of *Cyp17*.[86] The StAR protein is key for the regulation of steroidogenesis transporting cholesterol from the outer to the inner membrane of the mitochondria. Interestingly, the promoters of several steroid biosynthesis genes modulated by cAMP, including StAR, lack a consensus CRE.[87] Instead, control of StAR gene expression is modulated by interactions between several different transcription factors (reviewed in Ref. 82). Steroidogenic factor 1 (SF1; NR5A1) is a member of the orphan nuclear receptor superfamily that has been shown to be involved in the cAMP-regulated expression of many genes, and NR5A1 functions cooperatively with CREB–CREMτ to promote the transactivation of the StAR gene,[88] in addition to its role in induction of steroidogenic enzymes (reviewed in Ref. 83).

TESTOSTERONE

LH exerts its effects upon spermatogenesis principally via promoting testosterone secretion from the Leydig cell, with the androgen acting as a paracrine factor within the testis. Testosterone is a lipophilic steroid hormone that is essential for maintaining spermatogenesis and male fertility.[8,89] Testosterone deprivation studies performed in rodents have established that testosterone is essential for germ cells to progress beyond meiosis and that testosterone is required for the release of mature spermatids (reviewed in Ref. 8). Withdrawal of testosterone or knockout of the androgen receptor (AR; NR3C4) results in three major impairments to spermatogenesis. First, the integrity of the BTB is disrupted. The result of this insult causes meiotic and postmeiotic germ cells, formerly in a secluded specialized environment, to be exposed to autoimmune attack and cytotoxic factors.[90,91] Second, conversion of round spermatids to elongated spermatids is inhibited due to defects in cell adhesion that cause the premature detachment of round spermatids from Sertoli cells.[92–94] Third, fully mature spermatozoa cannot be released from Sertoli cells, and the germ cells are phagocytosed by the Sertoli cells.[94] Together, these findings confirm that testosterone is required for numerous processes that are essential to maintain male fertility.

Testosterone is the major androgen in the testis that regulates spermatogenesis. Androgen effects are mediated by AR, a 110 kDa protein encoded by a single gene on the X chromosome (Figure 16.6). The AR is a member of a large family of ligand-activated nuclear receptors. AR is a modular protein with four structurally and functionally distinct domains, including a poorly conserved N-terminal domain (NTD) encoded by exon 1, a highly conserved DNA-binding domain (DBD) encoded by exons 2 and 3, and a moderately conserved ligand-binding domain (LBD) that is produced from exons 4–8. A portion of exon 4 encodes a short hinge region that separates the LBD from the DBD and also contains part of a bipartite ligand-dependent nuclear

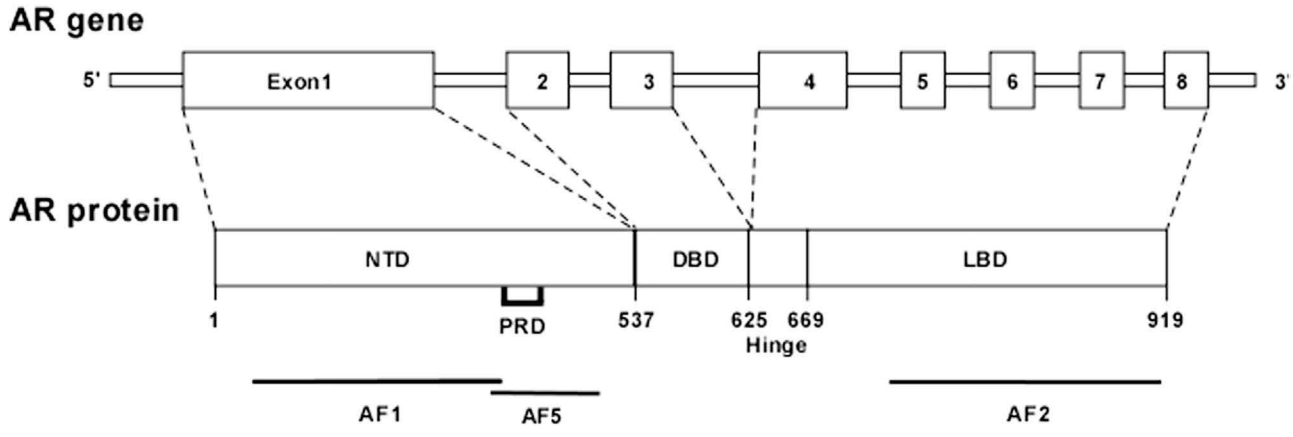

FIGURE 16.6 **Human androgen receptor (AR) structure.** The eight exons of the human AR are located on chromosome X bands q11–12. Exon 1 encodes the amino terminal domain (NTD), exons 2 and 3 encode the DNA-binding domain (DBD), and exons 4 through 8 encode the hinge region and ligand-binding domain (LBD). Also shown is the proline-rich domain (PRD) used to interact with Src kinase, as well as the transcription activating function domains 1, 2, and 5 (AF1, AF2, and AF5). Amino acid residues that separate the domains are shown below the AR protein.

localization signal (NLS) for AR nuclear transport (reviewed in Refs 95,96). Activation domains required for the regulation of gene expression are located in the NTD and LBD. The NTD also contains a proline-rich domain that is required to interact with and activate Src kinase.

AR Expression in the Testis

There are reports of AR expression in germ cells; however, the idea that AR is expressed in germ cells is not commonly accepted because there are other studies showing that AR is not detected in germ cells (reviewed in Ref. 97). Furthermore, at least three functional studies provide evidence indicating that if AR is expressed in germ cells, it is not required for spermatogenesis: (1) In one study, chimeric mice having both AR defective and wild-type germ cells produced pups from the AR defective germ cells[98]; (2) in another, AR defective germ cells transplanted into the testes of recipient wild-type mice rendered azoospermic by treatment with Busulphan were able to form colonies of donor cells undergoing spermatogenesis[99]; and (3) in a third, knockout of AR specifically in germ cells did not alter spermatogenesis or fertility.[100] Together, these results indicate that AR is not required in germ cells to maintain spermatogenesis. Instead, testosterone must support fertility by acting through somatic cells in the testis that express AR.

In the testis, AR is expressed in Sertoli, Leydig, PTM, arteriole smooth muscle, and vascular endothelial cells. In humans and rodents, AR is expressed in PTM cells at high levels from the fetal period throughout adulthood.[101–104] Adult Leydig cells also express AR at a constant level.[105] AR expression in the Sertoli cell is first detected in humans at the age of 5 months but remains weak until 4 years of age.[101–104] In mice and rats, AR in the Sertoli cell is first expressed 3–5 days after birth.[106–109] AR expression in postpubertal Sertoli cells is related to the stages of the cycle of the seminiferous epithelium. In men, AR levels are highest in stage III of the six stages, although AR was easily detected in all other stages.[110] In the adult rat, the expression of AR in the Sertoli cell is similarly cyclical. AR mRNA and protein expression progressively increases during stages II–VII and then declines sharply during or immediately after stage VII to become barely detectable in stages IX–XIII.[107,111,112] Because AR expression is greatest during stage VII, this stage is thought to be the most regulated by, and sensitive to, testosterone.[113] Various models of testosterone withdrawal from rats confirm that progressive germ cell loss begins during stage VII in the absence of testosterone.[8]

Testosterone Levels and Functions in the Seminiferous Tubule

Testosterone levels in the testes of men are 25–125-fold greater (340–2000 nM) than those in serum (8.75–35 nM). Testosterone levels are similarly elevated in the rodent testes.[114–118] One-third of testicular testosterone is tightly bound to sex hormone–binding globulin (SHBG, which has the same amino acid sequence as androgen-binding protein (ABP)) and is not readily available. However, two-thirds of testicular testosterone is free or bound to albumen and is bioavailable.[119,120] The bioavailable levels of testosterone in the testis far exceed those required for function as the binding affinity of testosterone for AR is approximately 1 nM, and the levels of testosterone required to regulate gene expression via AR binding to gene regulatory regions are 1–10 nM.[121] However, sperm counts decrease at a logarithmic rate as testosterone levels fall below 70 nM in the rodent.[122] Thus far, the biology underlying the necessity for the high levels of testosterone in the testis is poorly understood. One possibility is that the higher levels of testosterone may be required to activate recently identified nongenomic mechanisms of testosterone action that have been found to regulate processes required for spermatogenesis.[123]

Testosterone-Signaling Pathways

Genomic (also known as classical) testosterone signaling initiates with testosterone diffusing through the plasma membrane, followed by binding to AR that is often sequestered in the cytoplasm by heat shock proteins. When bound by androgen, AR undergoes a change in conformation that allows it to be released from the heat shock protein complex. To transmit classical testosterone signaling, AR then translocates to the nucleus, where it binds as a dimer to specific DNA sequences called androgen response elements (AREs) (Figure 16.7, pathway 1). AR binds to AREs using the DBD region encoded by exons 2 and 3, which form two zinc finger domains. AREs may be located in promoter regions near the transcription start sites of genes or at a distance far from the gene. When bound to the DNA, AR recruits co-activators or co-repressor proteins and protein complexes that together modulate gene expression. This classical pathway of testosterone action requires at least 30–45 min to alter gene expression and at least 1 h to produce nascent proteins.[125]

In addition to the classical pathway of testosterone signaling, there is evidence in Sertoli cells of more rapid, nonclassical (also called nongenomic) pathways of testosterone action. The nonclassical pathway requires seconds to initiate, and cellular effects can be assayed within minutes. Thus far, studies of nonclassical signaling by

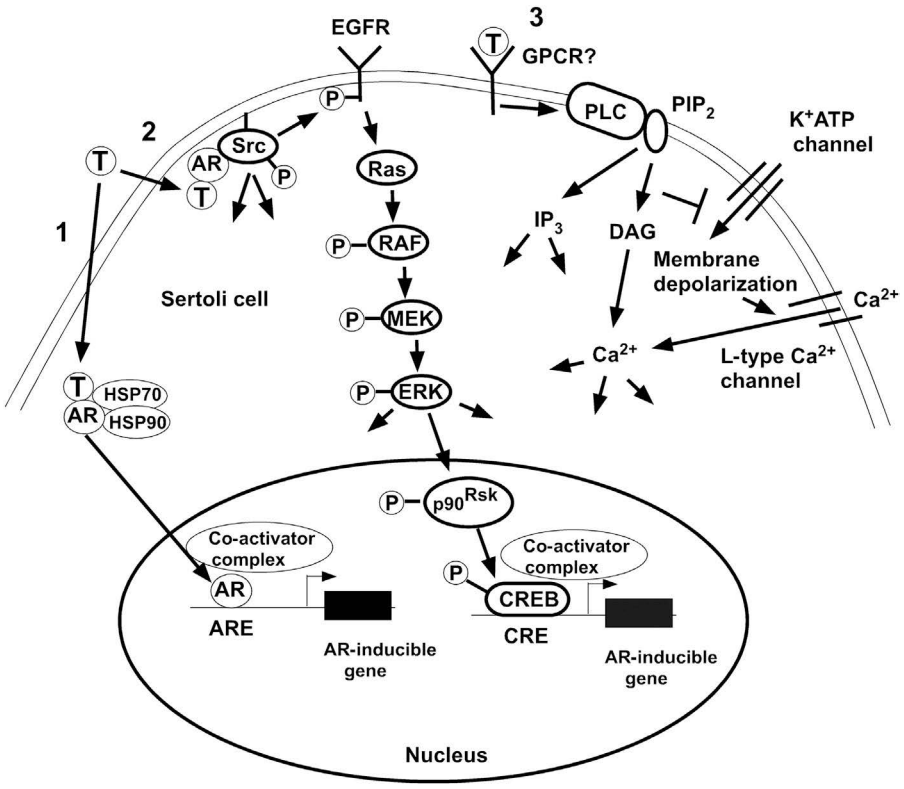

FIGURE 16.7 **Testosterone-signaling pathways.** (1) The classical testosterone-signaling pathway. Testosterone diffuses through the plasma membrane and binds with the AR. The AR undergoes an alteration in conformation that allows it to be released from heat shock proteins in the cytoplasm. AR then is able to translocate to the nucleus, where it binds to specific DNA sequences called androgen response elements (AREs). AR binding to an ARE allows the recruitment of co-activator and co-repressor proteins that alter the expression of genes to alter cellular function. (2) The nonclassical kinase activation pathway: testosterone interacts with the classical AR that then is able to recruit and activate Src, which causes the activation of the EGF receptor via an intracellular pathway. The EGF receptor then activates the MAP kinase cascade, most likely through Ras, resulting in the sequential activation of RAF and MEK and then ERK that activates $p90^{Rsk}$-kinase, which is known to phosphorylate CREB on serine 133. As a result, CREB-regulated genes such as lactate dehydrogenase A (*LDH-A*), early growth response 1 (*Egr1*), and *CREB* can be induced by testosterone. (3) The nonclassical Ca^{2+} influx pathway: testosterone interacts with a receptor in the plasma membrane that has characteristics of a G_q-coupled G protein–coupled receptor (GPCR). Phospholipase C (PLC) is activated to cleave PIP_2 into IP_3 and DAG. Lower concentrations of PIP_2 inhibit K^+_{ATP} channels, causing membrane depolarization and Ca^{2+} entry via L-type Ca^{2+} channels. *Source: Reproduced with permission from Ref. 124.*

testosterone have focused on two rapidly activated pathways in the Sertoli cells (Figure 16.7, pathways 2 and 3). First, testosterone acts via AR to activate a series of kinases, including Src and the mitogen-activated protein kinase (MAPK) cascade, as well as the CREB transcription factor and CREB-mediated transcription (reviewed in Ref. 123). Second, testosterone also can depolarize the Sertoli cell and cause Ca^{2+} influx, resulting in activation of intracellular signaling factors (reviewed in Ref. 126). It has not been established whether AR or another receptor is required to initiate this second pathway. Both classical and nonclassical signaling are discussed in greater detail in this section.

Cell-Specific Ablation of AR in the Testis

To examine the cell-specific roles of AR signaling, several groups have exploited the Cre–loxp systems to ablate AR in a cell-specific manner. At least five "floxed" alleles of AR have been generated[94,127–130] (reviewed in Refs 131,132), and the specific idiosyncrasies of this allelic series have proved far more informative than a single allele ever could. Two models target exon 1,[94,128] and systemic recombination of the floxed allele results in the expected phenotype, similar to the spontaneous AR mutant—the testicular feminized (Tfm) mouse.[133] However, one of these mutants has been engineered to retain a neomycin selection cassette that reduces AR expression in nonrecombined cells. These animals represent an important and novel model of partial androgen insensitivity, although interpretation of the cell-specific ablation of AR on this background is complex.[94] Far from proving problematic, however, this allele has provided a series of important observations,[91,134,135] especially when compared to similar cell targeting of AR in other floxed models of this receptor (reviewed in Ref. 131). Two further

models target exon 2.[127,130] In the absence of recombination, these floxed male mice are indistinguishable from nontransgenic males. In contrast, Cre-mediated ablation of exon 2 causes a frame shift mutation, which introduces a premature stop codon into the mRNA transcript that is quickly degraded. No AR protein is observed. The fifth floxed allele of AR deletes exon 3 upon Cre-mediated recombination.[129] Whereas deletion of exon 3 removes the second zinc finger of the DNA-binding domain, splicing remains in frame, and thus a truncated version of the AR protein is produced. The mutant protein is unable to bind to AREs in target genes and may have utility for discerning nonclassical roles of AR. Interestingly, in the absence of recombination, this allele confers slight hyperandrogenism on the mouse.[136] Together, these five AR alleles have been widely exploited to paint a very detailed picture of androgen signaling in the testis (reviewed in Refs 97,131,132,137–139).

AR in Sertoli Cells

The first use of the floxed AR alleles was to target AR in Sertoli cells, which have been acknowledged for many years as the key cell type supporting spermatogenesis. Three different groups published descriptions of independently generated Sertoli cell–specific AR knockout (SCARKO) models within 6 months of each other in 2004.[94,127,140] Although different floxed alleles of AR were employed, two of the three groups[127,140] chose to utilize the same Amh–MIS–Cre line, which is highly regarded for its specific and efficient Sertoli cell–targeting properties.[141] The third group generated an independent Amh–Cre line, which also targeted Sertoli cells with high efficacy.[94] Sertoli cell expression of AR begins at approximately postnatal day 4 in wild-type mouse testis.[142] However, both Amh–Cre lines ablated AR in fetal life. Thus, in all three models, Sertoli cells were never exposed to androgen–AR stimulation.

One of the most important outcomes from the SCARKO mouse studies versus those of total ARKO or Tfm mice was that restricted ablation of AR from Sertoli cells did not prevent testicular descent, due to continued androgen action at the level of the gubernaculam.[143] This result highlights the advantages provided by conditional gene targeting, as for the first time it was possible to examine the cell-specific role of androgens within the testis, without the confounding factor of cryptorchidism.

As both total-ARKO and Tfm mice display a significant reduction in Sertoli cell number, a similar reduction in Sertoli cell number was expected to arise from rendering Sertoli cells insensitive to androgens. Consistent with this hypothesis, testis weight was significantly reduced in SCARKO mice. Because Sertoli cells can simultaneously support only a fixed number of germ cells, a reduction in Sertoli cell number would reduce quantitative spermatogenesis, explaining the weight loss. However, surprisingly, this was not the case. SCARKO mice developed normal numbers of Sertoli cells that underwent appropriate maturation at the correct pubertal time point, demonstrating that Sertoli cell AR is dispensable for final Sertoli cell number and maturation.[144] The profound conclusion arising from this observation is that androgens must act via a different cell type to control the final Sertoli cell number, which was the first indication that a wider androgen-dependent paracrine-signaling network was at play in the testis.

Despite the Sertoli cell number being unaffected, SCARKO mice were completely infertile in all three models. Ablation of Sertoli cell AR did not impact the numbers of spermatogonia. However, spermatocytes were slightly reduced in number, and postmeiotic spermatids were significantly reduced or absent, consistent with a block in spermatogenesis during meiosis. This phenotype was later confirmed in a fourth model of Sertoli cell AR ablation.[145] These results were similar to the block in spermatogenesis observed in models with suppressed testosterone production.[146] This phenotype cannot be rescued in hpg.SCARKO mice treated acutely with exogenous androgens.[147] Together, these findings suggest that the primary effects of androgens acting via AR in Sertoli cells during adulthood are to support meiotic and postmeiotic germ cell development. In addition to the block in spermatogenesis, loss of Sertoli cell AR impacted several Sertoli cell–dependent functions, including defects in intact junctional complex formation, which perturbs the permeability of the BTB and compromises the immune privilege of the adluminal compartment[90,91,148,149] (Figure 16.8, pathway a–c).

In at least one model, ablation of Sertoli cell AR also leads to a significant reduction in the number of adult Leydig cells, suggesting that androgens function via Sertoli cells to influence Leydig cell development (Figure 16.8, pathway d). However, this reduction in Leydig cell number is compensated by increased testosterone production per Leydig cell, to maintain normal circulating testosterone concentrations.[150]

AR in Peritubular Myoid Cells

PTM cells interact intimately with Sertoli cells, for example to direct deposition of basement membrane components that surround the seminiferous epithelium.[151,152] Loss of PTM cells results in abnormal spermatogenesis,[153] although the roles that androgens play in this process remained a mystery for many years. In mice, AR is expressed in PTM cells throughout life,[154] and two models of AR ablation in PTM cells have been published. Differences in the choice of Cre recombinase lines used to target this cell type, however, resulted in widely different phenotypes. Further analysis suggested that one

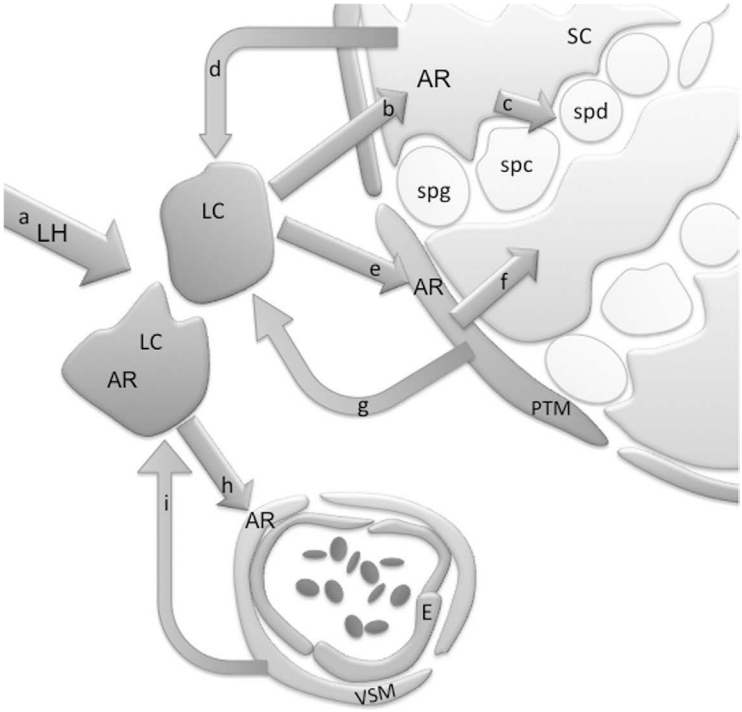

FIGURE 16.8 Androgen signaling within the testis. Androgen receptor (AR) is not expressed in germ cells, and as such, mediation of the testosterone signal on germ cells is via somatic cells that express AR. The cell-specific roles of AR signaling have been established through conditional gene targeting, revealing a complex paracrine signaling system at work in the testis that can be summarized as follows: LH enters the testis, binding LHCGR to stimulate testosterone production by Leydig cells (LC) (a). Testosterone binds AR in Sertoli cells (SC), promoting development of the blood–testis barrier (b) and supporting postmeiotic germ cell development (c). Unknown paracrine factors stimulated by SC-AR signal to control the final Leydig cell numbers (d). Stimulation of AR in PTM cells promotes PTM development and function (e), and the release of unknown paracrine-signaling molecules that support SC maturation and function, which in turn supports spermatogenesis (f). Furthermore, stimulation of AR in PTM cells also feeds back to promote differentiation of adult Leydig cells (g). Testosterone signaling to AR in vascular smooth muscle (VSM) promotes testicular vasomotion and fluid regulation in the interstitium (h), whereas AR activation in vascular smooth muscle cells supports overall Leydig cell function (i). E: endothelial cells, surrounding an arteriole; Spg: spermatogonia; Spc: spermatocyte; Spd: spermatid.

of the two models poorly targeted the PTM cells,[155] and thus the second model has become accepted as providing a more robust characterization of AR ablation from PTM cells.[154] In mice with cell-selective ablation of AR from PTM cells from embryonic day 17, testes are indistinguishable from littermate controls at postnatal day 12, suggesting that AR signaling in PTM cells is dispensable for initial testis development. However, at day 15, there was a significant reduction in testis weight, with gross and multiple impacts on testicular architecture and function.[154] Adult PTMAR knockout (PTMARKO) males are infertile, with significant reduction in numbers of spermatocytes and spermatids. The function of PTM cells in adults is directly impacted by the loss of AR, with changes in the expression and localization of several proteins such as the intermediate filament desmin and basement membrane protein laminin (Figure 16.8, pathway e). Sertoli cell function is also impacted, as evidenced by a reduction in lumen diameter (a measure of seminiferous tubule fluid secretion), and significant changes in gene expression of several key genes, including those previously identified as responsive to AR signaling within Sertoli cells[154,155] (Figure 16.8, pathway f). Loss of AR in PTM cells also impacts adult Leydig cell development. Whereas PTMARKO mice have normal numbers of adult Leydig cells (defined as 3βHSD positive cells), Leydig cell differentiation remains incomplete in PTMARKO mice, with significant numbers of precursor Leydig cells present in testes in adult mice[154,156] (Figure 16.8, pathway g). Interestingly, PTMARKO mice are able to maintain circulating testosterone concentrations within the normal range, but increased LH input is required. PTMARKO mice have a fourfold increase in intratesticular testosterone, suggesting that testosterone secretion by the Leydig cells within PTMARKO mice is perturbed,[154,156] or that the vascular system is impacted in some way that impairs movement of testosterone out of the testis.

AR in Leydig Cells

Data derived from a variety of sources identify several key functions of AR in Leydig cells, including the regulation of their final adult numbers, normal morphological development and maturation, and control of steroidogenic gene expression (reviewed in Ref. 157). However, much of these data derive from analysis of the ARKO mouse, which not only has a 40% reduction in numbers of Leydig cells compared to littermate controls, but also is limited by the ablation of AR signaling in all cell types of the body from fetal life, cryptorchidism, and low circulating testosterone. A Leydig cell–specific AR ablation (LCARKO) model has been reported,[100,158] although the phenotypic analysis of this mutant is complicated by the choice of Cre (anti-Mullerian hormone receptor 2 (AmhR2–Cre))[159] used to ablate AR. The AmhR2–Cre mouse has previously been utilized to target genes in both Sertoli cells and Leydig cells (reviewed in Ref. 160), and the reported LCARKO mutant displays some features consistent with SCARKO ablation models, which are therefore difficult to attribute to loss of AR specifically in Leydig cells. Thus, the functional role of AR signaling specifically within Leydig cells remains to be established.

AR in the Testicular Vasculature

Vasomotion is an androgen-dependent rhythmical contraction–relaxation mechanism present in several tissues, including the testis, that regulates fluid and nutrient exchange between the vascular system and peripheral tissues.[161,162] Direct evidence for the effector cell type of this androgen action has come from vascular cell–specific AR ablation models. Whereas AR expression in vascular endothelial (VE) cells appears to be dispensable for normal testis function, as shown with vascular endothelial androgen receptor knockout mice,[163] AR ablation from vascular smooth muscle (VSM) cells (smooth muscle cell-specific AR knockout mouse (SMARKO)) subtly impairs testis function.[164] Gross reproductive development is normal in SMARKO mice, but adult testis weight is reduced compared to that of littermates. Despite this, SMARKO mice are fully fertile. Cell-specific ablation of AR from VSM leads to a significant increase in interstitial fluid volume within the testis, due to impairment of testicular vasomotion[164] (Figure 16.8, pathway h). This is associated with slightly diminished Leydig cell function (Figure 16.8, pathway i), possibly a consequence of changes in testicular fluid exchange and microvascular blood flow within the testis.

Inducible AR Ablation and Adult Testis Function

One of the limitations of each of the published cell-specific ARKO models is that the timing of AR ablation is within fetal life. As a result, it is difficult to unequivocally assign phenotypic effects to perturbations in adult function. This situation is further complicated by the concept of fetal programming, where events in fetal life can influence phenotypic outcomes many weeks (in the case of rodents) or years (in the case of humans) later. Indeed, the concept of the Male Programming Window—the time period during fetal life in which androgen action is essential to program later developmental events—as demonstrated in the rodent,[165–169] is now widely accepted as a relevant paradigm for all male reproductive organs, including the testis.

To circumvent the temporal limitations of AR ablation and examine the role of androgens in adult testis function requires the separation of development and adult function. To achieve this requires the development of inducible models of AR ablation. A ubiquitously expressed tamoxifen-inducible Cre recombinase has been exploited to permit study of the testis following global ablation of AR in adulthood.[170] Analysis of the testis following inactivation of AR in adulthood revealed spermatogenic arrest when examined 50 days post treatment with tamoxifen. This was associated with decreases in expression of known AR target genes and impaired Sertoli cell function. However, tamoxifen, a potent estrogen receptor (ER) alpha agonist in the testis and elsewhere, itself resulted in changes in Leydig cell gene expression. In particular, control animals treated with tamoxifen showed changes in expression of genes associated with steroid hormone synthesis, along with a reduction in circulating FSH, LH, circulating testosterone, and intratesticular testosterone.[170] Whereas tamoxifen-induced ablation of AR (or, indeed, any gene in the testis) is a valid approach, the strategy provides additional challenges in terms of interpretation of phenotypic outcomes. Alternative approaches include doxycycline-inducible systems, cell-specific expression of dominant-negative forms of AR, and miRNAs targeted against AR; although each has been utilized in vitro, none has yet been exploited to target AR in vivo.

In males, it has long been thought that the AR in the testis is saturated with testosterone, because the concentrations of the steroid are orders of magnitude higher than in the circulation. Conceptually, therefore, modulation of AR abundance or AR signaling could provide the rheostat for androgen action. Whereas patients with completely versus partially inactivating mutations in AR demonstrate that this can be the case in terms of reduced AR action, it is only recently that the timing of AR expression has emerged as a paradigm for the study of AR action in the testis. Premature expression of *AR* mRNA in Sertoli cells has been induced by driving an *AR* cDNA transgenically from the *Abp* promoter. This transgene directs AR expression from e17,[145] while endogenous AR is detectable in mouse Sertoli cells at postnatal day 4.[142] Early expression of AR results in a reduction in Sertoli cell numbers in adulthood, despite increased expression of FSH and FSH receptor, and premature maturation of seminiferous tubules. There also was increased support for meiotic cells, but with reduced numbers of postmeiotic cells,[171] which is probably the result of increased AR expression causing the premature termination of Sertoli cell proliferation and the initiation of Sertoli cell differentiation. As a result, the fewer Sertoli cells produced support fewer germ cells. Importantly, this study also suggests that the close association between induction of AR expression and downregulation of *Amh* expression in Sertoli cells may be coincidental, with no direct causal link (at least in mice), which has been a long-held paradigm of interaction between the two hormone pathways.[172]

Testosterone-Regulated Gene Expression in the Testis

Testosterone may mediate gene expression changes via classical and nonclassical signaling pathways. Thus far, relatively few testosterone-regulated genes have been investigated to determine which pathway is required to alter their expression. Nonetheless, a number

of testosterone deprivation and replacement studies as well as AR mutant and knockout models have provided extensive information regarding testosterone-mediated gene expression in the testis.

Animal models with altered testosterone secretion have been used to identify testosterone-regulated genes. Several survey studies using various rodent models have been performed to assay gene expression in the presence and absence of testosterone signaling. In the first microarray studies that compared testis gene expression in normal 8-day-old neonatal mice versus mice injected with testosterone propionate, about 220 genes had expression changes of at least twofold, with 37, 45, and 50% of the genes being downregulated by testosterone 4, 8, and 16 h after androgen administration, respectively.[173]

Another study employed the *hpg* mouse model lacking FSH, LH, and therefore testosterone inputs. The mice were injected with testosterone propionate to increase testicular content of the steroid for 4, 8, 12, or 24 h. At each time point except for 24 h, testosterone resulted in more downregulated than upregulated genes. Groups of genes encoding milk proteins were activated, perhaps due to testosterone being aromatized to estrogen, which in turn upregulated pituitary prolactin secretion (discussed further in this chapter). Class I major histocompatibility complex (MHC) genes were activated that may contribute to the immunosuppression observed in testes.[174] A more recent study, also using an *hpg* model, found that 10 days of treatment with a nonaromatizable androgen (Dihydrotestosterone) increased the expression of Claudin 3 and Claudin 11 genes encoding transmembrane proteins required for the BTB and resulted in the formation of tight junctions in the *hpg* mice.[175]

Overall, the use of rodent models with altered testosterone levels in these initial studies identified many testosterone-regulated genes, with a high percentage being downregulated by the steroid. However, the genes identified by these survey studies showed relatively little overlap, the number of more highly regulated genes (> twofold) was limited, and few of the identified genes had known fertility phenotypes (reviewed in Ref. 138).

Identification of Testosterone-Regulated Genes Using AR-Deficient Models

A study using 20-day-old *tfm* mice having inactive AR in all cells identified 4700 differentially expressed genes in the testis as compared to normal mice.[176] From this database, 42 genes were found to be expressed by somatic cells of seminiferous tubules. Of the 42 genes, 26 were assayed: 20 were downregulated in *tfm* testes, and six were upregulated. Many of these genes were associated with vitamin A metabolism, solute transportation, or cytoskeletal function.[176] Unfortunately, this model is not ideal because it compares normal testes with those from *tfm* mice that do not descend into the scrotum and have arrested germ cell development. Thus, most of the genes identified were found to be of germ cell origin.

Studies comparing the transcriptional profiles of adult wild-type mice with AR hypomorph mice and AR hypomorphs having Sertoli cell–specific ablation identified 46 and 57 testis-expressed genes, respectively, that were regulated at least twofold by testosterone. About twice as many genes were found to be downregulated by testosterone than upregulated.[135] Genes involved in metabolic processes and signal transduction were identified as classes of genes found to be regulated via AR. In this study, it was determined that 65% of AR-regulated genes were associated with a conserved ARE within 6 kb of their transcription start sites, and at least 23% of the identified genes were previously shown to be regulated by validated AREs.[135] These findings confirm that testosterone and AR act directly via the classical signaling pathway through AREs to regulate a substantial percentage of testosterone-regulated genes in the testis. However, the lack of AREs associated with all testosterone-regulated genes suggests that some genes are regulated indirectly after initial stimulation of ARE-dependent genes, via posttranscriptional processes, or by other mechanisms such as nonclassical testosterone signaling.

AR-Dependent Regulation of Gene Expression in Sertoli Cells

In the first study of gene expression using SCARKO mice 10 days after birth, 40 genes showed a difference of at least twofold, with 28 downregulated by testosterone and 12 upregulated. Of the 40 differentially regulated genes, three (*Galgt1*, *PCI*, and epididymal protease inhibitor (*Eppin*)) are known to be required for fertility, but none completely block functions specific to seminiferous tubules, which are required for spermatogenesis.[155] *Galgt1* (*β1,4-N-acetylgalactosaminyltransferase*) encodes an enzyme required for the formation of complex gangliosides. Galgt1-deficient mice cannot complete spermatogenesis due to the inability to produce a specific class of fucoylated glycosphingolipids that may be required for the formation of the unique plasma membrane of haploid germ cells and for the transport of testosterone from Leydig cells to the seminiferous tubules and the bloodstream.[177,178] *PCI* encodes a serine protease inhibitor, and *PCI* knockout mice have spermatogenesis defects, including detached germ cells, disruption of the BTB, and damage to Sertoli cells; however, sperm are produced, but they are incapable of fertilization.[179] *Eppin* has not been shown to be required for spermatogenesis, but it is required for fertilization of the egg by sperm and has been the target of contraceptive development.[180] Functional analysis of a larger group of 692 differentially regulated genes found that of the 396 downregulated

genes, there was an overrepresentation of genes associated with MAPK activity and serine endopeptidase inhibitor activity. Functions overrepresented in the 296 upregulated genes included signal transduction, cell adhesion, Ca^{2+} binding, Ca^{2+}-dependent phospholipid binding, insulin-like growth factor (IGF) binding, and serine protease inhibition.[155]

A follow-up study using the same mouse model determined that differences in gene expression in SCARKO mice were first detected 8 days after birth.[142] Further analysis of gene expression in SCARKO mice 8–20 days after birth showed that genes encoding proteases, cell adhesion proteins, cytoskeletal elements, and extracellular matrix components were differentially expressed during the initiation of spermatogenesis, supporting the idea that the restructuring of seminiferous tubules and changes in cell junctional dynamics are triggered by testosterone during early puberty.[90,155]

Another group using a similar SCARKO mouse model and a more directed quantitative polymerase chain reaction (qPCR) assay approach found that genes encoding tight junction proteins of the BTB (claudin 11, occludin, and gelsolin), basement membrane components, tissue-remodeling factors, paracrine factors for support of germ cells, as well as transport proteins (transferrin, fatty acid–binding protein (FABP), and ABP) were regulated by AR 10.5 days after birth.[149] The genes identified and their expression differences from controls overlapped nearly completely with a similar qPCR-based study using the first SCARKO mouse model.[144]

A different SCARKO model was constructed using a juvenile spermatogonial depletion mutant (*jsd*) background. The *jsd* mice become sterile after the first few waves of spermatogenesis such that spermatogenesis does not subsequently proceed beyond the undifferentiated type A spermatogonia stage of development.[181] Thus, the creation of *SCARKO–jsd* mice permitted direct comparison of gene expression with *jsd* mice to determine the effects of AR knockout specifically in adult Sertoli cells in the absence of differentiating germ cells for both models. Compared to the *jsd* mice, 157 genes were downregulated and 197 genes upregulated in the *SCARKO-jsd* mice. Genes involved in cell adhesion, signal transduction, nuclear localization, and meiosis were differentially regulated in the absence of AR. Genes known to be expressed in differentiating spermatogonia were repressed in the absence of AR, which was consistent with the previous finding that suppression of testosterone in *jsd* mice supported spermatogonial differentiation.[134]

Genes Regulated by Classical Testosterone Signaling

Androgens mediate classical testosterone signaling by facilitating AR binding to AREs associated with androgen-regulated genes. The first AREs (classical AREs) were identified as inverted repeats of 5′-TGTTCT-3′-like motifs separated by three base pairs. These classical AREs can be occupied by AR as well as other steroid hormone receptors. An additional class of AREs (selective AREs) have been identified that bind AR more selectively. Selective AREs are direct rather than inverted repeats of 5′-TGTTCT-3″ (reviewed in Ref. 182).

Mice were engineered to express an AR that can bind classical but not selective AREs (the specificity affecting AR knockin (SPARKI) mouse). SPARKI mice display a 35% reduction in the size of the testis, and they are subfertile, as reflected by a 50% reduction in litter size. Spermatogenesis appears to occur normally up to meiosis in SPARKI mice because the number of spermatocytes is near normal. However, there are 53 and 56% decreases in the number of round and elongated spermatids, respectively. Sertoli cell numbers also are reduced by 32%. These results indicate that testosterone signaling through selective AREs contributes to Sertoli cell proliferation and/or survival. The percentages of germ cells completing meiosis and transitioning to become elongated spermatids were reduced 29 and 37%, respectively, in SPARKI mice. This reduced spermatogenesis efficiency in the absence of signaling from selective AREs is due to both the decreased ability of Sertoli cells to support germ cell maturation and the decrease in Sertoli cell number.[183] Analysis of testosterone-regulated gene expression in the SPARKI mouse testis revealed that expression levels of the *Rhox5*, *Tsx*, and *Drd4* genes were severely reduced, but expressions of *Spinlw1*, *Eppin*, *PCI*, and *Tubb3* were not affected. The gene expression studies suggest that in the testis, some genes are directly regulated through classical AREs, whereas other genes are regulated by selective AREs or by another mechanism. No studies of the specific effects of blocking testosterone regulation via classical AREs have been reported.

An Overview of AR-Regulated Gene Expression in the Testis

Overall evaluation of the various surveys of AR-regulated genes reveals at least four trends. First, all of the survey studies identify large sets of genes that are regulated by testosterone but relatively few that are regulated by more than twofold. This finding suggests that testosterone may maintain spermatogenesis via altering the expression of a broad spectrum of genes and that the sum of many small changes may be required to support fertility.

Second, of the differentially expressed genes, a large percentage appears to be downregulated by testosterone signaling. Downregulation of gene expression after testosterone stimulation is likely to occur in many instances due to indirect actions such as gene regulation downstream of the initial transcriptional regulatory event, due to post-transcriptional regulation, or in response to the activation of kinases by nonclassical testosterone signaling.

Third, there are few highly testosterone-responsive expressed genes that are commonly identified by the various models. For example, only the four highly regulated genes *Rhox5*, *Lrp4*, *Drd4*, and *Fhod3* are quantitatively regulated in the same direction in studies employing the SCARKO, SCARKO-jsd, and *tfm* models.[184] Also, few genes identified in the SCARKO model matched those that are rapidly induced by androgens in the testes of *hpg* mice or 8-day-old wild-type mice.[142] The lack of overlap is likely due to the differences in the ages of the mice, the duration of androgen stimulation or deprivation, hormone levels, and the germ cell constitution of the testes in the models that were used. Nevertheless, comparisons of differential gene expression across some models reveal some shared AR-regulated genes (Table 16.1). For example, several genes related to retinoic acid metabolism were identified in the *tfm* and AR hypomorph models, including alcohol dehydrogenase I, which is the rate-limiting step in the conversion of vitamin A to the potent signaling factor, retinoic acid. The transition from undifferentiated type A spermatogonia to differentiated type A1 spermatogonia, the entry into meiosis, and establishment of the cycle of the seminiferous epithelium are regulated by retinoic acid.[185–187] In the SCARKO and *tfm* models, seven highly regulated genes are found in common: *Rhox5*, *Drd4*, *Lrp4*, *Tsx*, and three serine endopeptidase inhibitors (*Eppin*, *Serpina 3N*, and *PCI*). Genes encoding cell adhesion proteins and cytoskeletal elements were also shared by the SCARKO and *tfm* models. Finally, *claudin 3*, which contributes to the formation of the BTB, is differentially expressed in both the AR hypomorph and SCARKO models (reviewed in Ref. 138).

The fourth trend observed from the gene survey studies of AR-regulated genes expressed in Sertoli cells is that their deletion rarely results in lack of sperm production, although there are examples of reduced fertility. Mice lacking the most highly inducible testosterone-regulated gene, *Rhox5*, which was identified in all models, are still capable of completing meiosis and retain some fertility.[188] As discussed in this chapter, the testosterone-regulated *Galgt1*, *PCI*, and *Eppin* genes are required for fertility but may not directly affect spermatogenesis.[177–180] Also, the lack of *Lrp8* causes sperm defects that develop in the epididymis.[188,189] Together, these findings reinforce the idea that testosterone support of fertility can occur via the regulation of functions that occur outside of the seminiferous tubules and that, in the absence of testosterone, it is likely that spermatogenesis is disrupted due to the combined effects on multiple genes.[184]

TABLE 16.1 Selected AR-Regulated Genes in Sertoli Cells, Their Functions, and Their Expression in the Absence of AR

AR-regulated gene	Function and expression without AR	
ADH1[a] Alcohol dehydrogenase	Conversion of vitamin A to retinoic acid	+
Aqp8[a]	Water transport	–
Car7[a]	Carbonic anhydrase	–
Cdo1[a]	Cysteine diogenase1	–
Claudin 3[a]	BTB	–
Claudin 11[a]	Adhesion protein	–
Drd4[a]	Dopamine receptor D4	–
eFABP	Fatty acid–binding protein	–
Eppin–Spinlw1[a]	Serine protease inhibitor	–
Fhod3[a]	Regulates actin cytoskeleton	+
Galgt1	Testosterone carrier	–
Gelsolin	Sperm release	–
Lrp4 = Corin[a]	Protease	–
Lrp8[a]	Apo-lipoprotein E receptor	–
MAKPK 13[a]	Kinase	+
Myoz 2[a] Myozenin 2	Binds actinin and filamin	–
Occludin	Adhesion	–
PCI = Serpin5[a]	Protein kinase C inhibitor	–
Rhox5[a]	Transcription factor	–
Scinderin	Actin severing	+
Serpina 3N[a]	Protease inhibitor	–
Tsx testis specific X-linked	Long noncoding RNA	–

Genes whose expression is decreased (–) or increased (+) in the absence of AR are denoted on the right margin.
[a] *Denotes genes shown to be regulated in more than one study of AR-defective or SCARKO mice.*

Nonclassical Testosterone Signaling

It has been proposed that only classical testosterone signaling is required for spermatogenesis because germ cell development is halted during meiosis in transgenic mice in which exon 3 of the AR containing a portion of the DNA-binding domain was removed.[145] However, the nonclassical activity of the exon 3–deleted AR mutation was not reported in the study. Analysis of nonclassical testosterone signaling in a Sertoli cell line lacking endogenous AR expression but expressing a recreated AR mutant lacking exon 3 showed that ERK phosphorylation did not increase in response to testosterone stimulation (WW, unpublished data). This result indicates that the removal of 39 amino acids of AR in this model may alter the structure of AR to inhibit both nonclassical and classical activity.

Testosterone activates two nonclassical pathways. In the first pathway, testosterone rapidly activates a series of kinases in Sertoli cells that are known to regulate spermatogenesis. Stimulation of Sertoli cells with levels of testosterone (10–250 nM) that are similar to or lower than

those found in the testis causes AR to transiently localize to the plasma membrane and results in AR interacting with and activating Src tyrosine kinase (Figure 16.7, pathway 2).[190] Studies employing RNA interference strategies and the use of AR-defective Sertoli cells indicate that AR and not a novel membrane receptor is required to transmit nonclassical testosterone signals.[191] Androgen stimulation triggers the direct association of the proline-rich region of AR (amino acids 352–359) and the SH3 domain of Src.[192] Testosterone-mediated activation of Src causes the phosphorylation and stimulation of EGFR via an intracellular pathway. Stimulation of EGFR is required to activate the MAPK cascade (Raf, MEK, and ERK) that causes $p90^{Rsk}$ kinase to phosphorylate the CREB transcription factor.[190] Activation of the nonclassical pathway induces the expression of CREB-regulated genes that are not regulated by AREs.[191] In contrast to the classical pathway, induction of ERK and CREB phosphorylation by testosterone is rapid (within 1 min) and can be sustained for at least 12 h.[191]

The release of mature sperm from Sertoli cells is regulated by Src kinase that is activated by nonclassical signaling. Culturing seminiferous tubule fragments in the presence of a Src kinase inhibitor decreased the relative numbers of sperm released. These results are consistent with earlier studies showing that during stages VII–VIII, when sperm are released, activated Src levels increase in the vicinity of the specialized Sertoli-elongated spermatid adhesion complex called the ectoplasmic specialization (ES).[193–195] Src also is known to phosphorylate focal adhesion kinase (FAK), β-catenin, and, *N*-cadherin proteins that contribute to the formation of the adhesion complexes between Sertoli cells and the mature elongated spermatids.[196–198] The redistribution of proteins required for the formation, dissolution, and reformation of the BTB may be dependent upon nonclassical testosterone signaling as testosterone stimulation of *hpg* mice caused the rapid redistribution of claudin 11 to the area of the forming BTB prior to activating the transcription of genes encoding BTB components.[175] Also, kinases that are known to regulate the dynamics of tight and adherens junctions[199–201] are regulated by nonclassical testosterone signaling.[190,202]

In the second nonclassical testosterone-mediated pathway (Figure 16.7, pathway 3), testosterone rapidly induces the influx of $[Ca^{2+}]$ into Sertoli cells within 20–40 s through L-type $[Ca^{2+}]$ channels.[203,204] Testosterone-mediated $[Ca^{2+}]$ influx occurs via the activation of an unidentified G_q-type GPCR and the subsequent activation of phospholipase C (PLC), which then hydrolyzes PIP_2 in the plasma membrane to produce IP_3 and diacylglycerol (DAG). The decrease in the levels of PIP2, an inhibitor of adenosine triphosphate (ATP)-mediated activation of K^+_{ATP} channels, promotes the closing of these channels, causing an increase in membrane resistance and depolarization of the cell. As a result, voltage-dependent L-type Ca^{2+} channels open and allow the influx of Ca^{2+}, which may alter many cellular processes.[205,206] Thus far, potential cellular targets and spermatogenesis processes regulated by the testosterone-mediated $[Ca^{2+}]$ influx pathway have not been investigated.[207]

Androgen Regulation of Micro-RNAs in Sertoli Cells

Testosterone regulates the expression of several micro-RNAs (miRNAs) in Sertoli cells that can decrease the half-life or translation of mRNAs that are highly expressed in Sertoli and germ cells. Using a testosterone-deprivation model in which mice were treated with the anti-androgen flutamide and the GnRH antagonist acyline, the expression of 28 miRNAs was found to be highly altered.[208] In the same study, many of the 28 miRNAs were similarly regulated in hypoandrogenic mice lacking LHβ. At least nine of the miRNAs were developmentally expressed such that their expression peaked on postnatal day 13 or 21 during the androgen-dependent steps of spermatocyte progression though meiosis I or the initiation of spermatid differentiation. Pathway analysis suggested that testosterone-regulated miRNAs targeted genes required for cell junction restructuring and cell signaling, two androgen-dependent processes required for germ cell development. Further analysis showed that the miR-471 miRNA binds to the 3′ UTRs and downregulates the Sertoli cell genes *Foxd1*, a forkhead–winged-helix transcription factor that is important in Sertoli metabolism, and desmocollin 1 (*Dsc1*), a desmosomal cadherin that is required for Sertoli–germ cell adhesion.[208] The downregulation of gene expression by testosterone-regulated miRNAs may be one explanation for the relatively high percentage of genes that are inhibited by testosterone signaling.

Proteomic Analysis of Testosterone-Regulated Meiotic Proteins

The completion of meiosis requires testosterone signaling via AR in the Sertoli cell (reviewed in Ref. 97). Thus far, gene expression surveys have provided relatively little information regarding the genes and proteins that are regulated during meiosis as a result of testosterone signaling through somatic cells. However, a proteomics study employing rat models having various levels of testosterone depletion and replenishment has provided long-needed information regarding the proteins and processes that are regulated in spermatocyte germ cells during meiosis.[209] The meiotic cell proteins that are indirectly regulated by testosterone include those having roles in metabolism and response to stress, including disulfide–isomerases, and heat shock proteins

as well as proteins associated with the unfolded protein response and DNA damage. Further study determined that spermatocytes are subjected to oxidative DNA damage after testosterone withdrawal. Consistent with the fact that androgen suppresses apoptosis, testosterone withdrawal altered the expression and posttranslational regulation of proteins involved in apoptosis. Proteins with roles in RNA splicing and processing as well as posttranslational processing and DNA repair also were identified. Finally, a number of proteins involved in cell division and meiosis were differentially expressed in response to altered testosterone levels.

FOLLICLE-STIMULATING HORMONE

FSH is a protein hormone that is produced by the anterior pituitary and transported to the testis via the circulation. In the testis, only Sertoli cells express the FSH receptor. Thus, all spermatogenesis-supporting functions of FSH are mediated through Sertoli cells. FSH binds to the curved inner surface of the FSH receptor ectodomain in a handclasp fashion resembling a right hand (receptor) grasping an American football.[33,210] The ligand-bound FSH receptors form dimers with the two ectodomains interacting with each other. Resulting signals from conformational changes in the seven transmembrane domains of the receptor are transmitted to the cytoplasmic portion of the receptor and elicit guanine nucleotide exchange in associated heterotrimeric G proteins.[33,211,212]

FSH Receptor Expression in the Testis

In the male adult rat, plasma FSH levels are relatively steady.[213,214] In contrast, the expression of mRNA encoding the FSH receptor is dependent on the stage of the seminiferous epithelial cycle, with levels being highest during stages XIII–II and lowest at stages VI–IX.[215–217] FSHR protein levels vary by more than threefold during the cycle in the rat, with the most receptors present in stages XIII–II and the fewest during stages VII–VIII.[215,218] As a result, there is a cyclical activation of FSH-mediated signaling pathways. For example, FSH-induced cAMP production closely follows the levels of FSH receptor, with the highest levels observed in stages XIV–V and the lowest levels in stages VII–XI.[219]

The expression of the FSH receptor is largely regulated at the level of transcription initiation from its promoter. Although binding sites for NR5A1, SMAD3, ETF, GATA1, and ETS transcription factors have been implicated in regulating *FSH receptor* transcription, an E-box element shared by the rat, sheep, and human *FSH receptor* gene promoters is a major determinant of transcription activity (reviewed in Ref. 220). USF1 and USF2, members of the E-box family transcription factors, bind to the E-box motif and activate the *FSH receptor* promoter in Sertoli cells.[221–223] Binding of USF proteins to the *FSH receptor* promoter increases during Sertoli cell differentiation.[224,225] FSH or cAMP transiently induces the expression of a USF inhibitor in Sertoli cells named ID2 (inhibitor of DNA binding and differentiation) to cause negative feedback on FSH receptor levels. The ID2 protein dimerizes with USF proteins and blocks USF DNA-binding activity, resulting in decreased FSH receptor promoter activity. However, long-term stimulation with FSH restores USF binding to the promoter, as well as promoter activity and *FSH receptor* mRNA levels.[225,226] This FSH-mediated mechanism to transiently decrease *FSH receptor* transcription may explain the cyclical changes in FSH receptor expression that occur in Sertoli cells during the cycle of the seminiferous epithelium in vivo.

Sequences required for activation of the *FSH receptor* gene in vivo are still not fully characterized, but extensive information in addition to that present in the promoter are required. Transgenic mouse studies have determined that a 413 kb region encompassing the *FSH receptor* gene plus 98 kbp upstream and 80 kbp downstream of the gene was not sufficient to provide appropriate temporal and spatial expression of the FSH receptor.[227] The binding of octomer transcription factor 1 (Oct1; POU2F1) to a site in the first intron of the *FSH receptor* gene may contribute to silencing expression of the gene in inappropriate cells.[228]

FSH-Signaling Pathways

FSH is capable of regulating at least five specific intracellular signaling pathways in Sertoli cells: the cAMP–PKA, MAPK, PI3K–AKT–mTOR–p70S6K, Ca^{2+}, and phospholipase A_2 pathways (Figure 16.9).

The cAMP–PKA Pathway

FSH binding to its receptor facilitates coupling of the activated receptor to a heterotrimeric G protein. The subsequent exchange of guanosine diphosphate (GDP) for guanosine triphosphate (GTP) on the G_s subunit leads to the dissociation of G_α from the receptor and the $G_{\beta\gamma}$ subunits. There are four families of G_α proteins (i.e., $G_{\alpha s}$, $G_{\alpha i/o}$, $G_{\alpha q/11}$, and $G_{\alpha 12/13}$) that are activated when bound by GTP. $G_{\alpha s}$ stimulates the adenylate cyclase enzyme to produce cAMP from ATP, whereas $G_{\alpha i/o}$ decreases adenylate cyclase activity and lowers cAMP levels (reviewed in Ref. 230).

Increased intracellular cAMP levels arising after FSH binds to its receptor cause the release of the catalytic subunit of PKA from the regulatory subunits and result in the phosphorylation of numerous PKA target proteins. One target of PKA is a family of transcription factors that bind to CREs, including the CREB transcription

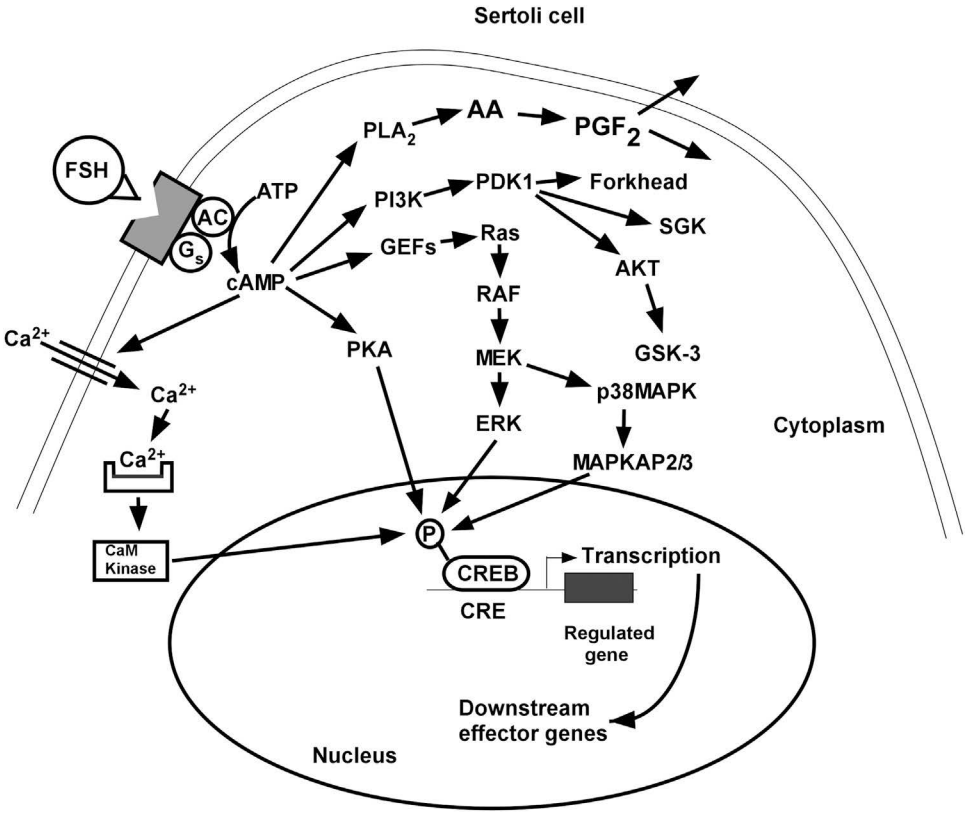

FIGURE 16.9 **FSH-signaling pathways.** Initially, FSH binding to the FSH receptor causes receptor-coupled G proteins (Gs) to activate adenylate cyclase and increase intracellular cAMP levels. Multiple factors can be activated by cAMP in Sertoli cells, including PKA that can phosphorylate a number of proteins in the cell and also regulate the expression and activity of numerous transcription factors, including CREB. FSH also causes Ca^{2+} influx into Sertoli cells that is mediated by cAMP and perhaps PKA modification of surface Ca^{2+} channels. Depolarization of the cell is also involved in Ca^{2+} influx. Elevated Ca^{2+} levels can activate calmodulin (CaM) and CaM kinases that have multiple potential downstream effects, including the phosphorylation of CREB. FSH activates or represses the MAPK cascade and ERK kinase in Sertoli cells in a developmental stage–specific manner. Inhibition of the pathway is not well characterized. Activation is most likely via cAMP interactions with guanine nucleotide exchange factors (GEFs) and activation of Ras-like G proteins followed by sequential activation of RAF, MEK, and ERK kinases. ERK is capable of activating CREB and other transcription factors, including SRF, and c-jun (not shown). In granulosa cells, FSH also activates the p38 MAPK. FSH and cAMP also likely act through GEFs to activate PI3K and then phosphoinositide-dependent protein kinase (PDK1) and PKB in Sertoli cells. Studies of granulosa cells identified Forkhead transcription factor (Forkhead), SGK (glucocorticoid-induced kinase), and GSK-3 (glycogen synthase kinase-3) as additional downstream targets of the PI3K pathway. FSH also mediates the induction of PLA2, the subsequent release of arachidonic acid, and the activation of eicosanoids such as PGE2 that may act as intracellular or extracellular signaling agents. *Source: Modified from Ref. 229.*

factor that is a central regulator of metabolic processes in Sertoli cells required to support germ cells. In Sertoli cells isolated from 15-day-old rats, FSH stimulation rapidly causes CREB to be phosphorylated on serine 133. Elevated cAMP levels increase CREB phosphorylation within 2 min, but CREB phosphorylation and activation are lost quickly (within 5 min) after the removal of stimulation.[231] The importance of phosphorylated CREB in Sertoli cells for the maintenance of fertility was demonstrated with in vivo studies in which a mutant CREB that could not be phosphorylated was overexpressed exclusively in Sertoli cells of rat testes. In seminiferous tubules infected with adenoviruses expressing the mutant CREB in Sertoli cells, disrupted spermatogenesis was observed due to the apoptosis of spermatocytes and subsequent loss of more than 75% of spermatids.[232]

The MAPK Pathway

The MAPK cascade is initiated with RAS stimulation of RAF kinases that phosphorylate MEK, which then phosphorylates ERK (Figure 16.9). FSH can activate the MAPK pathway rapidly and transiently via a $G_{\alpha s}$–PKA pathway and by a slower, more sustained mechanism through β-arrestins.[233] ERK then phosphorylates numerous proteins, including those that regulate cell adhesion, migration, differentiation, metabolism, transcription, and progression through the cell cycle.[234]

FSH activation of the MAPK pathway is thought to be a major promoter of Sertoli cell proliferation during the first 2 weeks of life in rats. In Sertoli cells, FSH activates ERK in proliferating Sertoli cells isolated from 5- to 11-day-old rats. FSH effects on ERK are reversed

coinciding with the end of the Sertoli cell proliferation period. Specifically, stimulation of 19-day-old Sertoli cells with FSH inhibits ERK.[235] Thus, whereas FSH initially supports proliferation, at puberty FSH begins to act to limit ERK activation and repress proliferation.

In the adult rat, FSH receptors are expressed in a cyclical manner such that low levels of FSH receptor and FSH signaling are present during stages VI–VIII. In the stages in which FSH receptor levels are low, less FSH-mediated inhibition of ERK would be expected. This idea is supported by the finding that phosphorylated ERK levels in Sertoli cells in vivo are elevated during stages VI–VIII.[193] The relief of FSH-mediated ERK inhibition may be part of a program that includes testosterone-mediated stimulation of ERK during stages VI–VIII.[202] ERK activity in Sertoli cells has been shown to be required for the progression of spermatocytes through meiosis.[236] ERK regulation of spermatogenesis-regulating processes is also focused in stages VI–VIII. During these stages, activated ERK may contribute to the disassociation of elongated spermatids from Sertoli cells (reviewed in Ref. 200), and disruption of the BTB is associated with activation of ERK.[237] Sertoli–germ cell anchoring dynamics are also regulated by ERK.[201] These findings suggest that FSH signaling during most of the cycle of the seminiferous epithelium may prevent the overactivation of ERK that otherwise would promote the proliferation of Sertoli cells in an already full epithelium and cause inappropriate changes to the BTB and Sertoli–germ cell adhesion.

Pathways Involving PI3K, AKT, mTOR, and p70S6K

Phosphoinositide 3 kinase (PI3K) signaling contributes to many processes, including cell cycle progression, cell growth, survival and migration, and intracellular vesicular transport.[238] G protein–coupled receptors such as the FSH receptor use associated G proteins to activate PI3K, a heterodimer composed of an 85 kDa regulatory subunit and a catalytic subunit of 110 kDa. PI3K then generates phosphorylated phosphatidylinositol intermediates that result in the activation of effector kinases, including AKT–protein kinase B (PKB).[238] FSH was shown to act through cAMP and PKA to activate AKT in a PI3K-dependent manner in Sertoli cells. The activation of PI3K contributes to metabolic processes in Sertoli cells that support germ cell functions. Specifically, FSH-mediated PI3K activation promotes: (1) the uptake of glucose that is required for conversion into lactate, (2) the induction of lactate dehydrogenase (LDH) that is required to produce lactate for germ cells, and (3) the production of γ-glutamyl transpeptidase (γ-GTP), a transporter of amino acids across the membrane and the secretion of transferrin required for iron transport to germ cells.[239] In immature (10-day-old) Sertoli cells, the PI3K pathway is required for FSH-dependent estradiol production, FSH-stimulated aromatase mRNA (*Cyp19a1*), and protein production derived from a ubiquitous NAPDH–cytochrome P450 reductase and a specific cytochrome P450 aromatase (encoded by the *Cyp19a1* gene) in Sertoli cells that irreversibly transforms androgens into estrogens.[240]

The specific pathways by which FSH activates PI3K and AKT in Sertoli cells have not been fully characterized. However, information from the related granulosa cells of the ovary provides potential signaling mechanisms. In granulosa cells, the seven-transmembrane domain of the FSH receptor has been shown to interact with the adaptor protein APPL1 (adaptor protein–containing PH domain, PTB domain, and leucine zipper motif) that, in turn, interacts with the p100α subunit of PI3K and AKT.[241] More recently, it was found that FSH signals through a preexisting growth factor receptor bound protein 2 associated binding protein-2 (GAB2) signalsome complex consisting of GAB2, PI3K, insulin response substrate 1 (IRS1), and PKA. FSH-mediated increases in cAMP levels and activation of PKA lead to phosphorylation changes for the proteins in the signalsome and activation of the PI3K–AKT pathway.[242]

FSH-mediated stimulation of PI3K also results in the activation of mammalian target of rapamycin (mTOR), which in turn activates p70S6K, which phosphorylates ribosomal S6 protein, resulting in increased mRNA translation and cell proliferation. FSH also can act via PKA to upregulate p70S6K activity and the phosphorylation of S6.[243,244] AMP-activated protein kinase (AMPK) is a negative regulator of p70S6K. FSH regulation of AMPK in Sertoli cells is not well characterized. However, AMPK, an inhibitor of granulosa cell proliferation, is inhibited by FSH in the ovarian cells.[245,246] Mutations that disrupt the AMPK–mTOR–p70S6K pathway in humans cause Peutz–Jeghers syndrome, which results in defective spermatogenesis, including severe loss of germ cells, vacuolated Sertoli cells, a Sertoli cell–only phenotype, and proliferating Sertoli cells that crowd the seminiferous tubules.[247,248] The knockout of upstream participants in the AMPK–mTOR–p70S6K pathway in Sertoli cells and the subsequent overactivation of mTOR and p70S6K signaling caused defects in Sertoli cell junctional complexes and cell polarity.[249]

FSH-mediated activation of mTOR and p70S6K also has important implications for the regulation of mRNA translation, as FSH causes the recruitment of mTOR and p70S6K to the 5′ cap of mRNAs and increases the phosphorylation of the eukaryotic initiation factor 4B, a target of p70S6K. The combined effects of this signaling pathway increase recruitment of eukaryotic initiation factors to the mRNA, facilitate mRNA association with polysomes enhance overall protein synthesis within 90 min of FSH stimulation, and may support mRNA-selective translation.[250]

Intracellular Ca^{2+} Pathway

FSH stimulation of immature Sertoli cells causes increases in the levels of intracellular Ca^{2+} within seconds.[251–254] One mechanism by which FSH-mediated changes in Ca^{2+} levels could affect Sertoli cell function is activation of calmodulin and Ca^{2+} calmodulin-dependent kinases that may alter the cytoskeletal structure of Sertoli cells, Sertoli–Sertoli tight junctions, and cell junction dynamics as well as the phosphorylation of transcription factors, including CREB.[255–259]

Studies of freshly isolated Sertoli cells held in suspension determined that FSH-mediated influxes of Ca^{2+} from external sources occurred via both voltage-gated and voltage-independent calcium channels.[252] In cultured Sertoli cells, FSH stimulation also caused Ca^{2+} release from intracellular stores that then resulted in Ca^{2+} influx due to Ca^{2+} release currents.[254] Under both conditions, it was found that increasing cAMP levels and PKA activity contributed to altering channel activity.[252–254] Further investigation determined that FSH causes a short (5 s) hyperpolarization of the Sertoli cell membrane that is dependent upon ATP-sensitive K^+ channels, followed by a prolonged depolarization (more than 6 min) that is dependent upon the uptake of Ca^{2+} by L-type voltage-dependent Ca^{2+} channels.[260–262] It has been proposed that FSH acts via G_i proteins to activate L-type voltage-gated Ca^{2+} channels required for Ca^{2+} entry and hyperpolarization of the plasma membrane and via $G_{\alpha s}$ G protein subunits to convert ATP to cAMP, which opens K^+_{ATP} channels.[263,264] Other studies argued against FSH acting through G_α or G_i to mediate calcium influx but found evidence for a phospholipase C-δ1 (PLC-δ1) pathway that activates T-type Ca^{2+} channels independently of intracellular calcium release.[265,266] It has been reported that FSH-regulated Ca^{2+} uptake through voltage-dependent Ca^{2+} channels is observed only in immature Sertoli cells and thus may be responsible for the differential responses of immature and adult Sertoli cells to FSH stimulation.[267]

Phospholipase A_2 (PLA_2) Pathway

FSH activation of PLA_2 leads to the release of arachidonic acid and its subsequent metabolism to prostaglandin E_2 and other eicosanoids that function as intracellular signals. As a result, adenylate cyclase activity and androgen aromatization are stimulated in Sertoli cells,[268] as well as Sertoli cell apoptosis and proliferation.[269] Further studies are required to better characterize the physiological importance of the PLA_2 pathway in Sertoli cells.

Desensitization of the FSH Receptor

Prolonged exposure of target cells to FSH results in a decreased response with time (desensitization). For example, a 2 h treatment with FSH reduces a subsequent FSH-mediated induction of cAMP production by 70% or more.[270,271] Desensitization can initiate within minutes of FSH binding to the FSH receptor due to phosphorylation of the first and third intracellular domains of the receptor by G protein–coupled kinases. Arrestin proteins are then allowed to bind to the receptor and uncouple the receptor from G proteins, preventing further signal transduction. Arrestins also facilitate internalization of the receptors by interacting with clathrin and concentrating receptors in clathrin-coated pits.[271–274] Long-term exposure of Sertoli cells to FSH also results in the upregulation of phosphodiesterase activity that decreases cAMP levels[275] and downregulation of FSH receptor expression.[276] The FSH-mediated activation of phosphodiesterase, the desensitization and downregulation of the FSH receptor, as well as the stage-specific control of FSH receptor levels suggest that limiting FSH signaling is important for Sertoli cell function. In support of this idea, a mouse transgenic model of constitutive FSH action displays elevated testosterone levels and is infertile.[277]

Differential FSH Signaling in Proliferating versus Differentiated Sertoli Cells

The function of FSH shifts during the development of Sertoli cells. FSH promotes the proliferation of Sertoli cells prior to puberty.[278,279] After puberty, when Sertoli cells do not divide or do so only very rarely in vivo, FSH acts through Sertoli cells to support germ cell viability.[280–282] There is evidence from the rat that FSH acts through the differentiated Sertoli cell to support the survival of spermatogonia,[280,283,284] although it is not clear whether FSH acts only to decrease apoptosis[280,285,286] or to also increase spermatogonia proliferation.[287,288]

FSH promotes Sertoli cell proliferation in rats beginning on day 19 or 20 of fetal life, but most Sertoli cell proliferation is halted 15–20 days after birth in the rat. During this period, circulating FSH levels and the expression of FSH receptors on Sertoli cells remain relatively constant.[278,289–294] Although FSH signaling potential apparently is unchanged, FSH can have opposing effects on proliferating and differentiated Sertoli cells. Alterations to FSH-mediated signaling pathways are thought to be responsible for the differing effects of FSH that occur during Sertoli cell differentiation.

During the proliferating and differentiating stages of Sertoli cell development, FSH effects on cAMP production and activation of the proliferation-promoting MAPK pathway are very different. In proliferating Sertoli cells cultured from 5- to 12-day-old rats, FSH had little effect on cAMP levels but activated the MAPK pathway in a PKA-dependent manner, as shown by increased phosphorylation of the ERK.[235] The FSH-mediated activation of ERK resulted in increased expression of cyclin D1 that

promotes proliferation.[235] In contrast, FSH simulation of differentiating 15- to 20-day-old Sertoli cells increased cAMP levels but decreased ERK activity.[235,244,295,296]

FSH also has been shown to activate the PI3K–AKT–mTORC1–p70S6K signal transduction pathway in Sertoli cells cultured from 8-day-old Sertoli cells. Furthermore, inhibition of the pathway blocked Sertoli cell proliferation.[297] In the 8-day-old Sertoli cells, FSH stimulation also decreased activity of the AMPK that is associated with increased expression of the cell cycle inhibitors $p19^{INK4A}$, $p21^{Cip1}$, and $p27^{Kip1}$.[297] In other studies, FSH stimulation of 5-day-old Sertoli cells increased the levels of PIP3, a downstream target of PI3K signaling. High basal levels of PIP3 were not further increased by FSH in 19-day-old Sertoli cells. These data suggest that FSH can induce PI3K activity in proliferating Sertoli cells, but PI3K activity is less responsive to FSH in differentiating cells.[244]

FSH-mediated increases in the levels of phosphatase and tensin homolog deleted in chromosome 10 (PTEN), an inhibitor of the proliferation-promoting PI3K–AKT signaling pathway, may contribute to blocking the proliferation of differentiating Sertoli cells. In differentiating Sertoli cells isolated from 19-day-old rats, FSH stimulation increases the levels of PTEN. Knockdown of PTEN by RNA interference allows FSH to induce the transcription of genes that are associated with proliferation.[298]

It has been known for some time that the effects of FSH on Sertoli cell proliferation is dependent in part on IGF1[299,300] and activin, which in the testis is produced by Sertoli, Leydig, and PTM cells.[301–304] FSH and IGF1 synergize to increase the activity of the growth-promoting AKT kinase in Sertoli cells,[305] and elimination of the IGF1 receptor in cultured Sertoli cells reduced proliferation, as indicated by BrdU incorporation.[306] More recent studies of mice lacking the insulin receptor and/or the IGF1 receptor confirm that FSH requires the insulin–IGF signaling pathway to mediate proliferative effects on immature Sertoli cells. In the absence of insulin–IGF1 signaling, Sertoli cell proliferation rates were reduced at embryonic day 17 and postnatal day 5, resulting in a delay of Sertoli cell maturation and in small testis.[307] Activin plus FSH stimulation cause synergistic increases in Sertoli cell proliferation,[282,308,309] and the combination of activin plus FSH is thought to prolong Sertoli cell proliferation beyond the period in which FSH is effective alone.[309] The decline in activin levels observed at puberty correspond with the halting of Sertoli cell proliferation and the initiation of differentiation (reviewed in Ref. 310).

The sum of all the changes in FSH regulation of signaling pathways during Sertoli cell development provides insight into the regulation of Sertoli cell proliferation and differentiation. However, the mechanisms responsible for causing the developmental stage–specific intracellular signaling changes in response to FSH have not been fully characterized.

Knockout of FSHβ and FSHR, and the Regulation of Sertoli Cell Proliferation in Mice

Historically, FSH has been shown to provide the major stimulus for Sertoli cell proliferation. Suppression of FSH levels in rats reduces Sertoli cell numbers,[311,312] whereas administration of FSH increases the number of Sertoli cells.[278,292,294,313–315] One time course of FSH immunosuppression in vivo found no effects on Sertoli cell numbers 3 days after birth, but the Sertoli cell population was decreased by FSH immunoneutralization at 9 days after birth. At the time point 18 days after birth, Sertoli cell numbers were not altered but germ cell apoptosis increased. These data indicate that FSH differentially affects Sertoli cells and (indirectly) germ cells in an age-dependent manner, promoting Sertoli cell proliferation during a time span centered around 9 days after birth but supporting only germ cell viability by day 18 in the rat.[280]

More recent studies from one group that employed knockout mouse models have provided information conflicting with the historical data that placed FSH as a central regulator of Sertoli cell proliferation in rodents. Specifically, comparisons of the numbers of Sertoli cells present in FSH-β knockout (FSHβKO) and FSH receptor knockout (FSHRKO) mice with wild-type mice 1 and 5 days after birth revealed no significant differences, suggesting that FSH signaling was not required for Sertoli cell proliferation in late-fetal and neonatal development.[316,317] The number of Sertoli cells was reduced in FSHβKO and FSHRKO mice between 5 and 20 days after birth and more dramatically in the adult. These data suggest that FSH acts only postpubertally to regulate the Sertoli cell number in mice. In contrast, both *tfm* mice lacking AR activity and AR null mice have significantly fewer Sertoli cells 1, 5, and 20 days after birth and in adulthood, suggesting that androgens are important mediators of Sertoli cell proliferation and/or survival,[316,317] although, as discussed in this chapter, the mechanism underpinning this remains unknown.

A second group studying FSHRKO mice has provided data supporting the idea that FSH contributes to prenatal Sertoli cell proliferation. In these studies, the number of Sertoli cells present at birth was reduced by 22% and BrdU incorporation in Sertoli cells was lower in the FSHRKO mice than in intact mice.[318] Other parameters of maturation at birth and 8 days after birth indicated that Sertoli cells were less differentiated in the FSHRKO mice. These results support the idea that FSH signaling contributes to Sertoli cell proliferation and differentiation in the fetal and neonate testis. However, there is a relatively small effect of FSHRKO on the number of

Sertoli cells in mice during one of the most prolific periods of Sertoli cell proliferation.[293]

Explanations for the differing paradigms of FSH regulation of Sertoli cell proliferation obtained from historical studies versus the new results from FSHβKO and FSHRKO mice will require further study. However, it should be noted that whereas the knockout studies were performed in the mouse model, most of the historical studies of FSH effects on Sertoli cell proliferation were performed using rats. One potential difference in these animal models is the patterns of serum FSH level present during development. In rats, FSH levels remain at or above adult levels from birth through 35 days after birth.[213,214] In contrast, serum concentrations of FSH in the neonate mouse are retained at low levels (2–3 ng/ml) before rising four- to fivefold to adult levels between 10 and 25 days after birth.[319] Other studies provide confirmation that FSH levels in adult mice are much lower than those in the adult rat.[320,321] Although direct comparisons of rat and mouse bioactive FSH levels were not performed in these studies, the data available indicate that rat FSH levels during the time period of Sertoli cell proliferation are at adult levels, whereas mouse serum FSH concentrations are at least fourfold lower than adult levels and 100-fold lower than those present in rats. Together, these data raise the possibility that FSH contributes less to testis development in the mouse and that perhaps mice and rats have differing strategies for supporting Sertoli cell proliferation. The developmental pattern of nonhuman primates best matches the rat model in that most Sertoli cell proliferation occurs immediately after birth and again during puberty only when FSH is present at adult levels.[322]

Gene Regulation by FSH Signaling

Surveys of FSH-regulated gene expression have been performed using various model systems, including FSH stimulation of cultured Sertoli cells,[323] immunosuppression of FSH in the rat in vivo,[280] FSH stimulation of *hpg* mice lacking gonadotropins,[324] and FSH stimulation of irradiated rats lacking germ cells.[325] Whereas the survey studies identified novel gene targets of FSH signaling, they also confirmed the regulation of previously described FSH-regulated transcripts required for normal testicular function. These genes include the FSH-stimulated *StAR* gene required for steroid synthesis, the cell cycle promoter *cyclin D2*, and *ABP*. Confirmed FSH-inhibited genes include IGF binding protein 3 (*IGFBP-3*), *HNF-3*/forkhead, endothelin-1, and cathepsin-L.[280]

Studies of gene expression in the testes of FSHβKO and FSHRKO mice indicate that most FSH regulation of genes that are required for supporting germ cells does not initiate until between 5 and 20 days after birth. These models demonstrated that FSH stimulates genes encoding the tight junction components ZO-1 and claudin 11, the tissue remodelers Cys-TE and tissue plasminogen activator (tPA), the ligand-binding protein (fatty acid binding protein), as well as the growth factors, platelet-derived growth factor A (PDGF-A) and kit ligand (also called the stem cell factor or Steel factor). Studies using the FSHβKO mouse also confirmed that FSH is required for the expression of the FSH receptor.[316]

Independent studies have identified other FSH-inducible genes in Sertoli cells that encode proteins having direct effects in supporting spermatogenesis, including DMRT, which has been implicated in sex determination and testis differentiation[326]; transferrin, which is required to transport iron to germ cells[327,328]; as well as VEGF,[323] which may act on receptors present on germ cells from the spermatogonia to round-spermatid stages of development.[329] FSH acts via cAMP and PKA to stimulate the transcription of the inhibin α subunit and secretion of inhibin B,[521–523] which in turn acts on the anterior pituitary to decrease FSH secretion and in the testis to counteract the actions of activins.[330]

FSH-mediated activation of PKA results in the phosphorylation and activation of the CREB transcription factor. CREB is able to activate transcription from the promoters of numerous genes in Sertoli cells. Two examples include the CREB gene promoter[231] and an internal promoter within the highly homologous CREM gene that results in the production of the inducible cAMP early repressor (ICER) that represses CRE-mediated transcription.[331,332] Rapid expression of ICER in response to elevated cAMP levels causes the transient downregulation of CREB transcription, but later ICER-mediated inhibition of ICER expression permits delayed induction of CREB promoter activity. The mutual control of CREB and ICER expression may explain the oscillating cAMP-mediated CREB mRNA expression that was observed in Sertoli cells during the spermatogenic cycle[333] and may provide for stage-specific expression of FSH-regulated mRNAs. Additional genes known to be regulated by FSH-inducible cAMP and CREB include lactate dehydrogenase (*LDH-A*), which controls the synthesis of the major fuel source for germ cells[334]; aromatase[335]; *tPA*[336]; and *IGF1*.[337]

Expression of kit ligand that potentiates the survival and expansion of spermatogonia[338] is also potentiated in Sertoli cells by FSH via cAMP. Elevated cAMP levels cause a complex of at least three proteins to bind to a cAMP-responsive −55 to +8 region of the *kit ligand* promoter by a mechanism that does not include CREB.[339,340] The kit ligand produced by Sertoli cells binds to its receptor c-kit on germ cells. The maintenance and mitosis of differentiating type A spermatogonia are absolutely dependent on Kitl and c-kit, but differentiation of type B spermatogonia into preleptotene spermatocytes is independent of c-kit.[341] Signaling through c-Kit also

promotes the progression into meiosis and survival during the pachytene stage of meiosis.[342–345] Kitl alters the expression of genes known to be up- or downregulated in spermatogonia during the transition from the mitotic to the meiotic cell cycle, which is consistent with the need for Kitl to activate c-Kit for the entry into meiosis.[346]

FSH rapidly and transiently induces the activity of the AP1 transcription factor by stimulating the transcription of the AP1 components c-fos and jun-B while inhibiting c-jun.[347] AR is also induced by FSH, and thus FSH regulates the androgen responsiveness of Sertoli cells.[348–351] In granulosa cells, FSH induces LRH1 (the orphan nuclear receptor liver homolog 1)[352] and HIF1 (hypoxia inducible factor 1) via the PI3K pathway.[353] The potential significance of FSH regulation of LRH1 and HIF1 has not yet been investigated in Sertoli cells. FSH stimulation also results in the translocation of the NF-κB transcription factors to the nucleus due to the release of NF-κB from its cytoplasmic anchoring partner IκB, most likely due to PKA phosphorylating IκB and marking it for degradation.[354–356]

Although studies of FSH-regulated gene expression have provided important information, they have been limited by the requirement to use models in which the germ cell complement is reduced or absent. Further understanding of FSH gene targets required to support germ cells will be forthcoming with the use of models in which FSH signaling can be transiently blocked in the adult Sertoli cell and stage-specific gene expression can be assayed.

FSH Regulation of GDNF in Sertoli Cells

FSH also upregulates GDNF (glial cell line–derived neurotrophic factor), a 15 kDa member of the transforming growth factor beta (TGF-β) superfamily that is essential for the proliferation of germinal stem cells.[357] GDNF was the first factor found to be required for the self-renewal and survival of SSCs.[358,359] Expression of GDNF in adult rat Sertoli cells in vivo is cyclical. The highest levels of GDNF mRNA are present during stages XIII through XIV and I in the rat,[360] corresponding to the stages in which FSH receptor levels and FSH binding by Sertoli cells are greatest[215,218] and immediately before and during the stages showing maximal replication of a type A single (A_s) class of spermatogonia that includes stem cells.[360,361] These data suggest that stage-specific increases in GDNF production by Sertoli cells likely cause proliferation of A_s spermatogonia that results in self-renewal. In contrast, during stages in which GDNF levels are decreased, division of A_s spermatogonia and their transit amplifying progeny tends to result in cells committed to becoming differentiated A1 spermatogonia.[360] It is possible that germ cells produce signals that inhibit GDNF expression in Sertoli cells to cause the stage specificity of GDNF production because *Gdnf* mRNA expression is increased in testes that are severely depleted of germ cells.[357]

Integration of the FSH and Testosterone-Signaling Pathways

FSH and testosterone both initiate signaling pathways that contribute to the support of spermatogenesis, and the hormones share some common characteristics. One characteristic that is shared by FSH and testosterone in young rats is the elevation of Ca^{2+} in Sertoli cells, although the mechanisms by which elevated Ca^{2+} might support spermatogenesis are not yet known.

In prepubertal rodents, either testosterone or FSH can independently increase the number of Sertoli cells,[362] although FSH is apparently more effective.[292,316,363–365] The similar stimulation of Sertoli cell proliferation by FSH and testosterone may be due to the shared ability of these hormones to activate the MAPK pathway in prepubertal Sertoli cells. In contrast, only FSH is capable of activating other proliferation-promoting signaling factors, including PI3K and AKT, which may explain the more effective proliferation-promoting properties of FSH.

Regulation of the CREB transcription factor may be another example of how testosterone and FSH synergize to provide the factors that are required to maintain spermatogenesis. Phosphorylated CREB in Sertoli cells was shown to be essential for spermatocyte survival.[232] FSH is about twofold more effective than testosterone in phosphorylating CREB.[191] The less efficient phosphorylation of CREB in the presence of testosterone alone may be one contributing factor responsible for the reduced fertility observed with androgens in the absence of FSH. Also, testosterone-mediated CREB phosphorylation would be restricted to only stages VI–VIII. For full fertility, it may be necessary for CREB to be phosphorylated during all stages of the spermatogenetic cycle, thus providing one explanation for the out-of-phase cycling of FSH receptor and AR expression that permits FSH and testosterone to enhance CREB phosphorylation throughout the entire cycle of the seminiferous epithelium.

FSH and testosterone also can act in opposition on specific signaling pathways to support spermatogenesis. In pubertal and postpubertal rats, FSH acts to inhibit testosterone-mediated activation of ERK in mature Sertoli cells. Testosterone-mediated activation of ERK is inhibited by FSH as FSH stimulation of Sertoli cells increases cAMP levels that activate PKA, which then blocks activation of RAF1, an initial member of the MAPK cascade[202] (see Figure 16.9). Because FSH receptor and AR levels are cyclically expressed in an out-of-phase manner during the cycle of the seminiferous epithelium, FSH would be expected to limit ERK activation during

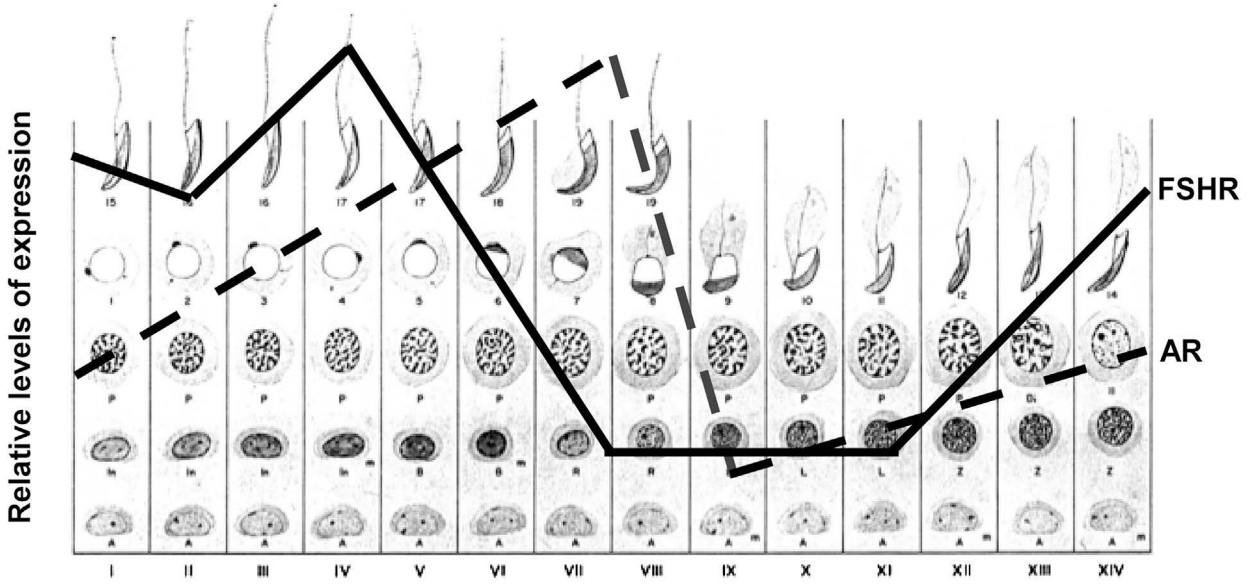

FIGURE 16.10 The expression patterns of FSHR and AR during the cycle of the seminiferous epithelium in rats. The relative levels of expression for FSHR (solid line) and AR (dashed line) are shown throughout the 14 stages (I–XIV) of the seminiferous epithelium cycle in the rat. *Source: Adapted from data provided by Refs 107,366.*

much of the cycle except during stages VII–VIII, when FSH receptor levels are lowest and AR levels are highest[107,111,112,215–217] (Figure 16.10). In agreement with this idea, activated ERK levels are elevated in stages VII–VIII adjacent to the heads of elongated spermatids in the process of being released and in adluminal regions adjacent to developing germ cells.[193] It is possible that this combination of FSH and testosterone signaling is one mechanism by which testosterone and FSH synergize to better support spermatogenesis. In this case, FSH would limit ERK activation in Sertoli cells to only the stages in which testosterone-mediated ERK activation is required for remodeling of Sertoli–germ cell attachments. In the absence of this synergism, the correct Sertoli–germ cell adhesion junctions might not be formed and germ cells would be released prematurely.

FSH, but not testosterone, has been shown to stimulate the PLA_2-signaling pathways in Sertoli cells (Figure 16.9), but thus far the function of PLA_2 signaling in maintaining spermatogenesis is not understood. Another difference between the two major hormonal regulators of Sertoli cells and spermatogeneis is that testosterone does not upregulate cAMP production in Sertoli cells.[191] These findings suggest either that elevated cAMP levels in Sertoli cells are not required for spermatogenesis or that redundant mechanisms are present. Finally, although FSH and testosterone activate some genes similarly, differences in their regulation of gene expression likely contribute to the synergistic effects of the hormones.

Testosterone and FSH also synergize to promote Sertoli cell proliferation. However, testosterone actions must be indirect because the proliferating fetal and neonatal Sertoli cells do not express androgen receptors (ARs).[107,109] Supporting this idea is the finding that knockout of AR in all cells reduces the number of Sertoli cells, whereas Sertoli cell–specific knockout of AR does not affect the population of Sertoli cells.[144] One possible explanation of these results is that androgen effects are mediated via PTM cells that express AR during fetal development.[109] Studies in nonhuman primates indicate that either FSH or androgen can cause Sertoli cell proliferation as treatment of juvenile monkeys with either FSH or LH resulted in a precocious increase in number of Sertoli cells, which is normally associated with the initiation of puberty.[362,367]

THYROID HORMONE

Thyroxine (T4) is the major form of thyroid hormone secreted by the thyroid gland, but 3,5,3-triiodo-L-thyonine (T3) is produced in target tissues by deiodination of T4.[368] Most actions of thyroid hormones are mediated by nuclear thyroid hormone receptors that are ligand-dependent transcription factors.[369] The thyroid hormone receptors (TRs) bind to specific conserved DNA sequence elements in gene promoters and recruit either co-activators or co-repressor proteins to regulate gene transcription.

Thyroid Hormone Receptor Expression in the Testis

Two genes (*Thra* and *Thrb*) encode the TRα1and TRβ1 receptors for T3 (reviewed in Ref. 370), and both receptors are expressed in Sertoli cells.[371–374] The TRβ1 receptor is also found in Leydig, PTM, and germ cells during neonatal development and in the adult.[374–376] Studies of transgenic mice lacking either TRα1 or TRβ1 receptors revealed that T3 acts predominately through the TRα1 receptor to regulate Sertoli cell proliferation and maturation.[377] Interestingly, TRα1 expression is high in rat Sertoli cell nuclei during the proliferation phase of Sertoli cell development, decreases after proliferation ceases, and remains low in adulthood.[375,376] Thus, the expression pattern of TRα1 matches the period of greatest response of Sertoli cells to thyroid hormone.[378]

Regulation of Testis Development by Thyroid Hormone

Knockout of TRα1 or expression of a dominant-negative form of TRα1 in only the Sertoli cells of transgenic mice results in increased testis weight and sperm production. Sertoli cell density also increased as a result of a higher proliferative index at postnatal days 0 and 3. These studies also suggested that T3 acts through the TRα1 receptor to downregulate the JunD–c-myc–Cdk4 pathway that promotes proliferation.[379]

Thyroid hormone is an established regulator of testis development prior to puberty. Hypothyroidism in neonatal rats results in impaired testicular development affecting testicular growth, germ cell maturation, and seminiferous tubule formation[380,381] that, when followed by subsequent recovery to euthyroidism, resulted in an 80–140% increase in adult testis size and daily sperm production.[382,383] The increases in testis size and sperm production were found to occur due to hypothyroidism-induced extension of the Sertoli cell proliferation period and delayed Sertoli cell maturation.[380,384–388] In contrast, hyperthyroidism results in premature halting of Sertoli cell proliferation, stimulation of maturation, and a 50% decrease in the size of the adult testis.[388] Further studies of neonatal mice established that T3 acts directly on Sertoli cells to decrease proliferation and initiate maturation.[389,390] Thyroid hormone also regulates Leydig cell differentiation and steroidogenesis in postnatal rat testis (reviewed in Refs 391,392).

The lack of T3 delays sexual maturation. In the hypothyroid state, completion of the first wave of spermatogenesis requires two- to threefold longer as compared to normal animals, but normal spermatogenesis does finally occur normally.[390] Prolonged depletion of thyroid hormone in the adult causes progressively degenerative changes to the seminiferous tubules, including decreased germ cell number and the release of fewer live sperm.[393,394] However, it is difficult to know whether the effects of prolonged hypothyroidism in the adult are due to the direct testicular effects of T3 depletion or the resulting decreased secretion of LH, FSH, growth hormone, and testosterone.[373,395–400]

Regulation of Sertoli Cell Number by Thyroid Hormone

One mechanism by which T3 limits the proliferation of Sertoli cells is by increasing the expression of cyclin-dependent kinase inhibitors (CDKIs) that are negative regulators of cell cycle progression. Specifically, the $p27^{Kip1}$ and $p21^{Cip1}$ CDKIs that are known to inhibit Sertoli cell proliferation[401,402] are regulated by T3. $p27^{Kip1}$ expression is increased and decreased by hyperthyroidism and hypothyroidism, respectively.[403] Furthermore, T3 stimulation of neonatal rat Sertoli cells increases the expression of the $p27^{Kip1}$ and $p21^{Cip1}$ proteins.[404]

Thyroid hormone also has been associated with the expression of the connexin 43 gap junction protein, and Sertoli cell–specific knockout of connexin 43 is linked to continued Sertoli cell proliferation that is maintained into adulthood, resulting in greater numbers of Sertoli cells. TRα1 levels are elevated in the Sertoli cell–specific connexin 43 knockout mice,[405] perhaps reflecting a less mature Sertoli cell phenotype. In agreement with these results, induced hypothyroidism in neonatal mice decreased connexin 43 expression, whereas T3 stimulation increased the size and number of gap junction plaques.[406]

Downstream Targets of T3 in Sertoli Cells

A compilation of genes regulated by thyroid hormone in the testis has been prepared elsewhere.[407] One thyroid hormone–regulated gene in Sertoli cells is aromatase (cytochrome P450 19 (*CYP19*)). The loss of aromatase activity is a marker for the completion of Sertoli cell maturation. Thyroid hormone decreases aromatase activity by directly inhibiting the transcription of the aromatase gene in Sertoli cells.[408] Thyroid hormone also downregulates the expression of neural cell adhesion molecule (NCAM), another marker of Sertoli cell maturation that is no longer expressed after differentiation.[409] Similarly, T3 decreases the expression of mRNA encoding Mullerian-inhibiting substance (MIS) (also known as anti-Müllerian hormone (AMH)), which is another transcript that decreases sharply with Sertoli cell maturation.[410] T3 increases androgen binding of Sertoli cells[411] and AR mRNA levels in immature rat Sertoli cells,[410] suggesting that T3 may contribute to the postnatal increase in AR expression that is associated with the maturation of Sertoli cells. Major components of the basement membrane

are regulated by T3 in cultured Sertoli cells, including laminin and entactin–nidogen (induced) as well as type IV collagen (inhibited),[412] raising the possibility that T3 alteration of the basement membrane may contribute to the differentiation of Sertoli cells. In Sertoli cells, thyroid hormone stimulates the conversion of glucose to lactate[390] that is used by germ cells as an energy source. The mechanism by which thyroid hormone stimulates lactate production is not known, but T3 increases the mRNA levels of the Glut1 glucose transporter, which are associated with elevated lactate production.[413]

Nongenomic Actions of Thyroid Hormone in Sertoli Cells

Thyroid hormones T3 and T4 use different mechanisms to promote amino acid accumulation[414] and stimulate protein synthesis in immature Sertoli cells.[415] T3 action is blocked by the protein synthesis inhibitor cyclohexamide, whereas T4 effects are unaltered by the inhibitor. These results indicate that T4 effects on protein synthesis are modulated by nongenomic pathways. Additional nongenomic actions of thyroid hormones have been reviewed recently.[416] These nongenomic actions include T4 stimulation of a rapid and transient Ca^{2+} influx into Sertoli cells.[415] One result of the Ca^{2+} influx is that the intermediate filament protein vimentin is hyperphosphorylated, causing increased accumulation of vimentin in the cytoskeleton.[417]

T3 Regulation of Leydig Cell Differentiation and Function

T3 receptors have been identified in Leydig cells, and thyroid hormone regulates the final number of Leydig cells as well as their steroid production (reviewed in Ref. 418). Chemically induced transient hypothyroidism in neonates results in increased numbers of Leydig cells in the adult testis[419,420] either by increasing the proliferation of existing Leydig cells[421] or by arresting Leydig cell differentiation and increasing the number of precursor mesenchymal cells that are generated and are available later for differentiation after euthyroidism is restored.[422] In contrast, hyperthyroidism accelerates Leydig cell differentiation and lowers the final population of Leydig cells.[423,424]

Acute T3 stimulation of cultured Leydig cells increases steroidogenesis, as shown by increased progesterone and testosterone production.[425–427] Short-term stimulation of Leydig cells also activates the transcription of the *StAR* gene that controls the first rate-limiting step for steroidogenesis. Chronic T3 treatment decreases *StAR* transcription and progesterone production in association with decreased delivery of cholesterol to the inner mitochondrial membrane. There is evidence that T3 regulates the *StAR* gene promoter via the NR5A1 transcription factor, but the mechanisms by which NR5A1 transactivation capabilities are altered by T3 are not yet understood.[426,428,429] In cultured Leydig cells, genes encoding steroidogenic enzymes also are regulated by T3, with *p450SSC* mRNA levels being upregulated but *p450C17* and *3β-HSD* mRNAs being inhibited after chronic T3 treatment,[428] in agreement with previous in vivo studies of hypothyroid rats.[430] *LHCGR* mRNA levels and LH binding to Leydig cells were similarly increased and decreased by acute and chronic T3 stimulation, respectively.[428]

ESTROGEN

Estrogen Production—Aromatase

The important role of estrogens in male reproductive development and function is now widely accepted (for reviews, see Refs 431–436). The classical pathway of estrogen action involves binding of the steroid hormone to the ligand-activated nuclear transcription factors ER-alpha (NR3A1) and ER-beta (NR3A2), which can function as independent homodimers or heterodimers, to bind specific target sequences, and EREs in promoters of target genes, as context-dependent transcriptional activators or repressors. Both receptors conform to the classical structure of nuclear receptors, with an N-terminal domain for co-factor interaction, a DNA-binding domain, a hinge region, and a ligand-binding domain.

The concentration of estrogens within the rete testis and testicular venous blood is significantly higher than in the general circulation in a variety of species, supporting a model of local estrogen production within the testis, although circulating estrogens can still impact testicular function via modulation of endocrine feedback mechanisms (for reviews, see Refs 433,435). In the testis, estrogens are generated locally via conversion from testosterone by aromatase. In the rat postnatal testis, aromatase is expressed in both Sertoli and Leydig cells, but with different temporal profiles. Immature Sertoli cells express high levels of aromatase during proliferation, with expression reducing at terminal differentiation.[437] In contrast, aromatase expression in Leydig cells increases as they mature, and Leydig cells are thought to be the major site of estrogen biosynthesis in the adult testis (reviewed in Ref. 438). However, aromatase is also produced by germ cells in the postnatal testis,[439] from the pachytene spermatocyte stage onward, making germ cells an important site of estrogen biosynthesis (for reviews, see Refs 431,433) that is responsible for a significant proportion of total testicular estrogen biosynthesis.[440]

Control of Aromatase Expression

The nuclear receptors NR5A1 and NR5A2 (LRH1) bind to recognition sites within the promoter region of the Cyp19 gene to drive tissue-specific expression of several aromatase transcripts, each with a unique 5′UTR but with a conserved open reading frame.[441,442] Mutation in Cyp19 is associated with male fertility disorders in humans.[434] In mice, ablation of aromatase (ArKO) results in progressive testicular degeneration.[443–445] Testicular anatomy and function are indistinguishable from those of littermate controls up to 4.5 months of age, suggesting that estrogens are dispensable for testis development and initiation of spermatogenesis.[445] Between 4.5 months and 1 year of age, however, a noticeable degeneration in seminiferous tubule integrity is apparent; it is associated with loss of postmeiotic germ cells, suggesting that chronic estrogen suppression perturbs spermatogenesis. Indeed, treatment with phytoestrogens is able to partially rescue the impacts on spermatogenesis.[443] ArKO mice sire fewer litters than control animals, although this could in part result from behavioral problems, as ArKO mice show reduced copulatory mounting behavior.[444] It is important to note one caveat to the information that the ArKO model provides in terms of understanding testicular estrogen signaling. ArKO mice not only have an absence of estrogens but also, because of the failure of aromatase action, have increased concentrations of testosterone within the testis. It is not known whether chronic overabundance of testosterone plays any part in the late-onset phenotype observed in these mice.

Transgenic mice overexpressing aromatase have also been generated.[446,447] These animals have increased circulating estradiol, combined with significantly reduced testosterone and FSH levels, and increased prolactin and corticosterone.[447] These animals display a variety of reproductive phenotypes from early postnatal life, including cryptorchidism, Leydig cell hyperplasia, dysmorphic seminiferous tubules, and disrupted spermatogenesis. However, because some phenotypes resemble those observed in models of exogenous estrogen exposure in perinatal life, it is challenging to dissect the relative impacts of the changes in the hormonal milieu as many of the phenotypes share similarities with models of hypoandrogenism (e.g., cryptorchidism and vestigial seminal vesicles). Chronic overexpression of aromatase in these mice is associated with continued Leydig cell hypertrophy and hyperplasia and the development of nonmalignant Leydig cell adenomas.[446] Furthermore, at 15 months of age, a marked increase in the numbers of activated macrophages was observed, which is associated with Leydig cell depletion and suggests activation of an inflammatory process not unlike orchitis, which is associated with cases of infertility in men.[446]

An important model that may resolve some of these confounding factors is the estrogen sulfotransferase (EST) knockout animal, which fails to deactivate estrogens via sulfation, resulting in increased local concentrations of bioactive estrogens without the confounding factor of reduced androgens.[448] EST is expressed abundantly in the testis of mouse, rat, and human, and has been localized to the Leydig cells in mouse,[449] where its expression is regulated by cAMP and androgens.[450] EST-KO mice exhibit a similar testicular phenotype of late-onset seminiferous tubule atrophy (associated with Leydig cell hypertrophy and hyperplasia and macrophage activation) to that observed in the aromatase-overexpressing mouse testis,[446,448] supporting the notion that the phenotype observed in the aromatase-overexpressing mouse is the direct result of increased local estrogen signaling. Together, these data suggest that either a reduction or increase in estrogens within the testis perturbs normal testicular function.

Estrogen Action

In postnatal life, both ER-α and ER-β are expressed within the testis. In the rodent, ER-α expression is restricted to the Leydig cell lineage,[451] whereas ER-β has a wider spatial expression[452] and is localized to cell nuclei of germ cells (spermatogonia, spermatocytes, and spermatids), PTM, Sertoli cells, and Leydig cells from early postnatal life.[453–455]

Whereas ERβKO males show no overt morphological differences from wild-type littermates and are fully fertile,[456] the testes in ER-αKO males develop normally but begin to degenerate from 20 to 40 days, and by day 150 the testicular seminiferous tubules are atrophic.[457,458] This testicular phenotype of the ER-αKO is complicated by the ablation of ER-α in the efferent ducts, loss of which leads to reduced fluid resorption within the efferent ducts[459] and fluid back-pressure into the testis, which may in part account for the testicular phenotype. This conclusion is supported by characterization of ENERKI mice,[460] which retain the ligand-independent activity of ER-α but lack classical estrogen-dependent signaling capability. These mice fail to develop the efferent duct phenotype seen in the ER-αKO mice, confirming that the efferent duct phenotype found in the ER-αKO mice is an estrogen-independent activity of ER-α.[460] However, the critical role of ER-α in the efferent ducts does not completely explain the observed testicular phenotype. Despite lacking the efferent duct defect, ENERKI mice also show similar late-onset testicular degeneration as the ER-αKO mice, with focal areas of seminiferous tubule atrophy apparent by 20 weeks of age (~day 150) and increasing in frequency over time.[460] This confirms a role for estrogen–ER-α signaling in control of testis function, although the global nature of these models

does not preclude an extratesticular site of action of estrogens, with concomitant knock-on effects occurring locally within the testis. Both the ER-αKO and ENERKI mice exhibit impacts on the HPG axis, which are proposed to result from a lack of negative feedback at ERs in the brain; a knock-on effect of this includes significant increases in circulating testosterone.[457,460] It therefore remains feasible that a proportion of the phenotype observed in the testes of these animals arises from perturbation of downstream factors.

However, a direct effect of ER-α in the testis has been recently elucidated. Mice carrying floxed alleles of ER-α have been described,[461] and conditional ablation of ER-α from Leydig (and a proportion of adrenocortical) cells has been carried out.[462] Loss of ER-α from Leydig cells enhances testosterone production.[463] Conversely, ER-α overexpression suppresses the cAMP-induced and Nur77-activated promoter activity of steroidogenic enzyme genes via the suppression of transactivation by Nur77.[463] Thus, estrogen signaling within Leydig cells appears to function as a modulator of androgen production.

Nongenomic Effects of Estrogens

In addition to acting directly on the DNA transcriptome, estrogens are able to mediate a rapid nongenomic effect on cells. The mechanism for this, however, remains contentious. Identification of a novel, widely expressed GPCR (GPR30; G protein–coupled estrogen receptor 1 (GPER)), with an apparent high affinity for estrogens,[464,465] provided new hope for understanding rapid estrogen signaling. Proving that GPR30 is responsible for nongenomic actions of estrogens, however, has proven problematic (for reviews, see Refs 431,466–468). Based upon the method employed, GPR30 has been localized to either the endoplasmic reticulum[465,469] or the plasma membrane[470,471] (reviewed in Ref. 468); GPR30 fails to bind radiolabeled E2, and E2 does not stimulate cAMP or calcium release.[472] Several mouse models with ablation of GPR30 have been generated,[473–476] but none displays (female) phenotypes similar to those of either the ER-αKO or aromatase knockout mice. Double and/or triple knockouts in combination with ER-α and/or ER-β have not been described; thus, the possibility of functional redundancy remains.

GPR30 has been shown to work with ER-α via EGFR–ERK–fos signaling to mediate estrogen-dependent proliferation of a spermatogonial cell line in vitro.[477] In the rat testis, GPR30 has been localized to pachytene spermatocytes and to spermatids, and has been demonstrated to work alongside ER-α to activate the EGFP–ERK–c-jun signaling cascade, triggering an apoptotic mitochondrial pathway with increased Bax and reduced cyclin A1 and B1, which are involved in the balance between cellular proliferation and apoptosis.[478,479] However, the role of GPR30 as an estrogen receptor remains far from proven[466]; the most recent data suggest that GPR30 in fact has a role as a collaborative partner to nuclear estrogen receptors, rather than acting as an estrogen receptor in its own right (reviewed in Ref. 467). Further investigations to fully elucidate the role of GPR30 both in general and specifically within the testis are clearly warranted.

To further assess the role of nongenomic estrogen signaling, a mouse model was recently created that expresses the E domain of ER-α at the plasma membrane in the absence of ER-α (membrane-only estrogen receptor (MOER)).[480] Liver cells from these animals retained the ERK and phosphatidylinositol response to 17-β-estradiol stimulation, which is lost in ERαKO mice, providing initial evidence of a wider influence of estrogens on cells via modulation of the Gq, PKC, and calcium-signaling pathways (reviewed in Ref. 481).

ACTIVIN AND INHIBIN

Components of the Inhibin–Activin Signaling System

The actions of testosterone and FSH to regulate spermatogenesis are mediated, in part, by members of the TGF-β peptide family. These family members include bone morphogenic proteins (BMPs), AMH, the growth and differentiation factors (GDFs), activin, inhibin, and the more distantly related glial cell line–derived neurotrophic factor (GDNF) (reviewed in Ref. 482). The important paracrine actions of TGF-β peptide family members in the fetal and neonate testis are well characterized (reviewed in Ref. 483). This section will focus on the inhibin and activin regulation of Sertoli cell proliferation and maturation as well as germ cell development in the testis because these factors are the major paracrine-acting members of the TGF-β peptide family in the pubertal and adult testis.

Inhibin proteins consist of a dimer of the α inhibin subunit with one of either activin β_A or β_B subunits forming inhibin A or inhibin B, respectively. Activins are composed of dimers of β_A or β_B subunits termed activin A ($\beta_A\beta_A$), activin AB ($\beta_A\beta_B$), or activin B ($\beta_B\beta_B$). Most of the cellular expression data for inhibin, activin, and their receptors has been collected from rodents. However, the available information indicates that the expression patterns for activins and their receptors in humans are similar to those described for rodents (reviewed in Ref. 484).

The principle site of inhibin B production is the Sertoli cell,[485–487] although there is evidence that Leydig cells can produce inhibin.[488,489] Activin A is produced by Sertoli cells, Leydig cells, and PTM.[301–304] Immunoreactivity for activin B has been identified in germ cells, including spermatogonia, spermatocytes, and round spermatids,

as well as Sertoli cells and Leydig cells.[319,490–492] However, it has not yet been demonstrated that germ cells are a physiologically relevant source of activins in the testis. Activin B appears to be a weaker agonist, which has been attributed to its lower affinity for the activin receptor complex.[493] There is little information available regarding the expression and function of activin AB.

Activins and other TGF-β peptide family members act through a receptor complex of type I and type II receptors. Activins can bind to two activin type II receptors (ActRIIA and ActRIIB) and three type I activin-like kinase (ALK) receptors: ALK2, ALK4 (by activin A), or ALK7 (by activin B).[494–496] The mRNAs encoding receptors for activin are present in Sertoli cells, spermatocytes, and round spermatids.[497,498] Type I and the type II ActIIB receptors are expressed in germ cells and in Sertoli cells.[498–500]

Activin binds first to an activin type II receptor that recruits a type I receptor. The type II receptor then phosphorylates and activates the type I receptor.[494] Activin-mediated activation of type I receptor kinase activity facilitates the recruitment and phosphorylation of the downstream signaling proteins, SMAD2 and/or SMAD3. The phosphorylated SMADs bind to SMAD4 to produce a complex that translocates to the nucleus, binds to specific promoters, associates with transcriptional co-regulators, and alters gene transcription (reviewed in Ref. 310). The relative levels of SMAD2 and SMAD3 as well as inhibitory SMADs such as SMAD6 and SMAD7 provide fine-tuning of transcriptional responses in response to activin stimulation.

A specific receptor for inhibin has yet to be identified. Inhibin can interact with activin receptors with the assistance of a third receptor subunit called TGF-β receptor III or betaglycan.[501] At the pituitary level, the betaglycan-enhanced binding of inhibin to type II activin receptors blocks activin binding and prevents dimerization with type I receptors.[502] However, similar inhibitory effects of inhibin in the testis are less well characterized.

Inhibin and Activin Functional Studies

Inhibin and activin have opposing actions on spermatogenesis. Injection of partially purified inhibin into the testis of Chinese hamsters decreased the numbers of differentiated spermatogonia in an FSH-independent manner.[503] In contrast, treatment of co-cultures of rat spermatogonia and Sertoli cells with activin A or activin B stimulated the proliferation of spermatogonia.[504] Activin A also stimulated DNA synthesis in spermatogonia and leptotene spermatocytes in cultured seminiferous tubule fragments from adult rats,[505] but inhibited the survival of SSCs cultured from adult mouse testes.[506] The "condensed-type" mitochondria of rat pachytene spermatocytes was maintained in culture by activin A, suggesting the ability to regulate meiotic development.[507]

Activin can independently increase Sertoli cell proliferation in the rat during the developmental window of 5–13 days after birth.[282,309,404] Activin acts directly on day 6–11 Sertoli cells to induce proliferation, but younger cells are unresponsive, although they respond to FSH.[282,404] Activin and FSH can act synergistically during the later stages of Sertoli cell development to increase proliferation.[282,308,309] The developmental stage specificity of activin signaling on Sertoli cell proliferation has been attributed to the upregulation of the ActIIB mRNA between 7 and 11 days after birth.[309] Furthermore, knockout of inhibin results in uncontrolled proliferation of Sertoli cells,[508] and the elevation of activin levels in vivo reinitiates Sertoli cell proliferation and dedifferentiation in adult mice.[509]

Developmentally regulated responses of Sertoli cells to activin also correspond to a change in activin signaling from the utilization of only SMAD3 by immature, proliferating Sertoli cells to the use of both SMAD2 and SMAD3 in maturing Sertoli cells.[510,511] Although not essential for fertility because *Smad3* null mice are fertile,[512] SMAD3 is likely a key mediator of activin-induced proliferation of Sertoli cells, and downregulation of SMAD3-mediated signaling is associated with transition to the terminal differentiation of Sertoli cells. Supporting this idea is the finding that removal of one or both copies of *Smad3* from mice lacking inhibin resulted in normal, terminally differentiated Sertoli cells and did not allow the formation of Sertoli cell tumors that occur due to elevated activin signaling.[513] Other consequences of activin A stimulation of cultured Sertoli cells include inhibition of androgen binding, decreased basal and FSH-induced AR mRNA expression, as well as FSH-mediated aromatase activity, but activin A did not alter FSH receptor expression.[302]

Inhibin and Activin Transgenic Mouse Models

Mice lacking the inhibin α-subunit due to deletion of the *Inha* gene (and therefore of inhibin production) develop Sertoli cell tumors because the loss of the activin signaling inhibitor results in unrestrained proliferation and failure to mature. These mice die from cachexia, a wasting syndrome.[508] Unfortunately, except for the finding that inhibin acts as a tumor suppressor, little information about the normal function of inhibin in the testis has been obtained from this model because of the disruption of the testis architecture by the tumors.

Deletion of the *Inhba* gene encoding the βA-subunit results in perinatal death and thus does not provide any information regarding the function of activin A in the testis.[514] Studies of mice lacking the *Inhbb* gene indicate that Inhibin B is not essential for spermatogenesis because these mice survive to adulthood, are fertile, and have a normal testis phenotype.[515] Knockout of the ActIIA

receptor that compromised activin and BMP signaling reduced testis size and delayed fertility onset. However, FSH levels were reduced in this model animal, causing assignment of activin functions to be more complex.[516]

To better understand the function of activin, the *Inhba* gene encoding the βA-subunit was replaced with *Inhbb* encoding the βB-subunit so that activin A was not expressed and only the less potent activin B could be produced. The mouse model, called BK mice, had lower testis weights and volumes as well as delayed onset of fertility, indicating that activin contributes to establishing spermatogenesis in the mouse. Sertoli cell numbers 7 days after birth were also lower, consistent with the known actions of activin in promoting Sertoli cell proliferation.[517] Studies of the BK mice also revealed that activin inhibits expression of the differentiation marker KIT by spermatogonia and the differentiation of spermatogonia.[518]

Further evidence for activin regulation of spermatogenesis was provided by transgenic mice overexpressing the human activin βA-subunit. In these mice, ectopic expression of the βA-subunit was observed in pachytene spermatocytes and round spermatids after 3 weeks of age. The mice were initially fertile but became sterile by 10 weeks due to the vacuolation of the testis as the number of spermatocytes and maturing spermatids were reduced, and no mature sperm could be produced.[519] Overexpression of the repressive activin-binding protein follistatin in mice caused a significant decline in testis weight even in transgenic lines with normal serum FSH.[520] Together, the transgenic models assessed thus far indicate that activin is not essential for fertility, but the hormone contributes to the processes of Sertoli cell proliferation and spermatogonia differentiation.

Regulation of Inhibin and Activin Activities

The production of inhibin and activin is regulated in reciprocal manners in Sertoli cells by FSH and cytokines. Expression of α-subunit mRNA in Sertoli cells is stimulated by FSH through cAMP and PKA.[521–523] FSH–cAMP–PKA also stimulate expression of the βB-subunit in Sertoli cells to a lesser extent.[524,525] The sum of FSH action on Sertoli cells is to increase inhibin B production. Sertoli cells also produce the βA-subunit that is not regulated by FSH.[302,526,527] In contrast, stimulation of Sertoli cells with the cytokines interleukin 1 (IL1) or tumor necrosis factor alpha (TNF-α) that are produced by Sertoli cells, germ cells, and macrophages[172] or via activation of Toll-like receptors (TLRs) inhibits the expression of the α-subunit and the βB-subunit, thus suppressing inhibin B production.[526–529]

βA-subunit expression and activin A secretion in rat Sertoli cells are potently stimulated via stress and inflammation signaling pathways through the TLRs and IL1 receptors that mediate the pro-inflammatory actions of IL1 and pathogenic molecules, including LPS and bacterial lipoproteins.[526,527,530,531] The TLRs and IL1 receptors interact with the adaptor protein MyD88 that activates a signaling pathway leading to TRAF6 and the activation of the MAPKs p38 and JNK as well as the transcription factors NF-κB and AP1.[532] TNF-α also activates activin A production using similar downstream signaling factors.[529,533] Together, these findings indicate that cytokines and proinflammatory signals decrease inhibin production but induce activin secretion. In contrast, FSH stimulates the production of inhibin B while inhibiting activin A.

As would be predicted from the FSH regulation of the α- and β- subunits of inhibin, the production of the peptides closely follows the cyclical changes in FSH receptor levels and FSH signaling within the seminiferous epithelium. Inhibin B and its subunits are constantly produced during the cycle, but expression is significantly reduced during stages VII–VIII, when FSH signaling is lowest.[492,534] Activin A and β_A subunit expression is low throughout most of the cycle but increases dramatically after stage VIII and reaches a maximum at stages IX–XI. Maximal β_B subunit mRNA levels are observed during stage XIII.[498,525] Further stimulation of activin subunit production may occur due to the production of inflammatory cytokines (IL1α and IL6) by Sertoli cells in response to the shedding of residual bodies of cytoplasm by maturing spermatozoa during the process of spermiation in stage VIII (reviewed in Ref. 535).

Activin-Regulated Gene Expression in Sertoli Cells

Gene expression analysis of activin-stimulated Sertoli cells cultured from 6- to 15-day-old mice revealed alterations in the expression of genes related to a diverse range of biological processes, including DNA and chromatin modification, the ubiquitin cycle, protein transport, RNA processing, and ion transport. Similar genes were found to be regulated in more focused studies of juvenile mice with excessive activin signaling and reduced activin bioactivity.[510]

Transduction of adult mouse muscle tissue with a virus expressing activin A increased serum levels of activin A by fourfold and reduced testis mass by 23%. The hypospermatogenic phenotype observed with this model is consistent with the failure of Sertoli cells to support spermatogenesis. Closer inspection revealed that the elevated activin levels potently disrupted tight junction formation and BTB integrity. The disruption of barrier formation may be the result of activin-mediated dedifferentiation of Sertoli cells that were found to display evidence of proliferation, expression of juvenile markers, and loss of mature markers. Microarray

analysis of Sertoli cells isolated from this model of elevated serum activin revealed that 425 genes were regulated by activin, including groups of genes associated with anchoring junctions, cell adhesion, regulation of cell division, as well as tissue morphogenesis metabolism and morphology. These results indicate that activin signaling must be strictly regulated to maintain mature Sertoli cell function.[509] The findings further support the idea that the decrease in activin levels observed during puberty contributes to the halting of Sertoli cell proliferation.[319]

OTHER HORMONES

Osteocalcin

Sex steroids have been known for many years to modulate skeletal development and integrity (reviewed in Ref. 536). However, until recently, a reciprocal mechanism whereby the skeleton influences sex steroid production remained elusive. Osteocalcin has recently been identified as a bone-derived hormone with significant functional impacts on sex steroid production by the testis, completing the circle of mutual interdependence between the skeletal system and gonad (for reviews, see Refs 536–538).

A suggestion of skeletal impact on the testis via osteocalcin was first identified in a mouse model with osteocalcin ablation.[539] Osteocalcin−/− mice show a severe decrease in testis and epididymal weights, and reduced sperm counts associated with a reduction in circulating testosterone (and increased circulating estrogens, possibly due to increased adiposity).[86]

The first direct evidence for a direct gonadal role of osteocalcin was derived from ex vivo cell assays in which a factor secreted by osteoblasts, but not other cells of mesoderm origin, was found to significantly increase testosterone production *by* Leydig cells and testis explants.[86] These observations were independently verified in vivo in a mouse model lacking osteoblasts, which displayed reduced circulating testosterone concentrations.[540] Osteocalcin was identified as the essential hormonal factor through additional culture models in which use of osteocalcin−/− osteoblasts resulted in a significant reduction of testosterone biosynthesis compared to control osteoblasts, which could be rescued by the addition of exogenous active osteocalcin,[86] an observation replicated in vivo in mice lacking either osteoblasts[540] or osteocalcin.[86] Furthermore, circulating testosterone concentrations were increased above controls in a transgenic mouse model (Esp−/−) where deactivation of osteocalcin is reduced. Esp or protein tyrosine phosphatase receptor type V normally functions to deactivate osteocalcin.

The cognate receptor for osteocalcin has been identified as the G protein–coupled receptor GPRC6A,[86] which is expressed on the surface of Leydig cells. GPRC6A−/− mice phenocopy osteocalcin−/− mice.[86,541] Osteocalcin is able to bind to the surface of Leydig cells (producing an intracellular increase in cAMP), but not to Leydig cells from GPRC6A−/− mice (associated with no increase in cAMP levels).[86] Interestingly, GPRC6A is not expressed in ovarian cells, which may explain the male-specific impacts on sex steroid production.

Upon binding to GPRC6A, osteocalcin causes an increase in intracellular cAMP, which promotes CREB phosphorylation and binding to promoter regions of the genes encoding the steroid biosynthesis enzymes StAR, Cyp11, 3β-HSD, and Cyp17 to drive transcription of these genes.[86]

Together, these combined data demonstrate that osteocalcin may be an important hormonal regulator of testosterone biosynthesis. Importantly, circulating LH was increased in osteocalcin−/− mice (which continue to have low circulating testosterone), suggesting that LH signaling is not sufficient to rescue inhibition of osteocalcin-directed testosterone biosynthesis, and thus that each of these two pathways acts at least somewhat independently. Further investigation of the clinical importance of osteocalcin for male reproductive health and fertility is an essential future requirement.

Insulin–IGF–Insl3 Signaling

Insulin is a member of the wider insulin–IGF–relaxin superfamily. Members share structural features, although sequence variability among family members is high. Insulin, IGF1, IGF2, and Insl3 are the key members associated with testis development and function. The impact of insulin–IGF–relaxin signaling on the testis has been established over a number of years, and forms a key part of the hypogonadism–adiposity–insulin resistance feedback system associated with certain aspects of metabolic syndrome (reviewed in Ref. 542).

IGF Signaling in the Testis

The IGF studied in most detail in the testis is IGF1 (also called somatomedin C) (reviewed in Ref. 543). Growth hormone (GH) is the main driver of IGF1 production throughout the body, with IGF1 mediating many of the trophic actions of GH. Indeed, IGF1 signaling is absolutely required for sexual development and attainment of reproductive competence, but absence of GH signaling generally does not preclude male (or female) fertility (reviewed in Ref. 544). IGF1 not only arrives at the testis from the systemic circulation but also is produced locally in the testis by both Sertoli and Leydig cells,[545,546] under

the control of gonadotropins as the testicular secretion of IGF1 increases following LH and FSH treatment.[547,548]

In postnatal life, IGF1R is localized to Leydig, Sertoli, PTM, and germ cells of the testis.[306,549] IGF1 signaling promotes postnatal Leydig cell development by functioning as an inhibitor of Leydig cell apoptosis. Phosphorylated IGF1R increases from birth to day 20 of postnatal life, remaining high in the adult testis. IGF1 decreases the level of apoptosis in Leydig cells at all stages of development. In contrast, the selective inhibitor of IGF1R, picropodophyllin, blocks this antiapoptotic effect in the presence and absence of IGF1, demonstrating that signaling via the IGF1R plays an important role in Leydig cell survival.[550] Consistent with this observation is that mice with null mutations in IGF1 display reduced numbers of adult Leydig cells,[551] which is associated with perturbed proliferation of progenitor and immature Leydig cells,[552] whereas excess secretion of IGF1 driven by increased endogenous GH in transgenic mice expressing human growth hormone–releasing hormone (GHRH)[553] significantly increases testis weight and testosterone production.[543] The mechanism underlying the antiapoptotic action of IGF1 involves the phosphatidylinositol 3-kinase–Akt pathway, and in immature Leydig cells, this growth factor enhances the expression of Bcl2 and cellular inhibitor of apoptosis proteins 2, while preventing activation of caspase-3 by cleavage.[550] Furthermore, investigations using IGF1 replacement and gonadotropin stimulation in IGF1 null mice suggest that the trophic role of LH on Leydig cell proliferation is in part mediated by IGF1 signaling.[551] Inactivation of IGF1 receptor in primary Sertoli cells leads to reduced Sertoli cell numbers, through both a reduction in proliferation and an increase in apoptosis, suggesting a similar trophic role for IGF1 receptor signaling in Sertoli cells.[306]

IGF1 signaling not only is important for Leydig cell development but also plays an autocrine role in the control of adult Leydig cell function.[554] IGF1 null mice initially increase androgen production compared to littermate controls between d4 and d12. However, the IGF1 null mice then fail to further increase androgen production, and they produce 30% of normal circulating androgens in adulthood.[555] IGF1 null mice display reduced Leydig cell expression of P450 SCC, P450c17, RLF–Insl3, 17β-HSDIII, 3β-HSD IV, and StAR at both days 35 and 50 (adult).[555] Whether these changes in gene expression result from developmental perturbation or indeed do represent the adult function of IGF1 remains an open question, and whereas IGF1 replacement in IGF1 null mice promotes Leydig cell proliferation, IGF1 treatment is unable to restore normal circulating testosterone concentrations.[551] These results suggest that the developmental role of IGF1 has knock-on effects on later testicular steroidogenesis. However, IGF1 does play a role in testicular steroidogenesis in adult Leydig cells. Studies suggest that IGF1 enhances LH-mediated stimulation of steroidogenesis in adult Leydig cells.[551,556] IGF1 has been shown to increase LHCGR density and promote P-450scc and 17 alpha-hydrolyase expression in cell culture.[557] In addition, in adult Leydig cell culture, regulation of StAR by IGF1 has been demonstrated to be mediated by the PKC pathway involving phosphorylation of both CREB and c-Jun.[558] In summary, IGF1 produced both locally and systemically is an essential factor in Leydig cell development and function, not only promoting Leydig cell proliferation but also supporting Leydig cell function in adulthood.

IGF2 is also known to also regulate cell proliferation, growth, migration, differentiation, and survival, mainly during embryogenesis (reviewed in Ref. 559). Data suggesting a role for IGF2 in postnatal testis development and function are limited, although IGF2 has been associated with early postnatal growth of the testis in humans.[560]

The physiological effects of IGFs are profoundly affected by six IGF-binding proteins (IGFBPs) that affect IGF signaling in a dose-dependent manner. In the testis, IGFBPs are produced by Leydig, Sertoli, and PTM cells, and of the six IGFBP proteins, only IGFBP1 is not produced within the testis (reviewed in Ref. 543). Establishment of the physiological impacts of IGFBPs in the testis is an ongoing process, although their role as a key modulator of testis function was established some time ago (reviewed in Ref. 561). IGFBP3 and IGFBP4 have been shown to impact Leydig cell function by inhibiting IGF1-induced androgen production,[562] whereas overexpression of IGFBP1 and IGFBP6 impacts testosterone secretion and spermatogenesis in vivo,[563,564] although this is likely to be due to modulation of the HPG axis at a central level.

Insulin

Along with IGF1, IGF2 and high concentrations of insulin also evoke phosphorylation of IGF1R and, like IGF1, enhance the expression of StaR by Leydig cells.[550] Transgenic overexpression of insulin in Leydig cells, driven by the Insl3 promoter, has minimal impact on the testis until 8 months of age, when a significant proportion of tubules in 8-month-old and a large majority of tubules in 12-month-old transgenic mice show impairment of spermatogenesis, with the formation of vacuoles, the release of premature germ cells, and focal as well as full degeneration of germ cells.[565] However, under normal physiological conditions or diabetic pathologies, the major site of action of insulin impacting testicular function appears to be at the level of the brain, through influencing pituitary LH secretion.[566]

Insl3

Insl3 is a member of the relaxin family of protein hormones. Several members of this family are associated with the testis (reviewed in Ref. 567) and are produced exclusively by both fetal and adult Leydig cells. Expression begins at the latter stages of Leydig cell development, and thus Insl3 has long been used as a biomarker of Leydig cell maturation.[568,569] Insl3 binds its cognate receptor, the GPCR LGR8–RXFP2[570] (reviewed in Ref. 571). LGR8–RXFP2 is expressed in the gubernaculum during fetal life, and loss of function of either Insl3 or LGR8–RXFP2 results in failure of gubernacular development and arrested testis descent.[572] In the postnatal testis, LGR8–RXFP2 is expressed in meiotic and particularly postmeiotic germ cells and in Leydig cells, although not in Sertoli or PTM cells,[573] suggesting that Insl3 signaling plays an important role in germ cell maturation. However, recent investigations of Insl3 signaling in the adult testis have revealed that specific ablation of LGR8–RXFP2 in male germ cells does not affect spermatogenesis, or fertility in adult males,[574] suggesting either that Insl3 plays a minimal role in spermatogenesis or that functional redundancy exists and the role of Insl3 can be compensated for by another, unknown factor.

Glucocorticoid Signaling

Glucocorticoids secreted by the adrenal in response to adrenocorticotrophic hormone (ACTH) can influence fertility at three levels: (1) in the hypothalamus, to suppress release of GnRH; (2) in the pituitary, to perturb LH and FSH secretion; and (3) within the testis, to influence steroidogenesis and spermatogenesis directly (reviewed in Ref. 575). The role of glucocorticoids in control of the reproductive axis has long been associated with reallocation of resources during the stress response; the concept is that direct suppression of the HPG axis gives a higher priority to an individual's survival than to their need to reproduce. Evidence in support of this concept is widespread. Men with Cushing's syndrome (chronic hypercortisolemia) present with low plasma testosterone.[576] A similar reciprocal relationship between increased glucocorticoids and reduced plasma testosterone is observed during anesthesia, surgery, anticipation of battle, or physical training[577,578] (reviewed in Ref. 575). Several cells of the testis express glucocorticoid receptor (GR), a member of the steroid receptor transcription factor superfamily (reviewed in Ref. 579), and as such are responsive to glucocorticoids. These include Leydig cells, macrophages, vascular endothelial cells, and PTM cells. Furthermore, GR is strongly expressed in spermatogonia and primary spermatocytes, suggesting a direct influence on spermatogenesis, but GR is not expressed in Sertoli cells or in more mature germ cells.[580]

In the postnatal testis, glucocorticoids function to directly inhibit transcription of the steroidogenic enzymes, P450SCC, StAR, and Cyp17.[581–583] Whereas ligand-bound GR functions primarily as a transcription factor, promoting or repressing expression of downstream targets by binding to glucocorticoid response elements (GREs) in the promoter region, the evidence for a mechanism of suppression of steroidogenesis by glucocorticoids invokes several nongenomic mechanisms. For example, ligand-bound GR has been shown to bind to and inactivate NR4a1 (Nur77), thus antagonizing a cAMP-mediated activation pathway leading to suppression of transcription of StAR.[584] A similar impact is seen on progenitor Leydig cells, where glucocorticoids inhibit production of androsterone, which suggests that Leydig cells are sensitive to glucocorticoid-mediated perturbation throughout postnatal development and not just in adulthood.[585]

A further mechanism underpinning glucocorticoid control of steroidogenesis is the finding that intracellular concentrations of glucocorticoids themselves may be controlled within Leydig cells. The two enzymes 11β-HSD1 and 11β-HSD2 act as either oxidoreductase or oxidative enzymes, respectively, to interconvert between corticosterone (in the rat; cortisol in humans) and its biologically inert derivative, 11-dehydrocorticosterone (11-DHC)[586,587] (cortisone in humans). 11β-HSD1 requires NADPH for reductase activity, which catalyzes the conversion of 11DHC to corticosterone, and NADP+ for oxidase activity that catalyzes the conversion of corticosterone to 11DHC. Meanwhile, 11β-HSD2 exploits a distinct cofactor NAD+ to catalyze its oxidase activity. Thus, variation of activity of these enzymes permits modulation of the concentration of glucocorticoids available to bind GR. This fast-acting mechanism works within Leydig cells in a cooperative cycle. 17β-HSD3 is able to couple with 11β-HSD1 and drive 11β-HSD1 oxidase activity, thus reducing the adverse effects of corticosterone on testosterone production. At the same time, 11β-HSD1 generates NADPH that promotes testosterone biosynthesis by stimulating 17β-HSD3, a situation that can perpetuate as long as androstenedione is available as a substrate. As availability of the substrate drops, the reductase activity of 11β-HSD1 increases, suppressing further testosterone production (reviewed in Ref. 588). The role of 11β-HSD2 is less well established, although it is believed to play a semiredundant role in supporting the oxidation of corticosterone to 11DHC, as a drop in 11β-HSD2 during aging is thought to expose Leydig cells to increased glucocorticoid-mediated suppression of steroidogenesis, which may prove an important factor in age-related reduction in testosterone production by the testis.[589]

Further to their role in suppression of testosterone production, chronic exposure to glucocorticoids can induce Leydig cell apoptosis[590] (reviewed in Ref. 591)

via a mechanism that includes activation of the Fas system, cleavage of procaspase-3, loss of mitochondrial membrane potential, and increased ROS generation.[592] Adenovirus microRNA-induced downregulation of GR expression in vitro alleviates the glucocorticoid-induced increase in apoptosis of Leydig cells, demonstrating the involvement of GR in this process.[593]

Glucocorticoid action also induces apoptosis of spermatogonia.[594] Although the mechanism for this remains unknown, germ cell apoptosis is associated with the induction of proapoptotic proteins such as Bax, an effect that can be blocked by inhibition of GR.[595] Whereas expression of GR within spermatogonia and primary spermatocytes suggests that germ cells are direct targets of glucocorticoids,[580,594] apoptosis of germ cells could simply be the result of suppressing testosterone production by the Leydig cells (a well-established mechanism for induction of germ cell apoptosis), as germ cells are most sensitive to dexamethasone-induced apoptosis in stages VII–VIII of the spermatogenic cycle, stages that are also consistent with androgen-dependent viability (reviewed in Ref. 575).

Mineralocorticoids

Very little is known regarding the role of mineralocorticoid signaling in the testis. The mineralocorticoid receptor (another member of the ligand-activated transcription factor superfamily) has been identified in Leydig cells, and aldosterone acting via MR has been shown to enhance LH-driven steroidogenesis via a synergistic pathway.[596] Further investigation of mineralocorticoid signaling is clearly warranted.

Leptin Signaling

The polypeptide hormone leptin is transcribed from the leptin (OB) locus produced primarily by adipocytes and acts in the brain to control key parameters such as regulation of body weight, food ingestion, body temperature, and metabolic rate associated with metabolism (reviewed in Ref. 597).

Leptin deficiency is also associated with male infertility in both humans and animals (reviewed in Refs 598,599). Leptin-deficient male mice are also sterile, and this is associated with impaired spermatogenesis, increased germ cell apoptosis, and upregulated expression of proapoptotic genes within the testes.[600] However, fertility can be restored by administration of exogenous leptin, suggesting that leptin provides functional support to spermatogenesis throughout life.[601,602]

Leptin acts at all levels of the HPG axis, and although leptin control of fertility is believed to be primarily mediated at the hypothalamic–pituitary level, leptin is also able to act directly within the testis (reviewed in Ref. 603). The testis produces endogenous leptin and expresses several isoforms of the leptin receptor (Ob-R). In 5-day-old mice, leptin is produced primarily in gonocytes and confined to spermatogonia at day 10. From day 20 onward, stage-specific expression of leptin is restricted to stages VII–XII of the cycle.[604] In situ hybridization analysis shows a scattered pattern of cellular expression of the Ob-R gene within the adult rat testis, including Leydig and Sertoli cells. In addition, assessment of the pattern of expression of Ob-R subtypes revealed that the long Ob-Rb isoform was abundantly expressed in adult rat testis. However, variable levels of expression of Ob-Ra, Ob-Re, and Ob-Rf mRNAs also were detected, whereas those of the Ob-Rc variant were nearly negligible[605] (reviewed in Ref. 604).

Leydig cells appear to be the key cell types responsive to leptin signaling (reviewed in Ref. 604). During postnatal development, prepubertal Leydig cells express the Ob-Rb form of leptin receptor and are thus a direct target of this hormone. Treatment with leptin in cell culture conditions reduces Leydig cell proliferation and induces premature expression of differentiation markers, suggesting that leptin may function to control final numbers of Leydig cells.[606] In cultured testis sections from adult rat, leptin decreased the hCG-stimulated mRNA expression levels of SF-1, StAR, and P450scc enzyme in a dose-dependent manner, but failed to alter those of 17β-HSD type III, suggesting that decreased expression of several upstream elements in the steroidogenic pathway may contribute to leptin-induced inhibition of testicular steroidogenesis.[605] Leptin's control of testosterone production is mirrored by testosterone-mediated suppression of leptin production by adipose tissue, which results in reduced LH secretion by the pituitary and reduced testosterone production by the testis.[607] Thus, leptin is able to modulate the testosterone-driven negative-feedback loop, acting via the HPG axis (reviewed in Refs 603,608). Evidence is emerging that other metabolic hormones such as adiponectin[609] and ghrelin[610–613] may play similar roles in testis function, strengthening the links between control of metabolic parameters and testicular function.

Prolactin

A further hormone that influences testis function is prolactin. Prolactin is produced in the anterior pituitary and, within the testis, by Leydig cells and germ cells.[614] Prolactin signals via the prolactin receptor, expression of which is controlled by NR5A1 in Leydig cells.[615] Prolactin functions in support of LH-driven testosterone production, both at the level of the pituitary and in the Leydig cells themselves, where it increases the activity of 3β-HSD (in rats) and 17β-HSD (in mice).[616–618] Administration of prolactin increases Leydig cell number[619] and circulating testosterone levels,[620] suggesting that

prolactin has a trophic role in testosterone production. However, despite prolactin receptors being found in Leydig cells,[621] where they appear to function to support expression of LHCGR,[622] a complete knockout of prolactin receptor in mouse has no observable testicular phenotype,[623] suggesting that while prolactin signaling supports testosterone production, it is not an essential requirement.

CONCLUSION

The testis has two major functions: the production of functional gametes and the synthesis of steroid hormones, in particular testosterone. These two roles are inextricably linked, as spermatogenesis is also dependent on a high concentration of intratesticular testosterone. In fact, it could be argued that the primary role of the testis is to produce testosterone, as testosterone synthesis proceeds in the absence of germ cells, but spermatogenesis cannot be completed without testosterone. Thus, the testis primarily functions as an endocrine organ that both influences other body systems to maintain homeostatic mechanisms and is itself responsive to stimuli received from extragonadal body systems. This complex hormonal milieu, coming together to act within an organ with a simple, primary, albeit essential role that is needed for the preservation of species, is both elegant and yet challenging to dissect. Despite this complexity, we already know much about how these interwoven stimuli and suppressors act (Figure 16.11).

Hormones and Spermatogenesis

Maintenance of spermatogenesis and male fertility is dependent upon the direct and indirect actions of numerous hormones. The two most critical endocrine effects are mediated by LH and FSH. Both of these hormones act indirectly to support germ cell development by acting on their respective targets—the Leydig cell and Sertoli cell, respectively—through signaling pathways that are relatively well characterized. The major function of LH signaling is to increase the efficiency of testosterone production, which in turn acts upon the Sertoli cell to facilitate the production of factors that are required for germ cells to pass through specific stages of development. FSH supports and complements testosterone actions to increase spermatogenesis efficiency. FSH acts in a similar manner to testosterone in Sertoli cells to cause the production of regulators of germ cell development and maintenance. The lack of direct action of the major spermatogenesis regulating hormones upon germ cells is an interesting mechanistic choice. Perhaps the additional level of control at the site of the Sertoli cell allows for better integration of signals from all hormone and environmental inputs. Other hormones and paracrine factors, including thyroid hormone and activin, respectively, increase spermatogenesis efficiency by regulating signaling pathways that control Sertoli cell growth and differentiation to determine the ultimate number of Sertoli cells and therefore the upper limit of germ cells and sperm that can be supported.

Hormones and Steroidogenesis

Another group of hormones (osteocalcin, IGF1, glucocorticoids, and leptin) provide fine-tuning and environmental input adjustments to spermatogenesis output. Interestingly, these hormones are similar in that in many cases, they act to alter testosterone production as a mechanism to regulate spermatogenesis efficiency. Whether their impact on spermatogenesis is the primary driver behind these hormones' abilities to modulate testosterone production, or is simply an endpoint response to increased testosterone production, remains to be established, but as discussed in this chapter, testosterone not only is required to support spermatogenesis but also acts bodywide to modulate a variety of parameters associated with male secondary sexual characteristics and adult homeostasis, perturbation of which is increasingly recognized to be associated with pathologies. The influence of these hormones on the testis may therefore reflect a wider signaling network, whereby the systems of the body are at least to some extent able to modulate testosterone production to their own ends.

Recently, the development of knockout mouse models with cell-specific ablation of hormone receptors has provided important physiological information regarding the spermatogenic processes that are regulated by individual hormones. However, often the knockout models are limited to providing information regarding initial developmental processes. In the future, additional inducible adult knockout models may permit a better understanding of hormone regulation throughout the spermatogenesis process and on wider testis function.

Unanswered Questions

A number of questions remain to be answered regarding the pathways by which hormonal signals regulate spermatogenesis. Although testosterone has been known to be essential for spermatogenesis for at least eight decades, the molecular mechanisms by which testosterone acts to maintain spermatogenesis are only beginning to be resolved. Important future questions to be answered include: How does testosterone act to maintain the BTB as well as Sertoli cell–germ cell attachment and germ cell release? What are the relative contributions of classical and nonclassical testosterone signaling in relation to testosterone-regulated stages of

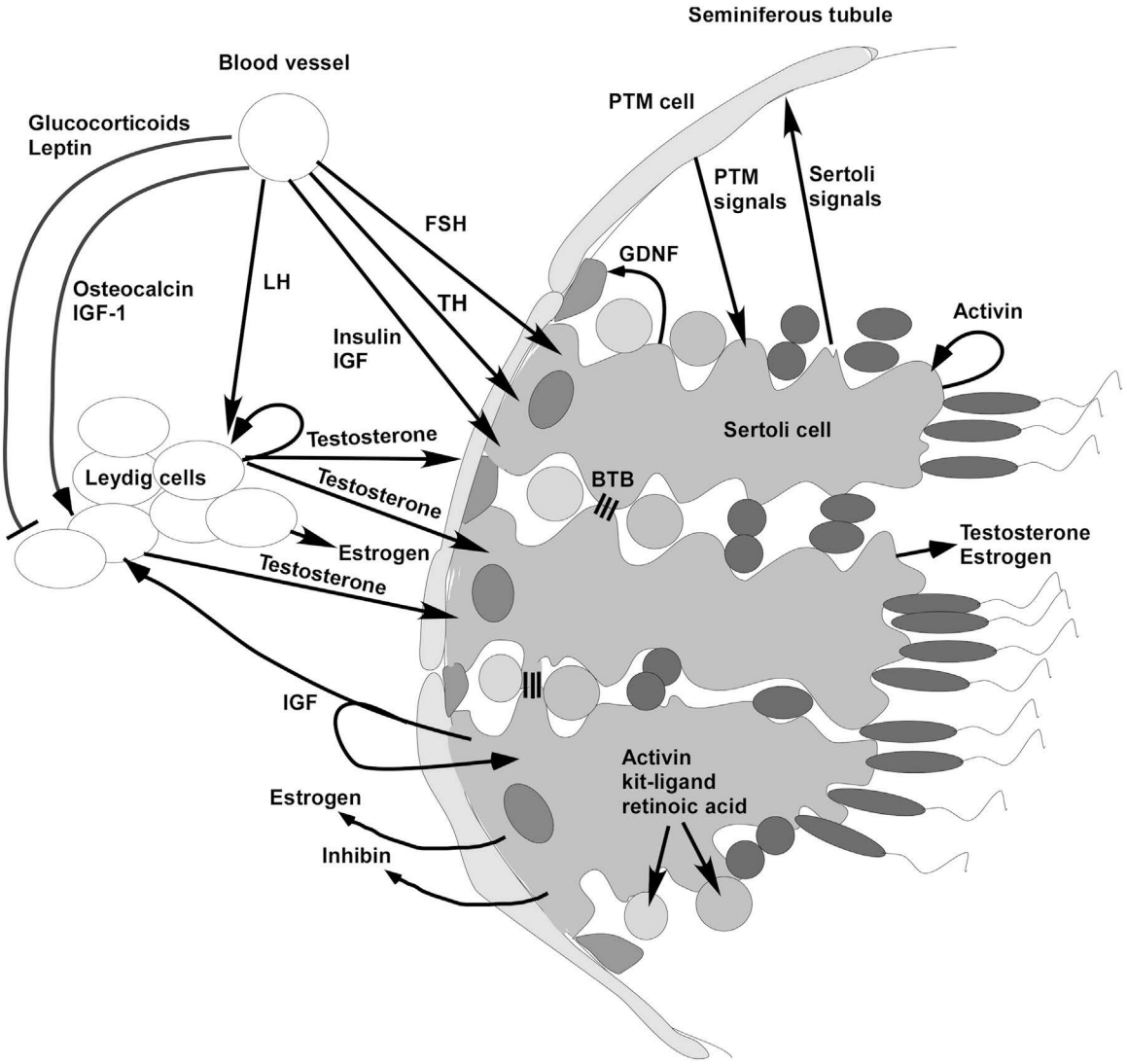

FIGURE 16.11 **Summary of hormone signaling in the testis.** Hormones derived from the circulation, including follicle-stimulating hormone (FSH) and thyroid hormone (TH), act on Sertoli cells to regulate Sertoli cell proliferation and support spermatogenesis. LH acts on Leydig cells to promote testosterone production. Osteocalcin and insulin-like growth factor 1 (IGF1) increase testosterone production, whereas glucocorticoids and leptin oppose testosterone production. Testosterone acts upon Leydig, peritubular myoid (PTM), and Sertoli cells to support the survival, development, attachment, and release of germ cells as well as maintain the blood–testis barrier (BTB). Estrogen produced by Leydig cells and Sertoli cells provides additional signals required to maintain fertility. Sertoli and Leydig cells produce activin that increases the duration of Sertoli cell proliferation and supports spermatogonia proliferation. In response to hormonal and paracrine signals, Sertoli cells produce glial cell line–derived neurotrophic factor (GDNF) to maintain spermatogonial stem cells, as well as activin, kit ligand, and retinoic acid that support the differentiation and proliferation of spermatogonia and entry into meiosis. Inhibin produced by Sertoli cells prevents uncontrolled Sertoli cell growth and provides feedback information to the pituitary gland to regulate FSH secretion. Less well-characterized signals are transmitted between PTM and Sertoli cells.

germ cell development? What is the relative importance of testosterone-regulated miRNAs in the regulation of spermatogenesis?

It has been established that FSH is not required to maintain fertility—at least in rodents. There are new arguments based on knockout mouse models that FSH contributes less than expected to Sertoli cell proliferation in mice. Hopefully, additional studies will clarify the relative contribution of FSH toward supporting the proliferation of Sertoli cells in the mouse versus the rat and primate. Another interesting new development is the finding that PTM cells relay testosterone signals to Sertoli cells prior to the expression of Sertoli cell AR. What are the signals being sent by the PTM cells? Also of interest is the function of estrogen in the testis. Does the loss of estrogen signaling only cause misregulation of fluid movement, or are there specific targets of estrogen in germ cells or supporting cells that regulate spermatogenesis? Finally, will the recent explosion in knowledge about the regulation of spermatogonial stem cell proliferation and differentiation

provide additional information about how hormonal signals regulate stem cell fate decisions and the maintenance of the stem cell niche?

Whereas the scientific community has generated an incredible amount of data relating to the influence, roles, and actions of individual hormones and paracrine factors in the testis, the future challenge is undoubtedly to work toward understanding the integration of these signaling systems, and how they act in concert to support testicular function. The experiments required to address this overarching question in vivo would be prohibitively expensive. It is the advent of increased computing power and the development of computer simulations that will allow integration of these disparate data sets and facilitate the identification of new testable hypotheses. Only by pooling these data to examine the system in its entirety, from the gross influence of circulating hormones to the cell–cell interactions and the intracellular signaling pathways, will we begin to comprehend the wonderful complexity and beauty that underpin the hormonal control of testis function.

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CHAPTER

17

The Epididymis

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INTRODUCTION

Since the publication of the chapter on The Epididymis in the third edition of *Knobil and Neill's Physiology of Reproduction* in 2006,[1] a continued striking series of events has marked the growth in our knowledge and understanding of the epididymis—a long, complex, convoluted duct connecting the efferent ducts to the vas deferens. We have progressed from having gained a basic appreciation of the structures, functions, and physiological regulation of this tissue to beginning to acquire an in-depth knowledge of the molecular events governing the development of the excurrent duct system and of the functions it controls. This tissue can no longer be considered the "abandoned child" of the male reproductive system.[2]

Five international conferences dedicated to this tissue have been held since 1988 (with a sixth one being planned) with proceedings ensuing for four of these.[3–6] A comprehensive, multi-authored volume dedicated to the epididymis has been published,[7] as well as a volume on changes in spermatozoa as they mature in the epididymis.[8] The yearly number of research publications on this tissue has increased at a remarkable rate. It has grown from a few hundred per year until the early 2000s to more than 1000 publications every year since 2005; PubMed currently lists over 15,000 publications related to the epididymis (over 24,000 if the efferent ducts and vas deferens are also considered as part of this system). Therefore, in preparing the current review, we have chosen (1) to narrow the scope by placing most of the emphasis on the epididymis itself, as opposed to the other components of the excurrent duct system, (2) to exclude some topics that have been extensively reviewed recently such as changes in spermatozoa during epididymal transit, innervation and vasculature of the duct system, and pathology of the epididymis, and (3) to focus on some of the more exciting recent developments in this field and provide supplemental information in an alternate format. Several of the subjects covered in the first and third editions are updated; however, due to space limitations, we have chosen not to include most of the plates that depict the histological organization and structure of the various epididymal epithelial cells from the first edition, but rather to place them on a Website (http://www.medicine.mcgill.ca/PhysiolReprodEpididymis/). Along with these plates, this Website contains color versions of plates from the third edition of this chapter and a number of tables, including some containing detailed information about specific epididymal proteins and genes as well as their regulation. We have intentionally chosen not to include such tables within the chapter not only because of space limitations but also because of the need to update this information regularly. We also felt that the review should focus on our ideas, philosophy, and viewpoints on what we believe is important and where the field should go.

As early as the fourth century BC the epididymis was described by Aristotle in his *Historia Animalium*, while the first recorded description of a dissected epididymis was made by de Graaf in 1668 in his monograph *Tractatus de Virorum Organis Generationi Inservientibus* (reviewed in Ref. 6). De Graaf noted that "…the semen … was watery and ash-like in the testis and becomes milky and thick in the epididymis".[9] Between 1888 and 1928 a number of scientists described the histological features of the epididymal epithelium, and surmised, from the images of secretion that they observed, that epididymal secretions

Knobil and Neill's Physiology of Reproduction, Fourth Edition
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"nourished" the spermatozoa in the epididymal lumen. In 1913, Tournade,[10] showed that spermatozoa released from the proximal epididymis were not motile when diluted in saline, but spermatozoa released from the distal epididymis were fully motile. In the bat, spermatozoa were observed to survive for several months in the cauda epididymidis during hibernation, presumably protected by epididymal secretions,[11] while rabbit spermatozoa survived in the epididymis for 30–60 days,[12] and bull spermatozoa for 2 months,[13] after ligation of the efferent ducts. The implication of these observations was that the epididymis conditioned the development of sperm motility and was important for sperm survival.

The consensus of these early investigations can be summarized by the last sentence of Benoit's classic 1926 monograph: "The role of epididymal secretions is to maintain sperm vitality, to permit the development of sperm motility, and possibly to protect them against noxious agents."[14] In a series of four papers between 1929 and 1931, Young[15–18] showed that during epididymal transit spermatozoa not only acquire a mature motility pattern but also become fertile; based on some poorly designed and interpreted studies, he concluded, erroneously, that the changes spermatozoa undergo during their transit through the epididymis represent a continuation of changes that start while spermatozoa are still attached to the germinal epithelium and are not conditioned by some specific action of the epididymal secretion.[19] Nevertheless, by 1931, most of the problems and questions relating to epididymal physiology had been recognized and most of the work done since then has been to provide the experimental evidence to flesh out the insights of earlier investigators.

Few studies were published on epididymal physiology between the 1930s and the 1960s. Those that appeared focused primarily on establishing the length of time necessary for spermatozoa to transit through the epididymis.[20,21] The apparent disinterest in the epididymis during this 20–30 year span is puzzling. After the work of Benoit in 1926[14] and Young in 1931,[15–18] there was a clear controversy that needed to be resolved. In 1965, only 43 papers on the epididymis were published. Whatever the reason for this disaffection, in 1964 Thaddeus Mann, in his book *The Biochemistry of Semen*,[2] refers to the epididymis as the abandoned child of the reproductive system.

In the mid to late 1960s a resurgence of interest in the epididymis was spearheaded independently by Bedford and Orgebin-Crist. They demonstrated that the key event in sperm maturation was not the passage of time, as proposed by Young, but exposure to the luminal environment of the epididymis.[22,23] Thanks to these and other studies, by the end of the 1960s, it was established that the potential for sperm motility and fertilizing ability is acquired as spermatozoa pass from the proximal to the distal epididymis; that the maturation process does not end with the acquisition of fertilizing ability, because spermatozoa that have just become fertile induce a higher rate of embryonic mortality when inseminated in vivo; that the maturation process is dependent on an androgen-stimulated epididymis; and that the maturation process includes changes in sperm organelles (reviewed in Ref. 24).

Since the 1980s, we have seen an explosion of studies on the presence, characterization, immunolocalization, and regulation of a large number of proteins and their mRNAs known to be specific to the epididymis, to be expressed at particularly high levels in some segments of the tissue, or to control key molecules that regulate epididymal function. The objective of many of these studies has been to develop ways of modifying epididymal function so as to alter the ability of this tissue to perform its function with respect to rendering spermatozoa fully mature and motile and to protect them from noxious factors, thus providing potential leads for male contraception or the management of male infertility. In the course of these studies, it has become clear that this tissue presents a novel model for studying cell-, segment-, and region-specific gene expression; for understanding mechanisms of aging; and for identifying processes for selective protection from infections and cancer.

DEVELOPMENT OF THE EPIDIDYMIS

Formation of the Mesonephric/Wolffian/Nephric Duct and Tubules

Many of the studies focusing on the specification and regulation of mesonephric duct and tubule formation have been examined in chick, frog, and zebrafish embryos, with some more recent studies using the mouse embryo. Space limitations prevent a comprehensive review of the development of the urogenital system, but there are several excellent resources with detailed descriptions of these events.[25,26] What follows is a basic summary of the embryonic and postnatal development of the epididymis.

The urogenital system is derived from the intermediate mesoderm, which, in the chick and mouse, is the result of a complex interaction between the intermediate and paraxial mesoderm. The expression of two key transcription factors, paired box 2 (*Pax*2) and *Pax 8* plays a vital role in this process because mice that lack these two genes fail to form a mesonephros and, therefore, later structures of the urogenital system; Bouchard et al.[27] suggest that *Pax 2* and *Pax 8* are critical regulators that specify the nephric lineage. At approximately gestation day 8 or 9 in the mouse and stage 16 in the developing chick, the mesonephric duct (Wolffian/nephric) develops as a cord of epithelial cells that express c-*Sim-1* (chick) and *Pax 2*[28,29] undergoes the formation of a lumen and rapidly extends throughout the length of the embryo in a cranial to caudal direction.

There is considerable evidence to suggest that, in some species, elongation of the nephric duct is the result of cell rearrangements rather than proliferation or changes in cell shape.[30] Interestingly, for proper nephric duct formation but not elongation, bone morphogenic protein 4 (BMP4) expression in the surface ectoderm appears to be critical.[31] Retinoic acid is also crucial for nephric duct formation as the duct does not form in retinaldehyde dehydrogenase 2 (*Raldh2*), a retinoic acid synthetic enzyme, knockout mice.[32] The *Tbx* family of T-box transcription factors may also play a role in the development of the Wolffian duct.[33] Interestingly, a case report in which a human fetus carried a mutation in the protein-tyrosine phosphatase 2 (*Shp2*) gene showed absent Wolffian ducts although the testes and external genitalia were present.[34] In another case report, a patient with unilateral absence of the epididymis and vas deferens was identified, but the precise gene mutation(s) in that patient is not known.[35]

As the mesonephric duct elongates, it induces the nearby mesenchyme to form the mesonephric tubules.[36] The tubules have a characteristic J- or S-shape and resemble developing nephrons. Without the Wolffian duct, the mesonephric tubules do not form.[31] It is clear that those cranial mesonephric tubules that lie in close proximity to the testis survive, and the distal ends grow toward the gonad, whereas the proximal ends contact the Wolffian/mesonephric duct. However, several fundamental questions remain. What are the mechanisms by which the mesonephric tubules are induced by the mesonephric duct? This process involves a complex mesenchyme to epithelial transition, and there is evidence to suggest that leukemia inhibitory factor (LIF), members of the wingless-type MMTV integration site (*Wnt*) family (*Wnt 6* and *Wnt 4*) and fibroblast growth factor (FGF) 2 are responsible for mesenchymal cell aggregation and the formation of the renal nephron (reviewed in Ref. 37). Whether this is recapitulated during the formation of the mesonephric tubules is unclear. Lambot et al. published an informative series of three-dimensional models of the mouse efferent ducts.[38] Alarid et al.[39] have shown that FGF 2 is important for epididymal development because the epididymis fails to develop when embryonic urogenital ridges and sinuses are cultured under the kidney capsule in the presence of FGF 2 antibodies. Studies by Sainio et al.[40,41] showed that the expression of the *Wilms Tumor 1* gene is important in the formation of the most caudal mesonephric tubules. Kitagaki et al. have shown the importance of FGF8, but not the tyrosine kinase receptor, rearranged during transfection (RET) during the development of the cranial mesonephric tubules and thus the efferent ducts. Loss of leucine-rich repeat containing G protein-coupled receptor 4 (LGR4) results in shortened and less convoluted efferent ducts. Further, the duct was infiltrated with immune cells, thereby blocking the duct and preventing flow of fluid and spermatozoa.[38,42,43] The *Von Hippel-Lindau* gene is important for normal mesonephric tubule development because mutations in this gene with an activation of hypoxic-inducible factor 1 (HIF1) and HIF2 result in maldeveloped efferent ducts that can result in the adult epididymis having cysts and microscopic epithelial nodular formations called tumorlets.[44,45] Notch signaling may also play a key role in setting up the boundary between the efferent ducts and the rete testis.[46]

What are the mechanisms by which only those tubules close to the gonad survive, yet others cranial and caudal to the gonad degenerate? Evidence suggests that the caudal tubules normally undergo apoptosis, so we can speculate that those tubules close to the gonad express the anti-apoptotic genes required to survive, but this remains to be established.

What controls the migration and differentiation of cells of the distal tubule into the rete testis? Earlier studies by Upadhyay et al.[47] provided evidence that, in the mouse, the cells within the distal end of the mesonephric tubules contribute directly to the rete testis and undergo differentiation, and that fusion between the mesonephric tubules and the testis did not occur. This finding is perhaps not too surprising in light of the extensive contribution of the mesonephros to the developing testis.

Which portions of the mesonephric tubule and duct form the discrete regions of the initial segment and the first part of the caput epididymidis[14] (Figure 17.1)? There is some evidence to suggest that cells of the Wolffian duct contribute to a small part of the proximal portion of the mesonephric tubule, but again it is not clear whether this represents the junction between the caput and the initial segment. For a more complete overview of the processes described above, the reader should refer to the reviews by Sainio,[40] Vazquez et al.,[49] and Jones.[50]

It is intriguing that the development of the mesonephric tubules may recapitulate the development of the renal vesicle into the renal proximal and distal tubules. It would not be too surprising to find that many genes that regulate this process also play a role in the formation of the efferent ducts and/or initial segment (see Ref. 51 for expression of genes in the mesonephric tubules). For example, members of the *Wnt* family (*wnt 4*)[52] and cadherins[53] play a role in the proximal/distal patterning of the renal proximal and distal tubules. Cadherin 6 is expressed in the proximal tubule progenitors, E-cadherin is expressed in the distal tubule and P-cadherin is expressed in the glomerulus.[54] The initial segment fails to develop in *c-Ros* mutants[55] and in *Sxr* (*XXSxr*) mice,[56] suggesting that the defect may lie in the origin and/or development of the mesonephric tubules. However, studies by Avram and Cooper[57] would suggest that *c-Ros* plays a critical role in the development of the initial segment during the postnatal period. Although it is well recognized that androgens play an important role in epididymal development, the initial segment fails to

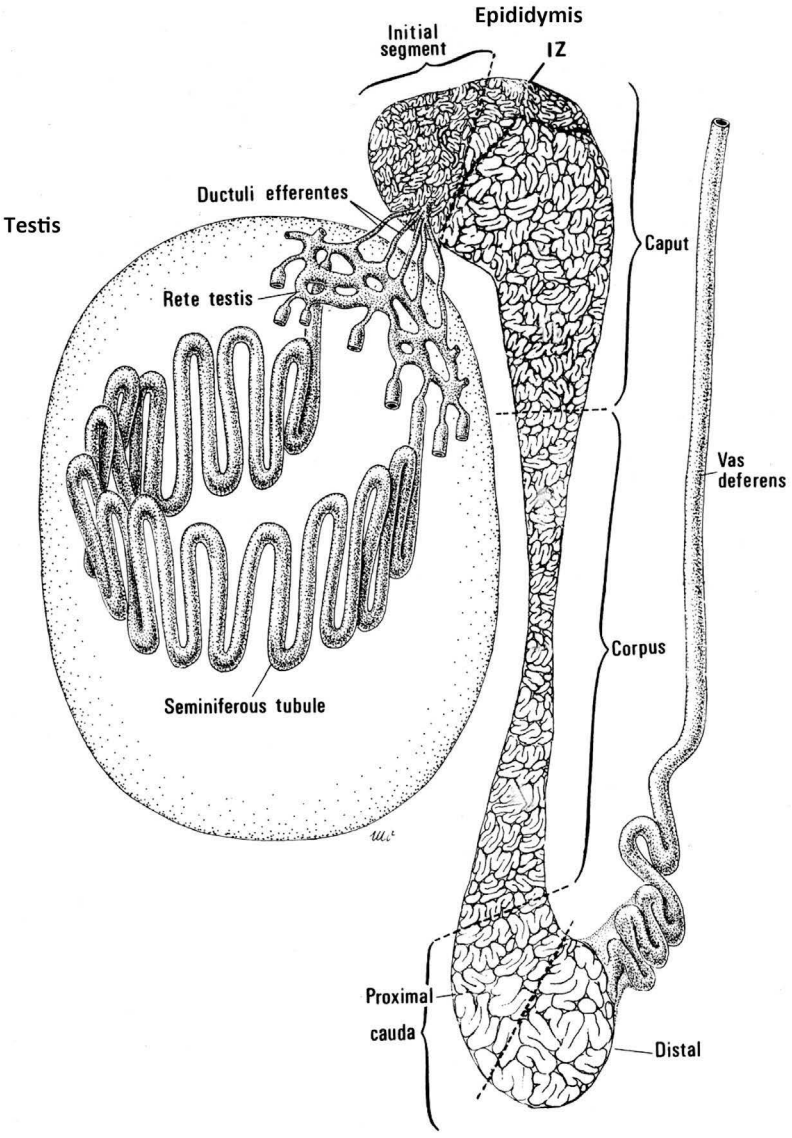

FIGURE 17.1 Diagrammatic representation of the testis and male reproductive tract showing a seminiferous tubule and the rete testis, the ductuli efferentes, the epididymis and vas deferens. The major regions of the epididymis, i.e., the initial segment, intermediate zone, caput, corpus, and proximal and distal cauda, are indicated. *Source: Adapted from Ref. 48.*

develop in a normal androgen environment in the *Sxr* mice;[56] the reason for this developmental defect is not known. This may suggest that additional factors, such as growth factors, may be important for mesonephric tubule/initial segment development. Interestingly, in both of these mutants, the prominent vascular system that supplies the initial segment also fails to develop,[55,58] indicating that there is an intimate relationship between epithelial and endothelial development. Again, this is perhaps not surprising in view of the formation of the renal glomerulus at the tip of the renal proximal tubule where endothelial cells are recruited to this site.

The embryonic origins of the different regions of the epididymis have received some attention; it appears that the efferent ducts are derived from the mesonephric tubules whereas the caput epididymidis to the vas deferens regions are derived from the mesonephric duct (reviewed in Ref. 50). However, the embryonic origins of the initial segment warrant further investigation because it is not entirely clear if the cells are of mesonephric tubule or duct origin. The cells of the initial segment are quite distinct from either the efferent ducts or the distal epididymal regions, but they function similarly to the efferent ducts in that they are actively involved in water reabsorption. Hence, it is possible that the initial segment may be of mesonephric tubule origin.

Elongation and Coiling during the Embryonic Period

Although there have also been some studies examining Müllerian duct elongation and coiling,[59] one of the last biological tubes to be examined regarding mechanisms and regulation of these parameters of growth/maturation is the Wolffian duct. Elongation and coiling are not trivial considering that the adult human epididymis is approximately

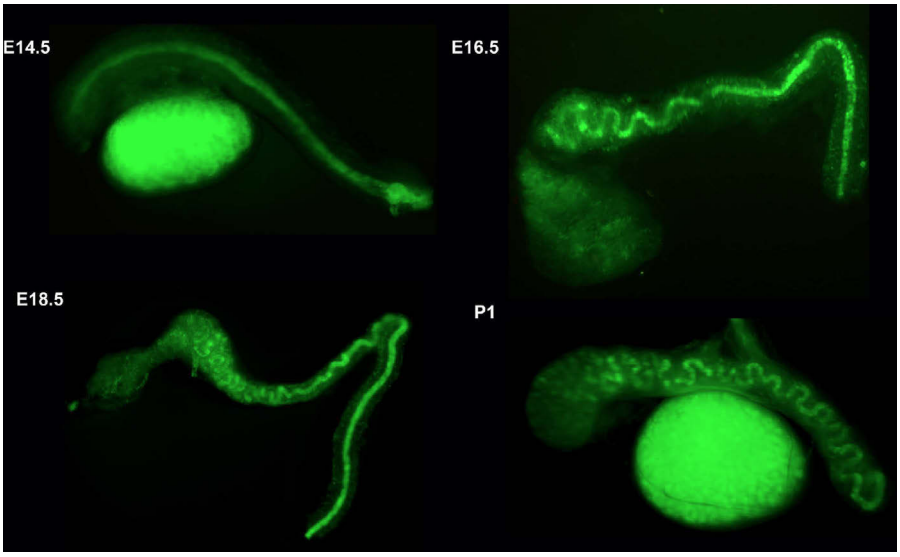

FIGURE 17.2 Wolffian ducts collected from hoxb7-gfp mice at different developmental stages, embryonic (E) days 14.5, 16.5, 18.5 and postnatal (P) day 1. This figure is reproduced in color in the color plate section. Note how the coiling proceeds from proximal to distal with three-dimensional coiling beginning around 18.5. *Source: Image from Ref. 60.*

6 m in length, the adult rat epididymis is approximately 3 m and the adult mouse epididymis is approximately 1 m.[1,60] Presumably, an epididymis that has failed to elongate and coil appropriately will lead to poor sperm maturation and, therefore, male infertility. Further, the failure of the vas deferens and portions of the epididymis to develop appropriately in patients with cystic fibrosis transmembrane conductance (*CFTR*) mutations[61,62] and the failure of the tubules from the human caput epididymal region to reach the testis (epididymal dysjunction)[63] can be hypothesized as a failure of ducts to elongate during development. Interestingly, Zvizdic et al.[63] showed that 30–79% of boys with an undescended testis also showed Wolffian duct abnormalities, of which 25% displayed epididymal disjunction.

Biological tubes can elongate via a number of mechanisms (see Refs. 64,65) and include cell shape change, cell rearrangements such as convergent and extension,[66] spatially restricted proliferation, generalized proliferation and cell recruitment, such as mesenchymal to epithelial transition.[65] There is evidence to suggest that the Wolffian duct elongates via generalized cell proliferation and cell rearrangements, but cell shape change cannot be ruled out.[60] Movement of mesenchymal cells into the Wolffian duct as a means to elongate the Wolffian duct does not seem to occur as shown by the absence of beta-galactosidase specific staining of mesenchymal cells (beta-galactosidase reporter gene driven by a mesenchymal cell-specific retinoic acid receptor beta promoter) in the Wolffian duct epithelium.[67]

Little is also known regarding the patterning of Wolffian duct coiling and how the epididymis reaches its final size. Figure 17.2 shows clearly that coiling begins around E16.5 in the mouse, but interestingly, coiling is only observed in the most proximal region. With time, coiling progresses more distally, with two-dimensional coiling first observed followed by three-dimensional coiling. This would suggest that a morphogenetic gradient is established and certainly inhibin beta A is a prime candidate as a modulator.[26,68] Given the role of members of the Hippo kinase family in dictating organ size[69,70] and the observation that members of this family have been identified in the developing Wolffian duct,[71] would suggest that this family may play a role in regulating size of the epididymis. As the Wolffian duct elongates and coils, it is held firmly by a proximal ligament attached to the kidney and basally to the gubernaculum. Presumably, these attachments will impart some tension as the Wolffian duct develops, and this may play a role in the regulation of cell proliferation, coiling, shape, and size. It has been shown that mechanical forces[72] and morphogen gradients[73] regulate the Hippo pathway in several tissues, and thus it is reasonable to propose that these regulatory mechanisms are acting in concert to regulate Wolffian duct development.

Although testicular luminal fluid factors appear to play a role during postnatal Wolffian duct development, they do not play a role during the embryonic period. Wolffian ducts elongate and coil in vitro without a testis although androgens are necessary.[60]

Postnatal Development

A more complete picture of the postnatal development of the epididymis has emerged over the years, with the majority of studies focused on the mouse, rat, and human, although other species have been studied, for example, bull, dog, rabbit, marmoset, and boar (reviewed in Ref. 74). Perhaps the most detailed description of postnatal epididymal development is that of the rat, and the studies by Sun and Flickinger,[75] and Hermo et al.[76] provide the basis for the following brief overview. There appears to be three major stages of epididymal development: the undifferentiated period, the period of differentiation, and the period of expansion.

By birth, the epididymis has undergone considerable coiling in the proximal regions (initial segment to corpus) and the cauda has yet to complete coiling (Figure 17.2). In the next 1–2 days, individual septa are seen delineating the tubule into segments.[60] The epithelium is undifferentiated and is characterized by columnar cells containing numerous mitotic figures. There is considerable growth of the rat epididymal duct from its embryological origins to postnatal day 15, when it reaches almost 2 m in length.[76,77] The period of differentiation has been examined extensively. Undifferentiated cells differentiate into the classically described cells of the adult epididymis: principal, halo, narrow (pencil), basal, and clear (light) cells; these cells as well as the newly identified dendritic cells are described below. Key changes in development in the rat are as follows: at postnatal day 14, there is the appearance of halo cells; on day 15, one observes narrow and columnar cells; by day 28, the columnar cells differentiate into basal and principal cells; from day 36 onwards, there is the appearance of narrow and clear cells and by approximately day 49, all epididymal cells are fully differentiated. Figure 17.3 outlines the differentiation of the rat epididymal epithelium from birth to adulthood. The period of expansion describes the continued growth of the duct and the appearance of spermatozoa in the lumen.

The mechanisms that regulate the growth and differentiation of the epididymal duct during the postnatal period are unknown, although it is clear that the expression of many epididymal genes is developmentally regulated. Certainly, luminal and circulating androgens (see below) play a critical role, but luminal fluid factors other than androgens also may be important. Early studies by Alexander (1972),[78] and Abe et al.[79] provided evidence that luminal contents produced by the testis and/or proximal regions of the epididymal duct may regulate epididymal epithelial differentiation, although the candidate regulators were not identified. There is evidence to suggest that testicular luminal fluid growth factors (e.g. FGFs) regulate initial segment function and that the same growth factors may regulate postnatal Wolffian duct development. It is tempting to speculate that luminal growth factors (e.g. BMPs, FGFs, NGFs) may also regulate epididymal epithelial differentiation. Loss of *Bmp4, 7,* and *8a*[80,81] and *Alk3*,[82] a gene encoding a putative BMP4 receptor, results in the loss of epididymal integrity during development. This suggests the importance of BMPs during epididymal morphogenesis. Certainly, luminal factors play a role in cell proliferation during the postnatal period because loss of such factors by ligating the efferent ducts leads to an immediate cessation of cell proliferation. Initially, it was suggested that a phosphatase, dual-specific phosphatase 6 (DUSP6), may be a key regulator of cell proliferation in this epididymal region, but results from Xu et al.[83] show that it played a greater role in the caput and corpus regions. It was hypothesized that the reason for region-specific regulation of cell proliferation was caused by differences in the balance of activities of pro- and anti-proliferative signaling pathway, of which members of the MAPK pathway played an integral role. Further, data suggested that the phosphatase and tensin homolog (PTEN) and members of the SRC family of kinases play a role in regulating cell proliferation in the initial segment.[83] SRC may also play a role in regulating cell proliferation in the cauda region.[84] In addition, the microRNA MiR-29a and nuclear autoantigenic sperm protein are potential regulators of epididymal cell proliferation during the postnatal period.[85]

Understanding the mechanisms of epididymal cell proliferation may provide clues as to why the epididymis rarely succumbs to cancer.

Role of Androgens

Interestingly, many of the findings showing that androgens play a role during Wollffian duct development have

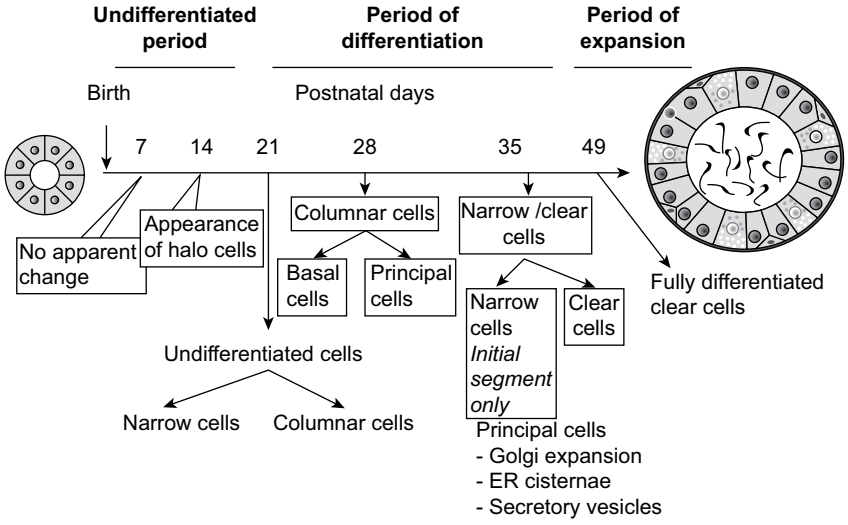

FIGURE 17.3 Diagram outlining the differentiation of the rat epididymal epithelium from birth until adulthood. Epididymal epithelial cells are undifferentiated until approximately day 21 when both narrow and columnar cells are first observed. At approximately day 28, the columnar cells differentiate into basal cells and principal cells. Narrow cells are seen within the initial segment only, but clear cells are observed throughout the epithelium from approximately day 36 onwards. By day 49, all epididymal epithelial cells are fully differentiated.

come from toxicological studies[86–93] and, more recently, from studies using androgen-knockout mice[94,95] or from the systemic administration of androgens.[96,97] However, the precise mechanisms underlying how androgens regulate downstream cellular targets that, in turn, regulate Wolffian duct development are not known.

STRUCTURAL ORGANIZATION OF THE EPIDIDYMIS

Anatomy

Seminiferous tubules converge to form the rete testis, which in turn gives rise to the efferent ducts (ductuli efferentes)—a series of tubules that, depending on the species, number from 4 to 20.[98,99] These tubules converge to form a single highly coiled duct, the epididymis (from the Greek for "on or adjacent to the testis") that is extremely long and varies from 1 m in mice,[100] 3 m in rats,[101] 3–6 m in man,[102] and up to 80 m in horses.[103]

The epididymis is usually divided into four gross anatomical regions: the initial segment, head (caput), body (corpus), and tail (cauda), as first described by Benoit in 1926.[14] Subsequently, a number of other schemes have been proposed for dividing the epididymis into different regions and/or segments, including a number of zones.[104–107] In the rat, there is an intermediate zone between the initial segment and caput epididymis with well-characterized cells.[108] However, in the present review, we will retain the most commonly used nomenclature for the four regions described above. In all mammalian species examined to date, each region of the epididymis is further organized into lobules separated by connective tissue septa. These septa serve not only as internal supports for the organ but have been proposed to also provide a functional separation between lobules, which allows selective expression of genes and proteins within individual lobules and may play a role in creating specialized septa-specific micro-environments where individual substances, such as growth factors, could have differentiated roles.[109–111] The extension of the epididymis is a straight tube, the vas deferens, which is surrounded by a very thick muscular layer. The vas deferens connects with the urethra which empties to the outside of the body. A schematic representation of the testis, efferent ducts, epididymis, and vas deferens is shown in Figure 17.1.

Epididymal Cell Types and Specific Markers

There are several types of epithelial cells that line the epididymis; some are located throughout the duct (e.g. principal cells), whereas others are found either exclusively or primarily in specific regions (e.g. narrow cells). We will provide a brief description of the major cell types along the epididymis, discuss the potential functions of these cells, and demonstrate the presence of specific markers for each cell type. Several detailed reviews of the histology of the epididymis for a number of species ranging from mouse to man and including dog, camel, elephant, opossum, bull, ram, hamster, mouse, and monkey have been published.[14,48,79,105,106,112–124] Together, these publications provide increasing evidence that similar regions and cell types are present in most mammals, including man. These cell types appear in the appropriate regions, share similar structural features and functions, including regional differences, and show very similar patterns of expression of some secretory proteins.[125–132] The comprehensive set of light and electron microscopy plates prepared for this volume are available at: http://www.medicine.mcgill.ca/PhysiolReprodEpididymis. Schematic representations of the organization of all the cell types and their known functions are represented in Figure 17.4. Key components of epithelial cells in the initial segment and in the remainder of the epididymis as observed at the electron microscope are depicted in Figures 17.5 and 17.6, respectively.

Principal Cells

The main cell type found in the epididymis of all mammals is referred to as the principal cell. These cells appear along the entire duct but show structural differences in each region.[48,134] The most striking feature of these cells is their highly developed secretory and

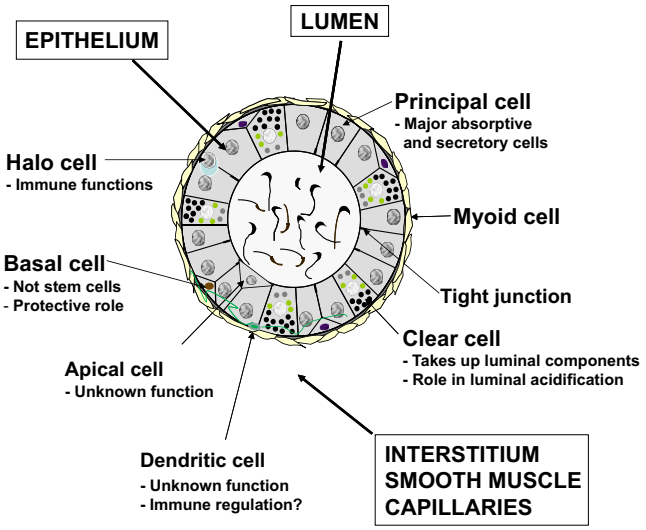

FIGURE 17.4 Schematic organization of the major cell types in a cross section of the epididymis as observed at the light microscope. The three epididymal compartments as well as the relative position and distribution of each of the main cell types are illustrated. The major function(s) associated with each cell type is also identified.

endocytic machinery and their basally aligned nuclei. Depending on the segment examined, principal cells comprise approximately 65–80% of the total epithelial cell population of the epididymis.[134] Both their structure and functions vary dramatically between the different segments.[48,114,135] These differences are reflected in the appearance and organization of their secretory apparatus (endoplasmic reticulum (ER), Golgi apparatus, and secretory granules) and endocytic apparatus (coated pits, endosomes, multivesicular bodies, and lysosomes). The precise characterization of the secretory granules, epididymosomes, and Golgi apparatus of these cells, as well as the identification of organelles and mechanism of uptake of luminal substances, have been well described.[133,136] In addition, one other major difference is the abundance of lipid droplets found only in the corpus epididymis (Figure 17.6), the exact significance of which is still poorly understood.

Principal cells synthesize a large number of proteins that are then either retained within the cells or actively secreted into the luminal compartment.[48,135,137–147] They also play an active role in endocytosing proteins found in the luminal compartment of the epididymis.[133] A schematic representation of these processes is depicted in Figure 17.7.

Apical Cells

Apical cells are found primarily within the epithelium of the initial segment and intermediate zone,[148–150] although they have been seen occasionally in other segments in aging rats.[151] These cells have a characteristic apically located ovoid nucleus and do not contact the basement membrane (Figure 17.5). They differ clearly from adjacent narrow and principal cells in terms of their protein expression profile.[149] However, little is known about the specific functions of these cells, aside from their ability to endocytose substances from the lumen, as revealed by the examination of these cells in β-hexosaminidase A knockout mice,[120] and the observation that they contain many proteolytic enzymes and a high density of mitochondria.[149,150]

Narrow Cells

In the rat and mouse, narrow (pencil) cells of the adult epididymis appear only within the epithelium of the initial segment and intermediate zone.[148,149] These cells are narrower than the adjacent principal cells, attenuated, and send a thin process of cytoplasm to reach the basement membrane (Figure 17.5). They are characterized by numerous apically located cup-shaped vesicles that are involved in endocytosis and function in secreting H^+ ions into the lumen by recycling to and from the apical plasma membrane.[151,152] Similar cells have also been reported within the same regions in numerous other species, including bovine, hamster, echidna, and humans.[112,116,117,127,151,153] Narrow cells are distinct from apical cells in their

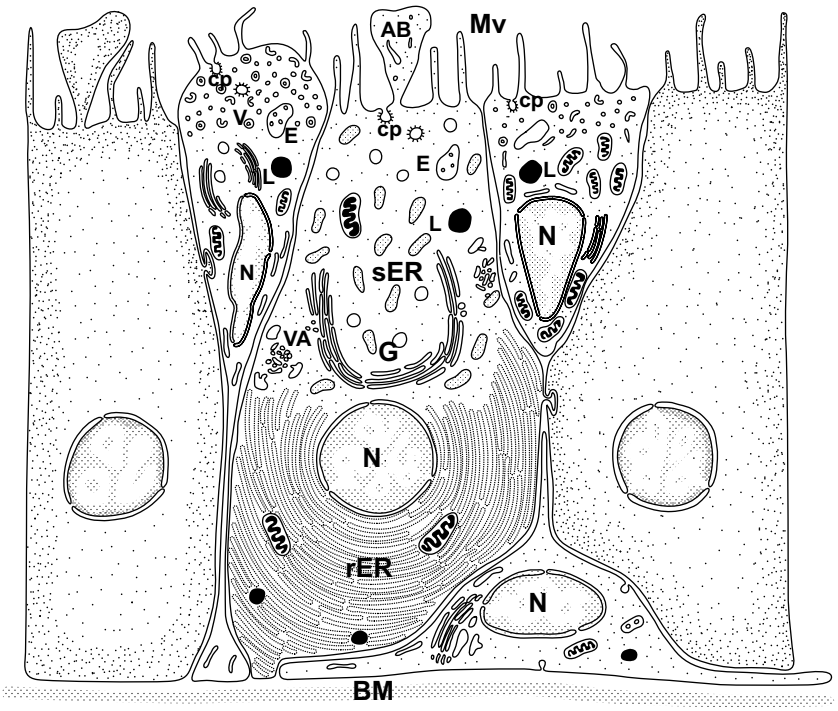

FIGURE 17.5 Schematic representation of cells found in the epithelium of the initial segment of the epididymis, as visualized in the electron microscope. Narrow cells, represented on the left, are elongated cells tapering toward the basement membrane (BM); they show numerous small apical cup shaped vesicles (v), coated pits (cp) and occasional endosomes (E) and lysosomes (L). Principal cells are columnar in appearance and show coated pits (cp), endosomes (E) and lysosomes (L), components of the endocytic apparatus. They also contain numerous parallel cisternae of rough endoplasmic reticulum (rER) located basally and dilated, irregularly shaped cisternae of ER sporadically dispersed in the apical and supranuclear regions of the cell (sER). The Golgi apparatus (G) is elaborate and located supranuclearly. Principal cells show blebs of cytoplasm emanating from their apical cell surface, referred to as apical blebs (AB) and small vesicular tubular aggregates (VA) occupying regions adjacent to the Golgi apparatus. Also illustrated on the right is an apical cell that, unlike the narrow cell, does not extend to the basement membrane and shows few coated pits (cp), apical vesicles, endosomes and lysosomes (L). A basal cell stretches along the basement membrane and sends a thin process up toward the lumen. N, nucleus; Mv, microvilli. *Reproduced with permission from Ref. 133.*

morphological appearance, relative distribution and expression of different proteins. They also differ dramatically from neighboring principal cells and display region specific expression of proteins such as the glutathione S-transferases (GSTs) and lysosomal enzymes.[149]

Clear Cells

Clear cells are large active endocytic cells present only in the caput, corpus, and cauda regions of the epididymis and are found in many species, including humans.[48,114,154] These cells are characterized by an apical region containing numerous coated pits, vesicles, endosomes, multivesicular bodies, and lysosomes and a basal region containing the nucleus and a variable amount of lipid droplets (Figure 17.6).[48,155,156] After injection of tracers into the lumen of the cauda epididymidis, the apical vesicles, endosomes, multivesicular bodies, and lysosomes of clear cells are found labeled, thus indicating an endocytic role for these cells; this endocytic activity is much greater in clear cells than in the adjacent principal cells, particularly in the cauda epididymis.[156,157] Clear cells normally take up the contents of cytoplasmic droplets released by spermatozoa as they traverse the duct.[48,156] Cytoplasmic droplets are formed at the time of the release of spermatozoa and contain Golgi saccular elements that may be involved in modification of the plasma membrane of spermatozoa.[158] Clear cells also endocytose a number of different proteins, but often in a region-specific manner.[137,141,159,160] These cells become abnormally large and filled with lysosomes after various experimental conditions that disrupt the normal functioning of the testis and epididymis.[134]

The acidification of the luminal fluid (see below) is thought to be mediated by clear and narrow cells. Indeed, key proteins for this process, H^+ATPase (or vacuolated V-ATPase), carbonic anhydrase II, and soluble bicarbonate sensitive adenylate cyclase (sAC), are selectively localized to these cells (Figure 17.8).[161,162] Further, ClC-5, a member of the voltage-gated CIC chloride channel family, is also expressed exclusively in clear cells and partially co-localizes with the H^+ATPase in their apical region.[163] Breton's group has proposed that, in narrow and clear cells, apical membrane accumulation of V-ATPase is triggered by a sAC-dependent rise in cAMP in response to alkaline luminal pH and that these cells play a specific role in luminal acidification.[164] Studies provide evidence that inhibition of RhoA or its effector ROCKII favors the recruitment of V-ATPase to the apical region of clear cells.[165]

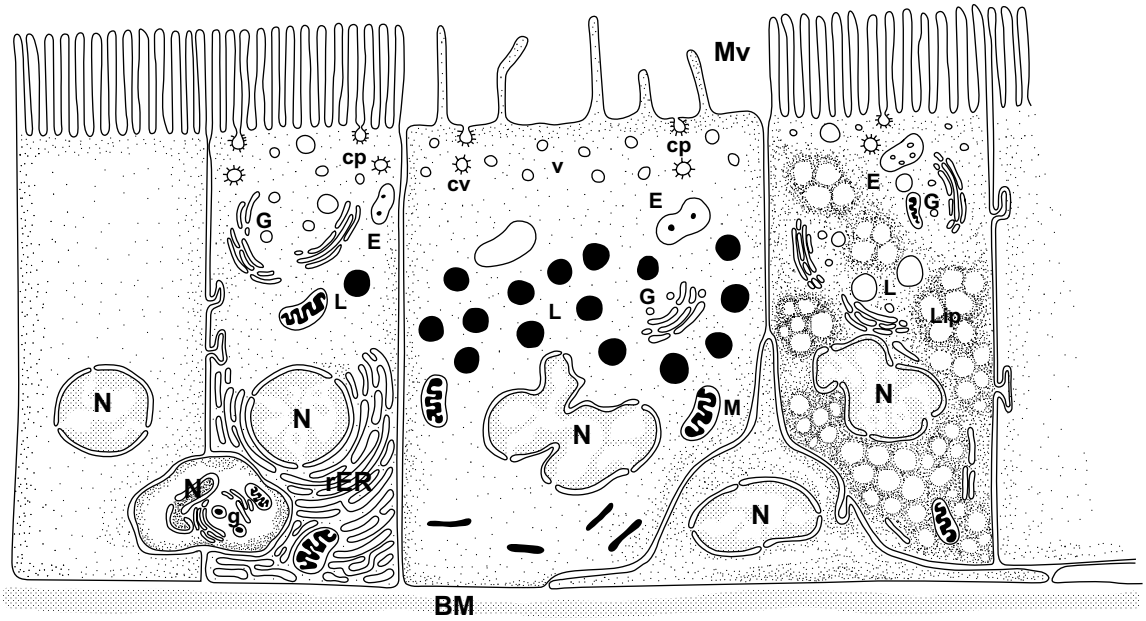

FIGURE 17.6 Schematic representation of a principal cell of the caput epididymidis on the left and a principal cell of the corpus epididymidis on the right, with a clear cell in between, as visualized in the electron microscope. Also represented is a halo cell and a basal cell. Principal cells of both regions contain coated pits (cp), endosomes (E) and lysosomes (L) and an elaborate Golgi apparatus (G). Rough endoplasmic reticulum (rER) occupies the basal region of the principal cell of the caput, while numerous lipid droplets (lip) occupy the cytoplasm of the principal cells of the corpus region. The clear cell shows few microvilli (Mv), but numerous coated pits (cp), small apical vesicles (v), coated vesicles (cv) endosomes (E) and lysosomes (L), all involved in endocytosis. The halo cell is inserted between adjacent principal cells, located basally and contains small dense core granules (g), while the basal cell stretches itself along the basement membrane (BM). N, nucleus. *Source: Reproduced with permission from Ref. 133.*

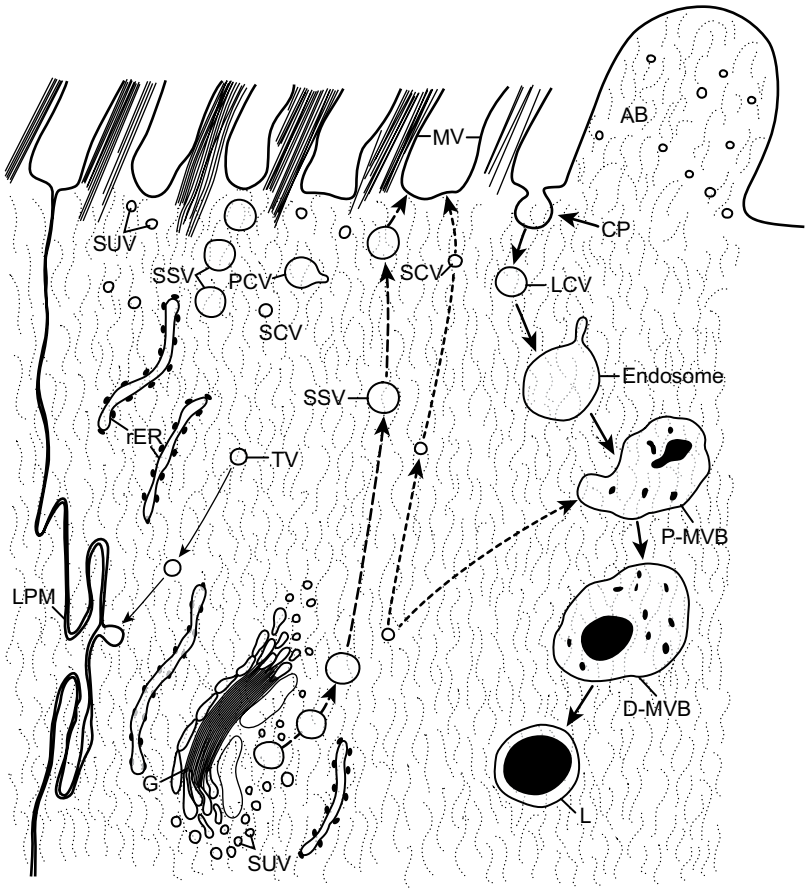

FIGURE 17.7 Schematic representation of the apical and supranuclear cytoplasm of a principal cell of the epididymis. The Golgi apparatus (G) is formed of several saccules packed on top of each other, from which the smooth surfaced secretory vesicles (SSV, 150–300 nm) and small coated vesicles (SCV, 60–70 nm) are derived. The SSV are destined for the apical cell surface with which they will fuse, delivering their content into the epididymal lumen in a regulated manner, by a process described as merocrine secretion. The SCV are targeted from the Golgi apparatus to multivesicular bodies (MVBs), delivering their lysosomal contents therein, while some may be destined for the cell surface. The endocytic apparatus consists of coated pits (CP), large coated vesicles (LCV), endosomes, pale (PMVB) and dense (DMVB) MVBs and lysosomes (L). Small uncoated vesicles (SUV, 60–70 nm) are also indicated, some of which may be Golgi derived and involved in constitutive merocrine secretion by fusion with the apical cell surface. An apical bleb (AB) is also indicated and contains mainly polysomes and small vesicles, the origin of which may be Golgi derived. Apical blebs appear to detach from the apical cell surface and upon fragmentation in the epididymal lumen liberate their contents therein for functions involved in sperm maturation. Mv, microvilli; PCV, partially coated vesicle; rER, rough endoplasmic reticulum; LPM, lateral plasma membrane; TV, small vesicles involved in the transcytosis or passage of substances from the lumen to the lateral intercellular space. *Source: Reproduced with permission from Ref. 133.*

Basal Cells

Basal cells appear in all species studied to date, including humans.[48,114,166,167] Hemispherical in appearance, they adhere to the basement membrane and do not have direct access to the lumen of the duct, although processes of these cells extend at times toward the lumen (Figures 17.5 and 17.6) as first reported by Veri et al.[167] and subsequently confirmed.[168] Like principal cells, basal cells are not thought to divide rapidly in adults nor are they thought to act as stem cells to replenish principal cells.[169]

Basal cells possess thin attenuated processes that extend along the basement membrane from their main hemispherical cell body to collectively cover a large proportion of the circumference of the epididymal tubule.[167,168] However, after ligation and castration, these cells transform into large bulbous dome-shaped cells that are closely packed together and show few short lateral processes.[170] Due to the dramatic decrease in size of the epididymal tubules after these treatments, it is likely that the shape and arrangement of basal cells in normal animals is governed in part by the volume and pressure exerted upon the tubular epithelium by luminal fluids and sperm derived from the testis. Consistent with this hypothesis is the observation that, in rats, during postnatal development, basal cells are transformed from dome-shaped cells to flattened cells that exhibit processes as spermatozoa and fluids arrive in the corpus and cauda epididymidis by days 49 and 56, respectively.[166]

Basal cells possess coated pits on the plasma membrane face that opposes the basement membrane and overlying principal cells, suggesting there is receptor-mediated endocytosis of factors derived from the blood or principal cells. Basal cells also show an accumulation of a secretory material within Golgi saccules and distinct secretory granules appear next to the Golgi apparatus, as seen in other typical secretory cells.[171] The destiny of the secretory material may be to regulate principal cell function or enter the circulation for functions as yet to be determined.

Studies from Wong's group (reviewed in Ref. 172) have proposed an additional function for basal cells; they suggest that these cells may have a role in regulating electrolyte and water transport by principal cells. This process is proposed to be mediated by the local formation of prostaglandins and requires the participation of the transient receptor potential (Trp) proteins. The latter serve as transmembrane pathways for Ca^{++} influx, while cyclooxygenase 1 (COX-1) is a

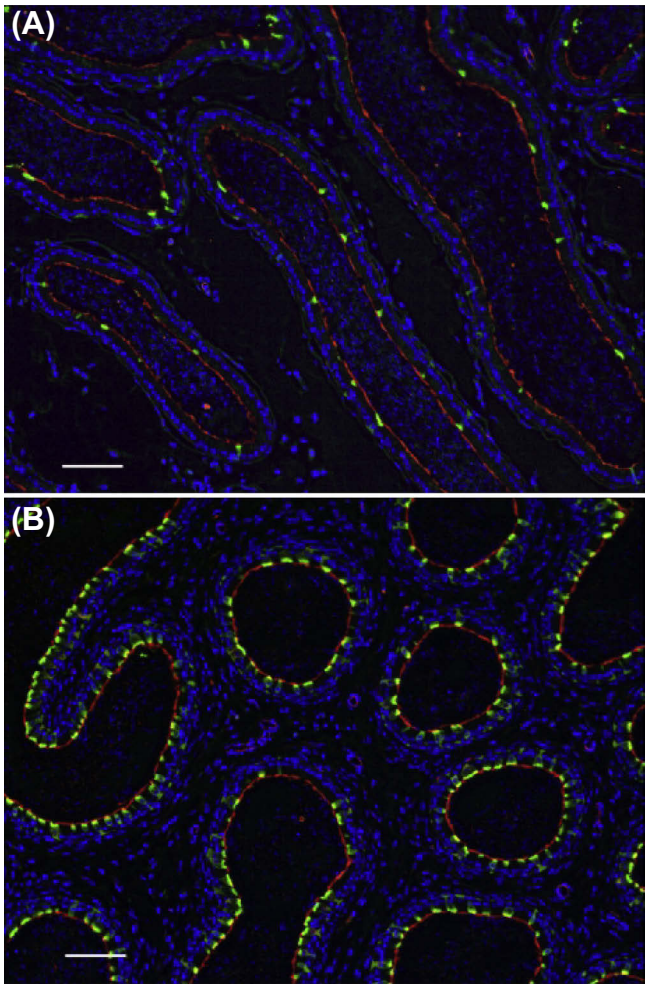

FIGURE 17.8 Relative numbers of clear cells in the rat caput (A) versus cauda (B) epididymidis. This figure is reproduced in color in the color plate section. Rat epididymis was stained for the V-ATPase B1 subunit (green) to label clear cells, and NHERF1 (red). Nuclei and spermatozoa were stained with DAPI (blue). NHERF1 is located in the apical membrane of both principal cells and clear cells. Clear cells positive for the subunit B-1 or V-ATPase are much more numerous in the cauda (B) than in the caput (A) epididymidis. Scale bars, 50 μm. *Source: Reproduced with permission from Ref. 164.*

key enzyme in the formation of prostaglandins. Both of these proteins are exclusively expressed in basal cells.

Halo Cells

Halo cells are small cells with a narrow rim of clear cytoplasm and are present throughout the epididymal epithelium (Figure 17.6).[48] These cells are usually located at the base of the epithelium and contain variable numbers of dense core granules. Halo cells have been described either as lymphocytes,[48] or monocytes;[173] these two cell types are difficult to distinguish using the light microscope due to their similarity in size and nuclear morphology. Although the exact nature of halo cells has been controversial since they were first described in 1957 by Reid and Cleland,[106] subsequent studies by Flickinger et al.[174] and Serre and Robaire[175] have resolved this issue by immunolabeling the main types of immunocompetent cells. It is now clear that, in young adult animals, halo cells consist of helper T lymphocytes, cytotoxic T lymphocytes, and monocytes, but not B lymphocytes. With age, there is a region specific increase in the number of each of these immune cell types, as well as the occasional appearance of eosinophils and B lymphocytes.[153] It is interesting to note that in the epididymal epithelium of young rats, the number of cells that stain for antibodies against monocytes-macrophages (ED1+), helper T lymphocytes ($CD4^+$) and cytotoxic T lymphocytes ($CD8^+$) is equivalent to the number of halo cells,[175] suggesting that halo cells are, under normal conditions, the primary immune cell in the epididymis (see also Chapter 19).

Dendritic Cells

Using specific markers for a sub-category of immune cells, recent studies by Da Silva et al.[176] have demonstrated the presence of another cell type in the epididymal epithelium. Dendritic cells are specialized antigen-presenting cells that are key regulators of immune responses in many tissues.[177,178] Although previously reported to not be present in this tissue,[179] several advances have allowed the clear demonstration that these cells are present in the epididymis. The finding that there are extensive networks of dendritic cells visualized in the epididymis of CD11c-EYFP[180] and CX3CR1-GFP[181] reporter mice, combined with the use of thick section (50-μm sections) for confocal microscopy allowed Da Silva et al.[176] to demonstrate that there is a network of these dendritic cells all along the epididymis, that there are multiple types of these cells in the epididymis, and that isolated CD11c+epididymal dendritic cells have strong antigen-presenting properties. These cells are likely to interact with both halo cells and the lymphatic system to serve in immune monitoring of the epididymis.

Cell–Cell Cross Talk in the Epididymal Epithelium

Each of the above cell types do not function in isolation. To create the specialized microenvironments needed to mature and store sperm throughout the epididymis (discussed below) requires extensive communication between adjacent cells and among different cells types. There are many such interactions. Basal cells form junctions with principal cells and halo and dendritic cells appear to monitor the entire epithelium.[48,182,183] We will focus on two such examples of this type of communication: the interaction between clear, basal and principal cells in regulating

intraluminal pH and, in the next section, cell–cell interactions in the formation of the blood–epididymis barrier.

Through an elegant series of experiments, the Breton laboratory has implicated a number of actors in clear, principal and basal cells that interact to regulate intraluminal pH in response to various stimuli (Figure 17.9).[184] These include sAC, proton-pumping ATPase (V-ATPase), guanylate cyclase (sGC), sodium bicarbonate cotransporter (NBC) in clear cells; protein kinase A (PKA) and cystic fibrosis transmembrane conductance regulator (CFTR) in principal cells, and angiotensin II receptor (AT2) prostaglandin E2 (PGE2) in basal cells. Briefly, it is proposed that basolateral activation of principal cells during sexual arousal triggers the secretion of bicarbonate and ATP into the lumen, a process that is mediated by CFTR and that then induces sAC in clear cells, resulting in proton-pumping ATPase (V-ATPase) accumulation in apical microvilli and consequent increase in proton secretion. Luminal angiotensin II stimulates AT2 in basal cells and induces the production of nitric oxide, which diffuses to produce cGMP in clear cells via activation of soluble guanylate cyclase (sGC). The cGMP triggers the apical accumulation of VATPase and increases proton secretion.

The Blood–Epididymis Barrier

Given the presence in spermatozoa of proteins that are recognized by the body as foreign, it only stands to reason that there should be a continuation beyond the testis of a functional barrier. The probable existence of a blood–epididymis barrier was discussed as early as 1976,[185] and several reviews describing different aspects of the barrier have appeared since.[186–194]

Structure

The junctional complex between adjacent epididymal principal cells is composed of apically located gap, adherens, and tight junctions. Tight junctions between adjacent principal epithelial cells at their luminal surface form the blood–epididymis barrier,[187,195] while gap junctions allow communication between adjacent principal cells. These tight junctions form a continuous belt around the cell, sealing the spaces between the epithelial cells, so that the luminal compartment and the intercellular spaces become separate physiological compartments.[196] The tight junctions begin to form at the time of differentiation of the Wolffian duct.[197] Using lanthanum nitrate as an electron opaque tracer that is blocked at tight junctions, the postnatal development of the blood–epididymis barrier was shown to be gradual; its

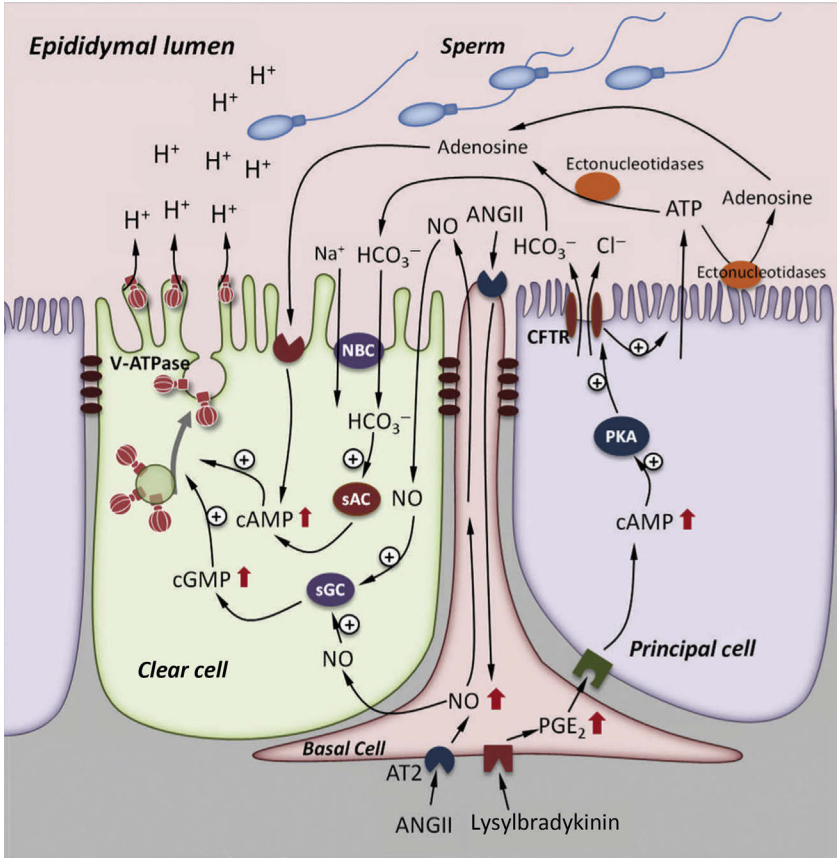

FIGURE 17.9 Schematic representation of cell–cell cross talk in the epididymis. Basolateral activation of principal cells during sexual arousal triggers the secretion of bicarbonate and ATP into the lumen, a process that is mediated by cystic fibrosis transmembrane conductance regulator. Stimulation of bicarbonate-sensitive adenylyl cyclase (sAC) and purinergic receptors in clear cells induces proton-pumping ATPase (V-ATPase) accumulation in apical microvilli, followed by an increase in proton secretion. Basal cells can extend a narrow body projection toward the lumen, and they form a new tight junction with adjacent epithelial cells. Luminal angiotensin II (ANGII) stimulates ANGII type II receptor (AT2) in basal cells and induces the production of nitric oxide (NO), which then diffuses out to produce cGMP in clear cells via activation of guanylate cyclase (sGC). The cGMP triggers the apical accumulation of VATPase and increases proton secretion. Simulation of basal cells by basolateral factors, including lysyl bradykinin, was shown to increase bicarbonate and chloride secretion in principal cells via prostaglandin E2 (PGE2) stimulation. NBC indicates sodium bicarbonate cotransporter; PKA, protein kinase A. *Source: Reproduced with permission from Ref. 184.*

formation was virtually complete by postnatal day 21 in rats.[196]

Changes in the structure of the junctional complex have been observed with the electron microscope when comparing the initial segment to the other segments of the epididymis. In the initial segment, the tight junctions span a considerable length of the apical plasma membrane and have few desmosomes.[187] As one progresses toward the caudal end of the epididymis, a general decrease in the number of tight junctional strands was noted; the span of merging plasma membranes was considerably reduced, but numerous desmosomes were found in the apical region.[187,197]

Junctional Proteins

Adheren junctions form a continuous belt and hold neighboring cells together through a family of calcium-dependent cell–cell adhesion molecules called cadherins, which mediate calcium-dependent homotypic interactions.[198–200] The cadherins have also been implicated in the formation and maintenance of tight junctions.[198,201–209] The cytoplasmic domain of cadherins forms a tight complex with several proteins, which either link cadherins to the cytoskeleton or are involved in signal transduction pathways. These include catenins, actinin, vinculin and zonula occludens (ZO-1). The cadherin-catenin complex is essential for cadherin mediated cell adhesion. Cyr et al.[210] reported the presence of E-cadherin and P-cadherin mRNA in the rat epididymis. Immunogold labeling using the electron microscope indicates that E-cadherin is localized in the extracellular space between the lateral plasma membranes of adjacent principal cells at the level of apically located junctional complexes in the adult rat epididymis,[187,211,212] as well as in the deeper underlying regions of the extracellular space between the lateral plasma membranes. Similar observations were noted in the human and mouse epididymis.[213–215] Although it was shown that in the normal adult rat epididymis, there was immunostaining for three anticatenin antibodies (alpha, beta-, and p120ctn) along the lateral plasma membranes (LPM) between adjacent epithelial cells,[216] the composition of the catenin-adhering junctional family of proteins and their relationship with cadherins remain to be established.

Claudins are another protein family involved in the formation of epididymal apical tight junctions. Claudin1 is found throughout the epididymis and is localized to epididymal tight junctions; it is regulated by androgens, but only in the initial segment of the tissue.[217] Claudin-7 is localized along the entire lateral membranes of the epididymal epithelium, including the apical junctional region throughout the epididymis, but claudin-8 is restricted to the apical junctional region.[218] The specific role of each of these various proteins in the formation of tight junctions throughout the epididymis remains to be established.

In addition to adherens and tight junctions, the epididymal junctional complex also contains gap junctions.[189,195,219,220] Gap junctions, made up of proteins termed connexins, mediate communication between cells by allowing small molecules to pass from cytoplasm to cytoplasm of neighboring cells, thereby metabolically and electrically coupling them together.[221] Connexin subunits oligomerize in the *trans*-Golgi network to form hemichannels or connexons. In the epididymis, gap junctions containing connexin 43 (Cx43) were first localized between principal and basal cells.[222] Although the presence of multiple connexins in specific cell types is not unique to the epididymis, the large number of different connexins[182,190,220,223–225] is suggestive of complex communication among epididymal cells. The structural aspects of the blood–epididymis barrier are referred to as the anatomical blood–epididymis barrier.

It is clear that the anatomical aspect of the blood-epididymis barrier is not static but highly dynamic. For example, basal cells have been shown to extend projections into the lumen[164,167] yet junctional complexes are maintained.[164] Presumably, the complexes must be formed de novo upon projection extension and rapidly degraded upon projection retraction.

Functions

The composition of epididymal luminal fluid is distinctly different from that of blood plasma. The blood-epididymis barrier keeps the two fluids in separate compartments.[144] The blood–epididymis barrier also maintains a specialized luminal microenvironment for the maturing spermatozoa by restricting the passage of a number of ions, solutes, and macromolecules across its epithelium.[186,226] For instance, molecules such as inositol and carnitine can be concentrated ten- to a hundred-fold in the lumen of the caput epididymidis, while others, such as inulin, L-glucose and albumin, are effectively excluded (reviewed in Refs 48,139). The blood–epididymis barrier carefully controls the microenvironment so that spermatozoa are bathed in an appropriate fluid milieu at each stage of maturation as they travel through each segment of the epididymis.[139] This is achieved by regulating the influx and efflux of solutes across the basolateral and apical membrane via specific transporters.[192] How each transporter is individually regulated and how they are coordinated to provide a unique and ever-changing microenvironment along the duct is unclear. This functional aspect of the blood–epididymis barrier is referred to as the physiological blood–epididymis barrier.[192]

In addition to the anatomical and physiological aspects of the blood–epididymis barrier, there is also an

immunological component.[191,192] This aspect of the barrier prevents the entry of immunoglobulins and immune cells into the lumen.[227,228] Further, the epididymis sequesters sperm antigens and so protects the spermatozoa as they progress along the duct. Very little is known regarding the immunological aspects of the blood–epididymis barrier compared to the blood–testis barrier[191,192,229,230] (see also Chapter 19) and more studies are obviously needed to understand how sperm are protected from the immune system. Interestingly, the epididymal interstitium has some immune protective abilities, although not as extensive as those of the interstitium of the testis.[229] Another possibility is that the dendritic cell network within the epithelium of the mouse epididymis[176] plays a role in the immunological blood–epididymis barrier, but this remains to be explored.

Although it would appear that the blood–epididymis barrier is resistant to the administration of various substances, such as gossypol[231] and estradiol,[232] the administration of the alkylating agent, cyclophosphamide (see later), to efferent duct ligated rats resulted in damaged spermatozoa.[233] This would suggest that this particular drug could enter the epididymal lumen and modify spermatozoa.[233] Treatment of adult mice with polychlorinated biphenyls (PCBs, Aroclor 1254) results in increased permeability of the blood–epididymis barrier and alters the expression of several tight junction proteins, such as occludin.[234] It has also been shown that there is a loss of integrity of the anatomical blood–epididymis barrier in mice that are mutant for Cathepsin-A, and this loss of integrity may be due to changes in the localization of claudins.[235] The inability of this barrier to maintain its tightness under conditions of stress, such as aging,[212] may help explain some of the deleterious effects of stressors on sperm function and fertility. However, many more studies are needed to establish the role played by this barrier in protecting spermatozoa from stressors and toxic substances.

FUNCTIONS TAKING PLACE IN THE LUMINAL COMPARTMENT

The main four functions of the epididymis are: the transport of spermatozoa, development of sperm motility, development of sperm fertilizing ability, and the creation of a specialized luminal environment conducive of the maturation process through the absorptive and secretory activities of the epididymal epithelium.

Transport of Spermatozoa

Once released in the lumen of the seminiferous tubule, spermatozoa are transported through the rete testis and efferent ducts and begin their journey down the epididymis. Several methods have been used to measure the duration of this transit. The most direct method is to incorporate a labeled isotope into the DNA of germ cells at the spermatogonia and preleptotene spermatocyte stages and follow the progression of the first wave of labeled spermatozoa down the epididymis. This approach gives the minimal time required for sperm passage through the epididymis. Total transit time, or transit through each epididymal segment, may also be estimated from the ratio of epididymal sperm reserves and daily testicular sperm production,[236] assuming that there is no sperm resorption and no difference in transit speed between segments. Despite some minor discrepancies between estimates obtained with these two techniques, it appears that, irrespective of the size of the animal, its sperm production, or its epididymal sperm reserves, the minimal time required for spermatozoa to transit through the epididymis is approximately 10 days (Figure 17.10, Table 1, supplemental material at http://www.medicine.mcgill.ca/PhysiolReprodEpididymis/). In most species, the average transit time of the majority of labeled spermatozoa is longer than that of the sperm vanguard; for example, in bulls the transit time is14 days as the average for the majority of sperm as opposed to 8 days for the leading vanguard.[236] As a result, there is some mixing of spermatozoa of different ages in the distal part of the epididymis.[237] There are two notable exceptions to the 10-day minimum transit time. In man and chimpanzee, the first labeled spermatozoa pass through the epididymis in 1 and 2 ± 1 days, respectively (Table 1 supplemental material at http://www.medicine.mcgill.ca/PhysiolReprodEpididymis/). These values give the most rapid transit time, but the average transit time is 12 days in man,[238] a value comparable to that found in other species.

When one follows the progression of labeled spermatozoa in the different regions of the epididymis, the transit time through the caput and the corpus is quite similar in all species studied, including man; most of the difference among species comes from transit through the cauda epididymis (Figure 17.10). Surprisingly, in species where this was tested, frequency of semen collection did not influence transit time through the epididymis markedly.[236,240,253] In bulls, daily sperm collection must exceed twice the daily testicular sperm production in order to observe an acceleration of sperm transit of only 3 days for the first wave of labeled spermatozoa,[236] while in rabbits different frequencies of ejaculation ranging from once per week to four times in one day had no effect on sperm reserves in the caput and corpus epididymis but dramatically reduced sperm reserves in the cauda epididymis and vas deferens.[254]

Spermatozoa enter the epididymis propelled by testicular fluid and possibly the beat of the ciliated cells of the efferent ducts. However, in the epididymis, the epithelium is lined by immotile stereocilia and the massive

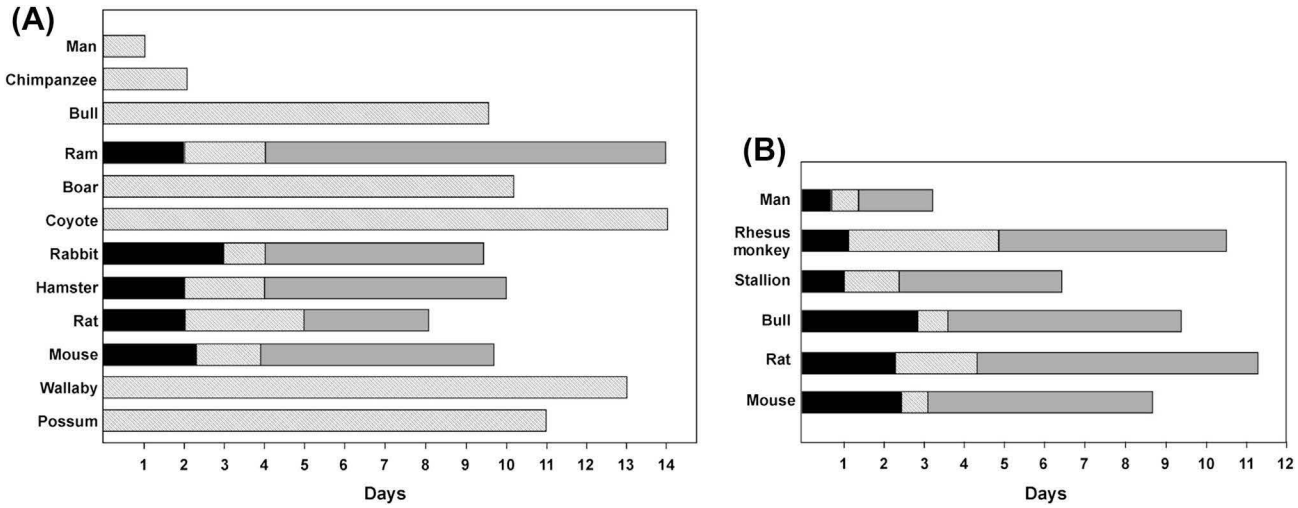

FIGURE 17.10 (A) Minimal time for epididymal transit determined by the progression of the first wave of labeled spermatozoa after isotope injection. Transport through the caput region (black bars), corpus region (light gray bars) and cauda region (dark gray bars) for the following species: (man;[238] chimpanzee;[239] bull;[236] ram;[240] boar;[241] coyote;[242] rabbit;[237] hamster;[243] rat;[244] mouse;[245] tammar wallaby and brushtailed possum[246]). (B) Duration of sperm transit through the whole epididymis and each segment determined by the ratio epididymal sperm reserves/daily testicular sperm production (man;[247,248] rhesus monkey;[249] stallion;[250] bull;[21,251] rat;[252] mouse;[245]).

fluid uptake taking place in the ductuli efferentes and the initial segment of the epididymis reduces drastically this fluid flow.[255] Sperm transport takes place against an increasing hydrostatic pressure gradient from testis to cauda epididymidis,[256] and proceeds even when fluid flow from the testis is prevented by ligation of the ductuli efferentes.[18,257]

The epididymis is surrounded by a smooth muscle layer of increasing thickness and alpha adrenergic innervation from proximal to more distal regions.[258] Therefore, the mechanism responsible for driving the contents through the lumen of the resting epididymis has been attributed primarily to the rhythmic muscular contractions of the smooth muscle lining the epididymal tubule.[259–262] Utilizing isolated epididymal tubules and measuring electrical activity via suction electrodes and injecting oil droplets into the lumen, the authors were able to show a relationship between the electrical and contractile activities of the epididymal tubule and the progression of the oil droplets. The droplets do not move in a linear fashion, but back and forth in a pendular manner. The movement forward starts when the spread of electrical activity approaches the droplet and stops or reverses when the electrical activity wanes or changes direction. Because the electrical pacemakers are randomly distributed and the electrical pulse spreads in both directions, droplets injected at the same time may spread in the lumen and follow different courses. The net distance covered during each pendular movement is small. Nevertheless, the droplets progress downward toward the vas deferens where the frequency of electrical activity is lower than in the caput epididymidis.[259,261] The rate of luminal flow is not uniform in the different segments of the epididymis.[260–262] The progression of droplets decreases from 420 mm/2 h in the initial segment to 64 mm/2 h in the distal caput and 25 mm/2 h in the cauda epididymis and vas deferens.[261] It is likely that the progression of oil droplets mimics that of spermatozoa in the epididymis. In bulls, labeled gold-coated beads injected in the rete testis are grouped in the distal caput epididymis 2 days after injection and in the cauda 5 days after injection, a transit time comparable to that of labeled spermatozoa; howeverbut 6 days after injection, beads are spread throughout the cauda.[236] Therefore, the progression of the epididymal luminal content is a dynamic process, resulting in a mixing of luminal content, and is controlled by the electrical and contractile activity of the epididymal tubule.

The smooth muscle contractions of the epididymal tubule and transit of spermatozoa therein are influenced by several factors, both hormonal and neuronal. Castration depletes epididymal sperm reserves,[263–265] and increases intraluminal pressure, contractility of the epididymis,[266] and sperm transport.[244] Testosterone treatment reverses the effect of castration, indicating that androgens control the contractility of the epididymal tubule to ensure an optimal rate of sperm transport. Estrogen, on the other hand, speeds up murine sperm transport drastically from 9.7 to 2.1 days[245] and act via a RhoA/Rho-kinase pathway.[267] Administration of chemicals that increase sympathetic tone, such as sibutramine (a neurotransmitter reuptake inhibitor), will also accelerate, up to 2-fold, the transport of spermatozoa through the epididymis,[268] while drugs that decrease sympathetic tone decrease sperm transport through the tissue.[269]

Contractility of the epididymal tubule is also influenced by prostaglandins.[270,271] These studies showed that physiological concentrations of $PGF_{2\alpha}$ increase the frequency and amplitude of contractions in proximal epididymis tubules while PGE_2 decreases contractions.[271]

Neurohypophysial peptides, such as oxytocin and vasopressin, and endothelin mediated by receptors present in the epididymis,[272–276] also increase epididymal contractility both in vitro,[277–281] and in vivo.[275,282] In several species, this results in an increase in the number of ejaculated spermatozoa and/or spermatozoa transported through the epididymis, including man.[275,283–289] Although the relative effect of oxytocin and vasopressin may vary among species, depending on the dose of the peptide or on the epididymal segment used in the in vitro studies,[282,289] it is clear that neurohypophysial peptides regulate both basal contractility of the epididymis and, upon release in the peripheral circulation around the time of ejaculation,[290–294] transport of spermatozoa through the vas deferens. Interestingly, the effect of oxytocin on epididymal motility is regulated by estrogens in part by an up-regulation of the oxytocin receptor gene and protein.[267,275] This may account for the estrogen induced accelerated sperm transport,[245] and suggests an interplay between steroids and neurohypophysial hormones in the regulation of epididymal contractility.

Neuronal regulation is also involved in epididymal contractility and sperm transport. This was first demonstrated by Simeone in 1933[295] using surgical sympathectomy and confirmed in later studies using either surgical[269,271] or guanethidine-induced chemical sympathectomy.[269,296,297] Surgical removal of a single neuronal ganglion, the inferior mesenteric ganglion that provides sympathetic innervation to the cauda epididymidis, is sufficient to slow down sperm transport through the epididymis.[298] Indeed, adrenergic and cholinergic drugs affect contractility of the epididymis both in vitro,[299] and in vivo.[300–302] Inhibition of phosphodiesterase 5 with drugs such as sildenafil citrate rapidly inhibits epididymal contractility presumably by blocking cGMP-hydrolysis thereby promoting relaxation of smooth muscle cells.[303] Temperature also affects epididymal contractility: a switch from scrotal to body temperature increases the frequency and spread of electrical activity of the smooth muscle of the epididymis,[304] and significantly speeds up sperm transport through the epididymis,[305] thus having potential deleterious effects on sperm maturation and fertility.

Although a neuromuscular mechanism may not be the only one responsible for sperm transport in the various segments of the epididymis and in all species, it appears to be the main mechanism responsible for sperm transport through the epididymis in mammals.

Maturation of Spermatozoa

Fertilizing Ability

In higher vertebrates, spermatozoa become functionally mature as they pass through the epididymis. They acquire the ability to move forward when released from the epididymis, ascend the female genital tract, undergo the acrosome reaction and bind to and penetrate the egg vestments, to finally achieve syngamy with the female gamete.

In all species examined thus far, a gradient of fertilizing potential is observed as spermatozoa traverse the epididymis (Figure 17.11). Although there is some species variation with respect to the exact site at which spermatozoa first gain their fertilizing potential, it is clear that to be competent to fertilize in vivo, spermatozoa leaving the testis have to pass through some part of the proximal epididymis.

This fertility profile can be altered somewhat using in vitro fertilization (IVF) techniques that bypass some or all steps in the normal fertilization process. For example, rabbit spermatozoa from the caput epididymis that do not fertilize more than 2% of the eggs after in vivo insemination,[23] fertilize 8% of the eggs after in vitro insemination.[320] A similar shift in fertility profile is observed when mouse spermatozoa are inseminated in vivo, in vitro, or after subzona injection (Figure 17.12). Testicular spermatozoa injected directly into the oocytes can even achieve a fertilization rate of 94% (Figure 17.12).[321] Fertilization also can be achieved when round spermatids are injected or electrofused with oocytes,[322] or when secondary or even primary spermatocytes are injected into the ooplasm.[321,323] However, the fertilization rates are lower than after injection of testicular spermatozoa.

Moreover, it appears that the acquisition of fertilizing potential is not a simple "on" or "off" state. Initial studies with rabbits,[23,308] rats,[314] and rams,[327,328] showed that after spermatozoa first gain the ability to fertilize eggs,

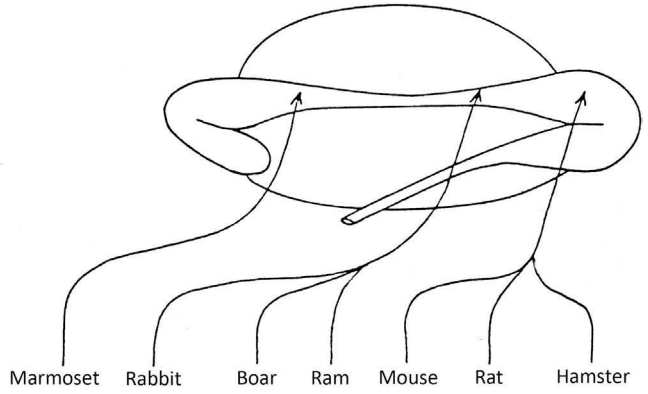

FIGURE 17.11 Approximate site within the epididymis where spermatozoa acquire their fertilizing ability. *Source: Information for each species, as brought together by Orgebin-Crist,[306] was obtained from the following sources: marmoset,[307] rabbit,[23,308,309] boar,[310] ram,[311] mouse,[312,313] rat,[263,314,315] and hamster.[316–319]*

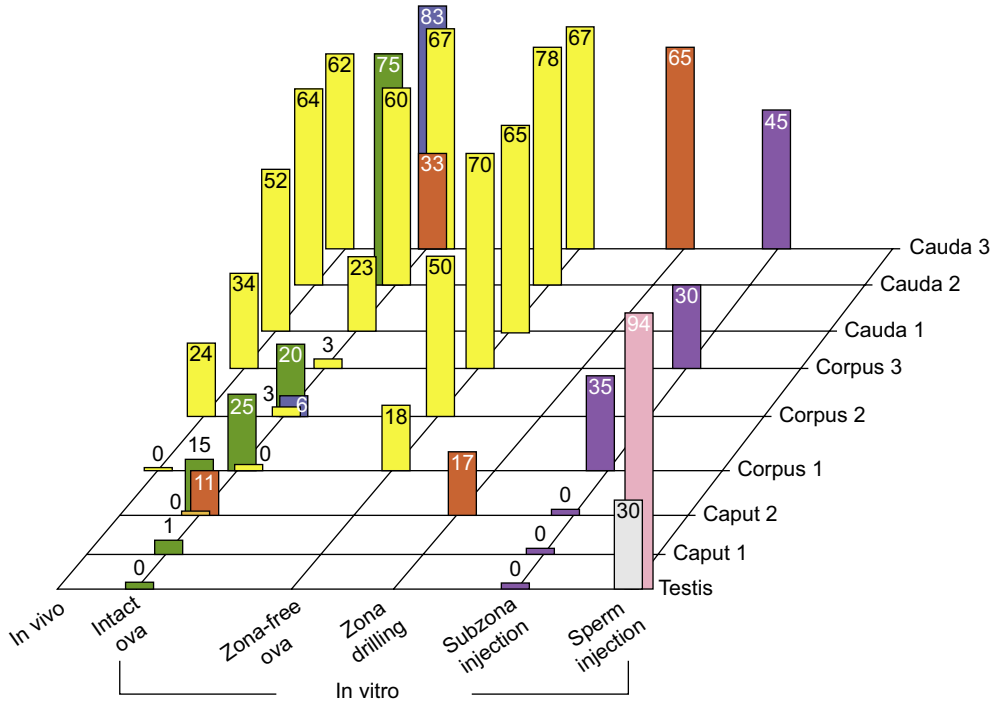

FIGURE 17.12 Fertilizing capacity of mouse spermatozoa from successive segments of the epididymis. The ability of spermatozoa to fertilize oocytes was assessed after either in vivo insemination (left column)[312] or in vitro[324] under different conditions: with intact ova,[312,313] zona-free ova,[313] after zona drilling,[325] or subzonal sperm injection or sperm injection.[326]

further transit through the epididymis is necessary for spermatozoa to acquire the ability to produce complete litters of viable offspring. Orgebin-Crist noted an increase in both pre- and postimplantation loss when rabbits were inseminated with corpus versus ejaculated spermatozoa;[23] this is apparently due to a delay in the fertilization of eggs,[23,329] and in the first zygotic division.[330] Although Overstreet and Bedford,[331] were unable to confirm these observations, subsequent studies in mice,[325] reported that only 8% of oocytes fertilized by caput epididymal spermatozoa were capable of developing to blastocysts in vitro compared with 48% of oocytes fertilized by cauda spermatozoa; Lacham-Kaplan and Trounson's group[324] confirmed that a high rate of embryonic arrest and retarded development occurs in mouse oocytes fertilized by epididymal spermatozoa that have just gained their fertilizing capacity. Although testicular spermatozoa and spermatids fertilize 94% and 37% of eggs, respectively after injection into the oocytes, only 54% and 28% of the fertilized eggs develop into live offspring when transferred into foster mothers.[321] Collectively, these experiments show that passage through the epididymis endows spermatozoa with the ability to ascend the female genital tract and interact with the egg. This maturation process can be circumvented since spermatid-injected oocytes develop into live offspring, but the chances of normal development are higher following injection of more mature spermatozoa.

The experimental protocol of insemination of equal numbers of spermatozoa from succeeding segments of the epididymis from one individual is obviously not possible in humans. However, in cases of obstructive azoospermia, epididymovasostomies have been done to recanalize the epididymis. The cumulative results show a low pregnancy rate when the anastomosis is done proximally and an increased incidence of pregnancies resulting from more distal connections.[332,333] These results, although not directly comparable with the animal studies, imply that in human, as in other species, there is a progressive maturation of sperm fertilizing ability in the epididymis. They also suggest that the fertility profile may be shifted more proximally when compared to other species. Pregnancies have been achieved even after reanastomosis of the efferent ducts to the vas, but the post-operative interval before pregnancy occurred was longer (2 years),[334] than after corpus-vas anastomosis (6–13 months).[333] If spermatozoa from the efferent ducts had the same level of maturity as spermatozoa from the corpus, one would expect the same postoperative interval, but the small number of cases precludes any generalization.

In cases of obstructive azoospermia or congenital vas agenesis, spermatozoa can also be aspirated from the epididymis and inseminated in vitro.[335] There is a statistical difference in fertilization rates with spermatozoa retrieved from different levels of the epididymis,[336] confirming the progressive maturation of human spermatozoa in the epididymis observed after reanastomosis.[332] After IVF and transfer, pregnancies have been reported with spermatozoa recovered from the corpus epididymidis,[337–339] and the proximal caput epididymis.[340,341] However, it should be stressed that the consequences of either pathological conditions of obstructive azoospermia or

congenital vas agenesis on the structure and functions occurring along the epididymal epithelium have not been investigated.

If immature human spermatozoa bypass normal sperm ascent in the female genital tract and sperm–egg interaction and are injected directly into the ooplasm (intracytoplasmic sperm injection, ICSI), they can form zygotes.[342–344] As in other species, the overall fertilization rate is similar or higher to that seen after conventional IVF.[344] Even testicular spermatozoa achieve fertilization rates of approximately 50%. Although several studies have reported fertilization of oocytes using round or elongated spermatids, the live birth rate using these methods is extremely low.[344–346] The overall fertility and delivery rates have been studied for several years and remained near 30% and 20%, respectively, for most countries.[347]

The congenital malformation rate and the developmental outcome of children born after ICSI with testicular, epididymal, or ejaculated spermatozoa are similar to that of children born after conventional IVF;[348–350] however, whether these ART procedures increase the rate of congential anomalies above that of the general population is not resolved.[351,352] Collectively, these clinical studies indicate that, as in other mammalian species, sperm capacity for optimal fertility in vivo increases during epididymal transit and procedures bypassing critical steps of the natural process of fertilization permit fertilization with immature spermatozoa. However, unlike other species, the fertility profile of human epididymal spermatozoa appears to be shifted more proximally because pregnancies have been reported with spermatozoa from the proximal epididymis.

Motility

Concomitant with the acquisition of fertilizing ability, a number of sperm characteristics undergo maturational changes. The first one to be recognized last century by Tournade[10] was the epididymal maturation of the sperm potential for motility. This was subsequently confirmed in numerous species of higher vertebrates from lizard,[353,354] to mouse,[355] rats,[314,315,356–362] hamster,[363–365] guinea-pig,[366] rabbit,[21,367,368] boar,[369] goat,[370] ram,[371,372] bull,[373,374] monkey,[375–377] and man.[126,378–380] The acquisition of motility is observed whether the testis and epididymis are located in the scrotum or in the abdomen, as in the elephant,[381] the hyrax,[381,382] and the armadillo.[382] The maturation of sperm motility potential involves both a quantitative increase in the percentage of spermatozoa that gain the potential for motility and a qualitative difference in motility patterns. Testicular spermatozoa are either immotile or display only a faint twitch of the flagellum. Spermatozoa released from the caput epididymis swim in a circular pattern, while spermatozoa released from the cauda move progressively and vigorously forward.

Other Maturational Changes

In addition to the development of the capacity for progressive motility, epididymal spermatozoa develop the capacity to undergo the acrosome reaction (mouse,[383] ram,[384] pig,[385] dog,[386] horse,[387] monkey,[388] and human[389,390]) recognize and bind to the zona pellucida and fuse with the vitelline membrane.[391–393] Concomitant with these functional changes, spermatozoa undergo structural changes during epididymal transit: migration of the cytoplasmic droplet along the sperm flagellum, acrosomal reshaping, changes in the sperm nuclear chromatin and some tail organelles, changes in the sperm plasma membrane, and fusion epididymosomes.[394–399] Collectively, these changes underpin the functional maturation and subsequent storage of spermatozoa in the epididymis, but none of these changes have been shown to be the only determinant of the acquisition of fertilizing ability.

Regulation of Sperm Maturation

Benoit[14] demonstrated that the function of the epididymis, and the maturation and survival of spermatozoa within it, depended on hormones secreted by the testis. After bilateral castration, not only the epididymal epithelium dedifferentiated, but epididymal spermatozoa died quickly, whereas after unilateral castration spermatozoa maintained their potential for motility on the castrated side 2 months after the operation. Experiments conducted by Bedford[22] and Orgebin-Crist[24,400] showed that while immature rabbit spermatozoa develop the capacity for motility and fertility when retained by ligatures in the proximal corpus, they do not become fertile when retained in the caput, although they develop a mature pattern of forward motility. This indicates that sperm maturation is a multi-step process and shows that, if the development of a mature pattern of motility is necessary, it is not sufficient to render a spermatozoon able to fertilize. It shows also that while some of the maturational changes such as motility, may be intrinsic to the sperm cell and develop with time, whereas others, such as the ability to interact with the egg, depend on the epididymal environment. The latter is conditioned by testicular androgens because sperm maturation occurs after castration, hypophysectomy, or in organ culture only when androgen is administered in vivo or included in the culture medium.[263,401–403]

Studies of co-incubation of epididymal spermatozoa with epididymal epithelial cell cultures have confirmed the role of the epididymis in promoting sperm maturation. In such cultures brushtail possum and tammar wallaby spermatozoa from the proximal caput underwent the morphological maturational change in head orientation accompanied by the development of progressive motility, normally only observed in vivo.[404,405] In hamsters, mice, and humans, co-cultures of immature

spermatozoa from the caput or corpus epididymis with cauda cells not only increased their motility, but also increased their capacity to bind to salt stored zona pellucida, to fertilize, and to support the development of embryos (sired by corpus spermatozoa).[300] These maturational changes are promoted by androgen-dependent factors from epididymal principal cells, because cocultures maintained in the absence of androgens fail to induce maturation.[406–408] The overwhelming evidence from all these experimental studies, both in vivo and in vitro, is that the final stages of the sperm maturation process in all mammalian species studied to date, including humans, depend on the epididymis.

However, reports of human pregnancies in two cases where the vasa efferentia was anastomosed to the vas deferens, therefore bypassing the entire epididymis, led to a challenge to the concept that the epididymis is necessary for the acquisition of fertilizing ability.[334] As discussed above, subsequent reports noted that the more distal the anastomic site, the greater the chances of fertility, confirming that the epididymis provides the milieu necessary for optimal fertility. Nevertheless, Temple-Smith[409] revisited the issue in elegant experiments using epididymovasostomies at different levels of the epididymis in rabbits and rats to assess the fertility of these animals. In both of these species, anastomosis of the distal initial segment to the vas, bypassing the distal caput, corpus and cauda epididymis, resulted in a significant reduction in sperm viability, motility, and fertility, confirming that spermatozoa require exposure to the epididymal environment for normal development of fertilizing ability. Interestingly, in rats, bypass of the distal caput and proximal corpus, either by epididymo-epididymostomy or graft of a vas bridge, had no effect on sperm motility, viability, or fertility. These studies show clearly that the epididymis plays a major role in the posttesticular maturation of spermatozoa, but they also show that exposure to the initial segment may be sufficient or that the lack of exposure to the epididymal regions where spermatozoa normally become fertile may be compensated by secretions from other epididymal regions. As discussed below, epididymal spermatozoa are exposed to a highly complex environment, but the nature of the specific changes induced by epididymal transit that are required to promote optimal fertilization still remains to be established.

Storage of Spermatozoa

The major site for storage of spermatozoa within the excurrent duct system of mammals is the cauda epididymidis. Though normal transit time in mammals through the cauda epididymidis is in the range of 3–10 days (Figure 17.10; Table 1 supplemental material at Website, see above), spermatozoa can be stored in this tissue for long periods of time extending beyond 30 days;[410] in bats, spermatozoa may be stored in this tissue for many months and retain their function.[411] Upon storage in the cauda epididymis, a loss in fertilizing ability occurs before a loss in motility.[412] Interestingly, spermatozoa that were aged in the male reproductive tract of rabbits, presumably in the cauda epididymis, induced a 10-fold higher incidence of chromosomal abnormalities in the resulting blastocysts than did their fresh counterparts.[413]

Based on studies in a number of mammalian species (reviewed in Ref. 414), Amann noted that 50–80% of spermatozoa present in the excurrent ducts were found in the cauda epididymis and that approximately 50% of these spermatozoa were available for ejaculation.[414] When animals (rabbits, stallions, and bulls) were at sexual rest, (i.e. seven or more days without ejaculation), the number of stored spermatozoa that were available for ejaculation was three to five fold greater than the daily sperm production rate and two to three fold greater than that found in a "typical" ejaculate; frequent ejaculation, that is one ejaculate every 1–2 days, did not result in a change in sperm production rate but did markedly decrease caudal epididymal sperm reserves and the number of spermatozoa in the ejaculate.[414] In contrast, man, whose sperm production rate is well below that of most other mammals, has a sperm reserve that is only about equal to the number of spermatozoa found in an ejaculate, whether at sexual rest or not.[414,415]

One would expect that the luminal environment required for the storage of spermatozoa in the cauda epididymis versus that required for the acquisition of fertilizing ability would be different. Though many differences have been described in the luminal make-up of ions, small organic molecules, proteins and glycoproteins between the cauda and the rest of the epididymis (see below), those special conditions that allow for the storage of spermatozoa in a quiescent state for long time periods in this tissue have not yet been elucidated, although changes in pH,[164,416,417] in several species and the presence of immobilin in the rat and hamster[416,418] have been proposed to be major factors.

Although it is clear that the major function of the distal segments of the epididymis is to store mature, live spermatozoa, other functions have been ascribed to this tissue. Abnormal appearing or dead spermatozoa have often been seen within the cauda epididymidis,[419,420] and the ability to recognize such spermatozoa and then to develop a mechanism to neutralize or destroy them has been proposed by several groups. Sutovsky et al.[421] proposed that the cell-surface ubiquitination of defective spermatozoa supplies the necessary signal for these cells to be phagocytosed by epididymal principal cells, thus providing a mechanism for sperm quality control. However, based on extensive histological observations of the normal epididymal epithelium and the rarity with which sperm phagocytosis is observed, it is apparent that

phagocytosis of spermatozoa is an unlikely mechanism for the disposal of the large number of spermatozoa produced daily.[422] The release from rat semineferous tubules of a large number of immature germ cells induced by the administration of a microtubule inhibitory agents such as colchincine results in the phagocytosis of immature germ cells by epididymal epithelial cells, but this is limited to the initial segment and is observed only when such stressors are applied.[423] In the mouse, Ramos-Ibeas et al.[424] reported that all epididymal segments have the ability to phagocytose immature germ cells, but that this is is a rare event under normal physiological conditions. Although these studies leave open the possibility that the epididymis may serve as the first selective barrier for the exclusion of abnormal gametes along the male genital tract, it is likely that if a quality control system is present in the epididymis, it is subtle and requires further investigation.

Studies from Olson's group[425,426] have identified a fibrinogen like protein (FGL2) that is secreted selectively in the proximal cauda epididymis of the hamster and that binds to and coats nonviable, but not viable, luminal spermatozoa. Unlike the hypothesis of Sutovsky's group, these investigators propose that the epididymis possesses a specific mechanism to identify and envelop defective spermatozoa with a protein complex containing the fibrinogen-like protein FGL2 that remains within the epididymal lumen. Both of these novel mechanisms require that epididymal epithelial cells have the ability to somehow recognize and tag defective spermatozoa. Although conceptually appealing, more extensive studies using different species and means of creating "abnormal" spermatozoa, such as drug treatments, are required to establish this as a function of the normal epididymis.

Jones[427] has hypothesized that following androgen withdrawal, either by orchidectomy or as a result of seasonal variation in seasonal breeders, a death pathway is activated that leads to the dissolution of spermatozoa. Such a mechanism would ensure that the epididymis is cleansed of defective and dead spermatozoa in preparation for the next breeding period. How androgen withdrawal would activate such a pathway remains to be determined, but it is interesting to note that many genes are activated in the epididymis after orchidectomy.[428,429]

In a series of studies on the evolution of the scrotum, Bedford proposed that the prime moving force behind the formation of the scrotum was the need to store spermatozoa at a low temperature;[305,430,431] he suggested "that migration to a scrotal site has been ordained primarily by the sperm storage region—the cauda epididymis—and that the function of the testis has only been an incidental factor in this evolutionary development."[431] This proposal was based on anatomical observations and on some physiological studies where the epididymis was placed abdominally while the testis remained in the scrotum; the rate of sperm passage through the cauda epididymis was found to increase about twofold in the rabbit after such a procedure. This hypothesis has been re-examined by several investigators. Werdelin and Nilsonne[432] proposed that the scrotum evolved before mammals and has been lost in many species because testicular descent is a "costly process" and that it is replaced when an alternative solution to the problem of the temperature sensitivity of spermatogenesis is available. Kleisner et al.[433] proposed that the diversity in the position of the testis-epididymis results from the need to provide conditions suitable for sperm development and storage. Although all of these theories are plausible, the fact remains that sperm are stored in the cauda epididymis, in most mammalian species, at lower than core or testicular temperatures.

Sperm Protection

It is clear that the blood–epididymis barrier allows for the production of a specialized luminal fluid microenvironment that is important for sperm maturation. However, another critical role played by this barrier is protection of the maturing spermatozoa. In addition, a series of elaborate defense mechanisms that help to protect spermatozoa from the immune system, harmful xenobiotics and reactive oxygen species have been developed in the epididymis. The defense mechanisms include restricting the types of compounds that can enter the epididymal lumen, the synthesis and secretion of specific proteins such as the defensins and defensin-like molecules, rapid elimination of potential harmful agents through the synthesis and secretion of antioxidant and conjugating enzymes, and the synthesis and secretion of antioxidant compounds such as glutathione and taurine. Because spermatozoa mature in a hyperosmotic environment, the epididymis also ensures that the spermatozoa are protected from potential rapid changes in osmolality and that spermatozoa can regulate their cell volume.

By 1950, it was shown that spermatozoa are highly susceptible to oxidative damage and that hydrogen peroxide was responsible for loss of motility and cell death.[434,435] Through a series of well-designed studies, Jones et al. clearly showed that when spermatozoa were incubated under aerobic conditions, they produced an organic peroxide and released a substance that was believed to be a lipid.[436–439] When incubated in the presence of lipid peroxides, spermatozoa became irreversibly immotile and released numerous intracellular enzymes. From these series of studies, it was suggested that incubation of spermatozoa under aerobic conditions resulted in lipid peroxidation by peroxides and/or lipid radicals that were responsible for structural damage, loss of motility, decline in metabolic activity and release of intracellular enzymes.

Since mammalian spermatozoa have a high content of polyunsaturated fatty acids in their membranes, they are highly susceptible to lipid peroxidation by reactive oxygen species (ROS).[438–441] It is crucial that spermatozoa be protected from the deleterious effects of too high a production of ROS as they progress along the epididymal duct; lipid peroxidation of membranes has been correlated with mid-piece defects,[442] decreased motility due to axonemal defects and reduced intracellular ATP levels,[443–445] as well as impaired capacity for fertilization (reviewed in Refs 442,446). There exists a delicate balance between the formation of ROS needed for sperm function and production of too much ROS that will damage sperm membranes and DNA Spermatozoa can protect themselves from ROS via the presence of superoxide dismutase, catalase, glutathione peroxidases, glutathione transferases, and other enzymes regulating the balance between the production and elimination of ROS.[445–448] These enzymes protect spermatozoa from lipid peroxidation by ROS via dismutation of the reactive oxygen to hydrogen peroxide and water; hydrogen peroxide is then rapidly converted to water via the enzymes catalase and/or glutathione peroxidase; alternatively it is conjugated to glutathione.[442,446,449–453]

In a continuing effort to prevent the oxidative damage to spermatozoa, the epididymis has developed an elaborate system to ensure that spermatozoa are protected as they mature along the epididymal duct. Each region or segment of the epididymis has developed its own sperm protective mechanisms primarily because (1) the metabolic activity differs from one region to the next, thereby producing different ROS species that need to be eliminated appropriately, and (2) spermatozoa are in a different state of maturity in each region, and therefore differ with respect to susceptibility to oxidative damage. For example, spermatozoa need to be especially protected from ROS as they enter the initial segment. The luminal fluid in this region is highly oxygenated,[454] the epithelial cells are surrounded by a dense capillary network,[455] with blood flow exceeding that of the distal epididymal regions,[456,457] and the initial segment cells are highly metabolically active (reviewed in Ref. 458). This unique combination results in the generation of ROS from several sources, including the endothelial and initial segment epithelial cells (reviewed in Refs 442,446). Therefore, it is not surprising that the initial segment expresses antioxidant enzymes. In the distal epididymal regions, the epithelial cells are still metabolically active, and the spermatozoa are continually exposed to an oxygen-rich environment; however, the degree of vascularization is less compared to the initial segment. Hence, spermatozoa are still subjected to ROS production, but the type of ROS may well be different from the types produced in the initial segment. Hence, the distal epididymal regions have alternative strategies to protect spermatozoa.

The major antioxidant enzymes present in the epididymis include superoxide dismutase,[459,460] gamma-glutamyl transpeptidase,[461–466] glutathione peroxidases,[467–471] glutathione transferases,[167,472–475] peroxyredoxins,[475–478] and indoleamine dioxygenase.[479] In addition, the lumen of the epididymis contains antioxidant molecules including glutathione, taurine, and tryptophan as the substrate for indolamine dioxygenase. Each of these antioxidant enzymes and molecules is found to varying degrees throughout the length of the epididymis.

Hence, the epididymis plays a critical role in the protection of spermatozoa from oxidative stress and from harmful xenobiotics. Interestingly, the processes by which the epididymis protects spermatozoa have also been considered to be a prime target for the development of a male contraceptive.[442]

The Microenvironment for Maturation, Storage and Protection

From the previous discussion it is clear that the epididymis plays an important role in sperm maturation. Although the actual time spent in the epididymis may be important for the maturation process, spermatozoa must still undergo considerable remodeling and be protected and stored in a specialized luminal fluid milieu. Crabo[255] first recognized the importance of the epididymal microenvironment, "The consensus today is doubtless that the epididymis actively promotes maturation of spermatozoa. The natural way for the epididymis to exert this influence would be via regulation of the sperm cells' environment, i.e., epididymal plasma: the environment in the caudal portion should favor maximal survival time of the spermatozoa which may be stored there for relatively long periods, without losing their fertilizing power and motility." In view of several published comprehensive reviews on the formation of the epididymal luminal microenvironment,[189,226,480–486] this section will summarize the contributions of ions, organic solutes and proteins to the specialized luminal fluid milieu. Although much is known concerning the composition of the epididymal luminal fluid, more studies are needed to understand the precise contribution of each component toward sperm maturation.

Perhaps the most under-appreciated role of the epididymis is the manner by which the epididymis forms the microenvironment. To do this formidable task, the epithelium must be structured in a way such that it prevents and regulates the entry of blood-borne substances into the lumen; has the ability to synthesize, secrete and absorb components; and is arranged so that spermatozoa come into contact with the appropriate environment

at the appropriate time (this is the most challenging aspect to understand because it implies that each cell along the duct knows precisely what its neighbor(s) is doing); and can respond to spermatozoa, which in turn regulate the microenvironment. Hence, there is a series of complex and ever-changing interactions between the epididymal epithelium, the microenvironment and spermatozoa from the beginning to the end of this tube that is several meters in length.

Water Movement and Its Influence on the Formation of the Luminal Microenvironment

Before discussing the composition of the luminal fluid microenvironment, it is important that water movement be discussed. The efferent ducts and the epididymis are water transporting epithelia. Hence, the concentrations of ions, small organic solutes and proteins are dependent on the movement of water into and out of the lumen. Thus, the measured concentrations of any ion, small organic solute or protein in epididymal luminal fluid are not only due to direct secretion into the lumen but also to the amount of water that is being reabsorbed. Water movement out of the duct is quite extensive. For example, the water transporting ability of the efferent ducts of the quail almost matches that of the rat renal nephron, while in the rat it is only 5–10 times less than that of the proximal kidney tubule.[375] Remarkably, in several species, 70–96% of the fluid leaving the testis is reabsorbed in the efferent ducts; for example, 96% is reabsorbed in rats;[487] 90% in boars;[255] 87% in the tamar wallaby;[488] and 96% in the elephant,[489] with concomitant declines in sodium and chloride concentrations but increases in potassium and hydrogen ion concentrations from the rete testis to the caput region. Within 24 h of efferent duct ligation in the rat, there is a remarkable increase of nearly 50% in testicular weight due to accumulated fluid, thus demonstrating how effective the efferent ducts and epididymis are at removing large volumes of water.[490] From the caput region onwards, 95% of the remaining 5–10% water is reabsorbed, ultimately increasing the concentration of spermatozoa from approximately 10^4/ml in rete testis to 10^9/ml in the cauda epididymis.

Hence, when analyzing the concentration of various ions, small organic solutes, and proteins, it is important to take into account the reabsorption of water. For example, the increase in the concentration of some organic solutes seen from the caput to cauda epididymis may not necessarily be due to an increase in the secretion of that solute but entirely to water movement. Examples of this include carnitine and inositol.[491,492] However, if water reabsorption is taken into account when calculating what the total protein concentration should be, a much higher value is calculated compared to the actual value measured.[493] This would indicate that considerable protein is indeed being reabsorbed, a well-known role of the epididymis. A simple analysis of this kind may help determine the extent, if any, of movement of any solute across the epididymal epithelia from proximal to distal regions. It should be emphasized that the measured concentration of any ion, small organic solute or protein in the luminal fluid of any epididymal region is not only a product of secretion, absorption, and water reabsorption but also of sperm uptake, degradation, and/or metabolism.

Ionic Microenvironment

Crabo[255] and Levine and Marsh[494] were the first to show that the ionic luminal fluid composition of the epididymal duct is distinctly different from blood plasma. However, through a more thorough analysis of epididymal luminal fluid collected by micropuncture, we now have the exact ionic composition to which spermatozoa are exposed as they progress along the duct.[485,495–498] Briefly, spermatozoa move from an environment in the seminiferous tubule, which has a pH of approximately 7.3 and lower sodium concentration but higher potassium concentration compared to blood plasma (reviewed in Refs 485,497), to an environment in the initial segment/caput region with pH 6.5 and sodium and potassium concentrations closer to those measured in blood plasma. Spermatozoa then move into a progressively lower ionic environment from the caput to the cauda but into a progressively higher organic solute and protein environment (reviewed in Refs 482,485,486,497,499). Sodium, chloride, calcium and magnesium concentrations decline from the caput to cauda epididymidis but phosphorus (as phosphate) and potassium increase in concentration (Figure 17.13). The physiological importance of the ionic microenvironment is unclear, but it may be involved in the development of sperm motility and in keeping spermatozoa in a quiescent state as they mature.[500–503] More studies are needed to test the hypothesis that ions play a critical role in sperm maturation.

Organic Solute Microenvironment

As well as analyzing the luminal fluid ionic microenvironment, Levine and Marsh[494] also measured the osmolality of fluid collected from each epididymal region and then compared these values to the sum of osmotically active ions in each region. They noted a difference. This osmotic difference increased from the caput to cauda epididymis to reach over 250 mOsm/kg water; the investigators suggested that this difference was due to the presence of organic solutes. Later studies by several groups showed that this was indeed the case with the identification of solutes such as glutamate, taurine, L-carnitine, myo-inositol, glycerylphosphorylcholine, phosphorylcholine, sialic acids and many other amino acids being present

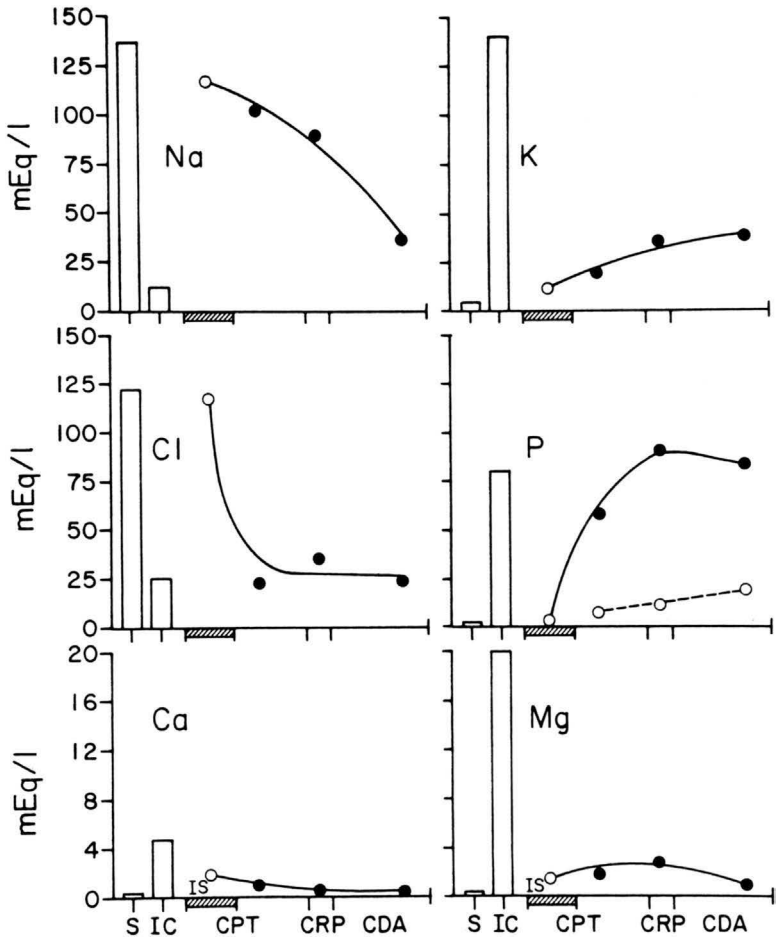

FIGURE 17.13 Serum, intracellular, and intraluminal concentrations of sodium, potassium, chloride, phosphorus, calcium, and magnesium in the rat epididymis. Serum (S) and intraluminal values are from Jenkins et al.[496] Intracellular (IC) values are those expected in typical somatic cells[504] and represent anticipated values in the epididymal epithelial cells; initial segment (IS), caput (CPT), corpus (CRP), and cauda (CDA) epididymidis. Data connected by hatched lines in the P panel are inorganic phosphate.[483]

in different concentrations through the epididymal duct of many species including man (reviewed in Refs 139,226,483,486).

The total concentration of organic solutes in the luminal fluid of the rat testis and epididymis increases from approximately 10–20 mM in the seminiferous tubule to 100–150 mM in the caput and to over 200 mM in the cauda epididymidis. The manner by which the epididymal epithelium forms an organic solute-rich environment is not completely understood, but there is considerable evidence to suggest that at least several transporters are involved in moving the solute from the blood into the lumen; in addition, the epididymis synthesizes and secretes solutes into the lumen. For example, L-carnitine is transported into the epididymal cells and lumen reaching concentrations as high as 50–60 mM in some species.[505–508] The high intraluminal concentration of L-carnitine translates into a remarkable 2000-fold gradient of L-carnitine across the epididymal epithelium. Several studies suggest that the distal caput, corpus and proximal cauda epididymidis are the regions involved in the active transport of L-carnitine.[509–513] The mechanism of L-carnitine transport in the epididymis is still not fully resolved, but it has been suggested that it involves an active transport system,[509,510,512,514] and that the transport is androgen-dependent.[512,515]

Some studies have suggested that L-carnitine is transported from blood into cells via the transporter OCTN2[516] and that its transcriptional activity is regulated by osmotic stress.[517] L-carnitine would then presumably move from cells into the lumen via the transporter CT2 that has been identified in the human epididymis.[518] Several transporters, such as OCTN2 and SMIT, a sodium-myo-inositol transporter, have *TonE* binding motifs on their promoters which bind a transcription factor TonEBP (Rodriguez and Hinton, unpublished observations) that is expressed during periods of hypertonicity; these data suggest that the organic solutes play a role in the regulation of water movement across the epididymal cell membranes. This is particularly important because in the epididymis of most species, spermatozoa are exposed to a hyperosmotic environment. Disruption of the ability of spermatozoa to regulate their cell volume has been suggested to cause male infertility.[519]

Although virtually nothing is known as to the roles of each solute, evidence suggest that these solutes play a critical role in the protection of spermatozoa and the epididymal epithelium from osmotic stress, similar

to the role played by these solutes in the kidney.[520,521] However, the high levels of organic solutes measured in the cauda epididymal fluid of several species would suggest that these high levels play a similar role in long term storage of spermatozoa in this region. Likewise, in bats that store spermatozoa in the cauda epididymis for up to 6 months, the spermatozoa are stored in a supra-physiologically high osmotic environment with luminal fluid osmolality being measured at greater than 1000 mOsm/kg water;[522] the mechanisms for generating and maintaining such a high osmolality are not known. Taurine and glutathione are both found in epididymal luminal fluid;[523,524] because both of these compounds have been implicated in protecting cells from oxidative stress, it would not be surprising if they have a similar role in the epididymis.

The transport of sugars and amino acids including inositol, 3-O-methyl-D-glucose, 2-deoxy-D-glucose (nonmetabolizable forms of glucose) and alpha-aminoisobutyric acid (AIB; a nonmetabolizable neutral amino acid) have been studied in the rat epididymis.[525–530] From these studies several observations were noted. First, glucose entered via a facilitated diffusion mechanism and this uptake appeared to be similar across all epididymal regions. Second, glucose transport was inhibited by the male infertility agents, alpha-chlorhydrin, 5-thio-D-glucose and 6-chloro-6-deoxy-glucose. Third, transport of inositol and AIB was highest in the initial segment compared to the rest of the epididymis. Finally, transport of inositol and AIB was saturable and, presumably, transport of inositol was via active transport whereas that of AIB was via a facilitated diffusion mechanism. Presumably, the glucose and amino acid needs of the epididymis reflect its metabolic needs and that of maturing spermatozoa. However, more studies are needed to examine the roles played by each solute. The role of known specific ion and organic solute transporters that have been identified in the epididymis will be discussed in greater detail later in this chapter.

The androgen luminal fluid microenvironment has received little attention recently but past studies have revealed that the spermatozoa are exposed to a complex rich androgen environment as they progress along the duct.[483] In several species, including rat, human, hamster, stallion and bull, the epididymal luminal fluid concentrations of testosterone and dihydrotestosterone (DHT) were shown to exceed the blood concentrations,[531–533] with DHT concentration being very high in the caput region. Presumably, the high luminal fluid androgen concentrations are maintained by androgen binding protein (ABP). It has been proposed that the ABP-androgen complex plays a role in epididymal function,[534] but confirmatory experiments have yet to be designed to test this hypothesis.

Protein Microenvironment

The protein composition of epididymal luminal fluid has been well studied for a number of species.[147,535–537] Spermatozoa are exposed to varying protein concentrations as they move along the seminiferous tubule (approximately 6–10 μg/μl) to the rete testis (approximately 1 μg/μl) to the caput and cauda epididymis (approximately 25–30 μg/μl). Because water is being absorbed along the epididymal duct a higher protein concentration would have been expected in the cauda compared to the caput epididymis. Hence, to account for this difference, proteins are presumably being absorbed and/or being degraded and/or being utilized by the spermatozoa. However, even though the net movement of protein is via absorption, many proteins are still being synthesized and secreted into the duct.

The proteomic profiles in epididymal luminal fluid have been analyzed by one and two-dimensional gel electrophoresis,[538] generating characteristic patterns of protein profiles for several species. Using an in vivo split-drop stopped-flow microperfusion technique together with two-dimensional gel electrophoresis and both silver staining and western blot analyses,[539] showed in vivo protein secretion into the lumen of discrete epididymal segments and regions. Later studies by Turner and colleagues,[540,541] showed similar data when the interstitium was perfused with radiolabeled methionine and luminal fluid collected by micropuncture and analyzed for radiolabeled proteins. Many of the proteins in the lumen of several species have now been identified using mass spectrophotometric methods.[536,537,542]

Epididymal luminal fluid contains a large repertoire of proteins including enzymes, growth factors, lipid-binding proteins, iron-binding proteins, proteins that may be involved in protection or in sperm–egg binding, and other proteins such as the lipocalins, clusterin, lactoferrin, or CTP/HE1.[543] Many of these families of proteins will be discussed below.

REGULATION OF EPIDIDYMAL FUNCTIONS

Hormones

In 1926, pioneering studies by Benoit demonstrated that the epididymis is dependent on an unknown testicular substance for maintenance of its structure and functions; he showed that the ratio of nucleus/cytoplasm in the mouse epididymis decreased after birth as the epididymis differentiated, and increased dramatically after orchidectomy, as the epididymis dedifferentiated.[14] This regulatory substance was identified five years later as testosterone.[544] Since then, more than a thousand papers have appeared on the response of the epididymis

in numerous mammals to androgen withdrawal and replacement on a wide variety of endpoints, ranging from morphological to biochemical and molecular.

The main approach to understanding the effects of androgen withdrawal on the epididymis has been removal of the testis. It is clear that this approach causes not only loss of androgens but also estrogens and any other testicular factor that may affect the epididymis. Orchidectomy causes a decrease in epididymal weight that is less marked than that of sex accessory tissues such as the prostate or seminal vesicles.[545,546] Unlike the case with other androgen-dependent male reproductive tissues, testosterone replacement, even at supraphysiological levels, only partially restores epididymal weight; this is due to the large proportion (nearly half) of the epididymal weight that is attributable to spermatozoa and the luminal fluid bathing them.[546,547] In the androgen-deprived state, spermatozoa do not gain the potential for motility, lose the ability to fertilize and eventually die.[14,263,548] Administration of antiandrogens, such as flutamide, to adult animals or to animals going through puberty, results in an accelerated sperm transit time through the epididymis, impairment of sperm motility and decreased ability of the cauda epididymis to store sperm[549,550]

After orchidectomy, the luminal diameter and epithelial cell height decrease and the intertubular stroma increases.[551,552] The smooth endoplasmic reticulum content is dramatically reduced, whereas the extent of the decline in the Golgi apparatus is less pronounced.[553–555] Morphological changes in principal cells suggest that these cells are particularly sensitive to androgen levels, in contrast to the other epithelial cell types, which appear to be less affected by orchidectomy.[157] The secretory function of principal cells becomes compromised in the androgen-deprived state. In addition to the virtual disappearance of endoplasmic reticulum from their apical cytoplasm, principal cells undergo a striking loss of apical microvilli from their surface, as well as lysosome accumulation, vacuolization, disappearance of vesicles from the cell apex, and increased endocytosis.[157,402] Epididymal androgen receptors and 5α-reductase activity are both decreased in the androgen-deficient state,[546,556–558] suggesting that the mechanisms of androgen action are compromised by androgen-withdrawal. Total epididymal protein, RNA and DNA content are reduced following orchidectomy but DNA concentration is apparently increased.[559] The relative increase in DNA concentration is attributed to the concomitant decline in cell volume; this is thought to be the principle mechanism by which the epididymal epithelium regresses after orchidectomy.

Restoration of circulating testosterone levels appears sufficient to reverse regressive changes in the caput, corpus and cauda epididymidis after orchidectomy but not in the initial segment, even when supraphysiological doses of testosterone are administered.[546,552,555,560–563] At three days following orchidectomy with testosterone or dihydrotestosterone (DHT) replacement to circulating levels, a time at which the prostate shows maximum rates of DNA synthesis, the epididymal labeling index, measured either by ^{3}H-thymidine incorporation or bromodeoxyuridine incorporation and mitotic index, is low except in the corpus region.[552,564] The small effect of androgens on mitotic index in the epididymis of the adult animal was similar for all cell types and distinguishes this tissue from other androgen-dependent tissues such as the prostate and seminal vesicles where androgens have a dramatic effect on proliferation.[552] This characteristic of the epididymal epithelium suggests that it may contain anti-proliferative signals that inhibit cellular proliferative capacity in response to androgen-stimulation or other stimuli. Interestingly, B-MYC, a transcription factor known to inhibit cellular proliferation, is highly expressed in the epididymal epithelium.[565]

Withdrawal of androgen by orchidectomy induces a wave of apoptotic cell death in the epididymis beginning in the initial segment and moving over several days to the cauda epididymidis.[110,566,567] Apoptosis in the initial segment seems to be caused by withdrawal of androgen and of luminal components coming from the testis and be p53 independent.[568] Using the entire epididymis, BCL-2, an anti-apoptotic factor, was found to be suppressed by 36 h after orchidectomy; this was followed by the appearance of Fas and DNA fragmentation in the epididymal epithelium at 48 h post-orchidectomy.[569] Mutant mice null for *Fas* showed no epididymal regression or DNA fragmentation after orchidectomy.[570] These data suggest that the regression of the epididymal epithelium after orchidectomy may be regulated through the FAS pathway.

Several studies point to the rapid action of steroids, as opposed to the slower, nuclear-receptor mediated response. For estradiol, it is now well established that such a mechanism is an integral component of hormone action[571,572] and involves steroid action at the cell membrane resulting in the activation of a signal transduction pathway. For androgens, Walker and his colleagues have demonstrated that such a mechanism exists for Sertoli cells[573] (see also Chapter 16). It has also been shown that androgen action can be as rapid as 1 min after exposure to androgen in an epididymal principal cell line.[574] The relative role of this signaling mechanism, as opposed to the more traditional nuclear receptor mediated mechanism remains to be elucidated.

In addition to androgens, many other hormonal factors have been postulated to play a role in regulating epididymal function; these are listed in Table 4 (supplemental material at http://www.medicine.mcgill.ca/PhysiolReprodEpidi dymis/). Of special note however, is estradiol. It has been known for many years that the administration of estrogens could affect the male

reproductive system in general[575–579] and the epididymis in particular.[245,580] With the advent of aromatase specific inhibitors and null mutation mice for the two isoforms of the estrogen receptor (discussed below), it is now clear that estrogens act as regulators of some specific epididymal functions;[581] however, much remains to be done to resolve exactly what role this steroid family plays.

Testicular Factors

In addition to being dependent on the presence of circulating androgens (reviewed in Ref. 578), the epididymis is also dependent on the presence of luminal fluid factors originating from either the testis and/or the epididymis itself. Without testicular luminal fluid factors many cells within the initial segment undergo apoptosis within 24 h.[566,567,582] Therefore, it is not surprising that ligating the efferent ducts results in changes in morphology and gene expression in the initial segment.[48,145,583] From these studies, it has been postulated that a factor(s) originating from testis is/are responsible for maintaining the integrity and survival of cells within the initial segment. This type of paracrine secretion has been termed lumicrine because the processes regulated are occurring within a duct/tubal system.[583] A general scheme depicting how factors secreted directly into the epididymis via the efferent ducts can affect activities of cells in the initial segment and how such effects can cascade down the duct is depicted in Figure 17.14. Perhaps the first studies demonstrating lumicrine regulation in the male excurrent duct system was those by Skinner and Rowson in the 1960s.[585,586] These investigators showed that the ampulla of the vas deferens weighed less, contained less fructose and was smaller in diameter following unilateral vasectomy compared to the ampulla on the control intact side. Skinner and Rowson[586] suggested that vasectomy prevented luminal fluid testosterone from reaching the ampulla, and, to test this hypothesis, they perfused the vas deferens of an orchiectomized ram with testosterone. Their data showed that the ampulla of the perfused side weighed more, contained more fructose and citric acid compared to the nonperfused control side. Similarly,

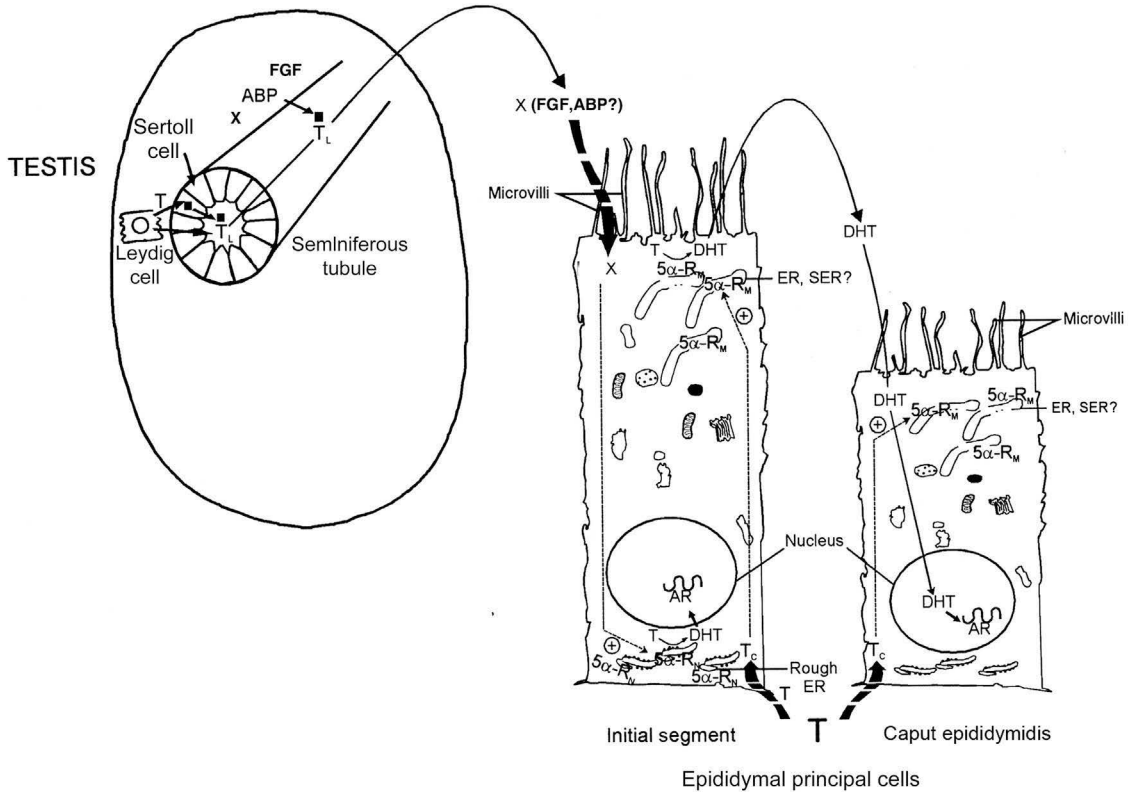

FIGURE 17.14 Proposed mechanism for the regulation of SRD5A and androgen action in the rat epididymis. Leydig cells synthesize testosterone (T), which stimulates the Sertoli cell to secrete ABP and other proteins into the lumen of the seminiferous tubule. In the initial segment of the epididymis, the paracrine/lumicrine regulator (e.g. FGF, ABP) is proposed to be the paracrine factor regulating the infranuclear localized steroid 5α-reductase enzyme (5α-R, SRD5A). In contrast, the apically localized 5α-reductase enzyme (microsomal 5α-reductase, 5α-R_M) found throughout the epididymis is under the control of circulating androgens. The synthesis of locally high concentrations of DHT in the initial segment of the epididymis provides an excellent mechanism for stimulating critically important androgen-dependent genes in this region of the epididymis and for providing the most active androgen to mediate androgen action in the rest of the tissue. ABP, androgen binding protein; 5α-R_N, nuclear SRD5A; 5α-R_M, microsomal SRD5A; ER, endoplasmic reticulum; SER, smooth endoplasmic reticulum; DHT, dihydrotestosterone; T, testosterone. *Source: Modified from Ref. 584.*

Abe et al.[79] show that ligation of the mouse corpus epididymidis results in abnormal differentiation of the principal cells downstream from the ligature. Hence, lumicrine regulation may not only occur between the testis and epididymis but also between regions of the epididymis including the vas deferens.

Although testicular luminal fluid steroids, androgens and estrogens, are the most obvious candidates for regulating initial segment function, several lines of evidence suggest that additional factors are important. Using a histological approach, Fawcett and Hoffer,[555] supported the idea that factors other than androgens are responsible for the maintenance of initial segment function because the morphology of the initial segment cells failed to return to normal following efferent duct ligation (EDL) and testosterone treatment. Likewise, Nicander and colleagues,[582] suggested that since the cells within the initial segment are highly mitotic, they are more dependent upon the presence of a mitogen(s) within testicular luminal fluid.

The first nonsteroidal molecule proposed to be a testicular factor acting on the epididymis was ABP.[533,587] This hypothesis stemmed from a series of studies demonstrating that the steroid 5α-reductase activity in the initial segment of the epididymis fell after EDL or unilateral orchidectomy; it could not be maintained by administration of testosterone, even at very high doses. Luminal fluid ABP was also proposed as the factor that enhances the function of ovine epididymal principal cells in culture,[588] appears to play a role in protein synthesis in the caput region,[589] and has the ability to bind and be internalized by epididymal principal cells.[590,591] However, although gamma-glutamyl transpeptidase mRNA IV (GGTIV) is also controlled in a lumicrine manner in the initial segment of the epididymis, ABP does not play a role in its expression.[592] Instead, fibroblast growth factors (FGFs) may be partly responsible for the regulation of expression of this gene.[592–594] It has been postulated that luminal fluid growth factors interact with their cognate receptors on the apical cell surface of initial segment cells whereupon a signal transduction pathway is initiated (e.g. MAPK, PI3 kinase); transcription factors are then activated, presumably by phosphorylation leading to transcription of target genes.[583,593] In support of this hypothesis soluble FGFs (2,4 and 8) have been identified in testicular luminal fluid, and FGFR-1 IIIc (both long and short forms) have been identified in the principal cells within the initial segment.[592,593] Further, the activity of the MAPK pathway and the expression of members of the PEA3 (ETV4) transcription factor family (ETV1, ETV4 and ETV5; ER81, PEA3, and ERM, respectively) are decreased following loss of testicular factors.[594–597] The cascade of signaling pathway events leading to initial segment cellular apoptosis following loss of lumicrine factors has been described.[596] From this study it appears that under normal physiological conditions, lumicrine factors maintain initial segment cell survival (prevent apoptosis) by maintaining the activation of the ERK pathway, suppressing stress-related signaling pathways and proapoptotic phosphatases and cell cycle inhibitors. Sipila and colleagues[598] also showed that etv4 mRNA expression was regulated by ERK signaling but not via protein kinase C, stress-activated p38, phosphoinositol 3-kinase nor p70 kinase cascades in an immortalized epididymal cell line. It is also very clear that the presence of the androgen receptor within the cells of the initial segment is vital for normal initial segment function.[94,599,600]

Another "factor" that has been postulated to regulate initial segment function is the presence of spermatozoa.[601] At first, cells seem to be an unusual "ligand", yet, upon closer inspection, there may be a sound basis for this hypothesis. Spermatozoa themselves may not be the "ligand", but it may be that the ligands that they carry and transport to the different epididymal regions that are important; many molecules are known to bind to spermatozoa and potentially shed in the epididymis. Interestingly, testicular spermatozoa stain positively for growth factor receptors;[602] it is tempting to speculate that upon entering the initial segment, growth factors dissociate from the sperm surface and are now available to stimulate their cognate receptors on the epididymal apical cell surface.

Lumicrine regulation is not necessarily restricted between the testis and the epididymis, but appears to operate along the entire length of the epididymis. An example is the potential function of BMP8A that is secreted by the initial segment and acts downstream in the cauda region.[603] Of further interest is that *RNase10*, which is highly expressed in the initial segment and under the regulation of lumicrine factors, is important for male fertility.[95]

MAJOR PROTEIN FAMILIES IN THE EPIDIDYMIS AND THEIR REGULATION

General Principles

The division of the epididymis into segments has more than anatomical implications. For many years, the differential expression of proteins and mRNA along the epididymis has become a hallmark of this tissue. Indeed, the large number of gene expression profiling studies,[71,432,475,604–616] proteomic analyses,[152,543,617–625] and microRNA and piRNA analyses[626–630] have all revealed that nearly all molecules have characteristic longitudinal expression profiles in the epididymis of all species examined. Data on the gene expression profiles of the different segments of the rat and mouse epididymis are available at http://mrgd.org/index.cgi. In addition, tables of some genes encoding distinct

classes of proteins (enzymes, signaling, DNA binding) that exhibit regionalized expression in the epididymis are provided in the supplemental material on this chapter's Website. Table 2 lists genes encoding secretory proteins which may perform direct or indirect roles in the sperm maturation process while Table 3 provides a list of genes that encode cellular proteins which likely perform regulatory functions (supplemental material at Website).

Although all of the above studies clearly demonstrate that there is a highly regionalized activation and deactivation of gene expression, the underlying mechanism(s) that allow for this regulation is unclear. Regionalized expression of transcription factors (see below) is beginning to provide some insight into this question; the relationship between the luminal, endocrine and cellular components that allow for such highly regionalized expression of genes, proteins and noncoding RNAs will require many more years to resolve.

Immunohistochemical staining of cross-sections of the epididymal epithelium for most proteins reveals a checkerboard pattern of expression. This characteristic pattern is usually seen when a given protein or mRNA is first expressed along the duct or when the expression terminates. The reason for this unique distribution of expression of genes and proteins in the epididymal epithelium is not clear. It can be assumed that adjacent principal cells either have different sensing mechanism for triggering the signal for expression of a given gene or protein, or, more likely, that principal cells function as a syncytium to coordinately optimize the level of expression of a given gene or protein. Three examples of this checkerboard pattern of expression are shown in Figure 17.15.

As one might expect based on their appearance and proposed functions, the different cells of the epididymal epithelium express different genes and proteins. Isolating each of the different cell types has been a challenge, but with the development of tools such as laser capture microscopy, it should become possible to resolve the profiles of genes and proteins expressed in each type of epithelial cell. Using immunohistochemistry, however, it has been possible to demonstrate that highly specific expression of certain proteins in principal, basal, clear, narrow and halo cells. In Figure 17.16, examples of the selective marking of each of these cell types is presented. More contemporary approaches now include labeling cells with an epithelial cell-specific promoter driving a reporter gene such as green fluorescent protein (GFP). The GFP-positive cells are then sorted, isolated and analyzed, e.g. Ref. 152. In order to provide potential tools to help identify cell types in vivo and in vitro, representative proteins expressed selectively in individual cell types of different regions of the epididymis are listed in Table 4 (supplemental material at Website); some of these proteins are secreted, while others are retained within the cell.

Functional Families

Ligands, Their Biosynthesis and Receptors

ANDROGEN RECEPTOR, ANDROGEN BINDING PROTEIN, AND STEROID 5α-REDUCTASES

The limiting factor in determining the action of testosterone on cells is not clear. The presence of both the

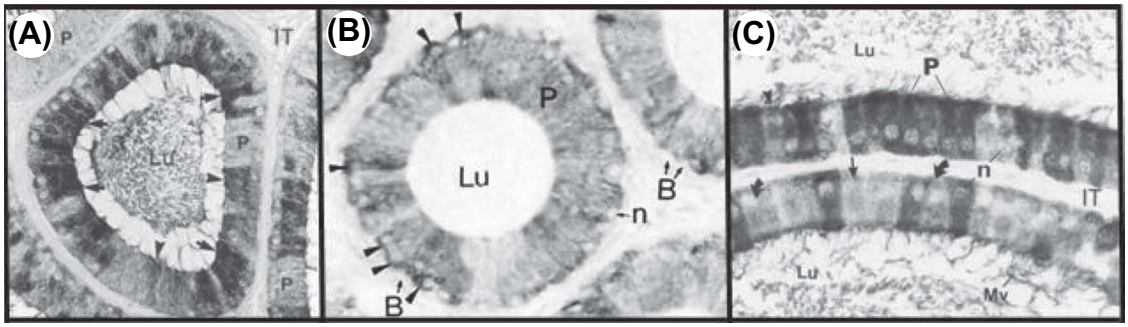

FIGURE 17.15 Representative examples of the checkerboard pattern of protein expression seen immunohistochemically along the epididymis. (A) High power light micrograph of tubules of the distal area of the initial segment immunostained with anti-immobilin. Although many principal cells are reactive, they show a variable staining pattern. Several principal cells are intensely stained throughout their cytoplasm (arrows), while others are moderately or weakly stained (arrowheads); a few are unreactive (P). The luminal content (Lu) is well stained. IT, intertubular space. ×350.[135] (B) High power light micrographs of the proximal initial segment of the epididymis stained for SDR5A. Epithelial principal cells (P), nuclei of principal cells (n), basal cells (B), and lumen of the duct (Lu) are shown. Intense staining in the infranuclear region of the principal cells is designated by the arrowheads. Magnification ×400.[452] (C) Higher power micrograph of portions of adjacent tubules of the caput epididymis immunostained with anti-SGP-2. Note intense immunoperoxidase staining reaction product over some epithelial principal cells (P). Other principal cells are moderately (arrowheads) or weakly (curved arrows) immunoreactive. A few principal cells are virtually unreactive (arrow). Note that the reaction product extends throughout the entire cell cytoplasm, i.e. basal, supranuclear and apical regions, leaving only the nucleus (n) unstained. The microvillar (Mv) border of the principal cells and sperm in the lumen (Lu) of the tubules are reactive. IT, intertubular space ×600.[632] *Source: Micrographs are modified from Refs 135,452,631.*

androgen receptor and an agonist are clearly necessary, but there are several lines of evidence that indicate that it is not primarily the absolute number of androgen receptors present but rather the amount of the ligand that is limiting. In addition, testosterone, a highly lipophilic molecule, is carried to sites distant from where it is made by binding proteins such as ABP. Testosterone can act directly on the androgen receptor in some tissues, such as muscle; in other tissues, such as some brain nuclei, testosterone needs to be aromatized to estradiol in order to mediate its action via the estrogen receptor; while still in other tissues, it is converted to a 5α reduced metabolite, DHT.[633] DHT binds to the androgen receptor with higher affinity than testosterone.[633–636] This latter mode is what occurs in many androgen dependent tissues such as the epididymis, prostate, seminal vesicles, and skin. The rate limiting step in the pathway leading from testosterone to its 5α-reduced metabolites is by steroid 5α-reductases (SRD5A1 and SRD5A2).

ANDROGEN RECEPTOR The first report that exogenously administered testosterone was converted to DHT and bound a cytosolic protein in the epididymis (the androgen receptor),[637] was nearly coincident with the discovery of a second androgen-binding component (androgen binding protein, ABP) in testis and epididymal cytoplasmic fractions.[638] Although both of these proteins bind androgen with high affinity, they are distinct molecules. For example, although they both have high affinity for DHT, the androgen receptor has a slow dissociation rate for DHT ($t_{½}$ 0 °C >4 days), while ABP dissociates at a much faster rate ($t_{½}$ 0 °C = 6 min). Androgen receptors have been found in the epididymis of all species examined to date (rat, rabbit, dog, ram, monkey, and human).[637,639–645] In all mammalian tissues, a single gene for the androgen receptor has been identified. In man, the gene spans approximately 90 kb, and it consists of eight exons on chromosome Xq12. The coded protein is approximately 110 kDa, and multiple transcripts are produced with variable lengths of a polymorphic region of CAG repeats in exon-1.[634,636,646,647] The gene contains two polymorphic trinucleotide repeat segments that encode polyglutamine and polyglycine tracts in the N-terminal transactivation domain of its protein; mutations in this gene can be associated with complete androgen insensitivity, but this depends on the severity of the mutation. Alternatively spliced variants encoding distinct isoforms have been described[647,648] but the consequences

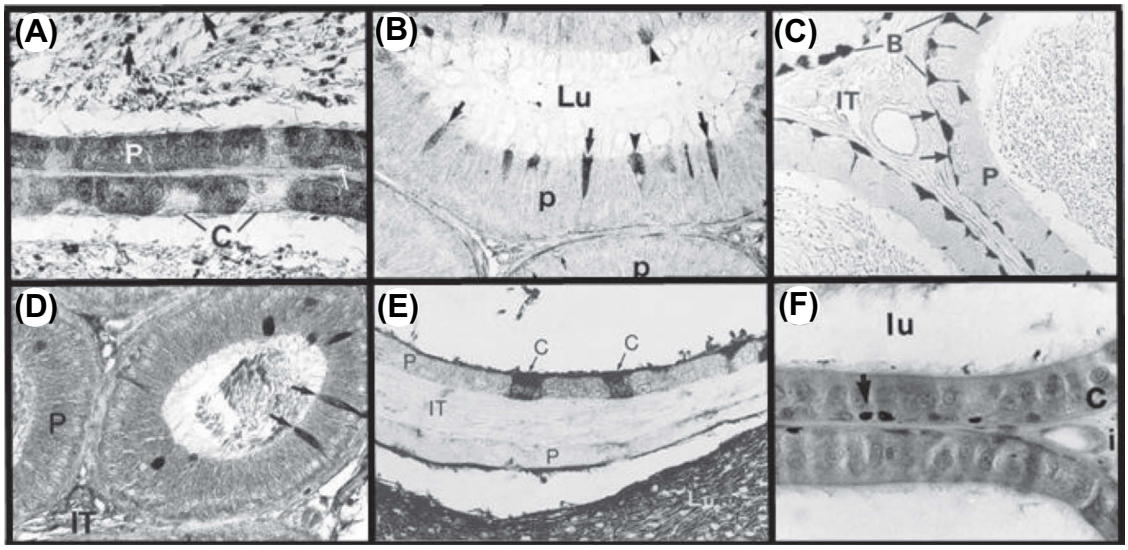

FIGURE 17.16 Cell specific expression of proteins along the rat epididymis. Examples of immunolocalization of proteins in each of the major cell types are presented. (A) Principal cells (junction of the caput-corpus of the epididymis) are immunostained for the glutathione S-transferase (GST) Yo subunit. The epithelial principal cells (P) are well stained, but basal (white arrow) and clear (C) cells are unreactive. Cytoplasmic droplets of spermatozoa (arrows) in the lumen are intensely reactive. ×365 [632]. (B) Apical cells (arrowheads) and narrow cells (large arrows) of the proximal initial segment of the epididymis are intensely reactive when immunostained with anti-Yo GST antibody, while principal cells (P) and basal cells (small arrows) are unreactive (Lu, lumen; ×320).[149] (C) Basal cells. The only cell type of the epithelium of the corpus epididymidis that is intensely reactive is the basal cell (B) when immunostained with antibodies to the Yf (Yp) subunit of GST. These cells often show slender processes (arrows) extending from the expanded main cell body along the basement membrane and, occasionally, toward the lumen (arrowheads). Principal cells (P) appear unreactive. Spermatozoa in the lumen are unreactive. IT, intertubular space. ×440.[167] (D) Narrow cells. Initial segment of epididymides immunostained with anti-carbonic anhydrase II antibody. Intense reaction is localized over narrow cells. P indicates principal cell; IT, intertubular space; and arrows, narrow cells (×350).[151] (E) Clear cells. Tubules of the distal area of the cauda epididymidis immunostained with anti-immobilin. Note that several epithelial clear cells (C) are intensely reactive throughout their cytoplasm, while other cells remain unreactive. IT, intertubular space; P, principal cells. X 375.[141] (F) Halo cells. Immunostaining with an antibody for helper T lymphocytes (CD4) in the cauda epididymidis. Arrow, CD4-positive cells; lu, lumen. X450.[175] *Micrographs are modified from Refs 141,149,151,167,175,632.*

for epididymal function of these alternate splicings have not been examined.

There are relatively small changes in the concentration of the mRNA for the androgen receptor along the epididymis.[584,649] When changes are noted, they often reflect a decline in the concentration of androgen receptors in the progression toward the distal end of the epididymis. For example, in the human, both the mRNA and protein for the androgen receptor are found throughout the entire tissue with high levels in the distal caput and proximal corpus, declining in the caudal region, including the vas deferens; interestingly the efferent ducts do not show any immunostaining while the vas deferens is only weakly positive.[641] An exception is the ram where androgen receptor concentrations are similar in the caput and cauda epididymidis but are less than 25% of these levels in the corpus epididymidis.[650] In both the rat and the goat, epithelial cells stain fairly evenly for androgen receptors throughout the duct.[651,652]

It is clear that principal cells stain heavily for androgen receptors, but the relative concentration of this receptor in basal, clear, narrow and apical cells is not known. However, the immunolocalization of androgen receptors in all epididymal epithelial cells has been reported for monkey, mouse, and goat,[645,653,654] while in the rat, it is apparent that clear cells do not stain for the androgen receptor.[558] Due to their origin in the immune system, and as one might anticipate, halo cells do not stain for the androgen receptor in any species examined. Peritubular cells occasionally display weak signals, while interstitial cells or blood vessels are consistently negative. When noted, the intracellular localization of the androgen receptor is distinctly nuclear.

Very few studies have focused directly on the mechanism of action of the androgen receptor in the epididymis; however, with the advent of several stable cell lines from this tissue,[598,655–658] some studies have been undertaken. Using the mouse PC-1 cell line, it was shown that DHT can cause a rapid response that is independent of the androgen receptor–DNA interactions and that this response is mediated by activation of the ERK pathway as early as 1 min after exposure to DHT;[574] interestingly, activation of the ERK pathway was blocked by hydroxyflutamide, indicating that early ERK activation was an androgen receptor-mediated response. Such a rapid action of androgen may ultimately act to modulate the transcription of genes regulated by AR in the nucleus.

The characteristics and mode of action of the androgen receptor as a transcription factor[552,633,636,659] in the epididymis seem to resemble those seen in other tissues. However, androgen signaling, although essential, is not sufficient for epididymis-specific gene expression since androgen receptor is expressed in many tissues. One mechanism proposed to explain the specificity of transcriptional responses to androgen is the tissue-specific combination of transcription factors binding to the promoter region of androgen regulated genes.[660] For example, the epididymal and prostatic lipocalin genes E-RABP and probasin are regulated by the androgen receptor. In the prostate, the transcription factor Foxα1 is required for probasin gene expression.[661] In the epididymis, Foxα2, but not Foxα1, is expressed, interacts with the androgen receptor and binds to the promoter of the E-RABP gene. Interestingly, overexpression of Foxα2 suppresses androgen activation of the E-RABP promoter, but increases activation of the prostate-specific promoter in an androgen independent manner.[662] Since Foxα1 and Foxα2 expression is restricted to the prostate and epididymis, respectively, it suggests that these transcription factors have distinct and different action on androgen receptor regulated genes in different male reproductive organs. It is tempting to speculate that these differential responses may have a role in the different response of these two organs to oncogenic transformation.

Little is known about the regulation of expression of the androgen receptor in the epididymis. In the goat, circulating androgens seem to be sufficient to regulate the expression of both androgen and estrogen receptors.[654] In the rat, androgen withdrawal achieved by suppression of LH by the administration of an GnRH antagonist results in a more extensive and rapid decline in androgen receptors in stromal than in epithelial cells; androgen replacement results in a complete restoration of androgen receptors, indicating that stromal cells are more sensitive than epithelial cells to the regulation of androgen receptor concentrations by androgen.[558]

ANDROGEN BINDING PROTEIN AND ITS RECEPTOR

Androgen binding protein (ABP) is one of the first proteins shown to be synthesized by Sertoli cells of the testis,[513] and has been found in several species, including the rat,[638,663] rabbit,[664] guinea pig,[665] ram,[639] monkey,[666] and human.[667] It is secreted in both the luminal and basal compartments of the seminiferous tubules, but the overwhelming majority, more than 80%, is directed toward the epididymis.[668] Plasma sex hormone-binding globulin (SHBG) shares the same amino acid sequence with ABP; these molecules differ only with respect to the types of oligosaccharides associated with them.[669] The biological significance of these differences is not clear in light of the fact that SHBG and a glycosylated mutant have similar binding characteristics to sex steroids.[670] ABP concentrations are highest in the caput and lowest in the cauda epididymal segments of most species examined, including primates; this distribution correlates well with DHT content.[139,671,672] ABP is synthesized and secreted by epididymal principal cells all along the epididymis,[673] but in several species it is also taken up into the epididymal principal cells of the initial segment and caput epididymidis,[663,674,675] through a

receptor-mediated process.[590,591,672,676,677] Testicular ABP production is regulated by androgens,[678] while that of the epididymis seems to be under the control of a testicular factor.[673]

Several functions have been ascribed to ABP. It has been proposed to act as a carrier of androgens to the epididymis.[679] Within the principal cells, testosterone is released from ABP and converted to the more potent androgen, DHT, by the enzyme SRD5A1 and 2. ABP has high affinity for 5α-DHT ($K_a = 1.25 \times 10^9$/M) and testosterone ($K_a = 0.5 \times 10^9$/M); rapid dissociation of testosterone from ABP (6 min) is consistent with a role for ABP as a carrier molecule for testosterone. In addition to its role as a carrier molecule, ABP has been suggested to be an androgen sink in the seminiferous tubules of the testis,[679] since the concentration of androgens in the seminiferous tubules needs to be higher than that in the peripheral circulation in order to maintain spermatogenesis[680–682] (see also Chapter 16). ABP has also been proposed to regulate the expression of SRD5A1.[452,533,588]

To unequivocally demonstrate a function for ABP in spermatogenesis or in the epididymis would require the development of a null mutation model; this has not yet been accomplished. However, mice overexpressing ABP,[683] show fertility defects stemming from spermatogenic dysfunction in the testis, which translates into reduced numbers of spermatozoa in the cauda epididymidis. Although in these transgenic lines, ABP protein levels are increased in the epididymis, the consequence of this on epididymal function is not evident.

Although it is clear that testicular ABP enters the principal cells of the proximal segments of the epididymis, the mechanism of internalization is still not fully understood. The presence of high affinity binding sites (K_a of 3.7/nM; 4.5×10^{11} sites/mg protein) on the apical surface of epididymal principal cells and the demonstration that the number of these receptors is fivefold lower in the cauda than in the caput of the epididymis have led to the proposal that ABP internalization is a receptor mediated process in both rats and monkeys.[591,672,676]

STEROID 5α-REDUCTASES The selective presence of radioactive DHT in epididymal cell nuclei after injection of radiolabeled testosterone,[684] and the demonstration using micropuncture studies that, beyond the efferent ducts, the predominant androgens in epididymal fluid are 5α-reduced metabolites of testosterone,[139] are two of the key findings establishing the central role of DHT, and hence steroid 5α-reductases, in mediating androgen action in the epididymis. The discovery that testosterone is rapidly converted by the epididymis to 5α-reduced products was first made almost simultaneously by two different groups.[685,686] The epididymis is one of the tissues with the highest level of activity anywhere in the body.[686]

In the mouse, rat, dog, monkey, and man, two 5α-reductase isozymes, with different tissue distributions, have been identified. SRD5A1 and SRD5A2 are the products of separate genes.[687,688] SRD5A1 is most abundantly expressed in the initial segment of the epididymis, with a positional gradient that is similar to the enzyme activity. Endocrine and developmental regulation of SRD5A1 show the following: (1) orchidectomy results in a decrease in type 1 mRNA levels in all epididymal segments; (2) after orchidectomy high-dose exogenous testosterone maintains SRD5A1 levels at control levels in all regions of the epididymis except the initial segment; (3) unilateral orchidectomy and efferent duct ligation cause a dramatic decrease in SRD5A1, selectively in the initial segment of the epididymis; and (4) only the type 1 transcript is developmentally regulated. Therefore, as for enzyme activity, the primary regulator of SRD5A1 expression in the initial segment is a lumicrine factor of testicular origin.[452,689]

Although several theories have been put forward regarding the relative role of these two isozymes,[690,691] little solid data exist to allow resolution of their relative roles. The consequence on reproduction in the mouse of a null mutation in *Srd5a1* is an effect on parturition,[692] but null mutations of either or both genes are compatible with male fertility.[690] However, no detailed studies on spermatozoa or on the epididymis of mice carrying null mutations for these genes have appeared. To understand the regulation of this enzyme activity in the epididymis, it is necessary to determine where and how the proteins are expressed, the translation, and, ultimately, the factors regulating transcription of the genes for this enzymatic activity.

5α-Reductase activity is expressed in a striking positional gradient as well as in different subcellular fractions in the adult rat epididymis.[587,693] The activity associated with the nuclear fraction is highest in the initial segment, where it is higher than in any other male reproductive tissue, and declines dramatically in more distal segments of the tissue. The hormonal regulation of epididymal 5α-reductase activity is complex.[452,689] This was the first enzyme shown to be regulated by testicular factors as well as by circulating testosterone, depending on which segment and subcellular fractions were examined. While nuclear fraction epididymal 5α-reductase activity is regulated in a lumicrine manner by a substance directly entering the epididymis via the efferent ducts, microsomal 5α-reductase activity is found throughout the epididymis but is present at a lower level and is regulated by circulating androgens.

The mRNA encoding SRD5A2 is found in high concentrations throughout the epididymis, with a somewhat elevated concentration in the caput epididymal segment.[452] Although in the epididymis SRD5A2 mRNA is present in abundance relative to that of SRD5A1, its

activity as a functional enzyme appears to be relatively poor. Unlike the developmental expression pattern of *Srd5a1*, characterized by dramatic increases occurring just before the first appearance of spermatozoa in the epididymis, *Srd5a2* expression does not show any significant developmental changes in any epididymal segment. Efferent duct ligation results in a dramatic decrease in *Srd5a1* expression in the initial segment, but causes a near doubling in that of *Srd5a2*. This differential regulation of the *Srd5a* transcripts within one tissue has not been observed in other rat tissues such as the prostate.[452]

In comparing the regulation of epididymal 5α-reductase enzyme activity and the expression of its mRNAs, it is apparent that a potential regulatory step is at the level of the protein itself. Post-transcriptional regulation has been proposed for several other epididymal proteins.[130,211,486,606,694–696] In the adult rat, SRD5A1 expression is intensely immunolocalized in discrete lobules of the proximal initial segment of the epididymis; a sharp decline in staining intensity occurs between the proximal initial segment and its adjacent region, followed by a progressive decrease in intensity beyond that point.[452] In all epididymal regions, SRD5A1 immunoreactive is localized specifically in the epithelial principal cells and is uniquely associated with membranous elements; the intracellular localization of the SRD5A1 changes as one moves down the epididymis. Savory et al.[697] have localized SRD5A1 to the nuclear envelope of both transfected COS cells and of rat liver. The infranuclear localized form of the SRD5A1 is regulated in a similar fashion to the *Srd5a1* and enzyme activity, that is, by a lumicrine factor of Sertoli cell origin.[452,698]

The genomic regulation of the expression of both 5α-reductase isozyme genes has also been investigated.[699,700] Characterization of the proximal promoter of *Srd5a1* displays all the features of a CpG island and has numerous Sp1 binding sites; it also has a bidirectional promoter activity in this region that is highest in the negative orientation. Promoter activity, in either orientation, is lost in Sp1 deficient cells and rescued following co-transfection with an Sp1 expression vector.[699] The *Srd5a2* promoter region has a noncanonical TATA box located upstream of the transcriptional start, has multiple Sp3 and Sp1 sites site, and is also GC rich.[700] The potential role of this GC-rich region is intriguing in light of the observation that the methylation pattern of the two genes appears to differ not only between each other but also between reproductive and non-reproductive tissues.[701] The genes for SRD5A1 and SRD5A2 are also differentially methylated in lymphocytes from normal and 5α-reductase deficient patients.[702] The methylation pattern of *SRD5A2* has been proposed as a marker for hepatocellular carcinoma,[703] but no studies on alterations of the methylation pattern of this gene in reproductive tissues have yet been reproted.

ESTROGEN RECEPTORS AND CYTOCHROME P450 AROMATASE

ESTROGEN RECEPTORS Starting in the 1970s, substantial evidence accumulated for the direct action of estrogens on androgen target tissues. Using binding studies, specific, high affinity cytosolic and nuclear estrogen binding proteins, presumably receptors, were identified in the epididymis by a number of investigators in mice,[704] rats,[705–707] guinea pigs,[708] rabbits,[709–712] dogs,[713] rams,[714] monkeys,[715,716] and men.[717] Through a comprehensive series of studies, Danzo's group established the presence of estrogen receptors in the rabbit epididymis and demonstrated both its developmental[718] and regional distribution;[708] however, enthusiasm for further studies on the estrogen receptor in the epididymis diminished when this same group demonstrated the presence of a protease, in the adult, that cleaves the DNA binding region of the estrogen receptor.[719]

The histolocalization of tritiated DHT and estradiol in the adult mouse epididymis revealed differential distribution of grains over nuclei in different segments of the excurrent duct system, as well as over different cell types.[704,719] These studies confirmed the earlier binding experiments and provided a more detailed localization of the putative receptors. Very strong labeling of the efferent ducts and initial segment of the epididymis was noted, with lesser binding in the more distal segments of the epididymis; nuclei of clear cells were more densely labeled with estradiol than with DHT, in sharp contrast to what was seen over nuclei of principal cells. Based on such data, and prior to the studies with null mutant mice, specific functions for estradiol in the epididymis were proposed.[48,570]

With the discovery of the existence of two estrogen receptors (ERα and ERβ, now referred to as ESR1 and ESR2), the availability of specific antibodies for these receptors as well as mice having null mutations for either or both receptors, our understanding of localization of estrogen receptors and their potential role(s) has grown rapidly.[581] ERα and ERβ are remarkably similar to one another in some respects(e.g. 95% identity in the DNA binding domain), but they have individual characteristics (e.g. less than 55% homology in the ligand binding domain), that clearly indicates functional differences.[720,721] The two isoreceptors have different binding affinity for estrogenic and antiestrogenic compounds,[722,723] different tissue distributions,[723] and different response elements.[724,725]

Although there are some conflicting data on the immunolocalization of ERα and ERβ along the efferent ducts and the epididymis presumably due to technical differences (e.g. antibody source, tissue preparation,[726,727]

and differences among species),[581] a general pattern is emerging. In the mouse, the epithelium of the efferent ducts shows strong ERα- and weaker ERβ -immunoreaction.[581,728,729] The initial segment of the epididymis shows some reactivity for ERα, primarily in narrow, apical and some basal cells;[653] in the caput, epididymidis, principal cells and clear cells stain positively, while in the distal regions only clear cells are immunoreactive. In the rat, ERα mRNA and protein are higher in the corpus region than in any other region of the rat epididymis.[581] In contrast, ERβ immunoreactivity is clearly observed all along the epididymis with more intense staining in the more distal regions (corpus and cauda).[581,729] The mRNA data are consistent with the observations made using immunohistochemistry.[581,726,730,731] In monkeys, baboons and men, both forms of the ER are present along the epididymis.[732–735] Interestingly, G protein–coupled estrogen receptor-1 (GPER, formerly known as GPR30) found on the cell membrane has also been found in the epididymis with the highest expression reproted for the corpus region.[581]

The first clear insight into the functional role of ERα in the efferent ducts and epididymis came through null mutation studies. The ERα receptor knockout mouse (αERKO) was developed by Lubahn et al.[736] and is infertile in spite of the presence, until puberty, of apparently normal seminiferous tubules; in the adult, testicular atrophy becomes evident.[737] Through an elegant series of studies, Hess's group,[738–740] has demonstrated that the cause of the infertility is atrophy of the seminiferous tubules due to increased back pressure resulting from the inability of the efferent ducts and initial segment of the epididymis to reabsorb the large volume of fluid secreted by the testis. This reabsorption process is under the control of estradiol, acting as a paracrine regulator of the efferent ducts. Further, it has been proposed that the action of estrogen on fluid reabsorption is mediated via the CFTR receptor.[741] The abnormal milieu created by the defect in fluid reabsorption contributes to abnormalities in sperm maturation and consequent infertility.[740] In contrast to the clear role of the ERα receptor, function for ERβ in the male reproductive tract awaits further investigation, as the ERβ knockout mouse is fertile and appears to have a normal testis and epididymis.[742]

CYTOCHROME P450 AROMATASE Cytochrome P450 aromatase (CYP19A1), localized to the smooth endoplasmic reticulum, is a terminal enzyme that irreversibly catalyzes the conversion of testosterone to estradiol. Both the absence of aromatase and an excess of estrogen result in male infertility.[743] There are several sources of estradiol in the adult male reproductive tract. In the testis of adults, both Leydig cells,[744,745] and germ cells[746] have been reported to actively synthesize estradiol. In germ cells, the enzyme is localized in the Golgi of round spermatids and throughout the cytoplasm of elongating and late spermatids.[581,743,747]

Early investigations of the presence of CYP19A1 in the epididymal epithelial cells indicated that this activity was absent;[748] however, no direct assessment of the enzyme was done in these studies. In several subsequent investigations, direct detection of the P450 aromatase mRNA, protein, and enzyme activity (formation of estradiol from testosterone), as well as the ability of specific aromatase inhibitors to inhibit estradiol formation, all led to the conclusion that epididymal principal cells, but not the smooth muscle cells surrounding the duct, have the ability to synthesize estradiol from testosterone in a number of species, including the rat,[749] monkey,[750] and man.[751]

In the epididymis, aromatization of the very high concentrations of testosterone to produce estradiol, could occur either in the luminal or the epithelial compartments. The presence of CYP19A1 in epididymal spermatozoa has been demonstrated in several species, including mouse, rat, bear, rooster, buffalo, and human.[746,752–756] The enzyme is localized to cytoplasmic droplets, and the staining becomes less intense as spermatozoa traverse the epididymis.[748]

RETINOIC ACID RECEPTORS

Retinoids, which include vitamin A, are highly potent molecules with pleiotropic action in a wide range of biological processes during development and in the adult.[757] For example, vitamin A deficiency (VAD) results in both keratinizing and non-keratinizing squamous metaplasia of numerous epithelia, including the epididymal epithelium.[758,759]

Given the importance of retinoids, it is not surprising that a complex system has evolved to control retinoic acid biosynthesis and metabolism.[760–762] Because retinoids are hydrophobic molecules insoluble in water, they require chaperones to ensure proper storage, transport, and uptake in the tissues. These chaperones, the retinoid binding proteins, are members of either a large superfamily of small intracellular proteins that includes numerous fatty acid binding proteins or another superfamily of secreted proteins, the lipocalins, which bind hydrophobic molecules. There are four intracellular retinoid binding proteins that are members of the fatty acid binding protein family (CRBP I and CRBP II: cellular retinol binding protein type I and type II; CRABP I and CRABP II: cellular retinoic acid binding protein type I and type II) and two extracellular retinoid binding proteins that are members of the lipocalin family (RBP: retinol binding proteins; E-RABP (LCN5): epididymal retinoic acid binding protein).

Within the nucleus, two classes of nuclear retinoic acid receptors named RAR and RXR have been identified and cloned. Each receptor class consists of three

receptor subtypes, α, β, and γ, and their isoforms. RXRs form heterodimers with RARs that are thought to be the functional units transducing the retinoid signal. The binding of RARs/RXRs to retinoic acid response elements (RARE) modulates gene expression and is likely to be responsible for the observed biological effects of retinoids.[763]

Most of the components of the retinoid-signaling pathway have been identified in the epididymis. Retinoids were quantified in the rat epididymis by high performance liquid chromatography (HPLC) followed by derivation of the HPLC-purified retinoids to confirm their identities.[764] In the epididymal tissue, the major retinoids identified included retinol, retinyl ester, all-*trans* retinoic acid and 9-*cis*-retinoic acid. The concentration of retinol and retinyl esters decreased from the caput to the cauda epididymidis from 2.7 to 1.7 nmol/g of tissue and 6.2 to 1.4 nmol/g tissue, respectively. The active retinoids, all-*trans* retinoic acid and 9-*cis*-retinoic acid (9-*cis*-RA) showed an inverse gradient with lowest concentrations in the caput (RA: 13 pmol/g tissue; 9-*cis*-RA: 2.9 pmol/g tissue) and highest concentrations in the cauda epididymidis. Using a bioassay, levels of 7 pmol/g of tissue have been reported in the murine epididymis.[765]

The cellular retinol and retinoic acid binding proteins are also present in the epididymis, but their localization is regionalized. CRBP is most abundantly present in the columnar epithelial cells of the proximal caput epididymidis of the rat; this tissue has one of the highest CRBP expressions in the body.[766–768] The mouse has the highest concentrations of CRBP in the proximal caput epithelium, but also has significant levels in the epithelium throughout the epididymis. CRABP II is found in the epithelium of the cauda epididymidis co-expressed with CRBP in both the mouse and the rat, while CRABP I is present in the smooth muscle of the distal cauda epididymidis.[769]

The retinoid-signaling pathway in the epididymis includes the retinoic acid receptors and the RA carrier protein E-RABP, synthesized and secreted into the lumen in the distal caput and found in the lumen from the caput to the cauda epididymidis. In the rat epididymis, RAR α, β, and γ transcripts have been detected by RT-PCR.[770] In situ hybridization and immunolocalization revealed region specific patterns of RARα mRNA and protein expression.[771] The highest levels of RARα transcript and RARα immunostaining were seen in the proximal caput epididymidis. The levels of RARα transcripts and RARα protein staining were higher in the initial segment, proximal caput and distal cauda. The lowest levels were seen in distal caput, corpus, and proximal cauda epididymidis.

It is clear that a remarkable retinoid homeostasis system has evolved to permit the transport of these hydrophobic molecules, their metabolism, their extracellular and intracellular sequestration, and delivery to the nuclear chromatin for optimum retinoid signaling in the epididymis. However, knowledge of the pattern of expression of the various components of the retinoid biosynthesis and metabolism pathway, although suggestive, is not sufficient to deduce the precise role of these regulatory factors or the site of action of RA in the epididymis. VAD has pervasive effect, affecting many organs during pre- and postnatal development and in adult life. Reproduction is affected both at the level of the testis where spermatogenesis is arrested and at the level of the epididymis where the epithelium develops squamous metaplasia. Because of this dual effect, it is difficult to ascertain from studies of VAD the physiological role of retinoids in epididymal function.

Gene inactivation has produced surprising results. Null mutants for transthyretin, a protein carrier of thyroxine found in serum, has a virtual loss of serum RBP; yet, the mice seem normal.[772] The CRABP I and CRABP II null mutant mice also appear normal.[773,774] The double knockouts of CRABP I and CRABP II have a higher death rate at 6 weeks than the wild-type mice, but the survivors appear normal.[774] Although each retinoid binding protein separately appears to be dispensable, the lack of phenotype may simply reflect that redundant mechanisms are built in within the organism.

The functional redundancy is well illustrated in the case of the retinoic acid nuclear receptors where six RXR isoforms and six RAR isoforms have been identified.[775] Null mutations of RARβ, RARα1, RARβ2, or RARγ2 appear normal. Null mutations of all RARα isoforms or all RARγ isoforms display some of the defects of the postnatal VAD syndrome. Only double null mutants of the RAR subtypes (RARα/γ, RARα/β_2, RARα$_1$/β_2, RARβ$_2$/γ) reproduce most of the VAD syndrome. Null mutation of RXRα induces some abnormalities, but double inactivation of RXRα and RARβ or RARγ increases the severity of the RXR phenotype. It appears that even though the different receptor isoforms are not truly functionally equivalent, they can substitute for one another under certain conditions. This functional redundancy may not be surprising in any gene family that has been conserved throughout vertebrate evolution and that has evolved by duplications from an ancestral gene.

Notwithstanding, an epididymal phenotype has been observed in transgenic mice after disruption of the RAR gene. Expression of a dominant negative mutant of RARα induced loss of organization of the columnar epithelium lining the cauda epididymidis and its transformation by squamous metaplasia. The aberrant transformation resulted in blockage and/or rupture of the duct with concomitant inflammatory response and, ultimately, infertility.[776] The RARα null mutant mice display not only aspermatogenesis, but also vacuolization of the epididymal epithelium,[777] and the RARα/γ

double null mutant display either severe dysplasia or complete agenesis of the epididymis and the vas deferens.[778]

In summary, given the pathological changes seen in the epididymal epithelium in VAD, the epididymal phenotypes seen in RAR null mutant mice and the presence of most of the components of the retinoid-signaling pathway, it is highly likely that retinoids play a regulatory role in epididymal function. However, there have been no recent studies on the role of vitamin A on the epididymis to support this hypothesis. Interestingly, the role of vitamin A during spermatogenesis has been and continues to be explored.[779–781]

OXYTOCIN RECEPTORS

The first demonstration of the presence of oxytocin receptors in the epididymis was by Maggi et al.[273] Since then, several reports on the localization, distribution, regulation and potential functions of these receptors in a variety of species have appeared.[272,274–276]

Although in the marmoset monkey, the oxytocin receptor seems to be primarily expressed in peritubular cells of the epididymis, in human, macaques, and ram, its localization is apparent in the epididymal epithelium and varies along the duct.[272,275] The expression in principal cells is seen only in the initial segment of the epididymis of primates and has a checkerboard appearance; throughout the rest of the duct, it is confined to peritubular cells of the epididymis.[275] In the ram, however, oxytocin receptors are found in both principal and basal cells throughout the epididymal duct, while peritubular staining is seen only in the more distal segments.[276] Since principal cells express oxytocin,[272,274–276,782,783] then presumably this hormone acts as paracrine regulator of epididymal sperm transit.

Two functions have been ascribed to the oxytocin/oxytocin receptor system in the epididymis.[784,785] As discussed above, the first is that of promoting contractility of the duct, as demonstrated both in vitro[277,786] and in vivo,[275] thus promoting sperm transport through the epididymis.[289] In addition, estrogens regulate both oxytocin and endothelin-1 induced epididymal contractions, which in turn regulate the activity of RhoA/Rho-kinase signaling.[267,275] The second function of this system involves the promotion of the formation of DHT by stimulation of 5α-reductase activity in the initial segment of the epididymis.[784,785,787] This function is of particular interest, given the very high 5α-reductase enzymatic activity in this segment of the epididymis (see above) and the presence of oxytocin in the luminal fluid of the ram and the rat.[782,783] The mechanism for this type of stimulation is not clear but has been postulated to be mediated by phosphorylation and activation of existing 5α-reductase due to tyrosine kinase activation through a coupling to the oxytocin receptor via G_i signal transduction.[788]

GROWTH FACTOR RECEPTORS

Fibroblast growth factor receptors (FGFR), platelet derived growth factor receptors (PDGFR) and vascular endothelial growth factor receptors (VEGFR) have all been identified in the epididymis. Specifically for FGFR, two splice variants FGFR-1 IIIc (long and short forms) have been identified in the principal cells of the initial segment.[593] Although all four major splice variants of FGFR (1–4) are present in initial segment tissue, it is only the FGFR-I IIIc variants that are present in principal cells. The remaining splice variants are presumably localized to the endothelial and interstitial cells. FGFs 2, 4 and 8, present in rete testis and epididymal luminal fluid,[592,593] are potential ligands for the apically placed receptor.[593,594,596] Interestingly, overexpression of FGF8b in the epididymis caused epithelial and stromal hypercellularity eventually leading to malignant transformation of the stroma.[789]

PDGF α and β receptors and their ligands (PDGF A–D) have been localized in the rat and mouse epididymis.[790] In view of the specific localization of PDGF-A in the epithelium and the PDGF α–receptor in the mesenchyme, the authors suggested that the PDGF-A/α-receptor system may be involved in epithelial–mesenchymal interactions during epididymal development. In the adult epididymis, PDGF-A and B and α and β receptors were identified in the epithelium only. Interestingly, in PDGF-A null mutants an epididymal phenotype was not observed during development but epididymal epithelial disruption was observed at postnatal day 25.[790]

VEGF and its receptors are highly localized in both the testis and epididymis, with c-fms-like tyrosine kinase (FLT-1) and fetal liver kinase (designated as kinase insert domain-containing receptor, KDR); VEGF receptors are identified by immunohistochemistry in the ciliated cells of the efferent ducts and vascular endothelial cells of the human epididymis, respectively.[791] Flt-1 was also observed in the lymphatics. Further, addition of VEGF28 to epididymal tissue in culture resulted in endothelial fenestrations and opening of interendothelial junctions. Since the ligand, VEGF, was localized to the peritubular and ciliated cells of the efferent ducts, and to the peritubular and basal cells of the epididymis, it was suggested that VEGF may play a paracrine role in the permeability of blood vessels via KDR and lymphatics via flt-1.[791] Overexpression of VEGF in the testis and epididymis results in male infertility due to spermatogenic arrest and increased capillary density in the testis and epididymis.[792]

Epidermal growth factor receptor (EGFR) has also been identified in the epididymis[793,794] and may play a role in the paracrine regulation of cell function within

tubular segments,[111] facilitate early response signaling pathways activated by DHT[634] and regulate connexin 43 in the human epididymis.[795]

Although not specifically expressed in the epididymal epithelium, the hepatocyte growth factor receptor, c-met, is localized to epididymal spermatozoa, with the ligand, hepatocyte growth factor (HGF), being secreted into the lumen by the epididymal epithelium. The putative action of HGF is maintenance of sperm motility.[796] Interestingly, the hepatocyte growth factor-like protein (HGFL) receptor, *Ron* mRNA, which encodes for is a receptor tyrosine kinase, is expressed in the epididymal epithelium.[797] Additional growth factor binding proteins and growth factor receptors that are expressed in epididymal epithelial cells include: insulin-like growth factor binding protein-rP1/mac25(IGFBP7),[798] latent transforming growth factor beta binding protein 2,[799] EGFR,[799] and growth hormone receptor/binding protein.[800] Microarray analyses have also shown the expression of numerous growth factor genes in the epididymis of several species.[604–616]

C-ROS (ROS1)

C-ROS is an orphan tyrosine kinase receptor that has received considerable attention since it was shown that *c-Ros* null male mice were infertile.[55] The kinase and extracellular domains of the *c-Ros* gene show similarities to the *Drosophila sevenless* (*sev*) tyrosine kinase receptor gene[801] (see also Ref. 802 for more information of the evolution of *c-Ros*). The *sev* gene was originally discovered in a *Drosophila* mutant that failed to express the R7 photoreceptor cell in the ommatidium.[803,804] Further studies demonstrated that the adjacent R8 photoreceptor cell expressed *boss*, the ligand for *sev*. Interaction between these two cell types was crucial for the development of these photoreceptor cells. Following this cell interaction, a signal transduction cascade involving RAS and activation of downstream target genes was initiated in the R7 photoreceptor cell. Interestingly, ectopic expression of *boss* or of constitutively active forms of *sev* causes the formation of ectopic R7 cells.[805,806] The overall sequence homology between c-ROS and SEV is quite low, but there are some very interesting structural similarities between them. For example, (1) both genes encode a large extracellular domain and a kinase domain, (2) sequence homology extends into the putative extracellular ligand-binding domain, (3) there is a short insert of five to seven amino acids in the same position in the kinase domain of both genes that is not found in other receptor tyrosine kinases, (4) there are several common exon-intron junction points between the two genes, (5) the kinase domains of both genes are 70% homologous[801,807–810], and (6) particularly striking is the analysis of Springer,[811] who showed a colinearity and spacing of β-propeller and EGF-repeat structures in c-Ros and *sev*. Interaction of ligand with β-propellers has been shown for other proteins.[812] The ligand of SEV is bride of sevenless (BOSS),[806,813] a seven-transmembrane G-coupled protein kinase, which has signal transduction properties of its own. Presumably, a *boss* homolog exists in mammals if c-ROS has similar function to SEV.

Although *c-Ros* is expressed in several tissues throughout development, including the lung, kidney and epididymis, it remains highly expressed in the initial segment of the adult mouse epididymis[55,810] and along the human epididymis.[814] A clue as to the function of c-ROS came from *c-Ros* knockout mice: the animals displayed an underdeveloped initial segment and male infertility. The infertility phenotype was due to spermatozoa having an angulated flagellum at the end of the mid-piece;[815] it was suggested that this defect was due to the failure of spermatozoa to regulate their cell volume.[815] This infertility phenotype was also shown to be cell autonomous; the defect was due to the passage of normal spermatozoa through the underdeveloped initial segment, resulting in the sperm phenotype, and not because of some inherent defect in the spermatozoa themselves. Further, the infertility phenotype was observed whether either the kinase domain or the extracellular domain of c-Ros was lacking.[55]

Although the functional role of c-ROS is not known, it has been suggested that it plays a role in the regulation of cell volume.[520,816] Although c-ROS itself may not have a direct effect on cell volume regulation, gene expression profiling suggests that several osmolyte transporters are compromised in the initial segment of the *c-Ros* null animals.[615] Certainly, the changes in pH of epididymal luminal fluid in the *c-Ros* null animals could also contribute to the infertility phenotype.[520] Further, it appears that at least inositol and glutamate sperm concentrations are lower in the c-Ros mice compared to controls, even though there are no differences in luminal fluid concentrations of the two osmolytes between the two groups.[520] Whether the lower intracellular levels of these osmolytes in spermatozoa is the cause of the infertility phenotype remains to be determined. Adding to the complexity of the problem is that the infertility phenotype may be related to an underdeveloped initial segment rather than directly to c-ROS. Since the adult mouse initial segment still expresses *c-Ros*, the challenge will be to silence this gene in the adult and then examine the fertility phenotype. This experiment needs to be done especially if c-ROS and its downstream signaling pathways are to be considered as potential targets for the development of a male contraceptive. Adding to this importance is the finding that the adult human epididymis also expresses *c-Ros* throughout its length.[814]

In an attempt to identify downstream targets of c-ROS, Keilhack et al.[817] used a yeast two-hybrid assay and identified several proteins that associated with

the kinase domain of c-ROS. These included the phosphatase SHP-1, phospholipase Cγ, the SH2 domain of c-Abl, Grb2, the phosphoinositide-3 kinase subunit p85α and the SH2 domains of SHP-2. To demonstrate the potential importance of the SHP-1 phosphatase in male fertility, viable motheaten (me^v) mice were tested for fertility because these mice contain a mutation in the SH2 domain of SHP-1. The male mice were infertile. Hence, downstream targets of c-ROS are clearly excellent candidates for male contraceptive development. Although Keilhack et al.[817] indicated that the infertility phenotype of me^v mice is similar to that seen in the *c-Ros* knockout animals, the authors do not present convincing data that there is a reduction in epithelial cell height in the initial segment of the *Shp-1* knockout animals similar to that seen in the initial segment of *c-Ros* knockout mice. Their data suggests that the initial segment has developed normally in this knockout. Hence, although male infertility is observed in both the *c-Ros* and *Shp-1* knockouts, the mechanisms by which infertility is achieved in each case are probably different. At this time, c-Ros remains a viable epididymal target for the development of a male contraceptive.[818]

HE6/GPR64

The *He6* gene encodes a novel human epididymal protein that comprises a seven transmembrane component with a large extracellular domain and shows high homology to the secretin/PACAP superfamily of G-protein coupled receptors.[819] In view of the large ectodomain, HE6 has also been considered to be a member of the LNB-TM7 protein family B-GPCR-related TM7 receptors with long terminal extensions.[820] This large domain also contains a GPCR proteolysis site (GPS) and therefore HE6 may exist as a two-subunit receptor. *He6* is located on the human X-chromosome and both rat and mouse homologs of *He6* have been cloned.[819,820] Immunolocalization studies show human and mouse HE6 localize to the apical surface of human epididymal cells with staining being more pronounced in the efferent ducts-caput region compared to the distal epididymal regions. A mosaic pattern of staining was also observed, as is often seen with localization of other epididymal proteins. Mouse HE6 was localized to the apical cell surface but highly restricted to the initial segment region.[820] The function of HE6 is yet to be determined, although a knockout study suggests that the protein may be involved in fluid reabsorption in the efferent ducts.[821] Further, studies have shown that HE8 localizes to subcellular domains involved in the process of fluid reabsorption.[822] Studies have also revealed a number of potential downstream targets of HE6 including Adam 28, claudin-10, EAAC1 and a novel gene, *Me9*.[823] HE6 would probably not be a good epididymal contraceptive target because of the blocked efferent ducts observed in the *He6* mouse knockouts.

It is interesting to note that *He6/Gpr64* is over expressed in Ewing sarcomas and up-regulated in carcinomas of the kidney, lung and prostate.[824] Regulating the expression of this gene may be important in preventing the very rare cases of carcinomas of the epididymis.

MINERALOCORTICOID AND GLUCOCORTICOID RECEPTORS

Previous studies have shown that the rat epididymis responds to aldosterone and aldosterone antagonists[825–828] and there is evidence for the presence of aldosterone receptors in the epididymis.[829–831] Interestingly, aldosterone receptors were identified in clear cells[829] indicating that aldosterone may be regulating water transport specifically in this cell type. Glucocorticoid receptors have also been identified in the epididymis.[831–833] The exact role of glucocorticoids and their receptors in the epididymis is unclear but there is some evidence to suggest they may play a role in innate immunity[834] (see also Chapter 19).

OTHER RECEPTORS

In addition to those receptors discussed above, the epididymis also expresses orexin receptors,[835–837] the urokinase-type plasminogen activator receptor (u-PAR[838]), apolipoprotein (apo) E receptor-2 (apoER2[839]), adrenomedullin receptors,[840] purinergic receptors,[841] guanylyl cyclase C receptor,[842] melatonin receptor,[843–845] vitamin D receptor,[846,847] LXR and ABCA1 receptors.[848] Interestingly, FSH receptor was identified in the mouse and human epididymis.[849] The precise biological role(s) played by any of these receptors in the epididymis is not clear.

Transcription Factors

An understanding of transcriptional regulation of epididymal genes has been limited to a few genes.[144–146,850–854] However, in these studies, several key transcription factors have been identified that may play an important role in the tissue-specific, region-specific and/or cell-specific expression of epididymal genes.

ETV4 (PEA3) FAMILY

One of the first major epididymal transcription factors to be examined was polyomavirus enhancer activator 3 (PEA3), which is a member of the Ets transcription factor family. This family is characterized by an 85 amino acid ETS domain[855] that encodes for a helix-turn-helix DNA binding motif. It is also part of a subfamily, referred to as the PEA3 family, which comprise PEA3, ERM, and ER81.[856–861] The consensus site for PEA3 is 5′-AGGAAG-3′ with GGAA being the core binding motif for the *Ets* family. Xin et al.[856] were the first to show

expression of *Pea3* in the epididymis; Drevet et al.[862] clearly demonstrated a role for members of the PEA3 family in regulating GGT IV and GPX5 gene expression in the epididymis. Performing standard in vitro promoter analyses of both GGT IV and GPX5 revealed that PEA3 acts as both a repressor and an activator of transcription. This was confirmed by Kirby et al. (2004),[863] using a novel in vivo electroporation technique. There were differences in promoter activity between the in vivo and in vitro studies when the same constructs were used, highlighting the importance of performing such an analysis under as near physiological conditions as possible. The arrangement of PEA3 binding sites along the GGT IV promoter may be unique to this gene because of the absence of binding motifs, for example, AP-1, which are known to regulate the activity of PEA3 in many other gene promoters, such as urokinase plasminogen activator (uPA) and matrix metalloproteinase.[864,865]

There appears to be redundancy among the PEA3 family members, as SouthWestern analysis[595] showed that multiple proteins identified as PEA3, and possibly ERM and ER81, bound to *GGT IV* mRNA PEA3 binding motifs. Further, *Pea3* knockout mice had a normal epididymal phenotype, supporting the idea that ERM and ER81 may take over the functions of PEA3. However, *Pea3* knockout male mice are infertile, but their infertility is due to impotence and/or failure to ejaculate spermatozoa.[866] Interestingly, the promoters of several genes that are highly expressed in the initial segment, including 5α-reductase type 2,[700] have PEA3 family binding motifs and each gene, including PEA3 family members, is regulated specifically by testicular factors. It will be important to generate initial segment-specific *Pea3* family member knockouts to determine whether PEA3 family members play an important role in the function of the initial segment and thereby male fertility.

CCAAT/BINDING PROTEINβ (C/EBPβ)

C/EBPβ is a member of the CCAAT/binding proteins that regulate genes that have various functions including cell cycle control, fatty acid metabolism, cellular differentiation, growth, tumorigenesis and apoptosis.[867] This six-member transcription factor family is characterized by having a highly conserved basic-leucine zipper domain at the C-terminus that is involved in dimerization and DNA binding. Interestingly, although heterodimerization can occur, the heterodimer C/EBPβ-C/EBPζ does not bind to the standard C/EBP consensus sequence but binds to a subset of promoters in genes involved in cellular stress. Hence, this family of transcription factors can function as either repressors or enhancers depending upon the physiological state of the cell.[868]

C/EBPβ is expressed in the proximal regions of the mouse epididymis and plays a role in the regulation of expression of the *Cres* (cystatin-related epididymal spermatogenic) gene.[869] Two putative C/EBP DNA binding motifs were identified in the first 135 bp of the *Cres* promoter. Data from gel shift assays, in vitro and in vivo promoter analyses and analysis of C/EBPβ knockout mice suggest that expression and activation of C/EBP is necessary for complete activation of *Cres* in the proximal caput region.[863,869] Further analysis showed that although C/EBPβ was the predominant form expressed in the epididymis, C/EBP α and δ were also expressed but to a lesser degree. C/EBPε was not identified.[869] Since the expression of members of the *Cres* gene family is dependent on the presence of testicular luminal fluid factors,[870] it is assumed that C/EBP family members are also regulated in a similar manner. As members of C/EBP family play a role during cellular stress, it would be interesting to examine their potential role during apoptosis of the initial segment following loss of testicular factors.

HOMEODOMAIN-CONTAINING TRANSCRIPTION FACTORS

Several classes of homeodomain-containing proteins are expressed in the mammalian epididymis; these include hox, PEPP-family (PEM), Prd-class and Pou-class genes.[71,850,871] Homodomain containing proteins are transcription factors that contain a highly conserved 60 amino acid DNA binding domain composed of three alpha helices with the third helix interacting closely with DNA The major role of *hox* genes is in organ development and in the establishment of segment identity along the anterior–posterior body axis.[872,873] The epididymis is a highly segmented organ and therefore it is not surprising that many *hox* genes are expressed in the developing epididymis.[71,871,874–876] Since the epididymis forms later in development, it is the *5′hox* genes that appear to play a major role in epididymal development compared to the earlier expressed *3′ hox* genes. Interestingly, hox genes are also expressed in the adult epididymis;[876] their precise function in the adult warrants further study.

A first indication that *hox* genes were expressed in the epididymis and played a role in epididymal development came from an analysis of reproductive tissues from *hoxa10* and *hoxa11* knockout animals.[874,877] One of the most intriguing phenotypes to be produced from these null mutations was the homeotic transformation of the distal epididymal regions. *Hoxa11* was expressed in the initial segment and in the more distal epididymal regions,[876] therefore, the observation of a distal epididymal phenotype in the *hoxa11* knockout was not surprising. However, it is not clear if the epididymis developed appropriately in this mutant because the initial segments of these animals were not analyzed. The regulation of *hox* genes in the epididymis is poorly understood; however, it is known that in many tissues, co-factors must be involved in the transcriptional activation of genes by hox

transcription factors. Two examples include MEIS family members and PBX,[878] both of which contain a homeodomain, *Meis 1a* and *1b* are expressed in the mammalian epididymis.[876]

A homeobox cluster termed *Rhox* has been identified on the X-chromosome. Although the majority of the members of this family are expressed in the testis, several are also expressed in the epididymis in a region-specific manner.[854,879,880] A putative homeodomain transcription factor called PHTF1 has been identified in the principal cells of the initial segment and the caput epididymidis regions.[881] Although this protein contains domains that would suggest that the protein is a transcription factor, immunolocalization studies show it to be localized to the endoplasmic reticulum saccules applied to the trans face of the Golgi system.

One of the most well studied homeodomain-containing transcription factors is PEM (RHOX 5), a member of the PEPP homeobox subfamily. Rao and Wilkinson[850] suggested that the *Pepp* genes were derived from the *Drosophila aristaless* gene. Of the *Pepp* gene family members, only *Pem* was expressed in the epididymis as well as in the testis.[850] *Pem*, like *hoxa11*, was expressed throughout development and into adulthood[882,883] from a "male-specific" proximal promoter. The expression of *Pem* in the epididymis was species dependent. In the mouse *Pem* was found mainly in the caput, whereas in the rat it is expressed throughout the duct increasing from caput to cauda. However, *Pem* was not expressed in the initial segment of either the rat or mouse.[850] Further, expression of *Pem* was androgen-dependent.[882,884] The function of *Pem* is not known because *Pem* knockout animals did not display an epididymal phenotype.[885] However, sperm did not display normal forward motility and subfertility was observed.[854,880]

FOXI1

FOXI1 is a member of the forkhead transcription factor that comprises over 100 members[886] and is characterized by a 100-amino-acid winged helix domain. It is also a highly conserved family being originally identified in the fly, *Drosophilia melanogaster*, but also found from yeast to human. Of interest to the epididymis is that a null mutation of *Foxi1* in mice leads to male infertility.[887] FOXI1 binding sites are found on the promoter of the B1-subunit of the vacuolar H^+-ATPase proton pump, and is also important for the activation of carbonic anhydrase II and the chloride/bicarbonate transporter pendrin (SLC26A4).[887,888] Because these transporters play a key role in proton homeostasis in epididymal luminal fluid, then their disruption leads to changes in pH which in turn leads to changes in the luminal fluid microenvironment that are necessary for normal sperm maturation.

Other transcription factors that have been identified in the epididymis include: SP1 and SP3,[699,700,889] c-FOS,[145] TST-1,[890] EMX-2,[891] LBX-2,[892] PAX-2,[893] ZFY,[894] NOR-1[895] in addition to those identified in gene microarrays.

Transporters and Channels

WATER AND ION TRANSPORT

Although the efferent ducts serve as a conduit for spermatozoa from the testis to the epididymis a major function of the efferent ducts is the reabsorption of water. The major driving force for water movement across the efferent ducts and the cauda epididymidis is the Na^+-K^+ ATPase located on the basolateral membrane.[896,897] However, since the activity of the Na^+-K^+ ATPase in the efferent ducts is less than that found in the cauda of the epididymis, the paracellular pathway also plays a critical role in sodium and water movement,[479] across the epithelium of the efferent ducts.

The involvement of an apically placed Na^+-H^+ exchanger, which drives sodium out of the lumen with water following, was proposed by Wong et al.[482] since fluid absorption is amiloride-inhibitable, isosmotic and flow dependent. So far four Na^+-H^+ exchangers (NHE1-4) have been identified in several tissues and three of them have been found in the epididymis;[898–901] such transporters appear to play a role in sodium and bicarbonate reabsorption and proton secretion. NHE1 and NHE2 are expressed on the basolateral surface and apical surface, respectively, throughout the epididymal duct, except that NHE2 is not expressed in the initial segment. Cheng Chew et al.[899] suggested that NHE1 is involved in bicarbonate secretion whereas NHE2 may be involved in sodium reabsorption. Studies by Breton's group[902] and others,[898,901] have shown that the expression of NHE3 varies throughout the length of the epididymis and is localized to the apical surface of principal cells only. Important to bicarbonate reabsorption are the presence of apical and cytoplasmic carbonic anhydrases. NHE3 appears to participate in bicarbonate reabsorption, similar to its role in the renal proximal tubule. Through the activity of apically placed NHE3, protons are secreted and sodium is reabsorbed. Carbonic anhydrase IV, which is also apically positioned, then converts the luminal protons into water and carbon dioxide, allowing the carbon dioxide to enter the cells. Cytosolic carbonic anhydrase II catalyzes the production of new intracellular bicarbonate and generates a proton. The proton is recycled back into the lumen via NHE3. Intracellular bicarbonate is then transported out of the cell across the basolateral membrane via the bicarbonate exchanger (AE2),[903] or the electrogenic sodium-bicarbonate co-transporter, NBC1.[903] The driving force for this whole process is provided by the low intracellular sodium concentration that is achieved by the basolateral Na^+/K^+ ATPase. In addition, maintenance of a high potassium concentration by the Na^+/K^+ ATPase induces an intracellular negative potential of about −70 mV. This drives

bicarbonate across the basolateral membrane via the NBC1 transporter, which transports three molecules of bicarbonate for every sodium molecule, resulting in the net transfer of two negative charges.

A major player in hydrogen ion secretion is vacuolar type H^+ATPase (also known as the V-ATPase), which is highly expressed in apical/narrow cells in the proximal regions of the epididymis and in the clear cells throughout the epididymis.[151,902–906] Further, using a proton-selective electrode, Breton and colleagues,[905] showed that the epithelium of the vas deferens secreted protons and that this was inhibited by bafilomycin, an inhibitor of H^+ATPase. This study clearly demonstrated that acidification of the luminal fluid within the vas deferens was due to this transporter. An acidic pH in the lumen of the epididymis and proximal part of the vas deferens contributes to keeping sperm in a dormant state during their storage period.[164,907] The importance of an acidic luminal pH in the establishment of male fertility was illustrated by the generation of *Foxi1* KO mice, which have a higher luminal pH compared to wild type mice.[887] FOXI1 is a transcription factor that regulates key acid/base transporters in clear cells, including subunits of the H^+ATPase, CAII and the chloride/exchanger pendrin.[887,888] *Foxi1* KO males are infertile due to the inability of their sperm to move up the female tract to fertilize an egg. To maintain an acidic luminal fluid microenvironment, clear cells respond to a luminal increase in bicarbonate by increasing their rate of proton secretion.[162] This is achieved via the bicarbonate-sensitive soluble adenylyl cyclase, sAC, which is enriched in clear cells. A sAC-dependent increase in cAMP induces the apical membrane accumulation of the H+ATPase in these cells.[162] This effect requires the participation of protein kinase A.[908] H^+ATPase-dependent proton secretion is regulated via recycling of sub-apical vesicles to and from the plasma membrane.[164] This is reflected in part by a very high rate of endocytosis in clear cells.[156,157] Recycling of the H^+ATPase may involve unique clathrin- and caveolin-independent mechanisms, as well as members of the SNARE protein family.[909] Both actin[165,910] and microtubules are essential for V-ATPase recycling to occur.[184,483]

The aquaporins (AQP) play a major role in water transport across the epididymal epithelium and, of the 13 isoforms, AQP 1, 2, 3, 5, 7, 8, 9 and 11 have been identified throughout the epididymis including the efferent ducts and vas deferens,[911–918] but not necessarily in every region and epithelial cells.[914,919,920] For example, AQP1, which is highly expressed in the efferent duct epithelium, is present only in the vasculature and is absent from the epithelium in the epididymis. AQP9 is clearly the most abundant of all aquaporins in the epididymis epithelium, and it is located in the apical membrane of principal cells. However, AQP5 and AQP11 are also expressed apically in some principal cells. The significance of such "redundant" aquaporin expression in the same membrane domain of these cells remains obscure. Although very few functional studies have been done on the aquaporins, studies by Wong and colleagues,[917] have shown that CFTR potentiates water permeability of AQP9 in oocytes and in the rat epididymis. The role of CFTR as a regulator of AQP9 function was further confirmed using epididymal tubules perfused in vitro.[921] In this study, CFTR was shown to co-localize with AQP9 in the apical membrane of principal cells and inhibition of CFTR reduced the permeability of AQP9. In addition, both proteins co-immunoprecipitated in a complex that also contained the scaffolding protein NHERF1. It was proposed that the c-terminal SVIM motif of AQP9 binds to the PDZ domains on NHERF1, which serves as a mediator for the interaction between AQP9 and CFTR. CFTR is expressed in the rat and human epididymis.[164,921–924] Besides CFTR's known role as a cAMP-activated chloride channel, its role as a regulator of AQP9 may, therefore, have ramifications in males with cystic fibrosis.[917] Other chloride channels have received little attention. However, a previous study by Isnard-Bagnis et al.[163] showed that the epididymis expresses members of *Cic* gene family of chloride channels. Studies from this group suggest that CIC-3 may be involved in chloride transport by principal cells and CIC-5 might play a role in the acidification of H^+ATPase-containing vesicles in narrow and clear cells.

Although net water movement is from lumen to cell in the proximal regions of the epididymis, water is secreted in the distal epididymis and plays an important role in ion and water homeostasis. Chloride and bicarbonate are again responsible for driving water from the cell into the lumen. Wong et al.[482] have undertaken extensive investigations recognizing the importance of peptide hormones in this process. These investigators have presented a novel model to explain anion secretion by multiple peptide hormones. For example, angiotensin II, bradykinin, endothelin, and arginine vasopressin (AVP) interact with their cognate receptors on the basolateral membrane, which in turn activates phospholipase A2 (PLA2) on membrane phospholipids resulting in the release of arachidonic acid. Prostaglandins are subsequently produced through the actions of cyclooxygenase I (COX-1) and isomerases. This process occurs within the basal cells. Prostaglandins then diffuse out of the basal cells and interact with their receptors on the basolateral membranes of principal cells, which in turn will cause an increase in cAMP resulting in activation of the apically placed CFTR channel and chloride secretion.[482] With the identification of the family of TRP channels in the epididymis, Leung et al.[172] suggested

that TRP3 provides the increase in intracellular calcium levels, which translocates PLA2 from the cytosol to phospholipids membranes, where COX-1 has been identified (Figure 17.17).

In summary, water secretion in the distal epididymis is driven by chloride transport whereas fluid reabsorption in the proximal epididymis is principally driven by sodium transport. As suggested by Wong and colleagues,[482] secretion of water may play a role in fine tuning water movement across the epididymal epithelium. This may be particularly important because at this moment there have been very few hormones that appear to play a role in regulating water reabsorption. Interestingly, luminal bradykinin increases AQP9-dependent transport via activation of the calcium-signaling pathway.[925] The dual activation of CFTR and AQP9 by this hormone might provide a synergistic activation of chloride-dependent water transport in the epididymis. In addition, estrogen has been implicated as a major regulator of water reabsorption in the efferent ducts.[726,738,926] As discussed above, the efferent ducts in ERKO mice failed to reabsorb water sufficiently, resulting in the accumulation of fluid and spermatozoa, testicular blockage and atrophy. Later studies by Hess and others suggest that androgens may also play a role in the regulation of water reabsorption by the efferent ducts.[581] If hormones such as estrogen and androgen play a role in water reabsorption in the efferent ducts, it will be of interest to identify the downstream targets of androgens and estrogens in order to uncover the mechanisms by which hormones regulate water movement.

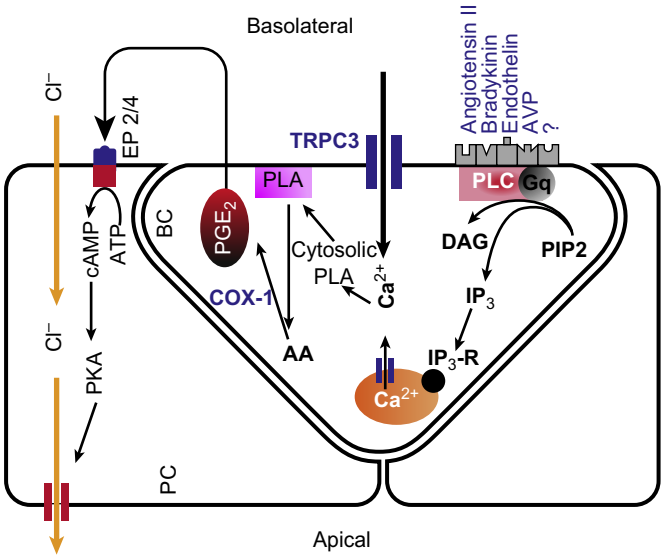

FIGURE 17.17 Schematic diagram illustrating the roles of COX-1 and Trp3 in the regulation of principal cell functions by basal cells in the rat epididymis. *Source: Modified from Ref. 172. Kindly supplied by Dr Patrick Wong.*

ORGANIC SOLUTE TRANSPORTERS

Few studies have focused on the physiology of organic solute transporters, although many have been identified in gene arrays.[433,475,607,611] This aspect of the review will only focus on those transporters that have been identified to function in the epididymis.

L-CARNITINE TRANSPORTERS In 1998, two independent laboratories reported the cloning of a high affinity L-carnitine transporter, OCTN2, isolated from human and rat small intestine cDNA libraries.[926,927] OCTN2 is a member of the organic cation transporter family. Thus far, only three members of this family, OCTN1 (SLC22A4), OCTN2 (SLC22A5) and OCTN3 (SLC22A21), have been shown to transport L-carnitine (reviewed in Ref. 928). OCTN2 transports L-carnitine against a concentration gradient in a sodium-dependent manner. In contrast, OCTN1 is a low-affinity, sodium independent carnitine transporter,[929,930] and OCTN3 is a high-affinity sodium independent carnitine transporter.[931,932]

The *Octn2* cDNA encodes a 557 amino acid protein with a predicted molecular mass of 63 kDa. Mutations in *Octn2* are responsible for primary carnitine deficiency, an autosomal recessive disorder characterized by cardiomyopathy and muscle weakness.[933–935] Hence, OCTN2 is a key protein involved in L-carnitine transport. OCTN2 is localized to the basolateral membranes of the rat epididymis in a region-specific manner, with highest mRNA and protein expression observed in epididymal regions involved in L-carnitine transport.[515] Therefore, OCTN2 is the prime candidate for transporting L-carnitine into epididymal cells.

The function of L-carnitine in the epididymis is unknown but the hypothesis that OCTN2 plays a role in the protection of osmotic stress in the epididymis is supported by an intriguing observation. Toshimori et al.[936] described an animal model for primary carnitine deficiency, the juvenile visceral steatosis (*jvs*) mouse. These mice exhibit symptoms similar to those of patients suffering from primary carnitine deficiency. Studies by Lu et al.[937] demonstrated that mutations in *Octn2* were responsible for the *jvs* phenotype, and complementation with human OCTN2, but not OCTN1, rescues the phenotype in the *jvs* mice.[938] Loss of OCTN2 results in the loss of integrity of the epididymal epithelium in *jvs* mice, especially within the corpus region, a site known to express high levels of OCTN2 and readily transport L-carnitine. Therefore, it would seem plausible that loss of epididymal epithelial integrity in *jvs* mice may be the result of osmotic stress. Further evidence to support this hypothesis comes from the studies of Cotton et al.[517] who showed that epididymal OCTN2 transcriptional activity is regulated by osmotic stress.

A putative protein that transports L-carnitine, CT2, from cells into the lumen has been identified.[412] Although initially thought to be a testis-specific transporter, immunohistochemical studies clearly demonstrate its presence on the apical surface of human epididymal cells.[516] The transporting characteristics and kinetics of CT2 are similar to those previously reported for an apical L-carnitine transporter in the rat;[939] we propose that they are, in fact, the same transporter. CT2 is a little unusual in that it can transport L-carnitine in either direction; transport is partially dependent upon the presence of sodium (in both directions), and the transporter has limited substrate specificity.[516] CT2 may have multiple functions despite its narrow substrate specificity. Certainly, from the distal caput to proximal cauda epididymidis, it functions to transport L-carnitine from cells into the lumen. However, it can also transport solutes from the lumen into cells,[939] suggesting that CT2 ensures that any L-carnitine that does enter the lumen proximal to the distal caput is rapidly removed and/or that it can transport other solutes yet to be identified.

TAURINE TRANSPORTERS Although not shown to specifically transport taurine into the epididymis, the taurine transporter TauT and the putative taurine channel phospholemman are expressed in the epididymis, with the more distal epididymal regions showing higher expression.[737] Although these studies were performed using the mouse epididymis, these data coincide nicely with the measurement of intraluminal concentrations of taurine in the rat epididymis; the values measured were: corpus (~6 mM) > cauda (~3 mM) > caput (~2 mM).[415]

GLUTAMATE TRANSPORTERS The expression of glutamate transporter *Eaac1* and the glutamate transporter-associated protein (*Gtrap)* have been identified in the epididymis.[940] Although the expression of both of these genes is high in the caput region where the highest concentration of glutamate is observed (~50 mM[523]), expression is also high in the distal epididymal regions. This would suggest that the protein is transporting glutamate into the lumen in the proximal epididymal region, that the protein is transporting glutamate out of the lumen in the distal epididymal regions because the intraluminal concentration of glutamate rapidly declines from the caput to cauda epididymidis,[523] and/or that the protein may be transporting other amino acids. Since many amino acid transporters are not specific for individual amino acids and some are bidirectional, then all three possibilities are plausible. Definitive functional studies are needed to confirm that either EAAC1 and/or GTRAP are responsible for the high intraluminal concentrations of glutamate. An alternative possibility for the high concentrations of glutamate is the metabolism of intraluminal glutathione by gamma-glutamyl transpeptidase.[941]

CE11/TSCOT/SLC46A2 A transporter called CE11 has been identified in the canine epididymis that has 12 transmembrane domains and functions as a co-transporter. The transporter has homology to the thymic stromal cotransporter (TSCOT; SLC46A2) with some similarity to a sugar transporter.[942] The transporter has been localized to the apical membranes of the canine epididymis and its function is not known.

ABC TRANSPORTERS ABC transporters (ATP-binding cassette) are a family of transporters that are responsible for the efflux of many xenobiotics and toxicants from cells and are expressed in a wide array of tissues.[943–945] ABCB1 is expressed in the clear cells of the epididymis, the epididymal endothelial cells and epididymal spermatozoa.[946] The exact role of this transporter is unclear, but its presence may be necessary for removal of xenobiotics for protection of the epididymal epithelium and spermatozoa.

Antimicrobial Peptides

Groups of epididymal proteins belonging to the defensin and defensin-like family and other proteins having antimicrobial properties were identified in the epididymis. The presence of antimicrobial peptides in both reproductive and non-reproductive tissues has long been acknowledged.[947–949] but recently several antimicrobial peptides have been shown to be epididymis specific. Some of these are members of the β-defensin family, beta-defensin-like peptides (e.g. bin1b, EP2 and HE2 variants), lactoferrin, cystatins, Eppin, and the cathelicidins (hCAP18, rCRAMP).[950–971]

The vertebrate defensin family of genes is comprised of two major groups, the alpha and beta defensins. The classic defensin motif consists of six cysteines linked by three intramolecular cysteine disulphide bonds, which differ in topology in the alpha and beta groups. Alpha defensins are produced mainly in neutrophils and Paneth cells, while the beta defensins are widely expressed in epithelial tissues, including those of the respiratory, gastrointestinal and reproductive systems and the skin. Defensins are synthesized as preproproteins that are subsequently cleaved to generate the active cationic peptides of approximately 3–5 kDa.[947,956] It has been suggested that, in the epididymis, a furin-like proprotein convertase is responsible for the cleavage.[957] The defensin genes are also clustered on certain chromosomes, chromosome 8 in human.[972,973] A study by Zaballos et al.[965] using the Celera mouse genome database found exons encoding 23 different β-defensins. Using a combination of RT-PCR and in situ hybridization, these workers found nine of

these genes to be highly specific for the epididymis and expressed in distinct epididymal regions. Gene microarrays have identified multiple defensins along different regions/segments of the epididymis.[608,611]

Members of the β-defensin and β-defensin-like family are expressed in the epididymal epithelium in a region-specific manner and appear to be secreted into the epididymal fluid as demonstrated by a variety of techniques including RT-PCR, in situ hybridization, western blot analysis and immunolocalization.[950,954,957–963,965–968,974,975] The androgen regulation of epididymal β-defensins is still unclear. The expression of *Bin1b* in the rat epididymis,[954] and *He2* expression in the non-human primate epididymis are androgen dependent.[950] Although rat β-*defensin1* is androgen-regulated in the initial segment and caput regions, this may not be the case in the more distal epididymal regions. To add to the complexity, *rat* β-*defensin1* expression in the initial segment/caput regions appears to respond slowly to androgen deprivation, suggesting that the action of androgens may be indirect.[960] *He2* expression in the human epididymis does not appear to be androgen regulated, but the subcellular distribution of the HE2 variants is androgen-regulated.[957] Expression of human β-*defensin 118* (previously called ESC42) in the non-human primate is primarily in the caput region and is androgen regulated. Hence, more studies are warranted to clearly define the androgen regulation of these genes.

Although one would suspect that members of the β-defensin family would be upregulated during inflammation, it turns out that, at least for rat β-*defensin-1*, this gene was not upregulated during inflammation of the epididymis induced by lipopolysaccharide from *Pseudomonas aeruginosa* or *Escherichia coli*.[960] Rat recombinant β-defensin 22, an epididymal-specific β-defensin, has been shown to have antimicrobial activity against *E. coli* and *Candida albicans*.[975] Similarly, rat epididymis-specific β-defensin 15[976] and BIN1b[977] have antimicrobial activity. An increase in resistance to infection was observed when *Bin1b* was overexpressed 50 times its normal level in the caput region of the mouse epididymis.[977] Further, these investigators showed a decrease in the levels of IL1α and IL1β in the caput region, suggesting a role for BIN1b in the regulation of inflammation.

Another β-defensin-related member that is beginning to receive considerable attention is SPAG11, which is found in the epididymis of several species.[978–981] The SPAG11B/D splice variant has been shown to bind to a tryptase alpha/beta1, a protein involved in inflammatory responses.[980]

The structure-function relationship of the defensins is complex. Outside of the defensin motif, their sequences vary greatly.[982] The antimicrobial activities of human β-defensin 3 (HBD3[983]) and HE2[957,967] are very much dependent on both charge and structure. In the case of HE2 expressed in the chimpanzee and in the human,[950,957] two major isoforms are found in the epididymis, HE2α1 and HE2β1, each related to the β-defensin gene family. HE2β1 clearly has the β-defensin motif and antibacterial activity, yet the processed form of HE2α1 does not have the β-defensin motif but does have antibacterial activity. This suggests that the human epididymis secretes a novel class of antimicrobial peptides.[957]

Cathelicidins are another group of antimicrobial peptides that has received attention recently. The human cathelicidin *hCap18* is expressed in the epididymis and associates with prostasomes (exosomes) in seminal fluid and with spermatozoa.[951,952,984] Although hCAP18 is processed in neutrophils by proteinase 3,[985] it is processed in seminal fluid by a prostate-derived protease, gastriscin, to generate a 38 amino acid antimicrobial peptide ALL-38. Presumably, this antimicrobial peptide plays an important role in preventing vaginal bacterial infections following intercourse and ejaculation. Cathelicidins have also been identified in other species, for example, rat and mouse cathelin-related antimicrobial peptide (CRAMP), and have been shown to have antimicrobial properties[887–989] Members of the family of lipocalins are actively synthesized and secreted by the epididymis of most mammals and have been proposed to play an active role in immune and inflammatory responses.[990,991]

In addition to their antimicrobial activity, several of these peptides have additional functions. For example, BIN1b[969,992] and β-defensin 15[976] appears to be involved with the acquisition of sperm motility in the epididymis and interfere with the ability of spermatozoa to fertilize an egg. It is intriguing that these peptides have evolved to have multiple functions in this tissue. Hence, future studies should help our understanding of how these peptides are indeed bifunctional, and of the mechanisms by which they promote sperm maturation and maintain antimicrobial activity.

Lipocalins

Several proteins binding and transporting small hydrophobic ligands and belonging to the lipocalin family have been identified in the epididymis. Some of these have been introduced earlier for their role in mediating retinoic action in epididymal epithelial cells. We will focus here on the secreted proteins that serve a carrier function.

EPIDIDYMAL RETINOIC ACID BINDING PROTEIN (E-RABP)

E-RABP is one of the major epididymal secreted proteins. It was first identified as one of the four prealbumin proteins migrating after polyacrylamide gel electrophoresis of rat and mouse epididymal cytosol,[993] and luminal fluid,[142,994] with an apparent molecular weight of 18,500. The mouse and rat proteins were subsequently

purified,[694,995] and the cDNA isolated.[694,996,997] In other studies, two retinoic acid binding activities were found after separation by ion exchange chromatography of rat and mouse caudal epididymal fluid.[998–1000] After purification and N-termini sequencing,[999,1001] these proteins were found to be identical to proteins identified by Brooks et al.[694] and Rankin et al.[1000] The predicted amino acid sequence, the ligand binding specificity, and the X-ray crystal structure showing that the protein has a β-barrel structure and a hydrophobic ligand pocket, indicated that E-RABP belonged to the lipocalin family of small extracellular hydrophobic binding proteins.[1002,1003]

E-Rabp is present as a single copy gene in both the rat and mouse. The gene structures are similar but not identical. The rat *E-Rabp* gene is unusual compared to other lipocalins in that it has eight exons instead of seven, the more usual number. The cDNA encodes a protein of 188 residues with alternative sites for signal sequence cleavage to give secreted proteins composed of 166 and 169 amino acid residues. In contrast, the murine gene has seven exons, as seen in other lipocalins.[1003] The cDNA encodes a protein of 185 residues, also with alternative sites for cleavage of the signal sequence to give secreted proteins of 163 and 166 residues. The exon/intron boundaries of the first seven exons are identical for rat and mouse and the first five exons are quite similar in size to the exons of other members of the lipocalin family. The murine gene is located within the [A3] region of chromosome 2. An analysis of the nucleotide sequence of the flanking regions of the *mE-Rabp* gene (*Lcn 5*) showed the presence of six new genes, *Lcn 8*, *Lcn 9*, *Lcn 10*, *Lcn 11*, *Lcn 12*, and *Lcn 13* that evolved by gene duplication. The new genes encode proteins of 175, 178, 182, 170, 193, and 176 amino acids, respectively, with a signal peptide of 16–21 amino acids, consistent with these proteins being secreted proteins.[1004,1005] Within this cluster of genes, *Lcn 5*, *Lcn 8*, *Lcn 9*, *Lcn 10*, *Lcn 12*, and *Lcn 13* are specifically expressed in the mouse epididymis. However, each gene has a distinct spatial expression within the epididymis and a different regulation. *Lcn 8* and *Lcn 9* are expressed in the initial segment only, *Lcn 5* is expressed in the distal caput only, while *Lcn 10* is expressed in the initial segment but also in the upper margin of the distal caput. The *Lcn 8*, *Lcn 9*, and *Lcn 13* genes are strictly dependent on testicular factors circulating within the luminal fluid. The *Lcn 5* and *Lcn 12* genes are dependent on androgens circulating in the serum, whereas *Lcn 10* gene expression is regulated both by circulating androgens and by testicular factors supplied from the luminal fluid.

A computational analysis of the human genome identified five putative genes (*E-Rabp* (*Lcn 5*), *Lcn 8*, *Lcn 9*, *Lcn 10*, and *Lcn 12*) encoding homologous lipocalins. These genes were localized on human chromosome 9q34.[1005,1006] The genomic organization, chromosomal arrangement, and orientation of these human genes were similar to those of the murine genes.

The cluster of lipocalin genes expressed specifically in the epididymis is localized within an area rich in lipocalin genes on mouse chromosome 2 and human chromosome 9.[1007] Interestingly, this epididymis-specific lipocalin cluster is flanked by other members of the lipocalin family, such as *Pgds*, and *Lcn 2*,[1005,1007] that are expressed in other tissues besides the epididymis.[1008,1009] This suggests that the epididymis-specific lipocalin cluster may contain a locus control region that enhances lipocalin gene expression only in the epididymis, while genes flanking either side of this epididymal gene cluster show expression in other tissues besides the epididymis.

More recently, a new member of the lipocalin family, *rLcn9*, has been cloned from the rat epididymis,[1010] mouse epididymal lipocalin promoters have been further characterized[852] as well as distinct epididymal lipocalins, for example Lcn6 and 12.[1011–1013]

LIPOCALIN TYPE PROSTAGLANDIN D SYNTHASE

Prostaglandin D Ssynthase (PGDS), formerly identified as β-trace, was first shown to be localized in the central nervous system and secreted in cerebrospinal fluid.[1014–1019] PGDS is also localized in the male genital tract where it constitutes one of the major secreted epididymal proteins in many mammals: mouse,[1020–1022] rat,[1008,1021–1023] hamster,[1021] ram, stallion,[1024,1025] bull,[1026] monkey,[1027] and man.[1028] The cDNA for PGDS was first isolated from a rat brain library,[1029] and subsequently from many species, including man and zebrafish.[1024,1026,1030–1035] The cDNA for L-PGDS encodes a protein of 180–190 amino acid residues with a signal peptide of about 20 amino acids removed in the mature protein and 2 N-glycosylation sites. The tertiary structure of the rat and mouse recombinant proteins identified PGDS as a lipocalin.[1027,1036] The gene organization is similar to that of other lipocalins in terms of number and size of exons and phase of splicing introns.[1037–1039]

Within the epididymis, PGDS distribution varies among species. In the mouse, *Pgds* mRNA is not detected in the initial segment, but is expressed in all the other regions of the epididymis,[793] while in the rat,[1008,1040] ram, stallion,[1024] and bull[1041] there is a higher expression of *Pgds* mRNA in the caput than in the cauda epididymidis. In rodents, the secreted protein accumulates in the caudal fluid of mouse, rat, and hamster,[1021,1026] while in ram and stallion the highest concentration of the protein is found in the fluid of the proximal epididymis.[1024]

PGDS expression is regulated by androgens, increasing in parallel with endogenous androgen levels during sexual maturation, decreasing after castration, and being restored with exogenous androgens.[1008,1022,1024,1040]

LIPOCALIN 2 (LCN 2)

Lipocalin 2, also known as NGAL, uterocalin, *neu*-related lipocalin, and 24p3, was originally identified as a component of neutrophil granules, but is also expressed

in epithelia in response to inflammatory signals.[1042] It is present in epididymal fluid, although it is not one of its major components. This protein is expressed in various organs but, within the male reproductive tract, is restricted to the epididymis.[1009] LCN 2 is a 25 kDa protein originally purified from human neutrophils.[1043] It is a 178 amino acid protein with glycosylation sites and a 20 amino acid signal peptide.[1044–1047] X-ray crystallography revealed that the protein has the typical lipocalin structure, but the calyx is shallower and broader than in other lipocalins.[1048] Within the epididymis, LCN 2 is expressed in the caput,[1009] under androgen regulation;[1004] the protein accumulates in the caudal fluid.[1009] Interestingly, and unlike E-RABP, sequential washing of spermatozoa with low salt and high salt left a substantial amount of LCN2 immunoreactivity in the Triton X-100 and the SDS sperm extract, suggesting that LCN 2 is bound to spermatozoa.[1009]

LIPOCALIN FUNCTION

Lipocalins form an ancient family of proteins widely distributed among species with the phylogenetic tree rooted in bacteria.[1049] *L-pgds* gene is found in fish, amphibians, and many mammalian species,[1027] and is believed to be the ancestral gene of the epididymal lipocalin cluster,[1005] while the *E-rabp* gene is found in reptiles,[1050] and mammals, including man.[1005] Genes preserved in different lineages are likely to possess important biological functions that are conserved from reptiles to primates. In addition, gene duplication provides genetic redundancy and improves the fitness of the organism.[1051] However, gene duplication also allows adaptation and functional innovation.[1052,1053] Species comparison, even over short evolutionary distances, reveals dramatic expansions and contractions of gene families.[1054–1058] It is all the more remarkable that the organization of the lipocalin gene cluster is conserved in mouse and man with similar gene number, order, and orientation.[1005] This implies that functional constraints have maintained the integrity of the cluster since their last common ancestor 75 million years ago.

The basic function of lipocalins, determined by X-ray crystallography, is to bind small hydrophobic ligands. The folding of the protein into a barrel structure with a deep calyx is well suited for this function. As extracellular secreted proteins, lipocalins function as carriers for these ligands, and their biological properties depend on their respective ligand. However, endogenous ligands are known for only a few lipocalins because the methods of isolation used disrupt the complexes. The endogenous ligands of LCN 1 (tear lipocalin) belong to a class of lipophilic molecules such as fatty acid, phospholipid, glycolipid, and cholesterol.[1059] By scavenging these molecules, LCN 1 may act as a general protection factor for epithelial surfaces. In vitro LCN 1 also binds retinol.[1060] In addition, LCN1 binds to microbial siderophores with high affinities and inhibits quite effectively bacterial and fungal growth under iron limiting conditions. Therefore, as discussed above, LCN 1 appears to be part of the complex defense system involved in protection against harmful molecules and microbial and fungal infections.[1061] LCN 2 is the only known endogenous ligand of the lipocalins cluster on human chromosome 9. Its ligand is a breakdown product of enterobactin, a siderophore that is used by bacteria for uptake of the essential nutrient iron.[1062] This is consistent with the potent antimicrobial action of LCN 2.[1063] LCN 2 is also upregulated in response to inflammatory stimuli.[1064,1065] This suggests that LCN 2 may have a role in the innate immune response to bacterial challenges in the epididymis. Mouse LCN 2 has also been reported to deliver ferric iron to spermatozoa through internalization,[1066] and to enhance sperm motility through an elevation in intracellular pH and an increase of intracellular cAMP accumulation.[1067] LCN 2 may have a dual function role in the epididymis.

Ligand affinity of the other lipocalins has been determined in vitro. L-PGDS binds all-trans or 9-*cis* retinoic acid and all-*trans* or 13-*cis* retinaldehyde, but not all-*trans* retinol.[1068] Also, it binds thyroid hormone, biliverdin, and bilirubin.[1069] However, similar to BIN1b and Eppin, discussed above, PGDS is a dual function protein. Within the cell, it acts as an enzyme producing PGD_2, a potent endogenous somnogen and pain modulator as well as anti-inflammatory agent, and after secretion, it functions as a lipophilic ligand carrier. This dual function may reflect subfunctionalization within the lipocalin cluster. After inactivation of the PGDS gene, the main abnormalities that occur are in the regulation of sleep and pain responses, but no obvious phenotype was reported in the male genital tract, reflecting perhaps a built-in redundancy in the lipocalin cluster.[1070] Nevertheless, PGDS has been reported to be a biochemical marker of sperm quality in human semen,[1071] and to be correlated with fertility in bulls.[1026] Another study failed to find the correlation statistically significant, although the lowest PGDS contents were found in rams and bulls with the lowest fertility and the highest content in animals with normal or high fertility.[1021] This suggests that PGDS affects male fertility, but its role is either not essential or can be compensated by other lipocalins in the cluster.

It is likely that retinoic acid is the endogenous ligand for E-RABP, which binds in vitro all-*trans* and 9-*cis* retinoic acid, but not retinol. E-RABP inactivation produced a phenotype similar to that seen in transgenic mice expressing a dominant negative mutation of retinoic acid receptor α, albeit with a lower severity and penetrance.[776] The transgenic mice expressing the dominant negative RARα were either infertile or had reduced fertility. The epithelium of the cauda epididymidis had undergone squamous metaplasia with tubule rupture, sperm leakage into the connective tissue, and inflammatory reaction. This indicates that interaction of retinoic acid with its receptor

is essential for the maintenance of the epithelium of the cauda epididymidis. These observations are consistent with E-RABP functioning as an intraluminal carrier protein delivering retinoic acid to the epithelium of the cauda, and the fact that the epithelium of the cauda epididymidis is most affected in VAD.[1072]

In summary, gene duplication of a PGDS-like lipocalin gave rise to a new epididymal lipocalin family as amniotes appeared during evolution. Internal fertilization is a common feature of amniotes and this process leads to complex and changing environmental conditions for male gametes. Gene duplication which led to an epididymal lipocalin gene cluster may have allowed an early adaptive response to a new environment; its conservation through 250 million years strongly suggests that the epididymal lipocalins have important functions.

Lipocalins on mouse chromosome 2 and human chromosome 9 are involved in immunoregulation, anti-inflammatory, and antibacterial responses.[990] As discussed above, the epididymis has a complex host defense system involving, in particular, the defensins. It is likely that the lipocalins of the epididymal cluster are part of this network, either by their antimicrobial action, as LCN 2, or by preserving the integrity of the luminal compartment of the epididymis as E-RABP (LCN 5).

CYSTATIN-RELATED EPIDIDYMAL SPERMATOGENIC (CRES) PROTEIN

CRES (*Cst8*) belongs to the cystatin family of proteins, albeit within its own subgroup because it does not contain the consensus sites for cysteine protease inhibition.[1073,1074] This protein has received considerable interest because of its putative antimicrobial role.[1075] Mice with a CRES null mutation have reduced fertility;[1076] this was due to the failure of spermatozoa to undergo capacitation. Addition of dibutyryl cAMP and 3-isobutyl-1-methyxanthine to the spermatozoa from *Cres* mutant mice reversed the defect. Further, CRES appears to play an important role in the formation of amyloid. The precise function(s) of these aggregations of amyloid, which are found within the epididymal luminal fluid, is/are unknown but Cornwall et al.[1074,1077] suggest roles in cell–cell communication, mediation of protein–protein interactions, delivery of proteins to the maturing spermatozoa and/or acting to remove secreted proteins from the lumen.

AGING

Effects of Aging on the Appearance of the Epididymal Epithelium

As discussed above, numerous studies have focused on the structure, function, and regulation of the epididymis during pre- and postnatal development and in the adult, yet very few investigations have been undertaken of the effects of senescence on epididymal structure and function. In the aging rabbit, Cran and Jones[1078] showed that there was an absence of spermatozoa in the epididymal lumen, an accumulation of lipofuscin pigmented bodies in principal and basal cells, cytoplasmic vacuoles in the distal segments, and intranuclear inclusions. In the cat epididymis during aging, Elcock and Schoning,[1079] observed occasional intraepithelial cysts and hyperplasia of the epithelium, as well as eosinophilic to amphophilic round cytoplasmic bodies in interstitial cells, and proteinaceous luminal debris. In the hamster, Calvo et al.[1080] found that the cauda epididymidis was particularly affected by aging; the epididymis of aged hamsters presented involutive changes including a decrease in tubular diameter in the cauda region. Principal cell ultrastructure showed the appearance of damaged mitochondria and bundles of filaments. The presence of large electron-dense vacuoles in some clear cells was unusual. Decreases in sperm concentration and an apparent decrease in motility were noted also. Decreased epididymal sperm counts, brown patches or lipofuscin, arteriosclerosis, lymphocyte infiltration, and the emergence of sperm autoantibodies have been reported in other apecies: rat,[1081,1082] monkey,[1083] and human.[1084,1085]

Results from many of these studies are difficult to interpret because separating direct from indirect effects can be challenging. Determining whether the age-dependent effects observed are due to aging of the tissue per se or to other conditions, such as decreased androgen prodcution, arthroscleroses or carcinoma in other tissues (e.g. pituitary) that impinge on the epididymis renders data interpretation difficult at best. The Brown Norway rat has been established as a valuable model for the study of the aging male reproductive system.[144,1086–1090] This strain of rat has a long life span, does not exhibit many of the age-related pathologies found in other rat strains, such as pituitary and Leydig cell tumors, does not become obese, and is the first rat strain to have had its genome sequenced;[1086,1087,1091] similar to humans, aging of the male leads to dramatic changes in the seminiferous epithelium and to decreases in spermatogenesis and steroidogenesis,[1091–1095] with no decrease in the concentrations of gonadotropins.

Using the Brown Norway rat as a model to study aging of the epididymis, several clear observations have emerged.[153,175,212] Although the luminal diameter and epithelial height were not affected by age, there was a marked progressive increase in the thickness of the basement membrane during aging. In young rats, the relative contribution of clear cells increased in the distal segments, resulting in a relative decrease in the numbers of principal cells, while halo cells were rare. With increasing age, there was a decrease in the proportion of principal cells and basal cells in

all segments of the epididymis, of narrow cells in the initial segment, and of clear cells in the corpus epididymidis. This decrease was accompanied by a proportional striking increase in the number of halo cells in each segment.[153]

Several dramatic changes occur along the epididymis in specific cellular structures during aging.[153] In the initial segment, basal cells emitted an extensive network of pseudopods extending downward toward the basement membrane, and the number and size of halo cells increased progressively during aging; whether aging results in an altered pattern of dendritic cells[176] has not been determined. In the caput epididymidis, principal and basal cells did not appear to change while clear cells changed dramatically; they appeared swollen and bulged into the lumen; their nuclei were irregularly shaped and displaced to the upper half of the cell; lysosomes, enlarged and dense, were often fused with lipid droplets; and the presence of lipofuscin became evident. In the corpus epididymidis, a remarkable increase in the size and number of lysosomes was seen in principal cells. The emergence of a localized region with large vacuoles reflected the major effect of age in the proximal distal corpus/proximal cauda epididymidis. Although some principal cells in old animals had a normal morphology, many others contained extremely large vacuoles. Endosomes and lysosomes often appeared to be emptying their contents within the large vacuoles, and debris from spermatozoa was found in giant vacuoles. Serial sections of these vacuoles revealed that entire spermatozoa were found in the vacuoles, thus leading to the hypothesis that mechanisms controlling intracellular trafficking are impaired as animals age.[153]

With increasing age, there was a dramatic increase in the number of halo cells (composed of monocytes, helper T lymphocytes, and cytotoxic T lymphocytes) throughout the epididymis in a segment specific manner.[175] There is a segment specific recruitment of cytotoxic T lymphocytes and monocytes-macrophages in the epididymal epithelium of aged rats whose epididymal lumen contain few spermatozoa. Thus, accumulation of damaged epithelial cells and antigens of germ cell origin, leaking through a dysfunctional blood–epididymis barrier, may contribute to the active recruitment of immune cells with age.[212] This age-dependent increase in the number of halo cells in the epididymis, in conjunction with the fact that the blood–epididymis barrier should protect from immunological attack, led to investigation of whether there are changes in the structure and function of this barrier with age in the Brown Norway rat model.[212] Based on changes in the distribution of several immunocytochemical markers of this barrier (occludin, ZO-1, and E-cadherin), as well as in lanthanum nitrate permeability, a clear breakdown of the barrier during aging was shown to occur selectively in the corpus epididymidis.

Molecular and Functional Effects of Aging in the Epididymis

Using both gene expression profiling (cDNA microarrays) and Northern blot analysis, segment specific changes have been found to occur along the epididymis during aging.[604,1095] The overwhelming effect was a decrease in gene expression during aging. In the initial segment, corpus and cauda epididymidis, more genes decreased in expression with age than did not change. Interestingly, the magnitude of the decreases in expression was considerably larger in the corpus and cauda epididymidis, where expression of 83% and 62%, respectively, of the genes decreased by greater than 50% with age. This is in contrast to the initial segment where only 31% of the genes expressed decreased by at least 50%; the caput epididymidis was the only segment in which the expression of a large proportion of the genes did not change with age. No transript showed increased expression with age in any of the four segments of the tissue; however, the expression of four transcripts was increased in a segment-specific manner.[604] These studies were done prior to the availability of rat whole transcriptome analysis or deep sequencing and should be revisitied with more in depth transcript analysis.

Nonetheless, changes in the expression of specific genes/gene families are of particular interest. The expression of the mRNAs for SRD5A (particularly the type 1 isozyme) is among the first to decline during aging, while the message for the androgen receptor is not affected.[1095] Changes in the expression of components of intracellular degradation pathways (i.e. proteasome components) are most dramatic in the distal epididymis. While in the initial segment and caput epididymidis, either no change or small decreases (less than 33%) are seen with age, in the corpus and cauda epididymidis essentially all proteasome components show remarkable decreases in intensity of expression (greater than 67%). Cathepsins, a family of cysteine proteases implicated in proteolytic processes, are abundantly expressed in the epididymis.[611,1095–1097] Of the six cathepsin transcripts detected in the epididymis, the relative intensity of cathepsin K was affected by age to the greatest extent in the initial segment, while that of cathepsin H and B decreased most in the more distal segments of the epididymis; cathepsin E transcripts decreased to below the level of detection in all regions of aged epididymides. Oxidative stress related genes, such as copper-zinc superoxide dismutase (Cu-Zn SOD1), showed a small decrease with age in the initial segment, but a very large decrease in the corpus epididymidis. The relative intensity of expression of

glutathione synthetase decreased dramatically with age in the initial segment, corpus and cauda epididymides, while epididymal secretory glutathione peroxidase (GPX5) showed the largest decrease in relative intensity in the initial segment and GPX4 showed the largest decrease in relative intensity in the cauda epididymidis.[604] The changes seen with age for many of these transcripts are consistent with what has been observed by others on the effects of aging in various tissues in a wide spectrum of animals from flies[1098] to mice (muscle[1099] and brain[1100]).

Mechanism(s) Underlying Aging of the Epididymis

Numerous hypotheses have been put forward regarding the underlying cause(s) of aging. These include the specific gene theory,[1101] such as the klotho gene,[1102] the telomere theory,[1103] altered gene expression due either to mutations[1104] or altered DNA methylation status,[1105] the network or immune theory of aging,[1106] and the free radical (oxidative stress) theory.[1107] However, there is no consensus regarding any one hypothesis, and too few studies have been done on the epididymis to demonstrate the primary role of any one or any combination of these theories. There is a significant recruitment of immune cells into the epididymis as animals age, and this recruitment is greatest in parts of the tissue exhibiting greatest damage.[175,212] Although it is possible that this immune response is a cause of the epithelial damage, it is perhaps more likely that the damaged tissue and ruptured blood–epididymis barrier are the attractants of immune cells. It was also shown that the amount of apoptosis seen in epididymides was higher in aged than in young mice, and that this increased rate of apoptosis could be prevented by the administration of testosterone.[1108]

There are several lines of suggestive evidence that point to the association of oxidative stress with changes induced by aging in the epididymis. These include region-specific accumulation of lipofuscin,[1109] changes in the distribution of glutathione S-transferases,[1110] formation of intracellular vacuoles,[153] and altered expression of genes associated with oxidative stress.[604] Therefore, it is reasonable to speculate that oxidative stress is a key factor in mediating the dramatic response of the epididymis to age. Knockout of *Nrf2* (nuclear factor-erythroid 2-related factor 2), the factor that regulates basal and inducible transcription of key genes for protection against ROS, results in an age-dependent decrease in sperm motility, further supporting the likely role of oxidative stress damage to the epididymis during aging.[1111] However, we still know little about whether there are changes with age in the ability of the epididymis to respond to stress or altered endocrine environments.

In addition, two issues have been addressed with respect to the effects of advancing age on epididymal functions: the first relates to tissue weight, sperm number, and the effect of sexual experience on sperm number in this tissue,[1112] and the second to the effects of testosterone administration on sperm number and sperm maturation status ("sperm profile").[1081] Sexual experience in rats was shown to lead to an increase in epididymal sperm counts, which peaked at 9 months but returned to within 5% of that at 3 months when animals were 24 months old. Only the weight of the epididymides increased between 3 and 24 months, reaching a value at 24 months that was 28% greater than that of the 3 month old animals. However, sexually inexperienced rats had a 31% decrease in epididymal spermatozoa between the ages of three and 24 months, while epididymal weight still increased by 25%.[1112] The lack of correlation between epididymal weight and epididymal sperm content is completely unexpected and provides an important clue to potential histological changes taking place within the tissue. The administration of exogenous testosterone could not prevent the decrease in sperm number, but did prevent the changes seen in sperm profile, suggesting that the epididymis of aged animals retains its ability to respond to endocrine manipulations.[1081]

Changes in Spermatozoa during Aging

Several clinical studies, as well as studies using models of animal aging, indicate that there are significant changes that occur in spermatozoa as males enter advanced age and that such changes have consequences for the progeny.[1113–1118] Determining whether these changes are due to alteration taking place in the testis or the epididymis is challenging. The existence of a paternal age effect on semen parameters, fertility, and anomalies in children demonstrates that the quality of spermatozoa and the genetic integrity of otherwise apparently healthy spermatozoa are not immune to the effects of paternal age.

Although genetic changes in spermatozoa during aging, such as non-dysjunction and autosomal dominant disorders that exhibit paternal age effects, such as Down's[1119] and Klinefelter syndromes[1120] and achondroplasia,[1121] are clearly of testicular origin, others, such as changes in the DNA methylation pattern (epigenetic changes),[1122] altered sperm motility and retention of cytoplasmic droplets are more likely to have an origin in the epididymis. Using the Brown Norway rat as a model for male reproductive aging, it was shown that there is a marked increase with age in the number of abnormal flagellar midpieces of spermatozoa, that the percentage of motile spermatozoa was significantly decreased in the cauda epididymidis of old rats, and that the proportion of spermatozoa that retained their

cytoplasmic droplet was markedly elevated.[1123] Furthermore, the extent of chromatin breaks is associated with advancing age and the ability to mount a response to oxidative stress decline with age.[1124–1126] Although it is now clear that germ stem cell number and quality decline with age[1127,1128] and that there are changes in the transcriptome of spermatognia and more advanced germ cells as a function of paternal aging,[1128,1129] it is likely that altered epididymal epithelial functions contribute to changes seen in ejaculated or cauda epididymal spermatozoa.

Another mechanism that may allow for sperm quality control has been observed to act at the level of the epididymis and involves the cell surface ubiquitination and subsequent phagocytosis of abnormal spermatozoa.[425] However, large-scale phagocytosis of spermatozoa by the epididymal epithelium is not observed in young or old animals.[426] The intriguing possibility that principal cells secrete into the lumen the appropriate components of the ubiquitin-proteasome pathway necessary to ubiquinate damaged sperm has been put forward.[1130] The mechanism(s) that would be used by the epididymis to identify such damaged sperm and its possible change in sensitivity with advancing age remain(s) to be determined.

The multiple effects of aging on progeny outcome, sperm morphology, the acquisition of and motility all indicate that the quality of spermatozoa is affected by aging.

THE EPIDIDYMIS AS TARGET FOR XENOBIOTICS

Xenobiotics that have been shown to act on the epididymis can be broadly divided into two major, somewhat overlapping, categories. The first consists of those chemicals for which the epididymis is the explicit target; a number of compounds that have the potential to act as part of a male contraceptive formulation fall into this group. The second is made up of therapeutic agents or known toxicants that are targeted principally at other organs but also act, as a side effect, on the epididymis. For some of these compounds, their effects on the epididymis are at the level of the epididymal epithelium, for others effects have been demonstrated on sperm traversing the epididymis, and for others still the site of action is mixed or not resolved. Various aspects of the action of drugs on the epididymis have been thoroughly reviewed.[818,1131–1136]

Demonstrating the direct action of chemicals on the epididymis is challenging because if, subsequent to administration or exposure to a chemical, there is an effect on the epididymal epithelium or on epididymal spermatozoa, it becomes essential to establish that such an effect is not mediated at the level of the hypothalamus, pituitary or testis. Furthermore, epididymal histopathology and sperm function tests have not been standard in drug development and the assessment of toxicants; hence, the action of xenobiotics on this tissue may often have been overlooked.

Chemicals for Which the Epididymis Is an Explicit Target

Selective modification of functions carried out either in or by the epididymis should be of major value not only in the development of male contraceptives, but also for the treatment of male fertility problems with an etiology that arises in the epididymis. Since spermatozoa reside a relatively short time in the epididymis and since no genetic transformation is known to occur as spermatozoa undergo epididymal maturation, the epididymis can be viewed as an ideal target for the development of male contraceptives or drugs that can block or enhance the maturation process. Although no therapeutic agent has yet been developed that meets these objectives, a large number of approaches have been pursued to attain these goals. Most of these agents can be grouped into drugs that act via modulating hormonal dependence, neuronal activity, or metabolism.

Hormonal Regulation

Although, as described above, many hormones and hormone receptors have been found in the epididymis in various species, we will focus on androgens and estrogens, hormones for which the consequences of manipulating the endocrine signal have been reported.

BLOCKING ANDROGEN ACTION

Several laboratories have determined the effects on the epididymis of agents that act as androgen receptor antagonists, such as cyproterone acetate or hydroxyflutamide.[1137,1138] These compounds successfully block epididymal functions and cause a reduction in tissue weight. Interestingly, unlike the case with other androgen-dependent tissues, such as the prostate or seminal vesicles, exposure to androgen receptor antagonists does not result in changes in intracellular androgen receptor localization in the epididymis.[1139] The advent of new antiandrogens, such as enzalutamide,[1140] is not associated with any tissue specificity as they still target the androgen receptor, as opposed to co-recptors that are more likely to be tissue specific. The therapeutic potential of these compounds as male contraceptives targeting epididymal functions is limited, however, because, as expected, the action of androgens in other target tissues was also blocked, producing side effects equivalent to a chemical orchidectomy.

BLOCKING THE FORMATION OF DIHYDROTESTOSTERONE

It has been established that DHT is essential for the maturation of spermatozoa and that the androgen found in the nuclei of epididymal cells is DHT.[637,1141] Thus, one approach to block androgen action in the epididymis is to inhibit DHT formation. Several compounds inhibit either one or both forms of SRD5A in many tissues, including the epididymis.[1142–1152] The first report was that of a family of 5,10-secosteroids that are suicide substrates (irreversible inhibitors) of the enzyme in vitro and have been shown to inhibit the epididymal enzyme.[1143] In a series of studies, Rasmusson's group[1144,1153] found that four aza steroids were effective inhibitors of steroid-5α-reductase in the prostate and a variety of other tissues. The lead compound, (diethyl-4-methyl-3-oxo-4-aza-5α-androstane-17 β-carboxamide, 4-MA), a competitive inhibitor of the epididymal enzyme,[1145,1152] ushered in a new era in potent inhibitors of this enzyme activity; this inhibitor of the epididymal enzyme was shown in one study to alter the fertility of male rats.[1153] The first commercially available inhibitor of 5α reductase, finasteride, also a member of the azasteroid family, has a predominant effect on the type two isozyme,[1154] while more recently developed agents, such as dutasteride or PNU157706, act as dual inhibitors.[1146,1155] Using PNU157706 as a model inhibitor, it was shown that treatment of adult rats with this agent results in pronounced effects on the expression of epididymal genes involved in signal transduction, fatty acid and lipid metabolism, regulation of ion and fluid transport, luminal acidification, oxidative defense, and protein processing and degradation, all processes essential to the formation of the optimal luminal microenvironment required for proper sperm maturation.[1148] Treatment of adult rats with this dual inhibitor had effects on both sperm quality and pregnancy outcome but did not result in an arrest of fertility.[1149] Interestingly, recent clinical observations[1156] associate the use of 5α-reductase inhibitors prescribed for benign prostatic hyperplasia or allopecia with a loss of fertility.

BLOCKING ESTROGEN ACTION

Although, as discussed above, the presence and selective localization of both ESR1 and ESR2 along the epididymis has now been established in several species, few studies have focused on elaborating the consequences of blocking estrogen action in the epididymis. It is clear that the removal of estrogen action (null mutations) in the efferent ducts resulted in a dramatic reduction in the fluid uptake capacity of that tissue and consequent infertility,[738] but less is know about the consequences of withdrawing estrogen from the epididymis. Some tantalizing lines of evidence are that selective blockade of estrogen action, whether by treating with an aromatase inhibitor or an estrogen receptor antagonist, resulted in blocking the oxytocin receptor in the rabbit epididymis,[278] and that by using an estrogen receptor α blocker, ICI 182,780, the apical cytoplasm of narrow and clear cells was affected.[1157]

Neuronal Regulation

An alternate means by which xenobiotics can affect epididymal functions is by either increasing or decreasing sperm transit time. Either administration of guanethidine, a drug that causes chemical sympathetectomy, or surgical sympathetectomy results in a marked increase in sperm transit time as well as in cauda epididymal weight without other apparent effects.[269,1158–1160] In contrast, several drugs accelerate transport of spermatozoa through the epididymis. In 1975, Meistrich et al.[245] demonstrated that treatment of mice with estradiol resulted in an increased rate of transport of sperm through the epididymis; similarly, diethylstilbestrol accelerates epididymal sperm transit in the rat.[1160] Administration of either chloroethylmethanesulphonate, a chemical that causes a reduction in serum testosterone, or hydroxyflutamide also led to accelerated sperm transit.[1159] Sulphapyridine, a metabolite of the antibiotic sulphasalazine, caused a marked reduction in fertility in both rats and man; although its mechanism of action has yet to be elucidated, it was also found to accelerate sperm transit through the epididymis.[1161]

Metabolism

Several compounds that act on enzymes in the cellular metabolic pathways have been tested as potential male contraceptives. The ones that have held the greatest potential will be reviewed below. However, this promising approach has yet to have a viable candidate.

α-CHLOROHYDRIN

In the late 1960s a series of studies showed that α-chlorohydrin, a monochloro derivative of glycerol, reversibly inhibits fertility in an array of species.[1162,1163] This drug is converted by glycerol dehydrogenase to 3-phosphoglyceraldehyde, an inhibitory analog of the substrate for glyceraldehyde 3-phosphate dehydrogenase (GADPH), and acts, therefore, at low doses, to inhibit this key metabolic enzyme in spermatozoa residing in the cauda epididymidis,[1164] and to reduce sperm motility.[1165] At higher doses, however, α-chlorohydrin caused the formation of spermatoceles in the caput epididymidis of rodents, resulting in the blockade of sperm transport through the tissue and the consequent atrophy of the seminiferous tubules due to increases in back pressure.[1166] Wong and colleagues,[1167] demonstrated that α-chlorohydrin blocked the resorption of sodium and water from the cauda epididymidis. Although

α-chlorohydrin inhibits a ubiquitous metabolic enzyme, its selectivity of action on spermatozoa is based on the fact that spermatozoa have a GADPH isoenzyme with a higher molecular weight that is not cytosolic.[1168] Nevertheless, severe toxicity (bone marrow depression, kidney, liver) was associated with this compound when it was tested in primates,[1163,1169] and, therefore, it will not be developed as a contraceptive for man.

6-CHLORO-6-DEOXY-GLUCOSE

In 1978, Ford and Waites,[1170] described another antifertility compound, 6-chloro-6-deoxy glucose, with the ability to inactivate spermatozoa from the cauda epididymides; it also acts by blocking glyceraldehyde-3-phosphate dehydrogenase.[1166] This compound was also found to affect different functions of the epididymis, but due to its neurotoxicity in primates (associated with its effects on glucose transport and inhibition of GADPH), it is unlikely to be accepted as a male contraceptive in humans.

GOSSYPOL

While gossypol, a chemical found in cotton seed oil, has been studied extensively as a potential male contraceptive agent,[1171,1172] its mechanism of action is still poorly understood. Although it has been shown to have a high contraceptive efficacy, its toxicity, probably mediated via a hypokalemic action, is likely to preclude the clinical use of gossypol as a "stand alone" contraceptive,[1173] it may have a role at low does in combination with steroidal contraception.[1174] Gossypol caused shedding of spermatozoa from the seminiferous tubules, separation of sperm heads from their tails, and a decrease in sperm number in the cauda epididymides.[1171] Studies by Romualdo et al.[1175] have shown that treatment of rats during the pubertal time window resulted in an increase in the appearance of round cells in the epididymal lumen; these cells were most likely of epididymal epithelial origin since they stained for cysteine-rich secretory protein CRISP-1, also known as protein E, a marker of epididymal principal cells. CRISP-1 staining was not found in testicular cells, providing further evidence that the epididymis is indeed a target organ for gossypol.[1175]

Chemicals Targeted Principally at Other Organs Which Also Act on the Epididymis

The epididymis has not usually been studied as one of the target tissues for the toxicological effects of drugs or toxicants, and consequently little is known about the importance of this tissue as a target for the toxic effects of xenobiotics. However, there are now a few instances where the epididymis has been demonstrated to be a target of drug action.

Cyclophosphamide

Cyclophosphamide, a widely used anticancer and immunosuppressive drug, has been shown to act as a male-mediated developmental toxicant.[1176] Administration of this drug resulted in an increase in the number of clear cells and in the number of spermatozoa with abnormal tails in the epididymis of rats.[134] By using several complementary approaches, Qiu et al.[233] demonstrated that the increase in post-implantation embryo loss observed among progeny sired by male rats subjected to short-term (4 or 7 days) treatment with cyclophosphamide was mediated by the action of this drug on spermatozoa while transiting through the epididymis. Whether the drug action was mediated via an effect on the epididymal epithelium or directly on spermatozoa is unresolved.

Fungicides

Several fungicides, including mancozeb, ornidazole, carbendazim, and benzimidazole, have effects on male fertility.[1177–1181] For some of these compounds, sufficiently detailed studies have been undertaken to indicate that there is a selective action at the level of the epididymis. For example, benzimidazole causes occlusion of the epididymis and the efferent ducts, and ornidazole causes a decrease in sperm motility. Whether the members of this family of drugs have a similar mechanism of action is not clear, but it is interesting to note that treatment with ornidazole caused an inhibition of glyceraldehyde 3-phosphate dehydrogenase, a similar action to that of 6-chloro,6-deoxy fructose and α-chlorohydrin.[929]

Methyl Chloride

Another mechanism by which a xenobiotic can affect the epididymis is by increasing the inflammatory response. Exposure of male rats to methyl chloride, an industrial gas, resulted in infertility (a high rate of dominant lethal mutations) that is associated with testicular degeneration, epididymal inflammation, and sperm granuloma formation. Chellman et al.[1182] demonstrated that the infertility was associated with epididymal inflammation and that treatment with an anti-inflammatory drug resulted in a reversal of these effects.

Sulfonates

Exposure to either ethyl dimethyl sulfonate or chloroethylmethanesulfonate results in what is commonly viewed as a selective destruction of Leydig cells and commensurate reduction in testosterone production.[1183] These chemicals also cause a reduction in fertility. Although, the effect on fertility may be ascribed to the reduction in testosterone, Klinefelter et al.[1184] clearly demonstrated that these chemicals have, at similar doses, a direct effect on the epididymis, independent of their action on Leydig cells. The epithelial height of cells

along the corpus and proximal cauda epididymidis was reduced, there was a reduction in the fertilizing ability of cauda epididymal spermatozoa and these spermatozoa had a reduction in protein content. Although the specific mechanism of action of this family of chemicals on the epididymis is still unresolved, a selective reduction in SP22, a protein that is specifically acquired by spermatozoa as they traverse the epididymis, has been reported.[1185]

In addition to the effects of xenobiotics seen after the exposure of adults, a number of studies have demonstrated that exposure during fetal, early postnatal or pubertal time windows can result in abnormalities in the epididymis (weight, histology) or in the spermatozoa (number, motility) present in this tissue. Some of these chemicals include dibutyl phthalate, a plasticizer,[91,1186] 2,3,7,8-tetrachlorodibenzo-rho-dioxin,[1187] and pesticides such as PCB 169 and methoxychlor.[1188]

CONCLUSION

In recent decades, we have witnessed an explosion of information on a wide range of facets of epididymal gene and protein expression and their regulation, effects of null mutation and targeted transgenic expression of specific molecules, secretion of proteins by epididymal epithelial cells and their interactions with spermatozoa, and mechanisms for creating an ever changing luminal microenvironment in the fluid surrounding spermatozoa in the duct lumen. Many of these advances have been attained thanks to the application of novel technologies that range from laser capture microscopy to gene expression profiling by microarrays, from in situ electroporation to tissue targeted gene deletions and the development of immortalized cell lines.

We are beginning to get a grasp of the remarkable complexity of the epithelium lining the epididymal duct and of the milieu it creates in the lumen to allow spermatozoa to become mature, be stored, and be protected. However, in spite of these great strides, some of the major questions relating to the epididymis remain unanswered. What mechanisms regulate the lobe/segment/region specific expression of genes and proteins along the epididymis? How can adjacent principal cells have such dramatically different levels of expression of various proteins? What are the actual functions of each of the highly differentiated cell types in the duct? Why have we not yet succeeded in isolating and immortalizing each of these cell types? Is there a specific protein secreted by the epididymis that allows spermatozoa to attain a mature state or does this critical event result from a series of multiple interrelated steps involving changes in proteins, sugars and lipids? What aspects of the cauda epididymal fluid allow sperm to remain dormant and functional for protracted time periods? What is the role of the assortment of antimicrobials being discovered in this tissue? Why is primary cancer of the epididymis so rare? These and other questions have been challenging reproductive biologists for many decades. With the plethora of new tools that have recently been developed and that will certainly continue to emerge, we are now poised to elucidate how this remarkable tissue works.

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CHAPTER

18

Accessory Sex Glands in the Male

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INTRODUCTION

The accessory reproductive organs in the male consist of a continuous series of paired ducts that transport sperm from the testis to the urethra (epididymis and ductus deferens, also known as vas deferens) and various glands that produce components of the seminal plasma. The accessory sex glands in the human male include the paired seminal vesicles, the prostate gland, the paired bulbourethral glands, and the glands of Littre (Figures 18.1 and 18.2). The main function of the sex accessory tissues collectively is to create the seminal plasma, the medium in which sperm are delivered in the ejaculate. The sex accessory tissues produce high concentrations of prostaglandins, fructose, citric acid, polyamines, zinc, and enzymes such as proteases and acid phosphatases. However, we do not have a clear understanding of the physiologic function of many of these substances or their relative importance to fertility. It is possible to remove sperm from the epididymis or testis and fertilize an ovum directly, thereby bypassing any exposure to the seminal plasma. Interestingly, rats remain fertile after surgical removal of either the ventral prostate or the seminal vesicles, but not both.[1] Thus, while the seminal plasma may not contain an absolutely essential ingredient for successful fertilization, the fluid and its components likely optimize conditions for sperm transport as they transit the female reproductive tract and maximize their chance at fertilization when they reach the ovum.

The variability of the anatomy and function of sex accessory tissues among mammals is striking. The seminal vesicles are prominent in the human, bull, and rat, but do not exist in the dog or cat. The prostate gland is found in all mammals. In the human and dog, it is a solitary structure, whereas rat and mouse prostates have distinct dorsal, ventral, and lateral lobes. Similarly, the seminal plasma varies widely among mammalian species. The average volume of ejaculate is approximately 70 ml for the horse, 4 ml for the bull, 9 ml for the dog, 3 ml for the human, and 250 ml for the boar. The human ejaculate clots and subsequently lyses within a short period of time, whereas the rodent ejaculate forms a solid copulatory plug. The major anion found in human ejaculate is citrate, whereas in the dog it is the chloride ion.[1] This wide variability is most likely the result of evolutionary pressure, perhaps due to differences in reproductive habits or environmental influences, but we can only speculate without further research.

Performing a literature search of the sex accessory glands reveals a difference in citations by orders of magnitude, with the prostate being far and away the most studied of these tissues. This is not unexpected, given the impact that the prostate has on public health. Prostate cancer is the most common noncutaneous malignancy in the United States, representing one-fourth of all such cancers, and is the second leading cause of cancer deaths in American men.[2] Additionally, nonmalignant growth of the prostate with age, benign prostatic hyperplasia (BPH), is seen in 40–50% of men at the age of 50, and over 80% of men in their 80s.[3] This prostatic overgrowth can lead to urinary symptoms and even obstruction. Up to half of all men by the age of 70 have urinary symptoms that warrant daily medication or surgery, and the majority of these symptomatic patients have an enlarged prostate as a cause of their suffering. The prostate is also susceptible to yet a third condition, an inflammatory process called prostatitis, which causes pelvic pain. Up to 16% of all men have this condition diagnosed in either an acute or chronic form at some time in their lives. The etiology of prostatitis is tied to a known bacterial cause in less than 10% of instances with the majority of cases being idiopathic. Studies have shown that between 2% and 12% of men older than 20 are currently experiencing prostatitis-like symptoms.[4,5]

Because of the enormous impact that the prostate has on men's health, it is rightfully the focus of much

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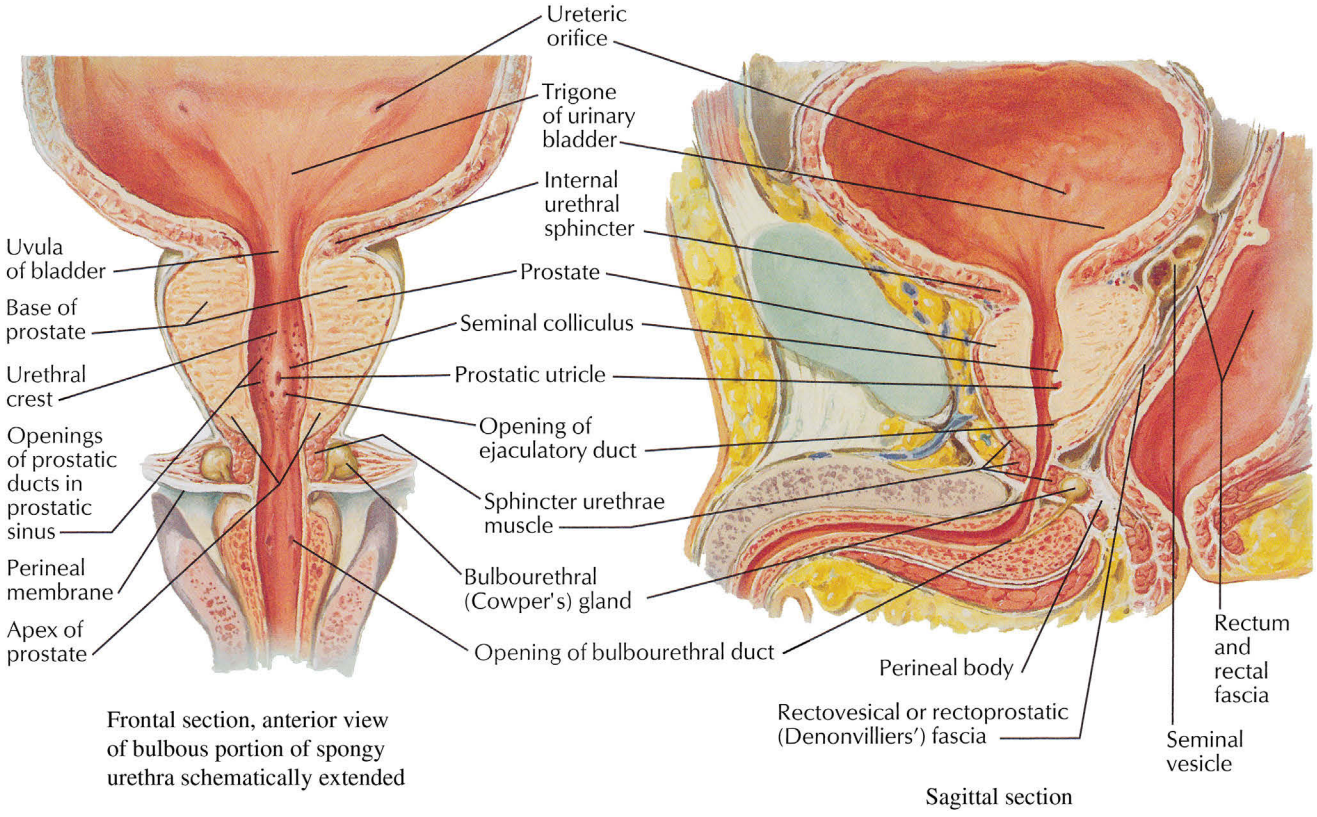
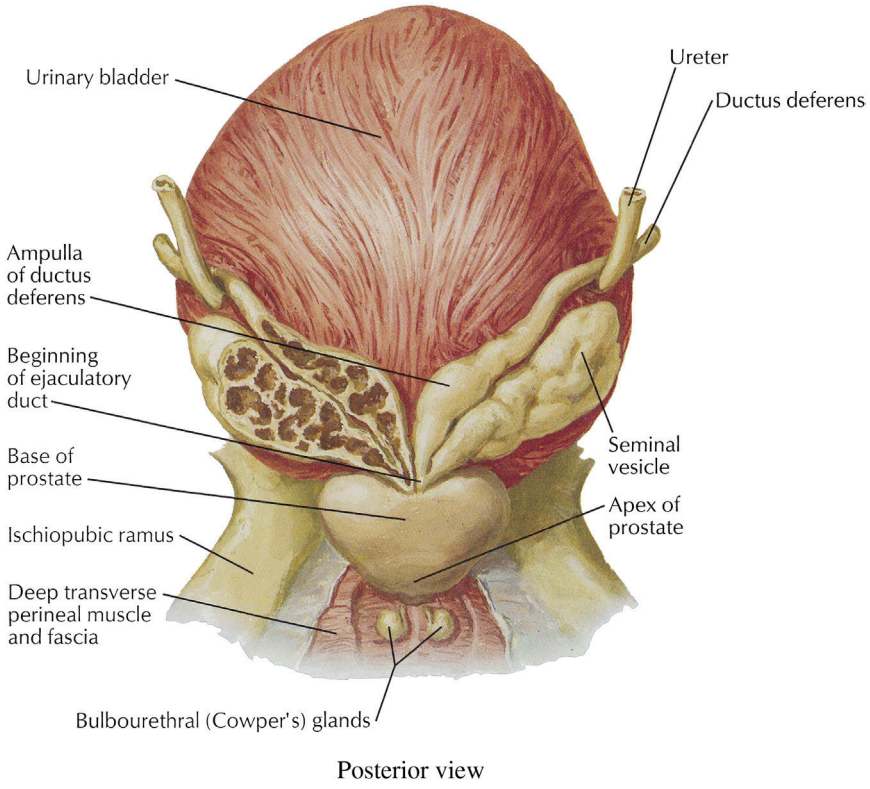


FIGURE 18.1 Anterior and sagittal views of male accessory sex glands in situ. *Source: From Netter FH.* Atlas of human anatomy. *Philadelphia: Saunders Elsevier; 2011. Plate 364.*

FIGURE 18.2 Posterior view of male accessory sex glands. *Source: From Netter, FH.* Atlas of human anatomy. *Philadelphia: Saunders Elsevier; 2011. Plate 364.*



more research than the other sex accessory tissues, and the prostate is the focus of this chapter as well. Nonetheless, there remains a need to conduct further research on the other sex accessory tissues, because they have a clear physiologic role and are less well understood. It is particularly notable that although the prostate has extremely high rates of disease, aberrant growth and cancers of the other male accessory sex glands are exceedingly rare. It has been suggested that the basis for differential disease predilection between the prostate and its immediate neighbor, the seminal vesicles, is their unique embryologic origin, with the prostate deriving from the endodermal layer and the seminal vesicles originating from the mesoderm.[6]

This review of the accessory sex glands will encompass their function, anatomy, hormonal regulation, and development including emerging evidence for perturbations by endocrine disrupting chemicals, all with an emphasis on the prostate gland where most information is available. We will also summarize diseases of the prostate and provide an overview of future directions and research needs.

History

The word prostate comes from the Greek word *προστάτης*, which means "one who stands before", "protector", or "guardian". The prostate was named for its anatomic position immediately preceding the bladder as a guardian or protector to the outside world. Herophilus provided the first known anatomic description of the prostate in the third century BC. He was a physician in Alexandria and performed dissections of the human body. Unfortunately, his writings were lost centuries later in the fire that destroyed the library in Alexandria in AD 391. Galen (AD 131 to 200), a physician in Rome, described urinary obstruction caused by a compressive mass within the prostatic urethra. Galen also noted that urinary obstruction could be due to inflammation or an abscess in the region of the bladder neck and prostatic urethra. Nicolo Massa, a Venetian physician, in 1536 provided the first accurate anatomic description of the prostate and its anatomic relationships to the bladder neck, seminal vesicles, and ampulla of the vas deferens.[7]

The first description of the pathophysiology of prostatic enlargement is attributed to Jean Riolan of France. In 1649, he observed that the urinary obstruction due to the enlarged prostate caused thickening of the bladder wall.[8] In 1769, Morgagni, the chair of anatomy at the University of Padua in Italy, added to the description a more detailed account of the changes that occurred from prostatic obstruction in both the bladder and the upper urinary tracts.[9] In 1786, John Hunter from Scotland observed that castration of prepubertal rats prevented prostate development, and that castration of adult rats caused atrophy of the gland.[10] This deeper insight into prostate developmental physiology was surprisingly ahead of its time.

Unfortunately, during the late 1800s and early 1900s, many less scientific theories arose in the medical literature regarding the etiology of prostate disease. The proposed causes of prostate disease included lascivious thoughts, horseback riding, high living, perverted indulgences, cold weather, alcohol, excessive intercourse, and masturbation. It might have been difficult for men in these times to discuss prostate-related symptoms, given the possible social implications of their disease.[11]

Over 100 years after Hunter's rat studies, the modern era of prostate research began with the pioneering work of Charles Huggins.[12] In 1941, Huggins discovered that prostate cancer regresses following androgen withdrawal, and established the role of sex hormones in prostate cancer growth. This was the first report of a regression of a cancer or tumor by chemical manipulation. It is for this work that he was awarded the 1966 Nobel Prize for Physiology or Medicine. He additionally studied the atrophic effects of androgen withdrawal on BPH. In 1944, Moore observed that patients who were either eunuchs or had pituitary infantilism had no evidence of BPH. Further observations in the 1940s led to the theory that BPH was a disease of hormonal imbalance, caused by decreased ratio of androgen to estrogen in the aging male. In 1969, Shutsung Liao discovered the androgen receptor (AR)[13]; he subsequently sequenced and cloned the AR in 1988.[14] In 1972, Cunha made the landmark discovery that embryonic urogenital mesenchyme induces adjacent epithelial cells to differentiate into the prostate epithelial phenotype.[15] To this day, the communication between stroma and epithelium and vice versa continues to be an important area of research.

From the early description of the prostate by Herophilus, to the modern discoveries linking prostate physiology and pathophysiology to hormones and stromal–epithelial cross-talk, these historical studies have become the foundation upon which prostate research is conducted today.

ANATOMY AND HISTOLOGY

Prostate Anatomy

The human prostate is a small walnut-sized gland situated at the base of the bladder between the symphysis pubis and the rectum (Figures 18.1 and 18.2). It is conically shaped, with the superior aspect abutting the bladder and the apex tapering inferiorly at the level of the internal urethral sphincter. The prostate completely

surrounds the pelvic urethra from the base of the bladder to the prostatic apex. The prostatic urethra is anatomically and functionally divided into two segments (proximal and distal) denoted by a sharp anterior angulation of the proximal segment at the verumontanum, a midline projection from the posterior wall of the urethra, also referred to as the seminal colliculus. The proximal urethra, which courses from the bladder to midway through the prostate along its 3 cm length, is surrounded by a smooth muscle sphincter, the preprostatic sphincter, which contracts during ejaculation preventing retrograde flow of semen into the bladder. The distal prostatic urethra begins at the verumontanum with a 35-degree anterior angle and is surrounded by the external urethral sphincter. Approximately 95% of the glandular prostatic ducts empty into the distal urethra at or below the verumontanum. The seminal fluid contributions from the seminal vesicles and vas deferens empty into the left and right prostatic sinuses via paired ejaculatory ducts. The ejaculatory ducts enter the prostate at its base, posterolateral to the urethra on the left and right, course approximately 2 cm inward, and empty into the prostatic urethra on either side of the verumontanum.

Although the human prostate is a single structure without the distinct lobes observed in rodents, McNeal has described a zonal anatomy of the adult human prostate (Figure 18.3) based on histologically distinct zones, and this anatomic description is the one most widely accepted today.[16] The prostatic urethra represents the primary anatomic reference point, and it divides the prostate into an anterior fibromuscular zone (30% volume) and a posterior glandular portion (70% volume) which contains the peripheral, central, and transition zones. The tubuloaveolar glands in the posterior prostate empty into the prostate sinuses via 20–30 ductules. The two principal regions of the glandular or posterior prostate are the peripheral zone (approximately 70% of the glandular volume) and the central zone (approximately 20% of the glandular volume). Anatomically, the central zone is a wedge-shaped area at the base of the prostate that narrows centrally and surrounds the ejaculatory ducts. The central zone comprises approximately 15–25% of the glandular volume in the prostate. The peripheral zone, comprising 70% of glandular prostate volume, surrounds the central zone at the base of the prostate and is in close association with the distal urethra in the prostatic apex. The glandular structure of these two zones is distinctive. The functional unit of a gland is a small, sac-like structure known as an acinus. The central zone contains irregularly contoured acini in close association with long, compact stromal elements, whereas the peripheral zone contains small, round, regular acini with loosely associated stromal connections. The transition zone, which accounts for less than 10% of glandular volume, lies adjacent to the lower portion of the proximal urethra and contacts the central and transition zone in this region. This zone, which consists of two separate lobules, is in intimate contact with the preprostatic sphincter. Histologically, the acini resemble those in the peripheral zone, surrounded by stroma that is dense and compact. A final region, the periurethral region, is comprised of tiny ductal branches scattered throughout the proximal urethral wall entirely confined within the preprostatic sphincter.

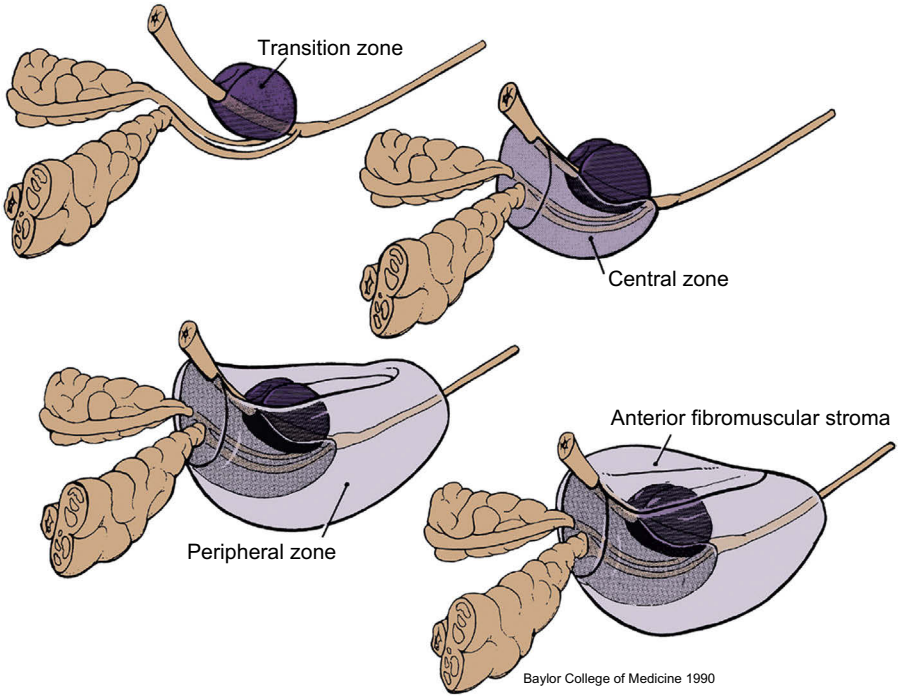


FIGURE 18.3 Zonal anatomy of the human prostate. The transition zone surrounds the urethra just proximal to the ejaculatory ducts. The central zone surrounds the ejaculatory ducts. The peripheral zone comprises the bulk of the posterior, lateral, and apical aspects of the prostate. The anterior fibromuscular stroma extends from the bladder neck to the external urethral sphincter. *Source: From Ref. 17.*

The inferior vesicle artery supplies the prostate, which is drained by three venous plexuses—one anterior and two lateral. The anterior plexus receives drainage from the deep dorsal vein of the penis and can be a source of significant bleeding during surgical removal of the prostate. Lymphatic drainage from the prostate travels primarily to the obturator and internal iliac nodes.[17] The prostate is innervated by both sympathetic and parasympathetic fibers via the cavernous nerves. Parasympathetic stimulation promotes prostatic secretion, whereas sympathetic stimulation causes contraction of smooth muscle within the stroma.

Prostate anatomy varies widely among the species, and comparative analysis has been thoroughly described by Dorothy Price.[18,19] The rodent is the most common research model to study prostate physiology and disease, and it has distinct anatomic and morphologic features from the human gland. The rodent prostate has three distinct paired lobes termed the ventral, lateral, and dorsal lobes, based upon their relative position in reference to the urethra (Figure 18.4). The rat lobes have increased complexity as compared to the mouse prostate, particularly in the lateral lobe, where two ductal types are observed in the rat, designated LP1 and LP2.[20] In addition, both species contain a coagulating gland, also referred to as the anterior prostate, which resides in the curvature of the paired seminal vesicles, and produces the seminal plug or coagulum that is not found in humans.

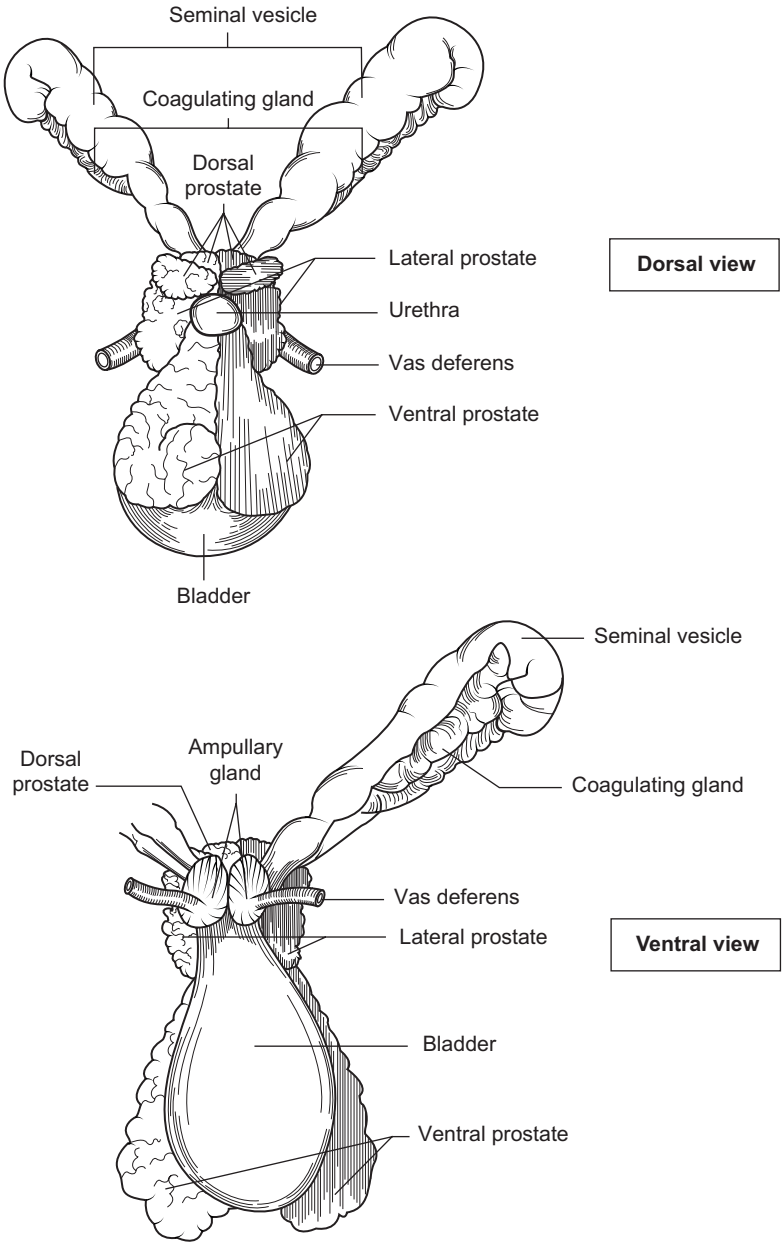


FIGURE 18.4 **Dorsal and ventral views of the rat prostate lobes and seminal vesicles.**

The ventral, lateral, and dorsal lobes of the rat prostate are each composed of a complex system of branched ducts that have a characteristic pattern unique to each lobe.[20] Together they contain 30–35 main ducts that drain the acini and empty secretions directly into the urethra along the anterior (dorsal lobe) to posterior (ventral lobe) axis.[21] The separate lobes have unique histology as well as lobe-specific secretions, which have been capitalized on to understand and model human prostate heterogeneity. The dorsal and lateral lobes resemble each other more than the ventral lobe, and the lateral prostate has been described as having the most histologic similarity to the human peripheral zone.[19] Although the rodent ventral lobes do not have a counterpart in the human (because the ventral buds regress during development) and have been suggested as a poorer model than the lateral and dorsal lobes, this distinction is somewhat artificial, because the lobular nature of the rodent prostate does not have a direct human counterpart. For example, recent studies have revealed that the rat ventral prostate has the highest expression of *Hox*b13, a homeobox gene essential for prostate identity,[22] which is significant since *HOX*B13 was recently identified as a key susceptibility gene for human prostate cancer.[23]

Prostate Histology

The mature prostate ducts contain three phenotypically and functionally distinct epithelial cell types—basal, luminal, and neuroendocrine cells—embedded in a fibromuscular stroma.[24] The exocrine product of prostate glands is produced by secretory luminal cells, which line the lumen of the glandular acini. Secretory cells are columnar and rest on a layer of basal cells over the basement membrane. They secrete a variety of proteins and products, which will be discussed later, into the lumen of the glands at the apex of the cell.[25] Basal cells are flat to cuboidal, nonsecretory epithelial cells. Human prostates have a continuous layer of basal cells with an approximate 1:1 ratio to secretory cells, while multiple other species have a discontinuous layer of basal cells with an average ratio of 1:7 basal to secretory.[26] Within the basal cell layer reside the prostate epithelial cell stem cells. An additional type of cell is seen in the epithelium, the neuroendocrine cell, which secretes hormones and cell-signaling factors in response to neural stimulation. These neuroendocrine cells are much less numerous and scattered sparsely throughout the acini and ducts.[27] Secretory epithelial cells in the human prostate are commonly identified by their expression of prostate-specific antigen (PSA); prostatic acid phosphatase (PAP); AR; homeobox gene *Nkx*3.1; and cytokeratins CK8 and CK18 but not CK5. Basal epithelial cells express CK5, CK14, and p63, and do not express PSA and PAP.[28–32] Neuroendocrine cells do not express PSA or AR, and can be identified by their expression of calcitonin and the neuropeptide chromogranin A.[33,34] The rodent prostate expresses similar cytokeratin and prostate-specific transcription factors with certain rodent-specific differences including variable expression among the distinct prostatic lobes.[35] In the rat, the ventral prostate expresses prostate-binding protein (PBP); the lateral lobe contains high levels of probasin and prostate-specific protein 94 (PSP94), also known as β-microseminoprotein and found in the human prostate; and the dorsal lobes contain low probasin levels and high levels of seminal vesicle secretory protein or SVS-II and DP-1.[36]

Secretory cells depend on androgen signaling for their survival and secretory activity and express high levels of AR.[37] Basal cells are largely AR negative,[38] although they may be androgen responsive through luminal cell and stromal cell paracrine signaling. Gap junction proteins exist between basal and luminal cells in the human prostate, which suggests an intimate level of communication. Additionally, the basal cells have junction-like structures between each other, which is reminiscent of the barrier in the testis and has led some to propose a similar blood–prostate barrier exists.[26,39] Little is known about the function of neuroendocrine cells; however, they express a wide variety of peptide hormones, biogenic amines, and carrier proteins. Interestingly, neuroendocrine cells are seen throughout the prostate glands at birth, and subsequently rapidly decline in numbers, only to reappear at puberty.[40]

The prostatic stroma is predominantly composed of smooth muscle and fibroblast cells, which contribute to both the structure and function of the prostate.[41] The smooth muscle cells provide the mechanical means by which prostatic secretions are expelled during emission.[42] Interestingly, the distribution of smooth muscle cells varies between species, with rodent smooth muscle being found strictly in a periacinar distribution and human smooth muscle found in the interductal stroma as well as periacinar. In addition to the mechanical roles of support and contractile elements, the stroma plays a critical role in epithelial homeostasis and development through the secretion of essential paracrine acting factors that drive epithelial proliferation and differentiation, as discussed in subsequent sections.[43] It is believed that aberrant growth with BPH and cancer is driven in part through reawakening of the embryonic growth potential of prostatic stroma, and evidence clearly shows a critical role for cancer-associated fibroblasts in driving prostate cancer progression.[16] The regional variations in epithelial proliferation and apoptosis along the prostatic ducts are associated with differing stromal organization. In the rodent prostate, the distribution of fibroblasts and smooth muscle cells varies along the length of a duct. In the distal regions, there is a discontinuous layer of fibroblasts between the smooth muscle cells, which surround the gland, whereas the stroma surrounding the proximal

duct has multiple continuous layers of smooth muscle with only a few rare fibroblasts interspersed.[41]

Prostatic smooth muscle cells consistently express AR, whereas fibroblasts do not. In the mature prostate, smooth muscle AR is believed to cause paracrine signaling, which helps maintain fully differentiated growth-quiescent epithelium.[38,44,45] Stromal growth factor expression has regional variation along prostatic ducts and is associated with differential epithelial responses.[41] It appears that, in general, fibroblastic signaling promotes epithelial proliferation, whereas smooth muscle signaling promotes cell death.

Seminal Vesicle Anatomy and Histology

In the adult human, seminal vesicles are paired structures posterior to the bladder and adjacent to the rectum (Figures 18.1 and 18.2). Positioned lateral to the posterior aspect of the prostate, the seminal vesicle lumens join with the ampullary region of the vas deferens, forming the ejaculatory ducts that transverse the central zone of the prostate and empty into the posterior urethra at either side of the verumontanum. The seminal vesicles are each 5–7 cm long with a volume of approximately 13 ml.[46] Unlike the prostate, the seminal vesicles are not branched structures and contain a singular common lumen; however, the glandular tissue is highly convoluted and infolded, thereby enabling its large secretory capacity. Similar to the prostate, the seminal vesicle epithelium consists of columnar secretory cells atop a discontinuous layer of basal cells; however, no neuroendocrine cells are found.[47] The epithelium is surrounded by a thick layer of smooth muscle cells innervated by sacral nerves S2–S4, and contracts during emission to expel the luminal contents into the ejaculatory ducts.

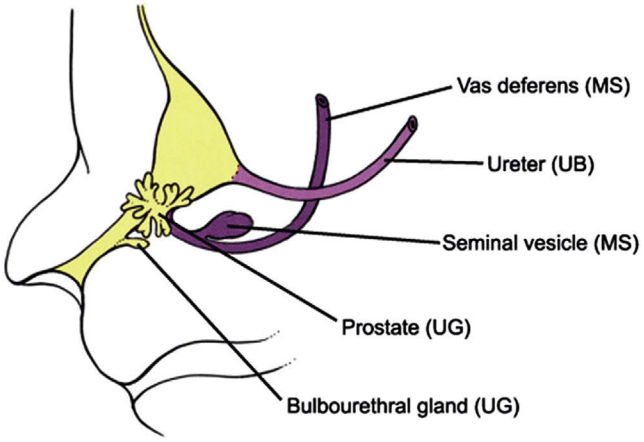


FIGURE 18.5 Development of human male accessory sex glands. The prostate and bulbourethral glands originate from endodermal urogenital sinus (UG) tissue, whereas the seminal vesicle and vas deferens derive from mesodermal mesonephric ducts (MS). The ureter develops from the ureteric bud (UB). *Source: From Park JM. Normal development of the genitourinary tract. In:* Campbell-Walsh Urology. *10th ed. Philadelphia: Elsevier Saunders; 2011. p. 58.*

Bulbourethral Glands and Glands of Littre

The paired bulbourethral glands, also known as Cowper's glands, are approximately the size and shape of peas and are located within the urogenital diaphragm immediately posterior and lateral to the membranous urethra (Figures 18.1 and 18.2).[48] They each contain tubuloalveolar glands, which empty into 2-cm ducts that drain into the bulbar urethra. They have a common developmental history to the prostate—both originating as epithelial buds from the urogenital sinus (UGS)—and are under similar androgenic regulation. The clear secretions of the bulbourethral gland perform primarily a lubrication function prior to and during the initial phase of ejaculation. An additional physiologic role is believed to involve immunoprotection to the reproductive tract, based on the high content of IgA and secretory component that is produced and secreted by these glands (see Chapter 19).[49] The glands of Littre are very small urethral glands that line the penile urethra. They are tubuloacinar mucinous glands that serve to lubricate the penile urethra prior to ejaculation.

EMBRYOLOGY AND DEVELOPMENT

Prostate Gland Development

In contrast to the seminal vesicles and vas deferens, which develop embryologically from the Wolffian ducts and are mesodermal in origin, the prostate gland originates from the UGS and is entirely an endodermal structure (Figure 18.5). It has been suggested that this endodermal embryologic origin may account in part for the high rates of aberrant growth and cancer in the prostate gland, whereas the other accessory glands have low rates of disease. A significant amount of information about prostate gland development at the morphologic, cellular, and molecular levels has been derived from studies using rodent models, while similar details for the human prostate are limited. This section will first discuss the embryologic and developmental details known for the human prostate and then proceed to the specifics of rodent prostate development including hormonal and molecular signaling that cooperate to regulate the process.

Human

Although the prostate developmental process is continuous, it can be categorized in distinct stages involving determination, initiation or budding, branching morphogenesis, differentiation, and pubertal maturation.[50]

Determination of the prostate occurs prior to clear morphological evidence of a developing structure and involves expression of molecular signals that commit a specific field of UGS cells to a prostatic cell fate. Phenotypic human prostate development commences as UGS epithelial cells form outgrowths or buds, which penetrate into the surrounding UGS mesenchyme in the anterior, posterior, and lateral directions caudal to the bladder. Prostate development occurs during the second and third trimester and is complete at the time of birth.[51–53] Primordia of the prostate gland appear during gestational weeks 10–12 in humans as solid outgrowths from the UGS immediately below the developing bladder. These epithelial buds penetrate the surrounding mesenchyme that accompanies the UGS. Stromal differentiation occurs as mesenchymal cells form lamina propria around the epithelial outgrowths.[54] The ducts grow rapidly in length, and arborize and lumenize, so that by weeks 14–18 there are approximately 70 tubuloacinar ducts that exhibit signs of secretory cytodifferentiation. In 1912, Lowsley classified anatomic lobes of the adult prostate based on his observation that prostatic epithelial buds originated in five different regions of the UGS that embryonically formed different regions within the gland: anterior, posterior, middle, and two lateral lobes.[51] Later studies have revealed that the distinctions that Lowsley observed in the fetal prostate do not appear to exist in the adult prostate, perhaps due in part to the regression of the anterior buds, and that embryonic extrapolations do not explain adult morphology. However, it is noteworthy that at the earliest embryonic stage, regional differences are found in the prostate. There is some evidence to indicate that the prostate also contains a small mesodermal contribution arising from mesonephric and paramesonephric tissues when buds penetrate dorsoposteriorly into the vicinity of the developing ejaculatory ducts.[55] This region would be contained within the central zone of the adult prostate.

The fetal prostate exhibits extensive squamous metaplasia in the acini of the developing central zone, which is evident at 22 weeks and gestational age and increases as the fetus matures.[56] This metaplastic activity peaks in the 36th week and declines thereafter, and the metaplastic cells are sloughed within the first weeks following birth. The significance of metaplasia is unclear, although it appears to be driven by maternal estrogenic actions. Trace amounts of prostatic secretions are observed as early as the 14th gestational week in the peripheral zone and increase in incidence and degree so that in the later weeks of gestation, several lumina are filled with mucopolysaccharides. Metaplasia and secretory activity appear to be mutually exclusive in the embryonic human prostate.

In humans, prostate development is largely complete at the time of birth, and the gland remains relatively dormant until puberty. At puberty, the prostate undergoes a rapid growth spurt (1–2 g before puberty to 12–20 g in a young adult), largely as a result of the pubertal surge in testosterone production (see Chapter 32). Quantitation of this growth spurt in baboons indicated that the increase in stromal mass preceded growth of the epithelial fraction, which concurs with the concept that mesenchyme and stroma are growth inductive for the prostate epithelium.[57] Prior to puberty, the prostate epithelium appears homogenous throughout the gland, whereas at puberty the epithelium in the central zone and peripheral zone undergoes final maturation and differentiation, becoming histologically distinct and heterogeneous. Prepubertally, only the proximal portion of the preprostatic sphincter is developed, and final maturation with distal extension to the verumontanum takes place during the pubertal growth spurt.

Rodent

In contrast to the human, the rodent prostate gland is rudimentary at birth and undergoes the majority of its development during the first 15 days of life. Immediately prior to birth, buds from the UGS epithelium penetrate into the surrounding UGS mesenchyme in the ventral, lateral, and dorsal directions to give rise to the distinct prostate lobes. Of interest, the coagulating gland (the anterior prostate) is a hybrid structure that originates embryologically from UGS epithelial cells that penetrate anteriorly into mesenchyme associated with the mesodermal seminal vesicles, to which they fuse. In the mouse, the initial outgrowth of epithelial buds occurs between fetal day 16.5–17.5 (f16.5–17.5) in a 19-day gestation strain,[58] while in the rat it occurs at f18.5 in a 21-day gestation strain.[20] At birth, the ventral, dorsal, and lateral rodent prostate lobes primarily consist of unbranched, solid elongating buds or ducts, and subsequent outgrowth and patterning occur postnatally. During this time, proliferation of epithelial cells occurs primarily at the leading edge of the ducts (i.e., distal tips).[44] Branching morphogenesis begins when the elongating UGS epithelial buds contact the prostate mesenchymal pads that are peripheral to the periurethral smooth muscle. At that point, secondary, tertiary, and further branch points are established with continued proximal-to-distal outgrowth and complexity.[59] Branching patterns are lobe-specific with ventral branching preceding that in the dorsolateral lobes by 3–4 days.[20] Morphogenesis of the entire complex is completed between postnatal days 15 and 30. Final growth and maturation occur at puberty (days 25–40), when circulating androgen levels rise sharply (see Chapter 30).

Epithelial and mesenchymal cell differentiation is coordinated with branching morphogenesis and occurs in the proximal-to-distal direction.[42,60,61] Epithelial differentiation from stem/progenitor cells into differentiated

basal and luminal cells has been documented in the rat prostate with changing patterns of cytokeratins as well as alterations in AR expression, an early marker of epithelial cell differentiation.[60] The process initiates between days 3–5 in the rat ventral prostate and about 2 days later in the dorsal and lateral lobes. Lumenization of the solid epithelial cords is concomitant with differentiation into basal and luminal cell layers and initiates in the proximal ducts around postnatal day 5, extending to the distal tips by about day 12. Between days 10 and 15 in the rat, functional differentiation commences, as defined by the synthesis of secretory products by differentiated luminal epithelial cells. Concomitant with epithelial differentiation, the prostatic mesenchyme undergoes differentiation postnatally. As UGS epithelial ducts penetrate into the prostate mesenchymal pads, mesenchymal cells condensate around the tip and form a distinctive pattern along the length of the basement membrane. Between postnatal days 3 and 5, cells adjacent to the ducts form a periductal layer of smooth muscle cells while interductal cells differentiate into mature fibroblasts.[60] Lying between the basement membrane and the periductal smooth muscle is an extremely thin single-cell layer of differentiated fibroblasts that thins in the distal direction.[62] As the prostate undergoes branching morphogenesis, a branched vascular bed forms in parallel with neovascularization forming within the prostatic stromal elements and capillary beds extending to the ductal basement membrane.[21,63]

Prostate Stem Cells

Adult prostate stem and progenitor cells have had a more thorough analysis than those found in the developing prostate. Consequently, our knowledge of hierarchical relationships and regulatory pathways is largely based on adult stem cells. Adult prostate epithelial stem cells have been identified in human and rodent prostate glands, where they play an essential role in tissue replenishment throughout life.[64–67] This rare cell type self-renews and has the potential to differentiate into the three distinct epithelial cell types, which are essential characteristics of bona fide stem cells. Adult prostate stem cells are relatively growth quiescent, occasionally dividing to self-renew and generate daughter progenitor cells. Studies across multiple systems as well as the prostate epithelium have characterized two types of stem cell divisions: (1) symmetric division, also known as symmetric self-renewal, which generates two identical stem cells; and (2) asymmetric division, which generates a single self-renewing stem cell and a daughter cell that has entered the earliest stage of differentiation (progenitor cell).[68–71] Unlike the stem cell, the daughter progenitor cell has transit-amplifying capacity through rapid cell divisions. As the progenitor cell proliferative potential is exhausted, it undergoes terminal differentiation.

Although prostate stem cells are present in several regions of rodent prostatic ducts, accumulated prostate stem cells with considerable growth potential have been found in the proximal region of ducts close to the UGS, and the survival of these cells does not require the presence of androgens.[72,73] Primitive proximal prostate stem cells that are able to regenerate functional prostatic tissue in vivo are also programmed to reestablish a proximal–distal ductal axis. In contrast, prostate stem cells in the distal region of the prostate duct have more limited growth potential and require androgens for survival.

Although the lineage hierarchy for the prostate epithelium has not been settled, epithelial differentiation of stem cells into differentiated basal, luminal, and neuroendocrine cells has been documented in the rodent prostate and in isolated human prostate cancer stem cells.[74–78] These studies on stem cell differentiation have been observed with changing patterns of cytokeratins, cell-specific markers, and alterations in AR expression, an early marker of luminal epithelial cell differentiation. Two models have been proposed for the prostate epithelial stem cell lineage into differentiated basal, luminal, and neuroendocrine cell types. In the traditional linear hierarchy model (Figure 18.6), self-renewing prostate stem cells residing

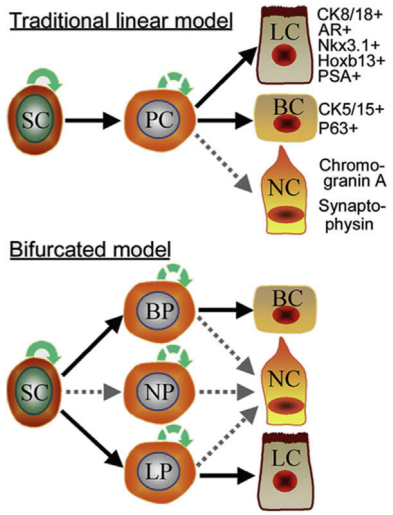


FIGURE 18.6 Prostate stem cell hierarchical models. In the traditional linear hierarchy model (top), self-renewing prostate stem cells (SC) give rise to intermediate, transit-amplifying progenitor cells (PC). These cells have high proliferative capacity and enter differentiation pathways to give rise to terminally differentiated luminal epithelial cells (LC), basal cells (BC), and neuroendocrine cells (NC). In the bifurcated model (bottom), a common SC with self-renewal capacity undergoes asynchronous cell division to give rise to lineage-restricted basal progenitor cells (BP) and luminal progenitor cells (LP). These PCs possess transient self-renewal capacity and terminally differentiate into BC and LC. The lineage of NC is unclear, and may arise from the hierarchical intermediate BP and LP that produce BC and LC, or it may have a separate neuroendocrine progenitor (NP) origin.

in the basal cell layer undergo asymmetric cell division, giving rise to daughter progenitor cells with high proliferative potential, referred to as transit-amplifying cells. In response to signals from the stem cell niche, these cells enter early differentiation pathways to eventually form separate basal, luminal, and neuroendocrine cells.[74,79,80] Phenotypic intermediate-type cells that co-express basal and luminal markers have been observed both in vitro and in vivo.[60,81] This suggests that basal and luminal cells are hierarchically related through common progenitor cells that give rise to differentiated basal cells and luminal cells. In the bifurcated model, basal cells and luminal cells represent separate epithelial cell lineages that originate from a common stem cell that undergoes asymmetric cell division to yield lineage-specific progenitor cells. These lineages may be sustained by intermediate transit-amplifying cells prior to final stages of terminal differentiation.

The lineage of neuroendocrine cells is unclear. Neuroendocrine cells may arise from the hierarchical prostate epithelial stem and progenitor cells that produce basal and luminal cells, or they may have a separate progenitor cell origin suggested for the bifurcated model. Critical to stem cell self-renewal, lineage commitment, and differentiation is the stem cell niche, or microenvironment, that is believed to provide signals that regulate these events.[82] This niche in both the developing and adult glands would include the mesenchyme/stromal and resident immune cells that communicate with stem cells through paracrine factors as well as neighboring epithelial cells that can communicate through gap junctions, cell adhesion molecules, and paracrine signaling.

Prostate epithelial stem and early progenitor cells are AR negative[83,84] and are thus not directly regulated by androgen action. As a result, any effects of androgens on prostate epithelial stem cell homeostasis and differentiation are most likely mediated through indirect actions via the stem cell niche that includes AR^+ stromal cells and in the mature prostate, AR^+ luminal epithelial cells.[85] Interestingly, recent data show that human prostate stem/progenitor cells express estrogen receptors ERα, ERβ, and GPR30 and show proliferative responses to estrogens, implicating their involvement in maintenance of the stem/progenitor pool.[86] Similar to stem cells in other systems, prostate stem cells also express several retinoic acid receptors (RARs and RXRs) through which retinoids promote differentiation.[84] This suggests that together, multiple signaling molecules may directly and indirectly contribute to the controlled equilibrium between self-renewal and differentiation in prostate epithelial stem cell populations.

Seminal Vesicle Development

The bilateral mesonephric or Wolffian ducts develop into the epididymis proximally (in relation to the testis); and the vas deferens, seminal vesicles, and ejaculatory ducts distally (Figure 18.5). Thus unlike the endodermal prostate, the seminal vesicles are of mesodermal origin. In the human, seminal vesicle development begins at 12 weeks gestation as a localized dilatation of the distal Wolffian duct. This dilatation expands into the saccular convoluted structure of the mature seminal vesicle. The entire Wolffian duct then achieves its characteristic thick investment of smooth muscle and relatively narrow lumen along its length. These confluent structures establish a channel that will carry fluid and sperm from the epididymis and vas deferens to the ejaculatory duct and urethra. The seminal vesicle joins the shared lumen of this channel at the level of the ejaculatory duct, which traverses the prostate posterolaterally and empties into the posterior urethra. The ejaculatory duct carries the combined contributions from the vas deferens and epididymis as well as the seminal vesicle.[87]

Morphogenesis of the rat seminal vesicle initiates in utero at f17 with two outgrowths budding anterior to the UGS. At birth, the rodent seminal vesicle is rudimentary consisting of a hollow tube surrounded by mesenchyme.[88] Unlike the prostate, the seminal vesicle contains a singular main duct that undergoes elongation and lateral branching during the postnatal period. *Fgf*10 signaling is essential for lateral branching. This was demonstrated in a mouse model with a spontaneous recessive mutation (*svs*) and subsequent *Fgf*R2iiib splice variant isoform that resulted in decreased lateral branching without a decrease in overall growth. The seminal vesicle structure primarily increases in complexity through extensive epithelial infolding into the glandular lumen that provides significant secretory capacity to the epithelium. The seminal vesicle mesenchyme differentiates into a thick layer of smooth muscle cells, which surrounds the ductal structure. A thin layer of fibroblasts differentiates between the smooth muscle cells and the basement membrane epithelium.[42]

Regulation of Accessory Gland Development

Androgens

The determination and initiation of prostatic and seminal vesicle development in the human and rodent fetus is entirely dependent upon androgens produced by the fetal testes. Surgical or chemical castration (i.e., antiandrogen administration) during critical periods of fetal life results in inhibition of prostate development.[18,88–91] However, the extent of inhibition depends on the timing of androgen ablation with removal prior to the time of budding in the murine or rat embryo blocking prostatic outgrowths altogether and removal after bud outgrowth reducing bud numbers in a temporal-dependent manner.[90,91] These findings indicate that although androgens are essential for prostate

determination, bud initiation, and maximal bud number and length, budding can continue to a large degree in the absence of testosterone due to irreversible commitment of the tissue. Rodent neonatal prostate explant cultures have further shown that although branching morphogenesis can occur in the absence of exogenous androgens, maximal organ growth with full branching as well as complete cellular cytodifferentiation are only realized with the addition of exogenous testosterone.[92,93]

In the 1970s, it was determined that the primary androgen responsible for prostatic development is dihydrotestosterone (DHT), the reduced metabolite of testosterone.[94] DHT is formed intracellularly in the prostate epithelium by 5α-reductase (SRD5A1 and SRD5A2) and has a higher affinity for AR as compared to the parent compound, testosterone.[13] Human males with 5α-reductase deficiency syndrome have complete absence of prostate morphogenesis, with normal development of the seminal vesicles and vas deferens, which empty into a blind vagina.[95] Similarly, treatment of pregnant female rats with a 5α-reductase inhibitor from f14-22 obliterated the formation of prostatic buds, an effect that could be reversed with concomitant administration of DHT.[96]

Androgen action is mediated through interaction with nuclear AR, which is a member of a nuclear receptor superfamily of transcription factors.[97,98] Evidence for the essential requirement of AR for prostate development comes from the observation of prostatic absence in mice or humans with complete dysfunctional AR.[99] AR is highly expressed in the UGS mesenchyme prior to and during prostate morphogenesis, whereas epithelial AR expression is induced after budding and branching morphogenesis has begun.[60,100] Classical tissue recombinant studies by Cunha demonstrated that AR in the mesenchyme, and not epithelial AR, are responsible for prostatic morphogenesis.[43,101] Although further analysis revealed that epithelial AR are required for expression of secretory proteins,[60,102] epithelial proliferation and cytodifferentiation appear to be largely driven by paracrine factors under mesenchymal AR control.[103] Nonetheless, mesenchymal differentiation into smooth muscle requires reciprocal signals from the epithelium, and because AR induction in prostate epithelium begins as early as postnatal day 1 or 2 (prior to cytodifferentiation of the epithelium and mesenchyme), it is possible that androgen-mediated actions in the epithelium are also involved in normal prostate morphogenesis.[60,104] AR inactivation restricted to murine prostate epithelial cells confirms the above model and also provides evidence that epithelial AR restricts epithelial cell proliferation and serves to maintain homeostasis of epithelial cell numbers.[70,105]

Estrogens

There is also clear evidence for a role of estrogens during prostate and seminal vesicle development. Although low levels of circulating estrogens are present throughout life in males, there are two time periods, during in utero development and upon aging, when males are exposed to relatively higher levels of circulating estradiol, which have been shown to impact the prostate gland. Estrogen receptors are expressed during early prostate and seminal vesicle development, with ERα expressed in developing prostate stromal cells[35,106] and ERβ localized primarily to luminal epithelial cells during early differentiation.[107] Extensive studies on the process of developmental estrogenization (referred to as estrogen imprinting or estrogen reprogramming) have shown that exposure to high-dose estrogens during the critical window of development inhibits prostate growth, leads to differentiation defects of epithelial and mesenchymal cells, and effects marked structural reorganization of the developing prostate.[108] Conversely, low-dose estrogen exposures during prostate development can produce opposite effects and increase prostate gland bud numbers and adult prostate size, indicating nonmonotonic dose responses.[109] The seminal vesicles are also influenced by early-life estrogens, which can program the structure in a manner similar to that in the prostate. The prostatic reprogramming events are mediated through stromal ERα, which in turn alters expression of multiple steroid receptors.[23,110] Downstream mediators of altered prostatic steroid signaling involve perturbed expression of several key transcription factors and secreted morphogens, including *Nkx*3.1, *Hoxb*13, *Bmp*4, *Fgf*10, and *Shh*, which are essential for normal prostate gland development.[111–113] Importantly, exposure to natural, synthetic, and environmental estrogenic compounds during fetal or neonatal development can lead directly to prostate neoplasia, dysplasia, and chronic inflammation with aging, if doses are high, and increase susceptibility to hormone-driven carcinogenesis with aging if doses are low.[112,114] It is thought that these lifelong changes as a function of brief, early-life exposure to estrogens are a result of epigenetic reprogramming of the prostate cells early in development that leads to altered epigenetic memory at the level of DNA methylation, histone modifications, and noncoding RNAs.[115] Whether estrogen reprogramming occurs in humans is unclear. However, squamous metaplasia in fetal human prostate epithelium is driven by maternal estradiol.[56] Furthermore, indicators of high estrogen levels during pregnancy, such as high birthweight and jaundice in the newborn, are associated with increased risk of prostate cancer, whereas indicators of low estrogen levels, such as preeclampsia, are related to decreased risk.[116] Together, this supports a growing body of evidence for a developmental origin for adult disease and extends this emerging paradigm to include prostate disease and cancer.

Retinoids

Retinoic acid and retinoids are derivatives of vitamin A and play an important role in organ development and tissue homeostasis. Their actions are mediated through retinoid receptors RARα, β, and γ and RXRα, β, and γ, which form RAR/RXR dimers that directly regulate target gene expression.[117] Retinoic acid receptors are expressed in a cell-specific manner in the developing rat prostate gland,[118] and retinoids play an important role in prostate bud initiation.[119]

Neural Regulation

It has been suggested that the developing prostate may have a neural regulatory component as well. Alpha-1 and beta-2 adrenergic receptors as well as M(1) cholinergic receptors are found at high densities in the prostate. Studies involving surgical denervation as well as botulinum toxin have given evidence that neural stimulation may be involved in prostate growth.[120,121] Additional study is needed in this aspect of development.

Developmental Genes

The branching morphogenesis that occurs in the developing prostate and to a lesser degree in the seminal vesicles is driven by time-specific and region-specific expression of key regulatory genes that are evolutionarily conserved throughout the animal kingdom. These conserved signaling pathways are seen repeatedly across species, suggesting that a common "morphogenetic code" or common set of rules exists that is used repeatedly in different combinations to result in formation of unique organs.[122] Similar pathways are seen among the different branched glandular structures within the same species; however, spatial and temporal differences, unique combinations of gene expression, and organ-specific genes result in unique structures. There are two major categories of developmental genes: those that encode for nuclear transcription factors and for secreted morphogens that effect responses through cell-specific expression of membrane receptors. Most of the available information derives from studies in the rodent prostate with limited evaluation undertaken in the seminal vesicles. This section will review nuclear transcription factors including common and organ-specific homeobox genes, as well as secreted signaling ligands, which provide the well-coordinated paracrine and autocrine communication between epithelial and stromal mesenchymal cells in the developing glands (Figure 18.7). It is important to note that several of these genes are direct downstream targets of steroid action, while expression of others is indirectly influenced by androgens, estrogens, and retinoids, which explains in part how these factors influence the divergent developmental processes.

Homeobox Genes and Transcription Factors

The homeobox superfamily of genes are involved in axis positioning and tissue determination, and several members of this group of genes have been identified as active within the developing prostate and seminal vesicles.[123] The common feature defining these genes is the homeobox, a highly conserved 180 base pair segment that encodes a 60 amino acid peptide segment that binds to specific regulatory regions of target genes.

Hox Genes

Hox genes are the largest and most extensively studied members of the homeobox superfamily and determine patterning in body regions from *Drosophilia* to humans. Mammals have 39 Hox genes, which are grouped into four clusters (A, B, C, and D) found on four separate chromosomes.[124] Similar genes in the separate clusters are considered paralogs and largely, although not always, have redundant functions. Expression of these genes, from the 3′ to 5′ end of each cluster, follows a strict pattern of spatial and temporal colinearity during embryogenesis. The 3′ genes (*Hox*1, 2, 3, …) designate anterior regions while the 5′ genes (*Hox*13, 12, 11, …) encode posterior or caudal regions. A generalized model

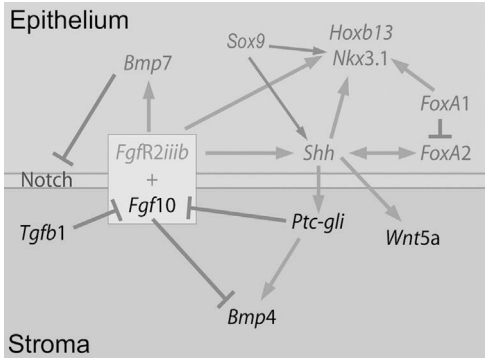


FIGURE 18.7 A schematic representation of regulatory networks between secreted morphogens and transcription factors in the epithelial and stromal cells at the distal signaling center of the developing rat prostate gland. *Fgf*10 (stromal) and *Fgf*R2iiib (epithelial) upregulate epithelial expression of *Shh* and *Bmp*7, which are involved in branching morphogenesis, as well as *Hox*b13 and *Nkx*3.1, which drive epithelial differentiation. Furthermore, epithelial *Sox*9 upregulates *Shh* and *Nkx*3.1 expression while *Fox*A1 stimulates *Nkx*3.1 and inhibits *Fox*A2 expression, which has reciprocal upregulation with *Shh*. In turn, secreted *Shh* upregulates *Ptc-gli* expression in adjacent mesenchymal cells, which downregulates *Fgf*10 expression, thus establishing a negative feedback loop for controlled growth. *Shh* and *Ptc-gli* also upregulate the growth-inhibitory *Wnt*5a and *Bmp*4 molecules in the mesenchyme, while *Fgf*10 downregulates their expression, which further serves to tightly control localized tissue growth. Stromal *Tgf*β1 suppresses *Fgf*10 expression, which may serve as a brake for growth as development nears completion. *Source: Adapted from Ref. 50.*

for regional tissue specification is that nested, partially overlapping expression domains of several genes in a Hox cluster determine segment identity.

As the accessory sex glands are the most posterior organs in the male, the posterior *Hox* genes, *Hox*9–13, are involved in their development and identity. The male accessory reproductive organs in the anterior-to-posterior order are the epididymis, vas deferens, seminal vesicles, coagulating gland, dorsal prostate, lateral prostate, ventral prostate gland, and bulbourethral glands, and a unique Hox code for each of these structures contributes to the organ-specific and prostate lobe-specific identities.[22] Studies in null mutant mice have demonstrated the functionally redundant *Hox*a13 and *Hox*d13 are necessary for prostate growth and branching, with compound mutants exhibiting normal testes and severely hypoplastic prostate tissue. Interestingly, in the mouse prostate, expression of *Hox*a13 and *Hox*d13 is highest during fetal life with a decline postnatally, whereas the rat prostate has the opposite pattern, with higher expression levels in the adult.[22,125–128] This implies that these highly conserved genes and morphogens actually function in different ways among species.

Although the A and D *Hox*13 paralogs are more strongly expressed in the mesenchyme with lesser expression in the epithelium, *Hox*b13 is only seen within the epithelial cells of mouse and rat prostates with expression rising in the postnatal period to peak levels in adult prostate epithelium.[22,129,130] Furthermore, while the A and D *Hox*13 paralogs are also expressed within the seminal vesicles, *Hox*b13 is only expressed in UGS-derived accessory sex glands, indicating relative specificity of this paralog within the male reproductive tract.[22] Importantly, *Hox*b13 has been shown to be essential for normal prostate epithelial cell differentiation, and its targeted expression is capable of driving undifferentiated prostate epithelial cells into an early differentiation pathway.[22,130] This is particularly notable in light of the recent discovery that *HOX*B13 in the human prostate gland is a susceptibility gene for prostate cancer,[131] with a germ-line mutation identified in 5% of patients with early-onset familial prostate cancer.[131,132]

In addition to Hox genes, several Hox protein cofactors are expressed in tissues that modify Hox gene specificity.[133] Of high relevance, Hox cofactors *Meis*1, *Meis*2, and *Pbx*1 were recently identified through a genomic screen as differentially expressed between matched patient specimens of seminal vesicles and prostate and that may explain, in part, the vastly different rates of cancer in these two structures.[134] Downregulation of *Meis*1, *Meis*2, and *Pbx*1 in prostate cancer tissue identified their critical roles as tumor suppressor genes. This contributes to the growing body of evidence that dysregulation of key developmental genes plays a key role in prostate cancer initiation and progression.

Nkx*3.1*

*Nkx*3.1 is a homeobox gene with expression limited to the UGS-derived prostate and bulbourethral gland epithelium.[135,136] In the fetal mouse UGS, *Nkx*3.1 is expressed at bud sites prior to bud formation, which suggests it has a role in prostate determination.[30,135] Null mutant mice exhibit defective branching patterns and perturbed epithelial differentiation,[30] and a sharp but transient peak of expression in the rat prostate epithelium during the second week of life concomitant with cytodifferentiation suggests involvement in that process.[112] Its continued expression in the adult prostate indicates its essential role in homeostasis.[137] Furthermore, multiple studies have shown *Nkx*3.1 to be a tumor suppressor gene, with loss of expression leading to prostate carcinogenesis and progression.[138]

*Nkx*3.1 expression is under direct androgen regulation and is upregulated by androgens in the adult mouse prostate, developing rat prostate, and a human prostate LNCaP cell line.[135,139,140] However, in the developing prostate, initial *Nkx*3.1 expression in the UGS epithelial cells is not likely a result of direct androgen action, because these cells are AR negative at that time. It has been shown that *Nkx*3.1 expression during prostate formation is dependent on epithelial sonic hedgehog (Shh) expression; thus its developmental expression is likely a function of gene regulatory networks operative at that time.[141]

Fox*A1 and A2*

Forkhead box genes (*Fox*) encode a superfamily of winged-helix transcription factors.[142] Multiple Fox genes are specific to endodermal-derived structures and are involved in their organ development.[143] *Fox*A1 (formerly *Hnf*3) is found in the developing prostate epithelium in the early stages of prostate formation and is involved in ductal morphogenesis and epithelial polarity and differentiation.[144,145] Although *Fox*A2 is only expressed at the mesenchymal interface of prostate epithelial cells during early budding and then subsides, *Fox*A1 expression in the rat and mouse increases with prostate development and is maintained throughout life.[144–146] In humans, continued *FOX*A1 expression is required for PSA expression, through interactions between *Fox*A *cis*-regulatory elements and AR on gene promoters.[146] In this regard, *Fox*A1 is now recognized as an essential pioneer factor for steroid receptors, where it guides AR binding to androgen response elements (AREs) throughout the genome.[147] Importantly, *Fox*A1 has been found to play a role in advanced prostate cancer.

Sox*9*

A member of the Sry box family, *Sox*9 has been found to be essential for normal prostate budding and branching morphogenesis.[148,149] *Sox*9 is expressed early in the fetal prostate epithelial cells, and *Sox*9$^{-/-}$ null

mutant mice fail to develop a ventral prostate and show deficient branching in the dorsolateral and anterior prostate lobes. Importantly, it was shown to be upstream of other critical genes in the developing prostate including *Nkx*3.1, *Shh*, and *Fgf*10.[148] SOX9 is expressed in human fetal prostate epithelium and localizes to basal epithelial cells in the adult human prostate.[150,151] As with several other developmental genes, aberrant SOX9 signaling has been found in prostate cancer cells, where it is frequently overexpressed and upregulates AR.[150]

Juxtacrine Signaling

NOTCH1/DELTA/JAGGED

The notch signaling pathway is highly conserved and involved in cell fate specification and patterning in developing tissues.[152] The notch receptor is a single-pass transmembrane glycoprotein that interacts with jagged/delta membrane proteins on adjacent cells. The pathway is activated by proteolytic cleavage of notch, which results in the intracellular domain translocating to the nucleus and interacting with transcription factors with subsequent changes in gene expression including *Hes*1, a known downstream target. Both *Hes*1 expression as well as nuclear localization of the intracellular domain of *Notch*1 have been demonstrated in mouse UGS epithelium and early prostate buds. Additionally, delta-like ligand1 (Dll1) localizes to epithelial cells where buds emerge, and *Jagged*1 expression localizes to both epithelial cells and mesenchyme at the time of budding.[153] These patterns demonstrate active *Notch*1 signaling at the time and place where bud initiation occurs, which suggests a potential role in prostate specification.

The newborn mouse prostate has high expression of *Notch*1 throughout morphogenesis and subsequently declines to low expression in adults.[154] *Notch*1 is initially expressed in all progenitor cells, but localizes to basal epithelial cells upon cytodifferentiation. Using secretase inhibitors to prevent *Notch* cleavage in neonatal rat ventral prostate organ culture results in markedly reduced branching morphogenesis and epithelial differentiation.[155,156] Recently, studies revealed a positive reciprocal regulatory loop between *Tgf*β and *Notch* in maintaining prostate basal stem cell dormancy,[157] and targeting the Notch pathway is a vibrant area of research for controlling prostate cancer growth and progression.

Secreted Signaling Molecules

In addition to the above transcription factors, multiple secreted signaling molecules play essential roles in bud initiation, branching morphogenesis and cell differentiation in the developing prostate. Specifically, combinations of Hedgehogs, Wnts, fibroblast growth factors (Fgf), and members of the transforming growth factor β family (Tgfβ), including bone morphogenic proteins (Bmp), and activins, are involved as secreted paracrine and autocrine signals in the complex interplay between mesenchymal and epithelial cells to drive and control prostate development. These positive and negative regulatory molecules are spatially and temporally regulated and communicate signals between cells via their cognate receptors. Here we highlight the key families known to be involved in this process.

Sonic Hedgehog

Sonic hedgehog (Shh) is a glycoprotein secreted by epithelial cells at the mesenchymal interface and is involved in proliferation, embryonic patterning, and cell fate determination in developing tissues.[158] *Shh* binds to membrane-bound patched (*Ptc*) receptors on adjacent mesenchymal cells, establishing epithelial-mesenchymal cross-talk. Binding of *Shh* to *Ptc* leads to activation of smoothened (Smo) and downstream activation of the Gli transcription factors. There are three known Gli transcripts in vertebrates: *Gli*1, *Gli*2, and *Gli*3. *Shh* has been shown to have both short-range and long-range actions dependent upon concentration gradients and regulates the expression of other morphogens in multiple structures including the prostate.[159] *Shh* plays several critical roles during prostate gland development, including an essential role for bud initiation, ductal elongation, maintenance of stem and progenitor cell populations, and normal epithelial differentiation. These findings have been reached through studies by multiple laboratories using $Shh^{-/-}$ null mutant mice, organ culture of murine and rat prostate lobes, tissue recombinants, and cell culture work, and the readers are referred to a review for further details.[50]

Shh is expressed in rodent prostate epithelial cells during prostate bud induction and rapidly declines over several days as morphogenesis is completed.[160–163] Its expression follows a spatial pattern, with initial broad expression throughout the bud epithelium transitioning to localized expression at the distal tips of elongating ducts.[163] The *Ptc* receptor is localized to mesenchymal cells adjacent to the distal tips of elongating ducts, with weaker *Ptc* expression seen within the epithelial cells, which is suggestive of an Shh autocrine action in the distal epithelium. *Gli*1, *gli*2, and *gli*3 exhibit a spatial pattern of expression as well, with *gli*1 and *gli*2 expressed in periductal mesenchyme along the length of the duct, while *gli*3 is only found in the mesenchyme adjacent to the tips.[161,163] The human fetal prostate expresses *SHH* with two strong peaks occurring at 10–13 weeks during active morphogenesis as well as at week 28 with corresponding elevated proliferation at that time. Additionally, the SHH signaling pathway genes including *PTC*1 and *GLI*1 have been shown to be active in the human fetal prostate, supporting its role in human prostate development.[164,165]

Androgens have been shown to upregulate prostatic *Shh* expression, but similar *Shh* levels in both male and female f17 UGS tissues and continued *Shh* expression in the male UGS despite AR blockade have cast doubt on the absolute necessity of androgens for developmental expression of *Shh*.[160,163,166] Additionally, *Shh* is expressed in fetal UGS epithelial cells and early prostate buds despite their AR^- status at that time. *Fgf*10 and *Sox*9 have been shown to be upstream of *Shh* in developing prostates, which supports the model of a complex regulatory network between secreted morphogens and transcription factors that ultimately regulates prostate development.[50] As with many developmental genes, strong evidence implicates dysregulation of *Shh* signaling during the process of prostate carcinogenesis and progression, and readers are referred to reviews and recent manuscripts on this topic.[167–169]

Fibroblast Growth Factor-10

Fgfs are a large family of secreted morphogens (23 known members) with a high affinity for heparin and glycosaminoglycans, which position them with interaction with Fgf receptors (FgfR) on target cells.[170–172] *Fgf*10 has been shown to play a critical role in initiation and directional outgrowth and branching of multiple branched structures including the prostate gland.[173,174] In the prostate, *Fgf*10 expression is spatially restricted to the distal aspects of the glands, where it is believed to function as a chemoattractant for elongating ducts and an inducer of ductal branching through stimulation of epithelial cell proliferation.[174] The expression pattern of *Fgf*10 in the prostate mesenchymal pad is broad during early budding stages and subsequently condenses around the elongating ducts with strongest expression at the distal tips during branching.[175] *Fgf*10 binds to the Fgf receptor splice variant *Fgf*R2iiib, a specific receptor for *Fgf*10, which is localized to epithelial cells in the distal tips of elongating rat prostate ducts.[175] This activates downstream intracellular signals in the *ras-raf-Mek-Erk*1/2 pathway to mediate its effects.[175] *Fgf*10 plays an essential role in prostate bud initiation, ductal elongation, and branching morphogenesis.[174–177] It accomplishes this through direct stimulation of epithelial cell proliferation as well as regulation of multiple growth stimulatory and inhibitor factors, which together permit localized domains of active cell division, leading to focal outgrowths and branchpoint formation.[175] *Fgf*10 expression declines to nadir levels as branching morphogenesis is complete, thus limiting continued growth of the tissue.

*Fgf*10 was initially described as prostatic andromedin, defined as a paracrine-acting, androgen-regulated, secreted mesenchymal factor that drives proliferation or differentiation of the epithelium.[178] Although subsequent studies have identified other secreted factors that are downstream of androgen action in the prostate, short-term organ-culture experiments have shown that *Fgf*10 as well as *Fgf*R2iiib are directly regulated by androgens during early morphogenesis, and as such, *Fgf*10 qualifies as a secreted androgen mediator.[140,175] Furthermore, *Fgf*10 signaling was shown to be essential in testosterone-mediated upregulation of *Shh*, *Bmp*7, and *Hox*b13, and downregulation of *Bmp*4 expression in the ventral rat prostate. Together, this provides compelling evidence that *Fgf*10 is an androgen-regulated paracrine factor that influences the expression of additional morphogenic genes. In turn, *Fgf*10 is upregulated by *Sox*9 and downregulated by *Shh-ptc-gli* and *Tgf*β1, contributing to a tight regulatory network of genes for controlled bud outgrowth and branching at specific sites.

Bmps/Tgfβ/Activins

Bone morphogenetic proteins (Bmp) are members of the Tgfβ superfamily, and act as inhibitors of proliferation during development.[179] *Bmp*4 is expressed broadly in the mouse and rat urogenital mesenchyme (UGM) before and during bud initiation, subsequently localizes to the periductal mesenchyme of elongating ducts, and declines to nadir levels shortly after birth.[112,180] $Bmp4^{-/-}$ is embryonic lethal; however, heterozygotic $Bmp4^{+/-}$ mouse ventral prostates exhibited increased branching tips consistent with *Bmp*4 acting as a growth inhibitor. Furthermore, organ culture studies with exogenous *Bmp*4 prevented ductal budding and outgrowth.[112,180] Androgens decrease *Bmp*4 expression, in part through *Fgf*10 signaling, indicating that androgens stimulate prostate growth not only through production of growth stimulatory factors but also by downregulation of growth repressors.[140] A second member of the bone morphogenetic protein family, *Bmp*7, has also been shown to play an inhibitory role in prostate development; however, due to expression in distinctly different cell compartments, the mechanisms of growth inhibition are likely different.[153] *Bmp*7 is expressed in mouse UGM prior to bud initiation and prostate epithelial cells postnatally. Null mutant $Bmp7^{-/-}$ mice exhibit a twofold increase in prostate gland branching, and organ cultures studies showed that exogenous *Bmp*7 restricts prostate growth and branching. *Bmp*7 represses *Notch*1 expression, and it is proposed that *Bmp*7 may restrict branching by limiting epithelial domains with *Notch*1 activity.

Bmp actions are locally regulated through *noggin*, a secreted inhibitor of *Bmp*4 and, to a lesser degree, *Bmp*7. *Noggin* binds Bmp ligands in the extracellular space, thereby blocking their ability to activate membrane receptors. Null mutant $noggin^{-/-}$ mice have complete absence of ventral prostate budding and much-reduced budding in the dorsolateral regions, which is likely due to the unopposed action of Bmps.[181]

*Tgf*β1 has also demonstrated a role in growth inhibition in the developing prostate. *Tgf*β1 expression is localized to the rodent periductal mesenchymal cells and the secreted latent protein, once activated extracellularly, drives their differentiation into smooth muscle cells.[182] Furthermore, paracrine actions on developing prostate epithelium play a role in initiating a differentiation program. *Tgf*β receptors *Tgf*βR1 and *Tgf*βR2 are found in both developing prostate stroma and epithelial cells. Organ culture studies with newborn mouse and rat prostates exposed to exogenous *Tgf*β1 exhibited an inhibition of testosterone-induced growth and branching.[183,184] Interestingly, the action of *Tgf*β1 varies along the proximal–distal axis, with inhibition of proliferation in the proximal ducts and stimulation at the distal tips.[185] *Tgf*β1 has been shown to both decrease *Fgf*10 expression as well as redistribute nuclear AR to the cytoplasm, which may contribute to the growth-inhibitory actions of *Tgf*β1.[185,186]

Activins, another member of the *Tgf*β superfamily, are also involved in prostate development. Activins are comprised of homodimers and heterodimers of βA and βB subunits that form activins A, AB, and B. Activin βA is expressed in mesenchymal cells adjacent to the distal tips of prostate ducts, and activin βB is expressed in mesenchymal and fibroblastic stromal cells. Upon differentiation of the epithelium into luminal cells, activin βB is strongly expressed. Exogenous activin A inhibits ductal branching and elongation in newborn rat ventral prostate lobes. Follistatin binds activins and counteracts their actions. Follistatin is expressed in the epithelium of both developing and mature prostates. Newborn prostate culture subjected to follistatin exhibited increased growth and branching, which supports the role of activins as an inhibitor of prostate development.[187] Together these findings support a balanced interaction between activins and follistatins in regulating ductal outgrowth and branching.

Wnt Genes and Secreted Signaling Regulators

Wnt gene products are a family of secreted glycoproteins involved in tissue patterning, cell fate, and proliferation throughout many different tissues.[188–190] The 19 known mammalian Wnt proteins are divided into two categories based on their downstream signaling pathway. Canonical Wnts signal via nuclear β-catenin/TCF-LEF and noncanonical Wnts function via Ca^{+}/PKC or RhoA/JNK. Both pathways involve initial binding to membrane frizzled (Fzd) receptors on target cells; however, the canonical pathway has a cell membrane coreceptor *LRP*5/6 not seen in the noncanonical pathway.[191] Wnt signaling is tightly controlled by multiple secreted negative regulators including Wnt inhibitor factors (WIF), secreted frizzled related proteins (sFRPs), and dickkopf (Dkk) proteins that locally antagonize Wnt actions. This complexity stresses the importance for tight control of Wnt signaling to effect normal development and maintain tissue homeostasis.[192,193]

The numerous functions of Wnt signaling in animal development include crucial morphogenic roles of many, if not most, organs; thus it is expected that this includes the prostate gland. The rodent developing prostate glands express all components of the Wnt signaling system with cell-specific expression of particular Wnt ligands as well as secreted regulators in a spatially restricted and temporal manner. Wnt ligands with high developmental expression, and thus an assumed specific role, include *Wnt*2, *Wnt*2b, *Wnt*4, *Wnt*5a, *Wnt*7a, *Wnt*7b, *Wnt*10b, *Wnt*11, and *Wnt*16.[50,93,194,195] Although the individual roles for each ligand have not been delineated, studies have shown a role for *Wnt*5a in positional identity of buds and an inhibitory role in duct elongation and branching.[113] Constitutive expression of stable β-catenin in prostate epithelium of transgenic mice resulted in epithelial hyperplasia and squamous metaplasia by 8 weeks of age with transdifferentiation to epidermal-like cell lineages suggesting that canonical Wnt signaling plays a key role in prostate cell determination, differentiation, and proliferation.[196] Canonical signaling through *Lef*-1 plays an important androgen-independent role in progenitor cell proliferation at distal tips of elongating buds.[197] Further, recent studies with conditional deletion of β-catenin in transgenic mice revealed an essential role for canonical Wnt signaling in early prostate lineage specification of UGS epithelial cells as well as initial bud outgrowth.[198] A role for β-catenin is also suggested for adult prostate epithelial proliferation, because nuclear β-catenin levels increase in proliferating prostate epithelium of castrated rat prostates following androgen replacement.[199] However, conditional deletion of β-catenin in the adult prostate did not markedly alter homeostasis, suggesting that sustained expression and β-catenin action is not essential in adult prostate epithelium.[198]

Equally important in mediating proper Wnt signaling and directing prostate morphogenesis are the specific secreted Wnt regulators. *SFrp*1 and *sFrp*2 were found to be highly expressed in the early UGM and prostate mesenchyme and somewhat later in ductal epithelium during bud initiation and elongation.[194,200] Using *sFrp*1$^{-/-}$ null mutant mice, this Wnt inhibitor was shown to be a key paracrine modulator of branching morphogenesis, epithelial proliferation, and differentiation through interference in noncanonical Wnt signaling.[201] *Wif*1, which localizes to developing prostate mesenchyme and elongating ducts, is an androgen regulated factor that increases ductal branching through basal cell proliferation.[202] The addition of Dkk1 protein, which specifically interferes with *Lrp*5/6, to prostate explant cultures increased branching, suggesting that canonical

Wnt signaling also plays an inhibitory role with regards to epithelial cell proliferation during development.[50,200]

FUNCTION OF SEX ACCESSORY TISSUES

The main function of the sex accessory tissues is the production of seminal plasma, the nongamete portion of the semen that serves as a buffered, nutrient transport medium for sperm as they are transported to the female genital tract. Although individual components influence the fertilizing capacity of sperm, it is recognized that seminal plasma substances are not required for fertilization of the egg by mature spermatozoa. Normal rates of fertilization are achieved in vitro using epididymal sperm from animals as well as from humans, obtained by testicular sperm extraction or TESE, a now routine procedure in assisted reproductive laboratories. However, it is accepted that the various components of seminal plasma greatly enhance spermatozoa's in vivo capacity for fertilization. Seminal plasma is slightly alkaline (pH 7.2–7.8), which helps to neutralize the acidic environment of the vagina. Seminal plasma is high in fructose, which serves as an immediate energy substrate for sperm at the time of ejaculation. Prostaglandins in seminal plasma increase female genital tract smooth muscle contractions, which aid in sperm transport, while additional factors are believed to prevent premature capacitation and activate enzymes necessary for oocyte penetration. Zinc and IgA serve as bacteriostatic factors while prostatic proteins prevent sperm from autoagglutination (Table 18.1).

TABLE 18.1 Human Seminal Plasma Components

Component	Concentration (mg/dl)
Phosphorylcholine	1000
Prostate-specific antigen (PSA)	50–500
Citric acid	376
Fructose	200
Spermine	50–350
Cholesterol	103
Prostatic acid phosphatase (PAP)	30–100
Phospholipids	83
Prostaglandins	10–30
Zinc	14
Immunoglobulins	
IgG	7–22
IgA	0–6
IgM	Detected

The normal human ejaculate volume is 1–6 ml and is comprised of mature sperm and seminal plasma. Although the average human ejaculate contains 100 million sperm per milliliter, spermatozoa represent less than 1% of the ejaculate volume, the rest being derived from the accessory sex ducts and glands. At the time of emission/ejaculation, the accessory sex glands do not contract simultaneously but rather in an organ-specific sequence. In man, a small initial fraction from the glands of Littre and bulbourethral gland is released prior to ejaculation and lubricates the urethra. Next, a sperm-rich fraction is expelled due to rapid peristaltic contractions from the distal epididymis and vas deferens, comprising about 5% of the volume. This occurs simultaneously with prostatic contractions and secretions, which contribute approximately 25% of the ejaculate volume. Together in humans, this first fraction is 0.3–0.5 ml and contains the greatest concentration of sperm. The last and largest fraction of the ejaculate originates in the seminal vesicles and varies from 1.0–2.5 ml or 75% of the ejaculate volume in man. Exceptions to this sequence include the bull, where accessory sex glands contract simultaneously, and the dog, which has no seminal vesicles.

The production of seminal plasma is under hormonal control and driven by the presence of androgens, which are always present in normal males. Seminal secretions that do not become the part of an ejaculate are thought to either be passively resorbed by surrounding tissues or expelled via the urethra during urination. During sexual activity, emission is the process by which spermatozoa and seminal plasma are expelled from the accessory sex structures into the urethra. Emission occurs due to contractile tissues in the vas deferens, seminal vesicles, and prostate, which are believed to be largely under alpha adrenergic control. Ejaculation is a separate process, also under sympathetic control, by which the contents of the urethra are expelled from the penis via the urethral meatus. Ejaculation is a penile event beyond the scope of this chapter and covered elsewhere in this textbook.

Coagulation/Liquefaction

Semen coagulates soon after ejaculation due to clotting factors produced by the seminal vesicles and forms a gelatinous mass. This coagulation process, while similar to blood coagulation, is unique to semen and is not inhibited by sodium citrate or heparin and does not involve prothrombin, fibrinogen, or the hematologic clotting factors. Under electron microscopy, coagulated semen contains a dense network of fibers, which trap and restrict the movement of spermatozoa.[203] The main protein products forming the fibrous coagulum are semenogelin I and II, which are produced in the seminal vesicles. The coagulated semen liquefies after 15–60 min

due to proteolytic enzymes produced in the prostatic secretions, including seminin, plasminogen activators, and PSA. This subsequent liquefaction frees the sperm, which can continue their journey to the uterus and beyond. This coagulation permits coating of the sperm with nutrients and factors that contribute to fertilization (e.g., capacitation factor) and also permits deposition of the sperm near the vaginal os. The subsequent liquefaction allows sperm to swim out of the coagulum and into the cervix and uterus. Absent or incomplete liquefaction process can lead to reduced fertility potential, and addition of α-amylase to the semen in the laboratory can rectify this problem. Some rodents produce additional coagulating products produced by a separate coagulating gland, leading to a hardened copulatory plug within the vagina, which prevents backflow of semen from the female reproductive tract.

Components of Seminal Plasma

Mammalian seminal plasma contains a large number of components and considerable variation exists between species. Some are present in the serum and are likely exudates from the circulation, whereas others are produced solely by the accessory sex glands, ducts, and testes, and are unique to seminal plasma. Due to the wide interspecies variability in sex accessory tissues and semen composition, this section will focus on human seminal plasma except where otherwise noted.

Zinc

Normal human seminal plasma contains over 100 times the concentration of zinc compared to serum with a mean value of 140 μg/ml.[204] This high level of zinc comes from the prostate, which has the highest concentration of zinc of any organ in the body.[205] There have been many theories regarding the physiologic role of zinc in the prostate and seminal plasma. There are many known zinc-containing metalloenzymes; however, the levels seen within the prostate would exceed that needed for such enzymes. Zinc is also known to bind multiple proteins,[206,207] and zinc in the seminal plasma may be involved in regulation of liquefaction by binding to semenogelin I and II.[208] Zinc may also serve as an antibacterial factor in both seminal plasma and non-ejaculatory prostatic secretions. In concentrations normally found in prostatic secretions, in vitro studies have shown zinc to be bactericidal against a variety of gram-positive and gram-negative bacteria. Additionally, expressed prostatic secretions from 36 normal men without bacterial infection had a mean value zinc concentration of 350 μg/ml compared to secretions in 15 patients with a documented history of chronic bacterial prostatitis with mean zinc levels of only 50 μg/ml.[207] Further study is needed to determine the precise cause and effect relationship between chronic bacterial prostatitis and zinc levels.

Citric Acid

The concentration of citrate in the ejaculate (mean, 376 mg/dl) is 500–1000 times higher than that in blood plasma. It is one of the major anions of human seminal plasma and is a potent binder of metal ions. Its concentration (20 mM) is similar to that of the divalent metals together, 13.6 mM (calcium 7 mM; magnesium 4.5 mM; zinc 2.1 mM). Seminal plasma citrate predominantly originates in the prostate, with a 100-fold less concentration found in seminal vesicle secretions.[209] The high levels of citrate have been linked to the decreased ability of prostatic mitochondria to oxidize citrate, which results in an imbalance between citrate production and oxidation.[210,211] Kavanagh[212] measured citrate and isocitrate levels and found the prostate to have a 33:1 ratio, whereas other tissues demonstrated 10:1 ratios. This finding suggests decreased aconitase activity as the reason behind such high citrate levels in prostatic secretions.

Fructose

Seminal plasma has a high concentration of fructose (mean, 200 mg/dl), which originates in the seminal vesicles.[203] Based on this, a fructose test on semen is used clinically to evaluate the seminal vesicle contribution to the ejaculate and evaluate potential ejaculatory duct obstruction. Additionally, this can aid in determining the etiology of azoospermia, because congenital absence of the seminal vesicles always occurs with absence of the vas deferens leading to ejaculatory azoospermia despite the potential presence of testicular spermatogenesis.[213] The seminal vesicles also secrete smaller amounts of other free sugars including glucose, sorbitol, ribose, and fructose, but the sum total of these is less than 1/20th the concentration of fructose. This high level of fructose is under androgenic regulation, but other factors such as blood glucose level, frequency of ejaculation, and nutritional status affect the seminal vesicle concentration. Because of the many factors involved, a wide variation in seminal plasma fructose levels can be seen in different semen samples from a single patient.[203]

Fructose in the seminal vesicles is produced from glucose, which undergoes aldose reduction to sorbitol, then ketone reduction to fructose. Fructose in the seminal plasma provides both an aerobic and anaerobic source of energy for spermatozoa. Additionally, cervical mucus has high concentrations of glucose with very little fructose, but sperm are capable of utilizing either sugar for energy.[203]

Phosphorylcholine

Mammalian semen is rich in choline produced mainly in the epididymis, and in the human, phosphorylcholine

predominates. Phosphorylcholine is a specific substrate for PAP, an enzyme that is active in the seminal plasma. This enzymatic reaction results in the rapid formation of choline in the ejaculate. These choline compounds are not metabolized by, nor do they affect the respiration of sperm, and their physiologic role is unclear.[214]

Spermine

Polyamines are basic, positively charged, small organic molecules. They are found in all eukaryotic cells and are believed to be involved in a variety of physiologic processes including cell proliferation. Spermine, a polyamine first discovered in semen, is found in seminal plasma at concentrations of 50–350 mg/dl and gives semen its unique odor. The prostate is the source of spermine and has the highest levels found in the body. The rate-limiting step in the synthesis of polyamines and spermine is mediated by the enzyme ornithine decarboxylase (ODC). ODC expression has been studied in BPH tissue and found to be increased suggesting a possible role for polyamines in that disease process.

Spermine has four positive charges, which bind strongly to acidic or negatively charged molecules such as phosphate ions, nucleic acids, or phospholipids. Spermine is created via a series of enzymatic reactions on other polyamines that proceed from ornithine to putrescine to spermidine to spermine. Spermine is oxidized enzymatically by diamine oxidase, which is found in the seminal plasma. The aldehyde products from this reaction are very reactive and toxic to both sperm and bacteria.[215,216] This is one reason why prolonged exposure of sperm to seminal plasma will reduce its fertilizing capacity.

Prostaglandins

In 1934, von Euler coined the term "prostaglandins" to describe the active components in seminal plasma in the assumption that they were produced by the prostate. Over 20 years later, Eliasson determined that the seminal vesicles were the primary producer of prostaglandins; however, the name stands to this day.[217] Prostaglandins are found in a wide variety of tissues, but the seminal vesicles have the highest levels by far in humans. The seminal plasma contains 15 different prostaglandins at a concentration of 100–300 μg/ml. Prostaglandins are 20-carbon hydroxyl fatty acids containing a cyclopentane ring and two side chains. They are classified into four major groups (A, B, E, and F) based on the structure of the cyclopentane ring and given a number based on the structure of the side chain (e.g., PGE3).

The E group of prostaglandins predominates in the male reproductive tract, while the F group predominates in the female. Prostaglandins have a variety of potent proposed physiologic effects in the male and are involved in erection, ejaculation, sperm motility, and testicular and penile contractions. Additionally, seminal fluid prostaglandins deposited in the vagina affect cervical mucus, vaginal secretions, uterine contractions, and thus rapid transport of sperm in the female genital tract independent of their motility.

Cholesterol and Lipids

Human seminal plasma contains 185 mg/dl of total lipids, 103 mg/dl of cholesterol, and 83 mg/dl of phospholipids. The phospholipids have been further characterized as 44% sphingomyelin, 12.3% ethanolamine plasmalogen, and 11.2% phosphatidylserine. It has been proposed that the ratio of cholesterol to phospholipid helps stabilize the sperm against temperature and environmental shock.[218,219]

Immunoglobulins

Immunoglobulins are found in the seminal plasma at levels lower than in blood. Specifically, IgG is measured from 7 to 22 mg/dl and IgA from 0 to 6 mg/dl. IgM has also been detected in the seminal plasma at very low levels.[220] Given the lower levels of immunoglobulins, it is theorized that these may simply be exudates; however, IgA has been localized more to the bulbourethral glands and suggests an immunologic component to the bulbourethral gland function.

Acid Phosphatase

Acid phosphatase activity is more than 200 times more abundant in the prostate than any other tissue. PAP is a 102-kd glycoprotein dimer with seminal plasma levels of 0.3–1 g/l. Interestingly, the level of PAP in the human prostate is 1000 times higher per gram of tissue than in the rat prostate. The substrate for PAP in the seminal plasma may be phosphorylcholine phosphate, which is rapidly hydrolyzed by the enzyme. However, the physiologic significance of the high levels of PAP and their function are not well understood.[221,222] There was previously greater interest in acid phosphatase assays in serum as a potential marker of metastatic prostate cancer; however, this interest has waned with the more sensitive and specific assay for PSA.

Prostate-Specific Antigen

PSA is a 33-kd glycoprotein that acts as a serine protease and is produced almost exclusively in prostate epithelial cells.[223] PSA is involved in lysing the ejaculatory clot with semenogelin as its substrate. The PSA gene (*hKLK3*) is a member of the kallikrein family of genes, which are all located on chromosome 19.[224] Clinically, PSA is used as an important serum marker for prostate cancer and prostate disease, because its presence in the circulation indicates a breakdown in the epithelial/basement membrane barrier. Unfortunately, while PSA is organ-specific, it is not cancer-specific and is found

in the serum of patients with BPH as well as prostatitis. PSA is found in seminal plasma in concentrations from 0.5 to 5 mg/ml, whereas PSA in serum of men 50–80 years of age without prostate disease is 1000-fold less, at 1.0–4.0 ng/ml.[225] The serum level of PSA in men with prostate cancer is generally elevated (>4.0 ng/ml), but not until its level is >10 ng/ml does this measurement suggest specificity for prostate cancer. As prostate cancer cells remain AR-dependent, even in the absence of androgens, circulating levels of PSA in patients with metastatic disease can reach into the hundreds and monitoring levels over time can be a useful indicator of progression and relapse after treatment failure.[226,227]

REGULATION OF THE ADULT PROSTATE

As with the developing accessory sex glands, the normal adult prostate, seminal vesicles, and bulbourethral glands are hormone-regulated and hormone-dependent tissues, requiring continued support for their homeostasis and functional activity. Although this is primarily mediated through androgens, other hormones influence these glands as well. Again, the majority of research on this topic has focused on the prostate, which will be highlighted here. In essence, the hormones that maintain the adult prostate can be divided into two categories, the steroidal and protein hormones. These hormones and their molecular mechanisms of action are described in greater detail in Chapters 9, 16 and 20.

Steroid Hormones

Androgens

The adult male accessory glands are entirely dependent on androgen action, mediated through AR, for homeostasis and functional activity. Removal of androgens results in rapid involution of the prostate epithelium, and to a lesser degree, stromal cells (within days) through programmed cell death or apoptosis.[228] This action is the basis for androgen deprivation therapy for the treatment of prostate cancer for which Charles Huggins was awarded the Nobel Prize in 1966.[229] Androgen replacement restores prostate size through rapid proliferation of stromal and epithelial cells and restores secretory capacity in the epithelium. Importantly, once prostate cell numbers reach baseline adult levels, negative feedback mechanisms, involving stromal–epithelial cross-talk through secreted growth regulators, result in growth cessation and prevent overgrowth of the gland. For the most part, these factors include many of the same mediators (e.g., *Tgf*β_1, *Bmp*4, and activin A) that regulate normal development as discussed above.[230]

Most androgens used by prostate cells are synthesized in the testes, although adrenal androgens can contribute to prostate health and disease. Testosterone enters prostate cells through passive diffusion as well as sex hormone binding globulin (SHBG)-facilitated transport and is rapidly and irreversibly metabolized to DHT by 5α-reductase (SRD5A1, SRD5A2) expressed in prostate cells.[231] DHT formation is highest in stromal cells and it rapidly diffuses into the prostate epithelium, although the epithelial cells also express this enzyme. Due to its higher affinity for the AR relative to testosterone, DHT is considered the primary androgen for the prostate, although testosterone can mediate similar effects.[232] The differential potency of AR for the separate androgens permits higher androgenic actions in the androgen-dependent prostate, seminal vesicles, and bulbourethral glands under physiologic circulating levels of testosterone.[233]

AR is expressed at high levels in all adult prostate luminal epithelial cells, while the basal and neuroendocrine cells are largely AR$^-$.[38,234] AR expression in adult prostate stroma is highest in smooth muscle cells with variable expression in fibroblasts.[38,234] Although prostatic endothelial cells are AR$^-$, vascular smooth muscle cells express AR and rapidly respond to androgen actions.[235] In the adult prostate, androgens primarily function to regulate the secretory activity of luminal prostate epithelial cells in addition to maintenance of homeostatic growth and survival. Thus the production of PSA, PAP, and multiple other secreted proteins in the human prostate (discussed later) and PBP and probasin in the rat prostate are under the direct regulation of DHT-AR at the promoter level of regulated genes. In contrast, androgen-mediated growth homeostasis of the adult prostate gland is primarily mediated through AR in stromal cells that regulate paracrine signaling between stromal and epithelial cells to control limited cell renewal and apoptosis. Of note, recent research has also determined that epithelial cell AR serves as a negative regulator of epithelial cell proliferation in the adult prostate, thus maintaining tight control over tissue size.[70]

Estrogens

Aging males are exposed to relatively higher levels of circulating estradiol, which have been shown to impact the prostate gland. In addition, estrogens are produced locally within the prostate via conversion of testosterone to 17β-estradiol by aromatase expressed within the prostate stroma[236,237]; thus estrogen action in the prostate can occur independent of serum levels of this steroid. The relative levels of free circulating estrogens increase as men age, which can increase estrogenic action in the prostate gland. Bioavailable testosterone levels decline in the aging male due to decreased production by the testis and increased SHGB levels combine to lower

free circulating testosterone.[238] However, circulating levels of free estradiol remain constant in the aging male due to an age-related increase in body weight and adipose cells, which express high levels of aromatase and peripherally convert androgens to estrogens.[239] The net result is a significant increase in the estrogen to testosterone ratio, allowing the balance between androgen and estrogen regulation of prostate growth to shift towards estrogen dominance. It has been proposed that increased estrogenic stimulation of the prostate in the aging male may lead to reactivation of growth and subsequent neoplastic transformation.[240,241]

Estrogen action in the adult prostate is mediated through ERα and ERβ, which are expressed in different cellular compartments of the prostate gland; ERα localizes to scattered prostatic stromal cells and ERβ is primarily expressed in prostate epithelium. This differential location as well as differential affinity of the two ERs for ligands, enhancers and co-activators may explain the diverse biological functions of estrogens within the prostate gland and may also be exploited for regulation of prostate disease. Recent reviews on ERs and their actions in the prostate gland provide extensive details of their expression, mechanisms of action, and functional roles.[242–244] In brief, ERβ is the predominant estrogen receptor expressed in both basal and luminal cells of the adult prostate gland, where it plays a role in maintaining the differentiated state of the epithelium. A study using βERKO mice ($ER\beta^{-/-}$) showed a shift in basal, intermediate, and luminal epithelial cell markers in the prostate towards a less differentiated gland that supports this function.[245] ERβ also has an important antiproliferative role in the prostate and participates as a brake for androgenic stimulation of prostate growth.[244,246] Further, it maintains prostate epithelial numbers in the adult prostate through targeted apoptosis of the basal and progenitor cell population, thus contributing to growth regulation of the adult gland.[247]

Retinoids and Vitamin D

Retinoic acid and retinoids are derivatives of vitamin A and play a major role in organ development and tissue homeostasis. Their actions are mediated through retinoid receptors RARα, β, γ, and RXRα, β, γ, which form RAR/RXR dimers that directly regulate target gene expression.[117] Adult human prostate stem and progenitor cells from disease-free donors express RARs and RXRs, and treatment with retinoic acid drives their differentiation into a luminal cell fate, indicating that retinoids maintain epithelial homeostatis in the adult gland.[84] Retinoic acid and its synthetic analogs trigger antiproliferative effects and differentiation in a variety of tumor cells, which has led to much research as potential anticarcinogenic agents. Many studies have explored the usefulness of retinoids in chemoprevention and treatment of prostate cancer, although mixed results have limited their widespread use in this regard.[248–250]

Vitamin D_3 is synthesized in the skin via ultraviolet light exposure and is also acquired in the diet. Following metabolism to the bioactive 1,25 hydroxyvitamin D_3 (1,25OH-D_3), this hormone binds to the vitamin D receptor (VDR), which forms an obligate heterodimer with RXRα to regulate gene transcription in target cells. Conversion of 25OH-D_3 to the active 1,25OH-D_3 by CYP27B1 occurs within the normal prostate and indicates that local production of this hormone may play a critical role in normal growth and differentiation.[251] VDR/RXRα are highly expressed in normal prostate epithelium where vitamin D_3 regulates hundreds of genes and possesses chemopreventive activities against prostate carcinogenesis.[252] Vitamin D_3 has antiproliferative and prodifferentiation effects on prostate epithelium, which is the basis for its chemopreventive actions. VDR null mutant mice produce more aggressive tumors when crossed with transgenic mice targeted for prostate cancer, further supporting the anticancer properties of vitamin D_3.[253] Recently, 1,25OH-D_3 was shown to induce cell cycle arrest in adult mouse prostate epithelial stem/progenitor populations and drive them towards luminal epithelial cell differentiation.[254] These findings for vitamin D_3, similar to the retinoid findings, are suggestive of a possible chemopreventive or therapeutic role by targeting adult prostate stem/progenitor cells, limiting their proliferation, and driving them into differentiated phenotypes. Whether this would be applicable to prostate cancer stem cells remains to be determined.

Protein Hormones

Prolactin

Prolactin (PRL) has direct effects on the prostate gland through PRL receptors (PRLR) expressed by prostate epithelial cells. Seminal work by Grayhack and others established that PRL drives prostate growth and enhances its secretory capacity.[255,256] Later studies revealed that PRL mediates these actions in part through upregulation of AR[257] as well as through direct and androgen-independent pathways.[258,259] Equally important, the rodent and human prostates produce their own PRL.[260,261] Extensive studies by Nevalainen have established that downstream actions of PRL in prostate cells are mediated through the *PRL-Jak*2-*Stat*5a/b signaling.[262]

Growth Hormone and Insulin-Like Growth Factor

Growth hormone (GH) has both direct and indirect actions on the prostate gland through GH receptors (GHR) expressed on epithelial cells as well through induction of hepatic synthesis of *Igf*-1. Clinical and epidemiological studies suggest that the GH/IGF axis is

important for normal human prostate growth. Chronic GH deficiency in adulthood is associated with reduced prostate volume,[263] whereas the prevalence of prostate hyperplasia was lower in GH-deficient patients as compared to controls.[264] Further, acromegalics have enlarged prostates that decrease significantly in size in response to somatostatin analogs or after surgical removal of a pituitary tumor to lower GH levels.[265]

Insulin-like growth factor-1 (Igf-1) is an important mediator of many of the biological activities of GH through *Igf*R expressed on target cells[266] and this is an important pathway for prostate development and homeostasis as well. *Igf*-1/*Igf*R signaling activates multiple downstream pathways, most notable p-*Akt* activation, leading to proliferation, differentiation, and proapoptotic actions. Conditional deletion of *Igf*1R in adult transgenic mice led to a hyperproliferative prostate epithelium indicating that the *Igf*-1/*Igf*R axis plays a key role in limiting cell proliferation.[267] Similarly, in normal adult human prostate epithelial cells, IGF promoted differentiation to luminal cells and not proliferation.[268]

Although there is ample evidence in human and animal studies that the *Igf*-1/*Igf*R axis plays an important role in prostate growth and homeostasis, it is important to note that not all of GH actions on the prostate are mediated through this downstream effector. Rodent models with deletion of the GH or GHR axis through spontaneous or targeted deletion have suggested a direct role of GH signaling independent of *Igf*-1 in maintaining normal prostate size and facilitating carcinogenesis and progression with aging.[269] This is supported by further studies that show that targeted deletion of hepatic *Igf*-1 is not sufficient to inhibit prostate cancer progression.[270] Taken together, these findings suggest that concomitant regulation of *Igf*-1 signaling and reduction in GH levels may be more effective in managing prostate disease.

Endocrine Disrupting Chemicals

Increasing evidence from both epidemiologic studies and animal models has implicated certain environmental chemicals as possibly influencing the development or progression of prostate diseases.[271–273] In most instances, these compounds are known endocrine disrupting chemicals (EDCs) and effects appear to be primarily, although not exclusively, linked to estrogen and androgen signaling, either by interacting directly with receptors or by influencing steroid metabolism and altering endogenous hormone levels.[271] The chemicals showing associations with aberrant growth and prostate cancer include certain pesticides (atrazine, chlorpyrifos, fonofos, phorate), arsenic, polychlorinated biphenols (PCBs), and bisphenol A (BPA). Unfortunately, these EDCs perturb homeostasis at low, environmentally relevant levels. because they hijack the endocrine system, which itself is geared to rapidly respond to low circulating levels of hormones in the picomolar and nanomolar range. This necessitates the widespread screening of manmade chemicals for hormone activity, a focused goal by the Environmental Protection Agency (EPA) and other regulatory agencies worldwide. Further, it demands that policymakers mandate effective screening strategies and regulations going forward (e.g., Toxic Chemicals Safety Act, or TSCA) to limit EDC exposures that may harm wildlife and human health.

DISEASES OF THE PROSTATE GLAND

Prostate Cancer

Prostate cancer is the most common noncutaneous malignancy in men in the United States. One in six American men will be diagnosed with prostate cancer and one in 36 will die of this disease, making prostate cancer a leading cause of cancer deaths (Figure 18.8), second only to lung cancer.[2] Prostate cancer poses a particular challenge to clinicians in that it has a high prevalence but is often an indolent disease. Autopsy studies have found prostate cancer in approximately 42% of American men 50 years or older who died of causes other than prostate cancer.[274,275] Prostate cancer is rarely diagnosed in men younger than 50, representing only 2% of cases, and has a median age of diagnosis of 68 years.[276,277] African American men have the highest incidence of prostate cancer worldwide, with a relative incidence of 1.6 as compared to Caucasian-American men. Furthermore, African-Americans with prostate cancer are more than twice as likely to die of the disease as Caucasian men.[2]

Prostate cancer is most likely to arise in the peripheral zone, which accounts for 70% or more of cases. The transition zone accounts for 10–20% of cases; and although the central zone comprises approximately 15–25% of the glandular volume in the prostate, only 1–5% of prostate cancers arise in this region.

Prostate cancer that has not metastasized is a curable disease, with most patients choosing either surgical removal through radical prostatectomy or radiation therapy. Unfortunately, both of these therapies have significant long-term risks associated with them, including impotence and incontinence, making it of critical importance to identify patients with indolent versus aggressive prostate cancer to best determine who would benefit from treatment versus surveillance. These indicators are not presently available, and continued research for blood and urinary markers as well as molecular markers in biopsy specimens is urgently needed. Aggressive prostate cancer that has progressed and metastasized is initially responsive to androgen

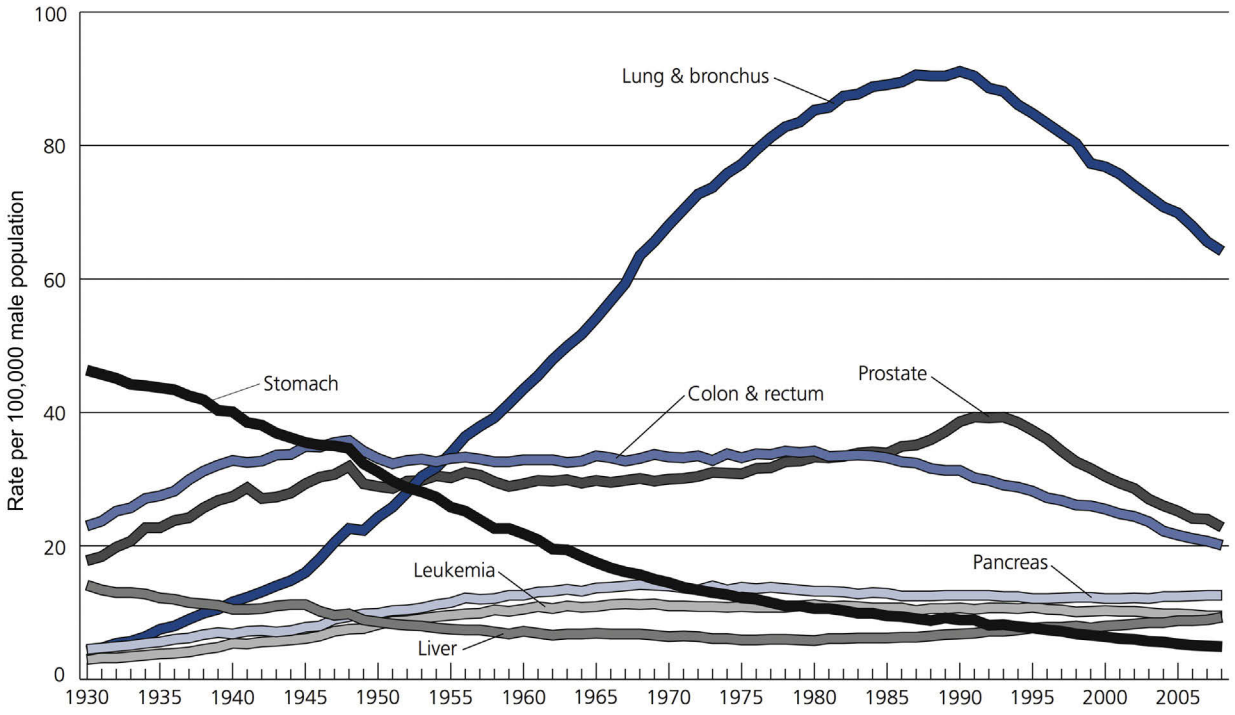


FIGURE 18.8 Age-adjusted American male cancer death rates (per 100,000, age adjusted to the 2000 US standard population) by site, US 1930–2008. Note: Due to changes in ICD coding, numerator information has changed over time. Rates for cancer of the liver, lung and bronchus, and colon and rectum are affected by these coding changes. *Source: From American Cancer Society; US Mortality Volumes 1930 to 1959, US Mortality Data 1960 to 2008, National Center for Health Statistics, Centers for Disease Control and Prevention.*

deprivation therapy, with patients going into remission for variable periods of time. However, prostate cancer almost universally becomes an androgen-independent disease and progresses despite androgen ablation after 12–36 months.[278–280] Although this castration-resistant prostate cancer (CRPC) is androgen-independent, it is important to stress that it remains AR-dependent in the vast majority of cases due to AR mutations, overexpression, ligand-independent activation, and autocrine production of local androgens by the cancer cells themselves. Thus new generation treatment modalities include, among other approaches, compounds that inhibit AR action and nuclear AR localization to prevent downstream signaling as well as novel steroid synthesis inhibitors.[281] The number of drugs available for CRPC has grown considerably in the past few years, with even more agents currently in trials, which will hopefully expand the arsenal of therapeutics against this challenging and deadly disease.

Prostate Cancer Stem Cells

A great amount of research effort over the past decade has gone into understanding the rare stem cell in the prostate gland and how its regulation and physiology may provide insight into the origin and treatment of prostate cancer. The cell of origin of prostate cancer, known as the tumor-initiating cell, appears to be heterogeneous and can include the differentiated basal, luminal, and perhaps neuroendocrine cells or involve transformation of the stem or early progenitor cells themselves, as has been shown in the hematopoietic system.[282–284] Regardless of origin, it is becoming clear that prostate cancers have stem cells of their own, which seed and maintain tumor growth. Evidence has shown that prostate cancer stem cells are highly resistant to hormonal and chemotherapy and are responsible for continuously seeding and maintaining growth, which may be the root cause of relapse after androgen ablative therapy ceases to work.[285] Learning more about normal prostate stem cells and prostate cancer stem cells in the future may lead to novel strategies for effectively treating prostate cancer.

Hormonal Influence

Aberrant AR signaling plays an essential role in both androgen-dependent and androgen-independent prostate carcinogenesis and progression. There are multiple factors that account for this, including AR polymorphisms, AR gain-of-function mutations, AR gene amplification, expression of AR variants lacking a ligand-binding domain, post-translational

modifications, alterations in AR co-regulators, and activation of AR through kinase cascades.[286–288] Androgens are also involved in mediating epithelial-mesenchymal transition of transformed epithelial cells, an initial step for metastatic spread.[289] Further, there is strong evidence for aberrant metabolism of androgens in prostate carcinogenesis and progression.[290] Readers are referred to these pertinent reviews for greater detail.

Estrogens play a significant role in the etiology and progression of prostate diseases in the aging male. There is a clear role for estrogens in mediating prostate carcinogenesis and prostate cancer progression, driving BPH, and as an underlying cause of some forms of prostatitis. These effects are mediated through ERα, ERβ, and the more recently characterized G-protein coupled receptor, GPR30, which facilitates estrogen actions at the cell membrane.[86,291–293] The readers are referred to recent comprehensive reviews on estrogen's action in abnormal growth and disease of the prostate.[242–244,294]

Elevated circulating levels of PRL that occur in some aging men have been shown to promote BPH and stimulate prostatitis in animal models.[295–297] Equally important, the rodent and human prostates produce their own PRL, and dysregulation of this signaling pathway can drive prostate carcinogenesis and progression.[260,261] Extensive studies by Nevalainen have established that downstream actions of PRL in prostate cells are mediated through the *PRL-Jak*2-*Stat*5a/b signaling.[262] Current work by this group is focused on utilizing this axis as an effective chemopreventive and therapeutic target for prostate cancer treatment.[262,298] Importantly, this approach shows potential for treating prostate diseases through a nonsteroidal axis, which universally fails due to the emergence of androgen-independent disease.

GH signaling has been shown to regulate proliferation of human prostate cancer cell lines, and their growth is slowed when transplanted in GH-deficient Little mice (*Ghrhr*$^{lit/lit}$) relative to controls.[299,300] These studies support the notion that GH signaling is important for the growth of advanced human prostate cancers.

In contrast, IGF treatment to prostate cancer cells stimulates cell proliferation, suggesting a differential response to this signaling axis in the transformed state.[268] Prospective epidemiology studies identified a positive association between serum IGF-I and prostate cancer risk,[301–303] and current clinical trials targeting this axis with specific antibodies and small molecules show therapeutic potential.

Benign Prostatic Hyperplasia

Nonmalignant growth of the prostate with age, BPH, occurs in 40–50% of men by age 50, and over 80% of men in their 80s.[3] This prostatic overgrowth can lead to urinary symptoms and, in severe cases, obstruction. Up to half of all men by the age of 70 have urinary symptoms that warrant daily medication or surgery, and the majority of these symptomatic patients have an enlarged prostate as a cause of their suffering.[304,305] BPH predominantly occurs in the periurethral transition zone, which can subsequently impinge on the urine flow through the prostatic urethra and become an obstructive process. BPH is a hyperplastic not a hypertrophic process, in which there is a net increase in the number of cells and not the size of cells.[306] This includes increased epithelial cell proliferation as well as stromal cell hyperplasia, which may underlie the disease through stromal–epithelial growth induction. Medical treatments for BPH and lower urinary tract symptoms include 5α-reductase inhibitors, which block the conversion of testosterone to DHT, resulting in a significant reduction in the volume of the prostate as well as symptoms in men with enlarged prostates.[307] The alpha-adrenergic blockers are another treatment option for BPH, which address the active component of obstruction by inhibiting α1 receptors on smooth muscle cells in the prostate and bladder neck, causing relaxation and improvement in symptoms in many patients.[308] Patients with symptoms refractory to medical treatment can benefit from a variety of procedures that all have the common goal of removing the offending enlarged portion of the prostate. These procedures include open surgical removal of the prostate adenoma and a variety of transurethral endoscopic procedures including transurethral resection of the prostate (TURP), laser ablation, microwave ablation, radiofrequency needle ablation, and electrical vaporization of the prostate.

Prostatitis

The prostate is also susceptible to an inflammatory process called prostatitis, which causes pelvic pain. Up to 16% of all men have this condition diagnosed in either an acute or chronic form at some time in their lives. The etiology of their prostatitis is tied to a known bacterial cause in less than 10% of cases, with the majority being idiopathic.[309] Prostatitis is classified into four categories: category I acute bacterial prostatitis, category II chronic bacterial prostatitis, category III chronic (nonbacterial) pelvic pain syndrome, and category IV asymptomatic inflammatory prostatitis, which is incidentally found as inflammation in a pathology specimen. Treatment depends on the category of prostatitis and includes culture-directed antibiotics, empiric antibiotics, nonsteroidal anti-inflammatory drugs, and alpha-adrenergic blockers particularly in patients with concomitant lower urinary tract symptoms. The etiology of nonbacterial prostatitis is unclear, with various proposed mechanisms including an autoimmune response, dysregulated cytokine production, a neurogenic cause, and a hormonal

basis involving elevated estrogens and prolactin with aging.[310] Further research is needed to better understand and treat this widespread disease.

CONCLUSION

The prostate continues to have an enormous impact on men's health due to its highly prevalent diseases: prostate cancer, BPH, and prostatitis. Multiple key areas of research have been identified to better understand the pathophysiology of prostate disease. Androgen signaling plays a crucial role in prostate development, growth, and homeostasis throughout life, and similarly, androgens and the AR are an integral part of prostate cancer growth and BPH. Other hormones such as estrogen, PRL, and GH, which have been less studied in relation to the prostate, contribute to physiologic and pathophysiologic processes. Disruption of endocrine signaling from environmental substances has also been implicated as a contributing factor in prostate disease. Future research is required to identify the molecular intricacies of this hormonal interplay that is intimately involved in dysregulated growth of this organ with aging. In addition to hormonal signaling, much more work is needed to better understand the exceedingly complex interactions between the stroma and epithelia of the prostate in development and normal maintenance as well as the communication breakdown that occurs in prostate disease. Another critical research concern is the prostate stem cell and prostate cancer stem cell, which may hold the key to future prostate cancer therapies. Our knowledge of prostate physiology and disease has grown by leaps and bounds since the first edition of this text in 1988, but many questions remain and the prostate continues to impact men's health greatly. It is hoped that continued research in this field may lead to future treatments and cures for these aging-associated diseases. In contrast to the prostate, relatively little work has been performed on the more enigmatic bulbourethral gland and seminal vesicle, largely due to their lack of disease. Nonetheless, a clearer understanding of all of the sex accessory glands' physiology and the interaction between sperm, seminal plasma, and the female reproductive tract may lead to better understanding of and treatment for male infertility.

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CHAPTER

19

The Immunophysiology of Male Reproduction

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INTRODUCTION

The Male Reproductive Tract as a Unique Immunological Environment

The male reproductive tract, and the male gamete especially, present a unique challenge to the immune system. The human testis continuously produces highly differentiated sperm derived from a pool of spermatogonial stem cells at a level of productivity and complexity matched only by the hematopoietic system. In contrast to the hematopoietic tissues, however, differentiated sperm first appear at the time of sexual maturation, long after the maturation of the immune system and the establishment of systemic immune tolerance (Figure 19.1).[1] In humans, the period between the editing of the lymphocyte repertoire and the first appearance of significant numbers of the earliest premeiotic germ cell (the spermatocytes) is generally more than 10 years. As a consequence, spermatogenic cells express many cell-specific proteins and other molecules that have the potential to be seen as "foreign" or "nonself" by the immune system.

We know that the immune system tends to view spermatogenic cells as foreign because of the relatively high incidence of autoimmune infertility among human populations. Even in developed countries with modern health care, sperm autoantibodies represent 5–10% of all male infertility,[2,3] while testicular biopsies from infertile men frequently display evidence of asymptomatic inflammatory reactions.[4,5] In other forms of autoimmune disease, such as type 1 diabetes or gastritis, the development of autoimmunity is due to disruption of the normal regulatory controls of the immune system, leading to reactions against antigens that normally are ignored. Only a limited number of antigens are involved and a specific dominant autoantigen usually has been identified.[6] In the case of the male reproductive tract, by contrast, autoimmunity generally involves antigens that would not be edited out of the self-reactive repertoire in the first place. Hence, autoimmune infertility generally involves multiple antigens as well as different antigens from one individual to another.[7–9] Moreover, infertility is not the only urological problem with an immunological basis. Chronic pelvic inflammatory disease in men, which may be accompanied by recurrent and even debilitating perineal or scrotal pain, is a serious and frequently intractable condition.[10–12] Infections may represent the initial cause of the majority of such cases, but the underlying mechanisms almost certainly involve an autoimmune component.[10]

Unraveling the origin of these immunologically-based disturbances of reproductive function is an important clinical goal, but the converse, and equally important, scientific question is: *What is it about the male reproductive tract that permits the continuous production of huge numbers of immunogenic cells expressing multiple autoantigens without apparent problems in the majority of individuals?* In most respects, the immune system within the male reproductive tract appears relatively normal, with effective lymphatic drainage and relatively free access of immune cells.[13,14] Certainly, there are some unique structural characteristics of the male reproductive tract that may contribute. For example, the male gametes are held at a substantially lower temperature than the rest of the body in species with scrotal testes and epididymides. There also exists a very effective blood–testis barrier that sequesters most of the spermatogenic cells in the testis.[15–18] Nonetheless, these physical elements cannot account for all the manifestations of immunological protection in the male reproductive tract.

Instead, studies over many years have confirmed that the male reproductive tract, and the testis in particular, constitutes a unique immunoregulatory environment. There are communication and regulatory networks that are common to both male reproduction and the immune system, providing many striking overlaps between the

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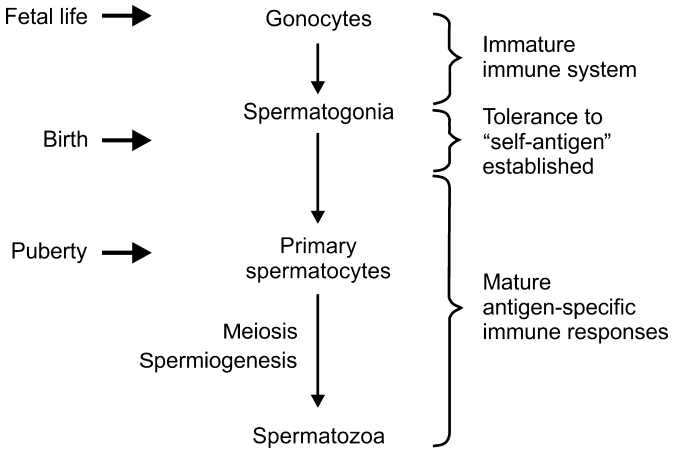

FIGURE 19.1 Developmental timeline of spermatogenesis and the maturation of the immune system. The majority of spermatogenic cells do not appear in the testis until the initiation of meiosis at puberty, whereas tolerance to self-antigen is largely established by the time of birth. As the development of spermatozoa from the spermatogonial stem cell population involves complex processes of nuclear reorganization (meiosis) and cellular differentiation (spermiogenesis) that are unique to the testis, there exists enormous potential for antigens of spermatogenesis to evade conventional tolerance mechanisms.

control of spermatogenesis and the processes of inflammation and immune activation. Part of this network includes local immunoregulatory and immunosuppressive mechanisms, which exist to provide protection for the endogenous antigens of spermatogenesis, protection that also extends to antigens expressed by foreign grafts inserted into the testis environment.[19] Immune cells that enter the environment of the male reproductive tract become functionally modified to restrict their proinflammatory activity and provide an immunologically constrained environment where antigen-specific immune responses are closely controlled. Balanced against this, of course, is the question of how the male reproductive tract is able to protect itself from recurrent infections and tumor development under these circumstances.

The observation that reproductive dysfunction is not only associated with local infection and its accompanying inflammation, but also with systemic disease, provides further evidence of an intimate relationship between the male reproductive tract and the immune system.[20,21] Many systemic illnesses are accompanied by a reduction in both serum androgen levels and sperm output, indicating that male sexual function and general well-being maintain a reciprocal relationship. It has been suggested that this represents a physiologically important mechanism, having evolved to limit reproductive activity during periods of illness. The possibility that acute inflammatory dysregulation of male reproductive function can lead to more permanent problems, such as autoimmune infertility or chronic inflammatory disease, also must be considered. It may even be the case that pre-existing hypogonadism predisposes men to inflammatory disease. At a fundamental level, the ability of the immune system and the male reproductive system to co-exist is no less essential to male reproductive success than the normal operation of the hypothalamic-pituitary unit or the critical interactions between somatic cells and spermatogenic cells. The use of the term *immunophysiology* in this context highlights the fact that the immunology and basic physiology of male reproduction cannot actually be separated.

Since the original publication of this chapter, nearly a decade ago, there have been considerable advances in our understanding of male reproductive immunophysiology. Most significantly, inflammation has moved to center stage in male infertility, with more testicular pathologies now being recognized as possessing inflammatory features,[22,23] and there is growing awareness that basic immunological mechanisms underlie normal reproductive tract function.[24] There has been a considerable increase in knowledge of the mechanisms responsible for controlling testicular immune responses, as well as advances in understanding of the unique immunology of the epididymis and excurrent ducts. The principal aim of this chapter is to provide a comprehensive overview of the field, including how it has developed, its basic tenets and mechanisms, and a broad outline of the current state of knowledge. The overview is confined to consideration of mammalian species, as particularly relevant to the human.

Historical Aspects

The study of male reproductive tract immunophysiology extends back to the very beginnings of endocrinology and immunology. Inflammation of the testis and its association with mumps parotitis was known in ancient times, having been described in the writings of Hippocrates (c. 460–377 BC). Studies on the transplantation of testes in domestic chickens, generally considered to be the earliest studies in the field of endocrinology, were undertaken in the eighteenth century by Hunter and by Michaelis (cited by Setchell, 1990),[25] although the first systematic experiments actually were reported by Berthold in 1849.[26] "Successful" transplants of mammalian testes had to wait until some time later.[27–29] In the early part of the last century prior to the discovery of the male sex hormone, testosterone, transplantation of male gonads, including transplants of animal gonads into humans, was undertaken by charlatans and serious researchers alike in the search for treatments to increase male health and virility.[30,31] It was quickly noted that, although the function of the interstitial tissue appeared to continue in these transplant experiments, tubule function was not preserved.[32] While there is no doubt that temperature and revascularization issues were

important limitations, it is now obvious that many of these experiments faced a crucial immunological impediment. Even today, grafts of testicular tissue generally are more successful in an immunologically compatible or immunocompromised host.[33] The concept of immunoregulation entered the story with the discovery that allogeneic testicular grafts could be made to survive much more successfully if tolerance had been induced by injection of the donor allogeneic cells at the time of birth in the donor,[34] or when transferred into the anterior chamber of the eye.[35,36] Sometime later, Billingham and others conducted a series of investigations into so-called immunologically privileged sites that led to recognition of the testis as a tissue that was particularly favorable for graft survival.[37]

Evidence that the spermatozoon itself is immunogenic to its autologous host dates back to the discovery of antibodies against sperm by Lansteiner[38] and Metchnikoff.[39] In the 1920s,[40] Guyer was able to produce infertility in rabbits and guinea pigs by passive immunization with sperm specific antisera, while Kennedy reported degenerative changes in the testis following active immunization with autologous sperm.[41] By the middle of the century, Voisin and colleagues were able to produce aspermatogenesis in guinea pigs by active immunization with testicular extracts.[42] Eventually, an association between sperm autoantibody formation and infertility in humans was noted.[43,44] Further experimental evidence confirming the possibility of autoimmune reactions against the sperm led to a general acceptance that these are an important cause of infertility in men.[2,45] In time, this led to the broader concept that the testis environment must provide protection for the spermatogenic cells through specific regulatory mechanisms.

Biological sex exerts a significant influence on the immune system. Gender differences in health and the greater prevalence, earlier onset, and severity of autoimmune diseases, such as systemic lupus erythematosus and rheumatoid arthritis, among women had been recognized long before a real understanding of autoimmunity existed.[46,47] A specific effect of the testes on the immune system was reported as early as 1898 by Calzolari,[48] when he found that castration of rabbits prior to puberty led to an increase or maintenance of the size of the thymus. This effect has been repeatedly confirmed and extended to include cell-mediated immunity and graft survival.[49,50] Many studies have shown that various immunological processes have gender-specific differences or can be affected by castration and/or sex steroid replacement. These data clearly established that products of the testis, and sex steroids in particular, regulate the immune system either directly or indirectly, setting the stage for the modern era of male reproductive immunophysiology.

STRUCTURE AND FUNCTION OF THE MALE REPRODUCTIVE TRACT RELEVANT TO IMMUNOPHYSIOLOGY

The anatomy and physiology of the testis and other components of the male reproductive tract are covered in detail elsewhere in this volume (see Chapters 3, 14, 16, 17, and 18). A brief outline highlighting the issues relevant to understanding the interface between the immune system and male reproductive tract is provided here.

The Testis

Structural Organization

Functionally and anatomically, the testis is separated into an avascular spermatogenic compartment, the seminiferous tubules, and a highly vascularized endocrine compartment, the interstitial tissue. The testis is enclosed by a fibrous capsule, but there is considerable species variation in the connective tissue of the testicular parenchyma. In the human, the testis is physically partitioned by connective tissue septa into discrete lobules containing the loops of the seminiferous tubules, which connect at both ends to the rete testis located along one pole of the testis.[51] The products of the seminiferous tubules are collected by the rete testis and transferred to the adjacent epididymis, which is connected to the rete testis via a series of efferent ducts. The testes of rodent species, such as rat and mouse, comprise only very loose connective tissue with no distinct septa separating the seminiferous tubules.[52] The interstitial tissue completely surrounds the seminiferous tubules (Figure 19.2), and contains the vasculature, lymphatic vessels, and nerves of the testis. The testicular blood supply arises from the abdominal aorta, and this produces a comparatively long and highly coiled spermatic artery that is particularly susceptible to physical insult and torsion in species with scrotal testes. The arterioles, capillaries, and venules of the testis thoroughly permeate the interstitial tissue surrounding the seminiferous tubules and rete testis. Consequently, these structures are close to an effective blood supply at all times in spite of the completely avascular nature of the spermatogenic compartment.

Unlike the capillaries of other endocrine glands, the majority of testicular capillaries are not fenestrated,[53] and the mechanisms whereby molecules enter and exit the testis via this route still await resolution.[54] There appears to be very little functional restriction on the exchange of even large molecules across this barrier and the interstitial fluid is very similar in its overall composition to that of the circulating blood.[55] The venous drainage of the testis via the spermatic veins is closely associated with the arterial supply, which together form a very effective countercurrent heat and solute exchange structure, called the pampiniform plexus.[56]

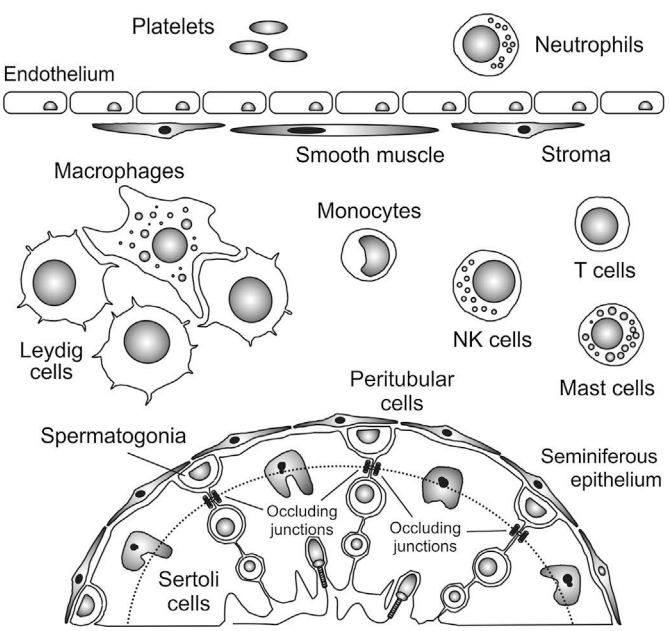

FIGURE 19.2 Immunological compartmentalization of the testis. The mammalian testis comprises three immunologically distinct compartments: the vascular compartment and intertubular (or interstitial) compartment are separated by a layer of nonfenestrated endothelium, while the intertubular and spermatogenic compartments are separated by a layer of peritubular myoid cells and by occluding junctions between adjacent Sertoli cells. These junctions constitute the blood–testis barrier, which further divides the seminiferous epithelium into a basal and an adluminal region (dotted line). The adluminal region contains the meiotic germ cells within a highly specialized microenvironment. Under normal conditions, monocytes, macrophages, T cells, NK cells and, in some species, mast cells and/or eosinophils have relatively free access to the intertubular compartment, but are entirely excluded from the adluminal region of the seminiferous epithelium. Neutrophils are confined to the vascular compartment except during specific immunological events.

The organization of the testicular lymphatics varies between species, ranging from irregular channels or sinusoids that are incompletely bounded by endothelial cells in rodents, to large discrete lymphatic vessels in humans, to very small, rapid flow lymphatics in porcine species.[52,57] These lymphatics completely invest the entire interstitial tissue and pass without restriction to local draining lymph nodes, principally the lumbar or para-aortic lymph nodes.[58,59] In the laboratory rat, drainage to the iliac and renal nodes predominates, but there may be some lymphatic drainage directly to the thoracic ducts without passing through regional lymph nodes.[13]

The most prominent cell type present in the interstitium is the Leydig cell,[60] which produces androgens.[61] Macrophages are commonly observed in the interstitium of most, if not all, species and many testes also contain variable numbers of mast cells and/or eosinophils.[62,63] Less numerous, but ubiquitous nonetheless, are the intratesticular lymphocytes: T cells and natural killer (NK) cells.[64–70]

The seminiferous tubules are bounded by a circumferential layer of peritubular cells and the basal lamina, which together form the limiting structure on which rests the Sertoli cells and the spermatogonia (Figure 19.2). Immune cells are occasionally seen within this boundary layer.[71] The Sertoli cell provides the structural framework for the organization of the seminiferous epithelium, but also plays a crucial role in supporting and directing the development of the spermatogenic cells. Adjacent Sertoli cells and spermatogenic cells maintain intimate contact at all times, with junctional and membrane specializations providing physical contact and communication.[72] At the time of puberty, cohorts of mitotically dividing spermatogonia begin to enter meiosis at regular intervals, moving away from the periphery of the tubule and becoming spermatocytes. Meiosis produces haploid round spermatids, which subsequently undergo considerable structural differentiation to become mature or elongated spermatids. Once these cells are released by the Sertoli cell into the tubule lumen they are called spermatozoa and fluid secreted by the Sertoli cells sweeps the released spermatozoa toward the rete testis.

The seminiferous epithelium is highly organized. As each cohort of spermatogonia becomes committed to the spermatogenic process, they displace earlier cohorts toward the lumen of the tubule, so that multiple generations of developing germ cells co-exist in each segment of the seminiferous epithelium. As a consequence of the regular intervals of spermatogonial commitment and the constant rate of the spermatogenic process itself, the generations form specific associations of germ cells at different levels of maturity within the epithelium. These associations constitute the stages of a recurring developmental sequence, called the "cycle of the seminiferous epithelium," which is also promulgated along the entire length of the tubule (see Chapters 16).[51] This organized complexity implies a high degree of communication and regulation across the generations as well as between spermatogenic cells and supporting Sertoli cells.

Elaborate occluding junctions between adjacent Sertoli cells form an intercellular barrier that is completely impermeable even to small molecules.[15,16] This constitutes the main component of the blood–testis barrier and separates the premeiotic and early meiotic cells in the basal region of the seminiferous epithelium from the adluminal spermatocytes and spermatids (Figure 19.2). In this way, a large majority of the developing germ cells

are sequestered within a highly specialized environment and effectively isolated from the vasculature and immune system. In contrast, the rete testis epithelium lacks both Sertoli cells and their highly specialized junctional specializations. The epithelial barrier restricting movement from the blood into the rete testis appears to be substantially less effective than that of the seminiferous epithelium, with the result that immunoglobulins and possibly even immune cells are able to cross the epithelium.[64,73]

Endocrine Regulation

Male reproduction is maintained by pulsatile secretion of gonadotropin releasing hormone (GnRH) by the hypothalamus, which stimulates concordant pulses of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) from the anterior pituitary.[74] In the testis, LH binds to specific G-coupled receptors on the surface of the Leydig cells, thereby stimulating adenylate cyclase to produce the intracellular second messenger, cAMP, and activating the cAMP-dependent protein kinase A (Figure 19.3).[61] This activation mobilizes cholesterol from intracellular stores, extracellular lipoprotein sources, or de novo synthesis from acetate, and stimulates the transfer of the cholesterol to the inner-mitochondrial membrane through the action of the steroidogenic acute regulatory protein (STAR).[75] Ongoing maintenance of steroidogenic enzyme expression is also under LH/cAMP control.[76] Once cholesterol enters the mitochondrion, it is metabolized to pregnenolone via the action of the cytochrome P450 cholesterol side-chain cleavage enzyme (CYP11A) residing on the inside face of the inner matrix membrane. Pregnenolone diffuses out of the mitochondrion to the smooth endoplasmic reticulum, where it may be converted to progesterone by 3β-hydroxysteroid dehydrogenase/Δ4-Δ5 isomerase (HSD3β). Pregnenolone and progesterone are first metabolized to their 17α-hydroxy forms and then to the weak androgens, dehydroepiandrosterone and androstenedione, respectively, by the action of steroid 17α-hydroxylase/17,20 lyase (CYP17A). Finally, androstenedione is converted to testosterone by the action of hydroxysteroid (17β) dehydrogenase (HSD17β), and dehydroepiandrosterone is converted to androstenediol and then testosterone, by the sequential actions of HSD17β and HSD3β. Testosterone is secreted from the Leydig cell and serves as the principal androgen in both the testis and circulation.

Both testosterone and FSH bind to specific Sertoli cell receptors to regulate spermatogenesis and Sertoli cell functions, including secretion of the protein hormone, inhibin.[77] In turn, testosterone and inhibin operate via a negative feedback loop to regulate LH and FSH synthesis and secretion at the pituitary and hypothalamic levels.[78] Withdrawal of androgens leads to rapid cessation of spermatogenesis, although the levels of intratesticular testosterone required to maintain qualitatively normal spermatogenesis are considerably lower than the intratesticular concentrations that normally exist.[79,80] Consequently, spermatogenesis can tolerate even relatively large declines in testicular androgen production with relatively minor losses of efficiency. In contrast, peripheral levels of androgens are critical; even small reductions can have profound effects on many androgen-dependent functions, including accessory gland function, secondary sex characteristics, and libido.[81] Peripheral androgen levels are dependent upon both Leydig cell production and testicular vascular function, so that interference with the vasculature of the testis can alter circulating testosterone levels quite significantly.[82] Conversion of testosterone and androstenedione to estrogens by the cytochrome P450 enzyme aromatase (CYP19A) in the Leydig cell and Sertoli cell is also required for normal development and function of the efferent ducts and epididymis.[83]

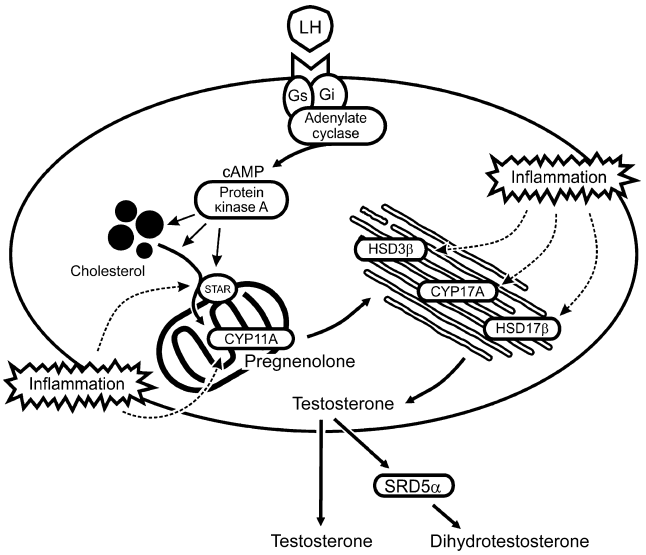

FIGURE 19.3 Regulation of testosterone biosynthesis in Leydig cells and sites of inhibition during inflammation. The gonadotropin, LH, binds to a G protein-coupled receptor on the cell surface, thereby activating adenylate cyclase, production of cAMP and protein kinase A activity. This stimulates the transfer of cholesterol from intracellular stores into the mitochondria through the action of the steroidogenic acute regulatory protein (STAR), where the cholesterol side-chain cleavage enzyme (CYP11A) converts the cholesterol to pregnenolone. Pregnenolone is converted to testosterone in the smooth endoplasmic reticulum by the enzymes, 3β-hydroxysteroid dehydrogenase/Δ4-Δ5 isomerase (HSD3β), steroid 17α-hydroxylase/17,20 lyase (CYP17A) and hydroxysteroid (17β) dehydrogenase (HSD17β). Testosterone is reduced by the action of the 5α-reductase enzyme (SRD5α) to the more potent androgen, dihydrotestosterone. Inflammation inhibits the activity of STAR and all the main enzymes of the steroidogenic pathway.

The Epididymis, Vas Deferens, and Accessory Glands

The epididymis comprises a long single, highly coiled epididymal duct lined primarily by columnar principal cells with extensive apical stereocilia. Testicular fluid

secreted by the Sertoli cells is largely reabsorbed by the epithelial cells of the efferent ducts and the proximal regions (caput) of the epididymis.[84] Sperm maturation occurs during transit through the epididymal duct and sperm are stored prior to ejaculation in the distal (cauda) region of the epididymis.[85,86] The cauda epididymis is connected to the vas deferens, a highly muscularized duct that drives the epididymal contents toward the urethra at the time of ejaculation. The testicular and epididymal secretions constitute only about 10% of the ejaculate, with the remaining 90% of the semen coming from the accessory glands: the seminal vesicles and prostate, in particular.[87] All the post-testicular ductal structures of the male tract and the accessory glands are dependent upon androgens for normal development and maintenance of function.[81] However, in contrast to the testis, conversion of testosterone to the more potent androgen, 5α-dihydrotestosterone (DHT), by the action of steroid 5α-reductase (SRD5α) is usually necessary in most other androgen-responsive tissues.[88]

It might be assumed that sperm spend the majority of their time in the testis and epididymis and appear only briefly within the vas deferens and urethra during ejaculation. Critically, however, some sperm may be retained within the tract for much longer periods, as spermatozoa continue to appear in the ejaculates of vasectomized men for several months even after a successful procedure.[89] In fact, the presence of intact sperm even has been noted in human prostate glands collected following prostatic surgery or postmortem, suggesting that such ectopic sperm may play a role in the etiology of prostatic inflammation and possibly even sperm autoimmunity.[90]

There is a blood–epididymis barrier restricting movement of molecules across the epididymal epithelium, although evidence suggests that this barrier is not as elaborate or effective as the blood–testis barrier.[72,86,91–93] In normal adults, circulating immunoglobulin appears to be restricted, although perhaps not entirely excluded, from passage into the epididymal fluid.[91] The most striking contrast with the seminiferous epithelium is the presence of macrophages and lymphocytes within the epididymal epithelium.[65,94–102] The presence of these cells suggests the existence of a very different immunological environment compared with that of the testis. Epithelial-type barriers, similar to that present in the epididymis, are found throughout the remainder of the reproductive tract.[72,93,103]

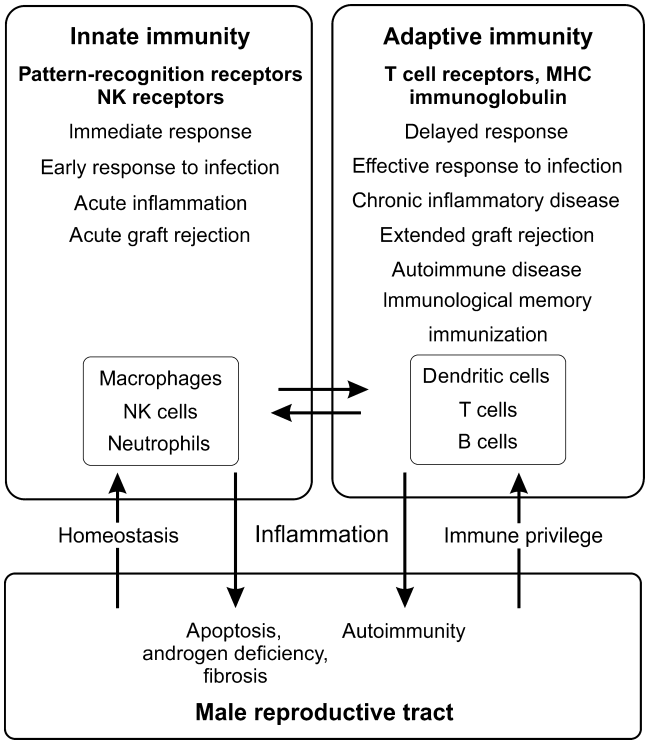

FIGURE 19.4 Components and properties of the innate and adaptive immune systems, and their interaction with the male reproductive tract. Innate immunity is the immediate response to infection and other external threats, subsequently activating the more delayed adaptive immune response. The male reproductive tract regulates innate and adaptive immunity in order to maintain protection against infections, while at the same time suppressing antigen-specific immunity to protect the spermatogenic cells (immune privilege). Activation of the innate and adaptive immune systems, due to infection or other inflammatory stimulus, has detrimental effects on male reproductive tract function, resulting in loss of androgens and spermatogenic disruption, and can lead to tissue damage, ongoing autoimmunity and infertility.

THE IMMUNE SYSTEM AND ITS ENDOCRINE CONTROL

General Principles

Fundamentally, the immune system provides protection for more complex animals from invading organisms that seek to exploit vulnerabilities or other opportunities to infect their host. This protection involves a complex suite of cells and molecules that allows the animal to identify and then eliminate the invading pathogen. In vertebrates, the immune system comprises an innate immune system, which generally recognizes uniquely conserved molecular patterns expressed by various pathogens, and the adaptive (or acquired) immune system, which specifically recognizes molecular patterns that are foreign to the host. Neither system operates in isolation and many of the cellular and molecular mechanisms overlap (Figure 19.4). The functions of both innate and adaptive immunity impact upon the male reproductive tract at multiple levels and have profound consequences for male reproduction.

The cellular components of the immune system are the leukocytes, or white blood cells. These cells and their products circulate continuously through the blood, lymph, and tissues in both surveillance and effector modes. The innate immune system is comprised principally of the mononuclear phagocytes (monocytes and

macrophages) and granulocytes or polymorphonuclear cells (neutrophils, eosinophils, basophils, and mast cells), but also involves cells more closely aligned with the adaptive responses (NK cells and dendritic cells). The cellular components of the adaptive immune system are the lymphocytes (T cells, B cells, and NK cells), and the "professional" antigen-presenting cells (dendritic cells and macrophages). In modern immunology, the cells of the immune system and their various functional subsets are primarily identified and even defined by expression of specific antigens, called cluster designation (CD) markers, recognized by well-characterized monoclonal antibodies (Table 19.1).[104]

The Innate Immune Response

The innate immune system provides the first line of defense against external threats through an inherent ability to recognize and rapidly respond to a broad range of pathogens and other immunogens, and by promoting the process of inflammation. Innate immunity plays a fundamental role in the response of the male reproductive tract to infections, but it also exhibits a much wider role in male reproduction because a number of regulatory mechanisms are shared by the innate immune and the reproductive systems.

Pattern Recognition Receptors and Activation of Innate Immunity

Activation of the innate immune response involves pattern-recognition receptors, which recognize specific motifs, or pathogen-associated molecular patterns (PAMPs), produced by bacterial, viral, fungal, and protozoan pathogens.[105] Unlike classical ligand receptors, these receptors are able to respond to multiple ligands that possess related, rather than identical, structures. The canonical pattern-recognition receptors are a family of transmembrane receptors called the Toll-like receptors (TLR), which are expressed on the cell surface and on intracellular endosomes.[106] There are several families of intracytoplasmic pattern-recognition receptors: the nucleotide binding and oligomerization domain (NOD)-like receptors (NLR), the retinoic acid-inducible gene (RIG)-like receptors (RLR), and the C-type lectin receptors (CLR).[107] Importantly, many of these receptors can also interact with endogenous molecules released by cell damage, called danger-associated molecular patterns (DAMPs), which include high-mobility group box 1 protein (HMGB1), heat shock proteins, extracellular matrix components, and nucleic acids.[108]

The TLRs are highly expressed by myeloid-lineage cells (monocytes, macrophages, and dendritic cells), but are also found on other leukocytes, epithelial cells, and stromal cells. There are 10 TLRs (numbered TLR1–10) in the human, but the laboratory rodents (rats and mice) possess an additional three TLRs (TLR11–13).[106,109] These receptors detect unique ligands of bacterial, viral, and fungal origin, such as bacterial and viral nucleic acids, bacterial lipopeptides, peptidoglycans, and lipopolysaccharides (LPS). LPS is a component of the cell wall of gram-negative bacteria, such as *Escherichia coli*, and the receptor for LPS is TLR4, which requires a co-receptor called MD2 (myeloid differentiation 2 protein), and the LPS-binding protein CD14 for full activation.[110,111]

The ligand-binding region of the TLRs is characterized by multiple N-terminal leucine-rich repeats, which facilitate detection of specific molecular patterns. These receptors are functionally related to the interleukin-1 (IL1) receptor (IL1R), with which they share a conserved cytoplasmic domain called the Toll/IL1R (TIR) domain.[106,112] Activation of the TLRs involves receptor dimerization and interaction of the TIR domain with an intracellular TIR domain-containing adaptor protein (Figure 19.5). In the case of all the TLRs, except TLR3, the adaptor protein is myeloid differentiation primary-response protein 88 (MYD88), which signals via IL1 receptor-associated kinase (IRAK) and tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6). This leads to activation of the p38 mitogen-activated protein kinase (MAPK14) and Jun N-terminal kinase (MAPK8), and nuclear translocation of the transcription factors, nuclear factor kappa B (NFκB), and activated protein-1 (AP-1).[113,114] This, in turn, induces expression of genes encoding the key pro-inflammatory cytokines and mediators, including both IL1α and β forms (IL1α and IL1β), TNF, IL6, IL8 (C-X-C motif ligand 8; CXCL8), IL12, inducible nitric oxide synthase (NOS2) and prostaglandin-endoperoxide synthase 2 (PTGS2; cyclooxygenase 2; Table 19.2).[115,116] In addition, TLR3 and TLR4 interact with the adaptor protein, TIR domain-containing adaptor molecule 1 (TICAM1), to activate TRAF3 and the transcription factor, interferon regulatory factor 3 (IRF3), resulting in production of the type 1 interferons (IFNα and IFNβ).[115] Some of the NLRs, which detect various bacterial PAMPs within the cytosol, likewise exert their actions via activation of NFκB and the MAP kinases, but a subset of the NLRs work by induction of the cysteine protease, caspase-1 (CASP1; interleukin-1β converting enzyme), through assembly of a large intracellular protein complex called the inflammasome.[117,118] Inflammasomes are generically composed of a pattern-recognition domain-containing protein, an adaptor molecule bearing a caspase activation and recruitment domain (CARD), and CASP1 itself, which activates the key pro-inflammatory cytokines, IL1β and IL18, by processing their inactive precursors (Figure 19.5).[118] These complexes are activated by various PAMPs and DAMPs, including bacterial toxins, viral RNA, and particulates, such as silica and uric acid crystals. Activation of the pattern-recognition receptors

TABLE 19.1 Cluster Designation (CD) Markers Relevant to the Male Reproductive Tract[a]

Marker	Gene Name, Common or Superseded Designation(s)	Function(s)
CD1	Ly-38, R3 (CD1D)	Nonclassical MHC; presentation of lipid and glycolipid antigens
CD3	T3, Leu 4	Signaling component of the TCR complex
CD4	T4, Leu 3	Co-receptor for recognition of MHC class II; component of the TCR complex
CD8	Ly-2, Ly-3, T8, Leu 2	Co-receptor for recognition of MHC class I; component of the TCR complex
CD11a CD11b CD11c	ITGAL, LFA-1, Ly-15, Ly-21 ITGAM, Mac-1, Ly-40 ITGAX, Leu M5	Integrin α chains; adhesion molecules, expressed on leukocytes
CD14	LPS-R	Lipopolysaccharide-binding protein complex co-receptor; TLR4 co-receptor
CD16	FCGR3, FcγRIII, Ly-17	Receptor for Fc fragment of immunoglobulin G; expressed on subset of NK cells
CD18	ITGB2, LCAMB	Integrin β2; adhesion molecule, pairs with CD11
CD25	IL2RA, Ly-43	IL2 receptor α chain; marker for activated and Treg cells
CD28	T44	Receptor for CD80 and CD86; expressed on activated T cells, NK cells
CD30	TNFRSF8	TNF receptor superfamily, member 8; expressed on activated T cells, B cells
CD40	TNFRSF5	TNF receptor superfamily, member 5; co-stimulatory receptor; expressed on antigen-presenting cells
CD45	PTPRC, LCA, Ly-5, T200	Protein tyrosine phosphatase, receptor type, C; leukocyte common antigen; expressed on all leukocytes
CD46	MCP	Membrane cofactor protein; complement regulatory protein
CD52	CAMPATH-1 antigen	Complement regulatory protein; Treg co-stimulatory co-receptor
CD54	ICAM1, Ly-47	Intercellular cell adhesion molecule-1; expressed on activated endothelial cells
CD55	DAF	Decay accelerating factor for complement; complement regulatory protein
CD56	NCAM1	Neural cell adhesion molecule-1; specific variant expressed on NK cells
CD59	MAC-IP	Membrane attack complex inhibition factor; complement regulatory protein
CD68	Macrosialin	Lysosomal membrane glycoprotein; expressed by dendritic cells, monocytes, some macrophages
CD80	B7-1, B7/BB1, Ly-53	Co-stimulatory co-receptor; ligand for CD28; expressed on antigen-presenting cells
CD86	B7-2, Ly-58	Co-stimulatory co-receptor; ligand for CD28; expressed on antigen-presenting cells
CD95	FAS, APO-1	Receptor for CD95 ligand; mediates apoptosis; expressed on activated lymphocytes
CD106	VCAM1	Vascular cell adhesion molecule-1; expressed on activated endothelial cells
CD126	IL6R	Interleukin-6 receptor subunit; pairs with interleukin-6 signal transducer
CD130	IL6ST, gp130	Interleukin-6 signal transducer
CD152	CTLA4	Cytotoxic T-lymphocyte-associated antigen 4; inhibitory receptor for CD80 and CD86; expressed on T cells
CD154	CD40LG	Ligand for CD40; co-stimulatory co-receptor; expressed on T cells
CD163	M130	Scavenger receptor for hemoglobin-haptoglobin complex; expressed on macrophage subset (M2)
CD206	MRC1	Macrophage mannose receptor 1; expressed on macrophage subset (M2)

[a]*Refer to text for full details. Note that some designations represent multiple protein members.*

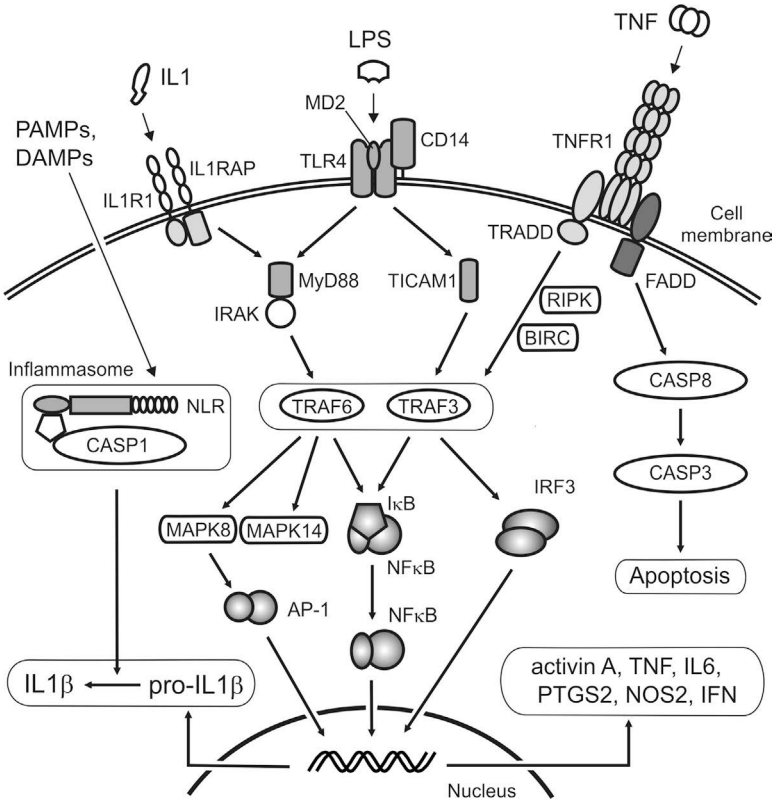

FIGURE 19.5 Schematic of inflammatory signaling pathways. Binding of bacterial LPS to Toll-like receptor 4 (TLR4), which also comprises co-receptor proteins MD2 and CD14, or binding of IL1 to the IL1 receptor (IL1R1) and engagement of the ILR acceptor protein (IL1RAP), leads to interaction with the adaptor molecules, MYD88 or TICAM1. Signaling via MYD88 occurs through the IL1 receptor-associated kinase (IRAK) and TNFR-associated factor 6 (TRAF6), leading to degradation of the NFκB repressor protein IκB, and activation of mitogen-activated protein kinases (MAPK8 and MAPK14). These kinases activate multiple downstream events, including production of the transcription factor AP-1. Signaling via TICAM1 occurs through TRAF3 and activates the interferon-induced transcription factor, IRF3. However, there is considerable potential for overlap between signaling pathways. Depending upon which adaptor molecule is engaged, binding of TNF to its receptor (TNFR1) can lead to activation of TRAFs via the TNFR-associated death domain protein (TRADD)-mediated pathway, via receptor interacting serine–threonine kinase (RIPK) or baculoviral IAP repeat containing protein (BIRC), or to the caspase activation cascade and apoptosis through the FAS-associated death domain protein (FADD). The transcription factors, NFκB, AP-1 and IRF3, translocate to the nucleus to induce transcription of inflammatory genes. Various pathogen-associated and endogenous molecules (PAMPs and DAMPs) bind to intracellular pattern-recognition receptors associated with the inflammasome, such as the NOD-like receptors (NLR), and activate caspase-1 (CASP1), which is required to process pro-IL1β into the active pro-inflammatory protein. Note that the pathways shown are highly simplified – not all intermediates or potential interactions are depicted.

TABLE 19.2 Key Enzymes Involved in Inflammation and Immunity in the Male Reproductive Tract

Enzyme Name	Gene Name	Common Names	Properties/Function
Nitric oxide synthase, type I	NOS1	Neuronal NOS (nNOS)	Ca/calmodulin regulated
Nitric oxide synthase, type II	NOS2	Inducible NOS (iNOS)	Transcriptionally and translationally regulated
Nitric oxide synthase, type III	NOS3	Endothelial NOS (eNOS)	Ca/calmodulin regulated
Phospholipase A2	Multiple genes		Hydrolysis of free fatty acid from membrane phospholipids
Prostaglandin-endoperoxide synthase 1	PTGS1	Cyclooxygenase 1 (COX1)	Constitutive, converts arachidonic acid to prostaglandin G/H
Prostaglandin-endoperoxide synthase 2	PTGS2	Cyclooxygenase 2 (COX2)	Inducible, converts arachidonic acid to prostaglandin G/H
Prostaglandin E synthase	PTGES		Converts prostaglandin H to prostaglandin E
Caspase 1	CASP1	Interleukin-1β converting enzyme (ICE)	Activation of IL1β and IL18
Caspase 3	CASP3		Mediates apoptotic pathway
Caspase 8	CASP8		Mediates apoptotic pathway
Indoleamine 2,3-dioxygenase	IDO		Metabolism of tryptophan, immunoregulation
Interleukin-1 receptor-associated kinase	IRAK		Activation of TRAF6, 3
IκB kinase	IKBK		Activation of NFκB
Mitogen-activated protein kinase 14	MAPK14	p38 MAP kinase	Stress/inflammatory signaling
Mitogen-activated protein kinase 8	MAPK8	JUN N-terminal kinase (JNK)	Stress/inflammatory signaling, activation of AP-1

(Continued)

TABLE 19.2 Key Enzymes Involved in Inflammation and Immunity in the Male Reproductive Tract—cont'd

Enzyme Name	Gene Name	Common Names	Properties/Function
Mitogen-activated protein kinase 3	MAPK3	Extracellular signal-regulated kinase 1 (ERK1)	Stress/inflammatory signaling
Mitogen-activated protein kinase 1	MAPK1	Extracellular signal-regulated kinase 1 (ERK2)	Stress/inflammatory signaling
Akt protein kinase	AKT	Protein kinase B	Cell survival, stress/inflammatory signaling
Tyrosine-protein kinase Src	SRC		Immunoregulation
Janus kinase	JAK		Activation of STAT
Phosphatidylinositol-4,5-biphosphate 3-kinase	PIK3	Phosphoinositide-3-kinase (PI3K)	Stress/inflammatory signaling
Protein kinase, cAMP-dependent	PRKA	Protein kinase A	Protein phosphorylation, signaling
Protein kinase C	PRKC		Protein phosphorylation, signaling

leads to functional activation of immune cells and sets the inflammatory response in train.

Inflammation

Inflammation is the immune system's initial response to a pathological challenge and involves major changes in homeostasis: fever, activation of immune cells, increased blood flow at the site of inflammation, and enhanced pain sensitivity.[119] Apart from specific pathogens, the inflammatory cascade can be triggered by tissue injury, insoluble particulates, activation of the plasma complement and clotting/fibrinolytic protease pathways, opsonized (antibody or complement-coated) particles or immune complexes, and intracellular components released by tissue damage. Monocytes and macrophages are by far the most effective promoters of the inflammatory response. Mast cells regulate vascular permeability, vasodilation, and leukocyte recruitment and play a critical role in allergic inflammation.[120] Neutrophils provide rapid and effective clearance of extracellular pathogens.[121] These immune cell types also exert a regulatory influence over the subsequent immune response.

In addition to the pro-inflammatory cytokines, the main mediators of the inflammatory process are pro-inflammatory prostaglandins and leukotrienes, neuropeptides such as substance P, vasoactive amines produced by basophils and mast cells (histamine and serotonin) and acute phase proteins from the liver, such as C-reactive protein and serum amyloid A. Local production of chemoattractive cytokines (chemokines), such as IL8 and chemokine (C–C motif) ligand 2 (CCL2; monocyte chemoattractant protein-1), together with upregulation of specific adhesion molecules on the endothelium (selectin E, selectin P, intercellular adhesion molecule-1 (ICAM1), vascular adhesion molecule-1 (VCAM1)) and on the leukocytes (selectin L, integrins), allow circulating neutrophils, monocytes and lymphocytes to specifically target and enter the affected tissues.[122] Resolution of the inflammatory response involves production of anti-inflammatory immunoregulatory cytokines, such as transforming growth factor-β (TGFβ) and IL10, some prostaglandins, late-acute-phase proteins, and activation of the hypothalamic-pituitary-adrenal axis to produce anti-inflammatory corticosteroids.[123–125] Long-term consequences of inflammation include increased tissue fibrosis and persistent alterations in the number, type, and activity of leukocytes within the affected tissue.[126]

The main effectors of the innate immune system are hydrolytic enzymes (e.g., lysozyme, serprocidins), antimicrobial proteins (e.g., complement, defensins), cytotoxic reactive oxygen species (ROS), NO, cytotoxic cytokines such as TNF, and the antiviral interferons.[119] Moreover, activation of the innate immune system leads to recruitment of the adaptive immune system.

The Adaptive Immune Response

The adaptive immune system involves complex cellular interactions that promote the functional maturation and expansion of regulatory and effector lymphocytes, thereby providing efficacy, specificity, and memory to the immune response, but requiring time to become effective. It operates through the ability to recognize and respond to molecular motifs (antigens), usually associated with proteins, that are not part of the normal host repertoire, and may indicate the presence of an external threat. Adaptive immunity is responsible for autoimmune reactions in the male reproductive tract that can lead to infertility and chronic inflammatory conditions. These reactions are mediated, but also regulated, by T cells, B cells, and NK cells, which have access to the male reproductive tract.

T-Cell and B-Cell Regulation and Functions

The adaptive immune system is dependent upon the unique ability of lymphocytes to generate a vast repertoire of cell surface receptors that can bind to almost any conceivable molecular surface, without ever having encountered the molecule before. On B cells, these receptors are surface-bound immunoglobulins, from which are derived the circulating antibodies. The core proteins of the T-cell receptor (TCR) are structurally related to the immunoglobulins, but the TCR itself is a complex of interacting surface proteins.[127] The diversity of these receptors involves extensive rearrangements of the basic immunoglobulin and TCR gene regions, which produce randomly reassembled genes encoding proteins, each with a very specific and unique topography.[128,129] Each precursor T cell and B cell expresses a surface receptor that is specific for a unique antigenic determinant and all their offspring (clones) will express the same receptor and specificity. B cells interact more or less directly with the antigenic molecule in situ. However, more precise regulation of the immune response involving T cells is determined by proteins of the highly polymorphic major histocompatibility complex (MHC), expressed on the surface of antigen-presenting cells.[130] Almost all cells in the body can act as antigen-presenting cells by proteolytically converting intracellular proteins, of either endogenous or infectious (e.g. viral) origin, into short antigenic peptides, which are then incorporated into a structural groove on the extracellular surface of the MHC protein complex during its assembly in the endoplasmic reticulum.[131] Some antigen-presenting cells (dendritic cells and macrophages) are able to phagocytose exogenous proteins, typically proteins of pathogenic origin, but also proteins derived from endogenous sources including the spermatogenic cells, and process these proteins for antigen-MHC complex formation. The TCR subsequently binds to the antigen-MHC complex on the surface of the antigen-presenting cell leading to the activation and proliferation of the T cell (Figure 19.6).

Typically, circulating T cells express one of the co-receptor proteins, CD4 and CD8, as part of their TCR, which permit them to recognize antigens associated with MHC class II or MHC class I molecules, respectively.[132] Antigens are presented to $CD4^+$ T cells by the professional antigen-presenting cells that express MHC class II antigens (dendritic cells, macrophages, and B cells).[133] On the other hand, $CD8^+$ T cells are recognized by MHC class I antigens, which are ubiquitously expressed. Activation of the T cell requires physical interaction between co-stimulatory ligand-receptor pairs, particularly CD28:B7 (CD80/CD86) and CD40:CD40 ligand (CD40LG), and production of either type 1 cytokines [IL2, IL12 and interferon-γ (IFNγ)] or type 2 cytokines (IL4, IL5, IL10 and IL13; Figure 19.6).[134,135] As a result of this complexity, T-cell

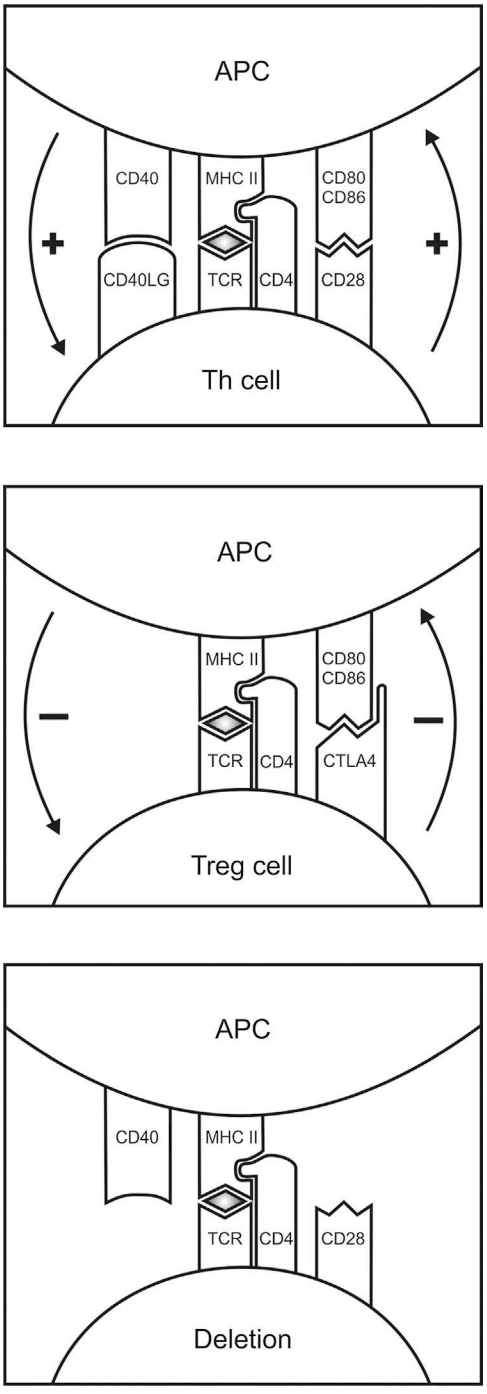

FIGURE 19.6 The antigen-presenting cell (APC)–T-cell synapse and the adaptive immune response. Recognition of the MHC class II-peptide antigen complex by the T-cell receptor (TCR) of a naïve Th cell together with engagement of the CD28:CD80/CD86 and CD40/CD40LG receptor/co-receptor pairs can lead to generation of Th1 cells, if type 1 cytokines (IL12 and IFNγ) are present. If interleukin-6 (IL6) and type 2 cytokines (IL4, IL5 and IL13) are present, Th2 cells are produced, and Th17 cells are produced when IL6 and transforming growth factor-β (TGFβ) are present. Engagement between the APC and T cell via the CTLA4 receptor produces an inhibitory response, as occurs in Treg cell interactions. Engagement of the APC and T cell in the absence of adequate co-stimulation or cytokine activity results in deletion or inactivation (anergy) of the Th cell.

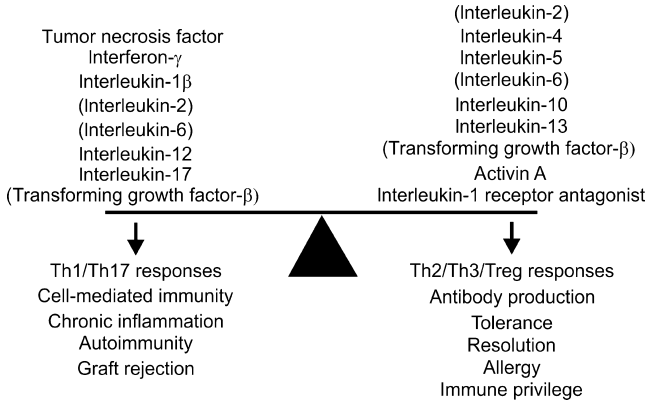

FIGURE 19.7 The cytokine balance and immune deviation. Cytokines can be designated either pro-inflammatory or anti-inflammatory/immunoregulatory, depending upon their predominant activities. The former group is associated with Th1 or Th17 type responses (cell-mediated immunity, autoimmunity) and the latter with Th2, Th3, or Treg type responses (antibody production, allergy, tolerance). It is important to note that several cytokines, such as IL6 and TGFβ, possess both pro- and anti-inflammatory properties, and fall within both groups. It is the combination of cytokines present that determines the T-cell response outcome.

activation can produce different outcomes depending upon the co-stimulatory molecules engaged and cytokines produced. Accordingly, naïve $CD4^+$ T cells may become type 1 helper (Th1) cells, which produce Th1 cytokines, direct the development of the cellular immune response involving cytotoxic $CD8^+$ T cells, and are associated with graft rejection, or they may become type 2 helper (Th2) cells, which produce Th2 cytokines, promote B cell development and antibody responses, and regulate allergic responses (Figure 19.7).[134,135]

In addition to the Th1 and Th2 cell subsets, which are primarily responsible for dictating the nature of the subsequent immune response to a particular antigen, there are a number of other T-cell subsets with specialized functions, which have been more recently described. The Th17 cell subset is functionally related to the Th1 subset but characteristically produces IL17, IL21, and IL22.[136] The Th17 cell is generated by exposure to IL6 and TGFβ and regulates protection against extracellular pathogens, including recruitment and activation of neutrophils, differentiation of B cells, and the inflammatory activity of epithelial cells.[137] However, these cells also have been implicated in the development of autoimmune disease and in allergy.[136,138] Absence of appropriate co-stimulatory molecule interactions and/or the presence of anti-inflammatory or immunosuppressive cytokines may lead to T-cell inactivation (anergy) and deletion or the generation of regulatory or suppressor T cells (Figure 19.6).[139,140] These T-cell subsets are responsible for regulating antigen-specific immunity and maintaining peripheral immune tolerance.

The development of B cells into antibody-secreting plasma cells following interaction with antigen requires specific Th2 cell help.[141] Once activated, these cells initially secrete multivalent IgM, but the cells gradually mature to produce high affinity IgG with the same antigenic specificity.[128] Finally, following the resolution of the immune response, at least some activated T and B cells clones persist as memory cells, with the result that lymphocyte responses to antigens generally develop much faster upon second exposure to the antigen.[142,143]

NK Cell and NK T-Cell Regulation and Functions

NK cells are lymphocytes that span the interface between the innate and adaptive immune systems.[144] These cells are developmentally related to cytotoxic $CD8^+$ T cells but are capable of recognizing and destroying transformed cells, such as virally-infected or tumor cells, without the need for prior sensitization by exposure to antigen. They can be activated by pro-inflammatory cytokines, notably IL12 and antiviral type 1 interferons.[145] They interact with their targets via a complex of stimulatory and inhibitory surface receptors, which allow them to recognize ligands uniquely expressed by transformed cells and to detect the lack of MHC class I expression, a characteristic of transformed cells.[144,146] Accordingly, NK cells are able to rapidly mobilize against pathogenic challenges long before T- and B-cell responses can develop. However, activated NK cells also participate in the adaptive immune response. They possess antigen-specific receptors, similar to cytotoxic $CD8^+$ T cells, and have been shown to regulate dendritic cell responses in a positive or negative manner, for example, by inducing dendritic cell death through contact-mediated lysis or by producing immunoregulatory cytokines, such as IFNγ, IL4, and TGFβ.[144,147]

NK T cells are a distinct cytotoxic lymphocyte subset that is defined by responsiveness to bacterial and mammalian glycolipid antigens presented by the nonclassical MHC class I molecule, designated CD1D.[148] These cells are considered to be T cells with NK activity, rather than NK cells per se, but they also appear to play an important role in immune regulation through production of either IFNγ or IL4/IL10.[149,150] Accordingly, both NK T cells and NK cells promote and mediate immunity to bacteria, viruses, and tumors, but are also capable of suppressing cell-mediated autoimmunity and graft rejection responses.

Immunological Tolerance and Regulatory Lymphocyte Subsets

The ability of randomly-generated, antigen-specific T and B cell clones to ignore antigens expressed by the host organism relies upon an effective cell-editing process, collectively referred to as tolerance. The tolerogenic process involves central and peripheral mechanisms, both

of which play a crucial role in protecting the spermatogenic cells and other antigenic components of the male reproductive tract from the immune system. Central tolerance occurs primarily in the thymus, during normal development, when expression of antigens by the thymic epithelium leads to functional deletion of self-reactive T cells by inactivation and apoptosis.[1] A similar mechanism mediates B-cell editing in the bone marrow.[151] It is now recognized that this process involves the promiscuous expression of many tissue-specific antigens in the thymic epithelium induced by the transcription factor, autoimmune regulator (AIRE).[152] Even sperm-specific antigens have been found to be expressed in the thymus under this mechanism.[153] However, this mechanism is not completely efficacious because autoreactive lymphocytes persist and may expand or become activated later in life, resulting in autoimmune disease.[154,155]

The mechanisms of peripheral tolerance, which need to operate effectively throughout life, are more complex. Peripheral tolerance involves functional deletion of autoreactive lymphocytes in the peripheral (secondary) lymphoid tissues due to weak antigen-stimulation in the absence of appropriate co-stimulation, production of blocking and anti-idiotypic antibodies, and development of antigen-specific regulatory and suppressor lymphocytes.[139,156] This involves an ongoing process of low-dose exposure of these cells to their antigen, accompanied by either modified co-stimulatory or specific immunoregulatory signals from antigen-presenting cells, regulatory lymphocytes, or other cell types in the vicinity.[157] This means, for example, that engagement of the peptide-MHC class II complex with the TCR in the absence of linkage of CD80/CD86 to CD28, in the presence of immunoregulatory cytokines, such as IL10 or TGFβ, or engagement by CD80/86 of the inhibitory T cell receptor, CTLA4, in place of CD28, leads to T-cell deletion by inactivation or apoptosis, and/or induction of regulatory/suppressor T-cell activity (Figure 19.6).[140,158] The best characterized, and arguably the most important, of these regulatory lymphocytes is the $CD4^+CD25^+$ regulatory T cell (Treg) subset, which expresses the transcription factor, FOXP3.[140] These cells can be induced by TGFβ and IL2, and selectively produce immunoregulatory TGFβ and IL10, but also appear to act via direct contact with antigen-presenting cells and other T cell subsets.[159,160] Both NK cells and NK T cells are also capable of antigen-recognition and immunosuppressive activity. Other T cell subsets implicated in immunosuppression through their ability to produce TGFβ and IL10 include Th3 cells, Tr1 (T regulatory 1) cells and γδ T cells.[161–163]

Autoimmunity and Rejection Responses in Immunity

Autoimmune disease generally represents the failure of tolerance. Somatic mutation of the antigen-receptor expressed by a T cell or B cell may result in the creation of new self-reactive clones, thereby subverting central tolerance.[164] Autoimmunity may result from an inflammatory response to an infection that damages or overwhelms normal mechanisms of self-tolerance, or where the infection involves organisms that express antigens that may cross-react with self-antigens (molecular mimicry).[165] Studies of disease models in humans and experimental rodents have also established that autoimmunity may have a genetic basis, as in the case of polyglandular autoimmune (PGA) syndromes. In humans, type 1 PGA is associated with a mutation in the AIRE transcription factor,[166] while type 2 PGA is related to a defect in regulatory T cell function.[167]

Failure of tolerance results in autoimmunity, but failure to induce tolerance in the first place lies behind transplantation rejection responses. The leukocytes of both the graft recipient and the donor tissue react toward their respective antigens, leading to the rejection of tissues that are not antigenically matched, in a process called graft-versus-host disease.[168] The response is said to be allogeneic if it occurs across genetic boundaries within the same species (allograft), while xenogeneic responses involve a graft and host belonging to different species (xenograft). The extensive polymorphism of the MHC, which is also called the human leukocyte antigen (HLA) complex in the human, is a major contributor to the allogeneic rejection response. Rejection responses typically involve the classical MHC class 1a (HLA-A, HLA-B and HLA-C) and MHC class II (HLA-D) antigens responsible for antigen presentation during T-cell activation.[131] On the other hand, nonclassical MHC antigens that are able to inhibit T cell and NK cell activity, such as HLA-G and HLA-E, are associated with suppression of the adaptive immune response and maintaining peripheral tolerance.[169] Consequently, both classical and nonclassical MHC are involved in regulating autoimmune responses, including autoimmunity in the male reproductive tract.

Initiation and Control of the Immune Response and Immunity

Antigen-specific immune responses are initiated in the secondary lymphoid tissues, particularly within the follicles and germinal centers of the draining lymph nodes and spleen or lymphoepithelial aggregates of mucosal tissues, where antigen-presenting cells come into contact with large numbers of T and B cells.[170] This implies that antigens normally must travel via the lymphatics to one of these organs, either by simple diffusion or carried by an antigen-presenting cell. Generally, immunologists do not consider that primary immune activation can occur within nonlymphoid tissues or assume that any response generated there is likely to be vigorous enough to lead to full participation by the immune system. However, there is some evidence that primary activation can

occur outside the lymphoid tissues, especially during graft rejection.[171,172]

Moreover, an alternative immune activation model has been proposed that combines elements of both the innate and adaptive immune system. This is called the danger hypothesis, which proposes that antigen-presenting cells responds to substances that cause or signal damage, rather than to those that are simply unrecognized.[173] These danger signals include CD40LG, the early pro-inflammatory cytokines IL1β and TNF, interferons, and heat-shock proteins, as well as substances that are normally only found inside cells (e.g. nucleotides, unmethylated CpG sequences in mammalian double-stranded DNA) and hyaluron breakdown products.[173] In this model, activation of the immune system occurs as a response to evidence of an extant threat rather than toward a specific feature of the threat itself. This mechanism may serve to explain the onset of certain autoimmune diseases.

Inflammation and immunity are damaging to the tissues in which they occur. Apart from the normal mechanisms of tolerance, the immune system needs to limit inflammation and immune responses to minimize such damage. Accordingly, inflammation triggers secretion from the adrenal gland of glucocorticosteroids, which repress expression of NFκB, inhibit the production of pro-inflammatory cytokines and mediators while stimulating the anti-inflammatory cytokines, IL4 and IL10, reduce expression of leukocyte adhesion molecules, and induce lymphocyte apoptosis.[124,174–177] The immune cells themselves produce other anti-inflammatory and immunosuppressive molecules, including prostaglandins D and J and lipoxins.[178,179] Moreover, activated lymphocytes have a limited lifespan, undergoing a process of activation-induced cell death following upregulation of the extrinsic apoptotic signal mediated by interaction of the FAS receptor with its ligand (FASL), eventually leaving behind a relatively small population of long-lived memory cells.[143,180] There are also specific mechanisms to inhibit antibody activity and promote antibody clearance, such as the induction of anti-idiotype (antitopographical) antibodies.[181]

Mucosal Immunity

Muscosal immunity concerns immunity at the interface between the external and internal environments. Mechanisms involved in mucosal immune responses are very similar to those of other tissues and lymphoid organs. Discrete lymphoepithelial aggregates (mucosa-associated lymphoepithelial tissue or MALT) within mucosal surface tissues allow interaction between antigen-presenting cells and lymphocytes to facilitate local immune responses.[182] Reactions to inhaled or digested antigens are controlled through a process called mucosal tolerance,[162,182] and failure of tolerance in the gastrointestinal and respiratory tracts may result in allergies. The mechanisms of mucosal tolerance involve clonal T-cell deletion or anergy and active suppression by regulatory/suppressor T cells.[183] Although $CD8^+$ T cells predominate in the mucosal epithelium, it is the $CD4^+$ T cells that are responsible for mucosal tolerance, producing IL4 and IL10 (Th2 cells), and TGFβ (Th3 cells).[162,183,184] Mucosal intraepithelial lymphocytes also express CD1D constitutively and can activate immunoregulatory NK T cells in the epithelium.[185]

A key feature of mucosal immunity is the isotype of the antibodies normally involved at mucosal surfaces. Tight junctions between the epithelial cells restrict passage of both antigen and antibody, so that antibody responses are dominated by secretory IgA, which lacks the ability to activate complement and possesses strong anti-inflammatory properties.[186] In order to cross epithelial barriers, a unique mechanism for transport of dimers of IgA through the epithelial cells involving a protein called secretory component is necessary.[187] Thus, IgA is generally the major immunoglobulin in secretions of mucosal surfaces. In mucosa, the epithelial cells also play an important role in host defense by producing many molecules associated with innate and adaptive immunity, including defensins, regulatory cytokines, TLRs, MHC antigens, leukocyte adhesion molecules, and co-stimulatory molecules.[188,189]

There is strong evidence that the male reproductive tract is a constituent of the mucosal immune system.[91,190] As far as the male reproductive tract is concerned, both IgA and serum-derived IgG appear in semen and bound to the sperm of men with autoimmune infertility.[3,45,191] It would be expected that the epithelial cells of the male urogenital tract might play a similar active role in regulating local immune responses. Although discrete MALTs have never been described within the male tract, the existence of similar or analogous structures cannot be entirely excluded.

Regulation of Immunity by Hormones and Gender

Corticosteroid Control of Inflammation and Immunity

Corticosteroids are essential physiological regulators of the immune response, acting through the ubiquitously expressed glucocorticoid receptor to exert negative feedback on the inflammatory response and control innate and acquired immunity at multiple levels.[124,174–177] Clinically, they are widely used in the treatment of autoimmune and inflammatory diseases. The particular relevance of corticosteroids for male reproductive function is the fact that they have inhibitory effects on testicular development

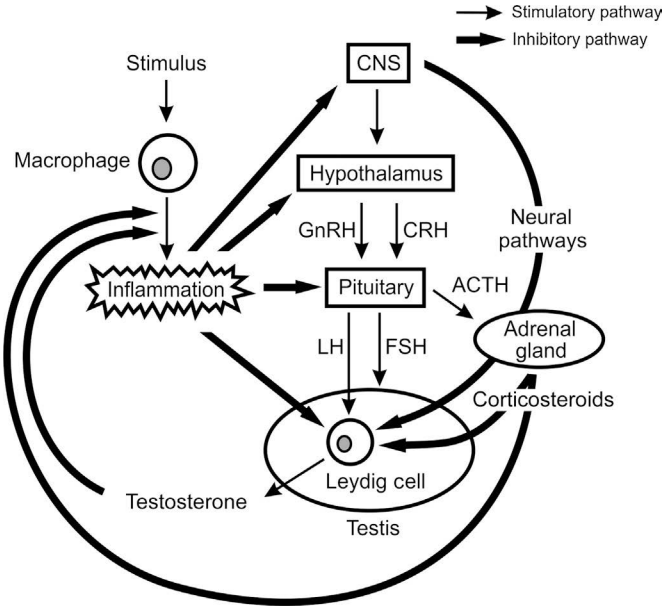

FIGURE 19.8 The interface between the hypothalamic-pituitary-gonadal-adrenal axis and inflammation. Regulation of the testes and adrenal glands is under the control of the central nervous system (CNS), which integrates information from the periphery and modulates secretion of the gonadotropin-releasing hormone (GnRH) and corticotropin-releasing hormone (CRH) by the hypothalamus. These hypothalamic peptides stimulate secretion of the gonadotropins (LH and FSH) and adrenocorticotropic hormone (ACTH), respectively. LH acts directly on the Leydig cells to stimulate steroidogenesis, while FSH controls the development and activity of the Sertoli cells. ACTH stimulates secretion of corticosteroids by the adrenals, which generally exert direct inhibitory effects on Leydig cell steroidogenic activity. Activation of macrophage function by an inflammatory stimulus triggers a cascade of events and secretions, which interact with the hypothalamic-pituitary-gonadal axis at all levels, inhibiting gonadotropin secretion and steroidogenesis and stimulating the hypothalamic-pituitary-adrenal axis. There is evidence that inhibitory regulation of steroidogenesis in response to inflammation also involves direct neural pathways from the CNS. As a consequence, inflammation profoundly inhibits the ability of the Leydig cell to produce testosterone. Eventually, steroids produced by the adrenals and testis exert feedback inhibitory effects on the inflammatory process, bringing about the resolution of the inflammation and recovery of testicular testosterone production.

and suppress Leydig cell steroidogenesis at all levels of the hypothalamic-pituitary-testicular axis with the result that activation of the hypothalamic-pituitary-adrenal axis during inflammation negatively impacts upon male reproductive function (Figure 19.8).[192]

Sex-Specific Regulation of Immune Function

The male reproductive system exerts a profound inhibitory effect on the development and function of the immune system. Compared with females, males have lower serum immunoglobulin levels, reduced cellular immunity and less effective responses to antigenic challenge, while the incidence of autoimmune diseases are generally far less common and less severe in males than in females.[193] This can be attributed, in part, to genetic and epigenetic differences between the sexes, as indicated by studies in sex-reversed mice, which established that XX-bearing mice displayed greater susceptibility to autoimmune disease than XY-bearing mice, independent of gonad type or hormones.[194] However, testis ablation and male sex steroid replacement studies have also established a critical role for products secreted by the testis in this divergence. Such studies demonstrated that androgens inhibit many immune parameters, particularly the size of the thymus and other immune tissues, lymphocyte number and activity, and antibody production, but also graft rejection, autoimmune responses, and resistance to infection.[195–197] These findings have been supported in more recent years by studies in transgenic animals lacking the androgen receptor,[198,199] and by clinical data from hypogonadotropic men.[200,201] Studies on macrophages and other androgen-responsive cell types in vitro have indicated that androgens are able to inhibit NFκB and expression of inflammatory genes, such as TLR4, IL1, and TNF.[202–204] On the other hand, androgens appear to stimulate neutrophil proliferation growth and function through activation of the extracellular signal-regulated (ERK) kinases (mitogen-activated kinase 3/mitogen-activated protein kinase 1; MAPK3/MAPK1) and production of the neutrophil growth factor, granulocyte colony stimulating factor.[198,205]

The classical androgen receptor is a cytoplasmic protein that binds androgens with high affinity, and translocates to the nucleus, where it acts as a transcription factor by binding to androgen-response elements in the promoter of responsive genes.[206] Studies by early researchers established that functional androgen receptors were primarily expressed on the stromal and epithelial cells of the immune tissues,[207,208] suggesting that the effects of androgens on immunity were exerted indirectly at the tissue level, rather than by direct effects on the circulating lymphocytes. In fact, the expression and relative importance of the classical androgen receptor on lymphocytes remains somewhat equivocal.[209,210] However, it is now clear that steroids can also interact with membrane-bound G protein-coupled receptors to trigger nongenomic responses in target cells.[211,212] Studies have shown that androgens can alter [Ca] fluxes in lymphocytes and macrophages via such membrane-mediated interactions,[213,214] and that this signaling affects gene expression and function in the target cells.[215]

Obviously, many questions remain concerning the detailed cellular mechanisms that mediate the actions of androgens on immune cells, but it is clear that these steroids have profound effects on immune responses and are capable of directly modulating these functions within the male reproductive tract and adjacent draining lymph nodes. Furthermore, this regulation may also involve other steroids produced by the testis, such as progesterone and the estrogens, which also have direct effects on immune cell function.[196,216] Estrogens, in particular,

have been implicated in regulating the differentiation and maturation of dendritic cells, and inhibiting their co-stimulatory activity.[217,218] There is even evidence that LH and FSH themselves directly regulate macrophage and lymphocyte growth and cytokine production acting through specific receptors expressed by these cells.[219,220]

Protein hormones and neuropeptides produced by the testis have direct effects on immunity. These include the pro-opiomelanocortin gene-derived peptides β-endorphin and α-melanocyte-stimulating hormone (α-MSH), which are produced by the Leydig cells and macrophages of the testis, and testicular GnRH.[221–223] The testis is a significant source of cytokines with immunoregulatory activity, specifically, members of the highly immunosuppressive/anti-inflammatory TGFβ family.[224,225] There is no doubt that exposure of circulating immune cells to these molecules within the testis, and/or their secretion into the blood and lymph, plays a significant role in local immunoregulation and sexual dimorphism of immune function.

IMMUNE CELLS OF THE MALE REPRODUCTIVE TRACT

Far from being a site where the immune system is restricted entry, macrophages, lymphocytes and granulocytes are characteristic features of the male reproductive tract (Table 19.3). There are substantial differences in the number and type of these cells within the different tissues and from species to species, which have important implications for understanding the immunophysiology of the male tract.

TABLE 19.3 Quantification of Immune Cells in the Normal Testis of Adult Rats and Humans[a]

Cell Type	Rat Testis ($\times 10^6$/g tissue)	Human Testis ($\times 10^6$/g tissue)
Macrophages	5–10	10–25[b]
Dendritic cells	0.2–0.3	Present
T cells	1–2	1.4–2.4
$CD8^+$ T cells	0.6–1.8	Present
$CD4^+$ T cells	0.2–0.3	Present
NK cells	0.6–1.0	1.0–2.8
Mast cells	Capsule region only	Present

[a]*Estimates based on data from stereological analysis of testes from Sprague–Dawley rats and from adult human testes with normal spermatogenesis (Hedger MP and Hayes RD, unpublished data).[69,70,226,227] The study of Vergouwen and colleagues[228] indicates that CBA/P mouse testes contain approximately 2–4 × 10^6 macrophages/g tissue, but there are no definitive quantitative studies of other leukocyte subsets in the mouse testis.*
[b]*Upper limit calculated from data obtained by Frungieri and colleagues[229] using a well-characterized monoclonal antibody against CD68. The observation that macrophage numbers in the normal human testis are at least as large, if not larger, than those found in the either the rat or mouse testis is consistent with nonquantitative observations using several macrophage markers.[67,230–232]*

Macrophages and Dendritic Cells in the Testis

Macrophages and dendritic cells are found in every tissue of the body. Although some dendritic cells have a lymphoid origin,[233] these cell types are chiefly derived from the circulating monocyte pool and are directed along tissue-specific developmental pathways by the influence of the local immunological environment.[234] As a consequence, macrophages, in particular, are extremely heterogeneous in appearance and function.[235,236] Superficially, the microglial cells of the brain and the Kupffer cells of the liver have little in common, but both cell types are macrophages and share common features: they express markers of the mononuclear phagocyte lineage, are actively mobile, phagocytic, cytotoxic, and are involved in tissue restructuring and antigen-presentation to $CD4^+$ T cells.

Distribution and Properties of the Testicular Macrophage Population

Early interest in testicular macrophages arose from studies on the accumulation of nondigestible tracers and radionuclides in the testicular interstitial tissue.[237,238] In spite of some early speculation that these cells might have a nonhematopoietic origin, studies by Miller and colleagues,[237,239,240] and subsequently by Hutson and colleagues,[241–244] established that the testicular macrophages share the classical characteristics of resident macrophages or tissue-fixed macrophages. They display the characteristic nuclear and cytoplasmic morphology of the mononuclear phagocyte lineage, are actively phagocytic, bactericidal and adherent in culture, and they express macrophage-specific enzymes, cytokine receptors, and surface antigen markers.[69,239,240,243,245–247]

There are substantial populations of resident macrophages in both the rat and mouse testes.[69,228,248–250] Other species with large numbers of testicular macrophages include the guinea pig, hamster, horse, bull, and human.[229,251–253] These cells are almost entirely confined to the interstitial tissue under normal (noninflamed) conditions. In the boar, which has a relatively sparse testicular interstitial connective tissue and very large numbers of Leydig cells, macrophages appear to represent a smaller proportion of total interstitial cells.[254] Curiously, the ram testis appears to possess only small numbers of recognizable macrophages, in spite of the general similarity of the testicular interstitial tissue and lymphatic organization in this species with that of the bull or human.[52,68] Testicular macrophages have been most intensively studied in the rat, with less extensive investigation in the mouse, and relatively limited investigations in other species. It generally has been assumed that the rat testicular macrophages are representative, but data suggest that there are significant functional differences even between rat and mouse testis macrophages. Further study on the macrophages of other species, particularly

the human and primates, is clearly necessary, and discussion of this complex cell type in the testis necessarily reflects the rather narrow available knowledge base.

In the rat and mouse, the ratio of macrophages to Leydig cells appears to be relatively fixed at approximately one macrophage to every four or five Leydig cells,[245,248–250] and macrophages display a very close physical and functional relationship with the Leydig cells. Ultrastructural studies have established the existence of highly-specialized cytoplasmic interdigitations linking the two cell types, indicating the potential for direct exchange of information and material,[60,240,244,255] while macrophages and Leydig cells undergo parallel alterations in morphology and cytoplasmic volume in experimental models of cryptorchidism and vasectomy in the adult rat.[256,257] During aging, testicular macrophages retain their morphological association with Leydig cells, but the cytoplasmic interdigitations are lost.[258] Aging testicular macrophages also acquire lipofuscin granules similar to those observed in aged Leydig cells.[258] Most surprising, however, was the discovery that macrophages play an essential role in normal Leydig cell development and in maintaining Leydig cell steroidogenic function in the adult.[208,259–265] It appears that the testicular macrophages have an integral role in testicular biology that goes beyond the typical functions of tissue-fixed macrophages in phagocytosis of cellular debris, connective tissue remodeling, maintenance of innate immunity, and regulation of adaptive immunity.

The immunological functions of the testicular macrophage population have been investigated in a number of studies, but the data are by no means comprehensive and many questions still remain. Although several studies have shown that the majority of rat macrophages express MHC class II molecules under normal conditions, indicating a capacity for antigen-presentation to helper and regulatory T cell subsets,[68,246,266] Tung and colleagues reported that strong class II expression in the normal mouse testis was restricted to cells located around the rete testis and was only upregulated in the interstitial tissue following immunization with testicular antigens in the presence of adjuvant.[267,268] This seems to indicate a fundamental difference in baseline activation status and antigen-presenting activity between the testicular macrophages of the rat and mouse. Human testicular macrophages appear to be more like those of the rat, with significant MHC class II expression found throughout interstitial tissue samples from normal men.[67,230] Studies of the ability of rat testicular macrophages to provide help for T cell activation in vitro indicate that these cells are deficient in co-stimulatory activity.[269,270] In the mouse testis, a lack of expression of the essential co-stimulatory molecules for T cell activation, CD80 and CD86, has been reported,[271] but this does not appear to be true for the rat.[272] With respect to inflammation, selective depletion of testicular macrophages in the rat with a macrophage-specific toxin exacerbated the testicular inflammatory response induced by hyperstimulation of the Leydig cells with pathological doses of the LH agonist, human chorionic gonadotropin (hCG).[273] Significantly, rat testicular macrophages display relatively poor expression of critical proinflammatory genes and proteins, including IL1β, TNF, IL6, IL12, NOS2, and macrophage migration inhibitory factor (MIF), in response to activation by bacterial LPS or regulatory cytokines[269,274–278]; however, these cells express constitutively high levels of the anti-inflammatory/immunosuppressive cytokine, IL10.[270] Examination of murine testicular macrophages indicates a similarly reduced pro-inflammatory capacity (Hedger MP and Winnall WR, unpublished data).

The accumulated data confirm that macrophages from both rat and mouse testes, although different in some functional respects, have reduced inflammatory and co-stimulatory activities. Macrophages displaying such properties are called alternatively activated or M2 macrophages (analogous to Th2 cells); they are characteristic of tumors and late-stage inflammation.[236,279] During inflammatory and immune responses, M2 macrophages serve to regulate the activity of pro-inflammatory or classical M1 macrophages to moderate cell-mediated immunity, and thereby play important roles in reducing autoimmune and rejection responses.

Heterogeneity of the Testicular Macrophage Population

Although testicular macrophages collectively display an alternatively activated phenotype, quantitative studies of macrophage numbers using specific antigenic markers in the rat testis indicate that testicular macrophages do not represent a homogenous population.[69,226,276] The majority of testicular macrophages in the rat express a molecule that is specific to tissue-fixed macrophages in most non-lymphoid tissues, recognized by the monoclonal antibody, ED2.[280] This molecule has been identified as CD163, a member of the group B scavenger receptor cysteine-rich protein superfamily.[281] It has been implicated in the stimulation of IL10 production by monocytes and macrophages following the endocytosis of hemoglobin:haptoglobin complexes, and is highly expressed by M2 macrophages.[282,283] However, a significant subset of testicular macrophages in the rat (about 15–20% of the total) do not express this marker. These macrophages can be identified by expression of the lysosomal antigen, CD68, recognized by antibody ED1, which suggests they may be recently recruited or even transient macrophages.[69,268,276,284] Moreover, about half of the $CD163^+$ subset appears to have lost CD68 expression. These data indicate the existence of several populations of macrophages in the normal rat testis, putatively representing different stages of development and/or functional states (Figure 19.9).[284]

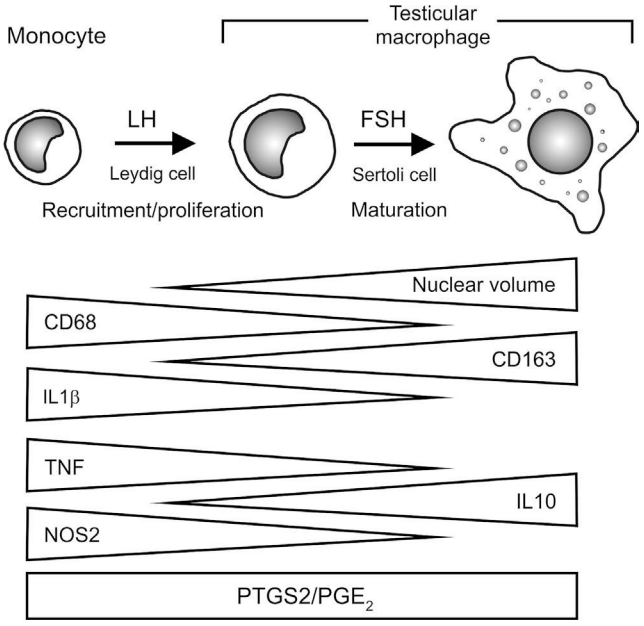

FIGURE 19.9 Maturation of the resident macrophage population of the rat testis. Macrophages in the rat testis are heterogeneous, corresponding to different stages of maturation from circulating monocytes through to a distinct testicular resident macrophage phenotype. This testicular phenotype is characterized by an increased nuclear and cytoplasmic volume, loss of the CD68 marker recognized by antibody ED1, upregulation of the resident macrophage surface marker ED2 (CD163), loss of ability to produce pro-inflammatory mediators and constitutive production of the immunoregulatory cytokine, interleukin-10 (IL10). Expression of the inducible form of prostaglandin-endoperoxide synthase (PTGS2) and the ability to produce prostaglandins, such as PGE_2, does not appear to change. Recruitment of macrophages to the testis and/or their proliferation is under the control of LH, acting via the Leydig cells, while maturation to the mature testicular phenotype appears to be FSH-dependent, indicating regulation by the Sertoli cells.

This heterogeneity may have functional correlates. Compared with $CD68^-$ macrophages, $CD68^+$ testicular macrophages possesses a distinctly smaller nuclear diameter and displayed overall higher levels of expression of NOS2 and CCL2 in normal and LPS-stimulated rats, based on immunohistochemical studies.[276,285] Expression of IL1β protein after LPS treatment appears to be confined to a relatively small subset of testicular macrophages.[274] Since the $CD68^+CD163^-$ macrophage subset increases in number in the testis after LPS treatment, it may be assumed that this subpopulation represents newly-arrived pro-inflammatory macrophages. However, when macrophages were isolated from non-inflamed rat testes on the basis of CD163 expression by flow cytometery, the $CD163^-$ subpopulation displayed very poor production of either proinflammatory cytokines or IL10 in response to LPS in vitro.[270] Altogether, these data suggest that the $CD68^+CD163^-$ subpopulation present in the normal testis comprises recently recruited or transient macrophages, and it is functionally distinct from the proinflammatory $CD68^+CD163^-$ macrophages that invade the rat testis following an LPS challenge in vivo.[276,286]

There is evidence for macrophage heterogeneity in mouse and human testes, as well. Studies have shown that macrophages isolated from the mouse testis are heterogeneous in their ability to produce TGFβ in vitro,[287] and approximately 20% express high levels of the alternative-activation marker, Ym1.[288] In the human testis, Frungieri and colleagues found that $CD68^+$ macrophages displayed substantial variation in expression of CD163.[229] All these observations are consistent with the hypothesis that the testis contains subsets of macrophages with varying pro-inflammatory capacities, although it appears that an alternatively activated, anti-inflammatory/immunosuppressive phenotype predominates under normal conditions.

Intratesticular Dendritic Cells

Dendritic cells, which are distinguishable from macrophages by their distinctive morphology and expression of specific functional markers, such as integrin αx (ITGAX; CD11c) and integrin αE2, have been observed in the rat, mouse, and human testis.[230,266,289–291] Dendritic cells are found throughout the interstitium, but they are much less prominent than the testicular macrophages. In spite of the common lineage, dendritic cells lack the efficient phagocytic and cell-killing capabilities of macrophages, although they are much more effective as antigen-presenting cells.[133] Crucially, dendritic cells are able to promote either cell-mediated immunity or tolerance to specific antigens, depending upon their maturity and activation status.[156] These cells are involved in directing immune responses within the testis and adjacent lymph nodes, and studies suggest that immature dendritic cells play a role in regulating tolerance in the normal testis.[271,272,292]

Recruitment and Regulation of Testicular Macrophages

At birth, there are relatively few macrophages in the testis, but macrophage numbers expand dramatically during testicular development, coinciding with proliferation of the adult Leydig cell population and the appearance of the meiotic spermatocytes.[228,248,249,293] Indeed, the development of both the testicular macrophage and the adult Leydig cell populations appears to be interdependent. There is considerable evidence that macrophages are required for adult Leydig cell development and function.[208,259,261,262,264] Conversely, studies from a number of groups have shown that the accumulation of macrophages during testicular development and maintenance of their numbers in the adult are dependent upon the action of pituitary LH and, more specifically, the Leydig cells themselves (Figure 19.9).[69,226,293–297] Depletion of the Leydig cells or suppression of their activity in the adult

testis by various means causes a progressive decline in the number of testicular macrophages.[69,226,294–298] Although there is evidence that macrophages in the testis possess LH receptors,[299,300] the balance of the data suggest that the Leydig cell is responsible, rather than a direct action of LH itself. Testicular macrophage depletion occurs in models in which serum LH is increased (e.g. following treatment with the Leydig cell toxin, ethane dimethane sulfonate (EDS))[69] or decreased (e.g. hypophysectomy, GnRH immunization, suppression of endogenous androgen with subcutaneous testosterone implants).[294–298]

The precise mechanisms involved in maintaining the testicular macrophage population are not known. Complete depletion of spermatogenic cells by cryptorchidism has absolutely no effect on testicular macrophage numbers in the rat,[226] suggesting that their regulation does not involve the seminiferous epithelium. Moreover, it does not appear that androgens are directly involved.[226,293,295] However, it is likely that direct contact with the Leydig cell membrane and/or nonandrogenic products of the Leydig cells may be responsible.[240,244] Studies have clearly indicated that normal development of the testicular macrophage population, as in other tissues, involves the macrophage growth factor, colony stimulating factor-1 (CSF1).[247,264,285,301] The chemoattractant cytokine, MIF, which is constitutively expressed by the Leydig cells, likewise may be involved.[226,278,302] Furthermore, intratesticular production of the chemokines, chemokine (C-X3-C motif) ligand 1 (CX_3CL1; fractalkine) and CCL2, is implicated in the recruitment of circulating monocytes under normal conditions and during inflammation, respectively.[285,286,303]

It remains an area of ongoing discussion whether resident macrophages found in most tissues under noninflammatory conditions are largely derived and sustained from the circulating monocytes or by proliferation within the tissue itself.[235,304] The significant population of macrophages expressing CD68, but not CD163, in the normal testis suggests that there may be constant recruitment of these cells from the monocyte pool.[284] On the other hand, there is evidence that testicular resident macrophages may undergo active proliferation by mitosis, at least under certain circumstances: during the early inflammatory phase following destruction of the Leydig cells by EDS[305] and during testis development in rats and mice.[293,306] Consequently, it is not absolutely clear whether the resident macrophage population of the testis, once established, is maintained by recruitment of new monocytes or by local proliferation. In fact, it is most likely that both processes are involved. Based on the extended persistence of radionuclides in rodent, canine, and human testicular macrophages, on the other hand, it would appear that most resident macrophages do not escape the testis alive.[237,238]

While initial evidence that testicular macrophages respond directly to FSH has been shown to be due to an experimental artifact,[307] a stereological examination of macrophage recruitment to the testis in GnRH-immunized rats given recombinant FSH replacement demonstrated that FSH stimulates an increase in macrophage nuclear volume.[294] This indicates an effect on macrophage activity in the testis, which is almost certainly mediated via the Sertoli cell, the only testicular cell type able to respond directly to FSH. It appears that, while Leydig cells are responsible for recruiting and maintaining the testicular macrophage population, the Sertoli cell may play a role in directing at least some of the testis-specific functions of these cells (Figure 19.9). These two somatic cells act together to recruit and modify the function of the testicular macrophages, thereby bringing about the unique resident macrophage phenotype found in this organ.

Lymphocytes in the Testis

It is a common misconception that lymphocytes are not found within the normal testis. In fact, lymphocytes are relatively prominent within and adjacent to the epithelium of the rete testis and are sparsely distributed throughout the testicular interstitial tissue in all species so far studied.[65–70,231] In the rat and human testes, lymphocytes actually represent about 10–20% of the total leukocyte population, although the proportion of these cells in the normal mouse testis appears to be somewhat lower (Table 19.3) (Hedger MP, unpublished data).[69,227] It is worth noting that considerable differences may exist among different strains of animals and between animals raised under different degrees of exposure to environmental pathogens, as lymphocyte populations are very sensitive to differences in genetic background and past immunological events. Functional characterization of these cells has received relatively little attention: T cells and NK cells, but not B cells, have been described in the normal rat, mouse and human testis.[65,67–70,227] Most evidence suggests that the intratesticular lymphocyte population is skewed toward major histocompatibility complex (MHC) class I restricted ($CD8^+$) cells and cells expressing NK cell markers (Hedger MP, unpublished data).[65,69,70,227]

Typically, T cells circulate through tissues as part of their surveillance function, and it is widely believed that activated T cells tend to recirculate in tissues where they initially encountered an antigen.[308,309] The phenotype of the majority of the T cells of the rat testis is certainly consistent with activated or memory T cells.[70] There is clear evidence that lymphocyte numbers increase in the testes of men with infertility and sperm autoimmunity,[66,232] and lymphocyte numbers in the rat testis appear to gradually increase with age while macrophage numbers remain relatively constant (Hedger MP, unpublished data). These data suggest that at least some of the T cells

in the testis are specific for testicular autoantigens. Alternatively, some of these cells may represent T cells that have been exposed to exogenous antigens within the testis environs during past infections.

The observation that there are substantial numbers of cells expressing NK markers in the testes of rats, mice, and humans is particularly significant (Hedger MP and Aridi DZ, unpublished data).[70] Following isolation from the normal rat and mouse testis, it was confirmed that these cells have specific NK activity against transformed cells in vitro, and that a significant proportion of these cells are actually NK T cells (Hedger MP and Aridi DZ, unpublished data). In addition, $CD4^+CD25^+FOXP3^+$ Treg cells are present in both rodent species, but are considerably more prominent in the mouse testis than in the rat. Immunohistochemistry has recently localized Treg cells to the human testis, as well.[310] The T cells and NK T cells from the rat testis were found to produce IL10 under both unstimulated and PMA/ionomycin-stimulated conditions, although the T cells displayed a very high proportion of anergic and apoptotic cells compared with T cells from other tissues (Hedger MP and Aridi DZ, unpublished data). These observations indicate that the lymphocytes found in the normal testis comprise cells involved in cell-killing and innate immunity, that immunoregulatory subsets (NK, NK T and Treg cells) are highly represented, and that intratesticular T-cell responses to activation are significantly abrogated.

In normal testes, lymphocytes are rarely, if ever, observed within the epithelium or lumen of the seminiferous tubules, although lymphocytes appear to be able to cross the epithelium of the rete testis.[64–66] Nonetheless, the number of lymphocytes and macrophages increases dramatically within the testicular interstitial tissue during testicular infection, experimental autoimmune orchitis, leukemic relapse, and testicular tumorigenesis.[311–316] This infiltration is generally associated with failure of spermatogenesis, and the immune cells may eventually breach the tight junctions of the blood–testis barrier and invade the seminiferous epithelium. Moreover, human infertility is often associated with increased numbers of intratesticular lymphocytes and macrophages, as evidence of ongoing inflammation even in the absence of an overt immune event.[4,5] It remains to be determined whether this is a cause or a response to testicular damage or failure, but there tends to be a relationship between the number of intratesticular lymphocytes and the extent of testicular disruption.

Mast Cells and Eosinophils in the Testis

Mast cells and eosinophils are specialized mediators and regulators of inflammation, particularly in the context of allergic responses.[120,121] Mast cells, in particular, are found in almost all tissues, including those of the male reproductive tract, but their distribution in the testis displays very distinctive, species-specific differences.[62] In the testes of the rat, mouse, dog, cat, bull, and deer, these cells are largely absent from the testicular parenchyma but are frequently associated with blood vessels in the testicular capsule. In contrast, mast cells are found throughout the interstitial tissue in equine and human testes,[62,71,317] while both mast cells and eosinophils are relatively abundant in porcine species.[62] The functional significance of these cells in the testis is not known, but it is reasonable to assume that they play a role in local innate immunity and possibly a role in the fine control of testicular blood flow. In humans, testicular mast cell numbers decline with advancing age.[317] However, increased numbers of intratesticular mast cells and increased mast cell expression of PTGS2 are associated with various forms of testicular failure, and they have been found to correlate with the severity of damage and increased NOS2 expression in the Leydig cells.[22,318–320] In contrast to mast cells and eosinophils, neutrophils are only found in the testis in conditions of testicular inflammation or damage.[276,314,321,322]

In the adult rat, mast cells are normally confined to the subcapsular region, but they proliferate dramatically throughout the testicular parenchyma following ablation of the Leydig cells by EDS.[69,323–325] The degree of proliferation appears to be under the control of the Leydig cells, suggesting that these cells produce an inhibitor of mast cell activity.[69,325] However, the increased mast cell numbers persist even after the Leydig cells recover and testis function returns to normal following EDS treatment. The species distribution of mast cells and eosinophils suggests some relationship with the level of aromatase activity of the Leydig cells,[326,327] and neonatal estrogen treatment increases mast cell numbers in the rat testis.[325,328] Stem cell factor (SCF), which is growth factor for both mast cells and Leydig cells that is produced by the Sertoli cell, also may be involved.[329,330] While eosinophils are not a feature of the normal rat or mouse testis, these cells were occasionally observed in the testes of GnRH-immunized rats.[294] It appears that the regulatory mechanisms for the two different granulocyte subsets in the testis may be quite separate and distinct.

Immune Cells in the Epididymis

In contrast to the testis, macrophages and lymphocytes in the epididymis are frequently observed within the epithelium, where they are commonly identified as halo cells, as well as the interstitial tissue.[64–66,94–100,331–334] Published immune cell subset data for the epididymis have been complicated by the fact that some studies have not clearly differentiated between lymphocytes and macrophages because the CD4 and CD8 antigens are also expressed by rat and human monocytes and

macrophages.[335,336] Studies in the mouse, where these antigens are more restricted to lymphocyte subsets, indicate that macrophages are the major epididymal leukocytes, located chiefly in the interstitial and peritubular regions, and that there appears to be a slight preponderance of MHC class II restricted $CD4^+$ T cells (helper and regulatory T cell subsets) over the $CD8^+$ T cell subset in the interstitial tissue, typical of blood and most other tissues.[95,96,99] Conversely, the intraepithelial lymphocytes are predominantly $CD8^+$ T cells, which is a common feature of mucosal epithelia.[65,96,98,334] This distribution of T cell subsets is consistent with the observation that the interstitial tissue macrophages express MHC class II antigens, whereas macrophages within the epididymal epithelium mostly do not.[96,99] As in the testis, development of the epididymal macrophages is dependent upon CSF1.[337] Studies have suggested that the basal cells, located adjacent to the basal lamina of the epididymis, exhibit structural and antigenic properties typical of macrophages.[94,338] The numbers of the basal cells expressing macrophage-specific markers in the mouse are increased by the presence of damaged sperm, and it has been speculated that these cells are actually a type of resident macrophages, which could play a role in regulating immunity in the epididymis.[338,339]

Recently, dendritic cells have been identified, using specific reporter-labeled fluorescence imaging, as a major component of the epididymal epithelium in the mouse.[102] These cells form a dense network in the basal region of the epithelium and extend their processes between the epithelial cells. They express characteristic dendritic cell and antigen-presenting surface markers, including ITGAX, CX_3CR1, MHC class II antigens and CD80/86, and possess effective antigen-presenting activity in vitro. These cells are completely distinct in morphology and functional properties from the conventional intraepithelial macrophages or the basal cells.

Immune cells are found in all regions of the epididymis, although there is a tendency toward larger numbers and activity of all leukocyte subsets within the peritubular zone and epithelium of the caput, compared with the cauda.[96–99,338] The number of intraepithelial macrophage and $CD8^+$ T cells increases preferentially in the more proximal regions of the epithelium during aging and increased spermatogenic disturbance in rats, as well.[100] Moreover, the intraepithelial dendritic cells appear to be particularly active within the proximal caput, such that their processes can extend all the way through the epithelium; they presumably can sample antigens within the epididymal lumen and present them to $CD4^+$ T cells in the epididymal stroma and local lymph nodes.[102] The lymphatics of the epididymis have been studied in detail in the rat and mouse; they show a distinct regional distribution favoring the cauda, but otherwise appear to be unremarkable.[340–342]

The very different distribution of leukocytes in the epididymis compared with the testis indicates the existence of a different immunological environment, where separation between the antigenic sperm and the local immune cells is less robust, and more dynamic interactions are possible. Obviously, separation of antigens and immune cells is not the principal mechanism protecting sperm from immunological attack in this organ. In fact, it has been speculated that the macrophages within the epididymal duct may even be responsible for phagocytosis of senescent and excess sperm, although the evidence for this is equivocal.[333,343–345] The immunophysiological functions of the macrophages and lymphocytes within the epididymis are only just being uncovered, and they represent a fertile area for future research.

Immune Cells in the Vas Deferens, Accessory Glands, and Urethra

The organization of the vas deferens is very different from that of the epididymis: it is not coiled but has an extensively convoluted epithelium and multiple layers of smooth muscle. Sperm are not stored in the vas, and the majority of sperm pass through the duct only transiently at the time of ejaculation, but this structure plays a special role in our understanding of autoimmunity in the male tract because of the widespread use of vasectomy as a contraceptive method. Approximately 70% of all vasectomized men develop sperm antibodies; in many of these men, the antibodies are persistent, thereby frustrating later attempts to restore fertility.[346,347] Sperm antibodies are likewise associated with obstructive azoospermia and congenital absence of the vas (CAV) in men,[348] and similar antibody responses occur in experimental animal models.[332,349–352] These observations clearly indicate that obstruction or damage to the vas deferens is a potent inducer of sperm autoimmunity.

Although less prominent than in the epididymis, intraepithelial lymphocytes, principally $CD8^+$ T cells, are found throughout the vas deferens, seminal vesicles, prostate, and urethra.[64,66,353–357] Macrophages appear to be relatively rare in the epithelia, but they are found in the stroma of these tissues even under noninflammatory conditions, where they are dependent upon CSF1 for their development.[337,353] Relatively little is known about the immunological properties of these cells, but a predominance of cytotoxic $CD8^+$ T cells is a common characteristic of all mucosal epithelia. While an immunoregulatory role of these lymphocytes in the reproductive epithelium remains to be determined, these cells are almost certainly involved in mediating cellular immunity at these sites.

It is self-evident that the leukocytes found in semen, even in men with no obvious infection or other inflammatory condition, must originate from the epithelium of the ducts and organs of the male reproductive tract.[358]

Logically, immune cells pass with relative ease across the epithelium in certain areas, thereby coming into physical contact with the sperm at the time of ejaculation. It is nonetheless curious that, while macrophages and lymphocytes are typically present, the leukocytes most commonly found in the semen are neutrophils, which are not a major leukocyte subset within either the stroma or epithelia of the male reproductive tract.[358–364] Because vasectomy dramatically reduces leukocyte numbers in the ejaculate, the epididymis and proximal vas deferens appear to be a major source of these cells, at least under normal conditions, but the accessory glands (seminal vesicles and prostate) are also implicated as a source of the seminal neutrophils.[364–366]

IMMUNOLOGICAL AND INFLAMMATORY MEDIATORS IN THE TESTIS

Inflammation and activation of the immune response has immediate and mostly negative effects on male reproduction and fertility.[20,21,367] It is widely assumed that this reproductive disruption is related to fever and the effects of raised body temperature on spermatogenesis in febrile patients, but there is actually very little experimental evidence to support this assumption. In fact, there is an enormous body of research to indicate that other inflammatory mechanisms can affect male reproductive function and are much more likely to disrupt reproduction.

More than 25 years ago, it was reported that rat testicular extracts contained a protein with properties similar to IL1, which eventually turned out to be IL1, produced by Sertoli cells rather than by intratesticular leukocytes.[368,369] Although generic molecules with inflammation-regulating activity, such as the prostaglandins, were already known to be produced by the reproductive tract, this discovery provided the first indication that an inflammatory molecule belonging to the immune system could be produced by a somatic cell of the male reproductive system. In subsequent years, it became increasingly obvious that many inflammatory and immunoregulatory cytokines could be produced by somatic and spermatogenic cells of the testis in response to an inflammatory stimulus, including IL1, IL6, TNF, and TGFβ family members and interferons. Moreover, once it became apparent that their production was constitutive, in the absence of any evidence of cryptic inflammation, or could be stimulated by physiological stimuli, such as hormones or cell–cell interactions, a role in normal reproductive physiology was anticipated. It is now very evident that inflammatory and immunoregulatory molecules produced by the testis are involved in regulating both immunological processes within the testis and many aspects of normal testicular function associated with fertility. The production and actions of the most prominent of these molecules are briefly outlined in this section of the review. Integration of their actions and the consequences for testicular immune responses and fertility are covered in later sections.

The Interleukin-1 Family and the TLRs

These molecules are related by the fact that the IL1 receptor and the TLRs belong to a family of dimerizing receptors that contain the conserved cytoplasmic TIR domain.[106,112] Both IL1 and TLR ligands act through similar signaling pathways to activate intracellular MAP kinases and the pro-inflammatory transcription factor, NFκB, and induce the expression of early pro-inflammatory genes, including IL1 (Figure 19.5).

Interleukin-1

The archetypical pro-inflammatory cytokine, IL1 is represented by two related proteins (IL1α and IL1β) capable of binding to the same receptor to exert an almost identical range of effects.[370] These proteins are produced by many cells types, but activated monocytes and macrophage are the major source of secreted IL1.[370] Both IL1α and IL1β are single chain proteins, with only about 25% homology at the mature protein level. They are synthesized as 31–33 kDa precursor proteins that are cleaved enzymatically to active 17-kDa forms (Figure 19.10). Both the precursor and mature forms of IL1α are biologically active, but the IL1β precursor is inactive. Consequently, production of active IL1β is a two-step process involving NFκB-induced transcription and translation of the IL1β precursor gene, followed by cleavage of the precursor to the mature cytokine by CASP1.[117,118] This processing of IL1β is linked to its secretion into the extracellular space. Conversion of IL1α does not involve CASP1, but the precursor can be cleaved by calcium-dependent membrane-associated cysteine proteases, or calpains, and extracellular proteases.[371] In contrast to IL1β, the majority of IL1α tends to remain within the cell or associated with the cell membrane, although it may be found in secretions as well.[370] It is generally believed to act as an autocrine growth factor or as a mediator of direct cell-to-cell communication, but the mechanisms involved are poorly defined. Both IL1α and IL1β differ from most cytokines in that they lack a signal sequence and their mechanisms of secretion remains to be elucidated.

Intratesticular Production of Interleukin-1

Intratesticular IL1α and IL1β have been observed in many species, including the human,[372] but have been most extensively studied in the rat. Endogenous production of IL1α appears in the rat testis at around 20 days of age, more or less coinciding with progression of the first wave of spermatogenesis.[369,373–376]

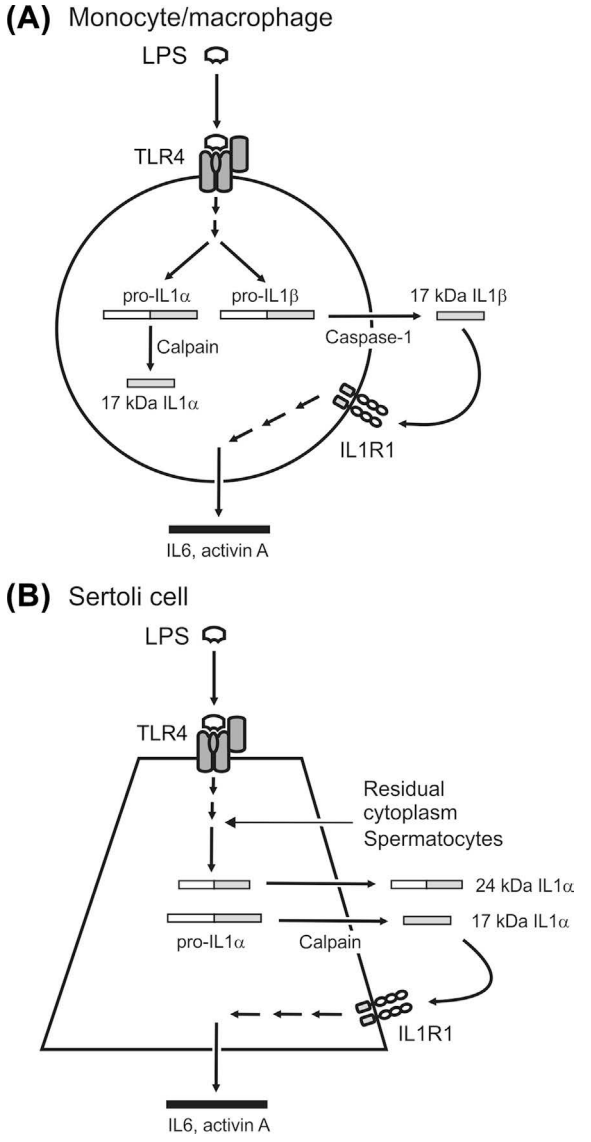

FIGURE 19.10 Comparison of the regulation and production of interleukin-1 (IL1), IL6 and activin A in the monocyte/macrophage and Sertoli cell. (A) Activation of the TLR4 complex on the surface of the macrophage by bacterial lipopolysaccharide (LPS) upregulates expression of both IL1α and IL1β, which subsequently are processed to their mature bioactive 17-kDa forms by the action of calpain and caspase-1, respectively. IL1α tends to remain associated with the cell, but IL1β is secreted upon cleavage and binds to the IL1 receptor (IL1R1) on the cell surface to stimulate production of other inflammatory cytokines, such as IL6 and activin A. (B) The Sertoli responds to stimulation by LPS, spermatocytes and the residual cytoplasm of released spermatozoa by producing IL1α, preferentially. Two alternate transcripts of IL1α are produced by the Sertoli cell, including a transcript lacking the calpain cleavage site domain, which encodes a 24-kDa form of IL1α with reduced bioactivity. Both forms appear to be secreted by the adult Sertoli cell and both are able to signal via the IL1 receptor to stimulate the production of IL6 and activin A.

This appears to be almost entirely due to the Sertoli cells, which synthesize and secrete both the mature 17-kDa IL1α molecule and a slightly larger 24-kDa testis-specific form of IL1α—the latter being the product of an altered mRNA transcript lacking the calpain cleavage site necessary for processing of the precursor (Figure 19.10).[377–379] Adult rat seminiferous tubule extracts also contain an immunoreactive 45-kDa IL1α protein that has yet to be fully characterized, but may be another variant of the precursor mRNA with one or more introns retained. These testis-specific transcripts are biologically active, although less potent than mature 17-kDa IL1α.[377,380] There is some evidence that rat spermatocytes and spermatids also express IL1α constitutively.[381]

Production of IL1α by the Sertoli is under control of the spermatogenic cells and shows a distinct cyclical variation that is related to the changes in spermatogenic cell associations as determined by the cycle of the seminiferous epithelium.[373,382–384] It is strongly stimulated by phagocytosis of the residual cytoplasm cast off by the spermatids when they are released into the tubule lumen.[375,376,385] Sertoli cell production and secretion of IL1α in vitro is induced by various inflammatory stimuli, including LPS and IL1 itself, but not by FSH.[376,385–388] Studies indicate that Sertoli cells from prepubertal rats produce, but do not secrete, IL1α, whereas Sertoli cells from adult rats are able to secrete biologically active IL1α.[369,379,389] The mechanism underlying this maturational change in ability of the Sertoli cell to secrete IL1α is unknown.

In contrast to IL1α, IL1β appears not to be produced in significant amounts in the rat testis under normal conditions, but is upregulated in a subset of testicular macrophages and the Leydig cells during inflammation.[274,369,373] Curiously, the majority of this secreted testicular IL1β is present as the precursor protein.[274] Studies have shown that Leydig cells express the mRNA for both IL1α and IL1β in vitro in response to stimulation by LPS or exogenous IL1,[390–392] and whole testes and isolated macrophages collected from LPS-treated rats or mice express IL1β mRNA,[393–395] and the immunoreactive protein.[396] Thus, while IL1β expression in the normal testis is relatively low, it increases during inflammation, possibly due to production by interstitial cells rather than by the Sertoli cells. The response of testicular IL1α to inflammation in vivo is less clear: although production by the Sertoli cell is upregulated by inflammatory stimuli in vitro, recent in vivo studies report either no response or reduction during systemic inflammation.[396,397]

Intratesticular Actions of Interleukin-1

Numerous studies have established that IL1, whether produced intratesticularly or external to the testis, is an effective regulator of Leydig cell steroidogenesis, spermatogonial and spermatocyte development, and various Sertoli cell functions associated with supporting spermatogenesis.

The actions of IL1 are mediated by the IL1 type I receptor (IL1R1), which belongs to the immunoglobulin superfamily of receptors, but also bears an intracellular TIR domain (Figure 19.5).[106,112] Following ligand binding, a second TIR domain-containing subunit, called the IL1R acceptor protein (IL1RAP), is recruited to form a complex that binds the MYD88 adaptor protein and activates IRAK, leading to NFκB nuclear translocation and MAP kinase activation. Moreover, IL1 is able to activate other signaling pathways through induction of phosphatidylinositol-4,5-bisphosphate 3-kinase (PIK3)/Akt protein kinase (AKT) and tyrosine-protein kinase Src (SRC), hydrolysis of GTP, phosphatidylcholine, phosphatidylserine or phosphatidylethanolamine, release of ceramide by neutral sphingomyelinase, and release of arachidonic acid by phospholipase A_2 (PLA_2).[370,398] There is an IL1 type II receptor (IL1R2), which lacks a TIR domain and can act as a decoy or inhibitory receptor.[399]

In cultures of Leydig cells from immature rats (10–20 days of age), IL1β caused a dose-dependent increase in DNA synthesis, and IL1α was much less potent than IL1β.[400] The effect of IL1β was not observed in Leydig cells isolated from older animals, suggesting that macrophage-derived IL1β could play a role in Leydig cell proliferation during prepubertal development.[400] The possible involvement of prostaglandins in this developmental regulation is indicated by the observations that IL1β stimulates the expression of PTGS2, both IL1 isoforms and IL6 in rat progenitor Leydig cells, and that prostaglandins act as intermediates in this stimulatory pathway.[401]

A review of the literature dealing with the direct effects of IL1 on testosterone production by mature Leydig cells in vitro reveals reports of stimulation, inhibition or even of no effect.[395,402–406] Presumably, these apparent discrepancies arise from differences in the experimental conditions or methodologies employed. On balance, studies suggest that IL1 is either stimulatory or has no effect on basal testosterone production by adult Leydig cells,[406–408] and there is general agreement that IL1 inhibits LH/hCG and/or cAMP stimulated testosterone production.[395,402,403,406,407,409] In the mouse, the major site of inhibition is at the level of CYP17A expression (Figure 19.3).[403,410] In the rat, IL1 appears to regulate Leydig cell steroidogenesis at the level of CYP11A, while STAR gene expression and protein synthesis are unaffected.[404,411] The effects of testicular IL1α on Leydig cell steroidogenesis may be dependent on both the variant of IL1α involved and on the stage of development of the Leydig cell. The 32-kDa IL1α precursor and the 17-kDa mature IL1α are both inhibitory to gonadotropin-stimulated or cAMP-stimulated steroidogenesis in mature Leydig cells, whereas the testis-specific 24-kDa IL1α splice variant has no effect.[380] In contrast, all three forms of IL1α stimulated testosterone production by immature rat Leydig cells. When IL1β was injected into the immature rat testis, Leydig cell steroidogenesis and serum testosterone levels were stimulated within 24 h, although Leydig cell activity had declined several days later.[412] No similar effect was observed in the adult testis. The signaling mechanisms mediating IL1 effects on the Leydig cells involve PTGS2 enzyme expression and PGE_2 acting as an intermediate.[402,413] Studies using Leydig cells from immature rats have also implicated the MAP kinases, MAPK14 and MAPK3/MAPK1, in the regulation of STAR and cholesterol mobilization.[380,414]

The IL1 receptor (IL1R1), along with the decoy receptor (IL1R2), has been localized to most testicular cell types in rat, mouse and/or human testis, including the spermatogenic cells and Sertoli cells.[415] It has been shown that IL1α is able to stimulate mitotic DNA synthesis in intermediate and B type spermatogonia and meiotic DNA synthesis in preleptotene spermatocytes.[382–384,416] In vitro, IL1 stimulates proliferation of prepubertal Sertoli cells[417] and regulates a number of activities of more mature Sertoli cell that support or control spermatogenesis, including aromatase activity,[418] production of glucose transporters, lactate and transferrin,[419–421] and production of IL1α, IL6 and the IL1 receptor.[375,385] IL1 also regulates production by the Sertoli cell of the inhibin B heterodimer and the homologous β-subunit homodimer, activin A, in an inverse relationship with FSH.[389,422] Considered more broadly, IL1 opposes the mostly stimulatory effects of FSH on Sertoli cell activity. Moreover, IL1 regulates the ability of the Sertoli cell to maintain contact with other Sertoli cells and developing spermatogenic cells, by altering the Sertoli cell cytoskeleton, and stimulates opening of the occluding junctions that make up the blood–testis barrier.[423] Regulation of Sertoli cell function by exogenous IL1 has been found to involve multiple pathway intermediates, including MAPK14, MAPK8 and MAPK3/MAPK1, PIK3/AKT, PTGS2/PGE_2 and NOS2/NO, although the more predictable participation of MYD88 and NFκB still awaits investigation.[417,424–427]

Interleukin-18 and Other Interleukin-1 Family Cytokines

The IL1 family comprises several structurally related proteins, which appear to have arisen by gene duplication.[428] The most closely-related to IL1α and IL1β is IL18, which is normally processed to its mature form by the inflammasome and the action of CASP1, like IL1β, but acts via a distinct IL18 receptor.[429] Both IL18 and its receptor have been found in the seminiferous epithelium of the rat, with IL18 mRNA and protein localized to spermatocytes and round spermatids.[430] Murine Leydig cells produce IL18, CASP1, and the IL18

receptor in response to stimulation by LPS,[431] and IL18 has been detected by immunohistochemistry in human testis, where its expression is reduced in impaired spermatogenesis.[432] Recombinant IL18 stimulates spermatogonial DNA synthesis in cultures of rat seminiferous tubules, without influencing germ cell apoptosis.[430] As is the case for IL1β, the majority of IL18 produced within the rat testis is in the precursor form,[430] indicating that the processing of these cytokines by the inflammasome/CASP1 in the testis is an area that merits more investigation.

The IL1 receptor antagonist (IL1RN) binds to the IL1 receptors but lacks the ability to transduce a signal, and so acts as a competitive antagonist of IL1 action.[433] It has been shown to be produced by mouse Sertoli cells, and its production is stimulated by FSH, LPS and IL1.[434] Male mice lacking IL1RN have higher intratesticular levels of IL1α and IL1β, along with reduced fertility and litter sizes and increased morphological abnormalities and premature activation of their sperm, although sperm number and motility is not affected.[435]

The TLRs

Studies have revealed that TLRs are widely expressed in the male reproductive tract (Table 19.4).[24,101] The other pattern-recognition receptor families have yet to receive much attention in this context, but several NLR and RLR family members have already been identified in the testis.[436,445,446]

Studies in the rat and mouse have established that Sertoli cells express TLR1-6 at significant levels, with relatively low expression of TLR7 and 13 in the mouse and of TLR 10 and 11 in the rat.[388,436–440] Quantitative examination of the TLR4 complex in rat Sertoli cells confirmed that the level of expression of TLR4 and its co-receptor, MD2, was similar in the Sertoli cell and testicular macrophage, but that Sertoli cells displayed low basal or LPS-induced expression of the TLR4 accessory protein, CD14.[388] Cytokine production responses to TLR1-6 ligands have been confirmed in both rat and mouse cells. The Sertoli cells produce appropriate cytokines, such as IL1α, IL6, and CCL2, in response to TLR4 activation by LPS,[379,388,436,437] and type 1 interferons in response to TLR3 or TLR4 activation[438–440]; however, the precise roles of specific signaling pathways in these responses requires further clarification, particularly with respect to the importance of the adaptor protein MYD88 and the transcription factor NFκB.[436–440]

The majority of studies on the effects of TLR ligands on Sertoli cells have employed LPS, which induces inflammatory gene responses in the Sertoli cells that are similar to those observed in macrophages.[24] However, LPS obtained from different bacterial strains can have quite different chemical composition and is frequently contaminated by other TLR ligands (e.g. bacterial lipoproteins

TABLE 19.4 Toll-like Receptor Expression in the Epithelium of the Male Reproductive Tract[a]

Receptor	Principal Ligands	Principal Pathogens	Cellular Location	Sertoli Cells	Epididymis	Vas Deferens
TLR1	Triacyl lipopeptides	Bacteria, mycobacteria	Cell surface	+++	++	+++
TLR2	Lipoproteins, peptidoglycans	Bacteria, mycobacteria, viruses	Cell surface	++++	++	+
TLR3	dsRNA	Viruses	Endosomes	++++	+++	++
TLR4	Lipopolysaccharides	Bacteria, viruses	Cell surface	++++	++	+
TLR5	Flagellin	Bacteria	Cell surface	+++	+++	+++
TLR6	Diacyl lipopeptides, zymosan	Bacteria, fungi	Cell surface	+++	+++	+
TLR7	ssRNA	Viruses	Endosomes	+/−	+	+
TLR8[b]	ssRNA	Viruses	Endosomes	−	+/−	−
TLR9	CpG DNA	Bacteria, viruses, protists	Endosomes	−	++	++
TLR10[c]	Unknown	Bacteria	Cell surface	+	+	−
TLR11[d]	Profilin	Bacteria	Endosomes	+	+++	+++
TLR12[d]	Profilin	Bacteria	Endosomes	−	ND	ND
TLR13[d]	Ribosomal RNA	Bacteria	Endosomes	+	ND	ND

ND, insufficient data available.
[a]*Consolidated data from published studies in the rat and mouse.*[388,436–444]
[b]*TLR not functional in rodents.*
[c]*TLR not expressed in mouse.*
[d]*TLR not expressed in human.*

and peptidoglycans).[447] This means that many studies in the literature using LPS actually describe responses involving multiple TLRs (usually TLR2 and TLR4). When highly purified LPS was used, rat Sertoli cells were more than 10-fold less sensitive to LPS than testicular macrophages, but they expressed similar levels of IL1α and IL6 and much greater levels of activin A when maximally stimulated.[388] These Sertoli cells also responded to the synthetic lipopeptide Pam3Cys (a specific TLR2 ligand) with a more prolonged pattern of gene expression. The need for relatively high doses of LPS to stimulate the Sertoli cell is probably related to the relatively low level of expression of the accessory protein, CD14, which serves to amplify the response to LPS in macrophages.[110] These data indicate that Sertoli cells respond to bacterial ligands acting through both TLR2 and TLR4, although they are less sensitive to these ligands in comparison with local macrophages and display a Sertoli cell-specific pattern of gene expression in response.

There have been few studies on the effects of TLR ligands on noninflammatory responses in the Sertoli cell: exposure of Sertoli cells to LPS in vitro directly inhibited lactate production and plasminogen activator activity, which are important functions for supporting spermatogenic cell development.[448] In other studies, LPS induced oxidative stress in Sertoli cells by increasing ROS production and reducing antioxidant activity,[449] while activation of TLR3, a receptor for viral double-stranded RNA, stimulated scavenger receptor expression and phagocytosis of apoptotic spermatogenic cells by Sertoli cells in culture.[439]

In rat and/or mouse studies, mRNA for TLR2, 3, 4, 7, 9, 10, and 12, along with low levels of MD2 and CD14, have been observed in Leydig cells; TLR2, 3, 4, 6, and 11 have been observed in peritubular cells; and TLR2, 3, and 4 have been observed in spermatogenic cells (Hedger MP and Winnall WR, unpublished data).[388,436,450,451] These cells also respond to TLR ligands in vitro. Leydig cells produce pro-inflammatory cytokines and type 1 interferons, but they reduce their sterodogenic activity when stimulated with LPS or a TLR3 agonist.[390,392,450] Peritubular cells respond to LPS by producing the macrophage chemoattractant, CCL2.[303] Most remarkably, activation of TLR3 has been reported to induce the inflammatory transcription factors, NFκB and IRF3, and a range of genes involved in inflammation and antiviral responses in mouse spermatogonia and spermatocytes.[451]

In the immune system, the TLRs and IL1 are fundamental regulators of NFκB activity. The regulation and functional role of this pro-inflammatory transcription factor in normal spermatogenesis is not fully understood at this time, but existing data suggest that there is an interaction between endogenously-expressed inflammatory molecules and NFκB in controlling seminiferous epithelial function. NFκB is constitutively present and active in the nucleus of cultured rat Sertoli cells and displays a cyclical localization within the nuclei of the Sertoli cells and spermatogenic cells throughout the cycle of the seminiferous epithelium.[452] Induced expression of NFκB stimulates androgen receptor expression, and therefore androgen responsiveness, in rat Sertoli cells,[453] but activation of NFκB in Sertoli and/or spermatogenic cells has also been implicated as an apoptosis-inducing signal in damage models, involving exposure to toxins, ischemia-reperfusion injury, and cryptorchidism.[454–457]

The TNF Family

Tumor Necrosis Factor

Members of the TNF family of cytokines are commonly membrane-bound homotrimers that can be released as soluble ligands by proteolytic cleavage. The canonical member of this family, TNF is a 17-kDa glycosylated polypeptide that is secreted principally by activated monocytes and macrophages, and binds to receptors that are present on most cells in the body.[458] This cytokine plays a central role in the initiation of the inflammatory response, and can exert pro-inflammatory or cytotoxic effects depending upon the receptor subtype engaged and the expression of specific adaptor proteins within the target cells (Figure 19.5). There are two families of TNF receptors (TNFR).[459,460] The TNFR type 1 family of receptors induce cell death through a motif in their cytoplasmic regions called the death domain (DD), and includes TNFR1, FAS and the death receptors (DR)3-6. When TNF binds to TNFR1, there is recruitment to the DD of the intracellular adaptor proteins TRADD (TNFR-associated death domain) and FADD (Fas-associated death domain), leading to activation of the cell death caspase pathway. However, the binding of TRADD can also lead to recruitment of the cellular inhibitor of apoptosis (baculoviral IAP repeat containing; BIRC) protein or receptor interacting serine–threonine kinase (RIPK), enabling the binding of TRAF2. This complex mediates activation of the NFκB pathway and the MAP kinases, MAPK14 and MAPK8. The TNFR type 2 receptors, which include TNFR2, TNFR superfamily member 8 (TNFRSF8, CD30) and 5 (TNFRSF5, CD40), and the lymphotoxin receptor, do not contain a DD in their intracellular domains, and instead associate with TRAFs leading to the activation of cell signaling events.

Intratesticular Production and Actions of TNF

In common with IL1, TNF regulates Leydig cell steroidogenesis and the spermatogenesis-supporting functions of the Sertoli cell, but TNF appears to act as a regulator of spermatogenic cell survival, rather than of proliferation or development. In situ hybridization studies in mice first identified the presence of TNF mRNA

in round spermatids, pachytene spermatocytes, and testicular interstitial macrophages.[461] Bioactive TNF was produced by the round spermatids in vitro and mRNA for the corresponding receptor was located on Sertoli and Leydig cells. Similarly, in the seminiferous epithelium of the rat, TNF has been localized to the spermatogenic cell compartment, and TNF receptors primarily localized to the Sertoli cells.[462] Treatment of isolated rat or mouse testicular macrophages with LPS also induces TNF secretion.[463,464]

The majority of studies performed using a variety of systems, including intact animal studies,[465,466] isolated primary cultures of Leydig cells,[410,467–469] and MA-10 tumor Leydig cells transfected with CYP17A-reporter constructs,[470] report inhibitory effects of TNF on Leydig cell steroidogenesis. Inhibition of LH/hCG binding, STAR expression, and cholesterol mobilization has been observed,[466,471] but these studies also indicate that inhibition by TNF occurs downstream of cAMP production at the level of steroidogenic gene expression (Figure 19.3). In cultured mouse Leydig cells, TNF had no effect on basal expression of CYP11A, but it suppressed basal expression of HSD3β and the expression of CYP11A, CYP17A, and HSD3β induced by analogs of cAMP.[410,468] The inhibitory effect of TNF on CYP17A gene expression was found to be mediated through protein kinase C, possibly by activation of MAPK8, MAPK3/MAPK1, and the transcription factors, NFκB and AP-1.[467,470,472] Several other studies have implicated the sphingomyelin/ceramide-dependent pathway in TNF-mediated inhibition of Leydig cell testosterone secretion.[466,469] Although the levels of testosterone and the mRNA levels of steroidogenesis-related genes were significantly lower after puberty in TNF knockout mouse testes than in wild-type testes, this may be attributed to increased levels of anti-Müllerian hormone in these animals.[473]

Within the seminiferous epithelium, TNF appears to play a complex role in the regulation of Sertoli cell and peritubular cell function, which is probably attributable to the ability of TNF to stimulate both cell-killing and cell signaling pathways. Many of the effects of TNF have been shown to involve the activation of NFκB and MAP kinases in the Sertoli cell.[453,474–478] Similar to IL1, TNF stimulates basal lactate production by cultured Sertoli cells, but generally antagonizes the actions of FSH on Sertoli cell function, including the stimulation of lactate production, aromatase activity and inhibin.[479–481] Nevertheless, TNF receptor subunit protein expression has been shown to be stimulated by FSH in porcine Sertoli cells.[482] TNF stimulates the expression of inflammatory cytokines, chemokines, and leukocyte adhesion molecules expression in both Sertoli cells and peritubular cells.[303,387,477,478] Furthermore, TNF stimulates plasminogen activator inhibitor expression in rat testicular peritubular cells, indicating that it plays a key role in controlling testicular protease activity.[483] In Sertoli cell monolayer cultures, TNF disrupts inter-Sertoli cell tight-junction assembly by inhibiting production of the junction protein, occludin, and by regulating matrix metalloprotease and protease inhibitor activity.[484] Delfino and colleagues have shown that TNF stimulates androgen receptor expression in Sertoli cells via upregulation of NFκB, which binds to several enhancer motifs in the androgen receptor promoter.[453]

Single nucleotide polymorphisms in the TNF gene cluster producing elevated TNF levels have been shown to be associated with reduced sperm count and sperm motility in infertile men,[485] but the outcomes for spermatogenesis of TNF action are not always so clear-cut. Paradoxically, TNF reduces spontaneous spermatogenic cell degeneration in cultured human and rat seminiferous tubules, apparently by regulating FASL and/or NFκB levels in the Sertoli cell, indicative of a germ cell survival role for TNF mediated through the Sertoli cell.[475,476] At first glance, this would appear to run counter to the observation that activation of NFκB in the Sertoli cell or germ cells induces spermatogenic cell apoptosis in various damage models,[454–457] and the studies that have identified a role for germ cell-secreted TNF in the disruption of spermatogenesis in response to cytotoxic injury of the Sertoli cell.[486] However, NFκB is also able to regulate and interact with pro-survival genes, such as the inhibitor of apoptosis (IAP) and BCL2 families.[459,487] The interactions between TNF, NFκB and different apoptotic regulators produces complicated outcomes for spermatogenic cell survival in different situations, and the responsible mechanisms invite further investigation.

FAS and FAS Ligand

The death receptor FAS and its ligand (FASL) most commonly act as membrane-bound trimeric ligand–receptor pairs mediating cell–cell interactions, and FAS–FASL binding on activated T cells is essential for moderating the immune response.[180] Typically, the DD in the cytoplasmic region of FAS recruits the FADD adaptor protein and induces T cell death via caspase-dependent apoptosis.[488] In the testis, FAS and FASL have been implicated in regulating spermatogenic cell apoptosis during testicular damage and, more controversially, in maintaining immune privilege.[489,490] Studies intended to localize FASL in the testis under normal conditions have produced conflicting results, which may be attributed to differences in detection methods, limitations of the reagents employed, and the fact that these molecules are readily inducible.[491,492] Thus, FASL has been described as being present in rat, mouse, porcine, and human Sertoli cells, and absent in most germ cells,[489,490,493,494] but others have reported that FASL expression in the rat seminiferous epithelium is confined to the germ

cells.[492,495] FAS has been identified on isolated mouse Sertoli cells,[496] but in intact testes it has been localized to spermatogonia and spermatocytes from the pubertal period onwards.[490,495,497]

Nonetheless, FAS expression is clearly associated with spermatogenic cells that are undergoing apoptosis.[475,486,490,497] Furthermore, FAS can be induced in the Sertoli cell by the action of TNF and IFNγ.[474,496] Expression of both FAS and FASL is upregulated in various models of seminiferous epithelium damage, indicating that this mechanism is important in regulating germ cell apoptosis in cases of physical and toxicological insult.[490,498] Induction of FAS and FASL is also implicated in the testicular response to inflammation.[499]

Interleukin-6

In contrast to either IL1 or TNF, IL6 is a cytokine with both pro-inflammatory and anti-inflammatory actions (Figure 19.7); for example, IL6 stimulates B cell growth and differentiation and promotes neutrophil activity and survival, but inhibits the effects of IL1 and TNF, and induces IL1RN and IL10 expression.[125,500] In the late inflammatory phase, it stimulates the acute-phase response and promotes fibrosis. Outside the immune system, IL6 plays crucial roles in hematopoiesis, liver and neuronal function, energy metabolism, and cancer. Cytokines of the IL6 type, which include leukemia inhibitory factor (LIF) and IL11, exert their action via binding to specific receptors that can form complexes with a common, ubiquitously-expressed membrane signal transducer, glycoprotein 130 (interleukin-6 signal transducer; IL6ST).[501] In lymphocytes and hepatocytes, IL6 binds to its specific membrane-bound receptor (IL6R; CD126), which has no intrinsic kinase activity and dimerization with IL6ST leads to activation of the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) and MAP kinase signaling pathways (classic IL6 signaling). However, all cells that express IL6ST are able to respond to IL6 bound to soluble IL6R, which may be produced from an alternatively-spliced mRNA transcript or released by proteolytic cleavage of membrane-bound IL6R, a process called trans-signaling.

In vitro, IL6 is constitutively produced by Sertoli cells and Leydig cells.[375,386,390,502–504] Production of IL6 in rat or mouse Sertoli cells is stimulated by a range of agents, including FSH, testosterone, IL1, TNF, and LPS, and is inhibited by INFG.[387,496] Phagocytosis of residual cytoplasm shed by late spermatids at the time of spermiation triggers Sertoli cell IL1α production, which stimulates IL6 secretion in an autocrine manner, by activating leukotriene production via the lipoxygenase pathway (Figure 19.10).[375,387] Leydig cells are strong producers of IL6 after stimulation by LH, LPS, or IL1 in vitro, and may be the major source of this cytokine in the testis.[390,503,504] Human peritubular cells express IL6 mRNA, but possibly not the protein, when stimulated by TNF,[478] and isolated spermatogenic cells from adult mice express IL6 mRNA and protein when stimulated with a TLR3 ligand, poly(I:C).[451]

Although not as intensively studied in the testis as either IL1 or TNF, it appears that IL6 regulates the function of somatic, spermatogenic, and immune cells. Both IL6R and IL6ST mRNA are expressed in rat Sertoli cells and are stimulated by IL1 and by IL6, but only the IL6R subunit is stimulated by FSH.[505] In the Sertoli cell, IL6 increases basal and FSH-induced transferrin and cyclic GMP secretion,[506,507] but reduces κ opioid receptor mRNA levels.[508] IL6 has been shown to act as an inhibitor of meiotic DNA synthesis in preleptotene spermatocytes.[509] An effect of IL6 on testicular steroidogenesis at the level of both the Leydig cell and the hypothalamic-pituitary levels is indicated by the observation that subcutaneous IL6 administration in men produced prolonged suppression of serum testosterone levels, without apparent changes in gonadotropins.[510]

The Transforming Growth Factor-β Family

Cytokines of the TGFβ family, similar to IL6, possess both pro-inflammatory/pro-fibrotic and anti-inflammatory/immunosuppressive properties (Figure 19.7), but also regulate growth and differentiation at a fundamental level throughout the entire body. They are mostly homo- or heterodimers that act by binding two transmembrane receptor kinases, denoted as type I and type II, and assembling them into an active heterotetramer.[511,512] This triggers a transphosphorylation cascade that begins with the activation of the type I subunit kinase by the type II subunit kinase, and is propagated by the canonical SMAD (small for body size/mothers against decapentaplegic homolog) protein signaling pathway, which in the case of TGFβ and activin involves SMADs 2, 3 and 4, and to non-SMAD-mediated signaling pathways.[511,513] These receptors are expressed in most cells and tissues.

Transforming Growth Factor-β

The three forms of TGFβ (TGFβ1, TGFβ2, and TGFβ3) found in mammals are disulfide-linked dimers of approximately 25 kDa in mass, derived from three structurally-similar subunits possessing intrastrand disulfide bonds that form a cysteine knot folding motif (Figure 19.11).[514] Heterodimers of the subunits can also occur but are less abundant. All three forms are differentially expressed by Sertoli cells, peritubular cells, and Leydig cells, particularly in the fetal and immature testis, although production appears to decline considerably during the establishment of complete spermatogenesis.[515,516] In the postpubertal testis, they also have been localized to the spermatogenic cells in a development-specific pattern of expression.[225,517,518]

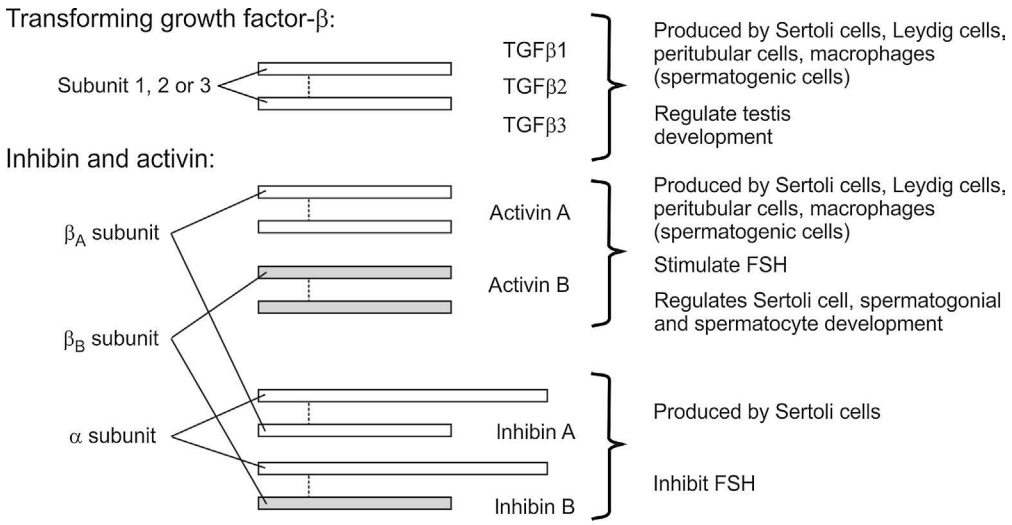

FIGURE 19.11 The transforming growth factor-β (TGFβ) and activin/inhibin family of cytokines. These cytokines are dimers of disulfide-linked subunits with considerable sequence and structural homology, and have diverse actions within the reproductive and immune systems. Three distinct subunits lead to production of homodimers (or less frequently heterodimers) of TGFβ1, 2, or 3. Dimerization of two homologous β-subunits lead to either activin A, activin B, or less commonly activin AB (not shown). Dimerization of the activin subunits with the closely related α-subunit produces the inhibins, which are produced almost exclusively by the Sertoli cells. Activins derived from the β_C-subunit (not shown), appear to play regulatory roles through formation of nonactivating homodimers and heterodimers.

The receptors for TGFβ are found in both somatic and spermatogenic cells.[225,517,519] In the fetal and immature testis, TGFβ has been implicated in controlling the development and apoptosis of gonocytes and spermatogonia, seminiferous tubule formation and Leydig cell differentiation, and Sertoli cell-peritubular cell interactions.[516,520–522] In the adult testis, TGFβ stimulates apoptosis and inhibits meiosis during spermatogenesis,[523–525] and inhibits Leydig cell steroidogenesis at the level of LH receptor number and signaling, as well as distal to cAMP production at the level of CYP17A expression.[526,527] Crucially, TGFβ2 and TGFβ3 regulate Sertoli cell tight junction dynamics, via the MAPK14 pathway, indicating a role for TGFβ-mediated signaling pathways in regulating the permeability of the blood–testis barrier.[528] Finally, TGFβ is a potent regulator of inflammation and the activity of immune cells, particularly of Th17 and Treg cells,[529,530] suggesting a role in controlling intratesticular immune responses.

Activin

The activins display a high degree of sequence and structural homology with TGFβ, operate through a similar receptor system, and signal via common pathways; nonetheless, they have their own characteristic range of biological actions.[531] There are four forms of activin subunits in mammals (β_A, β_B, β_C and β_E), but only the first three appear to be particularly relevant to the male reproductive tract.[224] Activins A and B are dimers of the β-subunits of inhibin A and B, which are themselves heterodimers of one of two β-subunits (β_A and β_B) with a common α-subunit (Figure 19.11).[532] Consequently, activin A ($\beta_A\beta_A$), activin B ($\beta_B\beta_B$), or activin AB ($\beta_A\beta_B$) forms exist, although most studies to date have concentrated on the activin A homodimer. Activin A is commonly believed to be the most biologically active form.[533] Whereas the chief physiological role of inhibin is to inhibit FSH at the anterior pituitary level, activin A and B stimulate FSH production.[534] Although both inhibin and the activins circulate in the blood, production of inhibin is almost exclusively confined to the Sertoli cell,[535,536] while the activins are produced in many different cell types and tissues, including the anterior pituitary, where they act as local regulators of FSH .[535,537–539]

Activins bind to one of two specific type II activin receptors (ACVR2A or ACVR2B), which are able to dimerize with a type I activin receptor serine/threonine kinase (activin receptor-like kinase, ALK).[512,540] As is the case for TGFβ, postreceptor signaling occurs via SMAD2/3/4, and several alternative signaling pathways, including inflammatory pathways involving MAPK14, MAPK8, and MAPK3/MAPK1.[512,513,541] Inhibin acts as a competitive inhibitor of activin because it can bind to the type II activin receptors but cannot bring about receptor subunit multimerization: this interaction of inhibin with the activin receptor is facilitated by a specific co-receptor protein called TGFβ receptor type III (TGFBR3), or betaglycan.[542] Activin C is a dimer of the structurally-related β_C-subunit, which does not appear to dimerize with the inhibin α-subunit and is unable to facilitate activin receptor signaling.[543,544] It is most highly expressed in the liver but is present in the testis.[545] Homodimers and heterodimers comprising the β_B- and β_C-subunits appear to act as weak competitive

agonists or antagonists of activin A.[533,544,546,547] Moreover, activin bioactivity can be effectively neutralized in the circulation and in tissues by an endogenous, high-affinity activin binding protein, follistatin.[548]

In the hematopoietic and immune systems, activin A is produced by activated monocytes, macrophages and dendritic cells, Th2 cells, bone marrow stromal cells, mast cells and neutrophils, and is stimulated by IL1, TLR ligands, and TNF, acting through MYD88/TRAF signaling, MAPK14 and MAPK8.[531] The β_A-subunit gene promoter comprises one or more AP-1 binding sites, but there do not appear to be any typical consensus NFκB sites within the proximal promoter.[549,550] Expression is stimulated in a synergistic manner by the Th2 cell transcription factor c-MAF and NFAT, a transcription factor that is expressed in immune cells.[551] Transcription of the β_A-subunit in response to cAMP probably involves the cAMP-responsive transcription factor, AP-2.[552] The presence of multiple phorbol ester-responsive elements (AP-1 and AP-2) in the promoter, and the stimulation of activin A production by phorbol esters in several cell types, also implicate protein kinase C in its regulation.[550,552] Multiple AP-1 and AP-2 sites have been identified in the promoter of the β_B-subunit, suggesting that activin B may be, at least in part, regulated in a similar manner to that of activin A.[552,553] In the murine liver, expression of the β_B-subunit is stimulated by systemic administration of LPS, implicating TLR signaling in its regulation.[554]

A large body of evidence suggests that activin A acts to promote inflammation and activation of macrophages and dendritic cells during the initial phase of the immune response, but suppresses immune responses once inflammation is established and activin A concentrations increase.[531] In cells of the monocyte/macrophage lineage that had not been activated, activin A induced nuclear translocation of NFκB, and production of pro-inflammatory mediators, such as IL1β, TNF, IL6, NO, and prostanoids.[555,556] However, in activated monocyte/macrophages, activin A inhibited processing of the IL1β precursor and production of TLR4, CD14, and pro-inflammatory mediators and increased production of IL1RN.[557,558] Similarly, activin A stimulated the recruitment and development of monocyte-derived dendritic cells,[559,560] but inhibited the ability of dendritic cells to mature and stimulate T cell activation.[561,562] Inhibitory effects of activin A on the antigen-presenting activity of macrophages have been observed as well,[563] and activin A stimulates the development of antigen-specific Treg cells.[564] Furthermore, activin A inhibits many of the actions regulated by IL1, IL6, and IFNγ, including T-cell and B-cell proliferation, monocyte phagocytosis, and production of acute-phase proteins.[557,565–567] Together with the fact that activin A is produced by Th2 cells and has been implicated in promoting various Th2-regulated immune responses, such as asthma and atopy,[568] mast cell recruitment, maturation, and activity,[569] and B cell immunoglobulin production,[555,570] these observations suggest that activin A may be a crucial Th2 cytokine. Likewise, the effects of activin A on macrophage inflammatory responses indicate an ability to switch pro-inflammatory M1 macrophages to an alternatively-activated, anti-inflammatory M2 phenotype. Finally, activin A has been shown to be both a fundamental regulator and an important intermediary in the process leading from inflammation to fibrosis, which is also associated with the M2 macrophage phenotype.[279,571]

In the adult testis, β_A-subunit mRNA or activin A protein is found in the Sertoli cells, Leydig cells, peritubular cells, resident macrophages, mast cells, and most spermatogenic cells, except spermatogonia.[224,572,573] In the adult rat, intratesticular fluid levels of activin A are 5–10 times higher than normal circulating concentrations.[274] In cultured Sertoli cells, activin A is stimulated by IL1 and TLR ligands (Figure 19.10), and inhibited by FSH and the cAMP/protein kinase A signaling pathway.[379,388,389,422,481,573] Immediately following spermiation, there is a spike in activin A production by the Sertoli cells, possibly driven by the surge of ILα produced by the Sertoli cell at this time.[572,573] Testicular macrophages, Leydig cells, and Sertoli cells respond to LPS by increasing β_A-subunit expression in vitro, although the Sertoli cells display a much more robust response and higher basal levels of production.[224,388] However, the actual role of activin A in testicular inflammation remains to be determined because the high intratesticular levels of activin A in the adult rat were not acutely affected by LPS treatment in vivo.[274] Significant production of activin B in the testis has yet to be confirmed: the β_B-subunit is produced by the Sertoli cell, but it is not clear whether any of this goes to form activin B homodimers, as inhibin B heterodimers are by far the major form of inhibin in the testes of most species, including the human.[422,574] The activin β_C-subunit has been immunolocalized to Sertoli cells, peritubular cells and Leydig cells, as well as spermatogonia, primary spermatocytes, and elongating spermatids.[545,575]

Activin receptors are expressed by most, if not all, of the somatic and spermatogenic cells of the testis.[572,576,577] Activin, together with TGFβ, exerts complex regulation of spermatogenic cell, Sertoli cell and Leydig cell development and function, and disorders of activin/TGFβ signaling are implicated in the onset of testicular cancer.[578] Depending upon the culture system employed, activin A exerts both stimulatory and inhibitory effects on spermatogonial cell proliferation,[579–581] and is able to maintain the condensed mitochondrial morphology found in spermatogenic cells beyond the leptotene stage of the first meiotic prophase.[582] Activin A regulates mature Sertoli cell function by disrupting tight junction formation and blood–testis-barrier function, and stimulating their

proliferative activity.[583] Activin A has been shown to regulate steroidogenesis by immature and adult Leydig cells in culture and by the MA-10 Leydig cell line,[584–586] although these effects were only evident after extended culture under non-physiological conditions. In mice, deletion of the β_C-subunit had no overt effect on testis function,[587] but overexpression of the β_C-subunit resulted in progressive hypospermatogenesis, which was proposed to be due to antagonism of activin A (and possibly activin B) in the testis.[547] Like TGFβ, activin is expected to be involved in controlling inflammation and immunity in the testis, by regulation of the maturation and activity of the intratesticular macrophages and lymphocytes.

The Interferons

The interferons are functionally-related cytokines with antiviral, antiproliferative, and immunomodulatory actions, comprising three main groups (α, β, and γ), based on their structural relationships and major cellular sources.[115,588] The type I interferons (IFNα and β) are produced by a broad range of cell types: typically, multiple forms of IFNα are produced by monocytes and macrophages, while two forms of IFNβ are produced by fibroblasts and epithelial cells, but they all exert their antiviral and antiproliferative effects via the IFNα receptor (IFNAR). The single type II interferon, IFNγ, is produced by NK and NK T cells, activated T cells and, under certain conditions, by macrophages and dendritic cells.[589,590] It acts through the IFNγ receptor (IFNGR), and regulates the activity of antigen-presenting cells as part of the adaptive immune response, in addition to its antiviral functions. In other words, IFNγ is a type II interferon, but a type 1 (Th1) cytokine. Production of type I interferons is normally stimulated through TLR3, which detects double-stranded viral RNA, and through TLR4, the receptor for bacterial LPS.[115] These TLRs upregulate the interferon-inducing transcription factor, IRF3, by engagement of the TICAM1 adaptor protein and interaction with TRAF3 (Figure 19.5).[106,115] Induction of IFNγ involves IL12 and IL18, type 1 interferons, JAK/STAT signaling, and the transcription factors, NFκB and AP-1, in a cell-specific manner.[589,590]

Viral infections stimulate type 1 interferon and IFNγ production by Sertoli cells, peritubular cells, Leydig cells, and testicular macrophages, leading to production of canonical antiviral proteins in the testis.[438,591–593] In vitro, type 1 interferon expression was induced by activation of TLR3 or TLR4 in murine Sertoli cells,[438,439] and by activation of TLR3 in murine spermatogenic cells.[451] Production of IFNγ in the testis has been implicated in both the regulation of immune privilege and onset of autoimmune orchitis.[594,595] Moreover, IFNγ stimulates Sertoli cell production of FAS and CASP1, and is implicated as the mediator of Sertoli cell and germ cell apoptosis under various conditions.[496,596] In a transgenic mouse model, overexpression of IFNβ in the seminiferous tubules caused spermatogenic cell loss and sterility.[597] These disparate observations are indicative of a complex relationship between the interferons, the local immune system, and spermatogenesis.

In a study on normal, healthy men, treatment with human IFNα significantly decreased serum testosterone levels.[598] This was most likely due to inhibition of steroidogenesis at the Leydig cell and hypothalamic-pituitary levels, because serum gonadotropin levels were unaffected. Both IFNα and IFNγ can inhibit testosterone production in primary cultures of porcine Leydig cells, and studies indicated that IFNγ inhibits cholesterol transport into the mitochondria, and inhibits expression of the STAR protein, CYP11A, and CYP17A (Figure 19.3).[599,600] In other experimental studies, human and murine IFNα inhibited steroidogenesis in rat Leydig cells.[601] These data indicate that interferons may contribute to the overall decline in steroidogenic function that is commonly observed in viral infections.[21,602,603] However, Dejucq and colleagues[592] have shown that Sertoli and Leydig cells in the rat testis strongly expressed IFNα and IFNγ during infection with Sendai virus, and that elevation in expression was associated with an increase in testosterone production by Leydig cells infected with Sendai virus in vitro. This result highlights the fact that responses to some infections may exert a stimulatory effect on steroidogenesis within the testis, even though factors that are inhibitory to steroidogenesis are induced.[604]

The Eicosanoids

The eicosanoids are formed by oxidation of the 20-carbon fatty acid, arachidonic acid, and encompass the prostaglandins (PGD, PGE, and PGF), prostacyclins (PGI), thromboxanes (Tx), lipoxins, and leukotrienes (LT) (Figure 19.12). These lipids are signaling molecules, fundamentally involved in physiological processes, including inflammation and immunity. They arise from hydrolysis of arachidonic acid from membrane glycerophospholipids, through the action of phospholipase A_2 (PLA2).[605] The rate-limiting step in the conversion of arachidonic acid to the prostanoids is cyclooxygenation, catalyzed by one of two closely related enzymes, PTGS1 and PTGS2 (Table 19.2). While PTGS1 is constitutively expressed, PTGS2 expression is inducible during inflammation, through the IL1/TLR and TNF signaling pathways.[606] Production of the specific prostanoid classes, however, requires the subsequent actions of specific prostaglandin synthases and thromboxane synthase.[607] In turn, the prostaglandins and thromboxanes interact with their respective membrane-bound G protein-coupled receptors to regulate cell growth, vascular smooth muscle tone, vascular permeability, and immune cell activity.[608] Prostanoids exert both pro-inflammatory and immunosuppressive actions through these receptor

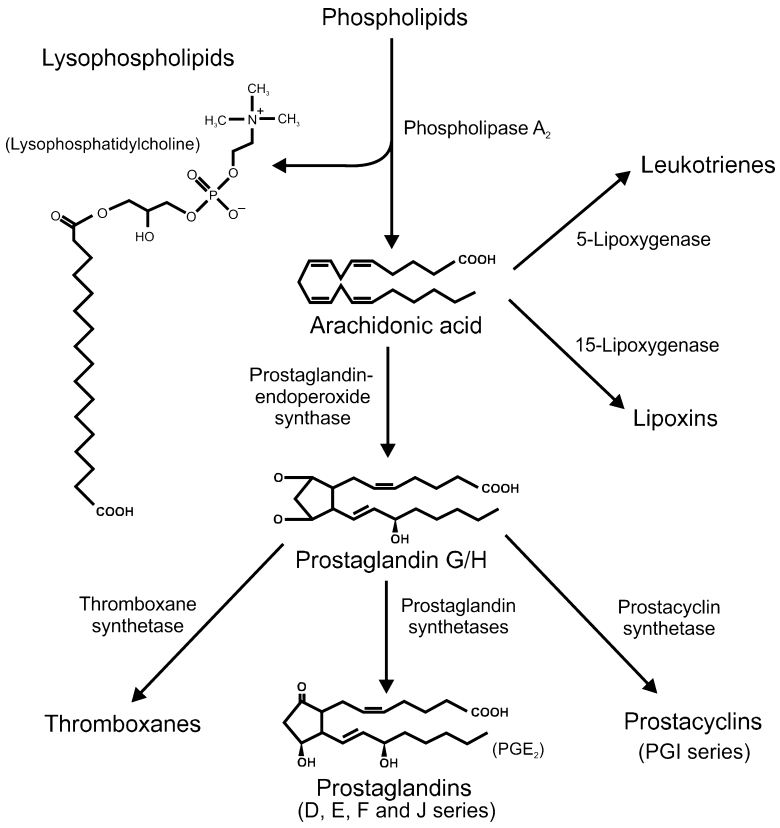

FIGURE 19.12 Synthesis of bioactive lipids. Cleavage of membrane-bound phospholipids by the action of phospholipase A_2 leads to production of arachidonic acid and lysophospholipids, such as the lysophosphatidylcholines. Arachidonic acid may be converted to prostaglandins, prostacyclins or thromboxanes via the intermediates prostaglandin G and H by the action of the rate-limiting prostaglandin-endoperoxide synthase (PTGS) enzymes. Alternatively, lipoxygenases may catalyze the conversion of arachidonic acid to leukotrienes or lipoxins. Most of the products in these complex pathways have specific regulatory activities, including profound effects on immune responses and the vasculature.

interactions, or through metabolism to other active immunoregulatory metabolites, such as the immunosuppressive ligand for peroxisome proliferator-activated receptor-γ (PPARγ), 15deoxy-PGJ_2.[609,610] An alternative synthetic pathway, catalyzed by the lipoxygenases, converts arachidonic acid to the closely-related leukotrienes and lipoxins, which likewise possess diverse metabolic, vascular, and inflammation-regulating activities.[178,611]

The enzymes responsible for prostanoid biosynthesis are expressed throughout the male reproductive tract.[612–615] Most testicular cells express both forms of PTGS and possess the capacity to produce prostaglandins in vitro,[615] although there are significant cell type-specific and species-specific differences in the relative levels of expression.[616–618] In the normal rat testis, PTGS2 is responsible for the majority of prostaglandin production, and chronic treatment with a specific PTGS2 inhibitor, celecoxib, considerably reduces intratesticular PGE_2 levels.[615,619] On the other hand, intratesticular PGE_2 levels are barely affected by acute LPS treatment.[615] This may be due to the fact that macrophages in the rat testis express PTGS2 at very low levels, relative to other testicular cells, but they are the only testis cell type to respond to an inflammatory stimulus with a significant increase in PTGS2 activity.[615] These data indicate a maintenance role for the so-called inducible PTGS2 enzyme in testicular function.

Prostaglandins, particularly PGE_2 and $PGF_{2\alpha}$, are clearly implicated in the control of Leydig cell development in the immature testis, production of pro-inflammatory cytokines by the Leydig cell, autoregulation of steroidogenesis in the adult, and the decline in Leydig cell function that occurs during aging.[401,620,621] However, the actual importance of prostanoids in regulating testicular function remains uncertain: male mice lacking either PTGS2 or PTGS1 are considered to be fertile,[622] and studies on the effects of prostaglandins or various PTGS inhibitors on spermatogenesis and fertility have produced conflicting outcomes.[618,619,623,624] Products of the lipoxygenase pathway, most notably LTB_4, have been implicated in the regulation of Leydig cell steroidogenesis by LH.[625,626] Furthermore, production of PGE_2 and $PGF_{2\alpha}$ induced by PTGS2 mediates the effects of IL1 on protein and lipid regulation by the Sertoli cell involved in supporting spermatogenesis and inflammation in the testis.[424,425] A relationship between expression levels of IL1β and PTGS2 in the testes of infertile men also has been reported.[627] These observations suggest that PTGS2 and prostanoid production mediate at least some of the effects of inflammation on testicular function.

Oxidative Enzymes, Nitric Oxide Synthases, and Reactive Oxygen Species

Reactive oxygen species are normal products of metabolism. They are produced continuously as byproducts of mitochondrial and microsomal electron transport

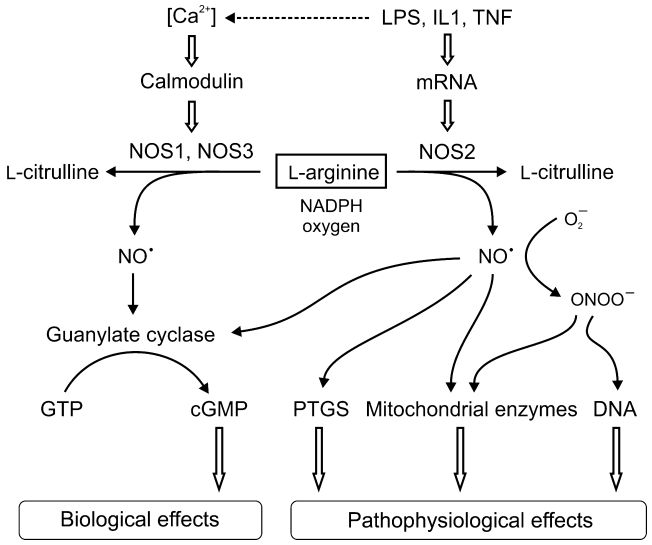

FIGURE 19.13 Regulation and actions of nitric oxide synthase (NOS) and NO production in normal physiology and pathophysiology. NO is produced by conversion of L-arginine to L-citrulline by NOS, and the freely diffusible NO radical (NO·) exerts a variety of physiological functions through regulation of cGMP-dependent protein kinases. The activity of the constitutively expressed isoforms, endothelial NOS (NOS3), and neuronal NOS (NOS1) are regulated through calcium-calmodulin. Synthesis of a constitutively active form of NOS (NOS2) is induced by inflammatory mediators and is responsible for the large upregulation of NO production during inflammation. High levels of NO· can be detrimental due to direct reaction with the heme group of prostaglandin-endoperoxide synthase (PTGS), mitochondrial enzymes and DNA, effects which can be potentiated by interaction with superoxide to produce the extremely reactive peroxynitrite anion. Peroxynitrite is the reaction product of NO and superoxide, and is one of the major cytotoxic agents produced during sepsis, inflammation and ischemia/reperfusion. Peroxynitrite decomposes to produce the hydroxyl radicals, which are potent oxidants.

reactions and other metabolic processes.[628] Cytochrome P450 enzymes, such as those that constitute the steroid synthetic pathways, produce ROS as a by-product of their catalytic mechanism.[629,630] However, rapid production of ROS as part of a respiratory burst by macrophages and neutrophils is also an important component of the early response to infection.[121,631]

The most important ROS are the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (HO·), nitric oxide (NO·), and peroxynitrite anion ($ONOO^-$)(Figure 19.13). During inflammation, their rapid production is stimulated by the activity of the enzymes NADPH oxidase and NOS2.[631,632] These small molecules are cytotoxic to microorganisms, although some play a more complex role in cell signaling processes, as well.[633] They also interact with vital intracellular macromolecules, such as proteins, lipids and DNA, to cause oxidative damage.[633,634] Cellular antioxidant systems that normally protect cells from oxidative damage include mitochondrial superoxide dismutase, catalase and glutathione peroxidases.[635] Excess production of ROS during oxidative stress, such as occurs during ischemia-reperfusion injury or excessive inflammation, can overwhelm these protective mechanisms, resulting in cell damage, and apoptosis or necrosis of affected cells, thereby contributing to the pathology of many diseases.[636]

At high levels, NO is extremely cytotoxic, especially as a precursor of the peroxynitrite anion, but at low levels NO acts as an intracellular and extracellular signaling molecule and has potent vasodilatory effects.[637] It can be produced by one of three related enzymes, neuronal NOS (NOS1), NOS2, and endothelial NOS (NOS3), which catalyze the conversion of L-arginine to L-citrulline and NO.[638] These enzymes are homodimeric proteins encoded by three separate genes. Both NOS1 and NOS3 are constitutively expressed enzymes whose activity is regulated through a calcium-calmodulin mediated mechanism, whereas NOS2 is a constitutively activated enzyme that is regulated at the transcriptional and translational levels by inflammatory mediators, including LPS, IL1, and TNF.[637]

Studies have reported the presence of NOS in Sertoli, Leydig, and peritubular cells, spermatogenic cells, testicular macrophages, and vascular endothelial cells of various species.[639–642] The NOS3 gene also produces a testis-specific isoform, TnNOS, which has been localized to Leydig cells.[643] In the normal rat seminiferous epithelium, NOS2 is expressed by elongating spermatids and pachytene spermatocytes, particularly during the stages immediately following sperm release, with relatively lower levels of expression in Sertoli cells and peritubular cells throughout the entire cycle.[641] Moreover, NOS2 is upregulated by LPS treatment in Leydig, Sertoli, peritubular, and spermatogenic cells, as well as a subset of the testicular macrophages.[276,641,644,645] This macrophage expression of NOS2 appears to be preferentially expressed by the infiltrating $CD163^-$ macrophages of the testis and is not detectable in the majority of resident macrophages.[276,641] Testicular cell NOS2 expression and NO production are also increased by inflammatory events induced by testicular ischemia-reperfusion injury or heat.[646–649]

Nitric oxide is an important regulator of spermatogenesis. Localized production of NO, by spermatogenic cells in particular, is implicated in control of the formation and disassembly of the Sertoli cell junctions that constitute the blood–testis barrier, as well as the junctional complexes involved in Sertoli-germ cell adhesion.[640] In transgenic mice lacking NOS2, pachytene and round spermatid apoptosis is significantly reduced, leading to an increase in daily sperm output and indicating a key role for NOS2 in limiting germ cell survival and/or the carrying capacity of the Sertoli cells.[647] Sertoli cell and Leydig cell numbers are also increased in these mice, indicating that developmental effects of NOS2 and NO are involved.[650] On the other hand, spermatogenic

damage caused by testicular torsion or cryptorchidism can be ameliorated by reducing NOS or NO levels.[649,651] Increased NOS2 is related to severity of testicular failure and mast cell numbers in infertile men.[320]

Treatment of murine Leydig cell primary cultures or MA-10 Leydig tumor cells with hydrogen peroxide causes oxidative damage, resulting in a marked reduction of the mitochondrial electrochemical gradient ($\Delta\Psi m$) and decreased STAR and HSD3β protein levels.[652–654] Elevation of endogenous ROS, particularly the superoxide anion and hydrogen peroxide, inhibits the activity of cytochrome P450 enzymes in the steroidogenic pathway, and plays a critical role in Leydig cell desensitization to hyperstimulation by LH or exogenous hCG,[629,630] which is used to treat delayed testicular descent in young boys.[655] In a similar manner, NO inhibits Leydig cell steroidogenesis directly, and treatment with NOS inhibitors counteracts the decrease in testosterone associated with inflammation or stress.[656–659] While this may involve oxidative damage via generation of reactive nitrogen species such as the peroxynitrite anion, it has been shown that NO inhibits steroid biosynthesis by binding reversibly to the heme group of cytochrome P450 enzymes.[656,660] Increased testosterone in NOS2-deficient mice may account for the developmental effects observed in these mice.[650] Moreover, NO is a potent vasodilator, and plays a role in the endogenous control of testicular blood flow and formation of the interstitial fluid, which also can impact upon Leydig cell function and spermatogenesis.[641,658]

Inflammatory Signaling Pathways and Testicular Function

It is now clear that inflammatory and immunoregulatory factors and pathways are constitutively active in the normal (noninflamed) testis, and that these factors and pathways have consequences for spermatogenesis and steroidogenesis. Clinically, there is a growing recognition that testicular failure, irrespective of etiology, correlates with increased inflammatory gene expression and immune cell infiltrates in infertile men.[4,5,22,23,229,318,320,372,432,627] *What is the actual physiological relevance of these experimental and clinical observations, and what might this mean for understanding the effects of infection, inflammation and immune responses on male fertility?* The physiological implications may be broadly separated into outcomes for effects on testicular steroidogenesis, which could lead to androgen deficiency problems, and more direct effects on spermatogenesis and sperm output.

Steroidogenesis

In terms of normal Leydig cell steroidogenesis, it may be anticipated that local production of cytokines, and small molecule inflammatory mediators, such as NO and prostanoids, will influence intratesticular androgen levels. These molecules may play a role in facilitating communication between cells of the seminiferous epithelium—the Sertoli cells, peritubular cells and spermatogenic cells—and the Leydig cell. Such paracrine actions have long been associated with local control of steroidogenesis, as a mechanism for modulating the response of the Leydig cell to stimulation by LH, in response to changing requirements of the seminiferous epithelium.[61,661] Moreover, since Leydig cells themselves produce these inflammatory regulators, they could be involved in autocrine regulation of steroidogenesis as well. Certainly, production of NO and other ROS, either by the Leydig cell itself or through its close physical relationship with the resident macrophages, is implicated in Leydig cell desensitization to stimulation by LH.[394,409,629,630] In terms of pathology, increased levels of inflammatory regulators, arising from either the circulation or the testis, during infection and inflammation, should similarly impact upon steroidogenesis.

Spermatogenesis and the Cycle of the Seminiferous Epithelium

Studies in the rat and mouse indicate that certain cytokines and inflammatory mediators are expressed in a dynamic manner throughout the spermatogenic process, in the complete absence of external drivers of inflammation. Specifically, there is a surge of production of ILα, IL6 and activin A by the Sertoli cell at the time of the release of mature spermatids from the seminiferous epithelium (spermiation), which is reflected in a concomitant increase in nuclear translocation of NFκB and production of TNF and NOS2 in the meiotic and postmeiotic spermatogenic cells.[373,375,384,452,461,509,572,573,641] This is a time when a number of critical physiological events occur within the seminiferous epithelium, including the phagocytosis of spermatid residual cytoplasm by the Sertoli cells, an increase in DNA synthesis associated with spermatogonial mitosis and the entry of preleptotene spermatocytes into meiosis, and the transition of these early spermatocytes through the tight junctions that normally constitute the blood–testis barrier.[18] The coincidence of these events is particularly significant because it has been established that ILα, IL6, and activin A are regulators of spermatogonial proliferation and differentiation,[382–384,416,509,579–581] while IL1α, activin A, TNF, and NO have all been shown to stimulate the opening of the blood–testis barrier by modifying the Sertoli cell cytoskeleton, inhibiting production of proteins involved in Sertoli–Sertoli and Sertoli-spermatogenic cell junctions and/or regulating protease and protease inhibitor activity.[423,484,583,640]

These observations suggest the existence of a fundamental mechanism, whereby the spermatogenic cells themselves drive inflammatory pathways within the seminiferous epithelium (Figure 19.14).[24,224] In this

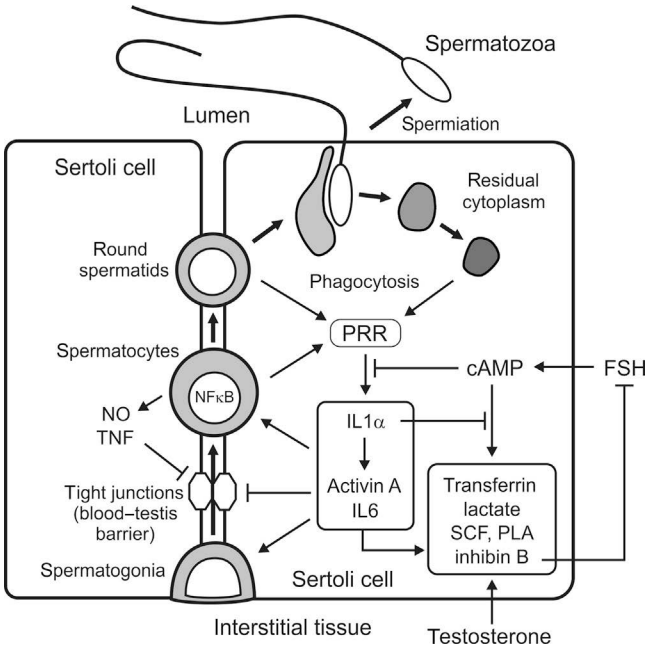

FIGURE 19.14 Hypothetical roles of inflammatory signaling pathways in control of spermatogenesis. Spermatogonia enter meiosis to become spermatocytes and pass through the tight junctions between adjacent Sertoli cells. At the end of meiosis, the resulting haploid spermatids undergo major structural differentiation and are released from the seminiferous epithelium as spermatozoa (spermiation), leaving behind most their (residual) cytoplasm, which is phagocytosed by the Sertoli cells. Spermiation coincides with a surge of production of inflammatory mediators, such as interleukin-1α (IL1α), IL6, and activin A by the Sertoli cells, and tumor necrosis factor (TNF) and NO by the spermatogenic cells, which regulate the proliferation and maturation of the nearby spermatogonia and spermatocytes, and reorganization of the tight junctions to allow spermatocytes to pass into the luminal compartment. This localized inflammatory response may be mediated through activation of pattern-recognition receptors (PRR) in the Sertoli cell by endogenous spermatogenic molecules. Degenerating spermatogenic cells may also drive these pathways at other stages of the spermatogenic cycle, and inflammation caused by infection will disrupt these processes. Inflammatory mediators also regulate the supportive functions of the Sertoli cells, such as the production of transferrin, lactate, stem cell factor (SCF), plasminogen activator (PLA), and inhibin B, which are stimulated by androgens and by FSH acting via cAMP. Although these separate signaling pathways control some functional outcomes in common, there is clear evidence for reciprocal inhibitory regulation between the signaling pathways as well.

model, phagocytosis of the residual cytoplasm at the time of spermiation stimulates inflammatory signaling and cytokine production in the Sertoli cells,[375,376,385,387,573] which subsequently induce NFκB nuclear translocation and inflammatory gene expression in the spermatogenic cells.[451,452,641] These induced inflammatory mediators, in turn, regulate the proliferation and differentiation of the spermatogonia and spermatocytes, and regulate the integrity of the tight junctions to allow the germ cells to pass through the blood–testis barrier. At the same time, these cytokines regulate the activity of the Sertoli cell and modulate the response of the cell to hormonal stimulation. This represents a feasible mechanism whereby the most recent generation of mature spermatozoa is able to communicate with and coordinate the activity of subsequent generations of spermatogenic cells.

In immunology, this type of inflammatory pathway activation is called sterile or non-pathogen-mediated inflammation, and similar mechanisms have been proposed for controlling activities in the epithelia of the intestine and lung, and in the vascular endothelium.[662–664] The actual trigger for this response in the Sertoli cell may involve the physical process of phagocytosis of the residual cytoplasm and activation of pattern-recognition receptors (PRR in Figure 19.14), such as the TLRs and the inflammasome.[108,117] These receptors are capable of detecting and responding to various endogenous intracellular ligands, including the nuclear protein, HMGB1, heat shock proteins, CpG-rich DNA and RNA, all of which are found in spermatogenic cells.[7,108,291,665]

Furthermore, some endogenously-produced inflammatory mediators, most notably IL1α, remain elevated within the Sertoli cell long after the spermiation event, which comprises only a brief period within the process of spermatogenesis.[373,375,384,452,461,502,509,641] This may be attributable to ongoing contact between the spermatogenic cells and Sertoli cell, mediated by pattern-recognition receptors on the cell surface, and/or by phagocytosis of degenerating cells, since many spermatogenic cells do not survive to complete spermatogenesis and are lost through apoptosis.[490,666] In support of this possibility, Zhang and colleagues have recently reported that damaged spermatogenic cells induce inflammatory gene expression, including IL1β, TNF, and IL6, in mouse Sertoli cells through activation of TLR2 and 4.[667] Such interactions may be driving other cyclical functions of the Sertoli cell, including the responsiveness of the Sertoli cell to FSH and androgen,[668,669] and the expression of regulators of cytokine activity, such as follistatin.[572,670]

The Significance of Inflammatory Signaling Pathways for Male Fertility

Studies on inflammatory processes in normal testicular function, or male reproductive function in general, should be tempered by two considerations. Firstly, because the mediators of inflammation and their signaling pathways are exquisitely sensitive to stimulation by microbial contamination, stress, or even the very act of cell isolation, it should not be assumed that expression levels are always indicative of a role in normal physiology. Many early studies of these processes may have been compromised by failure to eliminate endotoxin (endogenous LPS) contamination from the experiments, or the extreme sensitivity and nonquantitative nature of the detection methods used, such as RT-PCR. It is only when molecules have been repeatedly shown to be constitutively and substantially expressed in the absence of

exogenous inflammatory stimuli, and to have consistent and coherent effects on reproductive function in diverse studies, that we may assume that these molecules play a role over and above the usual response to infection or other immune activation events.

The second consideration lies in the fact that mice with transgenic deletions of many of these inflammatory and immunoregulatory factors, their synthetic enzymes or receptors, appear to be fertile.[671–674] However, this assessment may fail to take into account the fact that the reproductive phenotype is subtle, or even enhances fertility, as occurs in the NOS2-deficient mouse.[647,650] Sometimes, more careful examination of the reproductive tract and fertility reveals reproductive defects in male mice that are, nonetheless, capable of producing offspring.[264,435] Moreover, given the extraordinary complexity, diversity and redundancy of the immune system, effects may only manifest after compound deletions of multiple functions, or on certain genetic backgrounds. Some effects may not be evident under normal animal husbandry conditions, and may only become evident in older animals or in animals living in the wild exposed to environmental stresses, dietary deficiency or specific pathogens. Some effects may only become evident when other physiological processes are also compromised, such as occurs in male mice infected with *Taenia crassiceps*.[672] Wild-type mice with this infection display increased IL6 expression and become feminized due to excessive aromatase activity. This steroidogenic deviation is completely prevented in IL6 deficient mice, which otherwise have similar reproductive functions to those of wild-type mice. This observation is consistent with studies implicating IL6 as a regulator of aromatase activity,[675] but also provides an example of a reproductive knockout phenotype that only manifests itself following specific immunological events.

EFFECTS OF IMMUNE CELLS AND INFLAMMATION ON MALE REPRODUCTION

The Role of Macrophages in Leydig Cell Development and Steroidogenesis

Macrophages play an important role in Leydig cell development. During puberty, there is a close temporal link between the maturation of the adult Leydig cell population and the increasing number of testicular resident macrophages.[228,249,293] Moreover, specific depletion of the intratesticular macrophages inhibits the development of Leydig cells in immature rats, as well as the recovery of Leydig cells after EDS-treatment in adult rats.[259,261,262] The *op/op* mouse, which has an inactivating mutation in the CSF1 gene and consequently has reduced numbers of macrophages, also displays very poor fertility and reduced testosterone production due to developmental and steroidogenic abnormalities of the Leydig cells.[264,301] The influence of the testicular macrophages on Leydig cell growth and differentiation may involve specific macrophage-derived cytokines, such as IL1,[400] or the intercytoplasmic specializations between the two cell types, which appear very early in adult testicular development in the rat.[244]

Studies on the effects of resident macrophages on Leydig cell steroidogenesis under non-inflammatory conditions have produced inconsistent results. Stimulation of macrophage phagocytosis with an intratesticular injection of latex beads increased the steroidogenic capacity of Leydig cells in adult rats.[676] However, in macrophage-depleted adult rat testes, both a decline[263,273] and an increase[260,265] in testosterone production have been reported by different groups using essentially the same model. Moreover, medium collected from supposedly unstimulated testicular macrophages in culture have been shown to both stimulate[677,678] and inhibit[405,408,678] Leydig cell steroidogenesis in vitro. Similar discrepancies were observed in Leydig-macrophage co-cultures. The most likely explanation for these differences is that partial activation of the macrophages during isolation, or possibly endotoxin contamination, may have affected the results. Neuroendocrine influences also may be important; for example, studies of the bank vole revealed that testosterone production by Leydig cells from long photoperiod animals was more sensitive to stimulation by testicular macrophage conditioned medium and more sensitive to IL1α-mediated inhibition in comparison with short photoperiod animals.[679] On balance, however, the published data seem to indicate that resting testicular macrophages have a positive or trophic effect on Leydig cell steroidogenesis.

Testicular macrophages have been shown to express the cholesterol 25-hydroxylase enzyme and produce 25-hydroxycholesterol.[677] It has been proposed that testicular macrophages, due to their close association with the Leydig cells, provide 25-hydroxycholesterol as a substrate for testosterone biosynthesis, by-passing the need for STAR and supporting basal steroidogenesis. Interestingly, the 25-hydroxylase enzyme is negatively regulated by testosterone suggesting that there may be a feedback loop between the macrophages and Leydig cells.[680,681] Furthermore, 25-hydroxycholesterol has been suggested to be one of the factors which macrophages secrete in support of Leydig cell proliferation and development.[682]

Effects of Inflammation on the Leydig Cell and Steroidogenesis

Reduced testosterone production is an important mechanism underlying the inhibition of male fertility associated with inflammatory disease, regardless of whether or not infection is involved. This can involve inhibitory

effects at multiple levels of the hypothalamic-pituitary-testicular axis. Men with critical illness, burn trauma, sepsis, and rheumatoid arthritis have elevated serum TNF and IL1 levels and reduced testosterone.[367,683–685] Similar decreases in gonadal function have been reproduced in experimental animal models of chronic inflammation and systemic immune activation. For example, experimental adjuvant-induced arthritis causes a dramatic reduction in serum testosterone levels in rats,[686,687] and conditioned medium from testicular macrophages isolated from adjuvant-induced arthritic rats is inhibitory to Leydig cell testosterone production in vitro.[687]

The literature contains numerous experimental models that have been used to study the effects of inflammation on testicular function, although interpretation of the results can often be problematic. In general, studies employing bacterial or viral infections are complicated by the difficulties in distinguishing the effects of inflammation from the actions of the pathogen itself, or the subsequent immune response to the pathogen. Furthermore, although inflammation is characteristically inhibitory to Leydig cells steroidogenesis, increases in androgen production may be observed in some models of infection.[592,604] Likewise, experimental models involving active immunization, spontaneous autoimmunity, vasectomy, or tumors suffer from similar complications, where it is difficult to separate the inflammatory response from the immune response. More precise information can be obtained from studies using LPS, other TLR ligands, or inflammatory cytokines, to induce inflammation in the absence of a specific pathogen or antigen.

Lipopolysaccharide-Induced and Cytokine-Induced Inflammation Models

Intraperitoneal or intravenous injection of LPS in rats, mice, and rams or induction of sepsis with cecal matter in rats is followed by a significant decrease in intratesticular and serum testosterone levels.[322,658,688–691] There have been distinct differences reported in the dynamics and severity of this inhibition, from study to study and between different species. Responses in mice generally appear to be more rapid, larger, and more prolonged than those of rats, which showed a biphasic response at approximately 6 and 24 h after treatment with a brief recovery period in between.[322,393,689,690,692] Serum testosterone levels actually appear to rise in the boar following endotoxin treatment, as a result of increases in pulsatile LH secretion.[693–695] However, one should not conclude that all differences in experimental results obtained using LPS are simply due to differences between animal models. The LPS obtained from different bacterial strains, and even from different batches of the same strain, can have quite different chemical composition and potency, and is usually contaminated to varying degrees by other TLR ligands.[447]

What is evident is that systemic administration of LPS exerts predominantly inhibitory effects on Leydig cell steroidogenesis at the testicular level and at the hypothalamic-pituitary level, and inhibition may also involve peripheral responses to inflammation, such as corticosteroid production (Figure 19.8). In the rat model, suppression of steroidogenesis by LPS across a very broad range of doses was found to be due to inhibition of both LH secretion and a reduced capacity of the Leydig cells to produce testosterone in response to LH-stimulation.[322,393,696] Furthermore, the data indicated that the early phase of suppression of testosterone was due primarily to direct inhibition of Leydig cell steroidogenesis, whereas the later phase involved extratesticular mechanisms, including glucocorticoid-induced inhibition.[322,393]

Similar inhibitory effects to those caused by LPS on testicular steroidogenesis and serum testosterone levels can be induced in male rats by infusion of TNF alone.[465,466] In the human, Van der Poll and colleagues injected six healthy men with recombinant TNF and measured serum concentrations of gonadotropins, testosterone, and sex hormone-binding globulin (SHBG).[697] The TNF induced an early and transient increase in serum LH, whereas the concentrations of FSH remained unchanged. The increase in LH concentrations was followed by a transient decrease in serum testosterone levels after 4 h. LH had returned to control values when the testosterone levels reached a nadir, and SHBG was not affected. The results indicate that TNF affects the hypothalamic-pituitary-testicular axis at multiple levels in normal men.[697] In a similar study, serum testosterone was decreased without significant effects on LH levels in men treated with high doses of IL6.[698] In yet another study, male patients treated with high doses of IL2 as therapy for metastatic cancer were found to have significantly reduced serum testosterone levels.[699] Finally, multiple inflammatory markers, including IL1β, TNF, and IL6, are elevated in obese men and men with type 2 diabetes,[700,701] conditions known to be associated with hypogonadism and decreased circulating testosterone levels. Collectively, these experimental and clinical observations predict that in conditions characterized by elevated inflammatory mediators, in the absence of infection, there is a concomitant decrease in serum testosterone levels.

Effects of Inflammation on Leydig Cell Steroidogenesis at the Testicular Level

Most evidence suggests that the effects of LPS-induced inflammation on the Leydig cell itself are mediated through exposure to increased levels of pro-inflammatory molecules that inhibit steroidogenesis, most notably IL1, TNF, NO, and other ROS, and PGE_2, as outlined earlier (Figure 19.3). Although isolated Leydig cells express the LPS receptor subunits at relatively low levels,[388] and

there is evidence that they respond to LPS by increased expression of inflammatory cytokines, including IL1β and TNF,[431,450] it is extremely difficult to completely eliminate macrophages from Leydig cell preparations. Consequently, it remains unclear whether LPS can regulate Leydig cell steroidogenesis by direct action. Regardless of this uncertainty, there are several significant sources of pro-inflammatory molecules within the testis. The testicular macrophages produce IL1, TNF, NO, and other ROS, and PGE_2 when stimulated by LPS, although their production capacity is reduced compared with macrophages from other sites.[243,269,270,274,276,277,394,395,464,615,645,702] Sertoli cells, peritubular cells, and spermatogenic cells are also potential sources of these pro-inflammatory molecules.[369,381,461,615,641] The role of NO and other ROS may be particularly important: treatment of adult mice with LPS causes oxidative damage to Leydig cells, which manifests as a marked reduction of the mitochondrial electrochemical gradient, decreased STAR and HSD3β protein levels, and a fall in serum testosterone, similar to the effects of oxidation by hydrogen peroxide in cultured Leydig cells.[652–654] Moreover, treatment of rodents with NOS inhibitors counteracts the decrease in serum testosterone levels caused by stress and sepsis.[657,658] Curiously, pretreatment of adult rats with the PTGS2 inhibitor, celecoxib, reduced endogenous intratesticular PGE_2 levels, and partially reversed the inhibition of testosterone in response to LPS, without blocking the increased expression of IL1β, TNF, or NOS2 in the testis.[619]

An increase in peripheral levels of cytokines from the circulation due to activation of macrophages and other immune cells in the blood and tissues may also be involved in inhibiting Leydig cell steroidogenesis during LPS-induced inflammation. This may involve the same pro-inflammatory molecules that are produced in the testis following LPS treatment, but potentially other cytokines as well. For example, IL2, which is an autocrine T cell growth factor, inhibits gonadotropin-stimulated testosterone production by rat Leydig cells at the level of CYP17A,[703] but IL2 also stimulates IL1, TNF, and IFNγ.[704,705]

The Neural-Immuno-Endocrine Axis in Control of Testicular Steroidogenesis

In addition to direct effects of inflammatory mediators on the Leydig cells, testicular steroidogenesis is modulated during inflammation by neuroendocrine and neuroimmunological regulatory networks (Figure 19.8). These include the hypothalamic-pituitary-adrenal axis, central control of gonadotropin secretion from the anterior pituitary and neural inputs into the testis that regulate Leydig cell function by direct action or through changes in the testicular vasculature.

Activation of the hypothalamic-pituitary-adrenal axis and resulting production of corticosteroids are the key elements of the normal stress response that, among other things, modulates and limits the severity of the inflammatory response.[124,174–177] Moreover, corticosteroids inhibit LH and FSH secretion from the pituitary,[706] but also regulate steroidogenesis directly via specific receptors expressed on the Leydig cell surface,[255,707] acting principally through suppression of the critical steroidogenic enzymes, CYP11A, HSD3β and CYP17A, and induction of Leydig cell apoptosis.[192,708,709] The response of the Leydig cells to corticosteroids is modulated by the actions of corticosteroid 11β-dehydrogenase (HSD11β), and inhibition of Leydig cell function occurs when glucocorticoid levels exceed the capacity of this enzyme to metabolize them.[192] These mechanisms are activated, and impact upon testicular steroidogenesis, in LPS-induced inflammation.[393] Hypothalamic corticotropin-releasing hormone (CRH) also inhibits gonadotropin secretion during stress and inflammatory responses.[710]

Inflammatory mediators themselves can directly act upon the hypothalamic-pituitary unit to inhibit reproduction. Several studies have shown that LPS, IL1, IL6, and TNF can inhibit GnRH release and/or LH secretion at the level of the hypothalamus and pituitary,[711–714] although NO appears to have the opposite effect.[715,716] Furthermore, the observation that IL1 administered intracerebrally can reduce Leydig cell testosterone responses in the absence of decreased LH secretion, for example, in rats pretreated with a GnRH antagonist and therefore lacking LH secretion, led Rivier and colleagues to postulate that there is a direct neuronal connection between the brain and the testis that is activated by cytokines and other stressors within the central nervous system.[717,718] Injection of pseudorabies virus, a transganglionic retrograde tracer, into the testes labeled the spinal cord, the brain stem, and the hypothalamus, while spinal cord injury significantly reduced this staining, and abolished the ability of either IL1β or CRH administered centrally to inhibit testosterone responses to hCG.[719,720] Studies indicate that these agents stimulate a neural pathway within the brain, possibly involving central catecholamine pathways and NO, which suppresses Leydig cell function by inhibiting cholesterol transport protein levels, independent of effects on the pituitary.[720,721]

Furthermore, there is considerable evidence for the control of steroidogenesis by conventional neural pathways acting either directly on the Leydig cells or on the testicular vasculature. Leydig cell steroidogenesis and circulating androgen levels are extremely sensitive to reductions in testicular blood flow, as may occur during inflammation.[658,722] Moreover, Leydig cells can be directly inhibited by a number of neurotransmitters, including serotonin and the catecholamines and the testis is well-supplied by both catecholinergic and serotonergic innervation.[723–726] Activation of these neural pathways during inflammation, or alterations in other

neural pathways supplying the testis, may contribute to the overall suppression of Leydig cell steroidogenesis.

Vascular Inflammation and Leydig Cell Function

While changes in testicular blood flow affect Leydig cell function and steroidogenic output, disruption of blood flow can be a source of inflammation as well. Torsion of the spermatic cord, or experimental clamping of the testicular artery in laboratory rodents, renders the testis ischemic, and subsequent reperfusion causes an increase in oxidative stress and intratesticular inflammation, as indicated by increased intratesticular IL1β and TNF expression, activation of NFκB and stress-related kinase signaling pathways in the Sertoli cells, and neutrophil recruitment, resulting in decreased testosterone production and spermatogenesis.[82,457,727,728] Local increases in reactive oxygen and nitrogen species are also implicated in the suppression of Leydig cell steroidogenesis in this model.[648,729,730] The degree of damage (ischemia-reperfusion injury) is related to the duration of the torsion and resulting stress, and Leydig cell function is compromised in the long term.[82] Similar effects on Leydig cell steroidogenesis can be observed after treatment of rats with other vasoactive inflammatory mediators, such as serotonin and histamine.[731,732]

Effects of Inflammation on Spermatogenesis

Spermatogenesis is affected by inflammation through direct interactions with the inflammatory process and indirectly through alterations in hormone levels and the testicular vasculature. Specifically, this section is concerned with the effects of innate immune responses on Sertoli cell function and spermatogenesis, as distinct from antigen-specific reactions to the cells of the seminiferous epithelium.

Lipopolysaccharide-Induced Inflammation and Spermatogenesis

Acute systemic administration of LPS has been found to affect spermatogenesis in a number of species, resulting in apoptosis and progressive loss of spermatogenesis in the testis within days of administration, and reduced sperm concentration, motility, and viability for extended periods afterward.[322,499,688,693,694,696,733] In the adult rat, administration of a single dose of LPS across a very broad range of sublethal concentrations inhibited the formation of testicular interstitial fluid and suppressed intratesticular and circulating levels of testosterone, by inhibiting LH secretion from the anterior pituitary and reducing the capacity of the Leydig cells to produce testosterone in response to stimulation.[24,322,393,696] At the highest doses of LPS, this was followed by apoptosis of spermatogonia and spermatocytes, and premature release of spermatocytes and early round spermatids from the epithelium several days later.[322] Detailed stereological analysis established that the effects on spermatogenesis were quite specific.[696] At 24 h after treatment, there was a significant delay of spermatocyte development at the leptotene/zygotene phase of meiosis, followed within 6 days by premature release of these cells and the adjacent, but more luminally located, generation of round spermatids. There was also an increase in apoptosis of nearby spermatocytes and spermatids within the same timeframe.[696] These effects occurred more rapidly and were quite different from the effects of androgen withdrawal, which inhibits the release of mature spermatids into the tubule lumen and the integrity of the junctions between the Sertoli cells and midphase round spermatids.[734,735] In fact, lower doses of LPS caused a similar degree of inhibition of intratesticular testosterone levels to that caused by high dose LPS, without causing significant spermatogenic damage.[24,322] Moreover, even very high doses of LPS did not reduce intratesticular levels of testosterone in the rat much below 30% of control, well above the threshold necessary to sustain spermatogenesis in this species.[79,80,734]

Although there are elements of the LPS-mediated response that resemble damage caused by testicular heating, in fact, rodents have a very poor fever response to LPS and the doses of LPS that affect spermatogenesis in rats actually caused a fall in body temperature.[24,322,736] Nor was the pattern of spermatogenic degeneration consistent with vascular disruption. Administration of LPS did appear to alter blood flow through the rat testis, possibly as a result of vasodilation of the testicular arteries due to increased testicular NOS2 expression,[322,641] but there was no evidence of ischemia or ischemic damage.[276,322] Paradoxically, while LPS treatment caused an increase in vascular endothelial cell leakage in the testis, the inflammation was not accompanied by edema; in fact, interstitial fluid volume in the testis fell quite dramatically in this model.[322]

A primary cause of damage to spermatogenesis in LPS-induced inflammation could be the action of pro-inflammatory regulators on the seminiferous epithelium itself. This might be the result of increased levels of circulating molecules as well as their local production by intratesticular immune cells and somatic cells. In addition to the resident macrophage population, LPS stimulates an increase in intratesticular monocytes in the rat,[276,285] and neutrophils in the boar testis interstitium.[694,695] In vivo, LPS treatment upregulates testicular expression of pro-inflammatory genes, including CCL2, IL1β, TNF, IL6, and NOS2.[274,276,285,322,393,396,619,641,691,737–739] These molecules are constitutively produced by testicular macrophages, Sertoli cells, peritubular cells, Leydig cells, and/or spermatogenic cells, and their production can be stimulated by LPS in most, if not all, of these cells in vitro. Most importantly, these molecules are involved

in normal spermatogenic function, with direct and complex effects on spermatogonial and spermatocyte development, the tight junctions of the blood–testis barrier, and local immune cell activity, mediated via inflammatory signaling pathways within the seminiferous epithelium (Figure 19.14). Moreover, FAS and FASL, which have been implicated in regulating spermatogenic cell apoptosis under normal and pathological conditions, are also increased in the seminiferous epithelium of the LPS-treated mouse.[499] Overexpression of these regulators and universal activation of inflammatory signaling pathways in the testis, induced by LPS, would disrupt the normal regulatory processes underlying spermatogenesis. Critically, as most of the normal functions of the seminiferous epithelium are dependent upon androgen support, these effects of inflammatory disturbances could be exacerbated by the concomitant reduction in testosterone levels within the testis.

The severity of the LPS-induced inflammation appears to influence the resulting pattern of spermatogenic damage. In some studies in rats, high doses of LPS are associated with rapid and pronounced epithelial damage, and focal spermatogonial/spermatocyte apoptosis, within 3 days after LPS administration.[24,322] Studies have demonstrated an association between these earlier, more severe, damage events and testicular oxidative stress responses,[691,740] and anti-oxidants can have a protective effect on testicular damage responses to LPS in vitro and in vivo.[738,741,742] Responses include the induction of the stress proteins, heat shock protein 60 (HSP-60), HMGB1 and HMGB2, as well as increased lipid peroxidation, reduced antioxidant activities, mitochondrial dysfunction and spermatogenic cell apoptosis. Several of these induced molecules can activate TLR signaling as well,[24,108] potentially mediating additional damage.

In summary, the damaging effects of LPS-induced inflammation on spermatogenesis involve multiple mechanisms related to the severity of the stimulus and inflammatory response. These can include direct or indirect inhibition of Leydig cell function, vascular disruption and oxidative stress, as well as interference with the endogenous inflammatory pathways involved in normal regulation of the seminiferous epithelium (Figure 19.14). Differences in damage susceptibility may be attributable to actual differences in sensitivity to LPS at the cellular level, or the probability that circulating LPS may have variable access to the various compartments of the testis. For example, Sertoli cells are much less sensitive to stimulation by LPS than are testicular macrophages, possibly due to differences in expression of the TLR4 accessory protein, CD14.[388] Moreover, higher concentrations may be required to permit sufficient LPS to traverse the vascular and lymphatic endothelium of the interstitial tissue as well as the peritubular basement membrane and cell layers to directly affect seminiferous epithelial function. As would be expected, direct injection of LPS into the mouse seminiferous tubules, via the efferent ducts, caused extensive spermatogenic disruption,[743] but there have been no quantitative, comparative studies of the effects of systemic and intratesticular routes of administration to date.

Vascular Inflammation Models

Whereas LPS causes inflammation by direct activation, there are a number of clinical and experimental models of testicular inflammation that are secondary to vascular disruption; these include ischemia-reperfusion injury, vascular malformations (e.g. varicocele), heat or cryptorchidism, and hyperstimulation of the Leydig cells with LH/hCG. These models display similarities, but also some significant differences, to LPS-induced inflammation.

Experimental ischemia-reperfusion injury causes rapid apoptosis of the spermatogenic cells entering mitosis—spermatogonia and the very early spermatocytes—followed by progressive depopulation of the seminiferous epithelium.[744,745] Damage is proportional to the duration of ischemia and, when the procedure is performed unilaterally, the contralateral testis is also affected, which has been attributed to an autoimmune response or to reflex vasoconstriction via neural pathways.[746–750] Although Leydig cell function is compromised, this is not the primary cause of damage in this model.[82] Intratesticular inflammation and oxidative stress are implicated, however, because there is activation of NFκB, increased MAP kinase activity and production of IL1β and TNF in the affected testis, together with increased local ROS production and an increase in intratesticular immune cells.[457,727,729,751,752] Accordingly, treatment with antioxidants and anti-inflammatories can reduce the resulting spermatogenic damage.[753,754] The role of NO is less clear, because this molecule has both protective and damaging effects in ischemia-reperfusion injury models, and its impact on the testis may depend on the severity of the ischemic insult.[648,649,755] The most characteristic feature of experimental ischemia-reperfusion injury is a large increase in neutrophils in the testicular subcapsular venules of both the treated and untreated testis, and testicular damage can be reduced or prevented by blocking neutrophil recruitment or activity.[727,729,756–758] Thus, ischemia-reperfusion injury to spermatogenesis is an inflammatory event mediated primarily by infiltrating neutrophils, with altered blood flow and oxidative stress leading to apoptosis of early spermatogenic cells, in particular.

A varicocele is a dilation of the testicular veins and swelling of the pampiniform venous plexus, and is a cause of altered testicular blood flow, frequently observed in male infertility patients.[759] In both humans

and experimental animals, varicocele is associated with increased intratesticular NO levels and oxidative stress, an increase in intratesticular inflammatory markers, including IL1, and reduced spermatogenesis.[760–764] These observations clearly indicate that this apparently minor vascular anomaly can be a cause of inflammation and damage in the testis, although its clinical significance and the benefits of treatment are a source of ongoing discussion.

Administration of high doses of hCG is used in the treatment of delayed testicular descent in young boys and its impact on testicular function has been extensively studied in adult rats.[655] This treatment causes a hyperstimulation syndrome comprising a transient decrease in testicular blood flow, immediately followed by increased testicular blood flow and pressure, opening of the vascular endothelial cell junctions, and an increase in testicular interstitial fluid volume some 16–24 h later.[765] The syndrome is accompanied by accumulation of intravascular and interstitial neutrophils in the testis,[766,767] an increase in intratesticular expression of IL1β and other pro-inflammatory cytokines,[768] failure of mature sperm release, vacuolation of the seminiferous epithelium and apoptosis, and loss of spermatogonia and primary spermatocytes.[676,769] In rats, the response to hCG can be eliminated by depletion of the Leydig cells with EDS,[770] or depletion of neutrophils with a specific antiserum,[766] and exogenous IL1β is able to replicate most of the effects of hCG.[391,771] These observations suggest that this is an inflammatory response, possibly mediated via IL1β secreted by the Leydig cells. Interestingly, the vascular response is quite different to that of LPS-induced inflammation, which causes a massive reduction in interstitial fluid volume in the testis,[322] although some of the germ cell damage seen is similar in both models. It is significant that disruption of the seminiferous epithelium in both the hCG-hyperstimulation rat model and the ischemia-reperfusion model appear to be dependent upon recruitment of neutrophils to the testicular vasculature or interstitial tissue. Equally significant is the observation that both depletion of the testicular macrophages using liposome-encapsulated dichloromethylene diphosphonate[273] or stimulation of the macrophages with latex beads[676] exacerbate the effects of hCG on the testis, suggesting that the resident macrophages normally play a role in limiting the inflammatory response.

In general, there is a close relationship between vascular disturbance in the testis and inflammation, production of NO, and oxidative stress, leading to spermatogenic damage. These same processes are also implicated in other models of testicular damage, such as the response to heat or cryptorchidism, irradiation, and posttesticular obstruction events.[646,651,772,773] While Leydig cell function is frequently compromised in models of testicular inflammation, reduction of androgens is not the primary cause of spermatogenic failure. It is clear that inflammation has direct inhibitory effects on one or more stages of spermatogenic cell development. It also should be pointed out that, as in most other tissues, inflammatory responses in the testis do not appear to automatically produce autoimmune complications. However, inflammation can lead to increased fibrosis in the testis, presumably through increased production of pro-fibrotic cytokines, such as TNF, TGFβ and activin A, and increased testicular fibrosis is associated with spermatogenic failure and infertility.[318,774,775]

Inflammation in the Epididymis, Vas Deferens, and Accessory Glands

In comparison with the testis, there have been relatively few studies on inflammatory pathways or the effects of inflammation within the remainder of the male reproductive tract. These tissues display a high degree of regional, or segmental, specialization, corresponding to differential gene expression, leukocyte populations, and physiological functions.

Production and Actions of Inflammatory Mediators

The TLRs are found throughout the epithelium, and in immune cells, of the posttesticular reproductive tract.[109,441–444,776] Studies in the rat and mouse have produced some conflicting data, but overall indicate that expression levels of TLR1-6 in the epididymis are similar to those found in the testis, with expression progressively declining toward the vas deferens. On the other hand, expression of TLRs 7, 9, and 11 tends to be higher in the epididymis and vas deferens than in the testis (Table 19.4). This may have an influence on bacterial and viral pathogenicity in the different regions of the tract. The crucial TLR adaptor proteins, MYD88 and TICAM1, have been detected in all segments of the rat epididymis and vas deferens,[441] and the TLR4 co-receptor CD14 has been localized to the epididymis in the rat and human, where it appears to be more highly expressed than in the testis.[443,777]

All 10 human TLRs, as well as MYD88, TICAM1, and CD14, have been detected in human prostate samples.[778] The TLR2 and 4 proteins were localized to epithelial cells in the rat prostate,[779–781] and TLR2 and 9 have been shown to be expressed by human prostate cancer cell lines.[782,783] In the human prostate, TLR3 and 8 immunoreactivity was observed on the apical surface of luminal epithelial cells, and epithelial cells exhibited TLR9 in the penile urethra.[784] In rat seminal vesicles, mRNA for TLR2, 5, 6, 7, 8, 9, 10, and 11 were highly expressed, with low levels of the remaining TLRs.[776] Notably, expression of CD14 was higher in the prostate and seminal vesicles of the rat than in any other tissue of the male reproductive tract.[443] Studies have shown that sequence variants

in TLR4 and the TLR1-TLR6-TLR10 gene cluster in the human are associated with an increased risk of prostate cancer, suggesting a link between susceptibility to inflammation and tumorigenesis in this organ.[785,786]

Roles for TLR signaling in controlling functions of the epididymis and vas deferens, similar to those proposed for the testis, can be anticipated. The epithelium of the caput epididymis comprises numerous functionally active immune cells, particularly dendritic cells and macrophages, but also $CD8^+$ T cells.[96–98,102] This is consistent with the relatively high expression of TLRs in this region of the epididymis. In the regions of the epididymis closest to the testis, moreover, epithelial cell apoptosis is inhibited by repression of NFκB and STAT signaling and activation of the MAPK3/MAPK1 pathway, under the control of androgens, cytokines, or other secretions from the testis.[787,788] These pathways are normally regulated by pathways involved in TLR signaling, and the relationship between the TLRs and testicular factors in their control requires elucidation. Recently, it was discovered that activin A is highly expressed in the epithelium of the caput epididymis.[789–791] This expression is likely to be driven by TLR signaling, and because activin A is a critical regulator of macrophage, dendritic cell and T cell functions, one can assume that this cytokine influences immune cell activity and inflammation in the proximal epididymis. However, other functions of activin A also may be anticipated. In transgenic mice that overexpress the activin-binding protein, follistatin, fluid resorption and sperm transport in the efferent ducts and proximal epididymis is impaired, suggesting a role for endogenous activin in the control of fluid dynamics.[792] Furthermore, activin A has been shown to induce SRD5α in genital fibroblasts,[793] and the caput epididymis is the site of highest expression of this enzyme, indicating that activin A may regulate androgen activity in the epididymis.

In the more distal epididymis (corpus and cauda) and passing into the vas deferens, the pattern of cytokine and inflammatory regulator activity is different to that found in the caput epididymis (Figure 19.15). Activin A production progressively declines, while local production of follistatin increases to the point where follistatin expression levels are higher in the murine vas deferens than in any other tissue examined.[537,789,791,794] These observations suggest that controlling activin activity in the distal epididymis and vas deferens is functionally important. Significantly, the progressive reduction in activin bioactivity parallels the reduction in immune cell number and activity in the epithelium. Expression of TGFβ1 also appears to be greatest in the caput of the marmoset and mouse,[794,795] but displays a more distal distribution in the rat.[794,796,797] Furthermore, TGFβ3 was most highly expressed in the corpus of the rat epididymis, but TGFβ2 was not detected.[797] Removal of the testis or inhibition

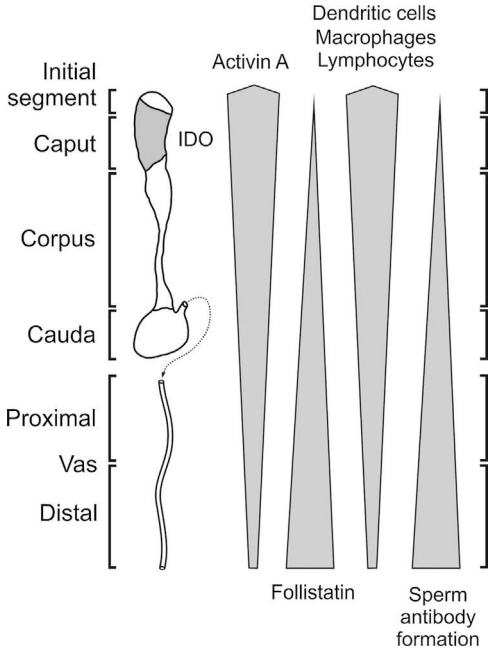

FIGURE 19.15 Region-specific immunity in the epididymis and vas deferens. Activin A and its binding protein, follistatin, are inversely distributed in a distinctive proximal-to-distal pattern, with highest levels of activin A expression in the caput, and highest follistatin expression in the distal vas. Elevated production of activin A coincides with increased numbers and apparent activity of intra-epithelial immune cells, most notably dendritic cells, but also macrophages and $CD8^+$ T cells. The immunoregulatory enzyme, indoleamine 2,3-dioxygenase (IDO), is highly expressed in the caput, with the exception of the region directly adjacent to the testis (initial segment). Susceptibility to sperm antibody formation following damage to the reproductive tract increases in a proximal-to-distal pattern, indicating a progressive decline in local immunoregulatory mechanisms.

of SRD5α activity increased expression of both TGFβ1 and TGFβ3, particularly in the caput.[796,798] These studies indicate species-specific differences in TGFβ expression that are, most likely, androgen-regulated. Functional roles for TGFβ in the epididymis or vas deferens have yet to be established, but a potential role for TGFβ1 in regulating tight junction formation between epithelial cells from the vas deferens has been demonstrated.[799] In direct contrast with activin A and TGFβ, NOS enzyme activity and PTGS2 expression are much greater in the cauda epididymis and vas deferens than in the more proximal regions of the epididymis.[614,616,800,801]

The data indicate that sperm are exposed to a dynamically changing immunoregulatory environment regulated by local inflammatory signaling pathways in the epithelial cells of the male reproductive tract. Moreover, there is evidence that the sperm themselves respond directly to inflammatory stimuli. Human sperm express TLR receptor proteins and respond to bacterial TLR2 and 4 ligands with reduced motility and increased apoptosis.[442,802,803] TNF has been shown to regulate sperm membrane lipid peroxidation and NO production, and

inhibit sperm viability and/or motility,[804–806] and MAP kinase signaling pathways in sperm are implicated in controlling motility, capacitation, and the acrosome reaction.[807]

Effects of Inflammation

There have been limited studies on the effects of inflammation, as distinct from infection, on cellular and molecular responses of the epididymis, vas deferens, or accessory glands. In rats, systemic administration of LPS induced activation of NFκB in the epididymis and increased several inflammation-responsive genes, including IL1β, although it was not clear that this was a response of the epididymal epithelium, since TLR4 expression was also localized to macrophages in the stroma.[443] In a similar study, no immediate morphological changes to the epididymis, at least at the light microscope level, were observed.[322] Injection of LPS directly into the caput epididymis of the rat, however, caused an increase in IL1β expression, as well as hyperemia, edema, and accumulation of immune cells in the interstitium, along with inflammatory damage to the epididymal epithelium, reduced sperm motility and decreased expression of several β-defensins.[808] Similarly, retrograde inoculation via the vas deferens using *E. coli* as a source of LPS caused a neutrophilic infiltration and edema in the cauda epididymis and proximal vas deferens, and elevated expression of IL1α, IL1β and IL4, although several other cytokines, including IL6 and TNF, were not significantly altered three days later.[809] The IL1 receptor has been detected in the murine epididymal epithelium, indicating the ability to respond directly to this fundamental pro-inflammatory cytokine.[415] An experimental left varicocele in the rat significantly reduced epididymal weight and diameter of the duct, together with increased apoptosis of principal cells, proportional to the duration of the varicocele.[810] Chronic treatment of mice with the anti-inflammatory PTGS2 inhibitor, nimesulide, caused epithelial cell damage and apoptosis in the vas deferens, and a large reduction in sperm motility, but not sperm count or viability.[811]

Treatment with LPS of the murine caput epididymal epithelial cell line PC1, which expresses TLR4, induced NFκB activation and expression of the pattern-recognition receptor for bacterial peptidoglycan, nucleotide-binding oligomerization domain 2 (NOD2).[812] Sequential stimulation with LPS and the peptidoglycan muramyl dipeptide enhanced TNF mRNA expression in these cells. Infection of rat cauda epididymal epithelial cells in culture with *Staphylococcus aureus* activated NFκB and MAPK14, production of IL1β, TNF and NO, and expression of mRNA for TLR2 and NOS2.[444] Similarly, treatment with LPS and IFNγ together stimulated NOS2 expression and NO production by cultured rat epididymal epithelial cells.[813] The epithelial cells of the prostate and seminal vesicles also respond to inflammation induced by LPS or bacteria, with activation of NFκB, increased TLR receptor expression, production of inflammatory mediators, such as NO, and various chemokines, resulting in immune cell infiltration in vivo.[103,779,781,814,815]

Overall, these studies provide clear evidence that inflammation both regulates and disrupts cellular responses throughout the male reproductive tract, with direct consequences for function and fertility. There are obvious parallels with the testis, but the underlying mechanisms in these tissues remain to be fully elucidated, and more investigation is warranted. This is particularly important when considering syndromes of chronic, antibiotic-resistant pelvic or perineal pain, such as chronic prostatitis, which are suspected to have an inflammatory basis, but lack a coherent etiology or pathology.[10–12]

Inflammation, Semen, and Seminal Leukocytes

In humans, seminal plasma is generally the only window available for investigating the immunology of the male reproductive tract. More invasive procedures such as biopsies are rarely performed, produce limited and selective samples, and have the potential to cause inflammatory reactions themselves. Much is made, therefore, of the relationship between measurements of leukocytes, antibodies and cytokines in seminal plasma, and fertility. However, while there is little doubt that sperm antibodies contribute to infertility, and that elevated cytokines are an indicator of ongoing infection, inflammation or other immune events, the diagnostic significance of cytokines and, especially, leukocytes in the semen is a subject of ongoing debate.

Many pro-inflammatory and immunoregulatory cytokines are found in samples of seminal plasma, even those from men with apparently normal fertility.[816] The list also includes molecules that modulate the activity of specific cytokines, such as the soluble IL2 and IL6 receptors, and follistatin.[817,818] This is not particularly surprising because these cytokines are present in most biological fluids and are modulated by the general health status of the individual. Elevated cytokine concentrations, both pro- and anti-inflammatory, in seminal plasma are commonly associated with inflammation, infection and increased leukocyte numbers in the male tract, but specific positive and negative associations with infertility of a noninflammatory nature have proven less consistent.[819–821] In a recent evaluation of the extensive literature on this topic, Pilatz and colleagues concluded that, while seminal cytokine profiles might be useful for diagnosis and monitoring of urogenital infections and inflammation, there was no obvious evidence for any associations with poor semen quality or infertility.[822] However, a relationship has been reported between NO

levels and oxidative stress in seminal plasma and infertility due to varicocele and other vascular anomalies, or prostatic inflammation/autoimmunity.[823,824]

An elevated number of leukocytes in the semen is usually considered to be an indication of infection, and the World Health Organization (WHO) has set an arbitrary level of 1×10^6/ml as the threshold of normality. In fact, leukocytes are present even in semen of fully fertile men, with many studies agreeing that 10^4–10^5/ml is a relatively normal value, and even in men with urogenital infections leukocyte numbers do not always reach the WHO level.[358,825] Indeed, leukocyte numbers alone may be a very unreliable indicator of the presence or absence of infection. The origin of these cells is also somewhat obscure. Evidence suggest that the epididymis or vas may be a major source,[364–366] but the primary leukocyte subset present in most semen samples are neutrophils.[358–364] Neutrophils are not a normal feature of the tissues of the tract, but may enter the semen via the accessory glands. This may involve the production of neutrophil-attracting chemokines, such as CXCL6 and CXCL9, by epithelial cells in the epididymis and prostate.[826,827]

Finally, the impact of these cells on fertility is poorly understood. Some studies have shown a relationship between leukocytospermia and impaired sperm function,[828–830] and data suggest that these cells might be a major source of ROS or other molecules causing sperm damage.[358,831,832] Other studies have failed to confirm a clear link between seminal leukocytes and infertility or sperm antibody formation.[358,362,833] It even has been suggested that they might play a beneficial role by the removal by phagocytosis of abnormal sperm or assisting in sperm capacitation.[343,362] It is fair to say that the role of leukocytes in semen is more complicated than might be expected, and many questions still remain.[358,364,833]

AUTOIMMUNITY IN THE MALE REPRODUCTIVE TRACT

Sperm Antibodies

Clinically, the most common immunological dysfunction of the male reproductive tract is the formation of sperm antibodies, which may inhibit sperm motility through agglutination reactions, target the sperm for immunological destruction in the male or female tract, or block essential surface receptors and molecules required for fertilization (Figure 19.16).[834,835] There is no single dominant antigen associated with sperm autoimmunity, and multiple antigens are usually involved.[7–9,291] These antigens have been extensively studied in experimental animals and in infertile patients, and include numerous sperm surface, as well as intracellular proteins, glycoproteins, and glycolipids.

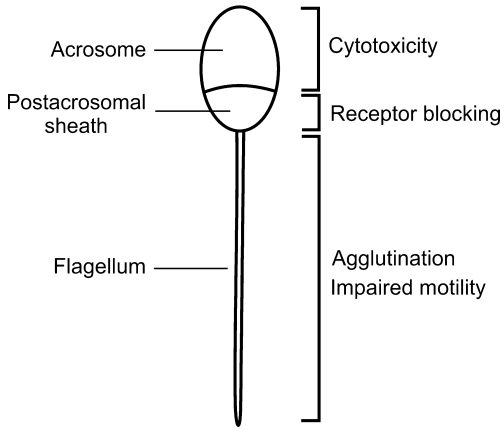

FIGURE 19.16 Sperm antibody binding sites and effects. The principal sequelae of antibody binding to spermatozoa are: (1) activation of immune cells against the sperm via complement or through interaction with immunoglobulin Fc receptors on phagocytes leading to cytotoxicity; (2) blocking or interfering with surface recognition molecules, in particular the egg binding receptors in the postacrosomal region; and (3) agglutination caused by cross-linking of sperm by multivalent antibody which may impede the ability of sperm to swim freely in the female tract.

The Origins of Sperm Antibody Formation

Physical damage to the male reproductive tract can lead to sperm antibody formation. This is best exemplified by the high incidence of sperm antibodies associated with vasectomy, where up to 70% of patients develop antibodies after the procedure.[346–348] Blunt trauma and testicular biopsy may cause sperm antibodies to form as well.[836,837] Sperm antibodies are associated with obstructive azoospermia and congenital absence of the vas deferens,[348,838] and with reproductive tract infection involving orchitis and/or epididymitis, although there is evidence that inflammation alone may not be sufficient to cause antibody formation.[839,840] There is a definite relationship between sperm antibodies and the existence of other autoantibodies, suggesting an important role for genetic factors in predisposition to sperm antibody formation.[841,842] A separate subset of antibodies directed against steroidogenesis-specific antigens expressed by the Leydig cells is associated with polyglandular autoimmunity, although sperm antibodies also may be found in patients with these conditions.[843,844]

In animal models, sperm antibodies can be induced by active immunization with spermatogenic cells or whole sperm, or with testicular or epididymal extracts. This procedure usually involves the use of adjuvants to boost the immune response, but protocols leading to antibody formation without adjuvants also have been reported.[845] As in humans, antibodies may develop following experimental vasectomy or vasal obstruction in rats, guinea pigs, mice, rabbits, and monkeys.[97,332,349,351,352,846,847] The strength of the response and its outcomes are very much strain-dependent. In some of these experimental models,

the formation of antibodies is associated with the development of orchitis and variable degrees of damage to the seminiferous epithelium, an outcome that is rarely, if ever, observed in humans under the same conditions.

Measurement and Clinical Significance of Sperm Antibodies

There has been considerable debate regarding the clinical significance of sperm antibodies or, perhaps more precisely, the tests used to measure them. Although antibody levels in seminal plasma or even blood are frequently quantified, it has been suggested that only antibodies that are actually bound to the sperm, and more specifically the anterior portion of the sperm, should be considered to be of real significance, and then only when a majority of the sperm are affected.[2,835,848,849] Hence, studies that use tests that detect sperm bound antibodies (e.g. agglutination and immunobead binding tests) are most likely to provide reliable results.[834,850,851] Although IgG can cross the reproductive tract epithelium at sites such as the rete testis to bind to sperm,[852] it also has been argued that only IgA antibodies are evidence of a specific mucosal immune response and should be considered of greater prognostic value in semen samples. The most rigorous approach to this issue involves confirmation that the antibody binding has functional consequences using a sperm function test, such as the mucus penetration assay, before accepting the pathological significance of the antibodies.[2,835,849]

Even allowing for the variation in methods employed, it appears that populations vary widely in the incidence of sperm antibodies. This may be due in part to genetic differences in susceptibility to antibody formation, although the prevalence of reproductive tract infections in the population and a lack of suitable treatment options are probably a stronger determinant. In developed nations, the incidence of sperm antibodies is around 5–10% of all infertility cases.[2,3] On the other hand, in parts of central Africa where treatment for reproductive tract infection is limited, or there is a reliance on self-medication and alternative therapies, the incidence of sperm antibodies among infertile populations is much higher.[853] In considering these figures, it also should be borne in mind that sperm antibodies may be present in the semen or blood of men of proven normal fertility.[854] Moreover, in the absence of any other underlying cause being evident, the presence of sperm antibodies tends to be assumed to be the cause of the infertility. It is quite possible that the incidence of infertility due primarily to sperm antibody reactions is lower than these values might suggest. While treatment with high dose corticosteroids has been used with limited success in the past to treat sperm antibodies, sperm washing coupled with intrauterine insemination, in vitro fertilization or intracytoplasmic sperm injection are the current methods of choice.[834,837]

Equally serious for the infertile couple is the development of sperm antibodies in the female tract. While largely outside the scope of this review, it is important to recognize that histocompatibility differences between partners may create potential problems for sperm survival and successful fertilization.[855–857]

Autoimmune Orchitis

The Etiology of Orchitis

In humans, isolated orchitis is usually associated with viral infections, most commonly postpubertal mumps.[858–863] Nonviral causes include mycobacteria (e.g. *Mycobacterium leprae* and *M. tuberculosis*) and parasites, such as Filariasis and Malaria. Bacterial orchitis is usually caused by retrograde ascent of urethral pathogens, such as *E. coli, Chlamydia trachomatis,* and *Neisseria gonorrhoeae*, and is almost invariably associated with inflammation of the epididymis (epididymo-orchitis). In both clinical and experimental models of infectious orchitis,[313,603,864] the immunological response to the infection may cause collateral damage to testicular cells, causing obstruction, fibrosis, and displacement or ischemia, and increases the risk of subsequent autoimmune reactions against testicular antigens. Autoimmune orchitis may also develop as a consequence of neoplasia, in response to an external antigenic challenge (e.g. vasectomy or active immunization), or may arise spontaneously, in the sense that no obvious cause can be discerned.[859,860,865] Whereas infectious orchitis resolves once the infection has been cleared, autoimmunity may lead to chronic inflammatory disease. In contrast with the incidence of sperm autoantibodies, however, autoimmune reactions against spermatogenic cells within the testis appear to be comparatively rare among most human populations.

While there are several animal models of spontaneous autoimmune orchitis, generally attributable to genetic or epigenetic causes, in the mink,[866] rats,[867] and dogs,[868] the incidence of such events have been difficult to establish in humans, given that the autoimmune event and its diagnosis might be separated by many years. There is evidence for immune complex formation in the basement membrane of the seminiferous epithelium,[869–871] and a high prevalence of asymptomatic testicular inflammatory lesions,[4,5] among infertile men. The problem lies in establishing whether these lesions are evidence of a past autoimmune reaction or secondary to inflammation from other causes. The testis is one of the organs that is affected in PGA syndromes, which are caused by failure of normal mechanisms of tolerance,[166,167,872] suggesting that other genetic causes for autoimmune orchitis may exist. There is some evidence of an association of orchitis with other autoimmune inflammatory diseases, particularly vasculitis (inflammation of the blood vessels) in men.[873] A genetic predisposition for development of

autoimmunity may require a secondary trigger, in the form of a precipitating infection, physical trauma to the reproductive tract or other inflammatory event, in order to proceed to actual autoimmune orchitis.

Experimental Autoimmune Orchitis

Several experimental animal models of autoimmune orchitis have been extensively studied by a number of groups, seeking to understand testicular immunophysiology and the causes of autoimmune infertility.[155,311,313–315,331,845,874,875] Most of these models involve immunization with testicular antigens, including spermatogenic cell, somatic cell, and extracellular matrix antigens, using complete adjuvants (containing active or inactivated microbial agents), or adoptive transfer of lymphocytes from animals with on-going autoimmune orchitis. However, at least one model of induced orchitis involving subcutaneous injection of syngeneic (genetically identical) spermatogenic cells into a susceptible strain of mice has been described.[876,877] A particularly interesting model of orchitis development occurs subsequent to vasectomy in some animals. Whereas vasectomy leads to sperm antibody formation alone in most strains of rats and mice, vasectomy results in a rapid progression to full-blown orchitis in the Lewis rat,[349–351] and SWR/J mouse.[878] Similar responses to vasectomy or epididymal obstruction have been observed in the rabbit,[879] guinea pig,[880] and rhesus monkey.[881] Finally, removal of the thymus gland around postnatal day three in mice causes polyglandular autoimmunity, which can include autoimmune orchitis similar to that found in humans,[155,874,875] indicating that immunoregulation by T cells is involved in the normal protection of at least some testis-specific antigens. These models have been crucial to the development of our concepts of the control of immune responses in the testis.

The majority of research has been focused upon autoimmune reactions to the germ cells, but testicular autoimmune disease also may involve the somatic cells. Autoimmune polyglandular diseases, most notably Addison's disease, are associated with specific immune reactions to steroidogenic cells throughout the body, including Leydig cells, thereby leading to hypogonadism.[843,882] This autoimmunity primarily involves reactions against the steroidogenic enzymes and hormone receptors,[883,884] although spermatogenic cell autoantibodies are commonly observed, as well.[844]

Although the models of experimental autoimmune orchitis differ in respect of the method of induction, there are common features in the progression of disease, which are summarized here. The onset of autoimmune orchitis in animals generally involves the appearance of testicular antibodies and immune complexes, followed by infiltration and activation of macrophages, dendritic cells and effector lymphocytes, invasion of the seminiferous tubules, sloughing of germ cells and focal necrosis, accumulation of neutrophils and/or eosinophils, and finally aspermatogenesis.[311,314,331,885–887] The epididymis and vas deferens are frequently involved. In adoptive transfer studies in mice, it was shown that $CD4^+$ T cells, but not antibody, can transfer orchitis from one animal to another, clearly implicating a cellular rather than humorally-mediated mechanism of disease development.[267,268,864] In adoptive transfer, moreover, the early disease almost invariably occurs in the region where the seminiferous tubules connect to the rete testis,[267,268] consistent with the fact that this is where access to the antigens of spermatogenesis is likely to be greatest,[64,73,267] and there is a greater concentration of MHC class II positive macrophages.[267,268,314,331,888] In the adjuvant-free model of induced orchitis, a similar pattern of disease initiation has been observed in this region.[889] Active immunization protocols using adjuvant, on the other hand, cause a more widespread induction of disease throughout the testis.[267,311]

Progression of autoimmune orchitis is marked by expansion and activation of the testicular dendritic cell and macrophage populations, which remain elevated throughout the course of the disease.[292,887] Increases in IL17-producing $CD4^+$ Th17 cells and $CD8^+$ T cells, and even Treg cells have been observed during the onset of autoimmune orchitis in the rat, and persist through the chronic phase, indicating that interaction between these cell subsets may determine the course of the disease.[885,886] Several immunodominant antigens have been identified in the rat, and include antigens that are not specific to the testis.[291] The pro-inflammatory cytokines, TNF and IFNγ, FAS and FASL, and NOS2 have been identified as potential causative factors in the development of orchitis, principally by stimulating antigen-presentation activity and spermatogenic cell apoptosis.[595,890–894] Although IL6 production by testicular macrophages increased during experimental autoimmune orchitis in rats,[895] and was implicated in the disruption of the inter-Sertoli cell tight junctions,[896] administration of IL6 has been shown to inhibit the progression of orchitis in LPS-resistant mice.[897] The progression of orchitis may also be inhibited by IL10 and by androgens.[898,899] The crucial requirement in the development of orchitis is to overcome the normal mechanism of systemic tolerance to testicular antigens, as well as the local immunoregulatory mechanisms.

Typically, immunological events induced in one testis can lead to damage in the contralateral testis, a condition sometimes called sympathetic orchiopathia. Such responses have been observed following a unilateral physical or toxic insult,[900,901] infection with *E. coli* or other pathogens,[902,903] and ischemia or ischemia-reperfusion injury.[746–748,904] This is usually attributable to generation of autoreactive lymphocytes and antibodies caused by events in one testis traveling to the other testis to initiate

disease, in a manner comparable to adoptive transfer of autoimmunity from one animal to another.

The precise reasons why some animal strains are more susceptible to the development of autoimmune orchitis remain to be fully explained, but it is obvious that susceptibility to autoimmune reactions to sperm and testis antigens is genetically determined.[905,906] Clinically, there is an association between sperm antibodies and other autoimmune antibodies,[841,842] and the development of sperm antibodies following vasectomy is strongly associated with the MHC.[907] In mice, specific orchitis susceptibility genes have been mapped to both MHC and non-MHC regions, and the loci linked to development of orchitis also have been shown to govern susceptibility to other autoimmune diseases, such as encephalomyelitis and diabetes.[908,909]

Autoimmunity in the Epididymis, Vas Deferens, and Prostate

Clinically, epididymitis is the most common intrascrotal inflammation, manifesting as pain, nodules, edema, urinary difficulties, fever, urethral discharge, and/or infertility, although it also can be asymptomatic. Acute epididymitis is most commonly due to retrograde ascent of urethral pathogens and sexually-transmitted bacterial infections—most notably *C. trachomatis* and *N. gonorrhoeae*, but also *Ureaplasma urealyticum*, *E. coli*, *Enterococcus faecalis,* and *Pseudomonas aeruginosa*.[860] Less frequently, systemic bacterial infections, such as *M. tuberculosis*, *Haemophilus influenzae type B*, and *Salmonella*, or nonbacterial infections, such as cytomegalovirus and filariasis, may be responsible, especially in children or immunocompromised individuals. Epididymitis also may have noninfectious origins in various medical procedures, the arrhythmia drug amiodarone, trauma, physical stress, vasectomy, urinary reflux, neoplasia, postinfectious and systemic inflammation, vasculitis, and systemic autoimmunity.[11,12,860,910,911] Vasitis is relatively rare and is usually caused by injury, bacterial infection, and following surgical manipulation, postvasectomy or following prostatectomy.[912] It may present as pain or be asymptomatic. If the inflammatory response in the epididymis or vas deferens is severe enough, this can lead to damage to the mucosal epithelium and/or obstructive lesions that may inhibit fertility. Prostatitis and vesiculitis are most commonly caused by bacterial infections, although antibiotic-resistant chronic prostatitis/chronic pelvic pain has suspected inflammatory, probably autoimmune, origins.[10,860,910,911]

Epididymitis is much more frequent than isolated orchitis, which may be a reflection of closer proximity to the external environment. However, different immunoregulatory mechanisms in the two organs may also be a contributing factor. Experimental evidence suggests that the epididymis may be more susceptible to inflammation and subsequent leukocytic infiltration than is the testis. For example, infiltration of lymphocytes and neutrophils into the interstitial tissue are frequently observed in the epididymis and vas deferens, but rarely in the testis, of aging mice.[913] In mice injected intravenously with *Bordetella pertussis* and adjuvant, neutrophils invade the stroma of the epididymis, vas deferens and accessory glands, but the testis is not affected.[914] In the alymphoplastic (aly) mouse, eosinophils and macrophages spontaneously accumulate in the stroma of the epididymis and vas deferens, but not the testis.[915]

Experimentally, epididymitis can be induced by retrograde inoculation with bacteria, such as *E. coli*, via the vas deferens, which leads to epididymitis, vasitis and orchitis, but is complicated by the presence of the bacteria itself.[95,809,916–918] Noninfectious models of epididymitis involve active immunization, neonatal thymectomy, and vasectomy. Epididymitis (actually epididymo-orchitis) and vasitis can occur following active immunization with testicular extracts in the presence of suitable adjuvants in rats, mice and guinea-pigs.[267,314,331] Passive transfer of T cells from mice immunized in this manner also leads to epididymitis, with testicular involvement initially confined to the region around the rete testis and the efferent ducts.[267,268,890] Subcutaneous injection of syngeneic testicular spermatogenic cells into mice in the absence of adjuvant causes orchitis without epididymitis,[876] but passive transfer of the lymphocytes from these mice after stimulation in vitro, favors the induction of epididymitis.[919,920] In other words, the transfer of T cells activated specifically against spermatogenic antigens selectively induces epididymitis in nonimmunized mice.[889] Removal of the thymus at 3 days of age in mice, which abrogates peripheral tolerance mediated by regulatory T cells,[921] initially causes epididymovasitis in the postpubertal period, followed by an increasing, but always smaller, incidence of orchitis many weeks later.[874,922] Likewise, mice with a targeted deletion of the immunoregulatory AIRE transcription factor developed sperm antibodies and leukocytic infiltrates in the epididymis, without orchitis.[923] Vasectomized A/J mice that have been immunized with syngeneic testicular spermatogenic cells develop epididymitis independent of orchitis,[924,925] suggesting that the epididymis can be the primary site of immune reactions following vasectomy, as well. The epididymis is also more susceptible than the testis to spermatic granuloma formation in humans and in experimental mice.[926,927] Taken altogether, these observations highlight a greater susceptibility of the epididymis to inflammation, relative to the testis, when responses to spermatogenic antigens occur. Accordingly, detailed studies in mice have established that the immunogenetics underlying susceptibility to autoimmune orchitis and epididymitis are quite distinct.[906]

Infection and inflammation in the epididymis is characterized by infiltration of macrophages, lymphocytes and neutrophils into the interstitial and peritubular zones, as well as the epididymal epithelium.[95,99,331,332,845,874,922,925,928] The consequences include reduced sperm productivity and function, increased sperm antibody formation, as well as damage to the epithelium and potential obstruction of the epididymal duct. Similar responses in the vas deferens and accessory glands may interfere with seminal fluid production and sperm transport. As in the testis, these disruptions of function may be due to the actions of the activated immune cells, but may also be due to altered epithelial cell or stromal cell function, through the activation of TLR signaling pathways, pro-inflammatory cytokines and other inflammatory mediators. Importantly, ongoing inflammation in these tissues, regardless of the cause, may be a source of chronic pelvic or perineal discomfort or pain.

IMMUNE PRIVILEGE IN THE MALE REPRODUCTIVE TRACT

Based on the data as outlined in previous sections, it is evident that the testis is an immunological target, and that autoimmune responses to spermatogenic antigens will occur given the right conditions, prompting the question: *What are the normal systems that exist within the testis to prevent or regulate development of autoimmunity there? In other words, what is the functional and physiological basis of "immune privilege" in the testis?* The concept of immunological privilege has generally been used by immunologists to describe tissues that are not readily accessible to antigen-presenting cells and circulating lymphocytes or that have deficient lymphatic drainage to local lymph nodes, such as the brain, the anterior chamber of the eye and the placenta.[37,873,929] Neither of these properties applies to the testis, and the most common misconception centers on the role of the blood–testis barrier.

The Immunological Role of the Blood–Testis Barrier

The actual location of the blood–testis barrier lies in tight occluding junctional specializations (zonulae occludens) between adjacent Sertoli cells in the seminiferous epithelium (Figure 19.2).[16,17] These elaborate junctions are quite distinct in organization from the normal adhering junctions and gap junctions found in most epithelia and are assembled at the time of puberty to form a complete seal separating the basal and adluminal compartments of the epithelium.[15,866] Their primary role is to create a unique physiological environment for the meiotic and postmeiotic germ cells, and all spermatogenic cells beyond the leptotene or zygotene spermatocyte stage are completely surrounded by the cytoplasm of adjacent Sertoli cells and are entirely dependent upon these cells for their support and regulation.[72,77,930,931] The process of movement of the spermatogenic cells themselves through the junctions during spermatogenesis does not involve a breach of junctional integrity.[72,931] Indeed, loss of barrier function is a precipitating factor in many forms of testicular failure, including spermatogenic damage during inflammation and infection.

A broader definition of the blood–testis barrier beyond the concept of a physiological barrier incorporates protection of the spermatogenic cells from harmful influences. Some definitions of the blood–testis barrier, therefore, also include the efflux-pump barrier system involving drug-transport proteins, such as p-glycoprotein and the multidrug resistance associated protein-1, on the capillary endothelium, peritubular myoid cells, and the basal aspect of the Sertoli cells.[932,933] While these elements may certainly be important for protection of the testis against toxic agents, their contribution to immunoregulation is likely to be marginal.

There are several lines of evidence that the blood–testis barrier does not account for all the manifestations of immune privilege in the testis. Studies have shown that spermatogenic cell autoantigens are not confined behind the Sertoli cell tight junctions, and are expressed by spermatogonia and the early spermatocytes.[934] Moreover, there is clear evidence that the barrier is incomplete in the epithelium of the rete testis. Significantly, orchitis can be passively transferred to naïve mice by lymphocytes from mice with active autoimmune orchitis.[267,268,919] In many seasonally breeding species, annual regression of both the later spermatogenic cells and blood–testis barrier occurs without inducing overt inflammation or autoimmunity.[72,866] Finally, the blood–testis barrier cannot explain the enhanced survival of grafts within the interstitial tissue.[14,266] On balance, the data lead to the conclusion that the blood–testis barrier does not prevent exposure of germ cell antigens to the immune system, indeed antibodies and lymphocytes specific for spermatogenic antigens may be a normal feature of the circulating immune repertoire.[854] Although the sequestration of a large proportion of the antigenic burden behind the blood–testis barrier no doubt plays a role in this process, it cannot be the primary explanation for functional immune privilege in the testis.

Evidence from Transplantation Studies

Perhaps the most intriguing aspect of immune responses in the male tract is the observation that allografts and even xenografts into the testicular interstitial tissue outside the blood–testis barrier are preserved for extended periods of time, possibly even

indefinitely.[14,266,935–937] This enhanced survival is not simply due to the reduced ambient temperature of the testis, as grafts to the equally hypothermic skin of the ear are not preserved,[266] while grafts continue to survive in testes that have been translocated to the abdominal cavity.[266,936,937] Moreover, the efferent lymphatics of the testis do not show any evidence of functional deficiency.[13] However, intratesticular parathyroid allografts fail to survive in rats sensitized against the donor antigens, while long-established intratesticular allografts are rapidly rejected following active immunization of the recipient with donor tissue.[14] These observations led to the hypothesis that local immunoregulation mechanisms are responsible for graft survival, and, more specifically, that the inductive phase of the immune response is suppressed within the testis and/or its draining lymph nodes. In other words, the immune system may be unable to recognize and/or respond to foreign antigens within the testicular environment.

It should be noted that studies on graft survival in the testes of laboratory rodents have been very successful, but the data in other species are less consistent. Similar transplant studies in the ram and cynomolgus monkey have proven unsuccessful.[938,939] Moreover, the type of graft also may be a factor. Selawry and colleagues were able to demonstrate survival of pancreatic islet allografts or xenografts in abdominally-located testes, but not in scrotal testes, of the rat.[937] Whether these differences in outcome are due to differences in testicular architecture, differences in lymphatic organization or vascularization, the size, health, and type of graft, the underlying immunogenetics of the donor and host, effectiveness of local immunoregulatory mechanisms, species differences in systemic immunity, or even the surgical procedures employed, remains to be answered.

Evidence suggests that testicular tissue and some testicular cells, in particular, have inherent characteristics that may make them more amenable to transplantation.[30,940,941] However, just as intratesticular grafts produce different outcomes in different studies, studies on transplantation of testicular tissue have met with variable degrees of success. Early observations were that fetal and postnatal testis tissues are viable as grafts under the kidney capsule of outbred rats, but that adult testis tissue is quickly rejected.[941,942] Perhaps surprisingly, transplantation of fragments of intact testicular tissue from numerous species under the skin of immunodeficient mice tends to be extremely successful, with vascularization, normal steroidogenesis and even complete spermatogenesis becoming established, although there is no exit for the spermatozoa that are produced.[943] The ability of spermatogenesis to occur in such grafts is probably related to the reduced temperature of the skin, but immunocompromised recipient animals are generally required to avoid rejection responses. On the other hand, adult testis allografts have been observed to survive under the kidney capsule in immunocompetent mice,[489] and allogeneic transplantation of spermatogenic cells, which were subsequently able to undergo normal spermatogenic development, have been successfully performed in several domestic species with intact immune systems.[944–946] Kimmel and colleagues observed that human testis xenografts to the murine kidney failed to survive in intact mice, but survival was possible in recipient mice with an inactivating deletion of MHC class II expression, thereby implicating a $CD4^+$ T cell-mediated rejection process.[947] Discovering the reasons why different models produce such widely different outcomes may provide a better understanding of the fundamental requirements for immunological privilege in the testis.

Immune Tolerance and Immunoregulation

It has become increasingly apparent that central and peripheral immune tolerance contributes to testicular immune privilege. This was first indicated by the fact that hypogonadism and sperm autoimmunity are frequently associated with the PGA syndromes, type 1 and 2, which are due to a mutation in the AIRE transcription factor that controls thymic antigen expression during immune development,[166] and a defect in regulatory T cell function,[167] respectively. Mice with a deletion of AIRE develop sperm antibodies and inflammatory lesions in the epididymis (O'Bryan MK, personal communication).[923] Exposure to testicular antigens during maturation of the immune system has been shown to reduce the severity of induced autoimmune orchitis in adult genetically-immunodeficient mice that have had their immune system re-constituted.[948] Moreover, the cluster of autoimmune endocrinopathies induced by thymectomy at 3 days of age in mice and rats frequently includes orchitis.[155,872,874,875,922] These disease models in humans and rodents establish a link between testicular autoimmunity and failure of tolerance, more precisely, a shift in the balance between autoreactive T cells and specific regulatory T cells.

Suppressor or regulatory T cells have experienced a major revival in interest in recent times, with most attention directed toward the $CD4^+CD25^+FOXP3^+$ Treg cell subset, but there is also evidence for $CD8^+$ regulatory T cell populations.[949,950] As would be expected, Treg cells can be found in both the normal and inflamed testis,[310,885] even if their actual role in the testis has yet to be firmly established. Nonetheless, several studies indicate that exposure of T cells to the testis environment induces an immune deviation response, that is, a switch from cell-mediated (Th1) type immunity to a Th2/Treg response that is predominantly immunoregulatory and tolerogenic (Figure 19.7). Injection of soluble antigen into the testis produces specific suppression of T cell-mediated

responses against the injected antigens.[951–953] Studies on pancreatic islet cell allografts in the mouse testis indicate that activated or memory T cells directed against graft antigens are destroyed when they enter the testis environment and that graft antigen-specific Treg cells are preferentially produced.[954,955] Isolation of a $CD4^+$ T cell line that was able to downregulate the development of adoptive transfer of autoimmune orchitis in mice has been reported,[956] and a crucial role for Treg cells in controlling the development of orchitis in response to vasectomy also has been demonstrated in mice.[957] A substantial increase in evidence linking Treg cells and other regulatory T cell subsets to the control of testicular immune responses can be anticipated in the near future.

FAS Ligand

In 1995, Bellgrau and colleagues[489] reported that expression of FASL was expressed by mouse Sertoli cells, and that mice deficient in either FASL or its receptor, FAS, did not show evidence of testicular immune privilege. In this study, FAS-FASL interaction was implicated in the prevention of antigen-specific responses within the testis, and subsequently, in other immune-privileged or immune-deficient sites.[958] It was proposed that T cells entering the testis and becoming activated by exposure to their respective antigen are immediately killed by interaction between FAS on their surface and FASL, either expressed on, or secreted by, the Sertoli cells. While this was an attractive hypothesis, a number of concerns regarding the FASL expression hypothesis as an overarching explanation for immune privilege arose. Several groups found that expression of FASL did not confer immunoprotection in their own studies in the testis or in other tissues, and in fact caused quite virulent inflammatory reactions in some cases.[959,960] FASL also appeared to be expressed at relatively high levels in epithelia of human tissues not generally thought of as immunologically privileged, such as the esophagus and lung.[961] Finally, Kimmel and colleagues reported being unable to detect FASL expression at all in normal human testes which had been flushed of all peripheral blood cell contamination prior to collection of RNA.[947]

The role of FASL in normal testis function or immunophysiology still remains unresolved. Several studies have suggested that, although FASL mRNA can be detected in Sertoli cells using very sensitive methods such as RT-PCR, the protein is not significantly expressed by the Sertoli cells in the normal adult testis, and in fact it may only be the spermatogenic cells that express the ligand constitutively.[492,959,962] In the immature rat and porcine testis FASL protein has been detected in Sertoli cells by immunohistochemistry,[490,963] but the specificity of FASL antisera used for this purpose has been challenged in at least one major review of the issue.[491] While it appears certain that modulation of FAS and FASL in the seminiferous epithelium plays an important role in regulating spermatogenic cell apoptosis, particularly in various testicular damage models,[490,772,964] a crucial role in maintaining immune privilege in the testis remains doubtful on the weight of evidence.

Major Histocompatibility Complex Expression in the Testis

Most studies have reported a characteristic absence of expression of both MHC class I and class II proteins on the cells of the seminiferous epithelium under normal conditions.[67,68,230,232,266,267,315,965–967] This suggests that spermatogenic cells may be able to avoid direct recognition by $CD4^+$ and $CD8^+$ T cells, which might be important for reducing the potential for antigen-specific immune responses in the seminiferous epithelium. On the other hand, there are some data to indicate that mRNA and protein for both MHC class I and class II molecules are expressed in human spermatozoa,[968–970] suggesting that mRNA present in the spermatogenic cells may be translated into protein at some time after they are released from the testis. This delayed expression of MHC molecules may play a role in protection of the sperm against infection or immune cells in the reproductive tract.

In contrast to the seminiferous epithelium, both MHC class I and II proteins are expressed in the testicular interstitial tissue. As would be expected, MHC class I expression is found on most interstitial cells, including the Leydig cells.[230,965] In the rat and human, testicular macrophages and dendritic cells express MHC class II throughout the interstitium,[239,246,266,965] but studies on the mouse testis indicate that expression of MHC class II is concentrated on cells in regions adjacent to the rete testis.[267,268] A greatly reduced number of MHC class II positive cells in the ram testis is consistent with the relatively low number of resident macrophages in this species.[68] In light of all the observations, however, it appears unlikely that a lack of MHC class II-positive antigen-presenting cells is a contributing factor in testicular immune privilege. Differences in the distribution of such cells, however, may be reflected in differences in susceptibility and development of autoimmune reactions between species, or possibly even between different strains. During the development of experimental autoimmune orchitis, which requires overcoming peripheral tolerance to intratesticular antigens, MHC class II expression and the cells bearing these molecules—dendritic cells and macrophages—are increased within the testis and in the draining lymph nodes.[292,887]

The nonclassical MHC class I genes, HLA-G and HLA-E, are associated with suppression of the adaptive immune response.[169,971] The soluble form of the HLA-G molecule has been localized by immunohistochemistry to Sertoli cells, spermatocytes, spermatids and some interstitial cells in the rhesus monkey testis,[972,973] and

expression of HLA-G and HLA-E has been detected in isolated human spermatogenic cells.[970] More recently, HLA-G has been detected in the seminiferous tubules and efferent duct epithelium of the human testis.[974] This molecule is associated with the placenta in protecting fetal alloantigens from the maternal immune system,[169,972] and its soluble form has been implicated in apoptosis of alloreactive $CD8^+$ T cells.[971] Its significance to the testis is entirely unknown at this time.

Immunological Responses in the Testis

It is evident that maintenance of peripheral tolerance is important for testicular immune privilege, and that loss of tolerance to testicular antigens leads to autoimmunity and testicular damage. Obviously, the MHC class II-expressing antigen-presenting cells of the testis must play a crucial role in this process, by creating a tolerogenic environment. The balance of data suggest that the majority of dendritic cells and macrophages in the mouse display reduced expression of MHC class II molecule and the regulatory CD80/CD86 co-receptors,[267,271] suggesting that they may be more likely to promote tolerance, but this does not appear to be true for the rat.[266,272] However, expression of other co-regulatory ligands and receptors, for example CD40LG or inhibitory variants of CD80/CD86,[975] have yet to be investigated in these cells. On the other hand, it is well-established that the macrophages of the rat and mouse display the anti-inflammatory/immunosuppressive M2 phenotype, manifesting as preferential production of immunosuppressive cytokines, such as IL10, and a reduced capacity for T cell activation.[269,270,275,287,288] This suggests that the predominantly immunosuppressive phenotype of the testicular macrophages is a critical element in maintaining testicular immune privilege, and that deviation of the macrophage and dendritic cell phenotype toward support of cell-mediated immunity is an essential event leading to autoimmune orchitis.[272,292,887,893]

Based on the existing evidence, it can be proposed that circulating $CD4^+$ effector T cells entering the testis are inactivated, deleted, or made tolerogenic by the resident antigen-presenting cell population. This model of tolerance in the testis is consistent with the presence in the testicular interstitial tissue of Treg cells,[310,885] but also of NK and NK T cells (Hedger MP and Aridi DZ, unpublished data).[70] Cells of the NK lineage are able to modulate dendritic cell activity to control adaptive immune responses.[976–978] These cells play a key role in promoting graft survival,[149,168] and have been implicated in the generation of $CD8^+$ regulatory/suppressor T cells in another immune privileged site, the anterior chamber of the eye.[150,979,980] Consequently, it is anticipated that intratesticular NK and NK T cells are involved in production of regulatory/suppressor T cells specific to the testis. Finally, a role for γδ T cells, a minor subset of T cells that possess an alternate TCR structure, has been invoked in suppressing autoimmune reactions in bacterial-induced autoimmune orchitis in the mouse, in part through production of IL10 and TGFβ.[313,903] This lymphocyte subset also appears to be involved in maintaining immune privilege in the eye.[981]

Testicular Immunoregulatory Mechanisms

Immune privilege, in the classical sense of providing extended graft protection, is restricted to a small number of tissues, so there must be some specific properties of the testis that create an environment where adaptive immunity is so closely regulated. Historically, the spermatogenic cells and Leydig cells have been implicated,[982,983] as they are cell types with functions that are unique to the testis. Early studies showed that spermatogenic cells can inhibit lymphocyte activity in vitro and in vivo.[983,984] More recently, Cheng and colleagues reported that the CD80/CD86 variant T cell regulator, programmed death-1 ligand-1 (PD-L1 or B7-H1), is expressed on murine spermatocytes and spermatids, and supports the survival of intratesticular pancreatic islet allografts.[985] However, contact between T cells and these cells behind the blood–testis barrier is unlikely, and complete disruption of spermatogenesis by translocation of the testis to the abdominal cavity does not appear to abrogate survival of intratesticular allografts or xenografts.[266,937,986] Nonetheless, even if the spermatogenic cells do not play a direct role in immunoregulation, it remains possible that these cells play a role by stimulation of immunoregulatory functions of the Leydig cells, and more importantly, the Sertoli cells.

The Role of the Sertoli Cell

There is little doubt that the Sertoli cell possesses unique immunoregulatory properties. Sertoli cells from immature rat, murine or porcine testes display extended survival as allografts or xenografts, and co-transplantation of Sertoli cells or testis cell mixtures containing these cells confers increased survival on a number of cell and tissue allografts and xenografts, including neural cells, pancreatic islets and skin.[987–990] Such procedures have even been used for protection of islet transplants in humans, with some success and controversy.[991] Although the immunoprotective properties of the Sertoli cells appear to be dependent upon their number and ability to form intercellular tight junctions and tubule-like structures, survival of co-transplanted cells adjacent to, but outside, these structures indicates that the protective mechanism does not require the creation of a physical barrier.[992] Protection appears to be an inherent property of the Sertoli cell, involving factors expressed on the surface or secreted from the cell (Figure 19.17).

Sertoli cells express comparatively low levels of classical MHC antigens, and produce the nonclassical MHC class I antigen, HLA-G, which would enhance

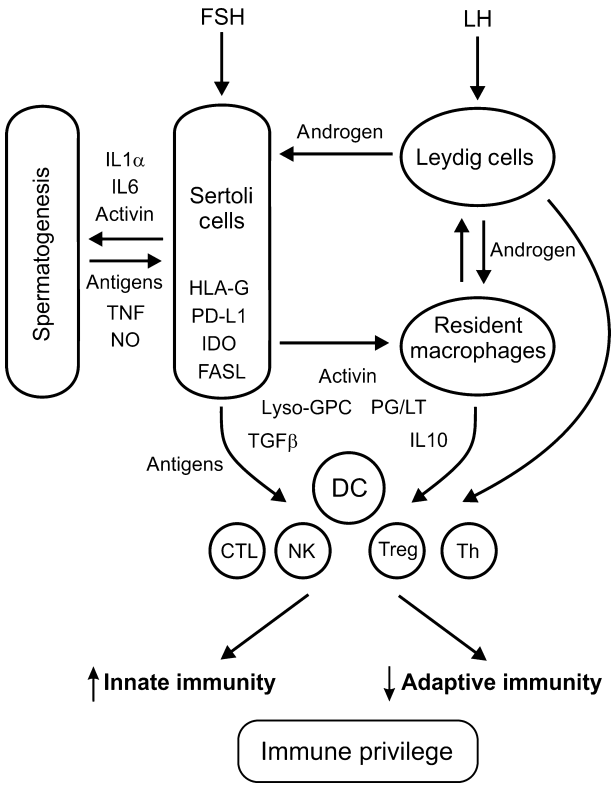

FIGURE 19.17 Intercompartmental interactions regulating steroidogenesis, spermatogenesis and immune responses in the adult testis. Within the seminiferous epithelium, the Sertoli cell supports and regulates spermatogenesis, through production of cytokines, including interleukin-1α (IL1α), IL6, and activin. Production of these cytokines is stimulated by the presence of the spermatogenic cells. Spermatogenic cells also produce tumor necrosis factor (TNF) and NO, which regulate Sertoli cell functions, such as the maintenance of the blood–testis barrier. Sertoli cells possess a number of immunosuppressive activities, which include expression of indoleamine 2,3-dioxygenase (IDO), HLA-G, programmed death-1 ligand-1 (PD-L1), and FAS ligand (FASL). Spermatogenic antigens released from the seminiferous epithelium, together with local production of immunoregulatory molecules, including IL10, transforming growth factor-β (TGFβ), activin, prostaglandins/leukotrienes (PG/LT), and lyso-glycerophosphatidylcholines (lyso-GPCs), create an environment that promotes tolerogenic responses (alternatively activated resident macrophages, Treg cells) and innate immunity (NK cells, cytotoxic T cell), while inhibiting cell-mediated immunity (helper T cells). Leydig cells are responsible for recruiting macrophages into the testis and may have further immunomodulatory actions through production of androgens, and other molecules with immunoregulatory actions, such as prostaglandins (PGs). DC, dendritic cells; NK, NK cells: CTL, cytotoxic lymphocytes; Th, helper T cells; Treg, regulatory T cells.

their potential to avoid detection by T cells and subsequent immune activation.[230,967,972,993] On the other hand, expression of ICAM1 and VCAM1 by the Sertoli cells suggests that these cells can selectively bind and interact directly with circulating lymphocytes.[477,994] Mouse Sertoli cells increase expression of PD-L1, but not positive co-stimulatory molecules, in response to IFNγ in vitro.[594] Human Sertoli cells were found to suppress MHC class II and inflammatory gene expression in human endothelial cell cultures stimulated with INFγ or TNF, and to inhibit the ability of these cells to stimulate spleen lymphocyte proliferation.[995] Finally, Sertoli cells are similar to macrophages in that they possess an enormous capacity for phagocytosis of senescent cells, cell debris, and other potentially antigenic complexes. Together with the inherent ability of the Sertoli cell to provide a highly supportive environment for cell growth and differentiation,[996] these characteristics no doubt all contribute to the unique graft-protecting abilities of the Sertoli cell.

Sertoli cells express and secrete a wide range of immunoregulatory molecules, several of which have been implicated in graft protection or immune privilege.[997] Studies have shown that Sertoli cells secrete lymphocyte-inhibiting activity in culture,[998,999] and are major sites of production of both TGFβ and activin A.[224,225] Production of TGFβ1 by co-transplanted Sertoli cells has been implicated in protection of syngeneic transplants of pancreatic islets in streptozotocin-induced diabetic rats and nonobese diabetic mice.[990,1000] There is evidence that FASL expression on the Sertoli cell may enhance graft survival, as well.[988,1000,1001] Sertoli cells produce several complement inhibitors, as well as inhibitors of granzyme B, which is a lytic molecule produced by cytotoxic lymphocytes.[1002–1004] Furthermore, IL6, which is secreted by the Sertoli cell under hormonal control, has a number of immunoregulatory properties, stimulating the production of anti-inflammatory cytokines by T cells,[125] and inhibiting the maturation of dendritic cells from circulating monocytes.[234] Cultured murine Sertoli cells progressively produce indoleamine 2,3-dioxygenase (IDO), an enzyme which catalyzes the metabolism of tryptophan, and which has been shown to stimulate dendritic cell-initiated tolerance,[1005] and to stimulate the development of Treg cells in tumors and in pregnancy.[1006,1007] Silencing of IDO with si-mRNA inhibited the ability of these cells to reduce diabetes in non-obese diabetic mice.[1006] Finally, mice with a Sertoli cell-specific deletion of the androgen receptor have a defective blood–testis barrier, the testis becomes infiltrated by macrophages, neutrophils, plasma cells, and eosinophils, and they develop antibodies against spermatogenic cells.[1008] This suggests that loss of androgen regulation in the Sertoli cell leads to a loss of testicular immune privilege.

The Role of the Leydig Cell and Androgens

It seems likely that Leydig cells actually promote inflammation-related responses of the testis, as these cells produce several inflammatory mediators constitutively (MIF, NOS2) or in response to inflammatory stimuli (IL1, IL6, and CCL2).[226,276,278,285,390–392,503,504,641,1009] These cells also play a central role in the regulation of interstitial fluid formation in both hCG-hyperstimulation and LPS-induced inflammation through control of endothelial cell permeability.[397,1010,1011] Conversely, early

studies established that mouse Leydig cells, but not other testicular cells, bound to lymphocytes, macrophages and eosinophils specifically in vitro,[1012,1013] and that the presence of rat or mouse Leydig cells could inhibit lymphocyte proliferation responses in vitro.[982] This binding may have been due, at least in part, to a variant of VCAM1 expressed on the Leydig cell surface,[1014] while inhibition of lymphocytes by Leydig cells in vitro appears to involve both direct contact and secretion of soluble inhibitory factors.[1015] The Leydig cell-secreted cytokine, MIF, is a potent inhibitor of cytotoxic T cell and NK cell activity,[1016–1018] and it may be that a function of this cytokine in the testis is to restrain cell killing in the absence of an overt immunological challenge. However, the most significant role of the Leydig cells in mediating immune control of the testis may lie in their ability to facilitate recruitment of resident macrophages in the testis (Figure 19.17).[69,226,293,296] Junctional specializations between Leydig cells and resident macrophages mediate close physical attachment of these cells in the normal testis,[240,244] and the immunoregulatory significance of these interactions has yet to be explored in any detail.

The principal endocrine function of the Leydig cell is the production of testosterone, and concentrations of this androgen are extremely high within the testicular interstitial tissue.[79,80] There is some evidence in vitro that androgens are able to regulate lymphocyte-mediated immunity by stimulating the production of IL10 by $CD4^+$ T cells, and stimulating the induction or proliferation of Treg cells.[197,210,213,214,899] A more general effect of androgens on the Th1/Th2 cytokine balance was demonstrated by the fact that administration of testosterone to hypogonadal men stimulated baseline IL10 levels and inhibited IL1β and TNF levels in serum.[1019] However, in vivo evidence for local immunoregulation by androgens in the testis is conflicting. Androgens do not appear to directly regulate macrophage or lymphocyte numbers,[226,227,295] and manipulation of Leydig cell function and androgen production by a number of methods had no effect on survival of parathyroid or pancreatic cell allografts in the rat testis.[935,936,986,1020] On the other hand, treatment with exogenous testosterone suppressed the progression of autoimmune orchitis in rats, by reducing the intratesticular accumulation of macrophages and $CD4^+$ T cells, and pro-inflammatory/Th1 cytokine expression, while increasing the number of Treg cells.[899]

Treatment with estrogen of the recipient rats prior to placement of parathyroid allografts was found to abrogate graft survival in the normal scrotal testis.[266] The problem with interpreting this finding is that estrogen inhibits androgen production by the Leydig cell, but probably has direct effects on immune responses and graft rejection in the testis as well. Estrogens regulate dendritic cell differentiation and activity,[217,218] and overexpression of CYP19A, leading to an elevated estrogen-to-androgen ratio causes progressively severe inflammation in the mouse testis, with massive proliferation and activation of intratesticular macrophages and testicular damage.[1021] Overall, it remains difficult to say what important role androgens and estrogens may play in regulating immune responses in the testis. Moreover, it should be noted that in addition to steroids, Leydig cells produce several other factors with lymphocyte regulating activity. These include the antiproliferative pro-opiomelanocortin peptides,[223,1022] and MIF, which notwithstanding its pro-inflammatory functions, inhibits the cell killing activity of cytotoxic T cells and NK cells.[1016–1018]

Soluble Immunosuppressive Activities and Bioactive Lipids

Rat testicular interstitial fluid is a potent inhibitor of T cell activation responses in vitro, in spite of the presence of substantial levels of locally-produced IL1, clearly indicating that soluble immunosuppressive factors are a principal influence on lymphocytes circulating through the interstitial tissue.[965,1023] Similar inhibitory effects on lymphocytes in vitro have been observed using whole testis extracts from mice.[1024] Early investigations established that this inhibition could not be attributed to androgens,[1023,1025] or to other candidate molecules known to be present in testicular interstitial fluid, such as PGE_2,[1026] TGFβ family members,[274,1027] or IL10.[1028]

Purification of this activity established that the lymphocyte inhibition was due to the presence of several lyso-glycerophosphatidylcholines (lyso-GPCs) in the interstitial fluid.[1029] These molecules are produced by the cleavage of a single fatty acid chain from plasma membrane phosphocholine-containing phospholipids via the action of PLA_2, and are known to possess potent anti-inflammatory and immunoregulatory functions (Figure 19.12).[1030] The actual mechanisms have proven difficult to study because lysophospholipids are cytotoxic at high concentrations.[1031] It still remains to be established whether the effects of lyso-GPCs on T-cell activity at physiological concentrations are mediated by a specific receptor, a direct physical interaction with the cell membrane, or both. However, it has been demonstrated that these lyso-GPCs are specific ligands for CD1D-restricted T cells, suggesting a role for these molecules in controlling NK T-cell responses and activity in the testis.[1032] Other lysophospholipds, such as lysophosphatidic acid and sphingosine-1-phosphate, for which G protein-coupled receptors are already known, have been implicated in regulating spermatogenesis and spermatogenic cell survival.[1033] The production, regulation, and activity of these molecules within the testis certainly deserve further study.

Prostanoids, which are derived from arachidonic acid cleaved from plasma membrane phospholipids by

the action of PLA_2, are widely produced throughout the testis due to the promiscuous expression of PTGS2 and PTGS1.[615] In culture, Sertoli and/or Leydig cells can produce all the classes of prostanoids: PGE_2, $PGF_{2\alpha}$, PGD_2, PGI_2 and TxA.[425,617,1034,1035] In the adult rat testis, PGE_2 and $PGF_{2\alpha}$ predominate, with PGD_2 detected at much lower levels; there is no comparable data for TxA or PGI_2.[1036] Direct evidence for the role of prostanoids in regulating testicular inflammation and immunity, however, is slight.

In macrophages from other tissues, PGE_2 has been shown to reduce pro-inflammatory activity and promote an alternatively activated (M2) macrophage functional phenotype.[1037–1039] Studies by Kern and colleagues confirmed that the addition of the broad PTGS inhibitor, indomethacin, to cultures of rat testicular macrophages stimulated their production of IL1β, IL6 and TNF in response to LPS-stimulation and blocked their inhibitory effect on peripheral blood lymphocyte proliferation in vitro.[269,277] It was suggested that PGE_2 might be responsible for the testicular macrophage phenotype that favors immune privilege. Working against this hypothesis, testicular macrophages produce little PGE_2 under normal conditions, and blocking PTGS2 activity in vivo or in vitro did not affect the production of pro-inflammatory cytokines in the testis in response to LPS.[276,615,619] However, chronic inhibition of PTGS2 did inhibit interstitial fluid formation in rat normal testes, even though this treatment ameliorated the loss of fluid that usually occurs during LPS-induced inflammation.[619] These data indicate that prostanoids are involved in control of vascular tone in the normal and inflamed testis. This suggests that the use of anti-inflammatories by men with marginal fertility might need to be considered as a factor when undertaking assisted reproductive therapies.

Spermatogenic Cell and Testis Transplantation

Since the pioneering work of Brinster and colleagues,[1040] the restoration of fertility in mice with congenital or chemically-induced absence of spermatogenesis, through transfer of isolated spermatogonial stem cells from fertile donors is now routine. This procedure usually involves injection of spermatogonia directly into the rete testis or efferent ducts of the recipient. Cross-species transplantation has only been fully successful in the case of rat spermatogonia to the mouse testis,[1041] although spermatogonial proliferation and even partial spermatogenesis in the murine testis has been observed for a range of species, including the human.[1042,1043] Spermatogenic development of transplanted stem cells in the testis of species other than the mouse also has been achieved, including rat into rat,[1044] and mouse into rat, bovine or monkey testes.[1045,1046] Successful transplantation of Sertoli cells[1047,1048] and Leydig cell stem cells[1049] have been reported in mice, using similar protocols.

Studies on spermatogenic stem cell transplantation generally employ a histocompatible or immunodeficient recipient. Allogeneic transplantation has been performed in a number of domestic animals and in monkeys with intact immune systems, causing minor inflammatory complications, but with some degree of success.[1050–1052] Transplantation of rat spermatogenic stem cells into immunocompetent mice, producing epididymal spermatozoa in a small number of animals, has also been reported.[1053] A characteristic feature of transplantation in immunologically intact animals appears to be an increase in peritubular macrophages and macrophage invasion into the seminiferous epithelium and tubule lumen.[1054,1055] Whether these cells may be serving an immunoprotective function, or are simply responding to an inflammatory stimulus from degenerating spermatogenic cells, is unclear. In a limited study of the immunological parameters affecting spermatogonial transplantation,[33] Kanatsu-Shinohara and colleagues found that allogeneic transplantation of mouse spermatogonia was only partially successful, with arrest at the round spermatid stage in highly disorganized tubules, mononuclear cell infiltration in the interstitial tissue and eventual rejection after several months. Mature spermatozoa and successful natural mating was able to be achieved by the use of donor stem cells from immature testes and treatment of the recipient animal with anti-CD4 and anti-CD8 antibodies or the immunosuppressive agent, rapamycin, to inhibit T cell activity.

The available data suggest that spermatogonial stem cell transplantation can be performed across allogeneic and xenogeneic barriers without rejection, but that the preparation of the donor cells and recipient and the transfer procedure itself may affect the outcome. This appears comparable to the variable success experienced in studies on engraftment into the interstitial tissue. It is not yet clear what the critical factors may be, but it should be noted that the spermatogonial cells normally are injected via the rete testis or efferent ducts, and into testes that lack mature germ cells. The rete testis is the most susceptible region of the testis for initiation of autoimmune reactions.[267,268,888] It is possible that the cytotoxic treatments used to deplete the endogenous spermatogenic cells may increase the risk of graft failure and rejection, or that the lack of endogenous spermatogenic cells may contribute to an absence or failure of tolerance to these cells, or alterations in the local immunoprotective environment. Possibly the most important factor that requires investigation is the composition of the spermatogonial cell preparation itself, and the degree to which it might be "contaminated" with resident testicular macrophages or Sertoli cells, as these have potent immunoregulatory properties.

The Definition and Significance of Testicular Immune Privilege

The original definition of immune privilege specifically applied to tissues where normal rejection responses against foreign tissue grafts, specifically allografts or xenografts, are reduced or prevented,[37,929] but the term is most commonly used to refer to a site where lymphatic drainage is deficient or access by immune cell is restricted. Sequestration of antigens of the central nervous system behind the blood–brain barrier is usually considered to be the best example of this.[1056] However, these are principally functional definitions, which emerged prior to discovery of the cellular and molecular mechanisms underlying immunological tolerance and the regulation of immunity. A more contemporary definition of immune privilege, which encompasses both of these traditional concepts as well as more recent immunophysiological principles, can be stated as "the extended survival of cells expressing antigens that under normal circumstances should provoke an immune response, as well as the mechanisms that contribute to this survival".[1057]

It appears that testicular immune privilege is dependent upon a number of overlapping mechanisms: (1) maintenance of central and peripheral tolerance, (2) the blood–testis barrier, (3) reduced immunogenicity of the testicular cells, (4) the anti-inflammatory phenotype of the resident macrophages, (5) immunosuppressive properties of the Sertoli cells and Leydig cells, and (6) local production of immunoregulatory/immunosuppressive cytokines, steroids and other bioactive lipids. The existence of multiple mechanisms may help to explain why orchitis and testicular autoimmunity affects only a minor subset of patients with PGA syndromes, and are not universally induced by day 3 postthymectomy in rats and mice. Moreover, based on the evidence from transplantation studies, it would appear that the ability of antigens within the testis to evade the immune system is not an absolute property of the testis or its draining lymph nodes, but is provisional upon certain criteria being met, such as the absence of testicular damage or other precipitating events. Tissues traditionally identified as being immunologically privileged, such as the testis and anterior chamber of the eye, may simply lie at one extreme of a range, as many of the mechanisms implicated in testicular immune privilege also exist to a more or lesser extent in most tissues. It might be most appropriate to think of immune privilege as simply a manifestation of a particularly effective immunoregulatory environment. The need for enhanced immunoregulation in tissues such as the testis or the eye may be related to the burden of the autoantigens present, and/or the need to prevent active inflammation causing damage to particularly susceptible tissues. While it is debatable whether autoimmunity to spermatogenic cells is more catastrophic to the organism than autoimmune gastritis, diabetes or thyroiditis, there must have been considerable evolutionary selection pressure applied to ensure that the immune system was restrained from attacking the post-meiotic germ cells the moment that they appeared during sexual maturation.

Immunology of the Epididymis and Vas Deferens

In contrast to the testis, the remainder of the male reproductive tract does not appear to be immunologically privileged, at least not in terms of extended graft survival.[1058,1059] This raises the question as to how sperm are able to continue to evade the immune system once they leave the testis environment. It is evident that the epithelial tight junctions of the epididymis and vas deferens are not as elaborate or effective as those of the blood–testis barrier.[72,86,91–93] Moreover, leukocytes—dendritic cells, macrophages and $CD8^+$ T cells, in particular—are commonly found within the epithelium of these tissues and appear to be able to have contact with the luminal contents.[65,96,98,102,334] Nonetheless, the fact that autoantigenic sperm can survive within the epididymis for considerable periods of time without eliciting an autoimmune response indicates that effective immunoregulatory mechanisms must be in operation.[101]

In general, the epididymis and vas deferens appear to be significantly more susceptible to inflammation and autoimmunity than the testis. Immune cell infiltrates are much more likely to appear in the epididymis than the testis in aging mice, in mice injected with *B. pertussis* and adjuvant, in mice with the alymphoplasia mutation, and in mice with a deletion of the AIRE transcription factor.[913–915,923] In addition, epididymitis or epididymovasitis precedes orchitis in the day 3 thymectomized mouse model, and following transfer of lymphocytes from mice immunized against spermatogenic cell antigens.[267,874,876] These observations suggest that the epididymis and vas deferens may be much more reliant on maintenance of tolerance to protect the sperm from immunological damage, compared with the testis.

Investigation of the incidence of sperm antibodies in men with obstructive azoospermia, congenital absence of the vas or following vasectomy indicated that lesions in the caput epididymis are associated with negligible antibody formation, whereas 66–100% of patients with obstructions in the cauda epididymis or postvasectomy had sperm antibodies (Figure 19.15).[348] This clearly indicates that obstruction or damage to the epididymis and vas deferens is a potent inducer of sperm autoimmunity, but that proximity to the testis reduces the severity of the response. Expression of inflammatory and immunoregulatory molecules and the distribution of immune cells in the epididymis and vas deferens is highly regionalized.

Specifically, there is a much greater number of dendritic cells, macrophages and T cells in the proximal epididymis, than in the more distal epididymis and vas deferens, and the dendritic cells appear to be more active.[96,98,102] Recently, it has been discovered that the immunoregulatory enzyme, IDO, is highly expressed in this region of the epididymis, and pro-inflammatory cytokine expression is higher than normal in the caput epididymis of IDO-deficient mice.[1060,1061] Moreover, IDO is regulated through the SMAD2/3/4 signaling pathway that is activated by activin A,[1005] and activin A levels are also highest in this region of the epididymis.[789] It can be proposed that activin A may drive IDO expression in the proximal epididymis, and together these immunoregulatory proteins induce a tolerogenic program in the intraepithelial dendritic cell and T cell populations. Although this hypothesis still remains to be confirmed, this would provide an effective mechanism for promoting tolerance to sperm antigens as they emerge from the immune privileged environment of the testis. The activity of Treg cells has been shown to control the pattern and severity of autoimmune responses to vasectomy in mice.[957]

Within the wider mucosal immune system, there is evidence that epithelial cells themselves can present antigen to activate immunosuppressive/regulatory T cell function,[188,1062,1063] and in this regard it is relevant that the principal cells of the epididymis are constantly absorbing large quantities of luminal fluid and its contents, including spermatozoa.[1064,1065] There is evidence that these cells also produce IL10.[809,1066] Moreover, epithelial cells of the epididymis and vas deferens express the glycosylphosphatidylinositol–anchored protein, CD52,[1067] which is a co-stimulatory molecule for Treg cell development,[1068] but may also play a role in protecting sperm against complement-mediated damage.[1069,1070] Consequently, removal of defective sperm from the epididymal lumen by the principal cells and presentation of their antigens to the intraepithelial lymphocytes might present an additional mechanism for controlling sperm autoimmunity in the epididymis, and possibly the vas deferens as well.

In addition to the tolerogenic mechanisms shared with the common mucosal system, there is evidence of other immunoregulatory mechanisms that may be more specific to the male reproductive tract. For example, immunosuppressive factors produced by the testis may diffuse into the epididymal fluid, and contribute to the unique immunoregulatory environment of the caput epididymis.[348] Modifications of the sperm surface membrane by epididymal secretions may act to obscure sperm antigens,[85] and expression of immunoregulatory molecules on the surface of the sperm itself, which include classical and nonclassical MHC antigens,[968,970] a CD4-like MHC ligand,[1071,1072] bacterial and viral TLRs,[451,802] and FASL,[492] also may play a role in evading immune responses in the epididymis and vas deferens.

Immunoregulation by Seminal Plasma

Seminal plasma is profoundly immunosuppressive, as defined by the ability to inhibit various T cell and NK cell activities in vitro.[1073,1074] This immunosuppressive activity has been proposed to play a role in preventing lymphocyte responses against sperm autoantigens in the male and female reproductive tracts,[856,1075] and, more recently, to prime the female immune system to tolerate paternal antigens on the developing fetus.[1076] The activity can been attributed to a number of specific and nonspecific factors, including prostasomes,[1077,1078] oxidized polyamines,[1079] prostaglandins of the E series,[1074,1080] nonspecific lymphocyte-suppressing proteins,[1081,1082] and immunoregulatory cytokines.[817,818,1083–1085]

Prostasomes are multilaminar vesicles secreted by the normal prostate, and are a major component of human semen.[1078] Pure preparations of prostasomes inhibit mitogen-induced T cell proliferation and inhibit macrophage phagocytic activity in vitro.[1077] The complement inhibitors, CD46, CD55 and CD59, have been found on the surface of prostasomes.[1086,1087] Seminal plasma also contains very high concentrations of the polyamines, spermine and spermidine.[1079] These polyamines are not immunosuppressive themselves, but are converted to their oxidized forms that are inhibitory of cell growth by the action of polyamine oxidase, an enzyme found in serum used in culture media.[1088] Oxidized polyamines are unstable and rapidly metabolized to the cytotoxic molecules, acrolein and putrescine.[1089] Prostasomes and polyamines are responsible for much of the apparent immunosuppressive activity of the ejaculate measured using lymphocyte cultures, but whether these factors have any physiological significance in terms of controlling immune responses in vivo remains speculative.[1090] On the other hand, following removal of the prostasomes and inactivation of polyamine activity in human seminal plasma samples from infertility clinic patients an inverse relationship between T cell inhibitory activity and the incidence autoimmune infertility associated with sperm antibodies has been observed.[1091]

Human seminal plasma contains extraordinarily high concentrations of PGE_2, PGE_1 and their 19-hydroxylated forms.[1092,1093] Aside from their well-characterized effects on vascular permeability and smooth muscle contractility, these hormones inhibit T cell proliferation, NK cell cytotoxicity and the pro-inflammatory activities of macrophages and T cells.[609,1094] They are produced throughout the male reproductive tract: the prostaglandins in seminal plasma largely come from the seminal vesicles, although the vas deferens also may be a major source because this tissue expresses extremely high levels of COX2 under normal conditions.[614,801] Following removal of the prostasomes and inhibition of polyamine oxidation, prostaglandins appear to be

responsible for most of the immunosuppressive activity of human seminal plasma.[284,818] Seminal plasma PGE_2 has been implicated in the promotion of tolerogenic responses in dendritic cells and the production of Treg cells.[1076,1095]

The cytokines with immunosuppressive/immunoregulatory activity that have been positively identified in human seminal plasma are TGFβ1 and TGFβ2,[1083,1085,1096] IL10,[817,1084,1097] and the activins, A and B.[1098,1099] The TGFβ in seminal plasma is derived from the distal tract, comprising the seminal vesicles and/or the prostate gland.[1096] IL10 is a product of monocyte/macrophages and T cells, but epithelial cells of the male tract are also a potential source of this cytokine.[809,1066] Although activin A subunit mRNA and protein is expressed in the human and mouse prostate,[537,1100] activin A levels in human seminal plasma are effectively eliminated by vasectomy, indicating a principally testicular or, more likely, epididymal origin.[789,1098] The most abundant and effective of the immunosuppressive cytokines in seminal plasma is TGFβ1, by a considerable margin.[818,1091,1101]

A positive relationship between the level of immunosuppressive activity in human seminal plasma and the presence of sperm antibodies has been observed,[818] but the actual relationship between immunosuppressive molecules in seminal plasma and immunological infertility in men remains to be established. It also should be borne in mind that these molecules have direct effects on the epithelia, stroma and leukocytes of the female genital tract.[1101,1102] Animal studies show an intense but transient inflammatory response in the endometrium at mating stimulated by seminal plasma and one consequence of this inflammatory response is the induction of a state of hyporesponsiveness to paternal MHC class I antigens.[1103] Insemination is causally linked to the activation and expansion of populations of lymphocytes mediating active immune tolerance at the embryo implantation site, specifically Treg cells, a process in which seminal plasma TGFβ1 and PGE_2 have been particularly implicated.[857,1101,1103] Semen may therefore play a critical role in providing the antigenic and environmental signals necessary to initiate an appropriate maternal immune response to the conceptus during pregnancy.

Other immunosuppressive/immunoregulatory molecules that are neither prostaglandins nor cytokines, have been detected in seminal plasma or accessory gland secretions from various species.[1081,1082] Some of these molecules have been identified, including the protein clusterin and a prostatic steroid binding protein in rats,[1104,1105] IgA,[816] and soluble HLA-G.[974] The actual significance of these molecules in immune regulation and autoimmune fertility requires further functional characterization.

INNATE IMMUNITY IN THE MALE REPRODUCTIVE TRACT: THE RESPONSE TO INFECTIONS AND TUMORS

If the male reproductive tract, and the testis in particular, is a site of reduced antigen-specific immune responses, then the question must be asked: *How does the genital tract avoid recurrent infections or the development of tumors?* Interest in the question has been stimulated by the fact that the male reproductive tract is a major site of transmission of human immunodeficiency virus (HIV),[1106] and by the observation that relapsing lymphoblastic leukemia in the testis following treatment is a frequent problem in male patients.[1107] While the progression of HIV in the male reproductive tract is very poorly defined, destruction of the spermatogenic cells is a characteristic feature of HIV infection in men.[1108] The testis is also suspected to be a sanctuary where HIV may be able to take refuge during therapy, possibly behind the blood–testis barrier, thereby subverting therapeutic effectiveness.[1109,1110] Studies by Jahnukainen and colleagues in rats have suggested that testicular relapse of leukemia may be due to the unique immunoregulatory environment of the testis and, specifically, the ability of Leydig cells to bind lymphocytes.[1111] Moreover, it appears that the immunological protection in the testis is extended not just to spermatogenic cell antigens and graft antigens, but also to tumor-specific antigens that would induce immune reactions elsewhere in the body.[1112]

As a result of these and other observations, it has been suggested that virally or tumorigenically transformed cells might be able to evade both the immune system and cytotoxic drugs by 'hiding-out' in the testis. The reality is, however, that infection of the testis is relatively rare compared with the remainder of the genital tract,[860] and testicular tumors are no more frequent than tumors in other parts of the body.[1113] Moreover, when they do occur, testicular tumors are accompanied by the expected mononuclear cell infiltrates, which are related to the size, progression and type of the tumor.[312,316] The effective deficiency in adaptive immune responses within the testis, therefore, implies that innate (pathogen-specific) immunity might have increased importance for dealing with tumors and infected cells at this site. Hypothetically, relying principally on mechanisms of innate immunity to provide protection against infections and tumors reduces the risk that inflammation will lead to antigen-specific immune responses and subsequent autoimmune damage to fertility. There is some justification for this assumption, based on studies of the male reproductive tract and from analogy with the rest of the common mucosal system. The mechanisms responsible broadly fall into cell-mediated responses and secreted molecules.

Cellular Responses

Although the environment of the male reproductive tract tends toward suppression of antigen-specific immunity and maintaining tolerance, these tissues are densely populated by effector cells involved in innate immunity, specifically macrophages and lymphocytes expressing CD8, which is expressed by both cytotoxic T cells and NK cells.[65,66,70,96,98,227,334,354,357] Significantly, NK cells are able to recognize and destroy infected or transformed cells without prior immunization, and so can act independently of the adaptive immune response. In addition to the testicular macrophages, both the Sertoli cell and Leydig cell are able to recognize and respond to bacterial pathogens directly,[376,386,387,390,392,994] through expression of specific TLRs on their surface. The importance of the TLRs in protecting against infection in the male reproductive tract is indicated by the fact that pathogens can increase their virulence by subverting the function of these molecules, for example, in the case of uropathogenic strains of *E. coli*.[275,436] Suppression of TLR expression throughout the male reproductive tract also appears to be a strategy utilized by HIV to evade immunity.[784]

All mucosa protect themselves through a number of nonimmunological mechanisms that support the integrity of the epithelial barrier. These include: tight junctions that restrict passage between cells, secretion of mucins and other antigenically inert molecules, control of local pH, recruitment of phagocytes (macrophages and neutrophils) to the epithelium, and production of lysozyme, ROS, proteases, complement components and antimicrobial cytokines, and peptides.[162,182,190,1063] It could be productive to investigate the role of these processes in resisting infection in the male reproductive tract.

Secreted Factors

Immunoglobulins

Immunoglobulin A is found in the fluid of all tissues of the male reproductive tract, but as vasectomy does not substantially alter IgA levels in semen, it appears that most comes from the urethra and accessory glands; more specifically, from the prostate gland.[1114–1116] Plasma cells secreting IgA have been found in the urethral gland,[354,1117] but most IgA in the reproductive tract is believed to be derived initially from the circulation.[190,355] Production of secretory component by the prostatic epithelium, which is required for transport of IgA across the mucosa, is androgen regulated.[1116] In men, bacterial infection of the reproductive tract is associated with a large increase in secretion of IgA in prostatic fluid.[1114] This anti-inflammatory immunoglobulin presumably plays an important first line of defense against infection in the male reproductive tract.

Interferons

These molecules possess a range of immunoregulatory and antiviral actions, including suppression of viral proliferation, cytotoxicity and, in the case of the type 2 interferon, IFNγ, stimulation of MHC class I and class II expression.[115,1118] The viral pathogen receptor, TLR3, which acts via the TICAM1 adaptor protein to induce production of type 1 interferons, is expressed by testicular macrophages, Sertoli cells, Leydig cells, peritubular cells and spermatogenic cells, and by epithelial cells of the epididymis and vas deferens.[438,441,442,450,451] In the testis, activation of TLR3 increases interferon production and interferon-inducible genes involved in viral protection in Sertoli cells, Leydig cells, and even spermatogenic cells.[438–440,450,451,597] Interferon production may also be induced through activation of TLR4. Testicular macrophages produce type 1 interferons constitutively, but viral infections upregulate their expression in both macrophages and somatic cells of the testis.[591,592] The strongest interferon response to infection occurs in the Leydig and Sertoli cells, but only the Leydig cells appear to express IFNγ under such conditions.

Defensins

Defensins are small (3–4 kDa) positively-charged antimicrobial peptides, which are able to disrupt bacteria, fungi, parasites, and some enveloped viruses by forming multimeric pores in the cell membranes of these pathogens.[1119] The defensins belong to one of two families, the α- and β-defensins (DEFA and DEFB).[1120] The β-defensins are produced by most mucosal epithelial tissues, but appear to be preferentially expressed in the urogenital tract, particularly in the testis and epididymis in the male, and their production is stimulated by TLR ligands and cytokines.[1121,1122] Genes encoding these peptides are expressed in discrete clusters along the length of the male reproductive tract,[794,1123] and a number of epididymal-specific β-defensins have been identified in the mouse and the rat.[1124–1126] Several of the β-defensins appear to be required for normal sperm maturation or for maintaining sperm motility in the epididymis, so that changes in their expression during infection also may contribute to sperm dysfunction.[808,1124,1127]

IMPLICATIONS, APPLICATIONS, AND THE NEXT 10 YEARS

This review has concentrated almost exclusively upon inflammation and immunity in the male reproductive tract as a means to understanding immune-based infertility and the impact of infection and inflammation on male reproduction. However, these processes have broader implications relevant to other areas of research and, in this last section, applications to clinical issues of

contraceptive development, transplantation medicine and chronic pain are briefly highlighted.

Immunocontraception

Conceptually, immunocontraception involving a vaccine that targets sperm antigens or reproductive hormones has the advantages of a potentially high degree of specificity and convincing proof-of-principle from the many patients with pre-existing autoimmune infertility. Although vaccines against gonadotropins actually have been trialed,[1128] it is unlikely that such an approach would be readily applicable to contraception in men. Other contraceptive vaccine studies have concentrated on antigens involved in critical sperm functions.[1129,1130] Such approaches raise concerns related to safety, efficacy and reversibility, but progress has been impeded largely by the issue of variability of immune responses after vaccination.[1131] Genetic differences in immune response genes in the human population and the complexity of the immune system itself might make it difficult to develop a single vaccine that works effectively enough in all men to compete with currently available hormone-based approaches to contraception. Another concern would be the risk of inducing more widespread autoimmune disease, since there is an established relationship between sperm autoimmunity and autoimmunity in general.[841,842] Furthermore, it may be difficult to develop a contraceptive vaccine that is entirely reversible. Nonetheless, the targeting of critical reproductive antigens to control fertility is a very attractive idea, which will most likely continue to engage reproductive immunologists. Most benefit from such studies may come from the identification and characterization of molecules with crucial functions in fertility which might be used as molecular targets for other drug agents that are not nonimmunologically-based.

Transplantation Medicine and Immunotherapy

One of the most obvious potential benefits from understanding the principles of immunoregulation as they pertain to the male reproductive tract is the possibility that these mechanisms might be applicable to controlling immunity at other sites. If there are immunoregulatory mechanisms that are more or less unique to the testis, for example, where extended graft survival has already been shown to be feasible, then elucidation of these mechanisms has the potential to lead to entirely novel methods of reducing graft rejection in general. The current methods used in transplantation medicine involve broad spectrum immunosuppressives, principally cyclic peptides of fungal origin that interfere with T cell signaling and prevent proliferation of these immune cells in a nonselective manner.[1132,1133] These drugs have considerable harmful side-effects, such as generalized immune suppression, nephrotoxicity and inherent tumorigenicity,[1134,1135] and require close monitoring and adjustment for the remainder of life. They also have damaging effects on testicular steroidogenesis and spermatogenesis.[1136,1137] Real alternatives or adjuncts to these agents, based on more physiological regulatory principles, would definitely be desirable. In particular, the unique immunological properties of the Sertoli cell raise hopes of an exciting new therapeutic opportunity in cell-based gene therapy.[989,1138] Such applications could include, not only the use of ectopic Sertoli cells to support pancreatic islet engraftment, for example, but also the employment of genetically-engineered Sertoli cell lines for delivery of therapeutic proteins. Time will tell if such a cell-based gene therapy approach is clinically viable.

Sterile Inflammation and Chronic Pain

An important concept that has arisen from studies on inflammatory pathways and immunity in normal male reproduction is the realization that dysregulation of these processes may be a cause of sterile, antibiotic-resistant inflammation and chronic pain. Chronic pelvic and perineal pain is normally associated with epididymo-orchitis, vasitis and/or prostatitis.[10–12,860,910,912] These conditions are usually due to infection or physical damage, but the ongoing production of endogenous ligands and danger signals, autoimmunity or alterations in regulatory signaling may cause inflammation to persist long after the infection has cleared or the trauma has been repaired.[10,12,1139] Prostatic hyperplasia and tumors also may be a cause, and inflammation may arise spontaneously in a susceptible subpopulation of patients. These chronic inflammatory pain conditions are poorly understood, and frequently difficult to treat.[12] Treatment alternatives are limited to standard anti-inflammatory and pain-alleviating measures, and the actual mechanisms involved are not well-characterized. Several prostanoids, including PGE_2 and PGI_2, have pain-inducing properties,[1140] and there is an association between chronic pelvic pain and production of the pro-inflammatory cytokines, IL1β, TNF, IL8 and IFNγ, in particular,[1139,1141] but research in this area is still in need of a rational hypothetical background. Given that these conditions are so poorly understood, extremely debilitating and frequently untreatable, they provide a real opportunity for effective future investigations.

CONCLUSION

In summary, the need for a close functional relationship between the male reproductive and immune systems is self-evident. Spermatogenic cells are susceptible to immunological responses and activation of immunity

against sperm or other elements of the reproductive tract can lead to androgen insufficiency, infertility or chronic inflammation. Inflammation and immune activation directly inhibit the hypothalamic-pituitary-Leydig cell axis at several levels, interfere with essential interactions between the Sertoli cells and spermatogenic cells and increase the potential for sperm antibody formation, a major cause of infertility in men. Nonetheless, spermatogenic cells normally are ignored by the immune system, as are grafts of foreign tissue placed within the testicular capsule. Traditional explanations for the protection of these cells, based on 'immune privilege' of the testis maintained by the blood–testis barrier or by exclusion of immune cells, are not consistent with either the histological organization of the reproductive tract or modern concepts of immunoregulation. A more realistic understanding of the control of immune responses in the male reproductive tract encompasses the activity of immunoregulatory macrophages and lymphocytes and peripheral tolerance, as well as active suppression of antigen-specific immunity by somatic cells involving regulatory cytokines, androgenic steroids and other anti-inflammatory and immunosuppressive factors. Dysregulation of this normal environment caused by infection, local or systemic inflammation, toxic insult, active immunization or deletion of regulatory T cells may activate the circulating immune cells, leading to a range of effects from temporary disturbance of spermatogenesis and steroidogenesis, all the way through to the creation of testis-reactive T cells and autoimmunity. Equally important for maintaining fertility, it appears that the restraints on antigen-specific immunity in the male reproductive tract are counterbalanced by enhanced local innate immune mechanisms and conventional mucosal immunity. In fact, it is increasingly evident that inflammatory signaling pathways are fundamental to the control of normal functions of the seminiferous epithelium, and possibly other functions throughout the male reproductive tract. Studies also suggest that establishment of male reproductive function is linked to the normal development of the local immune environment.

This review is intended to establish the concept that normal male reproductive function and the response to disease represent different facets of the same regulatory environment, involving complex interactions between somatic cells, resident immune cells and the circulating cellular elements of the immune system. The use of the term *immunophysiology* is intended to reflect this conflation of immunology and organ function. Unraveling these processes has obvious importance for issues related to male reproduction, such as autoimmune infertility, germ cell and testis transplantation, reproductive tract infection and chronic inflammatory pain. There is still a long way to go in order to understand these interactions completely, and how they impact upon health and physiology. Many unanswered questions remain, including:

- What are the local factors that regulate recruitment and functional modulation of macrophages and dendritic cells in the testis and downstream reproductive tract?
- What are the specific roles of T cell subsets in protecting intratesticular and sperm antigens?
- What is the relative contribution of local immunosuppressive mechanisms, such as the lyso-GPCs, toward this protection?
- What are the molecular details of inflammatory signaling in the seminiferous epithelium, and how do these impact upon reproductive health?
- What is the real clinical significance of leukocytes, cytokines and antibodies in semen?
- What are the mechanisms that regulate passage through the various testicular compartments, including the testicular capillaries and blood–testis barrier?

It is certain that the dramatic increase in interest, awareness and research activity in this area of male reproductive biology clearly means that there will be considerable new discoveries in the near future, with exciting and perhaps even totally unexpected implications and benefits. Indeed, such studies are crucial, in light of the increasing incidence of idiopathic male reproductive disorders,[1142] established and emerging infections with reproductive tract involvement (severe acute respiratory syndrome, resistant strains of tuberculosis),[863] and the resurgence of preventable diseases, such as mumps orchitis,[858] that threaten male fertility and reproductive health.

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CHAPTER

20

Gonadotropin Signaling in the Ovary

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INTRODUCTION

The gonadotropic hormones follicle-stimulating hormone (FSH) and luteinizing hormone (LH) initiate signaling events in ovarian target cells that govern the reproductive cycle and thus the continuation of a species. The ovarian follicle is the key structure that houses the oocyte. Upon appropriate stimulation by FSH and LH, follicles produce hormones that promote the development of secondary sex characteristics and that regulate the hypothalamic–pituitary axis as well as uterine receptivity. Somatic cells of the follicle contribute to hormone production and provide an environment supportive of growth and maturation of the oocyte. Additionally, the follicle must extrude the oocyte at ovulation to allow for fertilization, and remaining cells within the follicle must differentiate into cells of the corpus luteum that produce hormones necessary to sustain pregnancy. In the absence of FSH, follicles do not develop beyond the preantral stage, and animals are infertile. Similarly, in the absence of LH, ovulation does not ensue, corpora lutea do not form, and animals are infertile. Although there is a great deal of species variability, LH is also uniformly required to initiate progesterone production by the corpus luteum, which is necessary for implantation and the maintenance of pregnancy. Both gonadotropic hormones initiate their activities by binding to cell surface protein receptors. Through complex signaling pathways, FSH and LH initiate distinct, highly coordinated programs of gene expression that are only now beginning to be unraveled.

The existence of the gonadotropins was postulated based on the pioneering physiological studies of Long and Evans in 1922, and this work was quickly followed by the separation of pituitary extracts into the distinct fractions that would become known as FSH and LH by Fevold, Hisaw, and Leonard in 1931.[1] As approaches for the purification of the gonadotropins from various species were perfected in the 1970s, it became possible to begin detailed studies of their actions, leading to our fundamental understanding of the critical roles that they play in both male and female reproduction. A classic study by Greep et al. in 1942 showed that while purified FSH was sufficient to stimulate maturation of ovarian follicles to a preovulatory stage in hypophysectomized rats, LH was required to promote follicle ovulation.[2] These results form the basis for all of the subsequent studies reviewed in this chapter that now define the mechanisms by which FSH promotes follicular maturation and LH stimulates ovulation and oocyte maturation.

Early advances in our understanding of gonadotropin receptor localization and actions were made possible by the development in the 1960s by Hunter and Greenwood of a technique to iodinate hormones.[3] The development of conditional gene knockout technology in mice using Cre-Lox-mediated recombination in the late 1980s allowed investigators to establish the requirement for, and in some cases the function of, hormones, growth factors, receptors, and signaling molecules.[4] Mapping of intracellular signaling pathways regulated by the gonadotropic hormones and growth factors occurred with the development of commercially available antibodies specific to phosphorylated proteins in the 1990s.

In this chapter we review current knowledge about the signaling pathways by which FSH promotes follicle maturation and LH promotes ovulation and corpus luteum formation. We refer the reader to excellent reviews of the FSH[5–7] and LH receptors.[8–10] We focus on FSH and LH

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signaling in granulosa cells of immature preantral follicles and mature preovulatory follicles and in theca cells primarily of the rodent, although examples from other species are also discussed. We do not consider LH signaling pathways and the regulation of LH target gene expression in the corpus luteum; readers are referred to Chapter 23.

FSH SIGNALS PROMOTE FOLLICULAR MATURATION

Follicle Maturation

Initial formation and early growth of ovarian follicles occur in a gonadotropin-independent fashion and rely on a number of characterized locally acting regulatory proteins that signal between the oocyte and the somatic cells of the follicle.[11–14] Once the follicle acquires functional FSH receptors, it can respond to this pituitary gonadotropin to undergo further maturation in preparation for ovulation. Only a small subset of ovarian follicles survives to full maturity, with most being lost along the way to a programmed cell death process termed atresia.[15,16] FSH-stimulated maturation of ovarian follicles to a preovulatory phenotype encompasses not only an explosive increase in the proliferation of granulosa cells contained within the follicle, resulting in rapid follicle growth, but also granulosa cell differentiation. As a consequence, the follicle develops a fluid-filled antrum, although markers for antrum formation are not well established.

Recent microarray results and the Ovarian Kaleidoscope Database reveal that FSH regulates the expression of ~500 target genes[17,18] in granulosa cells that support follicle maturation. Well-known responses that are often used as markers for FSH-dependent granulosa cell differentiation include increased estrogen production resulting largely from expression of the rate-limiting enzyme in estrogen biosynthesis that converts testosterone to estrogen, P-450 aromatase (aromatase); increased progesterone production as a result of increased expression of the rate-limiting enzyme in progesterone production that converts cholesterol to pregnenolone, P-450 cholesterol side chain cleavage (SCC); increased expression of 3β-hydroxysteroid dehydrogenase (3β-HSD), which converts pregnenolone to progesterone; increased expression of membrane receptors for LH and epidermal growth factor (EGF), both of which are required for the ovulatory response; and expression of the α subunit of the hormone inhibin, which feeds back to the anterior pituitary to repress production of FSH.[19–23]

FSH also stimulates the transcription of genes that encode for intracellular signaling molecules, such as the regulatory (R) IIβ subunit of protein kinase A (PKA),[24] the A-kinase anchoring protein (AKAP) microtubule-associated protein 2D (MAP2D),[25] and serum glucocorticoid kinase (SGK).[26] FSH has also been shown to induce expression of the EGF receptor agonist epiregulin[27,28]; the transcription factors early growth response factor-1 (EGR-1),[29] gonadotropin inducible ovarian transcription factor-1 (GIOT-1),[30] and liver receptor homolog (LRH)-1[31]; the follicular-fluid associated protein pregnancy-associated plasma protein-A,[32] which is recognized to be the insulin-like growth factor (IGF) binding protein-4 protease[33]; phosphodiesterases (PDE) 4D1 and 4D2[34] to degrade cAMP; and vascular endothelial growth factor (VEGF), which is reported to be critical for antrum formation, granulosa cell proliferation, and estrogen production, and for increasing the vascularity of the theca cell layer.[35,36] FSH also inhibits the expression of calmodulin kinase IV (CaM kinase IV)[37] and 3,5,3′-triiodothyronine binding protein mRNA.[38]

FSH enhances expression at the protein level of immediate early genes such as JUNB, c-JUN, c-FOS, FRA,[39] and c-MYC,[40,41] although these increases could result in part from posttranslational modifications to enhance protein stability[42] rather than strictly transcriptional regulation. FSH also promotes increased expression of a number of genes involved in reorganization of the microtubule and actin cytoskeleton, such as β-tubulin, the heavy chain of kinesin, and tropomyosin-4.[36,43]

The predominant marker for granulosa cell proliferation is increased expression of cyclin D2.[44,45] Cyclin D2-deficient mice are infertile as a result of the inability of granulosa cells to proliferate in response to FSH.[44] It is interesting, however, that granulosa cells of cyclin D2–null mice differentiate to a preovulatory phenotype, and upon stimulation with an ovulatory concentration of LH, follicles do not ovulate but granulosa cells luteinize in follicles with entrapped ova.[44] While FSH is sufficient to induce certainly the majority of granulosa cell differentiation markers in serum-free primary granulosa cell cultures, expression of cyclin D2 requires not only FSH but also activin.[46–49] These results suggest that in the intact animal, granulosa cell proliferation requires both FSH and paracrine actions of activin.

Taken together these results indicate that the response of granulosa cells to FSH involves a complex and coordinated program of gene expression that comprises the regulation of hundreds of genes.[17,18] The stimulus to promote granulosa cell differentiation requires relatively low but constant levels of FSH; withdrawal of FSH or its mediator cAMP during the 48-h time course of granulosa cell differentiation (of the 4-day rodent estrous cycle) results in incomplete induction of differentiation markers.[50,51]

FSH and the FSH Receptor

FSH is a glycoprotein hormone consisting of two distinct subunits, an α subunit shared with the related

glycoprotein hormones LH, human chorionic gonadotropin (hCG), and thyroid-stimulating hormone, and a unique β subunit (Figure 20.1). FSH is produced in gonadotrope cells of the anterior pituitary, and its synthesis and secretion are highly regulated by neural, pituitary, and gonadal factors.[53,54] The FSH receptor is a seven membrane-spanning G protein-coupled receptor (GPCR) that belongs to the rhodopsin/β-adrenergic family of GPCRs.[55] GPCRs are characterized by the presence of intracellular and extracellular loops linked by transmembrane (TM) α-helices and by their ability to couple to one or more guanine (G) nucleotide binding proteins. The FSH receptor belongs to the subgroup of class A GPCRs that contains Leu-rich-repeat motifs in their extracellular domain as well as the AsnSerxxAsnProxx-Tyr motif in TM 7 and the DRY motif at the border of TM 3 and inner loop 2.[56] The extracellular domain contains at least eight Leu-rich-repeat motifs of approximately 24 residues[6] that are believed to provide structural integrity to the receptor.[5] FSH binds to the large extracellular N-terminal domain of the receptor,[57] although one report stated that the interior of the receptor is also important for both hormone binding and signal generation.[58] Upon binding of FSH to this receptor, the receptor becomes activated. Activation of GPCRs is believed to represent a conformational change that alters the orientation of the TM domains.[59] Circular dichroism studies detected a difference in the secondary structure of the receptor when FSH was bound compared with the unbound state, consistent with the generation of an altered conformation upon hormone binding.[60] Binding of a partially deglycosylated human FSH to the extracellular domain of the FSH receptor showed that specificity is mediated by both the FSH α and β subunits and that upon hormone binding, FSH undergoes a conformational change to a more rigid structure.[61] While these investigators provided structural evidence for the existence of FSH receptor dimerization,[61] a recent report observed a trimeric association between FSH and the extracellular domain of the receptor.[62] These investigators additionally suggest that FSH first binds to a high-affinity subdomain on the receptor, which results in the formation of a sulfotyrosine binding pocket on FSH, with sulfated Tyr335 of the receptor then being inserted into the FSH pocket, leading to receptor activation.[62]

The predominant intracellular signal generated upon FSH receptor activation is cAMP, consistent with receptor coupling primarily to the stimulatory G protein (Gs).[6] Although there are reports that the FSH receptor can also couple to a pertussis toxin-sensitive Gi protein to modulate cAMP production in a Chinese hamster ovarian cell line,[63] and to activate phospholipase C (PLC) upon receptor overexpression in human embryonic kidney (HEK) cells,[64,65] the physiological relevance of these results has not been established.

The FSH GPCR is encoded by a single, rather large gene that consists of 10 exons and 9 introns.[5,6,66] The extracellular domain is encoded by the first 9 exons; the C-terminal part of the extracellular domain, the transmembrane domains, and the intracellular C-terminal domains are encoded by exon 10.[66] This receptor is expressed in females only on follicular granulosa cells[6]; expression is absent from ovaries of postmenopausal women.[6] FSH receptor-null mice are infertile, and follicles do not develop beyond the preantral stage,[67,68] consistent with the view that this receptor is necessary

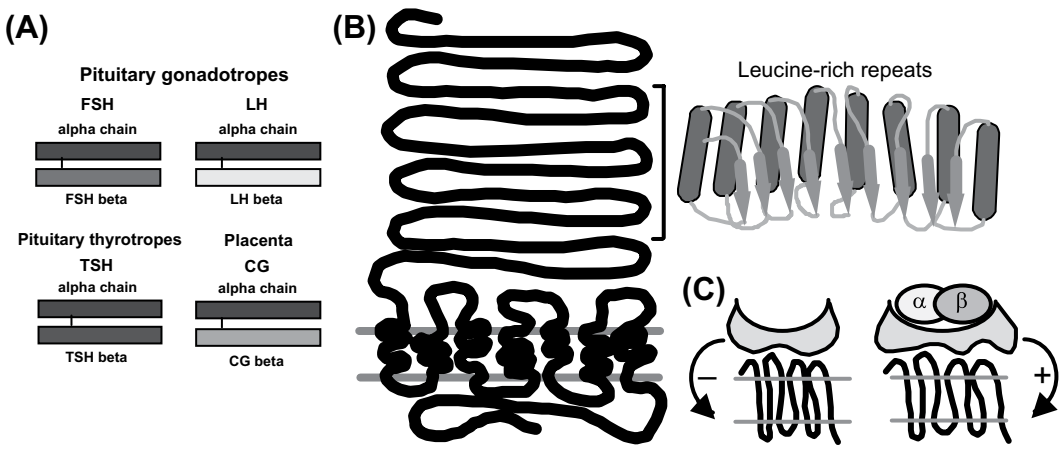

FIGURE 20.1 The gonadotropins and gonadotropin receptors. (A) A schematic of the family of dimeric glycoproteins hormones, which includes the pituitary gonadotropins FSH and LH, pituitary thyroid-stimulating hormone, and, in some species, placental chorionic gonadotropin. The α subunit is shared by all four hormones. (B) Generic schematic of the glycoprotein hormone receptors, indicating the large extracellular domain and the seven membrane-spanning domains characteristic of GPCRs. A significant portion of the extracellular domain is composed of leucine-rich repeats, each composed of a β-strand followed by an α-helix, and these are proposed to form a horseshoe-shaped domain as shown that likely plays an important role in ligand binding. (C) A model showing the view that the extracellular domain of the receptor plays a repressive role in the absence of ligand binding and that ligand interaction and ensuing conformational changes relieve this repression, allowing G protein activation. *Source: Modeled after Ref. 52.*

and sufficient to mediate the effects of FSH. FSH receptor expression is not detected in primordial follicles but has been detected on granulosa cells of preantral follicles from postnatal day 3 in rats; therefore, it is expressed at the very early stages of follicular development.[69–71] FSH receptor expression is increased by FSH or cAMP,[72,73] activin,[72–74] transforming-growth factorβ (TGFβ),[70] nerve growth factor,[75] and IGF-1,[72,76] and is abolished by the preovulatory LH surge that promotes ovulation.[69] Freshly harvested granulosa cells from preantral follicles of rats contain approximately 4500 receptors per cell.[77] Although the promoter of the FSH receptor gene contains an E-box, which is conserved among species and binds basic helix-loop-helix transcription factors such as upstream stimulatory factors 1 and 2, c-Myc and its dimerization partners Max or Mad, and hypoxia-inducible factor 1 (HIF-1),[78,79] and activation of the FSH receptor promoter requires steroidogenic factor (SF)-1[78,80] as well as binding of upstream stimulatory factors to the E-box, the mechanism for selective expression of the FSH receptor only in granulosa cells of female mammals has eluded identification. Exhaustive studies by Heckert and colleagues[7,79] revealed that elements required for cell-selective expression of the receptor are not present within the −5000 to +123 gene region surrounding the transcription start site.

Several naturally occurring inactivating mutations of the FSH receptor gene in humans have been described that result in arrested follicular development and hypergonadotropism. These include the following amino acid changes: Ile143Thr, Ala172Val, and Asp207Val in the extracellular domain, Lys584Val in the extracellular loop 3, and Arg556Cys in cytoplasmic loop 35. These mutations reduce or block FSH-stimulated cAMP generation, either as a result of poor hormone binding to the receptor and/or poor coupling to Gs. Directed point mutations in cytoplasmic loop 2 (Arg450His, Thr453Ala) either block or enhance receptor activation (Lys260Asp), consistent with the notion that this loop acts as a conformational switch.[81] A potential binding motif in cytoplasmic loop 3 for coupling to Gs (BXXBB, where B and X represent basic and nonbasic amino acids, respectively) has been identified.[81] A small molecule "negative allosteric modulator" that binds to the FSH-bound receptor in HEK cells overexpressing the FSH receptor and inhibits cAMP formation ($IC_{50} = 0.7 \pm 0.2\,\mu M$) by apparently stabilizing an inactive conformation of the receptor was recently reported, although the binding site for this compound was not identified.[82]

The only naturally occurring activating mutation identified for the human FSH receptor is Asp550Gly, located in cytoplasmic loop 3.[83] The FSH receptor with this mutation exhibits a modest increase in basal cAMP levels upon transfection into heterologous cells.[83] While the patient with this mutation exhibited normal testis function despite being hypophysectomized (because of a pituitary tumor), he received testosterone replacement therapy that might have contributed to the maintenance of spermatogenesis.[6,83] Directed mutation of Asp580His, a hot spot for activating mutations in other glycoprotein hormone receptors, results in enhanced granulosa cell proliferation, estrogen production, loss of small follicles, and development of hemorrhagic cysts in a mouse model.[84] It is surprising that more activating mutations of this receptor have not been identified.

Like most other GPCRs, the FSH receptor appears to exhibit desensitization or reduced FSH-stimulated cAMP production[65,85] upon exposure to saturating agonist, although granulosa cells likely never see saturating concentrations of FSH under physiological conditions. The mechanism of GPCR desensitization has been particularly well studied for the β-adrenergic receptor. Desensitization of the β-adrenergic receptor occurs rapidly after receptor activation and is characterized by both a rightward shift in the agonist dose–response curve and a decrease in maximal stimulation of adenylyl cyclase.[86] β-adrenergic receptor desensitization requires the phosphorylation of the activated receptor by a G protein-regulated kinase[87] and the consequent high affinity binding of arrestin2 (β-arrestin1) or arrestin3 (β-arrestin2) to the phosphorylated receptor.[88,89] Receptor phosphorylation functions to increase the affinity of the receptor for the arrestin.[88,90,91] Arrestins also play an integral role in receptor internalization based on their ability to bind both clathrin and β_2-adaptin of the AP-2 protein complex.[92–94] Thus, GPCR internalization often is linked to and follows receptor desensitization. Arrestins have also been shown in some cellular contexts to function as adaptors to bind signaling intermediates, such as upstream components in the mitogen-activated protein kinase (MAPK)/extracellular regulated protein kinase (ERK) and c-Jun NH2-terminal kinase (JNK) pathways, to redirect receptor signals to other pathways.[95]

The FSH receptor, upon overexpression (by approximately 10-fold) in a heterologous cell model, becomes phosphorylated in response to FSH on Ser/Thr residues in intracellular loops 1 and 3[96] in a G-protein-regulated kinase 2-dependent manner.[97,98] Mutation of the phosphorylatable residues in intracellular loop 1 leads to reduced receptor internalization.[96] Most of the internalized ligand-bound receptor recycles back to the cell surface, and intact hormone dissociates.[99] FSH also promotes the phosphorylation of a cluster of five Ser/Thr residues in the C-terminal tail of the receptor expressed in heterologous cells that stabilizes the binding of β-arrestin and is required for receptor desensitization but not for receptor internalization.[80] However, neither the rate nor the extent of FSH receptor internalization or FSH receptor desensitization has been studied under physiological conditions in granulosa cells.

There is also evidence that upon overexpression in a heterologous cell model, the FSH receptor not only binds β-arrestin[80] but also binds APPL1 (adaptor protein containing PH domain, PTB domain, and leucine zipper motif).[100] While these authors identified the binding site of APPL1 within intracellular loop 1 of the receptor (Lys376),[64] the physiological significance of this interaction is difficult to establish since the APPL1-null mouse is fertile.[101]

There is a report of an alternatively spliced FSH receptor that contains the first eight exons of the classic FSH GPCR plus a unique C-terminal extension that is hypothesized to traverse the plasma membrane a single time.[102] This receptor, upon expression in HEK cells, localizes to the plasma membrane and binds FSH in a specific and high-affinity manner but does not appear to couple to Gs to activate adenylyl cyclse.[102,103] This alternative FSH receptor is reported to be expressed in mouse ovaries[104] and to activate the ERK pathway in immortalized granulosa cells.[105] The physiological significance of this alternative FSH receptor to granulosa cell function remains to be elucidated.

FSH SIGNALING PATHWAYS THAT STIMULATE GRANULOSA CELL DIFFERENTIATION AND PROLIFERATION: cAMP AND PKA-DEPENDENT SIGNALING

The predominant intracellular signal generated by FSH-dependent signaling through its GPCR is cAMP (Figure 20.2), based on the ability of forskolin to mimic FSH's ability to induce such differentiation markers as progesterone synthesis and LH receptors[50,51,106,107] (reviewed in Ref. 19). cAMP signals predominately by activating the cAMP-dependent protein kinase (PKA).[108] PKA is a tetrameric holoenzyme that consists of two regulatory (R) subunits and two catalytic (C) subunits.

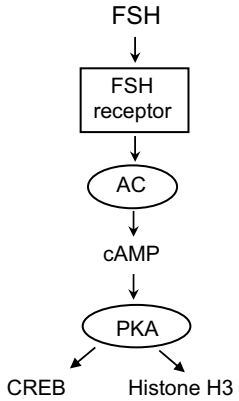

FIGURE 20.2 FSH signals via cAMP to activate PKA. Two PKA substrates in immature granulosa cells are CREB and histone H3.

PKA is activated upon binding of cAMP to the R subunits, resulting in the dissociation of the C subunits that function as active kinases to phosphorylate substrates.[109] Although representatives of an alternative family of cAMP effectors, consisting of cAMP-activated guanine nucleotide exchange factors also known as exchange proteins activated by cAMP (EPACs),[110,111] have been identified in rodent and human granulosa cells,[112] FSH either does not[113] activate or marginally (40%)[114] activates the EPAC target RAP1 in granulosa cells. Moreover, an EPAC-selective cAMP analog does not promote induction of aromatase,[115] a well-known marker of granulosa cell differentiation, or activation of the phosphatidylinositol-3 kinase (PI-3K) pathway.[116] Rather, in granulosa cells FSH activates PKA downstream of cAMP.[117] The cell-permeable selective PKA C subunit inhibitor peptide myristoylated-(Myr-) PKI, the active portion of the ubiquitous PKA inhibitor protein, inhibits the induction of a number of proteins induced by FSH, including MAP2D, inhibin-α, and progesterone producing enzymes.[113,118] PKI functions by binding to the substrate binding site of the C subunits of PKA, thus preventing other PKA substrates from binding, and preventing PKA from phosphorylating substrates.[119] While competitive ATP antagonists that are established inhibitors of PKA, such as H89 and KT5720,[120,121] also inhibit many of these responses initiated by FSH, including the induction of progesterone synthesis, aromatase, LH receptor, RIIβ, EGR-1, SGK, and MAP2D,[29,113,122] these inhibitors also inhibit other kinases, including p70 ribosomal S6 kinase (p70 S6 kinase) and RHO kinase (H89)[123] and phosphoinositide-dependent kinase 1 (PDK-1; KT5720).[124] Taken together, however, these results suggest that PKA is necessary for FSH-stimulated granulosa cell differentiation.

Two classes of PKA holoenzymes, PKA I and PKA II, exist based on the association of two possible RI subunits (RIα and RIβ) or two possible RII subunits (RIIα and RIIβ) with four possible C subunits (α, β1, β2, and γ).[109] PKA phosphorylates a large number of substrates in various cells.[125] Well-documented PKA substrates include CREB (cAMP response element binding protein),[126] PDE4D3,[127] SRC tyrosine kinase,[128] histone H3,[129] glycogen synthase kinase (GSK) α and β,[130,131] and L-type Ca^{2+} channels.[132]

But is PKA sufficient to promote granulosa cell differentiation? This remains a controversial topic that will be addressed throughout this review. The question of whether PKA is sufficient to drive granulosa cell proliferation has been addressed directly over the past 10 years by the Zeleznik group. Increasing intracellular levels of cAMP in preantral granulosa cells to levels equal to those stimulated by FSH either by transducing cells with adenovirally expressed constitutively active Gs[115] or constitutively active LH receptor[133] mimicked the ability of FSH to activate some (*Hsd3b2*, *Inha*) but not other

(*Lhcgr*, *Cyp19a1*) target genes to levels achieved by FSH 48 h posttreatment. However, cotransduction with constitutively active Gs and AKT (protein kinase B) rescued gene expression to levels equal to or greater than FSH,[115] suggesting that signaling via cAMP and AKT pathways is sufficient to activate FSH target genes. Correspondingly, while reducing FSH-stimulated cAMP levels ~ three-fold by transducing granulosa cells with adenovirally expressed constitutively active Gq greatly reduced the ability of FSH to activate key FSH target genes (*Lhcgr*, *Cyp19a1*, *Hsd3b2*, *Cyp11a1*), responses were rescued by the cell permeable cAMP analogue 8-bromo-cAMP.[134] Most compelling are results from a study in which granulosa cells were transduced with lentivirally expressed constitutively active PKA C subunit and compared to cells stimulated with FSH.[17] Microarray analysis of genes regulated 48 h posttreatment showed that the majority of genes were activated to the same extent by FSH and constitutively active PKA, although there were subsets that differed. The two caveats of this approach, as pointed out by the author, are: (1) constitutively active PKA cannot bind to AKAPs, thereby obscuring intracellular compartmentation, and (2) by 48 h, some FSH targets (*Lhcgr*, *Cyp19a1*) may begin to undergo downregulation in response to the potential of the constitutively active PKA C subunit to mimic the surge of LH. We conclude with our present state of knowledge that PKA appears to be sufficient to mediate granulosa cell differentiation, at least qualitatively. However, we expect that additional pathways activated by FSH contribute to optimal target gene regulation to yield a preovulatory follicle.

While most PKA in ovaries with preantral follicles consists of a PKA IIβ holoenzyme,[135] based on DEAE-cellulose chromatography, RIIβ knockout mice are fertile.[136] PKA Iα holoenzyme comprises less than 5% of the PKA activity detected in preantral follicle-dominated ovaries, although there is a large amount of C subunit–free RI.[135] The presence of substantial C subunit–free RI is surprising because RIα not bound to C is reported to be rapidly degraded[137] and may suggest the presence of activated PKA Iα, since free C subunit does not bind to DEAE-cellulose. Rodent ovaries do not express RIβ.[138] Taken together, these results suggest that the actions of FSH could be mediated by PKA Iα rather than by the seemingly more abundant PKA IIβ holoenzyme. Because RIα-null mice died at embryonic day 10.5,[139] proof that RIα is crucial to granulosa cell differentiation requires the generation of mice with an RIα deletion targeted to granulosa cells.

AKAPs Target PKA to Specific Subcellular Locations

The specificity of PKA action is accomplished by the targeting of PKA as well as its substrates to specific cellular locales by virtue of the binding of PKA regulatory subunits to a large family of AKAPs. It has been estimated that more that 75% of PKA holoenzymes are targeted to specific intracellular sites via association of PKA regulatory subunits with AKAPs.[109] RII subunits bind with nM affinity to AKAPs.[109,140] The domain on the AKAP responsible for RII binding comprises an amphipathic helix that binds the N-termini of the PKA-RII dimer.[141] AKAPs direct PKA to such locations as the mitochondria, Golgi apparatus, centrosome, nuclear envelope, and actin and microtubule cytoskeletons by the presence of specific subcellular targeting domains on each AKAP,[142–144] as depicted in Figure 20.3. Localization of PKA and one or more substrates to distinct regions within the cell is thought to promote both specific and efficient substrate phosphorylation in response to a stimulus.[144] AKAPs not only bind PKA but also function as platforms to coordinate signaling cascades by binding additional signaling proteins such as CaM, PDEs, protein phosphatases such as PP1 and PP2A, and protein kinases such as the tyrosine kinase ABL and the Ser/Thr protein kinase C (PKC) enzymes.[143,144] By confining PKA and specific substrates to isolated cAMP gradients at discrete cellular locations, not only are signaling cascades optimized but also their fidelity is maintained, thereby preventing inappropriate cross talk among pathways.[143]

Most known AKAPs anchor PKA II holoenzymes and exhibit at least a 100-fold lower affinity for PKA I holoenzymes.[143] Although there are a growing number of "dual specificity" AKAPs that readily bind PKA I holoenzymes, these AKAPs still exhibit a 10- to 25-fold preference for RII.[145] However, fibrous sheath protein 1,[146] the *Caenorhabditis elegans* $AKAP_{CE}$,[147] the peripheral benzodiazepine receptor-associated protein PAP7,[148] the neurofibromatosis 2 tumor-suppresser protein merlin,[149] sphingosine kinase interacting protein,[150] and GRB2

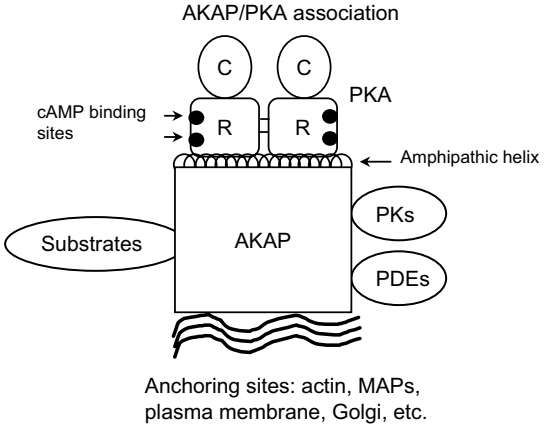

FIGURE 20.3 Schematic model of the association of AKAPs with PKA, other protein kinases (PKs), PDEs, substrates, and anchoring sites in cells.

(growth factor receptor binding protein 2) associated binding protein 2 (GAB2)[116] appear to preferentially bind RI and thus are PKA I AKAPs.

Preantral follicle-dominated ovaries express a large number of AKAPs, including AKAP-KL, AKAP79, Ezrin, AKAP-149, AKAP95, AKAP220, and AKAP100, based on RII overlay and western blotting results.[135] However, neither the selective link of each AKAP to individual PKA substrates and signaling pathways nor the cellular location of these AKAPs has been investigated. Thus, the cellular mechanism(s) by which each of the AKAPs present in granulosa cells modulates cellular functions is not known. We recently identified GAB2 as an RI AKAP and a direct PKA substrate in preantral granulosa cells that appears to orchestrate signaling of PKA into the PI-3K pathway, as discussed below, although formal proof of this link is lacking.[116]

PKA Targets in Undifferentiated Granulosa Cells: CREB and Histones H3 and H1

Defining the signaling pathways that are activated by FSH in granulosa cells and determining how PKA coordinates signaling into these pathways remains a central challenge in ovarian biology. Although details of the signaling pathways activated by FSH are just beginning to be elucidated, it is clear that FSH stimulates the PKA-dependent phosphorylation of the established PKA substrates CREB on Ser133[151,152] and histone H3 on Ser10.[117,118] Both FSH-stimulated CREB and histone H3 phosphorylations in granulosa cells are abrogated by the PKA inhibitor Myr-PKI, suggesting that PKA is the predominant kinase that phosphorylates these proteins (Figure 20.2). CREB and histone H3 are direct PKA substrates.[118,153]

It is interesting that in other cellular models, both CREB and histone H3 are phosphorylated on the same sites by protein kinases other than PKA. For example, CREB kinases in other cells include the PI-3K substrate AKT,[154] CaM kinases, the ERK substrates p90 ribosomal S6 protein kinases (RSK) and the mitogen- and stress-activated protein kinases 1 and 2 (MSK1 and -2), and the p38 MAPK substrates MAPK-activated protein kinases 2 and 3 (MK-2 and -3)[155,156]; however, inhibitors of these kinases do not reduce FSH-stimulated CREB phosphorylation in granulosa cells.[113,118] Similarly, in other cells histone H3 is commonly phosphorylated by the ERK substrate RSK2 and by the ERK and p38 MAPK substrate MSK-1.[157–159] However, inhibitors of ERK activation and of activated p38 MAPK do not reduce histone H3 phosphorylation in granulosa cells.[118] These data support the notion that PKA plays a unique role in granulosa cells to regulate signaling pathways.

FSH enhances the phosphorylation of histone H3 on Ser10 as well as the acetylation of Lys14, although it is not clear whether these modifications occur simultaneously or in a sequential manner.[118] The histone code hypothesis put forth by Strahl and Allis[160] stated that covalent modifications of core histone tails result in the remodeling of chromatin to affect downstream events. The best-known core histone modifications consist of the covalent addition of an acetyl group to Lys, the addition of one or more methyl groups to Lys or Arg, and addition of a phospho group to Ser or Thr. These additions occur primarily on the N-terminal tails of histones H3 and H4. Acetylation neutralizes the positive charge of the histone and phosphorylation adds a negative charge, thereby decreasing the affinity of histone for DNA.[161] The predicted result of histone acetylation and/or phosphorylation is the loosening of chromatin structure, resulting in increased access of select promoter regions to transcription factors and co-activators.[160,162] Methylation can correlate either with gene activation or gene inhibition and can occur on the same Lys residues that are acetylated, and Lys residues can be mono-, di-, or trimethylated, whereas Arg residues can be mono- or dimethylated. Arg methylation of histone H3 generally correlates with gene activation.[163] For histone H3, methylation of Lys4 correlates with gene activation, whereas methylation of Lys9 and Lys27 correlates with gene repression.[164,165] Histone acetylation is catalyzed by histone acetyltransferases, such as CREB binding protein (CBP), histone deacetylation is catalyzed by histone deacetylases, and histone methylation is catalyzed by methyltransferases.

Consistent with the histone code hypothesis, increased *c-Fos*, *Sgk*, and *Inha* promoter DNA is detected in the dual-phosphorylated and -acetylated histone H3-chromatin pools in response to FSH treatment of granulosa cells in chromatin immunoprecipitation (CHIP) assays.[118] Because the *c-Fos* promoter contains a functional cAMP response element,[166] signaling to activate *c-Fos* in granulosa cells probably reflects contributions from CREB. In this instance, the C subunit of PKA would function not only to phosphorylate CREB to promote binding of CBP[167,168] but also to phosphorylate histone H3, whereas CBP perhaps promotes the acetylation of histone H3 as well as recruitment of other co-activators and the basal transcription machinery.

Activation of the *Sgk* gene in response to FSH/PKA requires an Sp1 (specificity protein 1) binding site.[26] Because the phosphorylation and acetylation of histone H3 are linked to the rapid activation of the *Sgk* gene, perhaps co-activators with histone acetyltransferases activity as well as the C subunit of PKA complex with Sp1 at the *Sgk* promoter. It is possible that the stimulatory effect of PKA on *Sgk* transcription reflects H3 phosphorylation and chromatin reorganization in addition to direct phosphorylation of transcription factors. The mechanism by which PKA enhances the transcriptional activity of Sp1 is not clear, but as discussed later in "Cross Talk among FSH-Regulated Signaling Pathways and Transcriptional

Activators to Regulate Gene Expression," both ERK and the AKT target PKCζ are candidate kinases.[169] Histone H3 phosphorylation and acetylation is also linked to activation of *Inha* subunit gene expression. Activation of the *Inha* promoter requires synergism among PKA-phosphorylated CREB, SF-1, or LRH-1, and the co-activators CBP and the *Ncoa1* product steroid receptor co-activator-1 (SRC-1).[170] SF-1 and CREB constitutively bind to and interact (directly or indirectly) on the *Inha* promoter,[170] and CBP is recruited by and binds to phosphorylated CREB[167,168] and to SF-1.[171] The dependence on PKA for activation of the *Inha* gene has historically been assumed to be a consequence of the phosphorylation of CREB,[153] leading to CBP recruitment.[168,170] However, it is likely that a member of the cAMP-regulated transcriptional co-activator (CRTC) family also contributes to the ability of CREB to activate the *Inha* gene.[172] In response to elevated intracellular levels of cAMP, CRTC becomes dephosphorylated and translocates from the cytoplasm to the nucleus, where it binds to phosphorylated CREB and enhances its association with DNA.[172,173] It is also well established that PKA potentiates SF-1 transactivation activity. Although SF-1 can be phosphorylated by PKA in vitro, mutation of its consensus PKA phosphorylation site (Ser430Ala) does not affect the stimulatory effects of PKA on SF-1 transactivational activity.[174] It is now recognized that the transcriptional activity of SF-1 requires the co-activator β-catenin.[175,176] We have recently shown that FSH promotes the PKA-dependent phosphorylation of β-catenin on Ser552 and Ser675,[177] consistent with reports in other tissues,[178,179] and consequent activation of the β-catenin/T cell factor (TCF) artificial promoter-luciferase construct, TOPFlash,[177] as discussed below.

It is likely that the rapid phosphorylation of histone H3 on Ser10 by PKA and acetylation on Lys14 by CBP or other histone acetyltransferases constitute a necessary step in the transcriptional activation of many FSH responsive genes, leading to granulosa cell differentiation. FSH is also predicted to promote alterations in the methylation patterns of histone H3, although this H3 modification in granulosa cells preantral granulosa cells has not yet been reported. It is possible that histone H3, in its phosphorylated, acetylated, and possibly methylated conformation, functions as a scaffold to mediate the assembly of the multiprotein complex of transcription factors, co-activators, and basal transcription factors, which leads to transcription.[160] Based on results in a HEK cell line,[170] it is also expected that histone H4 is covalently modified in granulosa cells in response to FSH. Using the CHIP assay with an antibody to acetylated histone H4, it was shown that PKA increased histone H4 acetylation associated with the *Inha* promoter.[170] The increased histone acetyltransferase activity could result either from the recruitment of CBP[180,181] or the recruitment by CBP of a p300/CBP-associated factor PCAF.[182,183]

Histone H1 is also phosphorylated in granulosa cells in response to FSH, although with a slower time course than that of histone H3.[117] Histone H1 binds to the outer surface of the DNA that surrounds the core histones and to the stretches of linker DNA that connect nucleosomes.[184] Histone H1 is known to be an in vitro PKA substrate, with phosphorylation on Ser37,[129,185] and is phosphorylated on Ser37 in vivo in liver in response to glucagon treatment[129] and in response to forskolin addition to N18 neuroblastoma cells.[185] It is predicted that histone H1 phosphorylation contributes to chromatin remodeling, although less is known about the role of this histone in regulating transcriptional activation.

Because most of the known differentiation responses to FSH are mediated by PKA, it was initially assumed that the genes activated by FSH would all contain CREB-binding sites. However, cAMP response elements have been identified only in the promoters of a subset of FSH-regulated genes, including *Inha*,[186] *Cyp19a1*,[152] *Giot-1*,[30] *EGR-1*,[29] and *c-Fos*.[166] Therefore, FSH via PKA must phosphorylate additional targets to activate signaling pathways. Indeed, as discussed later, FSH also stimulates activation of the ERKs and PI-3K pathways in a PKA-dependent manner.

PKA Signals to Activate the ERKs in Undifferentiated Granulosa Cells

The ubiquitous ERKs, which belong to the MAPK family, are activated by a variety of receptor agonists. These kinases are classically activated by receptor tyrosine kinases such as the insulin, EGF, or IGF-1 receptors. Upon activation of these receptors, their consequent autophosphorylation creates specific binding sites for Src homology 2 (SH2) containing proteins such as GRB2.[187] GRB2, complexed with SOS, binds to the receptor tyrosine kinase, and the guanine nucleotide exchange factor SOS promotes activation of RAS by stimulating its GDP release. Active RAS then promotes activation of the Ser/Thr kinase RAF-1, which, in turn, phosphorylates/activates the ERK kinase MEK. MEK then phosphorylates ERK on Thr and Tyr residues, resulting in ERK activation. GPCRs activate ERKs generally by promoting the transactivation of a receptor tyrosine kinase,[188] resulting in its phosphorylation and consequent stimulation of the pathway leading to ERK activation, although the mechanisms of receptor tyrosine kinase transactivation vary among GPCRs and are incompletely understood.[189–191] While GPCR-generated cAMP is most commonly reported to inhibit ERK activation, especially in nonendocrine cells,[192] cAMP has been reported to activate ERK either via PKA by stimulating the phosphorylation of the small G protein RAP1, leading to the activation of B-RAF, MEK, and ERK,[193,194] or by directly binding to and activating the RAP1 guanine nucleotide exchange factor EPAC, leading to activation of B-RAF, MEK, and ERK.[110,111]

While FSH promotes the rapid albeit transient activation of ERK via a PKA-dependent pathway,[85,113,195] the mechanism by which PKA regulates ERK activity in preantral granulosa cells appears to depend in part on the extracellular matrix to which cells attach in vitro. When cells are attached to fibronectin, FSH does not promote the phosphorylation/activation of MEK; rather FSH regulates the association of a 100-kDa protein tyrosine phosphatase (PTP) with ERK in a PKA-dependent manner.[113] In the absence of FSH, a tonic stimulatory pathway promotes activation of the components of the ERK pathway upstream of ERK, including MEK; however, ERK activity is blunted by its association with a PTP that inactivates ERK (Figure 20.4). Consistent with this scheme, the MEK inhibitor PD98059 blocks FSH-stimulated ERK phosphorylation.[113] In the presence of FSH, PKA catalyzes the phosphorylation of the 100-kDa PTP that is complexed with ERK, resulting in dissociation of the phosphatase from ERK.[113] In the absence of the associated PTP, ERK is relieved from inhibition and is activated by the tonic stimulatory pathway.[113] This granulosa cell PTP is recognized by an antibody that recognizes PTP-SL, but based on its larger size and lack of recognition by other PTP-SL antibodies, the ERK-associated PTP in granulosa cells appears to be distinct from PTP-SL and has not been identified.[113]

Regulation of the tonic pathway that promotes activation of MEK in granulosa cells is poorly understood. Both extracellular Ca^{2+}, the EGF receptor, and the tyrosine kinase SRC (Rous sarcoma oncogene) appear to be necessary for FSH-stimulated ERK activation, based on the abilities of Ca^{2+} chelation with ethyleneglycotetraacetic acid, the EGF receptor inhibitor AG1478,[196] and the SRC inhibitor PP1 to abrogate this response.[113] Moreover, Ca^{2+} entry appears to be mediated by L-type Ca^{2+} channels, based on the ability of the L-type Ca^{2+} channel inhibitor nifedipine[113] to block FSH-stimulated ERK activation. Ca^{2+} entry also appears to be upstream of the EGF receptor and Src, because the EGF receptor inhibitor AG1478 and the SRC inhibitor PP1 block the ability of the Ca^{2+} ionophore A23187 to stimulate ERK phosphorylation[113] (Figure 20.4).

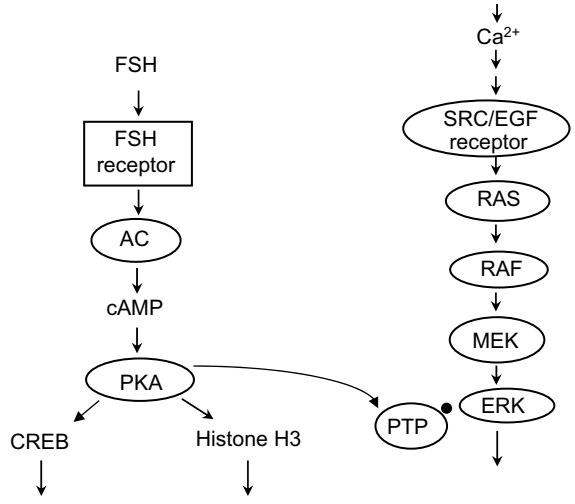

FIGURE 20.4 FSH via cAMP/PKA activates ERK by stimulating the phosphorylation and consequent dissociation of an inhibitory protein tyrosine phosphatase (PTP). A tonic pathway that includes Ca^{2+}, SRC, EGF receptor, RAS, and RAF promotes MEK phosphorylation.

Under similar experimental conditions, FSH-stimulated ERK activation requires PKA, SRC kinase activity, and the EGF receptor, although MEK phosphorylation in the absence of FSH (i.e., the tonic pathway) was not investigated.[185] In contrast, when cells are attached to serum-coated plates, FSH appears to signal via an EGF receptor and SRC-dependent route to activate RAS, leading to ERK activation.[107] There is also evidence that AMP-kinase (AMPK), which is active (phosphorylated on Thr172[197]) in the absence of FSH,[198] complexes with and inhibits ERK activity.[199] While FSH promotes the inactivation of AMPK,[199] the mechanism by which AMPK regulates ERK activity is not known.

Despite an incomplete understanding of the ERK activation pathway in granulosa cells, FSH-stimulated ERK appears to be necessary, but not sufficient, for induction of the MAP2D[113] and cyclin D2, based on protein/mRNA expression in the absence and presence of the MEK inhibitor, PD98059.[198,200] Similarly, this MEK inhibitor attenuates the FSH-dependent induction of the immediate early gene EGR-1 in granulosa cells,[29] suggesting that induction of EGR-1 by FSH requires MEK-dependent ERK activation.

FSH has also been shown to increase protein expression for a number of immediate early genes that comprise the AP-1 family, including JUNB, c-JUN, c-FOS, and FRA2,[39] as well as c-MYC.[40,201] JUNB has been shown in granulosa cells to regulate the expression of the *Inhb* promoter.[202] A recent report suggested ERK-dependent phosphorylation of these proteins stabilizes them by inhibiting their degradation; however, this response requires persistent ERK activation over a time course inconsistent with the expression of these immediate early genes.[42] Based on the transient activation of ERK in granulosa cells (which is generally undetectable by 1–2 h post FSH[113,195]), it is unlikely that the mechanism by which FSH increases the expression of these proteins rests with their ERK-dependent phosphorylation.

ERK can also phosphorylate or direct the phosphorylation of a number of transcription factors and co-activators that have been identified as participants in the induction of FSH target genes. Figure 20.5 reviews ERK targets that have been established in various cellular models.[156] For example, ERK is established to phosphorylate SF-1 on Ser203, resulting in recruitment of co-activators as well as driving the formation of a compact

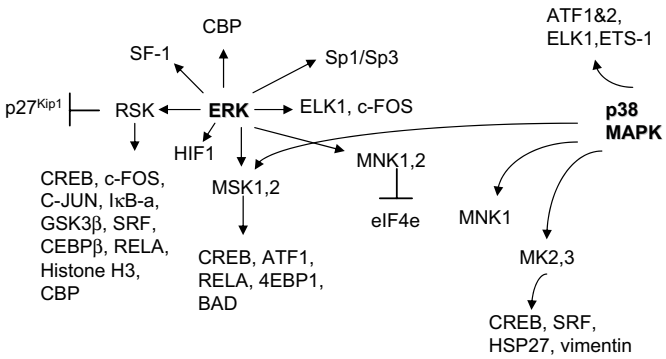

FIGURE 20.5 Summary of identified ERK and p38 MAPK substrates. Arrows indicate stimulation; perpendicular lines indicate inhibition of activity.

structure that results in formation of an active conformation.[203,204] ERK also phosphorylates Sp1/Sp3 on Thr266, thereby enhancing its DNA binding activity.[205] ERK is believed to phosphorylate CBP on Ser436,[206] and this phosphorylation has been suggested to be necessary for its recruitment of the AP-1 complex.[207] It is interesting that p300 lacks this phosphorylation site.[207] However, phosphorylation of CBP by ERK in vivo has not yet been reported, to our knowledge. ERK also phosphorylates a number of kinases, including RSKs 1–4, MSK1 and -2, and MAPK-interacting kinases (MNK1 and -2).[156] These kinases, in turn, phosphorylate a number of transcription factors, in a cell-specific manner, as reviewed in Figure 20.5. Only ERK-catalyzed RSK phosphorylation has been positively identified in granulosa cells treated with FSH,[118] although a number of additional ERK substrates are expected to be phosphorylated in granulosa cells in response to FSH. Although RSK can phosphorylate CREB and histone H3 in some cells, FSH-stimulated phosphorylation of these proteins is not inhibited by the MEK inhibitor PD98059,[118] indicating that neither CREB nor histone H3 is a RSK substrate in granulosa cells. Unphosphorylated RSK has been reported to sequester CBP and inhibit its histone acetyltransferase activity,[208,209] although this might be cell specific because RSK has also been reported to enhance co-activator activity of CBP.[210] An association between RSK and CBP has not been investigated in granulosa cells, but based on the number of transcription factors that require CBP in FSH-regulated signaling pathways in granulosa cells, including CREB,[167,211] HIF-1,[212] and FOXO1,[213] as described later, or that bind CBP, including SF-1[171] and SMAD,[214,215] regulation of the availability of CBP could be an important regulatory mechanism by which FSH enhances target gene expression. Thus, ERK signaling likely participates at least indirectly in the regulation of the expression of a number of FSH target genes by regulating the phosphorylation of a number of transcription factors and/or co-activators. The importance of the ERK pathway to follicle development is also evidenced by the expression of a constitutively active RAS (KRAS-Gly12Asp) in preantral granulosa cells that resulted in subfertile mice, reduced induction of *Lhcgr*, and development of abnormal follicles devoid of mitotic and apoptotic cells.[216]

Mammalian cells contain two additional MAPKs, the p38 MAPKs and the JNKs. Although activation of the JNKs by FSH to our knowledge has not been reported, the p38 MAPKs are activated by FSH in rat granulosa cells.[217–219] The effect of FSH is mimicked by forskolin[219] and appears to be PKA dependent, based on the ability of H89 to inhibit p38 MAPK phosphorylation,[219] although this result is controversial.[114,218] A downstream p38 MAPK target whose phosphorylation is increased by FSH is the small heat shock protein HSP27[219] (Figure 20.5). Although the significance of HSP27 phosphorylation in granulosa cells has not been delineated, upon phosphorylation HSP27 loses its ability to form oligomers and to block actin polymerization.[220] HSP27 is also recognized to inhibit apoptosis in various cellular models.[220] Rounding of granulosa cells, a characteristic response to FSH stimulation,[221] is inhibited by the p38 MAPK inhibitor SB203580, suggesting that the p38 MAPK pathway contributes to granulosa cell cytoskeletal changes.[222] Although FSH activates the upstream p38 MAPK kinases MKK3 and MKK6,[217] the cellular mechanism by which FSH, presumably via PKA, signals into this pathway is not known. Often, this pathway is activated by the small GTPases of the RHO family, leading to the activation of a number of possible kinases that direct activation of MKK3/6.[156] The potential ability of FSH to signal to a RHO family member has not been investigated to our knowledge.

The phosphorylation of a number of transcription factors in various cells is also regulated by p38 MAPK, as reviewed in Figure 20.5, and it promotes the phosphorylation of MNK1 (but not MNK2) and MK2 and -3.[156] HSP27 is actually phosphorylated by MK2 and -3 and not directly by p38 MAPK. CREB can also be a target of MK2 and -3 but is not likely to be phosphorylated by these kinases in granulosa cells in response to FSH, based on the inability of the p38 MAPK inhibitor SB203580 to affect FSH-stimulated CREB phosphorylation.[118] Other than HSP27, p38 MAPK targets in immature granulosa cells have not been identified.

FSH Increases Intracellular Ca^{2+} in Undifferentiated Granulosa Cells

FSH is reported to increase intracellular Ca^{2+} in a cAMP-dependent manner in porcine granulosa cells, and the Ca^{2+} appears to be derived largely from extracellular sources.[223–225] Ca^{2+} entry is inhibited with verapamil, an L-type Ca^{2+} channel blocker, suggesting that plasma membrane Ca^{2+} channels are opened by cAMP

or PKA.[225] Moreover, Ca^{2+} is required for FSH to activate the *Cyp11a1* promoter-reporter in porcine granulosa cells.[223] Although cAMP analogs that selectively activate PKA mimic the actions of FSH to raise intracellular Ca^{2+} levels in Sertoli cells,[226] equivalent studies have not been performed in granulosa cells. In contrast to reports on porcine granulosa cells, there are two reports that FSH does not mobilize intracellular Ca^{2+}, one in HEK cells heterologously expressing the human FSH receptor[227] and another in a rat ovarian granulosa cell line.[228] There is also a report that FSH increases intracellular Ca^{2+} in the human KGN cell line overexpressing FSH receptors.[64] In rat granulosa cells in culture, a Ca^{2+} signal is required for FSH-stimulated ERK activation, as discussed earlier.[113] Consistent with these data, Carnegie and Tsang[229] reported that ethyleneglycotetraacetic acid and the Ca^{2+} channel blocker verapamil inhibit FSH-stimulated progesterone production and the Ca^{2+} ionophore A23187 stimulates progesterone production in rat granulosa cells. Taken together, these results suggest that Ca^{2+}contributes to the actions of FSH. Whereas FSH appears to promote the entry of Ca^{2+} into porcine granulosa cells in a cAMP-dependent manner, the regulation of intracellular Ca^{2+} in rat granulosa cells is less clear.

Ca^{2+}-dependent downstream signaling pathways have also been studied in granulosa cells. CaM kinases II and IV are present in immature rat and porcine granulosa cells,[37,230] and FSH promotes a decline in CaM kinase IV expression to undetectable levels by 24 h in rat granulosa cells.[37] Mice deficient in CaM kinase IV exhibit impaired follicular development and ovulation.[37] Cotransfection of active CaM kinase IV with a *Cyp11a1* promoter-luciferase construct into porcine granulosa/luteal cells resulted in a significant increase in basal transcription of this gene that was blocked by a dominant negative CREB mutant.[230] Cotransfection with CaM kinase II was ineffective.[230] This result suggests that CREB is a possible CaM kinase IV target in porcine granulosa/luteal cells. Ser133 on CREB is a recognized CaM kinase IV target in neuronal cells.[231] Although these results suggest that CaM kinase IV plays a role in the basal transcription of *Cyp11a1* in mature granulosa cells, the role of CaM kinases in immature cells is less clear. An early report by Conti and collaborators[232] showed that FSH treatment of rat granulosa cells activated a cAMP-PDE that required Ca^{2+}/CaM. Additionally, FSH-stimulated progesterone production in rat granulosa cells is reported to be inhibited by the CaM inhibitor R24571,[229] although FSH-stimulated ERK activation (Hunzicker-Dunn, unpublished data) is not inhibited by the CaM kinase II inhibitor Kn62.[233] Thus, additional studies are required to clarify the role of Ca^{2+} and its regulated kinases in FSH actions.

PKA Signals to Activate the PI-3K Pathway in Immature Granulosa Cells

There is abundant evidence that FSH promotes the activation of PI-3K, leading to activation of AKT and downstream targets in granulosa cells that promote cell survival, translation, proliferation, and differentiation[113,115,218,234–236] (Figure 20.6). The PI-3K inhibitors wortmannin and/or LY294002 block FSH-stimulated induction of target gene products that characterize differentiated granulosa cells, including the LH receptor, MAP2D, VEGF, inhibin-α, and RIIβ.[235] In addition, constitutively active AKT enhances and dominant negative AKT blocks FSH-stimulated induction of the LH receptor, aromatase, inhibin-α, and/or 3β-HSD.[115,237] These data suggest that FSH-dependent activation of the PI-3K pathway is obligatory for the induction of critical genes that characterize the differentiated granulosa cell phenotype.

PI-3K. PI-3K consists of a 110-kDa catalytic subunit and an inhibitory 85-kDa regulatory subunit.[238] PI-3K is most commonly activated downstream of receptor tyrosine kinases such as the insulin receptor, IGF-1 receptor, and EGF receptor, which, upon ligand binding and

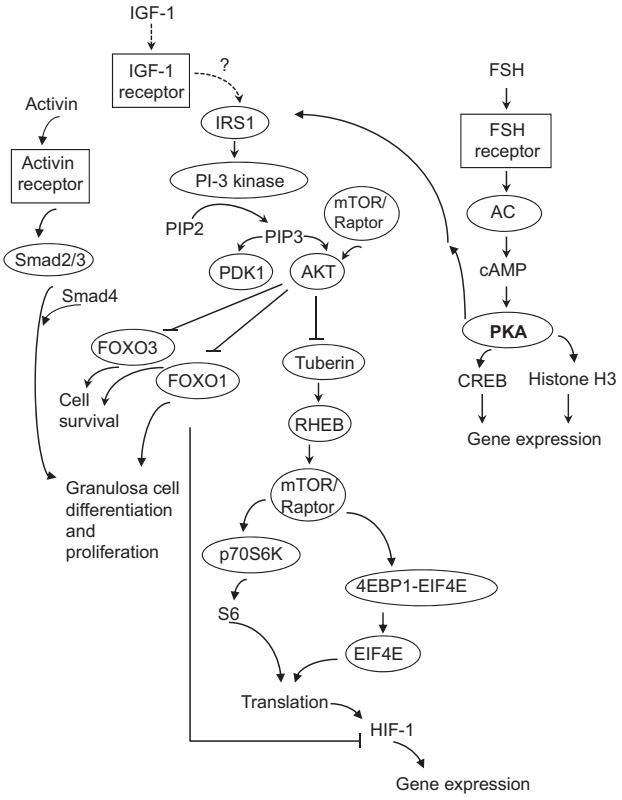

FIGURE 20.6 FSH via cAMP/PKA signals to activate PI-3K, leading to mTOR1-stimulated translation and inhibition of FOXO1 and FOXO3 targets. FSH and activin synergize to promote gene expression. Multiple arrows indicate more than one step in pathway. Potential contribution of IGF-1 and IGF-1 receptor is indicated by dashed lines.

dimerization, phosphorylate adaptor proteins (insulin receptor substrates 1/2 [IRS 1/2] or GAB 1/2) on specific Tyr residues. Activation of PI-3K catalytic subunit occurs on binding of both SH2 domains of the regulatory subunit to dual phosphorylated TyrXXMet (where X represents any amino acid, p-YXXM) motifs either on the adaptor protein IRS 1/2 or GAB 1/2.[238] While the catalytic subunit of PI-3K also contains a RAS binding domain, the contribution of active RAS to PI-3K activation under physiological conditions is not clear.[238] Activated PI-3K phosphorylates the 3′-OH position of the inositol ring of phosphatidylinositol 4,5-bis phosphate (PIP2), generating phosphatidylinositol 3,4,5-trisphosphate (PIP3). Increased concentrations of PIP3 at the plasma membrane create high affinity binding sites for PDK-1 and AKT through their pleckstrin homology domains, localizing both kinases in juxtaposition. PDK-1 then phosphorylates AKT on Thr308; AKT is subsequently phosphorylated on Ser473 by mTORC2 that consists of the mammalian target of rapamycin (mTOR)/Rictor complex, resulting in the full activation of AKT and its translocation to the nucleus and other cellular locations.[239] The mechanism by which GPCRs activate PI-3K is less-well understood and often involves direct activation of the 110-kDa catalytic subunit by Gβγ subunits.[238]

Consistent with the classic manner in which the PI-3K pathway is activated, in granulosa cells exogenous IGF-1 activates the PI-3K pathway via the IGF-1 receptor tyrosine kinase.[218,235,237,240] That IGF-1 is important for follicular function is evidenced by the fact that IGF-1-null mice are infertile; follicles of IGF-1-null mice do not progress beyond the preantral stage of development.[241] Although granulosa cells of preantral and antral follicles are the primary site of IGF-1 synthesis,[242] FSH does not promote IGF-1 expression by rat granulosa cells.[76,243] A recent report showed that granulosa cells express a basal level of IGF-1.[237] It is well established that FSH and IGF-1 strongly synergize to activate a number of FSH target genes, including those for *Cyp11a1*, *Cyp19a1*, and *Lhcgr*.[47,243–246] However, IGF-1 alone promotes only a minimal activation of these target genes,[47,243,247] indicating that additional FSH targets/responses are required to induce the full differentiation response.

The cellular mechanism by which FSH activates the PI-3K pathway in granulosa cells is beginning to be unraveled (Figure 20.6). FSH-stimulated AKT phosphorylation is detected within 5–10 min of FSH addition to rat granulosa cells.[116,235] Signaling by FSH to activate the PI-3K leading to the phosphorylation of AKT in granulosa cells is mimicked by cell-permeable cAMP analogs or the adenylyl cyclase activator forskolin[115,218,235] and is mediated by PKA.[116] PKA promotes the phosphorylation of IRS-1 on Tyr989, a canonical YXXM binding site for the regulatory subunit of PI-3K, resulting in phosphorylation of AKT on both Thr308 and Ser473.[116] These phosphorylations of IRS-1 and AKT are all abrogated by the specific PKA inhibitor PKI. The adaptor GAB2 appears to coordinate signaling of PKA to PI-3K, as depicted in Figure 20.7. GAB2 is present in granulosa cells in a preformed complex with IRS-1 and the PI-3K heterodimer. GAB2 is an RI-AKAP and a direct PKA target, and is rapidly phosphorylated on Ser159 in response to FSH. Overexpression of GAB2 enhances FSH-stimulated AKT phosphorylation.[116] However, there remain many unanswered questions, including the identity of the tyrosine kinase that phosphorylates IRS-1 on Tyr989, the mechanism by which PKA apparently activates this tyrosine kinase and/or inhibits a tyrosine phosphatase, and the mechanism by which GAB2 appears to coordinate this signaling cascade.

A recent report has shed new light on the contribution of IGF-1 to the FSH signaling pathway that leads to AKT phosphorylation. Granulosa cells, cultured in the presence of transferrin, selenium, insulin, amino acids, and a cholesterol source, express ~1 ng/ml IGF-1 under basal conditions.[237] FSH or dibutryl cAMP coupled with autocrine-generated IGF-1 signal via the IGF-1 receptor tyrosine kinase to activate AKT to induce *Cyp19a1*.[237] Addition of selective IGF-1 receptor antagonists or transduction of cells with lentivirus expressing short hairpin RNAs for IGF-1 or the IGF-1 receptor markedly reduced the ability of FSH to induce *Cyp19a1*. Also, AKT phosphorylation (Ser473) detected 1 h post FSH was abrogated by a selective IGF-1 receptor antagonist (NVP-AEW541[248]).[237] Together, these results suggest that in some manner FSH in combination with IGF-1

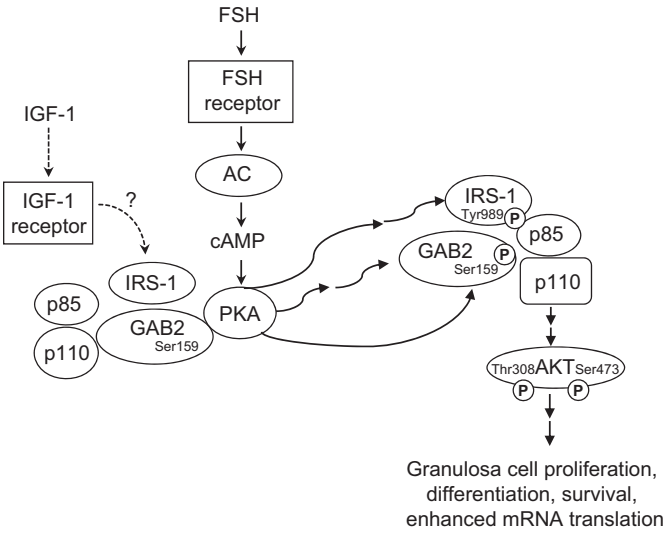

FIGURE 20.7 Mechanism by which FSH via PKA activates PI-3K leading to AKT activation. Multiple arrows indicate more than one step in the pathway. Potential contribution of IGF-1 and IGF-1 receptor is indicated by dashed lines.

activates the IGF-1 receptor to signal to AKT.[237] Additional studies are necessary to clarify the contributions of IGF-1 and the IGF-1 receptor to FSH signaling into the PI-3K pathway.

While the overexpression of constitutively active RAS (Gly12Asp) in immature granulosa cells elevates basal AKT phosphorylation,[216] transduction of granulosa cells with an adenovirus expressing a dominant negative RAS (Ser17Asn) does not prevent AKT phosphorylation on Ser473 or Thr308.[116] Taken together these results support the current view that RAS is not a major regulator of PI-3K activity under physiological conditions.[238]

AKT Substrates. General downstream targets of AKT in a variety of tissues can include the transcription factor CREB, the proapoptotic protein BAD, the phosphodiesterase PDE3β, nitric oxide synthase (NOS), the translation antagonist tuberin, GSK3β, the p53 E3 ubituitin ligase MDM2, the RAB-GTPase activating protein AS160 that regulates the cellular location of glucose transporter 4, select members of the FOXO transcription factor family, D-type cyclins, the cell cycle inhibitor $p27^{Kip1}$, the translation elongation factor E2F, and others[154,239] (Figure 20.8). Thus, AKT can signal to regulate transcription, translation, metabolism, cell survival, and differentiation, in a cell-specific and agonist-dependent manner.

CREB. In rat granulosa cells, FSH-stimulates CREB phosphorylation on Ser133. However, CREB phosphorylation is mediated by PKA in granulosa cells, as reviewed earlier, and not by AKT. This conclusion is based on the inability of the PI-3K inhibitor wortmannin[249] to reduce CREB phosphorylation[113] and the inability of CREB to be further phosphorylated in granulosa cells transduced with constitutively active AKT.[115]

Tuberin and Translational Regulation. FSH also stimulates the phosphorylation of tuberin, a product of the tuberous sclerosis (TSC) tumor suppressor gene[235] (Figure 20.6). In its unphosphorylated state, tuberin (TSC2) in a complex with TSC1 (Hamartin) is active and inhibits translation.[250] The tuberin–TSC1 complex functions as a GTPase-activating protein for the protein RAS homolog enriched in brain (RHEB).[251] This TSC complex stimulates the hydrolysis of the GTP bound to RHEB, converting RHEBGTP to RHEBGDP, resulting in the inactivation of RHEB. Phosphorylation of tuberin on Thr1462 by AKT results in an inhibition of the GTPase-activating protein activity of the TSC1–TSC2 complex.[251] As a result, RHEB is maintained in its active conformation. By a poorly understood mechanism, RHEBGTP promotes activation of mTORC1 (mTOR/Raptor complex). mTORC1 signals to at least two substrate proteins to stimulate translation, p70 S6 kinase, and a binding protein that sequesters the eukaryotic translation initiation factor eIF4E (eIF4E-BP1).[252–254] Phosphorylation of p70 S6 kinase on Thr389 leads to the phosphorylation of the ribosomal protein S6. Phosphorylation of S6, one of 30 ribosomal proteins that along with the 18S rRNA comprises the 40S ribosomal subunit complex, stimulates the translation of mRNAs containing a 5′-oligopyrimidine tract.[255] Phosphorylation of eIF4E-BP1 on Ser65 and other sites frees eIF4E[256,257] to bind to the 5′-methyl cap of mRNAs.[255,258] This stimulates cap-dependent translation and is the rate-limiting step in translation initiation.[258]

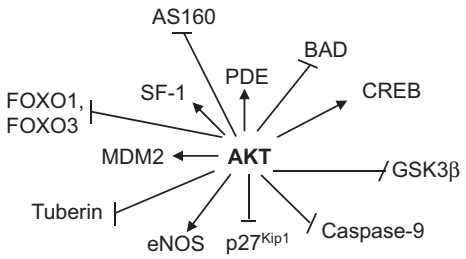

FIGURE 20.8 Summary of identified AKT substrates. Arrows indicate stimulation; perpendicular lines indicate inhibition.

FSH, via PI-3K/AKT, stimulates the phosphorylation of tuberin on Thr1462 within 10 min, leading to the activation of mTORC1 and phosphorylation of its substrates p70 S6 kinase and downstream S6 protein as well as 4E-BP1 in rat granulosa cells.[235] That mTORC1 activation is crucial for activation of a subset of FSH target genes is evidenced by the ability of the mTORC1 inhibitor rapamycin[252] to inhibit the induction of MAP2D and RIIβ protein expression as well as promoter-reporter activities for *Lhcgr*, *Vegf*, and *Inha*.[235] These results suggest that a very rapid effect of FSH in granulosa cells is to stimulate translation. Indeed, it has been suggested that activation of the PI-3K and ERK pathways in general preferentially promotes translation rather than transcription.[259] mTORC1-dependent targets of translation whose expression is increased in various cellular models include the cyclins, resulting in increased cell proliferation.[260,261] Certainly a future area of research should be the identification of FSH-targeted translation products via some of the newer proteomics approaches or via ribosome profiling.

There is also a report that FSH promotes the phosphorylation of tuberin via an ERK-dependent, AKT-independent pathway that leads to phosphorylation of p70 S6 kinase and increased cyclin D2 mRNA that is partially reduced by the mTORC1 inhibitor rapamycin, using granulosa cells seeded on serum-coated plates.[262] While the ERK target RSK has been reported to phosphorylate tuberin,[251] it is not clear how the mTORC1 pathway leads to enhanced cyclin D2 transcription.

It is quite interesting that IGF-1 also stimulates the phosphorylation of AKT, tuberin, p70 S6 kinase, and S6 protein in rat granulosa cells,[235] but as already indicated, IGF-1 does not stimulate expression of FSH target genes,[47,243] at least to levels observed with FSH. This

result suggests that additional pathways activated by FSH but not by IGF-1 are required for FSH target gene expression.

HIF-1α. One of the mRNAs whose translation is increased by FSH downstream of AKT and tuberin in granulosa cells is the transcription factor HIF-1α.[235] HIF-1 is a heterodimer, consisting of HIF-1α and HIF-1β, the latter also known as the aryl hydrocarbon receptor nuclear transporter. HIF-1 is a member of the basic helix-loop-helix/Per/aryl hydrocarbon receptor nuclear transporter/Sim family of transcription factors that bind to a modified E-box on DNA promoters.[263] HIF-1β is constitutively expressed; HIF-1α protein levels are regulated. HIF-1 is best known for its regulation of cellular responses to hypoxia.

Under normoxic conditions, HIF-1α is hydroxylated on two critical proline residues by oxygen-dependent prolyl hydroxylases, triggering HIF-1α degradation.[263,264] Hydroxylation of the Pro residues creates a binding site for the protein product of the von Hippel–Lindau tumor suppressor gene, VHL. VHL is an E3-ubiquitin ligase and promotes the polyubiquitination of proline hydroxylated forms of HIF-1α, leading to their degradation by the proteosomal system. Cobalt has been shown to inhibit the interaction of HIF-1α and VHL[265] and is often used experimentally to stabilize HIF-1α protein levels. HIF-1 activity is also regulated by a second oxygen-dependent mechanism. Under normoxic conditions, a protein called "factor inhibiting HIF-1" catalyzes the hydroxylation of an Asn residue in the C-terminal transactivation domain of HIF-1α, preventing the binding of its co-activator p300/CBP. Under hypoxic conditions, the activity of the oxygen-dependent prolyl hydroxylases is reduced, and HIF-1α protein is stabilized. The activity of the asparaginyl hydroxylase is also reduced under hypoxic conditions, thereby allowing the binding of p300/CBP to stimulate HIF-1 activity. The prolyl hydroxylases require iron as a cofactor, so agents that chelate iron such as desferrioxamine inhibit the propyl hydroxylases, thereby stabilizing HIF-1α protein levels.

HIF-1α protein can also be regulated at the level of translation upon activation of the PI-3K pathway by insulin or IGF-1.[266,267] In these studies, expression of HIF-1α protein correlated with activation of HIF-1 DNA binding activity and/or HIF-1-dependent reporter gene activities. Inhibitors that abrogate the ability of these growth factors to stimulate the phosphorylation of p70 S6 kinase and 4E-BP1 block the increase in HIF-1α protein.[266,267]

In rat granulosa cells, FSH promotes an increase in HIF-1α protein accumulation under normoxic conditions (Figure 20.6), which is detected in the presence either of a proteosomal inhibitor or $CoCl_2$.[235] FSH also promotes an increase in HIF-1 activity, detected using a minimal hypoxia response element linked to the luciferase reporter in rat granulosa cells. Moreover, co-expression of a HIF-1 dominant negative construct, lacking its DNA binding and transactivation domains,[268] reduces the ability of FSH to activate promoters for *Lhcgr*, *Vegf*, and *Inha*, as detected using promoter-reporter assays.[235] These results suggest that HIF-1 activity is required for FSH to activate *Lhcgr*, *Vegf*, and *Inha* genes. However, although each of these genes contains apparent HIF response elements and VEGF is an established HIF-1 target, only *Vegf* in granulosa cells has been shown to be a direct HIF-1 target.[247]

While there is some evidence that the granulosa cell environment may become increasingly hypoxic as the follicle grows,[269] hypoxia does not appear to be a prominent contributor to the ability of FSH to induce target genes such as *Lhcgr* (E. Donaubauer and M. Hunzicker-Dunn, unpublished).

IGF-1 also promotes an increase in the accumulation of HIF-1α protein in rat granulosa cells to levels equivalent to those achieved with FSH; however, interestingly IGF-1 does not enhance HIF-1 activity, based on a promoter-reporter assays.[247] This result shows that the mere presence of HIF-1α protein is not sufficient to generate an active HIF-1 transcriptional factor. Thus, additional unidentified signaling events independent of the PI-3K pathway that are activated by FSH are required to "activate" HIF-1.

Transcriptional activity of HIF-1α requires binding of CBP/p300 to the C-terminal transactivation domain.[264] The binding of CBP/p300 is negatively regulated in an oxygen-dependent manner by the hydroxylation of Asn803 by "factor inhibiting HIF-1".[264] HIF-1 transcriptional activity in a number of cellular models is also positively regulated by ERK, although whether ERK directly phosphorylates HIF-1α is controversial.[247] Moreover, activation of VEGF transcription in various cell models requires cooperation between HIF-1 and an AP-1 complex.[212] Thus, ERK could contribute to HIF-1 activity by phosphorylating HIF-1α protein, by phosphorylating CBP, or by phosphorylating members of the AP-1 family, as summed in Figure 20.5. In rat granulosa cells, *HIF-1* promoter-reporter activities and the interaction of HIF-1α with the *Vegf* promoter in a CHIP assay are inhibited by the MEK inhibitor PD98058, consistent with the potential contribution of ERK phosphorylation to HIF-1 transcriptional activity.[247] Moreover, IGF-1 in rat granulosa cells does not activate ERK phosphorylation, suggesting that the inability of IGF-1 to promote HIF-1 transcriptional activity is the result of the lack of ERK activation by IGF-1.[247] However, transient expression of a constitutively active MEK was not sufficient to rescue IGF-1-stimulated HIF-1 activity.[247] Based on our evidence that ERK activity in immature granulosa cells is restrained by a PTP whose activity is inhibited by FSH,[113] the potential contribution of ERK needs to be reevaluated in cells transduced with adenovirally expressed constitutively active ERK.

FOXO1 and FOXO3. A second AKT target in rat and porcine granulosa cells is FOXO1.[48,112,234,270,271] FOXO1 is a member of the forkhead O class of transcription factors that was originally identified by its disruption in the pediatric tumor rhabdomyosarcoma.[272] Three of the members of this family are AKT targets: FOXO1, FOXO3, and FOXO4.[273] FOXO-regulated genes control the cell cycle, cellular metabolism, and cell death. These transcription factors bind to DNA as monomers at the insulin response element consensus sequence TT(A/G)TT(T/G)(A/G)(T/C) on target genes. In the absence of signaling through the PI-3K pathway, these FOXO transcription factors are bound to DNA and are active. They can function as transcriptional activators to stimulate such target genes as the cell cycle inhibitor $p27^{Kip1}$, glucose-6-phosphatase activity,[274] and IGF binding protein-1, as well as transcriptional repressors to suppress such target genes as cyclin D1 and D2.[273] Upon phosphorylation by AKT, FOXO1, -04, and -03 exit the nucleus and are targeted for degradation. Three Ser/Thr residues on FOXO proteins are phosphorylated by AKT. For FOXO1, phosphorylation of Ser256 diminishes DNA binding and is necessary for the phosphorylation of Thr24 and Ser319.[275] Phosphorylation of Thr24 disrupts association of FOXO1 with CBP and stimulates binding to 14-3-3 proteins, and phosphorylation of Ser319 stimulates nuclear export.[273]

FOXO factors also bind nuclear receptors, including those for estrogen (preferentially ER-α over ER-β), progesterone, androgen, thyroid hormone, glucocorticoid, and retinoid acid, presumably via the conserved LeuXXLeuLeu domain located in the C-terminal region of the FOXO protein.[273] Although the actions of the FOXO factors can require binding to DNA, there is also evidence that they can affect transcription of target genes independent of their DNA binding activity, possibly via their association with co-activators such as CBP or with steroid receptors.[276] FOXO factors can also be acetylated, possibly by CBP, a modification that is thought to activate FOXO transcriptional activity, although the effect of acetylation is controversial (reviewed in Ref. 273).

There is accumulating evidence from a number of cellular models that FOXO factors promote cell cycle arrest.[273,277] Cell cycle arrest can occur as a result of increased expression of the cyclin-dependent kinase (CDK) inhibitors $p27^{Kip1}$, $p21^{Cip1}$, and p130, and/or by decreased expression of the CDK or their binding partners cyclin D or cyclin E. In general, progression from G_1 to the S phase of the cell cycle is mediated by increased expression of cyclin D, which partners with CDK 4/6, followed by increased expression of the CDK 2 partner cyclin E. Hyperphosphorylation of the retinoblastoma protein catalyzed by CDK 4/6 and CDK 2 relieves inhibition of the transcription factor E2F by retinoblastoma, allowing for the expression of genes necessary for DNA replication and S phase entry.

FOXO transcription factors have been reported to inhibit cell cycle progression by enhancing transcription of $p27^{Kip1}$ and other CDK inhibitors, by repressing cyclin D transcription, and/or by increasing transcription of the unconventional cyclin G2, which inhibits the cell cycle.[278] Porcine granulosa cells undergo proliferation upon stimulation with FSH and insulin.[270] Transduction of porcine granulosa cells with a dominant negative FOXO1 truncation mutant, which lacks its transactivation domain, increases entry of these cells into the S phase of the cell cycle by redirecting the compartmentalization of $p27^{Kip1}$ from the nucleus to the cytosol and decreasing $p27^{Kip1}$ content.[270] Although $p27^{Kip1}$ is known to be phosphorylated on Thr157 by AKT, resulting in its redistribution from the nucleus to the cytosol,[279–283] the mechanism by which FOXO1 directs relocation of $p27^{Kip1}$ to the cytosol followed by its apparent degradation is not known.

It is not known if FSH promotes a redistribution of $p27^{Kip1}$ to the cytosolic fraction in rat granulosa cells upon FOXO1 phosphorylation as occurs in porcine granulosa cells.[270] However, $p27^{Kip1}$ does not show any evidence of downregulation, and interestingly, the phosphorylatable Thr157 residue believed to be responsible for exit of $p27^{Kip1}$ from the nucleus is not conserved in rodents.[279,280]

In contrast to porcine granulosa cells, proliferation of rat granulosa cells and the obligatory induction of cyclin D2[45] and the S-phase marker proliferating cell nuclear antigen, under primary serum-free culture conditions, does not occur in the presence of FSH alone or FSH plus IGF-1.[44,47,49] However, as shown in Figure 20.6, in the presence of activin, cyclin D2 mRNA expression is strongly enhanced by FSH under serum-free culture conditions.[46,227,276] These results suggest that FSH, directly or indirectly, stimulates the expression of activin or a similar TGFβ-family ligand and that both of these ligands converge to stimulate cyclin D2 expression and consequent granulosa cell proliferation. Rat granulosa cells are reported to produce activin subunits throughout early follicular development.[284] However, activin levels produced under culture conditions are apparently too low to synergize with FSH, possibly due to contributions from the activin inhibitor follistatin and/or to utilization of activin subunits by inhibin-α, as these cells also produce follistatin and inhibin.[285]

FOXO1 functions to repress activation not only of the *Ccnd2* (cyclin D2) gene but also to repress activation of a subset of FSH targets that characterize the preovulatory phenotype, including *Nr5A1* (SF-1), *Nr5A2* (LRH-1), *Cyp19a1* and *Cyp11a1*, *Inha,* and *Ereg* (epiregulin).[48] The list of genes repressed by FOXO1 in immature granulosa cells has been expanded to include genes required for cholesterol biosynthesis and steroidogenesis as well as *Lhcgr*.[271] Phosphorylation of FOXO1 by AKT is necessary but likely not sufficient to achieve the induction of

these FSH target genes, as evidenced by the established regulation of *Inha*[170,186] and *Cyp19a1*[152,286] promoters, for example, by a number of transcriptional activators. The ability of FOXO1 to repress activation of these FSH target genes is based on studies in which granulosa cells were transduced with an adenoviral vector encoding a constitutively active FOXO1 mutant in which the three AKT phosphorylation sites were mutated to Ala (A3-FOXO1). Direct binding of FOXO1 to the cyclin D2 promoter was evidenced by CHIP assays, this association was abrogated upon stimulation of cells with FSH, and the association was enhanced and not regulated in cells transduced with the constitutively active A3-FOXO1 mutant.[48] However, it is not known if other FSH target genes regulated by inactivation of FOXO1 are repressed directly or indirectly by FOXO1.

FOXO1 also represses the ability of FSH to promote the accumulation of HIF-1α, as evidenced in cells transduced with A3-FOXO1 compared to empty adenovirus, and thus activation of HIF-1 transcriptional activity[247] (Figure 20.6). While the FOXO1 target is likely a ubiquitin ligase, this ligase has not been identified. The ability of FOXO1 to repress the accumulation of HIF-1α in the absence of FSH may be a mechanism to prevent inappropriate actions of HIF-1 in granulosa cells.

The induction of *Ccnd2*, *Nr5a1*, *Inha*, *Cyp11a1*, *Cyp19a1*, and *Ereg* was also abrogated in granulosa cells transduced with a dominant negative Smad2/3 mutant.[48] These results suggest that induction of these proliferation and differentiation responses requires not only relief from repression by FOXO1 but also the contribution of the Smad2/3 transcription factors (Figure 20.6). However, the mechanism by which Smad2/3 cooperates with the AKT pathway leading to FOXO1 phosphorylation to promote expression of at least a subset of FSH target genes has not been elucidated. It is interesting that active (unphosphorylated) FOXO1 can complex with phospho-Smad to promote expression of the cell cycle inhibitor $p21^{Cip1}$ in neuroepithelial and glioblastoma cell lines.[287] In granulosa cells, however, phospho-Smad2/3 functions upon inactivation of FOXO1 to drive proliferation. Thus, the cross talk between these two pathways is cell specific.

A similar repressive function in follicular maturation has been attributed to FOXO3, but at a much earlier stage of follicular development. Unlike FOXO1-null mice, which die at embryonic day 10.5,[288] FOXO3-null mice show global activation of primordial follicles, resulting in oocyte death and depletion of follicles as a result of increased granulosa cell mitotic activity.[289] These results suggest that FOXO3, most likely in oocytes, functions to suppress maturation of primordial follicles.

FOXO3 has also recently been shown to promote expression of the pro-apoptotic protein Bcl-2-interacting mediator of cell death/Bcl-2-related ovarian death agonist (BIM/BOD) in porcine granulosa cells.[290] CHIP assays place FOXO3 on the *Bim(Bcl2l11)* promoter in the absence of FSH.[290] FSH via the PI-3K/AKT pathway stimulated the phosphorylation of FOXO3 on Ser253, resulting in its inactivation and exit from the nucleus. Consistent with the positive effect of FOXO3 on BIM/BOD expression in the absence of FSH, FSH reduced expression of BIM/BOD.[290] These results suggest that FOXO3 is a transcriptional activator of *Bim*, and that FSH reduces the expression of the pro-apoptotic protein product to promote granulosa cell survival.

Owing to the embryonic lethality and premature ovarian failure of global FOXO1[288] and FOXO3[289] knockouts, respectively, FOXO1 and FOXO3 were recently selectively deleted from granulosa cells.[291] While targeted deletion of FOXO1 resulted in a subfertile mouse (some mural granulosa cells retained FOXO1 expression), targeted deletion of FOXO3 did not reduce fertility. However, dual targeted deletion of FOXO1 and FOXO3 generated an infertile mouse in which follicle growth was arrested at the preantral/early antral stage of follicle development.[291] Deletion of FOXO1/FOXO3 would be expected to reduce expression of those genes that are positively regulated by FOXO1/FOXO3. However, enhanced expression of genes that are repressed by FOXO1/FOXO3 might not occur due to the absence of positive transcriptional regulators. These double mutant mice exhibit severely reduced serum FSH levels[291] and thus would not be expected to express genes repressed by FOXO1/FOXO3. Granulosa cells of the double mutant mouse exhibited reduced apoptosis, associated with reduced expression of the FOXO3 target[290] *Bcl2l11* (that encodes for BIM/BOD) and *Bmp2* that encodes bone morphogenic protein 2 (BMP2), a protein selectively expressed in follicles undergoing apoptosis.[291] Granulosa cells of these double mutant mice also exhibited reduced expression of a number of apparent FOXO1/FOXO3 targets, including *Igf1* (insulin-like growth factor 1), *Ctgf* (conective tissue growth factor), *Amh* (anti-Mullerian hormone), and *Nr0b1* (nuclear receptor subfamily 0, group B, member 1; Dax1),[291] the expression of which likely contributes to the immature granulosa cell phenotype (in the absence of FSH). Studies with isolated granulosa cells showed that the positive effects of (active) FOXO1/FOXO3 on the expression of these genes was enhanced by activin, consistent with the interaction of FOXO1/FOXO3 with pathways downstream of activin.[291] It is interesting that activin signaling pathways appear to converge both with the positive effects of FOXO1/FOXO3 on target genes that maintain granulosa cells in an immature state (in the absence of FSH)[291] and with FSH signaling pathways that promote inactivation of FOXO1/FOXO3 to drive granulosa cell maturation.[48]

GSK3β and the WNT signaling pathway. An additional AKT target in many cells is the Ser/Thr protein kinase GSK3β (Figure 20.8). GSK3β is active when the PI-3K pathway is silent. Active AKT phosphorylates GSK3β on Ser9, resulting in the inactivation of GSK3β.[292] One of the GSK3β targets in a number of cellular models is the D-type cyclins. In the absence of signaling through the PI-3 kinase pathway, GSK3β phosphorylates the D-type cyclins, leading to their ubiquitination and degradation.[293] Activation of the PI-3K pathway promotes AKT-catalyzed phosphorylation of GSK3β, thereby rescuing D-type cyclins from degradation. Although there is evidence that FSH promotes the phosphorylation of GSK3β in rat granulosa cells,[218] it is not known if the stability of cyclin D2 in granulosa cells is also regulated by GSK3β. However, based on evidence that FSH stimulates only a modest two- to three-fold increase in cyclin D2 promoter-reporter activity in transiently transfected granulosa cells compared with the robust increase in cyclin D2 protein expression,[48] it is likely that FSH also stabilizes cyclin D2, possibly via inactivation of GSK3β.

GSK3β can also be phosphorylated on Ser9 by a number of other Ser/Thr kinases, including PKA, PKC, and the ERK substrate RSK, to reduce its activity.[294,295] Although FSH activates PKA, AKT, and RSK in granulosa cells,[113,115,117,118,218,234,235] FSH-stimulated GSK3β phosphorylation is strongly inhibited by PI-3 kinase inhibitors,[218] suggesting that GSK3β is predominately an AKT substrate in granulosa cells.

In addition to regulating the stability of the D-type cyclins, GSK3β is recognized to phosphorylate a number of transcriptional activators, including β-catenin, CREB, HIF-1, NF-κB, and AP-1, as well as structural proteins and metabolic and signaling proteins such as MAP2, kinesin light chains, glycogen synthase, eIF2B, PKA RIIβ subunit, and protein phosphatase 1.[294,295] Thus, GSK3β potentially regulates glucose production, translation, transcription, cell survival, and cell motility. Generally, GSK3β substrates are initially phosphorylated by another protein kinase, priming the substrate for phosphorylation by GSK3β.

The phosphorylation activity of GSK3β is also regulated by its association with a specific protein complex that includes its substrate, β-catenin, the obligatory priming kinase, and other proteins. This pathway is regulated by the paracrine/autocrine factor WNT.[296] In the absence of the WNT ligand, GSK3β is complexed with a scaffold protein (AXIN), casein kinase 1α, adenomatous polyposis coli (APC), and β-catenin, as depicted in Figure 20.9. In this complex, β-catenin is targeted for ubiquitination and proteosomal degradation as a result of a priming phosphorylation by casein kinase 1α on Ser45 followed by phosphorylation by GSK3β on Thr41, Ser37, and Ser33.[296] WNT signaling through the plasma membrane Frizzled receptor/lipoprotein receptor-related proteins 5 and 6

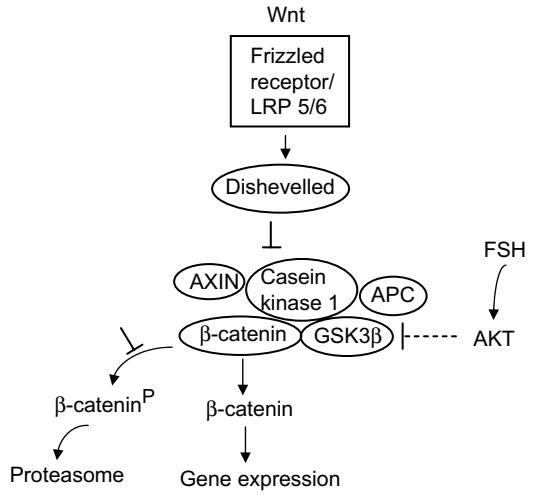

FIGURE 20.9 Schematic diagram of the rescue of hypophosphorylated β-catenin from proteosomal degradation by WNT signaling. A potential route for FSH to inhibit phosphorylation of β-catenin via AKT is depicted.

together with the scaffolding protein Dishevelled inhibits GSK3β activity by a poorly understood mechanism. As a result, β-catenin is not phosphorylated and escapes from degradation. Nonphosphorylated β-catenin translocates to the nucleus where it functions as a transcriptional co-activator. While β-catenin is best known for its ability to bind TCF/lymphoid enhancer factor (LEF) and to activate TCF/LEF–regulated genes,[297] β-catenin is also required for the transcriptional activation of SF-1.[175,176]

Immature granulosa cells express Wnt2, Wnt4, Frizzled1, Frizzled4, and Dishevelled2 mRNAs.[298,299] Conditional deletion of Wnt4 from granulosa cells using *Amhr2-Cre* reduced the number of antral follicles ~ 50%,[300] suggesting that Wnt4 contributes to follicular maturation. However, to our knowledge, there is no evidence that FSH enhances expression of Wnt4 to activate the canonical β-catenin pathway in granulosa cells. The Richards' laboratory used an alternative approach to evaluate the contribution of β-catenin to FSH-signaling pathways. Conditional expression of a dominant stable β-catenin in granulosa cells of small antral follicles (driven by *Amhr2-Cre*), in which exon 3 that contains the N-terminal phosphorylation sites required for degradation was excised, generated the formation of either abnormal follicles that lacked oocytes and were highly vascularized or cystic structures as well as some normal follicles, resulting in subfertile mice.[301] A microarray analysis of ovaries expressing this dominant stable β-catenin compared to control ovaries revealed abnormal overexpression of Wnt/β-catenin signaling antagonists such as AXIN as well as bone and neuronal cell markers.[302] Conditional expression of the dominant stable β-catenin in granulosa cells of follicles at a slightly later stage of maturation, driven by the *Cyp19-Cre*,

showed that β-catenin inhibits apoptosis and facilitates FSH-stimulated follicular growth by enhancing expression of *Fshr*, *Cyp19a1*, *Ccnd2*, and *Nr5a1* without affecting *Lhcgr* expression.[303] The ability of β-catenin to regulate expression of *Cyp19a1* agrees with previous reports in granulosa cells that β-catenin is required for transcriptional activation of SF-1 on the *Cyp19a1* promoter.[286,304] Fan et al.[303] showed that FSH activated the artificial TCF/β-catenin promoter-reporter TOPFlash, and that FSH enhanced the nuclear accumulation of nonphosphorylated β-catenin. The authors postulate that the mechanism by which FSH enhances nuclear accumulation of nonphosphorylated β-catenin may be via AKT-dependent phosphorylation/inactivation of GSK3β,[303] as shown in Figure 20.9. An alternate pathway by which FSH regulates β-catenin activity is discussed later in "Cross Talk among FSH-Regulated Signaling Pathways and Transcriptional Activators to Regulate Gene Expression." In contrast to enhancing FSH responses, persistent expression of the dominant stable β-catenin repressed LH-induced oocyte maturation, ovulation, and luteinization by inhibiting expression of LH target genes and thus compromised fertility.[303] The negative effect of the dominant stable β-catenin was at least in part due to reduced phosphorylation of CREB, MEK, and ERK in response to hCG, although it is not clear how dominant stable β-catenin interferes with CREB and MEK/ERK phosphorylations.

SGK. FSH and forskolin also induce the immediate early gene SGK[118,122,218] and stimulate its apparent phosphorylation, based on an upward shift in mobility on sodium dodecyl phosphate polyacrylamide gel electrophoresis.[199] SGK bears similarities to AKT, as it is activated in a PI-3K-dependent manner by PDK-1[305] and phosphorylates many of the same substrates phosphorylated by AKT, including members of the FOXO family.[306] However, recent evidence suggests that the primary function of this kinase is to regulate the abundance of Na^+, K^+, and Cl^- ion channels by preventing their proteosomal degradation[307,308] by phosphorylating and inactivating a ubiquitin ligase.[309] SGK is induced in granulosa cells in a PKA-dependent manner, and its induction is prevented by the PKA inhibitor H89.[106] SGK is not induced in granulosa cells by IGF-1.[199] In contrast to the signaling pathway that regulates the FSH-stimulated induction of SGK in granulosa cells, FSH-stimulated phosphorylation of SGK in granulosa cells is abolished by PI-3K inhibitors wortmannin and LY294002, consistent with FSH signaling to stimulate phosphorylation/activation of SGK via the PI-3K pathway.[199] However, neither the substrates nor the function of SGK in granulosa cells is known.

Apoptosis-related PI-3K Substrates. The PI-3K pathway is also known to regulate cell survival. Indeed, addition of the PI-3K inhibitor LY294002 to rat granulosa cells induces apoptosis.[310] Up to 99% of the ovarian follicles undergo apoptosis primarily at the early antral stage of development (in rats).[311] Follicles survive and proceed to final stages of maturation only if they are rescued from apoptosis, predominately by FSH. It is not surprising that apoptosis is a highly regulated event in the ovary and that a number of proteins in the apoptotic pathway have been identified as being highly expressed in ovarian cells.[290,311]

There are two major pathways that lead to apoptosis: one directed by the death receptors of the tumor necrosis family (TNF) and one directed by the release of cytochrome c (and other proteins) from mitochondria as a result of various cellular stresses such as heat shock, oxidative stress, and DNA damage.[312] In both of these pathways, cysteine aspartic acid-specific proteases (caspases) become activated and cleave proteins that are crucial to cellular function, resulting in programmed cell death. Initiator caspases are activated in response to a proapoptotic signal, and they in turn activate effector caspases that catalyze substrate proteolysis, leading to cell death.

FSH appears to inhibit apoptosis primarily by controlling the release of cytochrome c from mitochondria, as depicted in Figure 20.10. The proteins that regulate this pathway are members of the BCL-2 (B-cell leukemia/lymphoma 2) family and share one or more BCL-2 homology (BH) domains.[313,314] Group I, the anti-apoptotic proteins that contain four BH (BH 1–4) domains, includes BCL-2, BCL-XL (extra large), MCL-1(myeloid cell lymphoma-1), and DIVA. They prevent apoptosis by binding to and blocking the activity of the Group II pro-apoptotic BH123 domain proteins BAX, BAK318 and possibly BOX (BCL-2–related ovarian killer).[315] BAX and BAK, upon activation, oligomerize at the outer mitochondrial membrane and create pores for the exit of cytochrome c. Group III BH3 domain-only proteins stimulate apoptosis upon receipt of an apoptotic stimulus most likely by inhibiting the interaction of Group I anti-apoptotic proteins with Group II pro-apoptotic proteins[314] and include BAD (BCL-XL/BCL-2–associated death promoter), BID, PUMA, BIM (aka BOD, BCL-2-related ovarian death agonist), and NOXA. These proteins are restrained in the absence of an apoptotic signal at the transcriptional (BIM, PUMA, NOXA), posttranslational (BAD), or precursor (BID) levels.[313] They bind with selectivity and high affinity to Group I anti-apoptotic proteins, effectively freeing Group II pro-apoptotic (BH123) proteins to oligomerize and stimulate cytochrome c release.[314]

Upon release of cytochrome c from the mitochondria, cytochrome c complexes with APAF-1 (apoptotic protease activating factor-1), resulting in the oligomerization of APAF-1 and recruitment of procaspase-9.[312] Caspase-9 becomes activated as a result of an autocatalytic event, forming the active apoptosome, leading to the activation of effector caspases-3 and -7, which promote the

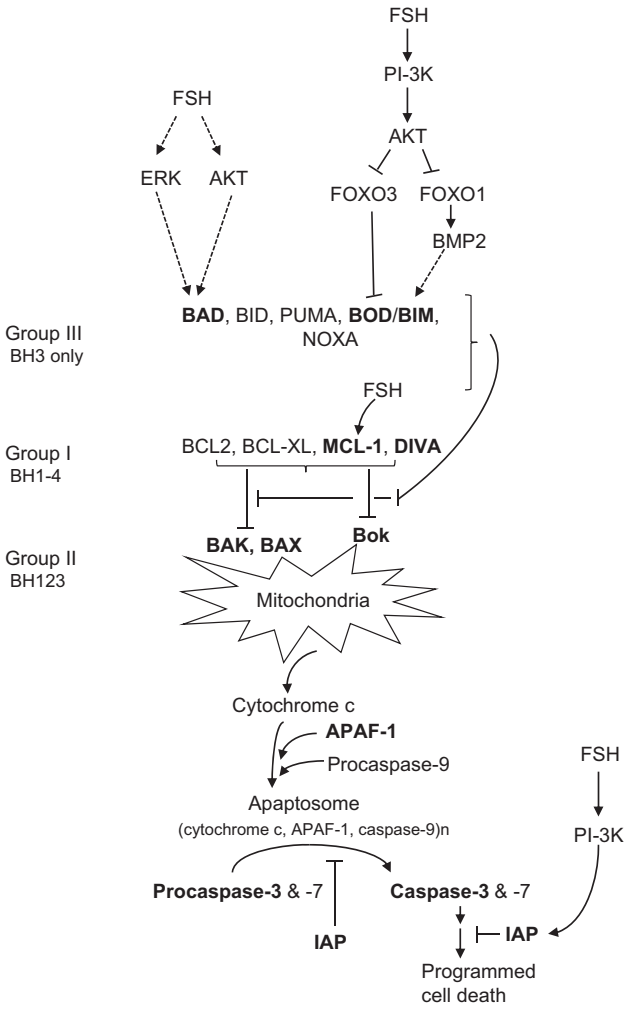

FIGURE 20.10 Schematic diagram of the mitochondrial-dependent apoptotic pathway in ovarian cells and sites at which FSH has been reported to inhibit (perpendicular lines) or stimulate (arrows). Bold names represent proteins identified in granulosa cells. Dashed lines represent expected but not demonstrated regulation.

proteolysis of a large number of proteins crucial to cell survival and consequent cell death.[312] PI-3K–stimulated AKT has been reported to phosphorylate and inactivate caspase-9[316]; however, the AKT phosphorylation site found in human caspase-9 is not conserved in rat, mouse, or monkey caspase-9.[317] Thus, regulation of caspase-9 phosphorylation/activity at least by AKT does not appear to be a widespread mechanism by which PI-3K signaling promotes cell survival. Although IGF-1 has been shown in porcine granulosa cells to inhibit apoptosis,[240] a definitive role for IGF-1 in rat granulosa cell apoptosis, to our knowledge, has not been reported.

The fate of a cell is determined by the balance between survival and apoptotic signals. Transgenic mice overexpressing the Group I anti-apoptotic protein BCL-2 in the ovary show decreased follicular apoptosis and increased folliculogenesis; however, BCL-2 does not appear to be expressed in the ovary.[318] Rather, ovaries appear to express predominately MCL-1, and expression of MCL-1 is increased by treatment of rats with the FSH receptor agonist pregnant mare's serum gonadotropin (PMSG).[319] While increased expression of MCL-1 likely contributes to the ability of FSH to promote cell survival, the mechanism by which FSH increases MCL-1 expression was not explored. Yeast two-hybrid analysis showed that MCL-1 interacted with BAK, BAX, and BOK (Group II BH123 pro-apoptotic proteins) to inhibit apoptosis, and with BAD and BIM (Group III BH3-only pro-apoptotic proteins) to promote apoptosis, consistent with the current view of the mechanism by which Group III proteins promote apoptosis. Ovaries also express high levels of the Group I anti-apoptotic protein DIVA.[298]

Yeast two-hybrid studies with an ovarian cDNA library showed that a mutant, nonphosphorylated BAD (Group III BH3-only protein) interacts with MCL-1, thereby diminishing the anti-apoptotic effect of MCL-1.[319] The phosphorylation of BAD prevents its interaction with MCL-1, thereby allowing MCL-1 to inhibit apoptosis, and promotes its binding to 14-3-3 proteins. BAD is phosphorylated on Ser136 by AKT, and on Ser112 and Ser155 by PKA or RSK in a cell-specific manner.[320] Phosphorylation of Ser155 is thought to be rate limiting and to require priming phosphorylations at Ser112 and Ser136.[320] Based on the ability of FSH to activate PKA, AKT, and RSK, it is likely that BAD phosphorylation contributes to the ability of FSH to promote granulosa cell survival. However, FSH-stimulated BAD phosphorylation has not been formally demonstrated in granulosa cells.

FSH has recently been shown to promote granulosa cell survival by inhibiting the transcription of *Bcl2l11* that encodes BIM/BOD, a BH3-only Group III pro-apoptotic protein.[290] In the absence of FSH, FOXO3 is a transcriptional activator *Bcl2l11*.[290] The effect of FOXO3 appears to be facilitated by FOXO1 activation of *Bmp2* expression; BMP2 also enhances expression of *Bcl2l11* to promote apoptosis.[291] FSH via the PI-3K/AKT pathway stimulates the phosphorylation of FOXO3 on Ser253, resulting in its inactivation and exit from the nucleus.[290] AKT-stimulated FOXO1 phosphorylation in response to FSH is also expected to reduce expression of BMP2 and consequently of *Bcl2l11*, although this has not been demonstrated. Together, these results show that in the absence of FSH, FOXO3 and FOXO1 via BMP2 are activators of *Bcl2l11* expression. FSH reduces the expression of BIM/BOD in a PI-3K/AKT–dependent manner by regulating the activity of FOXO3 and likely of FOXO1 (Figure 20.10). Thus, in the absence of FSH, FOXO1 and FOXO3 contribute to granulosa cell apoptosis.

Granulosa cells express APAF-1 and, in the absence of gonadotropin support, exhibit increased activation of caspase-3.[321] There is also evidence that gonadotropins decrease APAF-1 expression[294] (Figure 20.10), a response expected to be anti-apoptotic. Although granulosa cells

of caspase-3-null mice show attenuated apoptosis, these cells do undergo apoptosis, suggesting that other effector caspases can compensate for caspase-3.[322]

Additional regulation of the apoptotic pathway occurs via a family of inhibitors of apoptosis (IAPs) that act as substrate inhibitors of caspases and block the activation of both effector caspases and, at higher concentrations, initiator caspases.[297] Of these IAPs, the X-linked IAP (XIAP) is reported to be the most potent.[297] FSH has been shown to increase expression of XIAP in rat granulosa cells[236,310] (Figure 20.10). Based on evidence that the addition of XIAP antisense oligonucleotides to granulosa cells enhances apoptosis,[310] it is likely that the FSH-dependent regulation of XIAP expression contributes to the anti-apoptotic effect of FSH.[323] There is also a report that AKT interacts with and phosphorylates XIAP, protecting it from ubiquitin-directed degradation.[324] These results suggest that the regulation of XIAP is crucial to the ability of FSH to rescue granulosa cells from programmed cell death and that FSH potentially regulates XIAP at multiple levels. However, FSH-stimulated XIAP phosphorylation via AKT has not been demonstrated in granulosa cells. A second group of regulators of the apoptotic pathway function as inhibitors primarily of the initiator caspases. One of the members of this group of proteins is FLICE inhibitory protein, or FLIP.[297] Although FSH has not been shown to regulate the expression of FLIP in granulosa cells, the prosurvival effects of TNF-α in rat granulosa cells are accompanied by increased expression of both XIAP and FLIP.[325,326]

NF-κB. The PI-3K pathway can also signal to regulate cell survival by promoting the nuclear translocation of the ubiquitous transcription factor NF-κB. Seven proteins comprise the NF-κB family: p105 and its proteolytic product p50, p100 and its proteolytic product p52, RELA (p65), RELB, and c-REL.[327] These transcription factors function as heterodimers and bind to κB response elements on target genes. More than 200 genes have been reported to be regulated by NF-κB.[328] The prototypical NF-κB transcription factor complex consists of p50 and RELA.[327] p50/RELA exists in the cytosol as a latent transcription factor complexed to an IκB inhibitor, as shown schematically in Figure 20.11. The family of IκB inhibitors include IκB-α, the prototypical IκB inhibitor, as well as IκB-β, γ, and ε.[327] In response to growth factor or cytokine stimulation, the IκB kinase (IKK2; which exists in the cytosol in a complex with IKK1 and the scaffold protein NEMO) is activated and phosphorylates the IκB inhibitor, targeting IκB for ubiquinitation and proteosome-mediated degradation.[327] With the dissociation of IκB, the nuclear localization signal of RELA becomes exposed and p50/RELA translocates into the nucleus. Transcriptional activation of this complex, however, additionally requires the phosphorylation of RELA in a cell-specific manner.[327] RelA kinases include

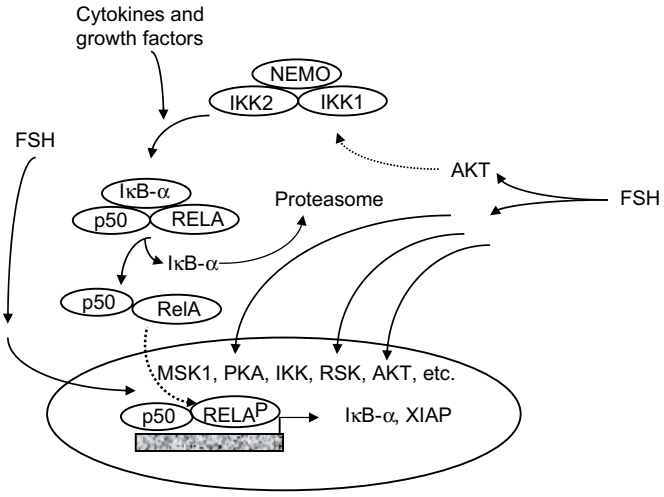

FIGURE 20.11 Schematic diagram of the pathway leading to NF-κB-dependent activation of target genes. Potential sites of FSH stimulation of this pathway are indicated. Dashed lines represent expected but not demonstrated regulation.

PKA, MSK1, RSK, AKT, PKCζ, casein kinase 2, IKK, and NF-κB-activating kinase.[327,329] Phosphorylation of RELA generally increases the binding of co-activators, like CBP, and/or DNA binding activity.[313]

FSH has been shown to promote the translocation of RELA from the cytoplasm to the nucleus of rat granulosa cells (as shown in Figure 20.11) and to increase NF-κB–DNA binding activity, detected by formation of a protein–DNA complex using the consensus κB binding site and granulosa cell nuclear extracts in mobility shift assays.[236] Additionally, the DNA–protein complex could be supershifted by antibodies to p50 and RELA, consistent with the presence of these proteins in the protein–DNA complex.[236] Inhibition of the nuclear translocation of p50/RELA with SN50, a drug that binds to and blocks the nuclear localization signal of NF-κB, inhibited the ability of FSH to induce the anti-apoptotic protein XIAP and suppressed NF-κB–DNA binding activity.[236] These results suggest that the caspase inhibitor XIAP may be an NF-κB target in granulosa cells (Figure 20.11). Pretreatment of granulosa cells with the PI-3K inhibitors LY292004 or wortmannin inhibited FSH-stimulated activation of p50/RELA DNA-binding activity as well as XIAP expression, suggesting a role for PI-3K in this pathway.[236] However, these authors did not determine whether the PI-3K inhibitors blocked translocation of NF-κB or affected its transactivation activity. Because AKT has been reported to enhance IKK activity[292] to phosphorylate IκB and to promote transactivation of RELA,[315] either/or both sites of action of PI-3K is possible. However, these authors did not detect the phosphorylation of IκB-α or its degradation during the first 30 min after treatment with FSH, in contrast to results with TNF-α-treated granulosa cells.[236] Moreover, modest (2.5-fold) overexpression of a dominant

negative IκB-α (which had its IKK phosphorylation sites mutated from Ser to Ala) did not inhibit the ability of FSH to increase p50/RELA DNA-binding activity or XIAP expression but did inhibit the ability of TNF-α to increase p50/RELA DNA-binding activity.[236] One interpretation of these results is that FSH regulates the nuclear translocation of p50/RELA by an alternative pathway independent of IκB phosphorylation. Because the transactivation of RELA can also be enhanced by PKA,[313] it is tempting to speculate that FSH-stimulated PKA also participates in RELA transcriptional activation (see Figure 20.11). Additional studies are thus required to clarify the mechanism by which FSH enhances NF-κB activity and to identify additional NF-κB target genes in granulosa cells.

NOS. An additional AKT substrate is endothelial nitric oxide synthase (eNOS; *Nos3*). NOS catalyzes the oxidation of L-arginine to nitric oxide (NO) and L-citrulline. NO regulates a number of biological functions including apoptosis, cell migration, and angiogenesis.[330] Phosphorylation of eNOS on Ser1177 by AKT results in its activation.[331] A major target of NO is the soluble guanylyl cyclase, the activation of which results in the production of cGMP.

Ovarian cells have been shown to express both eNOS (*Nos3*) and inducible NOS (iNOS; *Nos2*) in both a gonadotropin-dependent and -independent manner. Both eNOS and iNOS appear to be expressed in theca and stroma of immature rat follicles in the absence of gonadotropin stimulation[332]; eNOS, but not iNOS, is also expressed on the surface of oocytes.[332] The injection of PMSG to immature rats or addition of FSH to porcine granulosa cells induces the expression of eNOS, but not iNOS, in mural granulosa cells (~2.5-fold).[332] hCG injection to immature rats further increases eNOS expression 5–7 fold.[332] While iNOS-null mice do not exhibit a striking reproductive phenotype,[333] eNOS-null mice exhibit reduced rates of ovulation and oocyte maturation (>50%), and a five-fold increase in estrogen at a time when estrogen is 50% lower than proestrus peak levels in wild-type mice.[333,334] eNOS expression in response to both PMSG and hCG is regulated, in part, by nuclear orphan receptor LRH-1 (*Nr5a2*).[335] Conditional deletion of *Nr5a2* from granulosa cells, driven by *Amhr2-Cre*, results in reduced *Nos3* expression at 40 h post PMSG and 4 h post hCG, and CHIP assays place *Nr5a2* on the *Nos3* promoter 40 h post PMSG.[335] Conditional deletion of *Nr5a2* from granulosa cells also prevents the fall in estrogen that normally precedes ovulation, as discussed below in "Luteinization," consistent with the notion that LHR-1–regulated *Nos3* expression contributes to the LH/hCG-induced down-regulation of *Cyp19a1*. These results suggest that eNOS is most likely activated in response to the preovulatory surge of LH.

The signaling pathways that regulate eNOS activation to generate NO have not been studied in granulosa cells, although regulation by the PI-3K pathway downstream, at least of LH/hCG, is likely, based on evidence that the phosphorylation of eNOS on Ser1177 by AKT is activating.[331] FSH has been shown to produce only a minimal increase in cGMP levels,[336] and direct activation of soluble guanylyl cyclase in rat granulosa cells results in reduced FSH-stimulated cAMP production, estrogen, and inhibin-α synthesis.[336,337] These latter results suggest that it is unlikely that FSH signals to activate guanylyl cyclase activity in mural granulosa cells. However, FSH-dependent NO signaling to regulate pathways other than guanylyl cyclase cannot be excluded.

Testosterone Synergizes with FSH

It is common for investigators to include either testosterone or estrogen in primary granulosa cell cultures, based on evidence that they enhance FSH actions.[19] However, the mechanisms by which either estrogen or testosterone contributes to FSH-stimulated responses are undefined. A recent study showed that conditional deletion of the androgen receptor from preantral follicles, using *Amhr2-Cre*, resulted in subfertile mice that ovulated ~75% fewer oocytes, with ovaries that contained fewer preovulatory follicles and corpora lutea, and increased preantral and atretic follicles.[338] Follicles grew more slowly and appeared to be halted at the preantral stage of follicle development, consistent with the notion that the androgen receptor in conjunction with FSH is required for follicular maturation to the preovulatory state. In agreement with these results, addition of 1 μM testosterone to primary granulosa cells for 96 h promotes a striking (>15-fold) increase in *Cyp19a1* mRNA expression that is mediated, in part, by increased expression of *Nr5a2* (LRH-1), and increased association of *Nr5a2*, and not *Nr5a1*, with the *Cyp19a1* promoter, as evidenced by CHIP assay.[339] Testosterone plus FSH yields a synergistic increase in *Cyp19a1* mRNA expression.[339] Estrogen elicits a more modest ~two-fold induction of *Cyp19a1* mRNA while the testosterone metabolite 5α-dihydrotesterone (DHT) is ~50% as effective as testosterone.[339] In contrast to these studies, there are reports that DHT inhibits follicular maturation.[19] Indeed, Menon's group showed that DHT (~ 25 nM, 24 h) inhibits FSH-stimulated ERK, Tuberin, and p70 S6 kinase phosphorylations resulting in reduced induction of cyclin D2 expression in granulosa cells.[262] The actions of DHT were mediated, in part, by promoting the phosphorylation of AMPK on Thr172, thereby activating this kinase.[199] While AMPK co-immunoprecipitates with ERK, the mechanism by which active AMPK inhibits FSH-stimulated ERK activation was not investigated.[199]

CROSS TALK AMONG FSH-REGULATED SIGNALING PATHWAYS AND TRANSCRIPTIONAL ACTIVATORS TO REGULATE GENE EXPRESSION DURING GRANULOSA CELL DIFFERENTIATION

As reviewed in the preceding sections, FSH regulates gene expression to inhibit apoptosis, drive proliferation, stimulate steroidogenesis, and promote a complex differentiation response that enables granulosa cells to respond appropriately to the LH surge. FSH accomplishes this array of functions by regulating posttranslational modifications of proteins that function as transcriptional activators, co-activators, repressors, or co-repressors, and of proteins that remodel chromatin to modulate gene activation. The majority of the responses to FSH appear to be mediated by PKA, as reviewed earlier. PKA can directly phosphorylate transcriptional activators, like CREB, and co-activators, like β-catenin, as discussed below, or can initiate signaling cascades that promote posttranslational modifications of transcriptional activators, such as GATA-4/6, co-activators, repressors, and co-repressors. PKA also enhances the translation of preexisting mRNA, such as that for HIF-1α, and newly transcribed mRNA. Figure 20.12 reviews some of the FSH target genes that are regulated directly or indirectly downstream of PKA-regulated transcriptional activators, co-activators, and repressors, as described in preceding and subsequent sections. As we will review below, it is apparent that no single transcriptional activator is sufficient to activate a target gene, and that a number of pathways converge on the promoters of these genes to activate multiple transcriptional activators and co-activators, to remodel chromatin, and to remove repressors and co-repressors. FSH also represses expression of target genes, such as those that stimulate apoptosis (*BIM*),[290] as previously discussed; however, this topic is less well defined. FSH also stabilizes mRNA, at least for the *Lhcgr*; this FSH response is also poorly understood.[340] In this section, we will summarize our current understanding of the convergence of signaling pathways on various transcriptional regulators to stimulate or inhibit expression of select FSH target genes.

LH Receptor

The rate of *Lhcgr* transcription and *Lhcgr* mRNA stability are increased by FSH.[340] While cAMP is required to stabilize *Lhcgr* mRNA in granulosa cells, the mechanisms of its actions are complex and poorly understood.[340] Transcriptional regulation of *Lhcgr* has been more extensively investigated. cAMP responsiveness of the 5′-*Lhcgr* promoter was attributed, in part, to three Sp1 binding sites.[341] Sp1 is a common PKA-dependent transcriptional activator for a number of FSH target genes, including *Sgk*,[26] *Egr-1*,[29] and *Ereg*.[27] Sp1 binds to "GC"-rich motifs, and its transcriptional activity is enhanced upon phosphorylation at a number of Ser/Thr residues by various kinases including ERK or PKCζ downstream of PI-3K.[169] As both the ERK and PI-3K pathways are activated in a PKA-dependent manner in granulosa cells, as previously reviewed, Sp1 could be regulated by either or both kinases. Consistent with a role for ERK in FSH-stimulated expression of EGR-1, FSH-stimulated Sp1 binding to the *Egr-1* promoter is reduced by pretreatment of granulosa cells with the MEK inhibitor PD98059.[29] However, the ability of either ERK or PKCζ to phosphorylate Sp1 and regulate its binding to FSH target genes has not been demonstrated in granulosa cells.

We recently showed that the co-activator β-catenin contributes to the ability of PKA to activate *Lhcgr*

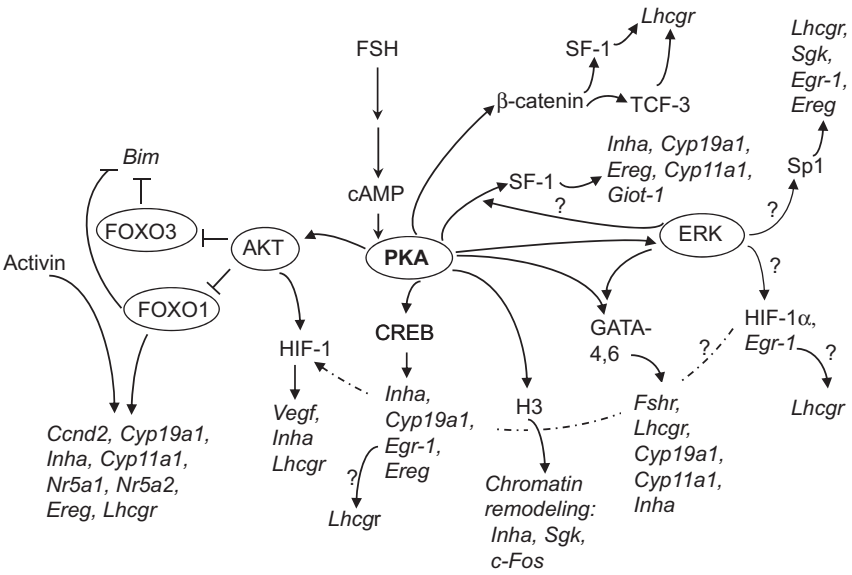

FIGURE 20.12 Composite regulation of FSH-responsive gene targets. Question marks (?) for ERK signaling reflect inhibition by a MEK inhibitor but not proof of target phosphorylation. Regulation of Lhcgr by Egr-1 is suggested by literature.

transcription[177]. Transduction of granulosa cells with an adenovirus that expresses a mutant SF-1 that cannot bind β-catenin[342] blocks the ability of FSH to promote *Lhcgr* mRNA expression. This result suggests that SF-1 is necessary for *Lhcgr* mRNA expression and that FSH activates β-catenin. FSH stimulates the PKA-dependent phosphorylation of β-catenin on Ser552 and Ser665, sites that have been linked with the ability of β-catenin to act as a co-activator for transcriptional factors,[178,179] and activation of the TCF artificial promoter-reporter TOPFlash, consistent with a previous report.[303] In support of these results, transduction of granulosa cells with an adenoviral β-catenin mutant that mimics phosphorylation of these sites (Ser552Asp, Ser665Asp) enhanced *Lhcgr* mRNA expression in FSH-treated cells.[177] The ability of β-catenin to function as a co-activator for SF-1 is now recognized in a number of cell models.[175,176,286] SF-1 is also necessary for expression of other FSH target genes, such as *Cyp19a1*, *Ereg*, *Cyp11a1*, *Inha*, and *Giot-1*. We hypothesize that the PKA-dependent phosphorylation of β-catenin contributes to the PKA-dependent regulation of these genes and is the predominant mechanism by which β-catenin activity is regulated in granulosa cells. However, this hypothesis has not been formally tested for genes other than the *Lhcgr*.

Results also suggest that β-catenin functions in granulosa cells to promote expression of *Lhcgr* mRNA by displacing co-repressors from TCF3 bound to the *Lhcgr* promoter.[177] This conclusion is based on data showing that an adenoviral dominant negative TCF,[343] in which the β-catenin binding site was deleted, abrogated the ability of FSH to induce *Lhcgr* mRNA. This suggests that β-catenin functions not only as a co-activator for SF-1 but also competitively displaces a member of the co-repressor Groucho-related genes family to activate TCF3.[344] Consistent with these findings, CHIP results showed that TCF3 is constitutively associated with *Lhcgr* promoter, and that FSH stimulates association of β-catenin phosphorylated on Ser552 and Ser675 as well as SF-1 with *Lhcgr* promoter.[177] Microarray results suggest that TCF3 is a common activator of a number of FSH target genes, such as *Inha*, *Cyp19a1*, and others, but additional experiments are required to confirm this hypothesis.

Lhcgr mRNA expression also appears to require expression of the immediate early gene EGR-1, based on evidence that follicles of EGR-1-null mice do not acquire LH receptors and thus cannot ovulate.[345,346] EGR-1 is a zinc-finger transcription factor that binds GC-rich enhancer elements.[347] FSH transiently increases EGR-1 expression in granulosa cells.[29] Activation of the *Egr-1* promoter is PKA dependent and involves ERK-dependent binding of Sp1 and Sp3 and phosphorylated CREB, as evidenced by electromobility shift assays and promoter mutational analyses.[29] Evidence that EGR-1 regulates *Lhcgr* expression has only been provided indirectly by electrophoretic mobility shift assays and by the ability of overexpressed EGR-1 to modestly enhance *Lhcgr*-promoter luciferase activity in a Leydig cell line.[346] Additional studies are required to define more completely the regulation of *Lhcgr* by EGR-1.

Lhcgr mRNA expression also appears to require HIF-1 and relief from repression by FOXO1, both of which are regulated downstream of PKA and PI-3K signaling. Although formal evidence for the regulation of *Lhcgr* mRNA expression by HIF-1 is not available, indirect data showed that dominant-negative HIF-1α missing its DNA binding and transactivation domains abolished FSH-stimulated *Lhcgr* promoter-luciferase reporter activity.[235] *Lhcgr* has also been identified as a FOXO1 target whose FSH-stimulated mRNA expression is reduced in cells transduced with adenoviral A3-FOXO1.[177,271]

In summary, pathways that contribute to the transcriptional activation of this gene include: PKA via either ERK or PKCζ to phosphorylate/activate Sp1; PKA to phosphorylate/activate β-catenin to activate both SF-1 and TCF3; PKA via PI-3K to phosphorylate/inactivate FOXO1; PKA via PI-3K and another pathway, possibly ERK, to promote accumulation of HIF-1α and activate HIF-1; and PKA to phosphorylate/activate CREB plus ERK or PKCζ to phosphorylate/activate Sp1 to promote expression of the immediate early gene EGR-1. Finally, we hypothesize that PKA also promotes remodeling of chromatin associated with *Lhcgr* gene by stimulating the phosphorylation of Ser10 and acetylation on Lys14 of histone H3, as previously discussed for the *Inha* promoter.[118] However, there is no direct evidence that either of these histone H3 modifications is linked to the *Lhcgr* promoter.

Inhibin-α and Aromatase

The expression of both inhibin-α and aromatase have been extensively studied regarding the combinations of factors required to promote their transcription. Unlike the *Lhcgr* promoter, the *Inha* promoter contains a functional nonconcensus CREB binding site as well as an SF-1 binding site. SF-1/LRH-1, both of which bind to the same DNA element,[348] and cAMP/PKA synergize to activate the *Inha* promoter.[170,349] The SF-1 and CREB sites are adjacent to each other on the promoter, and although SF-1 and CREB can interact in extracts, it is not known if this interaction is direct.[170] The co-activators CBP and SRC-1 further increase *Inha* transcriptional activity in a mouse granulosa cell line, and the association of both with the *Inha* promoter is enhanced by forskolin, as evidenced by CHIP assays.[349] Recent studies suggest that the LIM domain protein four and a half LIM domain 2 (FHL2) also functions as a co-activator of SF-1/LRH-1 and CREB in granulosa cells.[350] FHL2 is recruited to the *Inha* promoter in a granulosa cell line in response to

elevated cAMP levels, although the mechanism of this recruitment was not investigated.[350] Unlike CBP and the co-activator SRC-1, FHL2 lacks enzymatic activity and may function as a scaffold to integrate the association of other co-activators.

CHIP assays also revealed that while SF-1 is associated with the *Inha* promoter in the basal state, forskolin stimulates the release of SF-1 and binding of LRH-1 (which is induced by forskolin) in a granulosa cell line.[349] These cAMP-dependent associations of SF-1/LRH-1 with the *Inha* promoter, as well as cAMP-stimulated *Inha* promoter-luciferase activity, were inhibited by the MEK inhibitor PD98059,[349] possibly reflecting ERK phosphorylation of SF-1[203] and LRH-1.[351] The mechanistic basis for the dynamic associations of SF-1 and LRH-1 with the *Inha* promoter is not known, but might reflect distinct posttranslational modifications or distinct associations of the co-activator β-catenin[175,176] with SF-1 versus LRH-1. While SF-1 and LRH-1 tend to have overlapping actions,[352] selective regulation of the *Cyp19a1* expression by LRH-1 in testosterone-treated granulosa cells has been reported and seems to be due to preferential expression of LRH-1 by testosterone.[339]

The ability of cAMP to activate the *Inha* promoter thus likely reflects actions at a number of sites. cAMP-activated PKA phosphorylates CREB on Ser133, promoting recruitment of CBP.[167,211] PKA also promotes the phosphorylation on Ser10 and acetylation on Lys14 of histone H3, as previously discussed, thus remodeling chromatin in the region of the *Inha* promoter.[118] PKA is also recognized to potentiate SF-1 transcriptional activity both via the phosphorylation/activation of β-catenin and via ERK-mediated phosphorylation of SF-1, as previously described. *Inha* expression is also regulated by HIF-1, based on the ability of a dominant-negative HIF-1α to reduce significantly the ability of FSH to activate *Inha* promoter-reporter activity.[235] Additionally, expression of constitutively active FOXO1 in granulosa cells reduces the ability of FSH plus activin to activate *Inha* expression,[48] although there is no formal evidence that FOXO1 represses *Inha* expression.

There is also accumulating evidence that GATA transcription factors regulate a number of FSH targets, including *Inha* in granulosa cells. GATA factors are zinc-finger DNA binding proteins. GATA-4 and -6 are highly expressed in granulosa cells,[353] and promoters of several FSH target genes contain functional (*Inha*, *Nr5a1*, *Hsd17b*, *Cyp11a1*, *Cyp19a1*, *Star*) or putative (*Lhcgr*, *Hsd3b1*) GATA regulatory elements.[354,355] GATA-4 is phosphorylated on Ser261 directly by PKA[356] and on Ser105 by ERK.[357] *Inha* gene expression is activated by GATA-1 in testicular cell lines,[358,359] and PKA-phosphorylated GATA-4 enhances an *Inha* promoter-reporter construct in CV-1 cells.[356] GATA-4 also regulates expression of *Cyp19a1* in granulosa cells.[360] FSH stimulates the association of GATA-4 with the *Cyp19a1* promoter, as shown by CHIP assays, and GATA-4 siRNA reduces the ability of FSH to promote *Cyp19a1* mRNA expression. A nonphosphorylatable Ser105 GATA-4 mutant prevents the induction of *Cyp19a1* mRNA by FSH, and the MEK inhibitor U0126 abolishes FSH-stimulated phosphorylation of GATA-4 that was transfected into granulosa cells.[360] That GATA-4 is required for follicular maturation was recently demonstrated with conditional deletion of both GATA-4 and GATA-6 in granulosa cells with the *Cyp19-Cre*.[353] Mice are infertile, follicular maturation is arrested prior to antrum formation, and granulosa cells exhibit reduced *Fshr*, *Lhcgr*, *Cyp19a1*, and *Cyp11a1* mRNA. Together these results show that GATA-4 is necessary for follicular maturation and that its transcriptional activity is regulated by PKA.

FSH also regulates the expression of a protein, DAX-1 (dosage-sensitive sex reversal, adrenal hypoplasia critical region, on the X-chromosome, gene 1, *Nrob1*), that functions as a transcriptional repressor of Nr5a1 (SF-1) and Nr5a2 (LRH-1).[342] FSH reduces DAX-1 mRNA and protein levels, and overexpression of DAX-1 has been shown to prevent activation of *Giot-1* promoter activity by SF-1 in granulosa cells.[30] Expression of the *Cyp19a1* gene is also inhibited by DAX-1,[361] suggesting that for those genes that require binding of SF-1 or LRH-1, decreased expression of DAX-1 may be a conserved response to FSH. However, the mechanism by which FSH downregulates *Dax-1* mRNA has not, to our knowledge, been investigated.

Taken together, FSH-stimulated target gene activation requires input from a number of pathways, as summarized in Figure 20.12. Inhibitors of PKA, PI-3K, and MEK abrogate the ability of FSH to activate select target genes. The requirements for both PKA-phosphorylated CREB as well as for SF-1 and/or Sp1 and/or GATA-4/6 to promote transcriptional activation of a number of FSH target genes coupled with their dependence on the PI-3K pathway explains, in part, why activation of the PI-3K pathway by IGF-1 is not sufficient to induce FSH target genes. Although some of the transcription factors and co-activators required to activate a small subset of FSH target genes have been identified, much needs to be learned regarding the combinatorial requirements for transcription factors and co-activators and how the activity of these factors is regulated. Moreover, it is likely that additional signaling pathways are regulated by FSH. Finally, we know very little about how FSH-dependent pathways converge with the many signaling systems involved in initial formation and early growth of the follicle, as discussed in Chapter 21. Thus, there is a great deal remaining to be elucidated regarding how FSH signals to promote follicular maturation.

LH SIGNALS THAT REGULATE THECA CELL FUNCTIONS

Follicles are exposed to relatively low levels of LH as follicle maturation proceeds. The resulting increased production of androgens provides the substrate for aromatase in granulosa cells to synthesize estrogen. In addition to facilitating the actions of FSH, these increasing levels of estrogen act in a positive feedback fashion on the brain to induce the preovulatory LH surge. In pathological conditions such as polycystic ovarian syndrome, theca cells produce elevated levels of androgens. Thus, an understanding of how LH regulates androgen production is important.

LH has been shown to enhance mRNA expression of the transcription factor required for cholesterol biosynthesis, sterol regulatory element binding transcription factor 1a (*Srebf1a*), and HMG-CoA reductase(*Hmgcr*), the rate-limiting enzyme in cholesterol biosynthesis.[362] LH also induces 17α-hydroxylase/C17-20-lyase (CYP17; *Cyp17a1*), the rate-limiting enzyme in androgen biosynthesis in theca cells,[363] and the low-[364] or high-[365] density lipoprotein receptor, depending on the species, to provide cholesterol for steroid biosynthesis. While the signaling pathways and transcriptional regulation of LH-regulated genes in theca cells have not been intensely investigated, recent studies have placed SF-1 on both the *Cyp17a1* and *Star* promoters, and GATA-6 on the *Cyp17a1* promoter, as shown by CHIP studies.[366] There is also evidence that the nuclear orphan receptor NR4A1/NUR77 contributes to the ability of LH to induce genes required for androgen biosynthesis including *Star*, *Cyp11a1*, *Cyp17a1*, and *Hsd3b2*.[367] Overexpression by adenoviral transduction of *Nr4a1* increases and knockdown of *Nr4a1* reduces the expression of all of these genes in response to LH. The induction of these mRNAs as well as that of *Nr4a1* is mimicked by forskolin, consistent with a cAMP-regulated pathway.[367] LH via a PKA-dependent step has been shown to signal into the AKT, Tuberin, mTORC/Raptor pathway to stimulate the phosphorylation of p70 S6 kinase, leading to the upregulation of cyclin D3, *Hsd3b1*, *Cyp11a1*, and *Cyp17a1*.[368] Although this pathway is generally reserved for regulating translation,[250] in theca cells p70 S6 kinase phosphorylates CREB on Ser133. CHIP assays place CREB on the *Cyp17a1* promoter.[368] The requirement for the mTORC/Raptor pathway to regulate expression of *Hsd3b1*, *Cyp11a1*, *Cyp17a1*, and *Ccnd3* is based on inhibition by the mTORC/raptor inhibitor rapamycin and by mTORC siRNA studies.[368]

Thus, while some of the signals leading to enhanced transcription of genes that promote androgen production have been identified, the persistent regulation of these pathways during the life of the follicle until the surge of LH remains incompletely understood. It is quite interesting, as discussed below, that the signaling pathways initiated by LH to regulate gene expression in theca cells are quite distinct from the pathways used by LH to regulate ovulation and corpus luteum formation.

THE LH SURGE PROMOTES OOCYTE MATURATION, OVULATION, AND CORPUS LUTEUM FORMATION

A Brief Overview of the Physiology of Oocyte Maturation, Ovulation, and Luteinization

Follicles are exposed to a single high level surge of LH, driven by the hypothalamic release of gonadotropin-releasing hormone that promotes ovulation and differentiation of mural granulosa and theca cells (but not cumulus granulosa cells) into luteal cells (luteinization) and triggers final maturation of the oocyte. These responses to LH require the induction of more than 500 genes.[369]

Cumulus Expansion. Preceding ovulation, the cumulus granulosa cells and oocyte become surrounded by a hyaluronan-rich extracellular matrix that facilitates the expansion/movement of cumulus granulosa cells within this matrix. Cumulus expansion and oocyte maturation require, for example, the induction of hyaluronic acid synthase-1 *Has-2*, pentraxin-related protein 3 *Ptx3*, and TNF-α-stimulated gene-6 *Tnfaip-6*.[370] Mice that are deficient in *Ptx3* and *Tnfaip-6* exhibit ovulation defects,[371,372] consistent with a link between cumulus expansion and ovulation.

Ovulation. Ovulation is a complex, incompletely understood process that results in the breakdown of the follicular wall and release of the oocyte and surrounding cumulus granulosa cells. The oocyte and surrounding cells escape through the mural granulosa cell layer, the basement membrane, the theca cell layer, and ovarian surface epithelium. LH induced genes that are required for ovulation include the cyclic nucleotide degrading phosphodiesterase *Pde4d*[34], *Ptgs2* that encodes the rate-limiting enzyme (prostaglandin-endoperoxide synthase) in prostaglandin synthesis,[373] and *Pgr* that encodes the progesterone receptor[374] and its downstream targets pituitary adenylate cyclase activating polypeptide (*Pacap*),[375,376] lysosomal protease cathepsin L (*Ctsl*),[377] and the matrix metalloproteinase *Adamts-1* (a disintegrin and metalloproteinase with thrombospondin-like repeats).[378]

Oocyte maturation. Oocytes are restrained in prophase I of meiosis until just before ovulation. Breakdown of the nuclear membrane, also known as germinal vesicle breakdown, marks an oocyte that has reinitiated meiosis. Completion of the second meiotic division after fertilization results in the production of two polar bodies and a single haploid oocyte.

Luteinization. Luteinization is associated with a cessation of granulosa and theca cell proliferation and is characterized by hypertrophy of both granulosa and theca cells[379] as well as by increased steroid hormone synthesis and secretion. Luteinization requires the downregulation of cyclin D2 (*Ccnd2*),[45] and expression of the rate-limiting enzyme in progesterone synthesis encoded by *Cyp11a1*,[380] the cholesterol-mobilizing protein *Star*,[376] and cell cycle inhibitors $p21^{Cip1}$ and $p27^{Kip1}$[45,381] encoded by *Cdkn1a* and *Cdkn1b*, respectively. The surge of LH also terminates the expression of select FSH target genes that were required for follicular maturation but are not required for luteal function. For example, the following genes are transcriptionally silenced, as discussed below, in response to LH: the FSH receptor (*Fshr*),[69] estrogen receptor β (*Esr2*),[382] *Cyp19a1* (aromatase),[383] *Inha* (inhibin-α),[384] and *Foxo1*.[112] The LH receptor[385] (*Lhcgr*) is transiently downregulated by the LH surge; however, the mechanism is not via reduced transcription, as discussed below. The expression of other FSH targets whose functions are required either during luteinization or for luteal function are not affected by the surge of LH include, for example, *Map2d*,[25] *Cyp11a1*,[380] and *Sgk*.[112] Some of these responses are also variable among species; for example, inhibin-α is repressed by the LH surge in the rodent,[384] but inhibin A is actively secreted during the luteal phase in the primate reproductive cycle.[386]

LH and the LH Receptor

The pituitary gonadotropin LH shares a common alpha subunit with FSH, but its synthesis and secretion from pituitary gonadotropes is under distinct regulation. For many of the studies discussed in this chapter, the related placental gonadotropin hCG (Figure 20.1) is used as an agonist of the LH receptor. The LH receptor, like the FSH GPCR, contains nine Leu-rich-repeat motifs in its extracellular domain and is classified in subgroup A of the rhodopsin/β-adrenergic family of GPCRs[9,387] (Figure 20.1). These Leu-rich-repeats contribute to hormone binding and structural integrity of this receptor.[388] The LH receptor is encoded by a gene with 11 exons and 10 introns.[6] Exons 1–7 encode the regions of the large extracellular domain that are essential for high affinity binding of LH receptor agonists and the placental-derived hCG.[389,390] Exons 9 and 10 also encode the extracellular domain, but this region appears to be less important for agonist binding.[386] Exon 11 encodes the seven TM domains and the C-terminal tail. The LH receptor in female mammals is expressed in the ovary in theca cells of ovarian follicles, in mural granulosa cells of mature preovulatory follicles, and in corpora lutea. LH receptor-null mice are infertile; their ovaries contain only early antral follicles and lack preovulatory follicles or corpora lutea.[391]

The absence of follicular development to a preovulatory stage in LH receptor-null mice likely reflects, at least in part, the reduced levels of theca-derived androgens and thus granulosa-derived estrogens,[391] and the apparent requirement of these steroid hormones for final follicular maturation. Granulosa cells also express androgen receptors.[392] Androgen receptor-null mice exhibit increased granulosa cell apoptosis and defective cumulus cell expansion in response to the LH surge,[393] and conditional deletion of the androgen receptor from granulosa cells results in subfertile mice that ovulate ~75% fewer oocytes.[338] Together these results suggest a role for androgens in granulosa cells distinct from their function as an aromatase substrate. Although granulosa cells express primarily ER-β and theca cells express ER-α, mice null for ER-α or ER-β ovulate in response to exogenous gonadotropins, although ovulation efficiency is reduced, suggesting that estrogen facilitates ovulation.[394] Incomplete follicular maturation in the LH receptor-null mice could also result from effects of basal LH on granulosa cells distinct from those described for the surge of LH.

In the absence of agonist, GPCRs are believed to exist in the membrane primarily in an inactive conformation that is in equilibrium with an active conformation.[59] Agonist binding is thought to stabilize the receptor in the active conformation. Consistent with this notion, and unlike the FSH receptor, a number of mutations in the LH receptor have been identified that stabilize the receptor in an active conformation in the absence of agonist.[395] The first human mutation of the LH receptor identified was Asp578 in TM 6 in patients with familial male-linked precocious puberty.[396] This mutation results in a constitutively active LH receptor, which in males leads to precocious puberty. For reasons that are not clear, females with this LH receptor mutation do not exhibit abnormal fertility.[395] This may reflect a stringent requirement for postpubertal levels of FSH to induce LH receptor expression in the female ovary. The absence of a phenotype in women with constitutively active LH receptors is especially unexpected in light of the clear phenotype in mice overexpressing a longer-lived LH mutant protein, which is characterized by precocious puberty, anovulation, and resulting infertility.[397] TM 6 and its extension into the cytoplasm as the third intracellular loop have been defined as hot spots for LH receptor mutations that promote a constitutively active receptor, and more than 16 mutations have been identified in this region.[395] Additionally, Ser431 in TM 3,[398] Arg442 in the second intracellular loop,[399] Asp397 in exoloop 1,[400] and Lys583 in exoloop 3[401] of the rat LH receptor appear to contribute to the stabilized agonist-dependent active conformation of the LH receptor to generate cAMP (without affecting agonist binding), whereas Lys583 in exoloop 3 also appears to stabilize the agonist-stimulated active state

of the receptor to generate inositol trisphosphate (IP_3).[402] A large number of inactivating mutations of the LH receptor in women have been reported.[395] These women exhibit amenorrhea and no preovulatory follicles or corpora lutea, consistent with the established functions for LH, and do not respond to exogenous hCG.

The LH receptor in granulosa cells is induced by FSH[403]; LH receptor synthesis then ceases in response to the surge of LH, as discussed below, and is reinitiated with the formation of the corpus luteum. Hormonal regulation of the induction of LH receptors in corpora lutea is species dependent,[404,405] consistent with the high degree of species specificity in the factors that regulate formation, maintenance, and regression of the corpus luteum. Theca cells appear to express LH receptors coincident with the morphological formation of this cell layer at very early stages of follicular development[69]; however, it is not known if LH receptor expression is constitutive in these cells or is regulated by a paracrine factor produced either by the oocyte or by adjacent granulosa cells.

The LH receptor couples to activate Gs,[406,407] uniformly resulting in activation of adenylyl cyclase to increase synthesis of cAMP.[408] The LH receptor has also been shown to activate Gi,[409–411] Gq/11, and G13, based on the ability of LH to stimulate binding of a GTP photoaffinity analog to these Gα proteins.[411] Although LH receptor activation can stimulate Gi to inhibit adenylyl cyclase activity upon expression in a heterologous cell model,[412] LH-stimulated adenylyl cyclase activity in porcine follicular membranes is not increased by pretreatment of membranes with pertussis toxin, which uncouples GPCRs from Gi.[409] Thus, the physiological significance of LH receptor coupling to Gi is not known. Constitutively active LH receptors expressed in heterologous cells also activate PLC, resulting in elevated levels of IP_3 and Ca^{2+}.[408,413,414] Similarly, when LH receptors are expressed at sufficiently high concentrations, receptor activation raises intracellular Ca^{2+} and activates PLC.[415–417] This response is pertussis toxin insensitive[410] and thus most likely mediated via activation of Gq/11. In rat granulosa cells, which express approximately 10,000 receptors per cell,[411] LH raises intracellular levels of IP_3 presumably via activation of PLC.[418] Thus, signaling through cAMP appears to be the predominant but not the only second messenger through which the LH receptor can modulate responses.

Although the exact residues that bind Gs on the LH receptor have not been identified, the C-terminal portion of the third intracellular loop extending into TM 6 domain is important, based on the ability of a synthetic peptide corresponding to this region to activate adenylyl cyclase in the absence of LH receptor expression.[419] Truncation of the C-terminal region of the rat LH receptor at residue 653 does not affect agonist-stimulated adenylyl cyclase activation but hampers receptor-stimulated PLC activation, suggesting that the G protein that activates PLC couples to a distinct region of the receptor from that which signals to Gs.[420]

The LH receptor also exhibits desensitization and, in heterologous cell models, relatively rapid internalization in response to saturating levels of agonist. However, the preovulatory LH surge does not promote rapid internalization of the LH receptor in granulosa cells. Rather, cell surface receptor numbers around the time of ovulation decrease with a T1/2 of approximately 8.5 h.[421–423] Although this receptor can be phosphorylated on up to five serine residues located in the C-terminal tail[424,425] in a G protein-regulated kinase-dependent manner,[426,427] phosphorylation of the human and porcine receptors does not contribute to either uncoupling from Gs/adenylyl cyclase[424,428–430] or receptor internalization.[431] Similarly, for the rat LH receptor, mutation of the serine residues in the C-terminal tail retards the rate of desensitization but does not affect the extent of LH receptor desensitization.[420,424,426] These results are consistent with evidence that truncation of the C-terminal tail of the LH receptor does not prevent LH receptor desensitization.[432] A protein that interacts with the C-terminus of the human LH receptor, the type I PDZ domain protein GIPC,[433] appears to regulate levels of cell surface LH receptor during hormone internalization and to play a role in ligand recycling.

Thus, unlike the β-adrenergic and rhodopsin GPCRs, in which high affinity binding of arrestin to the receptor is facilitated by receptor phosphorylation,[89,434] binding of arrestin to the LH receptor and consequent uncoupling from Gs appears to depend on receptor activation rather than phosphorylation.[431,435–437] Indeed, arrestin2 binds with pM affinity to a synthetic peptide corresponding to the third intracellular loop of the porcine LH receptor to promote desensitization.[438,439] The accessibility of arrestin2 to the LH receptor in porcine membranes and in HEK kidney cells stably transfected with the murine LH receptor is regulated by the association of arrestin2 with the small G protein ADP-ribosylation factor (ARF)6.[439,440] LH receptor activation promotes activation of membrane-delimited ARF6[441] by ARNO (ARF-nucleotide binding site opener) or a similar ARF6 activator,[442] releasing arrestin2 from its membrane docking site.[438] The binding of arrestin to GPCRs has been shown to sterically hinder the ability of the receptor to bind Gs.[439] Consistent with this model, both Gs binding and arrestin2 binding appear to require the C-terminal portion of the third intracellular loop of the LH receptor.[419,438] Although this pathway for ARF6-dependent desensitization has not been tested in other cellular models, overexpressed ARNO is reported to stimulate arrestin-dependent β-adrenergic receptor internalization, and an inactive ARF6 that cannot bind GTP inhibits β-adrenergic receptor internalization.[443] These results

suggest that ARF6 might play a more universal role in regulating arrestin availability to GPCRs.

Coupling of the LH receptor to Gs is also regulated in a poorly understood manner by PDE4D.[34] Unexpectedly, in granulosa cells of PDE4D null mice, the immediate (detected by 1 h) and the sustained (over 6 h) cAMP synthetic response to an ovulatory concentration of hCG (both in vivo and in vitro) is significantly blunted (and not increased), whereas the response to the adenylyl cyclase activator forskolin (in vitro) is equivalent compared with that of wild-type mice.[34] This result suggests that PDE4D in some manner facilitates coupling between the LH receptor and Gs to activate adenylyl cyclase and that in the absence of PDE4D, coupling between LH receptor and Gs is impaired. LH receptor-Gs coupling is likely regulated by the sustained levels of PDE4D induced in granulosa cells by FSH rather than that induced by LH.[34] How PDE4D enhances LH receptor-Gs coupling remains to be elucidated.

MECHANISMS BY WHICH LH STIMULATES OOCYTE MATURATION, OVULATION, AND LUTEINIZATION

LH-Regulated Signaling Pathways in Preovulatory Cells

The remainder of this chapter will focus on our current understanding of the cellular mechanisms by which LH stimulates oocyte maturation, ovulation, and luteinization. The reader is also referred to Chapter 22 on ovulation. We begin by summarizing LH-stimulated signaling pathways; however, additional information on specific pathways is provided in the context of the following sections.

cAMP- and PKA-Dependent Signaling to CREB and ERK. The LH receptor is believed to signal predominately via Gs to activate adenylyl cyclase and raise intracellular levels of cAMP. Consistent with this notion, the adenylyl cyclase activator forskolin mimics LH to stimulate expression of a number of LH target genes.[29,364,444–447] The primary cAMP target is PKA. Indeed, ovulatory concentrations of LH/hCG in preovulatory follicles lead to activation of PKA,[448] and the specific PKA inhibitor PKI[119] or the selective PKA inhibitor H89[123] inhibits the induction of a number of LH targets or responses.[29,364,448]

As in preantral granulosa cells, hCG or forskolin treatment of preovulatory granulosa cells stimulates the phosphorylation of the PKA target CREB on Ser133.[151,449] Although LH-stimulated CREB phosphorylation is undetectable when granulosa cells are pretreated with the selective PKA inhibitor H89,[424] it is possible that other pathways downstream of PKA also contribute to CREB phosphorylation. LH target genes that contain cAMP response elements and have been shown to bind CREB include EGR-1,[29] ICER,[450] and StAR.[451]

As will be highlighted in the following discussion, one of the most important signaling pathways by which LH regulates gene expression is via the ERK1/2 (MAPK3/1) pathway (Figure 20.13). The initial route to ERK is via PKA, based on the ability of H89 and Myr-PKI to strongly inhibit LH-stimulated ERK activity in mural granulosa cells.[452] However, in contrast to regulating the association of a PTP with ERK as is observed in preantral granulosa cells,[113] PKA signals to regulate ERK activity in preovulatory granulosa cells at a step upstream of MEK. While LH stimulates MEK phosphorylation,[452] the site of regulation by PKA has not been elucidated in preovulatory granulosa cells. In MA-10 and primary Leydig cells, LH signals via PKA into the ERK pathway to activate RAS.[452,453] However, the relevant direct PKA substrate in either of these cell types has yet to be identified.

Sustained ERK activation is required for LH responses that lead to oocyte maturation, ovulation, and luteinization. This is achieved by the ability of LH to promote activation of an EGF receptor located in both mural and cumulus granulosa cells in a PKA-dependent manner.[451,454] The EGF receptor family consists of the EGF receptor (ErbB1, HER), ErbB2 (Neu, HER2), ErbB3 (HER3), and ErbB4 (HER4); granulosa cells express ErbB1, ErbB2, and ErbB3.[455] ErbB1 binds EGF or EGF-like ligands to promote homo- or heterodimerization and activation of its tyrosine kinase activity.[456] An elegant series of studies have shown that LH generates a group of EGF-like ligands that include amphiregulin, epiregulin, and β-cellulin that are synthesized as transmembrane precursors and then shed as mature proteins by cleavage at the membrane surface by a metalloproteinase[454] (Figure 20.13). LH is believed to promote both the de novo synthesis of these ErbB ligands, based on inhibition by puromycin, and to activate a metalloproteinase, based on inhibition by pan-metalloproteinase inhibitors, to generate these EGF-like ligands in a PKA-dependent manner in mural granulosa cells.[451] The ErbB1 ligands promote activation of ErbB1 on both mural and cumulus granulosa cells to signal through the canonical EGF receptor pathway to activate ERK.[456] The functional significance of the ERK pathway to LH actions is discussed below. The ErbB1 ligands also signal via the EGF receptor to activate p38 MAPK (MAPK14), also discussed below.

LH also stimulates expression of the EGF-like factor neuroregulin-1, via an ERK-dependent pathway, and this ligand binds ErbB3.[455] The ErbB3 receptor appears to signal predominately to the PI-3K/AKT pathway in granulosa cells, based on the ability of neuroregulin-1 to enhance AKT but not ERK phosphorylation.[455] Both amphiregulin and neuroregulin-1 contribute to luteinization by enhancing expression of genes required for progesterone production and oocyte maturation.[455]

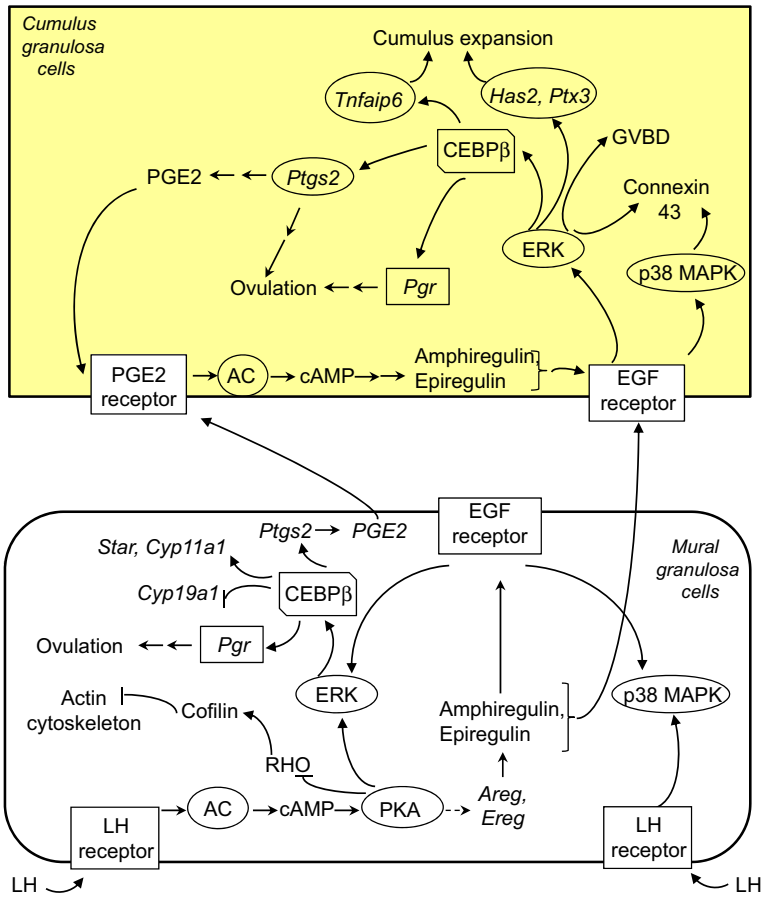

FIGURE 20.13 LH signals that regulate ovulation, cumulus granulosa cell expansion, and luteinization.

LH signaling to p38 MAPK. hCG signals to rapidly phosphorylate p38 MAPK(MAPK14) in preovulatory granulosa cells[452] as well as its upstream kinases MKK3 and MKK6.[217] However, at least the initial signaling into the p38 MAPK pathway appears to be independent of PKA, based on the inability of H89 to modulate hCG-stimulated p38 MAPK phosphorylation in cells in which hCG-stimulate CREB phosphorylation was blocked by H89.[452] The functional significance of the p38 MAPK pathway activated by hCG and ErbB1 ligands is discussed later.

LH Increases Intracellular Ca^{2+} but Does Not Appear to Activate PKC. It is well established that the LH receptor, upon expression in a heterologous cell, activates PLC to raise IP_3 levels and/or intracellular Ca^{2+}.[408,410,417,457] The rise in intracellular Ca^{2+} in one report was shown to be independent of the concomitant rise in cAMP and thus likely dependent on IP_3.[457] In rat granulosa cells, LH is reported to increase IP_3.[458] In porcine theca cells, LH has been shown to generate an oscillatory Ca^{2+} signal that is not inhibited by pertussis toxin (thus independent of Gi) but is inhibited by the Ca^{2+} chelator ethyleneglycotetraacetic acid and by the PLC inhibitor U73211.[459] The rise in intracellular Ca^{2+} likely activates a CaM kinase, and LH-stimulated progesterone production is reduced by 50% by the CaM kinase inhibitor Kn93.[444] Ca^{2+} has recently shown to be required for hCG/amphiregulin-stimulated increases in the activity of the protease calpain-2 in cumulus granulosa cell–oocyte complexes.[460] Active calpain-2 promotes degradation of proteins within the focal adhesion complex of cumulus cells, leading to cell detachment, and is believed to contribute to cell movement within the cumulus cell–oocyte complex.[460] It is also possible that LH activates one or more PKC isoforms.

PKC consists of a number of isoforms[461]: the conventional PKCs (α, β, and γ) require Ca^{2+}, diacylglycerol, and phosphatidylserine for activation; the novel PKCs (δ, ε, η, θ) do not require Ca^{2+}; and the atypical PKCs (ζ, ι/λ) require only phosphatidylserine for activation. Subovulatory concentrations of LH plus 200 nM PMA (phorbol myristate acetate), which binds the diacylglycerol binding site on conventional and novel forms of PKC, mimic the effect of an ovulatory concentration of LH to promote progesterone production, *Ptgs2* induction, and luteinization of granulosa cells[462]; 200 nM PMA mimics the surge of LH to stimulate expression of LH targets such as the *Pgr*[463] and *Egr-1*.[29] However, in contrast to these results, hCG treatment of granulosa cells does not appear to promote activation of the

conventional or novel isoforms of PKC.[449] The ability of LH to activate the atypical forms of PKC has not been thoroughly investigated, but LH is reported to stimulate the membrane translocation of the atypical PKC isoform PKCζ, and this pathway is proposed to mediate the induction of the immediate early transcription factor *NGF1B/EGR-2* in these cells[464] that is implicated in *Cyp19a1* gene repression,[465] as discussed later.

LH and Insulin/IGF-1 Synergize to Activate AKT and LH Target Genes. There is abundant evidence in preovulatory granulosa and theca cells that LH/hCG synergizes with insulin/IGF-1 to promote target gene expression. This synergism is observed in granulosa cells with respect to stimulation of progesterone production,[466,467] *Star* expression,[468,469] *Ldlr* (low-density lipoprotein receptor) promoter-reporter activity,[364] and, surprisingly, cAMP production.[468,469] Synergism is also evident in vivo with conditional deletion of the lipid phosphatase PTEN (phosphatase and tensin homolog) that degrades PIP3, as shown by increased numbers of ovulated oocytes and increased litter size.[470] Synergism is reported in theca cells for the *Cyp17a1* promoter-reporter.[363,468] Co-transfection with the PKA inhibitor PKI inhibited both LH stimulation and the LH/hCG synergism with insulin/IGF-1 in regulation of *Star*[364] and *Ldlr*[364] promoter-reporter activities, consistent with LH signaling to these targets via PKA. Synergism between these pathways to stimulate *Ldlr* promoter-reporter activity was also inhibited with the PI-3K inhibitors wortmannin and LY24002.[364]

Insulin/IGF-1 are expected to signal through their canonical receptor tyrosine kinase pathways to activate IRS-1/2, PI-3K, and AKT.[238] However, the cellular mechanism by which LH regulates the PI-3K/AKT pathway is poorly understood. There is one report, using an in vivo model in which LH was infused into the vena cava of diestrus rats, that investigated signaling to AKT.[469] These authors showed, through immunoprecipitation of indicated targets from ovarian extracts, that LH stimulates the tyrosine phosphorylation of the cytokine receptor-associated tyrosine kinase Janus kinase 2 (JAK2), the phosphorylation of JAK2 targets signal transducer and activator of transcription (STAT)-1 and -5b, the tyrosine phosphorylation of IRS-1 and its association with PI-3K, and AKT phosphorylation. They also demonstrated that LH stimulates the tyrosine phosphorylation of SHC,[469] an adaptor protein that binds to phosphorylated tyrosine residues and associates with GRB2/SOS to activate RAS[471] and leads to an activation of ERK.[469] Moreover, these authors reported that LH and insulin synergized to increase all these responses except for ERK activation and tyrosine phosphorylation of STAT-1.[469] Additional studies are required to confirm these observations and to determine the mechanism by which LH signals to regulate these phosphorylation events.

Cumulus Granulosa Cell Expansion and Oocyte Maturation

Oocyte Arrest. Oocytes are arrested in prophase I of meiosis prior to the surge of LH, as shown in Figure 20.14. Arrest is initially maintained by the absence of at least the maturation promoting factor (MPF) kinase CDK1,[472] and possibly other proteins in oocytes. Oocytes acquire competence to resume meiosis sometime between preantral and antral stages.[473] Arrest is then maintained by elevated levels of cAMP and cGMP within the oocyte.[474] cAMP levels sufficient to maintain meiotic arrest appear to be the product of signaling by the orphan GPCRs GPR3[475] and GPR12[476] to Gs and adenylyl cyclase. Downregulation of GPR3 and GPR12 promotes meiotic resumption.[476] The ligands that activate these receptors remain unidentified. A second key player is the oocyte-specific cAMP hydrolyzing phosphodiesterase PDE3A, whose activity is inhibited prior to the LH surge primarily by cGMP,[477] thereby maintaining elevated intra-oocyte levels of cAMP. PDE3A-null mice ovulate but are infertile because oocytes do not undergo germinal vesicle breakdown and thus remain arrested.[478]

cAMP via PKA maintains oocyte arrest by inhibiting the activation of maturation promoting factor MPF, which consists of the cyclin-dependent kinase CDK1(CDC2) and Cyclin B. PKA maintains CDK1 in

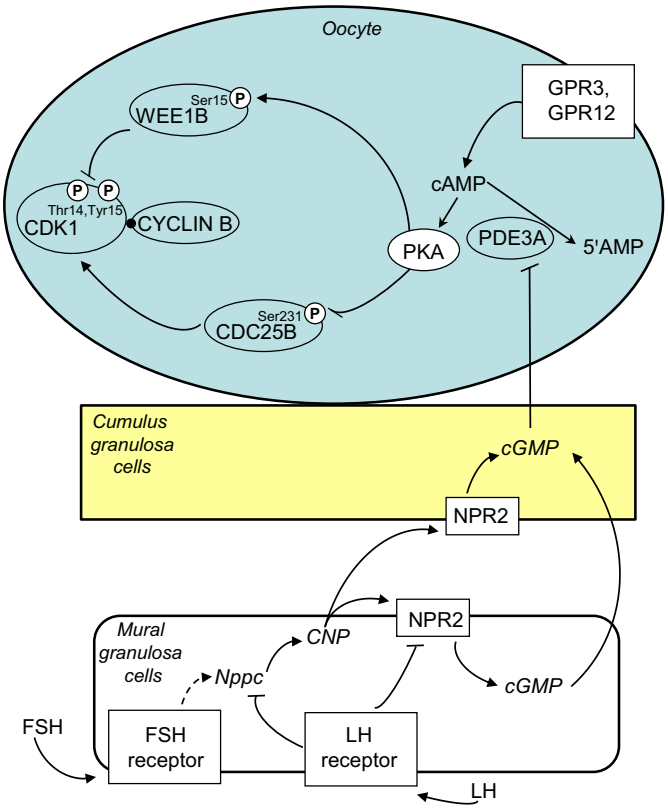

FIGURE 20.14 Mechanism by which LH promotes oocyte maturation.

an inactive conformation that resides in the nucleus by phosphorylating/activating the oocyte-specific dual-specificity kinase WEE1B on Ser15.[479] WEE1B phosphorylates CDK1 on Thr14 and Tyr15, inhibiting its activity.[480] PKA also maintains CDK1 in an inactive conformation by inhibiting the dual-specificity phosphatase CDC25B that can dephosphorylate Thr14 and Tyr15 on WEE1B. Phosphorylation of CDC25B on Ser321 promotes the association of CDC25B with 14-4-3 proteins in the cytosol, thus restricting accessibility to the substrate CDK1.[481]

Recent studies have elucidated key pathways that elevate levels of cGMP within cumulus granulosa cells to inhibit oocyte PDE3A. FSH enhances the expression of natriuretic peptide precursor C (*Nppc*) mRNA in mural granulosa cells.[482–485] The active peptide, C-type natriuretic peptide (CNP), is the ligand for the membrane guanylyl cyclase receptor NPR2 (natriuretic receptor B). NPR2 is expressed predominately in cumulus granulosa cells, to a lesser extent in mural cells, and not in oocytes.[482] The addition of CNP to cumulus–oocyte complexes elicits a rise in cGMP levels in both cumulus granulosa cells and oocytes; meiotic arrest is not maintained in oocytes from mice that are null for either *Nppc* or *Npr2*.[482] cGMP is believed to diffuse from the granulosa cell continuum into oocytes via gap junctions. Mice deficient in the major gap junction protein that connects cumulus cells and oocytes, Gja4 (Connexin 37), are infertile.[486] Expression of NPR2 in cumulus cells and the inner layer of mural granulosa cells is stimulated by oocyte-derived bone morphogenic protein 15 (BMP15), growth differentiation factor 9 (GDF9), and fibroblast growth factor 8B (FGF8B).[482]

Response to LH. The surge of LH rapidly reduces mural granulosa cell NPR2 activity[484] and more slowly reduces the expression of NPPC,[483] resulting in reduced levels of CNP in cumulus cells[484] (Figure 20.14). Additional pathways, described below, contribute to a rapid decrease in levels of cGMP in the oocyte.[477,487] The cGMP-dependent inhibition of the cAMP-hydrolyzing PDE3A is relieved, cAMP is hydrolyzed to 5′AMP, and PKA activation ceases.[474] As a result, CDC25B relocates to the nucleus[488] to dephosphorylate/activate CDK1, and meiosis resumes.

LH promotes rapid activation of ERK in mural granulosa cells via a PKA-dependent pathway[449] (Figure 20.13). LH also promotes the PKA-dependent[451] transactivation of the EGF receptor (ErbB1) in mural granulosa cells, leading to the sustained activation of ERK in mural and especially cumulus granulosa cells.[23,451,489] As described earlier, LH enhances expression of EGF-like ligands, especially epiregulin and amphiregulin, by mural granulosa cells. These EGF-like ligands act in an autocrine manner on mural granulosa cell EGF receptors and in a paracrine manner by diffusing through follicular fluid/extracellular spaces to activate EGF receptors in cumulus granulosa cells.[23] As a result, EGF receptor activation in cumulus cells is somewhat delayed compared to that in mural cells.[23,487] Expression of the EGF receptor on cumulus granulosa cells is regulated by oocyte-derived GDF9 and BMP15 via SMAD2/3 dependent pathways[490]; expression of EGF receptors in mural granulosa cells is regulated by FSH.[19]

Consistent with a major role for amphiregulin and epiregulin as mediators of LH actions, recombinant amphiregulin or epiregulin has been shown to mimic many of the responses elicited by LH, including cumulus expansion,[369,491] oocyte maturation,[487] and induction of genes required for ovulation, such as *Pgr* and *Ptgs2*.[369] Moreover, these responses are consistently inhibited by the EGF receptor antagonist AG1478 and are blunted with reduced expression paradigms for the EGF receptor.[369,487,489,492]

Amphiregulin has also been shown to promote a rapid decline in cGMP levels via mechanisms that are poorly understood but do not appear to be via activation of cGMP phosphodiesterases.[477,487] The pathway is inhibited by the EGF receptor antagonist AG1478, but cGMP levels are still greatly reduced in granulosa cells of mice with a conditional deletion of the EGF receptor, suggesting the presence of an alternative secondary pathway by which LH rapidly reduces cGMP levels.[487]

One of the major targets activated downstream of amphiregulin/EGF receptor in cumulus granulosa cells is ERK (Figure 20.13). Activated ERK closes the gap junctions that connect adjacent cumulus granulosa cells by directly phosphorylating connexin 43 on Ser255 and Ser262,[487] an event that contributes to, but apparently is not required for, oocyte maturation.[493] Activated ERK is necessary[369] but not sufficient for oocyte maturation and does not appear to contribute to the rapid fall in follicle cGMP induced by amphiregulin.[487] Activated ERK is required for expression of key cumulus expansion genes, such as *Has2* (hyaluronan synthase) and *Ptx3* (pentraxin-related protein), that generate the mucified, hyaluronan-rich matrix that contributes to the expansion of the cumulus cells surrounding the oocyte.[23,369] Activated ERK contributes to the expression of C/EBPβ (CCAAT-enhancer binding proteinα/β), and directly phosphorylates C/EBPβ.[369] ERK-induced C/EBPβ is necessary for amphiregulin to induce expression of genes required for ovulation, including *Pgr* that encodes for the progesterone receptor[369]; *Pgr* null mice do not ovulate.[374] C/EBPβ is also required for amphiregulin to promote expression of *Ptgs2* that encodes the rate-limiting enzyme for PGE2 synthesis.[23] PGE2 appears to function in a critical autocrine regulatory loop that increases the expression of amphiregulin and epiregulin by cumulus granulosa cells that in turn functions to sustain the actions of these EGF-like ligands[491] (Figure 20.13). Cumulus granulosa cells of *Ptgs2*-null mice express greatly reduced levels

of epiregulin and amphiregulin in response to an ovulatory concentration of hCG[491] and *Ptgs2*-null mice do not ovulate.[373] PGE2 via its receptor (EP2 subtype, *Ptger2*) and a cAMP/PKA pathway also promotes expression of *Tnfaip6* that contributes to cumulus expansion.[370,494] C/EBPβ is also required for expression of the EGF-like ligand type III neuroregulin I that, together with amphiregulin, apparently contributes to the fidelity of the expression of cumulus expansion genes.[455] C/EBPα/β-null mice exhibit normal cumulus expansion but fail to ovulate.[495] These results suggest that C/EBPβ targets are not required for cumulus expansion.

A potential mechanism to downregulate or modulate expression of C/EBPβ targets following ovulation was provided by evidence that theca and subsequently granulosa cells express a basic leucine zipper transcription factor, NFIL3 (nuclear factor interleukin-3), that can bind to the same response elements as C/EBPβ and CREB.[496] These proteins are expressed 8–24 h post hCG, and appear to act as repressors by competing with C/EBPβ and CREB for binding sites on both *Ptgs2* and *Areg* promoters. Overexpression of NFIL3 by adenoviral transduction of granulosa cells greatly reduces expression of *Areg*, *Ereg*, *Pgr*, and *Ptgs2* mRNA.[496]

The importance of ERK activation to oocyte maturation and ovulation was elegantly demonstrated with the conditional knockout of *Mapk1/3* (ERK 2/1) from granulosa cells using the *Cyp19a1-Cre*.[369] Mice did not exhibit cumulus expansion in response to hCG, due to a lack of expression of cumulus-expansion genes, and did not ovulate due, in part, to the absence of *Pgr* and *Ptgs2* expression (Figure 20.13). The absence of ERKs in cumulus granulosa cells prevents exogenous amphiregulin from stimulating cumulus expansion genes and genes required for ovulation. Oocyte maturation is also prevented, although how ERK contributes to this process is not clear. Follicles exhibit the normal initial increases in amphiregulin and epiregulin in response to hCG, but the induction is not sustained, likely as a result of the absence of PGE2 resulting from the absence of *Ptgs2* gene expression.

The conditional knock-in of a constitutively K-RAS, driven by *Cyp19a1-Cre*, that leads to activation of the ERK signaling pathway results in defects in cumulus expansion, oocyte maturation, and ovulation.[216] The majority of luteinized follicles exhibited entrapped oocytes. These results support a critical role for the appropriate regulation of ERK activation in response to the LH surge.

EGF receptor activation also appears to signal to activate p38 MAPK (MAPK14), based on reduced p38 MAPK phosphorylation (~50%) with conditional deletion of EGF receptor from granulosa cells.[487] p38 MAPK also appears to contribute to the phosphorylation of connexin 43, based on reduced phosphorylation of both Ser262 and Ser255 (50–60%) in follicles pretreated with the p38 inhibitor SB202190. The conditional deletion of p38 MAPK from granulosa cells had no effect on fertility.[497] However, while cumulus oocyte complexes respond normally to amphiregulin with the induction of cumulus expansion genes, *Areg* and *Ptgs2*, they fail to respond to PGE2, suggesting that the PGE2 regulatory loop in cumulus cells is mediated by p38 MAPK. Mural granulosa cells appear to compensate for the reduced expression of amphiregulin on cumulus cells by expressing elevated levels of amphiregulin and epiregulin.[497] One interpretation of these data is that while p38 MAPK contributes to EGF receptor-regulated pathways in cumulus cells, it is not essential.[474]

Ovulation

Ovulation requires both *Pgr* and *Ptgs2* induction by the LH signals that transactivate the EGF receptor leading to ERK activation and expression of C/EBPβ, as discussed earlier. PDE4D-null mice do not ovulate as a consequence of severely reduced expression of both *Pgr* and *Ptgs2*.[34] Reduced expression of these genes likely reflects the altered cAMP production that is two- to three-fold reduced and no longer transient. However, oocyte maturation and luteinization appear to be normal. Similarly, in anovulatory PGR-null mice, *Ptgs2* is induced, *Cyp19a1* is downregulated, luteal cells express *Cyp11a1*, and entrapped oocytes are functional.[377] Remodeling of the follicle wall is also believed to require one or more metalloproteinases, based on the ability of pharmacological inhibitors of this family of enzymes to block follicle rupture.[498] ADAMTS-1, a PGR target,[378] has recently been identified as a metalloproteinase that is required for follicle rupture; ADAMTS-1-null mice exhibit a 75% reduction in the rate of ovulation.[499] The proteoglycan versican is a major target of ADAMTS-1. Versican is induced by the LH surge in mural granulosa cells by a pathway that is independent of *Pgr* and *Ptgs2*, it binds hyaluronan, and relocalizes to the granulosa/theca cell boundary and to the cumulus oocyte complex.[500] ADAMTS-1-null mice exhibit a 75% reduction in versican cleavage at the granulosa/theca cell interface and within expanding cumulus cells.[500] ADAMTS-1-null mice also fail to exhibit the characteristic invagination at the theca cell border that is involved in vascularization of the follicular structure required for corpus luteum formation.[500] The remaining 25% of versican cleavage is presumably mediated by additional unidentified proteases.

The absolute requirement of *Pgr* expression for ovulation suggests that its ligand, progesterone, is required to activate PGR-regulated genes.[501] Progesterone production by mural granulosa cells requires induction of *Cyp11a1* and *Star*.[380,502] Reduced progesterone biosynthesis decreases ovulation rates.[503] Progesterone accumulation in granulosa cell medium also requires

dynamic reorganization of the actin cytoskeleton.[504] The key downstream target whose phosphorylation is regulated by hCG is the actin depolymerizing factor cofilin. Cofilin phosphorylated on Ser3 does not bind actin filaments, while dephosphorylated cofilin binds actin and enhances actin dynamics. hCG in a PKA-dependent manner, independent of ERK, inhibits the pathway from RHOGTP via RHO kinase and Lim kinase that stimulates the phosphorylation of cofilin on Ser3. hCG promotes inactivation of RHO (converting RHOGTP to RHOGDP), leading to the dephosphorylation/activation of cofilin. Transduction of granulosa cells with an adenoviral cofilin phospho-mimic, cofilin Ser3Glu, reduces hCG-stimulated progesterone production by 70%.[504] These results show that the surge of LH also regulates intracellular signaling pathways distinct from those leading to transcriptional regulation (see Figure 20.13).

Ovulation and cumulus expansion also require the orphan nuclear receptor LRH-1 (*Nr5a2*).[335] Conditional deletion of *Nr5a2* from granulosa cells, driven by *Amhr2-Cre*, abrogates LH-induced expression of *Ptgs2* and its target *Tnfaip6*, thus blocking cumulus expansion, as well as expression of *Star* and *Cyplla1*, thus preventing progesterone production and ovulation.[335] *Nr5a2*-null mice also fail to downregulate *Cyp19a1*, resulting in elevated estrogen production. The LH-induced downregulation of estrogen expression appears to be required for *Ptgs2* expression, based on evidence that *Sult1e1*-null mice, that fail to express the enzyme estrogen sulfotransferase that inactivates estrogen, exhibit elevated estrogen and reduced expression of both *Ptgs2* and *Tnfaip6*.[505] One relevant LRH-1 target in granulosa cells that appears to be required for downregulation of *Cyp19a1* is *Nos3*.[335] *Nr5a2*-null mice expressed reduced levels of *Nos3* mRNA, and CHIP assays show direct binding of LRH-1 to the *Nos3* promoter.[335] As discussed under the AKT target eNOS, *Nos3*-null mice exhibit reduced ovulation rates and oocyte maturation, and a marked increase in estrogen levels following the LH surge (rather than a decrease).[333,334] Together these results suggest that LH signals via the nuclear receptor LRH-1 not only to enhance expression of *Star* and *Cyplla1* but also to promote expression of *Nos3*. LH also potentially promotes activation of the *Nos3* product eNOS that is some manner is required for the downregulation of *Cyp19a1* and consequent upregulation of *Ptgs2*. However, the mechanism by which LH regulates LRH-1, how eNOS contributes to *Cyp19a1* downregulation, and how elevated estrogen inhibits expression of Ptgs2 are not known.

Luteinization

Luteinization defines a program of terminal differentiation that converts mural granulosa cells and theca cells to luteal cells. Proliferation ceases, the structure formed following ovulation becomes highly vascularized, and luteal cells produce high levels of progesterone. Genes specific to granulosa cells are downregulated, and genes required for luteal function are upregulated.

The LH surge suppresses granulosa cell proliferation by promoting downregulation of cyclin D2, encoded by *Ccnd2*,[45] and upregulation of cell cycle inhibitors $p21^{Cip1}$ and $p27^{Kip1}$,[45,381] encoded by *Cdkn1a* and *Cdkn1b*. Conditional deletion of ERK1/2 from granulosa cells prevents cell cycle arrest and luteinization.[369] Although luteinization is associated with cell cycle arrest, luteinization and exit from the cell cycle are independent responses to the surge of LH. Thus, in both $p27^{Kip1}$-null[381,506] and $p27^{Kip1}/p21^{Cip1}$ double-null mice,[381] preovulatory follicles undergo ovulation and luteinization to form a corpus luteum in response to the LH surge even when granulosa cells continue to proliferate, as evidenced by retention of cyclin D2 expression and high bromodeoxyuridine incorporation in cells expressing *Cyp11a1*. In cyclin D2–null mice, follicles do not ovulate, potentially due to reduced cumulus cell numbers,[507] but granulosa and theca cells still luteinize in follicles with entrapped ova.[44] Similarly, in PDE4D and C/EBPα/β-null mice, granulosa and theca cells luteinize without release of oocytes.[34,369]

Progesterone production is the hallmark of a functional corpus luteum. The preovulatory surge of LH accordingly induces the persistent and elevated expression of the rate-limiting enzyme in progesterone synthesis *Cyp11a1*[380] and the cholesterol-mobilizing protein *Star*.[376] Expression of both genes is blocked by conditional deletion of *Mapk1/3*[369] and *Cebpa/Cebpb*[495] from granulosa cells and reduced in mice conditionally expressing partially inactive EGF receptors.[492] These results show that ERK activation downstream of LH-stimulated EGF receptor transactivation is necessary to promote expression of both *Star* and *Cyp11a1*. Expression of both genes, however, requires input from additional signaling pathways.[48,353,355,367,369,495]

Luteinization involves the silencing of a number of genes induced by FSH that define the preovulatory phenotype, such as *Inha* and *Cyp19a1*. The surge of LH induces permanent silencing of the *Inha* gene in the rodent. Repression of inhibin expression during the periovulatory time window permits the secondary surge of FSH on the morning of estrous that contributes to follicle recruitment.[508] Reduced *Inha* transcriptional activity is initially accomplished by the rapid, albeit transient, induction in mural granulosa cells of the immediate early gene *Crem* (cAMP response element modulator) that encodes the inducible cAMP early repressor ICER.[346] ICER, along with CREB, C/EBPβ, and NFIL3 are basic leucine zipper transcription factors that bind DNA and homo- or heterodimerize to activate or repress transcription. As shown by CHIP assays, ICER competes with

phospho-CREB-Ser133 homodimers for occupancy of the cAMP response element on the *Inha* promoter.[509] As a result, the multiprotein complex that promotes transcription assembled on the CREB-occupied *Inha* promoter is disassembled, and *Inha* transcription ceases. Consistent with the transient induction of ICER, a second immediate early gene C/EBPβ is induced by 1 h and persists for 24 h that continues to repress transcription from the *Inha* promoter.[510] C/EBPβ-null mice do not form corpora lutea[511] and do not exhibit *Inha* downregulation following injection of hCG to PMSG-primed mice.[510] Although C/EBPβ can form inactive heterodimers with CREB, the predominant repressive mechanism appears to be via the binding of C/EBPβ homodimers to a nonconcensus binding site.[510]

Recent results show that histone modifications, which alter the accessibility of DNA to transcriptional activators, temporally followed by DNA methylation that further reduces binding of transcriptional activators, additionally contribute to LH-induced *Inha* silencing.[512] CHIP assays showed that histone H3 trimethylated on Lys4 (H3K4me3), a modification linked to active genes, was enriched on the *Inha* promoter in preovulatory cells, obtained 48 h post PMSG, and then declines 18–20 h post hCG. Conversely, histone modifications linked to repressed genes, H3K27me3 and H3K9me3, are low in preovulatory cells and substantially increase in ovulatory cells and further increase in luteal cells obtained 6 days post hCG.[512] DNA methylation within the cAMP response element, as determined by bisulfite sequencing, is low in preovulatory and ovulatory cells and markedly enhanced in corpora lutea. It is hypothesized that the repressors like ICER and C/EBPβ recruit histone-modifying enzymes that promote methylation of Lys27 and Lys9, and that these histone modifications contribute to recruitment of DNA methylation enzymes. Together these modifications prevent the binding of CREB and other transcriptional activators to *Inha*, resulting in its silencing.

Cyp19a1 is also downregulated as a result of the LH surge, at least in part as a negative-feedback pathway to release the hypothalamic–pituitary axis from stimulatory effects of estrogen that initiated and sustained the surge of LH. Mechanisms of repression at the promoter are less well defined for *Cyp19a1* compared to that for *Inha*. The immediate-early orphan nuclear receptors NR4A2 (nuclear receptor subfamily 4 group A member2)/NURR1 and NGF1B (nerve growth factor 1B)/EGR-2 have been implicated in the negative regulation of the *Cyp19a1* gene after the LH surge, using human granulosa-like tumor cell line, KGN, based on promoter-luciferase results and siRNA(silencing RNA)-mediated downregulation of *Nurr1*.[465] *Nurr1* and *Egr-2* mRNAs are rapidly and transiently upregulated in gonadotropin-primed rat ovaries in response to hCG, the former via a PKCζ pathway.[438] A member of the *Jun* family of immediate early genes may also contribute to the downregulation of *Cyp19a1* by binding to the cAMP response element usually occupied by phospho-CREB(Ser133).[513] The redistribution of CREB from the nucleus to the cytoplasm during luteinization may also contribute to the downregulation of targets such as *Cyp19a1*.[122]

ERK is required for *Cyp19a1* downregulation. ERK1/2-null mice do not exhibit *Cyp19a1* downregulation in response to an ovulatory concentration of hCG[369] (Figure 20.13). C/EBPα/β-null mice exhibit the same phenotype,[495] consistent with evidence that expression and activation of C/EBPβ is dependent on ERK1/2 activation[369] and with evidence of the repressive actions of C/EBPβ.[510] Likewise, granulosa cells expressing EGF receptors with compromised receptor tyrosine kinase activity exhibit decreased C/EBPβ and fail to downregulate *Cyp19a1*[492] (see Figure 20.13). Activation of the EGF-receptor/ERK1/2/C/EBPβ pathway is also required for the downregulation of *Fshr*[369,492] and *Lhcgr*,[369] and for the upregulation of *Cyp11a1*,[369,492,495] *Star*,[369,495] the estrogen-inactivating enzyme *Sult1e1*,[369] and runt-related transcription factor *Runx2*.[495,514] *Cyp19a1* downregulation also appears to be a consequence of LRH-1-induced *Nos3* expression,[335] as discussed earlier in "Ovulation," although the mechanism by which eNOS contributes to *Cyp19a1* expression is not known. As with *Inha*, the downregulation of *Cyp19a1* is linked with inactivating histone modifications, while the upregulation of *Star* is linked with activating histone modifications.[515] Similarly, DNase1 protection assays suggest that hCG enhances chromatin condensation for *Cyp19a1* and reduced chromatin condensation for *Star*.[515]

The ovulatory surge of LH promotes a transient downregulation of LH receptors that recover some 48–72 h later, in a species-specific manner, with the formation of the corpus luteum. LH receptor downregulation in granulosa cells is relatively slow, with a T1/2 of at least 8.5 h,[421–423] compared to that of most other GPCRs.[86,516] LH receptor downregulation in response to an hCG bolus has been extensively studied, as described below, using day-5 corpora lutea from superovulated pseudopregnant rats as a model. Recent studies have used primary cultures of human granulosa cells with equivalent results. A decline in *Lhcgr* mRNA is first detected ~ 6 h post hCG; *Lhcgr* mRNA is undetectable by 12 h post hCG, by northern analysis.[517] However, unlike the downregulation of *Inha* and *Cyp19a1*, there is no change in the rate of *Lhcgr* transcription. Rather, hCG promotes increased *Lhcgr* mRNA degradation.[518] Enhanced *Lhcgr* mRNA degradation is mediated by a 50 kDa LH receptor mRNA-binding protein (LRBP)[519] identified as mevolanate kinase.[520,521] Suppression of LRBP expression abrogated the ability of hCG to downregulate *Lhcgr* mRNA, suggesting that LRBP is not only

necessary but sufficient for this regulation.[522] LRBP binds an 18-base pair sequence within the coding region of *Lhcgr* mRNA.[519] LRBP binds *Lhcgr* mRNA at the ribosome, yielding an untranslatable complex that is targeted for degradation.[523] *Lhcgr* mRNA downregulation and the two-fold upregulation of LRBP protein detected 12 h post hCG is prevented by H89, consistent with a PKA-dependent response.[524] *Lhcgr* mRNA downregulation is also reversed by the ERK1/2 inhibitor U0126. The requirement of ERK1/2 activation for *Lhcgr* mRNA downregulation is supported by results with ERK1/2-null mice that fail to show a decline in *Lhcgr* mRNA post hCG.[369] The relevant ERK1/2 target has not been identified, but might be LRBP itself, especially since LRBP is readily detectable in granulosa cell extracts in the absence of hCG, consistent with regulation by a posttranslational mechanism.[524] While the mechanism by which surface LH receptors are downregulated in granulosa cells and corpora lutea following a bolus of LH or hCG has been clearly defined, the physiological consequence of blocking this response, especially in granulosa cells, has not to our knowledge been investigated.

MOLECULAR BASIS FOR THE DIFFERENTIAL REGULATION OF TARGET GENE EXPRESSION BY FSH IN PREANTRAL AND LH IN PREOVULATORY GRANULOSA CELLS

There have been many advances in the seven years since we last reviewed the cellular signaling pathways by which FSH and LH regulate target gene expression. Cyclic AMP/PKA play a major role in the actions of FSH and LH in granulosa cells. Despite the fact that these two gonadotropins are structurally related, act on similar receptors, and signal predominantly through cAMP-dependent mechanisms, FSH and LH elicit very different responses in preantral versus preovulatory granulosa cells. For example, FSH acts via cAMP/PKA to activate transcription of target genes such as *Cyp19a1*, *Inha*, and *Lhcgr* in preantral granulosa cells but does not stimulate expression of the *Ptgs2* or *Pgr*, whereas LH has the opposite effects in preovulatory granulosa cells and stimulates the downregulation of *Cyp19a1*, *Inha*, and *Lhcgr*, and activates transcription of the *Ptgs2* and *Pgr* genes. Thus, a central question in reproductive biology is how the same second messenger/effector exerts such different responses during the continuum of granulosa cell differentiation. Are the different responses attributable to the activation of distinct signaling pathways, such as only PKA for FSH and PKA plus PKC for LH, or to the duration of signaling? Or, do the different responses of the two cell types reflect the fact that preovulatory granulosa cells have differentiated and contain a different proteome, including distinct AKAPs, kinases, phosphatases, transcription factors, and other regulators important in generating their unique response?

To address the question of different signaling pathways for FSH versus LH, Zeleznik's group[115,133] cleverly investigated target gene expression after adenoviral transduction of preantral granulosa cells with constitutively active LH receptors (Asp578His). Results from these experiments showed that the LH receptor expressed in preantral cells partially mimics the responses of FSH and induces *Inha* and *Hsd3b2* but interestingly does not induce or minimally induces *Cyp19a1* or *Lhcgr*, even when cAMP levels are equivalent. These results suggest that these two GPCRs not only couple to overlapping signaling pathways but also appear to couple, perhaps more efficiently, to different signaling pathways to regulate distinct cellular events in preantral versus preovulatory granulosa cells.

Alternatively, rather than there being different second messengers generated by FSH versus LH receptor activation in preantral versus preovulatory granulosa cells, differences in the amount, duration, or cellular location of cAMP may distinguish FSH signaling from that of LH signaling. It is well known that sustained FSH at relatively low concentrations is necessary to stimulate granulosa cell differentiation,[106] whereas a bolus of LH is required to stimulate ovulation. Indeed, it has been proposed that the actions of LH and FSH are distinct in mature versus immature granulosa cells as a result of the ability of LH receptor activation in mature cells to generate greater levels of cAMP.[525] Although this view is supported in mature, preovulatory granulosa cells by the observation that high doses of recombinant FSH can substitute for LH/hCG in promoting ovulation and luteinization in the rat,[526] high levels of cAMP in immature cells do not seem to mimic actions of the LH surge in mature cells.[115,133] To our knowledge, it is not known if overexpression of the constitutively active LH receptor in immature cells promotes induction of genes characteristic of mature cells, such as *Pgr* and *Ptgs2*; however, the reduced expression of *Cyp19a1* and *Lhcgr* are reminiscent of downregulated genes in mature cells.

FSH and LH receptor activation also regulate the expression of the cAMP degrading PDEs.[527] PDEs certainly participate in LH receptor signaling because PDE4D-null mice exhibit an ovulation defect.[527] However, the contribution of the PDEs to the distinct signaling by FSH and LH receptors in immature and mature granulosa cells is not fully understood. There is also abundant evidence for the cellular compartmentalization of cAMP in various cellular models.[143,528] Consistent with the conceivably restricted production of cAMP in select cellular sites, PKA is recognized to be anchored to select cellular locations by the growing family of AKAPs.[142] Potentially, then, the distinct expression

of one or more AKAPs between immature and mature granulosa cells could also localize PKA and distinct PKA substrates to different cellular locals and account for distinct signaling in immature versus mature cells by FSH versus LH receptors. That PKA regulates ERK activity by distinct mechanisms in preantral versus preovulatory granulosa cells,[113,449,529] and the more prominent role of ERK signaling in preovulatory versus preantral cells[369] indicates that these two cell types are indeed quite distinct and that preovulatory cells are not simply preantral granulosa cells that express, for example, LH receptors.

CONCLUSION

As reviewed in this chapter, tremendous progress has been made in understanding mechanisms of gonadotropin action in regulating ovarian follicle maturation, oocyte maturation, and ovulation, and luteinization. The structures of FSH and hCG are now known to atomic level resolution; there is a fundamental understanding of the gonadotropin receptors and how they selectively bind FSH and LH; it is clear that cAMP serves as the key second messenger for nearly all gonadotropin actions in the ovary; substantial inroads have been made in defining the signaling pathways activated by gonadotropins; and many of the target genes activated by FSH or LH have been identified and their physiologic functions explored. In addition, genetic studies in mice and naturally occurring mutations in women have verified the fundamental importance of the gonadotropins and their receptors to ovarian function and allowed new insight into the precise stages and processes in which they act. Finally, some insight into what differentiates FSH and LH action in the ovary is now being obtained.

Despite this wealth of information, much remains to be learned about gonadotropin signaling pathways in the ovary. While the crystal structure of FSH in complex with the extracellular domain of the FSH receptor was recently reported,[62] the complete structures of the gonadotropin receptors, extending to the full-length proteins, are needed to gain further insight into hormone-binding selectivity and into the conformational changes that underlie receptor activation and G protein coupling. The recent ability to solve crystallographic structures of several model GPCRs sets the stage for these future challenges. Studies to more fully understand the tissue- and cell-specific expression and hormonal regulation of the gonadotropin receptors are also important. Although some of the major signaling pathways downstream of gonadotropin-regulated cAMP generation have been explored in depth, as discussed in this chapter, others are just beginning to be appreciated, and more detailed analyses are required. New advances in proteomics are likely to be important here, particularly methods to carefully examine the phosphorylation status of kinase target proteins in ovarian cells and to more fully characterize the kinases and phosphatases involved in particular regulatory events. As discussed, limited data also point to activation of additional signaling mechanisms via cAMP-independent pathways in ovarian cells, and this is an area worthy of further investigation. A wealth of new information is accumulating from gene expression profiling and other high throughput analyses with respect to potential target genes activated by gonadotropin signaling, and verifying these targets, establishing mechanisms of their regulation, and exploring their functions in the ovary promises to be a significant focus of future work.

Cross talk between signaling pathways is an often discussed but poorly understood aspect of signaling that is likely to be increasingly important as we begin to understand the molecular details of individual pathways. It will be particularly valuable to understand how gonadotropin signaling integrates with the many other signaling pathways important for follicle formation and maintenance. For example, TGFβ family proteins play numerous roles in cell communication in the follicle, but little is known regarding how these pathways impact gonadotropin signaling and vice versa. Similarly, understanding how other signaling pathways influence the fundamental processes of cell proliferation, cell differentiation, and cell death in the ovary, and how gonadotropin action might modify these events remains a key challenge.

We raised the issue of spatially regulated signaling as it relates to the AKAPs and their roles in determining cell-specific responses to cAMP, but the broader issue of spatially restricted signaling domains is likely to be an important one that applies to many pathways and contributes in a significant way to tissue- and cell-selective responses. Further characterization of such spatial domains in ovarian cells is warranted. This also reinforces the view that signaling principles learned in generic cell systems are not always applicable to granulosa or theca cells and must be tested in the ovary. The temporal aspects of regulation are likely to be just as important. We broadly define for the purposes of this discussion preantral and preovulatory granulosa cells as distinct stages of differentiation in the follicle that respond differentially to the gonadotropins, yet the reality is that there is a continuum of differentiation states that defines the precise nature of the response to gonadotropins or other stimuli.

Gene disruption technologies in mice have been an integral component of studies of gonadotropin signaling pathways. A significant number of gene knockouts yield reproductive phenotypes, and as discussed throughout the chapter, these mice have been invaluable in establishing roles for particular signaling proteins and pathways

in the ovary. However, many gene disruptions are not particularly informative with respect to reproduction because of early lethality or broad spectrum phenotypes that impact reproduction in an indirect fashion. Thus conditional disruption approaches, including both ovary-specific and temporally regulated knockouts, are likely to be very informative for deciphering details of gene and protein functions in gonadotropin-dependent signaling. In the same vein, recent advances in RNA interference technologies that include in vivo delivery hold much promise for downregulating selective gene products in targets like the ovary so as to establish their functional importance.

Finally, studies of gonadotropin action in the ovary continue to be informed by clinical investigation and genetic analysis of reproductive disorders, and this is an area that will surely receive much more attention in the future. Naturally occurring mutations in the gonadotropins and their receptors provide a rich context in which to learn about interaction with and activation of the gonadotropin receptors. An increasing number of mutations in signaling molecules and transcription factors exhibit some reproductive phenotype, and these are tremendously informative in inferring the functional importance of particular genes and pathways. Even complex diseases that impact the ovarian reserve, leading to premature ovarian failure, or that impact follicle maturation, leading to polycystic ovarian syndrome, are now being explored in genetic detail, and aspects of gonadotropin signaling will likely come into play in these as well. Thus, the future holds much promise for better understanding the molecular details of how FSH and LH function as master regulators in the maturation, ovulation, and luteinization of the ovarian follicle.

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CHAPTER

21

Follicular Development: Mouse, Sheep, and Human Models

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INTRODUCTION

The first scientists in gonadal biology were likely farmers in antiquity that spayed and neutered female and male animals in order to, respectively, control fertility and behavior. Aristotle (384–322 BC) believed that the male provided the "seed" that grew within the female "soil."[1] Galen (129–199 AD) and Soranus of Ephesus (c. 50 AD) described ovarian structures and possibly believed that a female seed existed.[2] After Galen, there was not much written about reproduction for over 1000 years. By the sixteenth century, ovaries had been described by Leonardo Da Vinci, Berengario da Carpi, Andreas Vesalius, Gabrielle Fallopio, and Hieronymus Fabricius. Andreas Vesalius also appreciated the existence of follicles within the ovary and described what was likely a corpus luteum. In the seventeenth century, William Harvey proclaimed "ex ovo omnia," or everything comes from the egg.[3] However, Harvey was most likely referring to the products of conception rather than the germ cell, as he did not believe that the ovary had much to do with conception. Niels Stensen and Regnier de Graaf believed that the whole follicle was the egg that became fertilized in the ovary and that only the fertilized egg escaped the ovary, while others at that time thought that follicular fluid was female semen.[4] It took another 150 years for von Baer in 1827 to discover that the egg within the follicle was the "female seed." The seventeenth century also saw the rise and fall of preformationists, who believed that we are derived from miniature versions of ourselves (i.e., we are all preformed).[5] Although preformationists are considered incorrect, we now know that oocytes store proteins and RNAs necessary for fertilization and early embryogenesis, and hence we are "preformed" to a certain degree at the molecular level. In this chapter, we will focus on the current understanding of ovarian folliculogenesis and, where appropriate, touch upon some of the controversies that exist in the field.

Folliculogenesis is the process by which the female germ cell develops within the somatic cells of the ovary and matures into a fertilizable egg.[6–8] Follicle development is complex and involves the integration of signals from multiple organ systems. While early folliculogenesis appears to be directed by signals within the ovary, endocrine hormones from the pituitary are necessary for folliculogenesis to proceed beyond these early stages. In turn, the ovary produces a number of feedback and feedforward hormones that regulate hypothalamic and pituitary function. Within the ovary, cross-talk between the oocyte and the somatic-derived granulosa and theca cells occurs at every stage for normal follicle development.[9] Advancing genetic and genomic technologies have accelerated our understanding of the genetics and physiology of reproduction. Complete genome sequences are available for many organisms, including the human, rat, mouse, chicken, cow, pig, dog, sheep, and cat, and, together with rapidly improving sequencing technologies, are allowing a much faster discovery of reproductively relevant

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naturally occurring mutations in humans and other animals. In addition, large-scale sequencing of cDNA libraries generated from a variety of human and rodent tissues, including newborn and adult ovaries and isolated oocytes, is leading to the unprecedented discovery of many novel genes that are critical for reproductive processes.[10–13] Large-scale projects to determine the functional significance of the genome have shown that a substantial portion appears to either bind various regulatory proteins or transcribe small and long noncoding RNAs in addition to the protein-coding genes.[14] In addition, genome-wide association studies identified multiple loci that associate with menopause and highlight the importance of DNA repair and immune pathways in reproductive senescence.[15] Many of the loci contain genes not previously known to be involved in ovarian development or folliculogenesis, and they provide new targets for future studies that will have relevance to mechanisms that lead to ovarian senescence. These large-scale genomic projects provide a necessary infrastructure for basic investigators to overlay reproductive developmental pathways.

Genomic manipulation through engineered deletions, point mutations, duplications, and genomic rearrangements in the mouse have allowed investigators to generate new lines of mice lacking key reproductive genes that serve as in vivo models for human diseases, including infertility. The technology to delete mouse genes and generate nonfunctional loci (gene "knockouts") or novel expression patterns (gene "knockins" and transgenic overexpression lines) has resulted in more than 400 mouse models with reproductive phenotypes[16] (Table 21.1). These mice are invaluable for various reasons: they allow us to study physiology in order to infer the functional importance of a particular gene, they can give us insight into the compensatory mechanisms that occur when the particular gene is deleted, they can be used to model treatments of reproductive pathologies, and they can also be used to narrow the candidate genes for particular human disorders. One of the most instructive examples has been the mouse mutants for follicle-stimulating hormone (FSH) and its receptor, FSH receptor (FSHR), which closely mimic humans with mutations in these genes[21–24] (as discussed in this chapter).

A number of searchable internet-based databases are available for investigators interested in genes that have a reproductive function. Jackson Laboratories (http://www.jax.org) maintains an extensive website with complete mouse genome informatics where information can be queried under individual genes, and it includes detailed information about the expression and phenotypes of spontaneous and targeted mutations as well as relevant references. The Ovarian Kaleidoscope database (http://ovary.stanford.edu) keeps up-to-date information on the expression, function, and regulation of genes expressed in the ovary.[25] The National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) has databases of genomes, sequences of many transcriptomes, and invaluable tools for analyzing and discovering genes important in ovarian development. Other online resources include the Mammalian Reproductive Genetics database (http://mrg.genetics.washington.edu), a database of the genes and literature relating to reproduction that is searchable by phenotype or expression pattern; and the GermOnline database (http://germonline.unibas.ch), a database of microarray expression data for genes relevant to mitosis, meiosis, and gametogenesis.

This chapter focuses on the development and analysis of mouse models for the study of follicle development. Although the emphasis is placed on mouse models, relevant studies in primates, including humans, and sheep are included where data are available. Understanding reproduction through the manipulation of the mouse genome has helped us delineate the function of genes important in mammalian ovarian, follicular, and oocyte development. These models are useful for some clinically relevant reproductive disorders and diseases in humans; however, it is also important to bear in mind that species-specific differences in mechanisms exist. Despite these concerns, studies among various species are helpful in delineating general themes that drive ovarian folliculogenesis.

MORPHOLOGY OF FOLLICULOGENESIS

Murine Folliculogenesis

Folliculogenesis is a highly regulated developmental sequence resulting in the growth and differentiation of the oocyte and associated somatic cells. A commonly used classification of oocytes and follicles for mice is that of Pedersen and Peters.[26,27] This classification is based on oocyte size, granulosa cell numbers, and follicular morphology (Figure 21.1). The classification is useful as a means of uniformly describing stages in follicular development. In addition, the commonly used names primordial, primary, secondary, preantral, and antral follicles often are vaguely defined but are useful for general purposes. Neither nomenclature is ideal, as not all follicles with the same oocyte size class share the same developmental fate.

In mice, oocytes enter meiosis during embryonic life, and at birth, some oocytes are in the transitory stages of prophase I (pachytene and early diplotene), while others have entered late diplotene and dictyate, where they will remain arrested until meiosis is again resumed shortly before ovulation. Centrally located within the newborn mouse ovary are oocytes that have been surrounded by a flat, squamous, epithelial-derived layer called the pregranulosa cells; these represent primordial follicles. Subsequently, primordial follicles populate the surface of the developing and adult ovaries. Once formed, primordial follicles are thought to represent the only available source of oocytes during the entire reproductive life span of

TABLE 21.1 Female Reproductive Phenotypes in Genetic Mouse Models

Gene	Name	Mouse Knockout Model: Female Reproductive Phenotype	References
SECRETED FACTORS AND RECEPTORS			
Acvr2	Activin receptor IIA	Infertility; follicles arrest at the early antral stage due to defects in pituitary function	257
Bmp15	Bone morphogenetic protein 15	Background-dependent subfertility; defects in ovulation and fertilization; enhanced defects when haploinsufficient for *Gdf9*$^{+/-}$	260
Bmpr1b	Bone morphogenetic protein receptor, type 1B	Infertility; defects in cumulus cell physiology and endometrium	261
Ccnd2	Cyclin D2	Infertility; granulosa cells do not proliferate in response to FSH	262
Cga	Glycoprotein hormones, alpha subunit	Infertility; FSH and LH deficiency	263
Fst	Follistatin	Complete loss of germ cells by birth; granulosa cell–specific knockout has early ovarian failure	264,265
Fshb	Follicle-stimulating hormone beta subunit	Infertility; preantral follicle block	22
Fshr	Follicle stimulating hormone receptor	Infertility; block prior to antral follicle formation	24
Gdf9	Growth differentiation factor 9	Infertility; arrest of follicle growth at the primary stage	266
Gnrh	Gonadotropin-releasing hormone	Infertility; hypogonadotropic hypogonadism	267
Ghr	Growth hormone receptor	Delayed puberty and prolonged pregnancy	268
Igf1	Insulin-like growth factor 1	Infertility; preantral follicle block	269
Inha	Inhibin alpha	Infertile; develop gonadal sex cord tumors with 100% penetrance	41
Inhba	Inhibin/activin beta A	Perinatal lethality	257
Inhbb	Inhibin/activin beta B	Extended gestation; unable to nurse offspring;	258
Lep	Leptin	Infertility in *ob/ob* mice; hypogonadotropic hypogonadism	270
Lhb	Luteinizing hormone beta	Infertility; hypogonadal; absence of large antral follicles and corpora lutea	271
Lhcgr	Luteinizing hormone/choriogonadotropin receptor	Infertility; folliculogenesis blocked at the antral stage	272,273
Ngfb	Nerve growth factor beta	Reduced primary and secondary follicles	189
Ntrk2	Neurotrophic tyrosine kinase receptor, type 2	Reduced number of secondary follicles and a decrease in granulosa cell proliferation	195
STEROIDS AND RECEPTORS			
Cyp19a1	Cytochrome P450, family 19, subfamily a, polypeptide 1 (aromatase)	Infertility; hypertrophied stromal cells; defects in ovulation; hemorrhagic cysts	274
Esr1	Estrogen receptor 1 (alpha)	Infertility; hemorrhagic ovarian cysts and uterine defects	275
Esr2	Estrogen receptor 2 (beta)	Subfertility; reduced litter sizes and granulosa cell defects	276
Ar	Androgen receptor	Subfertility, disrupted folliculogenesis, premature ovarian failure	277–280
STRUCTURAL PROTEINS/MISC.			
Gja4	Gap junction membrane channel protein alpha 4 (connexin 37)	Infertility; no large antral follicles; defects in ovulation; oocyte defects	281
Gja1	Gap junction membrane channel protein alpha 1 (connexin 43)	Neonatal lethality; neonatal ovaries have germ cell deficiency	282
Ptx3	Pentraxin related 3	Subfertility; defects in ovulation and fertilization	283
Tnfaip6	Tumor necrosis factor alpha–induced protein 6	Infertility; defects in cumulus cell physiology	284

(Continued)

TABLE 21.1 Female Reproductive Phenotypes in Genetic Mouse Models—cont'd

Gene	Name	Mouse Knockout Model: Female Reproductive Phenotype	References
TRANSCRIPTION FACTORS			
Figla	Factor in the germline alpha	Infertility; oocyte loss by postnatal day 2	90
Foxl2	Forkhead box L2	Infertility; absence of secondary follicles and oocyte atresia; follicular depletion	202,203
Foxo3	Forkhead box O3	Infertility by 15 weeks of age; widespread activation of follicle pool	135
Foxo1/3	Forkhead box O1 and forkhead box O3 (conditional)	Conditional *Foxo1/3* deletion in granulosa cells causes infertility and impaired folliculogenesis	285
Hesx1	Homeobox gene expressed in ES cells	Pituitary dysplasia	286
Lhx3	LIM homeobox protein 3	No development of anterior or intermediate lobes of pituitary gland	287
Nobox (Og2x)	Newborn ovary homeobox	Infertility; oocyte loss by postnatal day 14; disrupted primordial-to-primary transition	103
Lhx8	LIM homeobox protein 8	Infertility; oocyte loss by postnatal day 7; disrupted germ cell cyst breakdown, primordial follicle formation, and primordial-to-primary transition	96,100
Sohlh1, Sohlh2	Spermatogenesis and oogenesis specific basic helix–loop–helix 1 and 2	Infertility; oocyte loss by postnatal day 14; disrupted germ cell cyst breakdown, primordial follicle formation, and primordial-to-primary transition	96,98,99
Nr5a1	Nuclear receptor subfamily 5, group A, member 1 (SF1)	Gonadal agenesis; fail to produce FSH and LH	288,289
Prop1	Paired-like homeodomain factor 1	Infertility; multiple anterior pituitary defects and hypogonadism	290,291
Pit1	Pituitary specific transcription factor 1	Infertility; multiple anterior pituitary defects and hypogonadism	292
Otx1	Orthodenticle homolog 1 (Drosophila)	Hypogonadism; age-specific defects in folliculogenesis and fertility	293,294
Taf4b	$TAF_{II}105$ RNA polymerase II, TATA box binding protein (TBP)-associated factor	Infertility; arrest of follicles at preantral stage	295

The Phenotypes of representative female homozygous null mice are described above. There are over 300 genes with mouse mutations that affect ovarian function. Additional citations can be found in the text and in Refs 296–299.

the female. Controversy exists about whether postnatal oogenesis exists, with some investigators interpreting their studies to suggest that a population of oocyte-generating stem cells exists in adult ovaries,[28,29] while others deny such a possibility.[30,31] It remains to be determined whether the challengers of the current dogma (i.e., a fixed oocyte pool) can provide evidence to address criticisms and experimental findings that are difficult to explain if postnatal oogenesis occurs and contributes significantly to overall ovarian physiology. Primordial follicles are accepted by many in the field to represent the "ovarian reserve," or fixed number of germ cells formed following initial arrest in meiosis I that are available for the production of mature, fertilizable oocytes during the reproductive life of the female. Currently, primordial follicle physiology is under intense investigation as defects in the size or activation of the ovarian reserve are thought to limit reproductive life span in women.

Newborn mouse ovaries are densely packed with oocytes, most of which are present in clusters with no evidence of surrounding granulosa cells[27] (Figure 21.2(A),(D)). These oocytes have various terms, including "naked" oocytes, germ cell cysts, clusters, nests, or syncytia, and correspond to Type 1 follicles in the Peters and Pedersen classification.[27,32–34] Germ cell cysts disappear as primordial follicles appear, and the total number of oocytes declines as many oocytes undergo cell death. By postnatal day 3, a class of primary follicles is discernable by the presence of cuboidal granulosa cells and growth of the oocyte beyond 20 μm. These follicles correspond to Type 3a follicles in the Pedersen classification (Figures 21.1, 21.2(B),(E)). Numerous primordial follicles now replace the substantial number of germ cell cysts at the periphery of the ovary. At postnatal day 7, most of the germ cell cysts have disappeared and primordial follicles are the most abundant follicular type, but primary and secondary follicles are present in the medullary region (Figure 21.2). Secondary follicles (Type 4) (Figures 21.1 and 21.2(B),(E)) are distinguished from primary follicles as having more than one layer of granulosa cells as well as acquiring an additional somatic cell layer,

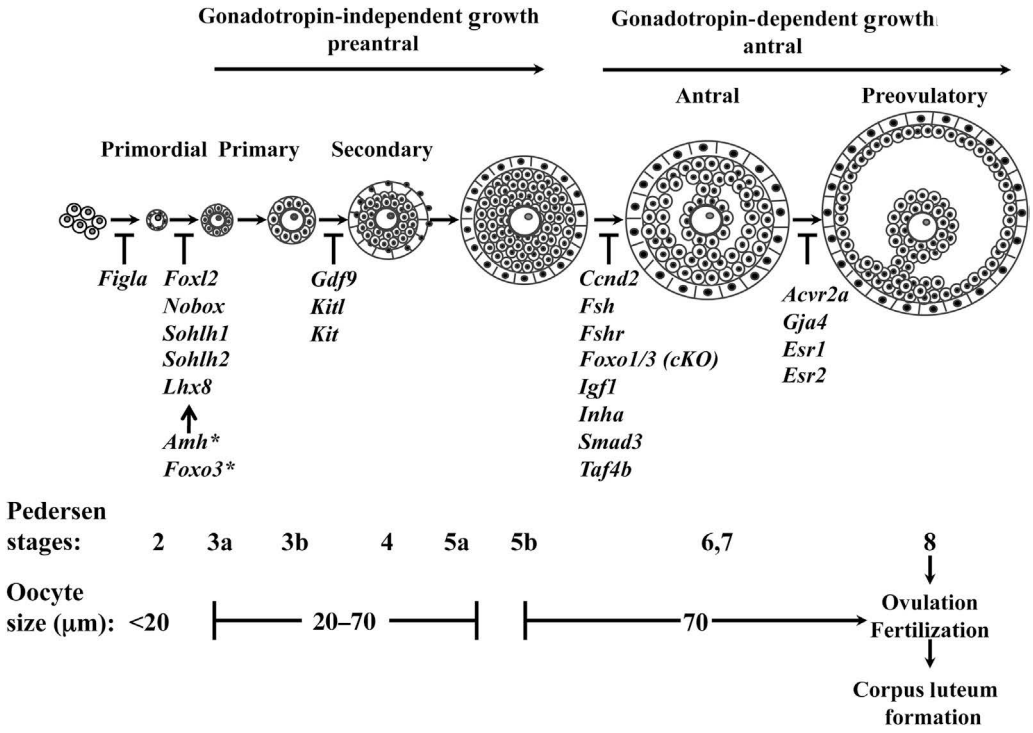

FIGURE 21.1 **Classification of mouse follicles.** From left to right, postnatal follicle development begins at the primordial stage to the antral stage. Prior to antrum formation, follicle growth is independent of pituitary gonadotropins. Following ovulation, granulosa cells undergo luteinization to become the corpus luteum. Pederson classification (Types 2–8) is shown for each stage. Corresponding oocyte diameters for each type are also indicated. A subset of genes implicated at various stages of follicular development is also listed when initial gross disruption is observed in respective mouse knockouts. **Amh* and *Foxo3* knockout mice show accelerated transition from primordial to primary follicles.

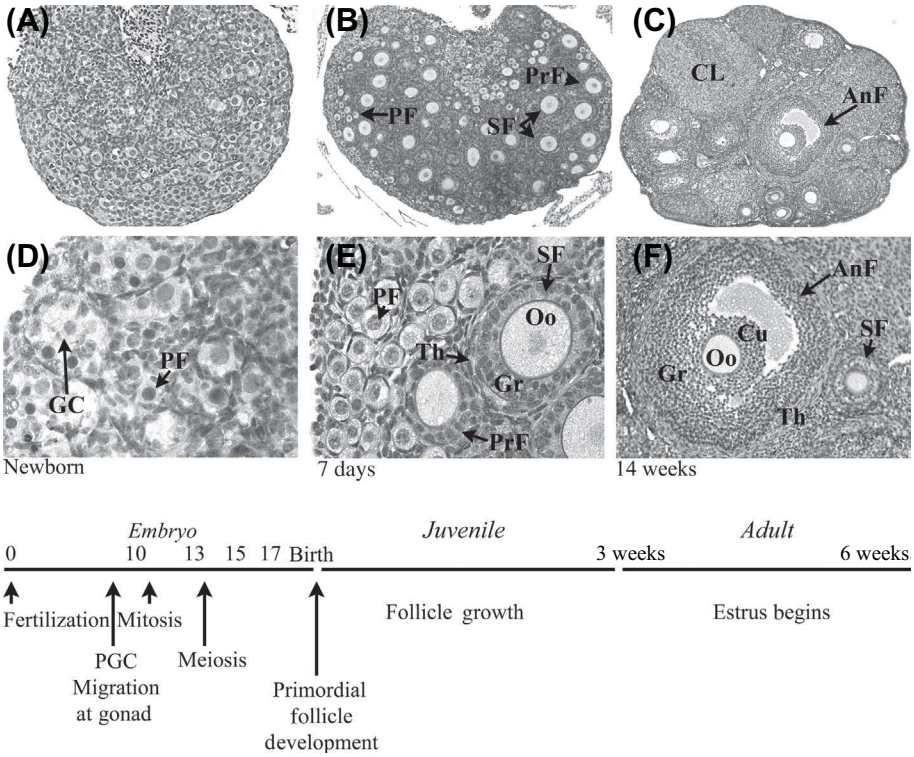

FIGURE 21.2 **Folliculogenesis in the mouse.** Light micrographs depicting follicle stages are shown in panels (A)–(F) above the timeline. Follicle development begins embryonically when primordial germ cells migrate to the developing gonad. By E11–13, the PGCs undergo mitotic division, and by E13.5 they begin meiosis. Oocytes will arrest in meiotic prophase I until ovulation. *Panels (A) and (D)*: Newborn ovaries contain oocytes in germ cell cysts (GCs) and are beginning to form primordial follicles (PFs). *Panels (B) and (E)*: By 7 days after birth, primary follicles (PrFs) and secondary follicles (SFs) have developed. Primary follicles contain a growing oocyte and are surrounded by cuboidal granulosa cells. These further develop into secondary follicles by proliferation of granulosa cells and the acquisition of a thecal cell layer (Th). A 4–5-day estrous cycle begins at 4 weeks of age, and pituitary gonadotropins are required to develop follicular stages beyond the secondary stage. *Panels (C) and (F)*: Adult ovaries contain multiple-stage follicles, including antral-stage follicles (AnF) and corpora lutea from previous ovulations (CL). Granulosa cells from antral follicles are divided into two types: mural granulosa cells (Gr) that line the follicle wall, and cumulus granulosa cells (Cu) that surround the oocyte (Oo). GC: germ cell cyst; PF: primordial follicle; PrF: primary follicle; SF: secondary follicle; AnF: antral follicle; CL: corpus luteum; Oo: oocyte; Gr: granulosa cell; Cu: cumulus granulosa cell; Th: thecal cell. Micrographs not shown to scale.

the theca. The thecal cell layer begins to form in growing follicles around the basement membrane and ultimately differentiates into the theca interna and theca externa. The theca interna layer differentiates into theca interstitial cells, whereas the theca externa layer differentiates into smooth muscle cells. Theca development also coincides with the development of numerous small blood vessels, presumably via angiogenesis.

A postnatal day 14 ovary looks similar to a day 7 ovary but is larger and contains multiple secondary follicles. Follicles are also degenerating at this stage, as lack of appropriate pituitary FSH levels does not support further follicular development. By postnatal day 21, Type 6 follicles are also discernable. In a Type 6 follicle, the oocyte reaches its final diameter of approximately 70 μm, and is surrounded by multiple layers of granulosa cells that contain scattered areas of interstitial fluid. Eventually, these scattered areas of fluid coalesce to form an antral cavity. This also divides the granulosa cells into two populations, mural and cumulus. Granulosa cells that surround the oocyte are called the cumulus granulosa cells, while granulosa cells that line the basement membrane are called mural granulosa cells. Cumulus and mural granulosa cells differ not only in their location but also in their expression of different molecular markers.

Antral follicles are highly differentiated endocrine structures, characterized by the formation of fluid-filled cavity (antrum) next to the oocyte, and they correspond to Type 6–8 follicles in the Pedersen classification (Figures 21.1 and 21.2(C),(F)). These follicles are responsive to gonadotropins and participate actively in the hypothalamic–pituitary–gonadal (HPG) axis. Unless rescued by FSH, the granulosa cells of most early antral follicles undergo widespread apoptosis and ultimately follicular death.[35,36] In dominant follicles, luteinizing hormone (LH) stimulates thecal cell androgen production, while FSH stimulates mural granulosa cell proliferation, aromatization of androgens to estrogens, and LH receptor expression.[37–40] Follicle-derived estrogens exert positive feedback on both the hypothalamus and pituitary to trigger the midcycle gonadotropin surge of LH that precedes ovulation. Inhibin, secreted by granulosa cells, also acts as an endocrine hormone and feeds back to the pituitary to inhibit FSH secretion.[41] The complicated web of interactions between the oocyte, granulosa cells, thecal cells, pituitary, and hypothalamus ultimately selects follicles that will ovulate.

The LH surge triggers release of the oocyte from meiotic arrest, breakdown of the follicle wall, and extrusion of the cumulus–oocyte complex. The cumulus cells surrounding the oocyte undergo a process of expansion and extracellular matrix production. The oocyte completes meiosis I, releases its first polar body, and progresses to the metaphase stage of meiosis II, where it arrests again until fertilization. Granulosa cells remaining in the postovulatory follicle undergo luteinization, a process that involves cell cycle exit, cellular hypertrophy, acquisition of steroidogenic morphology, and expression of cytochrome P450 cholesterol side chain cleavage. This results in formation of the corpus luteum (Figure 21.2(C)), which is a highly vascularized, transient endocrine organ that produces progesterone, which is essential for uterine preparation and maintenance of pregnancy (see Chapter 23). In mice, the entire process of the primordial follicle to maturity takes approximately 3 weeks—considerably shorter than the several months thought to be required in humans.

Human Folliculogenesis

Primordial follicle formation in humans occurs in utero.[42–44] Primordial follicles are observed in almost all ovaries after 20 weeks of gestation, whereas preantral and antral follicles are observed from 26 weeks onward. Three types of very small follicles can be defined in primates as well as other mammals: primordial follicles, follicles with mixed cuboidal and squamous granulosa cells (intermediate follicles), and primary follicles. It has been suggested that despite this histologic variation in granulosa cells of early follicles, human follicles with an oocyte nuclear diameter less than 19 μm can be considered resting.[45,46] Unfortunately, no molecular markers are known that differentiate resting from early-growing follicles, and "resting" may not be an accurate term, because transcription of many oocyte-specific genes still occurs.[47–49]

In newborn human females, approximately 400,000 follicles remain out of 6–7 million that were present midway (20 weeks) through gestation.[45,50,51] The newborn human ovary measures approximately 1.3 cm × 0.5 cm × 0.3 cm and weighs less than 0.3 g.[52,53] Follicular maturation and atresia occur prenatally and throughout childhood. Because ovulation does not occur, corpora lutea and corpora albicantia are absent in the prepubertal ovary, and all maturing follicles undergo atresia in a manner identical to that occurring in adults. By puberty, almost 90% of the initial pool is depleted, presumably by apoptosis, although other types of cell death have been detected in the rodent ovary.[54–56]

Beyond age 37, the number of follicles declines to approximately 25,000, and the rate of follicle loss increases so that by age 50, approximately 1000 follicles remain.[57,58] Thus, only approximately 400 follicles will ovulate during a woman's reproductive life, with the vast majority never reaching the mature stage.[59] Adult ovaries are ovoid, measure approximately 4 cm × 2 cm × 1 cm, and weigh approximately 7 g. In contrast, menopausal ovaries weigh half as much and contain few if any primordial follicles and a wide variety of stromal-like cells, including luteinized stromal cells.[60,61]

PRIMORDIAL FOLLICLE FORMATION

During fetal ovarian development, oocytes are contained within germ cell clusters or cysts (GCCs), which form by both clonal division and aggregation of oocytes.[62] Each GCC contains eight or more germ cells, with intercellular bridges connecting some of the oocytes. While loss of intercellular bridges through deletion of *Tex14* (testis-expressed gene 14), an inactive kinase required for intercellular bridge stability, reduces the number of germ cells at birth in females by approximately half, female fecundity is not affected.[63] During primordial follicle formation, projections from the somatic cells penetrate between the oocytes in GCCs, leading to constriction of the intracellular bridges and eventual enclosure of oocytes by the somatic cells.[32,64] GCC breakdown occurs during a relatively short time span in mice, initiating around embryonic day 17.5 (E17.5) and completed by postnatal day 4.[32] In sheep, primordial follicle formation occurs at days 75–100 of gestation,[65] between 70 and 90 days in pigs,[66] and after approximately 120 days of gestation in humans.[67]

The molecular events responsible for the breakdown of germ cell cysts and the concomitant formation of primordial follicles in the newborn ovary are still being defined. Breakdown of germ cells cysts is associated with germ cell death via apoptosis and autophagy, as well as progression through meiotic prophase I.[32,68] It is not clear how these processes are linked or regulated with respect to determining the size of the primordial follicle pool. Many studies have relied upon in vitro culturing of intact ovaries with recombinant ligands or small-molecule inhibitors to determine their effects on primordial follicle assembly. These studies have identified multiple signaling pathways that regulate this process, including steroid hormone signaling and signaling by members of the TGFβ family such as activin and anti-Müllerian hormone (AMH). Several comprehensive reviews covering this topic are available.[69–71] The remainder of this section highlights several pathways that regulate primordial follicle assembly.

Steroid Hormone Signaling

It has been suggested that high levels of maternal estradiol and progesterone inhibit primordial follicle assembly, and that in rodents the drop in steroid hormone levels after birth initiates follicular assembly.[72] Progesterone reduces the rate of primordial follicle assembly in mice and rats,[72,73] possibly through the reduction in germ cell apoptosis.[72] However, progesterone receptor (*Pgr*) knockout mice appear to have normal follicle development, but are infertile due to ovulation and uterine defects.[74] Thus, progesterone's effect on primordial follicle assembly may act through a nonclassical mechanism.[72] In other species, such as sheep, cattle, and human, primordial follicle assembly takes place during fetal development at times of high estradiol and progesterone levels. Therefore, if steroid signaling suppresses primordial follicle assembly in these species, additional mechanisms of regulation must occur, perhaps including the expression of hormone-binding proteins that may sequester steroid hormones and diminish their bioavailability.[75] In cattle, while maternal serum levels of steroids are high, progesterone and estradiol levels decline in fetal ovaries with increasing age.[76] In addition, estradiol and progesterone inhibit follicle formation in cultures of fetal bovine ovaries,[77,78] suggesting that steroid hormone regulation of primordial follicle assembly may be conserved to some extent in mammals. Therefore, a mechanism to limit steroid activity in fetal ovaries may have evolved to allow for prenatal primordial follicle formation.

Treatment of newborn mouse ovaries with estradiol, synthetic estrogens such as diethylstilbestrol (DES), or phytoestrogens such as genistein leads to the formation of follicles with multiple oocytes, termed polyovular follicles, or multiple oocyte follicles (MOFs).[73,79–81] The effects of genistein are likely mediated by the estrogen receptor beta isoform (ERβ) because polyovular follicle formation is prevented in estrogen receptor beta (*Esr2*) knockout mice but not estrogen receptor alpha (*Esr1*) knockouts.[82] DES and estradiol decrease expression of the protein subunits required to produce activin (*Inhba* and *Inhbb*), growth factors with important regulatory roles in follicle development (see the section Activins), and thus activin may mediate the effects of estrogen on primordial follicle assembly.[79] In line with this hypothesis, conditional deletion of the activin βA subunit in granulosa cells results in the formation of polyovular follicles.[83] In turn, it has been shown that activin induces expression of the estrogen receptors *Esr1* (*ERα*) and *Esr2* (*ERβ*), suggesting a feedback loop between estrogen and activin signaling.[84]

Notch Signaling

Notch signaling is a developmentally conserved metazoan-signaling pathway that mediates signaling between neighboring cells. *Notch* encodes a transmembrane receptor that binds to a membrane-bound ligand (Jagged (JAG1 and JAG2) or Delta-like 1 (DLL1), DLL2, and DLL4) on an adjacent cell. Binding of the ligand results in proteolysis of the Notch intracellular domain (NICD), which translocates to the nucleus and interacts with transcription factors of the C-promoter-binding factor 1/suppressor of hairless/Lag-1 (CSL) family. Of the four Notch paralogs (*Notch 1–4*), *Notch2* is most abundantly expressed in the ovary and is expressed in the pregranulosa cells of primordial follicles.[85] Oocytes express *Jag1*, the most highly expressed of the six ligands in the ovary.[85,86] Chemical inhibition of Notch signaling in cultures of newborn ovaries results in reduced germ cell cyst breakdown and a

lower percentage of primordial follicles.[85] Deletion of lunatic fringe (*Lfng*), an *N*-acetylglucosaminyltransferase that regulates Notch interactions with its ligands, causes infertility in mice and the presence of polyovular follicles in ovaries.[87] Perturbations of Notch–Jagged signaling have also been detected in mice null for neurotrophic tyrosine kinase receptor 2 (*Ntrk2* or *TrkB*) (see the section Nerve Growth Factor and Receptors).[88] *Ntrk2*$^{-/-}$ mice die within 2 weeks of birth, but when ovaries are transplanted to the kidney capsule and allowed to develop, the ovaries show reduced follicle assembly and massive oocyte loss.[89] *Ntrk2*$^{-/-}$ ovaries have reduced *Jag1* expression and Notch target gene expression, including *Hes1*, *Hey2*, and *Myc*.[88] Overexpression of *Jag1* in *Ntrk2*$^{-/-}$ oocytes in vitro increases *Myc* expression in granulosa cells, restores follicle growth, and causes granulosa cell proliferation.[88] These data support a model in which neurotrophin signaling acts via the Notch-signaling pathway to mediate oocyte–granulosa cell development, although how these interactions may lead to the formation of primordial follicles is still unresolved.

Factor in the Germline (*Figla*)

A mutation in the mouse helix–loop–helix transcription factor, factor in the germline alpha (*Figla*), causes a disruption of primordial follicle formation and the subsequent loss of most of the oocytes by postnatal day 2.[90] FIGLA is an oocyte-specific protein, which binds promoter elements (E-box) in the oocyte zona pellucida (ZP) genes to regulate their expression in vitro.[91] ZP proteins, encoded by *Zp1*, *Zp2*, and *Zp3*, are a major component of the glycoprotein-rich matrix that surrounds the developing oocyte and is required for fertilization. The ZP may also act as a barrier to the exchange of growth factors between the oocyte and the immediately adjacent granulosa cells.[92] Single *Zp1*, *Zp2*, or *Zp3* knockout mice are able to form primordial through antral follicles, and therefore, individual loss of the ZP genes cannot account for the observed phenotype of the *Figla* mutant mice.[93–96] It is likely that FIGLA regulates expression of other genes that are critical for follicle formation and oocyte survival. Indeed, microarray data analysis between wild-type and *Figla*$^{-/-}$ ovaries identified POU domain, class 5, transcription factor 1 (*Pou5f1/Oct4*); E2f transcription factor 5 (*E2f5*); signal transducer and activator of transcription 3 (*Stat3*); lymphocyte cytosolic protein 1 (*Lcp1*); cytoplasmic polyadenylation element–binding protein 3 (*Cpeb3*); ELAV (embryonic lethal, abnormal vision, *Drosophila*)-like 2 (*Elav2*); and cytoplasmic polyadenylation element–binding protein 1 (*Cpeb1*) as downregulated genes in the knockout.[97] Interestingly, depletion of oocytes in *Figla*$^{-/-}$ leads to overexpression of several testis-specific genes, a phenomenon also seen in the *Nobox*$^{-/-}$ ovaries[97,98] (see the section Newborn Ovary Homeobox Gene). These data suggest that *Figla* and other oocyte-expressed transcription factors such as *Nobox* may be important for ovarian determination by suppressing male-specific genes. This idea is further supported by data that ectopic expression of *Figla* in male germ cells suppresses expression of testis-associated genes, such as tudor domain containing 1, 6, and 7 (*Tdrd1*, *Tdrd6*, and *Tdrd7*).[99]

The human homolog of FIGLA also has restricted expression to germ cells, forms a heterodimer with the E12 protein, and binds to the E-box of the human Zp2 promoter.[47,100] *FIGLA* is expressed as early as 14 weeks of gestational age, with a dramatic increase in transcripts by midgestation (20 weeks gestational age, i.e., the time of primordial follicle formation in humans), suggesting a similarly conserved function of the human and mouse FIGLA proteins. Mutations in *Figla* can be detected in a subset of women with premature ovarian insufficiency,[101] a condition characterized by hypergonadotropic hypogonadism prior to age 40.

Newborn Ovary Homeobox Gene

Nobox is an oocyte-specific homeobox gene discovered in a screen to identify oocyte-specific genes using a computer database–based "in silico" subtraction of expressed sequence tag (EST) databases, and it was one of the first oocyte-specific homeobox genes to be discovered.[102] *Nobox* mRNA and protein are expressed in both germ cell cysts and primordial follicles and throughout folliculogenesis.[102,103] Female mice lacking NOBOX cannot form growing follicles, have an arrest in folliculogenesis at the transition from primordial to primary follicles, and are sterile.[103] Unlike *Gdf9* null ovaries, *Nobox*-deficient ovaries rapidly lose oocytes so that by 2 weeks after birth, very few follicles are found (Figure 21.3(C)). It is of interest to note that shortly after birth, *Nobox*$^{-/-}$ ovaries contain more oocytes in germ cell cysts and less in primordial follicles as compared to the wild-type ovaries. At day 7 after birth, *Nobox*$^{-/-}$ ovaries still have the majority of oocytes in the form of cysts, with only a few primordial follicles and no secondary follicles; by day 14, most oocytes are degenerating. Ultrastructural examination of *Nobox*$^{-/-}$ ovaries at E16.5 and shortly after birth revealed early postnatal defects in the breakdown of germ cell cysts.[64] In wild-type mice, the somatic cells surrounding the cysts produce long projections that penetrate between oocytes, leading to the breakage of the cytoplasmic bridges connecting oocytes and the separation and somatic envelopment of individual oocytes. Further invasion of somatic cells between the oocytes correlates with the formation of individual primordial follicles.[64] In *Nobox*-deficient mice, somatic cells have a limited number of very short projections as compared to the wild type, and do not show peri-oocyte invasion. This results in the formation of highly abnormal syncytial follicles

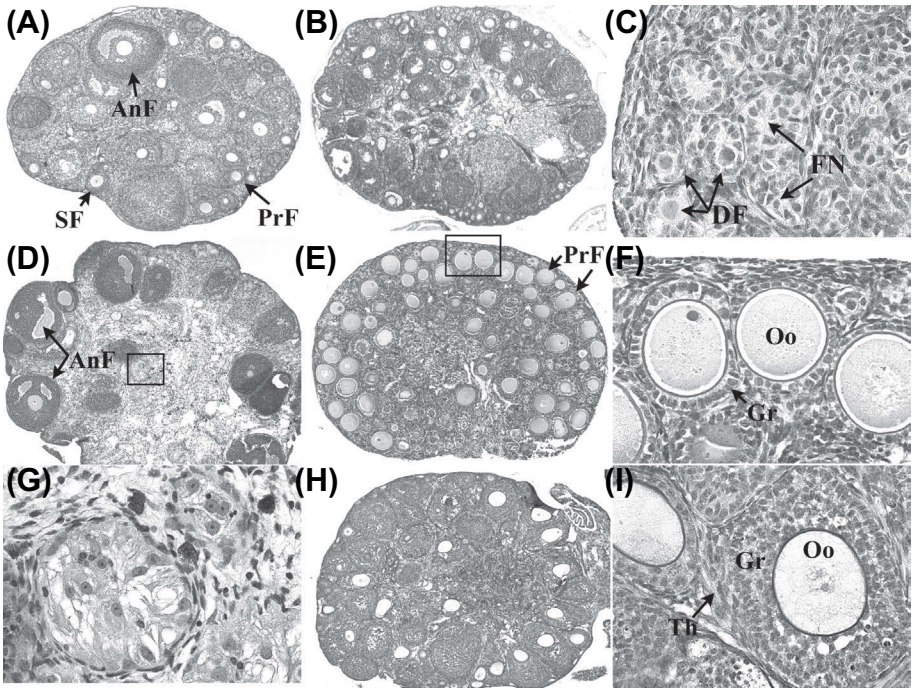

FIGURE 21.3 Ovarian histology for various mouse knockout models. Light micrographs of adult ovarian sections from knockout models. *Panel (A)*: Adult wild-type ovaries demonstrate follicles from many stages. *Panel (B)*: Adult *Fsh*$^{-/-}$ mutant ovaries lack antral follicles. *Panel (C)*: 14-day-old *Nobox*$^{-/-}$ ovaries contain only follicular nests and degenerating oocytes. *Panel (D)*: *Esr1*$^{-/-}$ *Esr2*$^{-/-}$ double-mutant ovaries containing many advanced follicle types as well as structures that resemble male seminiferous tubules (boxed and shown at higher magnification in *panel (G)*). *Panel (E)*: *Gdf9*$^{-/-}$ null ovaries arrest at the primary follicle stage. *Panel (F)*: Magnification of boxed *Gdf9*$^{-/-}$ follicles shown in *panel (E)*. *Panel (H)*: Double-mutant *Gdf9*$^{-/-}$ *Inha*$^{-/-}$ ovary demonstrates partial rescue of the *Gdf9*$^{-/-}$ phenotype. *Panel (I)*: Higher magnification of the *Gdf9*$^{-/-}$ *Inha*$^{-/-}$ showing multilaminar follicles and a thecal cell layer. PrF: primary follicle; SF: secondary follicle; AnF: antral follicle; Oo: oocyte; Gr: granulosa cell; Th: thecal cell; DF: degenerating follicle; FN: follicular nest. Micrographs not shown to scale.

composed of clusters of unseparated oocytes surrounded by 2–3 layers of somatic cells. These defects in germ cell cyst breakdown are in part responsible for abnormal formation of primordial follicles and associated oocyte loss. Persistent expression of *Stra8* (simulated by retinoic acid 8), a gene that controls meiotic initiation, in the *Nobox* knockout newborn ovary signifies that *Nobox*-deficient oocytes are immature at the molecular level, despite forming primordial-like follicles.[98] Moreover, *Nobox* deficiency likely affects the production of factors that oocytes use to communicate with surrounding somatic cells.

Nobox encodes a homeobox gene, and such genes encode transcription factors that play critical functions during organogenesis and can be either ubiquitous or cell type specific in their expression and function. Because *Nobox* expression is restricted to oocytes, it was hypothesized that NOBOX regulates expression of genes preferentially expressed in oocytes. Consistent with this hypothesis, it was demonstrated that the NOBOX deficiency preferentially disrupts expression of multiple oocyte-specific genes, including *Gdf9*, bone morphogenetic protein 15 (Bmp15), *Pou5f1* (*Oct4*), ret finger protein-like protein 4 (*Rfpl4*), and Maloney sarcoma oncogene (*Mos*). Overall, when comparing *Nobox* knockout and wild-type ovaries, 28 (74%) of 38 genes that downregulated more than fivefold in the absence of *Nobox* were preferentially expressed in the oocytes, whereas only five (15%) of 33 genes that upregulated more than fivefold in the absence of *Nobox* were preferentially expressed in oocytes.[98] NOBOX appears to directly regulate a subset of genes preferentially expressed in the oocytes, and seems to act as an activator of genes necessary for both folliculogenesis (*Gdf9* and *Bmp15*) and early postfertilization embryonic events (NLR family pyrin domain containing 5 (*Nlrp5/Mater*), DNA methyltransferase 1 (*Dnmt1*), zygotic arrest 1 (*Zar1*), and nucleoplasmin 2 (*Npm2*)).[98,104,105] In addition, pluripotency-associated genes, *Pou5f1* and sal-like 4 (*Sall4*), are drastically downregulated in *Nobox*-deficient ovaries, whereas testes-determining gene *Dmrt1* is overexpressed. These findings indicate that *Nobox* is likely an activator of oocyte-specific gene expression and suggest that the oocyte plays an important role in suppressing expression of male-determining genes, such as *Dmrt1*. The mechanism of follicle loss in *Nobox* null ovaries has yet to be determined, although it appears to be independent of known members of the apoptotic cascade (i.e., B-cell leukemia/lymphoma 2 (*Bcl2*), Caspase 2 (*Casp2*), BCL2-associated X protein (*Bax*), and Bcl2-like 2 (*Bcl2l2*)), which are normally expressed in *Nobox* null ovaries. It is possible that other oocyte-specific genes are essential for oocyte survival, and elucidation of their pathways in *Nobox*-deficient ovaries will be of great interest. The overall hierarchy of oocyte-specific transcriptional control in early folliculogenesis is still unknown; however, the list of important players keeps growing (Figure 21.4).

A human homolog of *Nobox* exists and mimics the expression observed in mice.[48] Human *NOBOX* expression within the ovary is oocyte specific, and observed from the primordial-stage ovarian follicle through to the metaphase II (MII) oocyte. *NOBOX* is a candidate gene for idiopathic (no known cause) and nonsyndromic (the

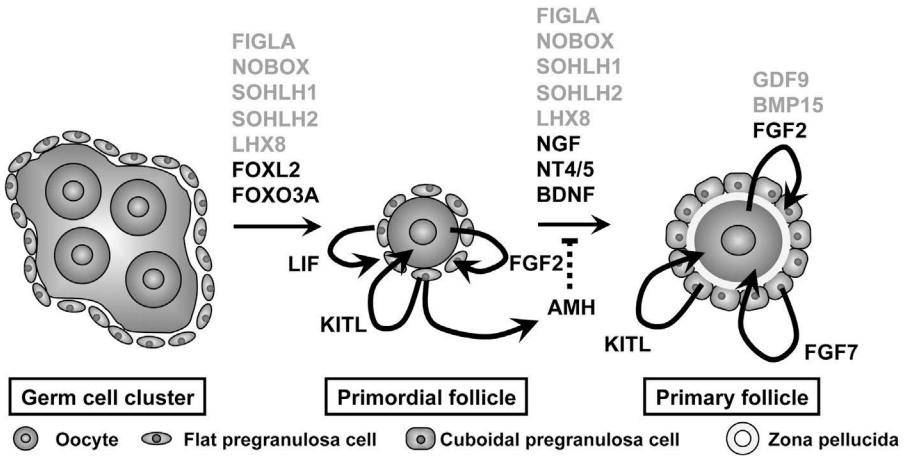

FIGURE 21.4 Early folliculogenesis. Germ cell cysts break down after birth, and primordial follicles form. Germ cell–specific factors considered part of the same pathway are shown in a lighter font. The factors are listed at the site of proximate action as derived from mouse transgenic and ovarian culture studies. LIF: leukemia inhibitory factor; KITL: kit ligand; FGF2: fibroblast growth factor; AMH: anti-Müllerian hormone; FGF7: fibroblast growth factor 7; NGF: nerve growth factor; NT4/5: nerve growth factor receptors; NOBOX: newborn ovary homeobox gene; FIGLA: factor in the germline alpha; FOXL2: forkhead box L2; GDF9: growth differentiation factor 9; BMP15: bone morphogenetic protein 15; BDNF: neurotrophic factor; NT4/5: neurotrophin 5; FOXO3A: forkhead transcription factor; LHX8: LIM homeodomain 8; SOHLH1: spermatogenesis and oogenesis helix loop helix 1; SOHLH2: spermatogenesis and oogenesis helix loop helix 1.

phenotype is confined to ovarian failure) human ovarian insufficiency. Indeed, several human studies have associated *NOBOX* mutations and deletions with premature ovarian insufficiency. Several groups have reported that mutations or deletions in the *Nobox* locus associate with primary ovarian insufficiency in humans.[106–109]

Spermatogenesis and Oogenesis Helix–Loop–Helix Transcriptional Regulators 1 and 2 (*Sohlh1* and *Sohlh2*)

In addition to *Figla*, two other basic helix–loop–helix (bHLH) transcriptional regulators have been implicated in early folliculogenesis. *Sohlh1* and *Sohlh2* are important bHLH germ cell specific transcription factors implicated in gonadal development. Both are strongly expressed in germ cell cysts and primordial follicles, moderately expressed in primary follicles, while not expressed in secondary follicles. The expression patterns of *Sohlh1* and *Sohlh2* are unique, as compared with *Figla*, *Nobox*, and *Lhx8*, which are expressed throughout folliculogenesis. This may indicate that *Sohlhs* play important and unique roles in primordial follicle formation, activation, and survival. Ovaries from *Sohlhs* mutant mice are completely devoid of follicles by 3 weeks postnatal age.[110–113] The defect lies in primordial follicle formation, and surrounding pregranulosa cells do not proliferate. *Sohlh1*$^{-/-}$ and *Sohlh2*$^{-/-}$ mutant mice have very similar ovarian phenotypes, with perturbed expression of nearly identical genes as assessed by RNA arrays, including several germ cell–specific genes such as *Gdf9*, *Bmp15*, *Zar1*, *Lhx8*, and *Nobox*.[110,111,113,114] The lack of granulosa cell proliferation in *Sohlh1* and *Sohlh2* knockouts can, in part, be explained by the downregulation of *Kit* and *Gdf9*, but unlike *Gdf9*$^{-/-}$ knockout ovaries, *Sohlh1*$^{-/-}$ and *Sohlh2*$^{-/-}$ oocytes grow to a size of greater than 30 μm in diameter without conversion of flat pregranulosa cells to the cuboidal cell type expected for oocytes of that size. These results are consistent with premature oocyte activation, which ultimately leads to the loss of follicles in mutant mice. SOHLH1 and SOHLH2 proteins can form homo- and heterodimers, and these dimers may play a role in the transcriptional control of early folliculogenesis. *Sohlh2* transcripts can be detected as early as E12 in mice, while *Sohlh1* transcripts are expressed later, at E16. Whether SOHLH2 and SOHLH1 regulate each other is unclear, and both SOHLH1 and SOHLH2 are downregulated in each other's deficiency. The nearly identical phenotypes in the two knockouts indicate that these two critical molecules act along similar, if not identical, pathways.

LIM Homeodomain Protein 8 (*Lhx8*)

Lhx8 is a homeobox gene that is preferentially expressed in oocytes and critical in folliculogenesis.[110,114] *Lhx8* encodes a LIM homeodomain protein that is expressed in the embryonic ovary from E18.5 throughout folliculogenesis. LIM proteins are found in invertebrate and vertebrate eukaryotes, and they have diverse biochemical functions. The LIM protein superfamily includes protein kinases and GTPase-activating factors, transcription factors, and transcriptional co-activators implicated in cellular architecture and intracellular signaling.[115,116] SOHLH1 transcriptional regulator binds the *Lhx8* promoter, as shown by chromatin immunoprecipitation

experiments,[110] and likely regulates *Lhx8* activity directly. *Lhx8* is therefore downstream of the SOHLH1–SOHLH2 pathway, and unlike *Sohlh1* and *Sohlh2* genes that also regulate spermatogonial differentiation in males,[117] *Lhx8* is only involved in the regulation of the female germline. Other regulators of *Lhx8* may include *Yin Yang1* (*Yy1*), a multifunctional transcription factor that can act as a transcriptional repressor and activator. *Lhx8* transcript expression is diminished in ovaries made conditionally deficient of *Yy1* by *Zp3*-driven *Cre* expression.[118] *Lhx8*-deficient mice are infertile due to lack of oocytes by postnatal day 7, with an early block in the formation of primordial and primary follicles. Several germ cell–specific genes known to be essential for oogenesis, such as *Gdf9*, *Bmp15*, *Nobox*, and *Figla*, are downregulated in *Lhx8*$^{-/-}$ovaries,[110,114] and many of the genes downregulated by *Lhx8* are also perturbed on *Sohlh1* and *Sohlh2* knockout RNA expression arrays. These results argue that *Lhx8* likely shares *Sohlh1* and *Sohlh2* pathways. The nature of signaling molecules and pathways that regulate expression of *Figla*, *Sohlh1*, *Sohlh2*, and *Lhx8* is unclear, and remains an important area of investigation.

Zinc Finger GATA-like Protein 1 (*Zglp1*)

Zinc finger GATA-like protein 1 (ZGLP1, also known as GLP1) is a nuclear protein containing two zinc fingers, one of which is highly homologous to the conserved GATA zinc finger DNA-binding motif. *Zglp1* is primarily expressed in the somatic cells of the developing gonads at E13.5 and granulosa cells of the adult ovaries.[119,120] Analysis of developing *Zglp1* null mouse ovaries shows that germ cells are appropriately specified and migrate normally to nascent gonads. Initial studies indicated that germ cell loss occurs as early as E17.5, at the time when oocyte meiosis is becoming arrested and prior to the formation of primordial follicles.[119] Subsequent studies suggest that the loss of germ cells begins at around E13.5 and is completed by birth.[120] Mutant mice have defects in the expression of genes associated with meiotic entry; however, it is unclear whether meiosis is perturbed and whether decreased gene expression is simply a reflection of fewer germ cells. Pregranulosa cell formation and function appear to be normal as *Foxl2* mRNA and protein expression, laminin deposition, and pregranulosa cell appearance are unaffected in *Zglp1* null embryonic ovaries as compared to wild-type ovaries. ZGLP1 can repress *Gata4* and *Gata6* expression; however, the role of GATA-regulated signaling in pregranulosa cells is unknown (see the section GATA Transcription Factors). *Zglp1* mutants exhibit one of the few known ovarian phenotypes where the major block occurs between the onset of meiosis and primordial follicle formation. Conditional inactivation would clarify the roles of *Zglp1* in later stages of folliculogenesis.

Prenatal Exposures to Toxins and Transgenerational Effects

The idea of fetal origins of disease stemmed from Barker's hypothesis that fetal growth restriction can lead to adult disorders,[121] and led to many subsequent studies that associated differential fetal exposures and chronic disease. The fetal gonad is a dynamic organ with many critical events, such as primordial germ cell proliferation, sex determination, meiosis, and epigenetic reprogramming, occurring during development. Fetal exposure to environmental toxins, mainly endocrine disruptors, may lead to impaired reproductive outcomes and ovarian pathology.[122–124] Interestingly, environmental toxins such as vinclozolin, an agricultural fungicide, and dioxin, a chemical contaminant generated from combustion and industrial processes, reduce the size of the primordial follicle pool in the offspring and subsequent generations, including the F3 generation, when administered to pregnant dams during gonadal sexual development.[125] These findings argue that transgenerational effects operate in the ovary. Mechanisms behind transgenerational inheritance are unclear, although epigenetics likely plays an important role.[122] It is also unclear whether transgenerational effects are physiologically significant or relevant to the human condition of premature ovarian insufficiency and polycystic ovarian syndrome (PCOS).

THE PRIMORDIAL TO PRIMARY FOLLICLE TRANSITION

The regulation of the nongrowing pool of primordial follicles is one of the most poorly understood processes in ovarian biology. The transition from primordial to growing follicle occurs spontaneously in newborn mouse ovaries cultured in medium in the presence of 10% fetal bovine serum.[126,127] After 8 days of culturing newborn ovaries, primary and secondary follicles form. Oocyte–granulosa cell complexes isolated from the 8-day organ cultures of newborn ovaries can be used to produce oocytes capable of fertilization, embryo formation, and live pups, albeit at a very low efficiency. Unlike mouse ovaries, follicular development in vitro occurs more rapidly in baboon ovaries, with many primary follicles observed by day 2 of culture and a much-diminished number of primordial follicles.[127,128] Removal of ovaries from their natural milieu may eliminate a potential inhibitor of follicular growth present in the surrounding tissue, peritoneum, or blood. On the other hand, it is possible that current culture media provide a much richer source of nutrients and oxygen, leading to premature activation of follicular growth.

Numerous factors accelerate the development of follicles in vitro. Primordial follicles from 4-day-old newborn

rat ovaries cultured in vitro develop faster when basic fibroblast growth factor (FGF), kit ligand (KITL), leukemia inhibitory factor (LIF), nerve growth factor (NGF), or bone morphogenetic protein 4 (BMP4) are introduced into the culture.[129–133] Typically, without treatment with growth factors, 50% of follicles grow in vitro; this rises to 60% upon treatment with particular growth factors. While small, the increase in growing follicles is statistically significant and indicates that numerous factors potentially enhance follicular growth.

PI3K–AKT and Tsc1–mTORC1 Signaling Pathways

The phosphatidylinositol 3-kinase–protein kinase B (PI3K–AKT) pathway is a conserved signaling pathway that regulates cell growth and survival in many different cell types. Regulation of primordial follicle activation by the PI3K pathway was first discovered when the transcription factor, forkhead box O3 (*Foxo3*), was deleted in mice. FOXO3 localizes to the oocyte nucleus during primordial follicle assembly, and is exported to the cytoplasm during oocyte activation.[134] In *Foxo3* null mice, primordial follicles form normally but do not undergo arrest. Thus, the entire pool undergoes global activation, resulting in premature follicle depletion and sterility by 15 weeks of age.[135] In addition, $Foxo3^{+/-}$ female mice have premature reproductive senescence due to an increased rate of primordial follicle activation.[136] Conditional deletion of *Foxo3* in oocytes of primordial follicles using the *Gdf9* promoter-driven *Cre* expression results in a similar phenotype, and confirms that the loss of FOXO3 in oocytes, as opposed to the somatic cells, is responsible for the activation of primordial follicles.[134]

AKT phosphorylates FOXO3 and causes FOXO3 cytoplasmic sequestration and degradation. Phosphatase and tensin homolog (PTEN) antagonizes PI3K, and removal of PTEN activates AKT, which in turn phosphorylates a wide range of intracellular targets, including FOXO3. It is therefore not surprising that oocyte-specific deletion of *Pten*, using *Gdf9* promoter-driven *Cre* expression, results in global activation of the primordial follicle pool.[137] Inducible conditional mutations in *Pten* have also been generated in primordial follicles of adult mice, and these mice also show enhanced primordial follicle activation, indicating that the PI3K pathway is important throughout the reproductive life span.[134] Oocyte loss in oocyte-specific *Pten* mutant ovaries may occur in part by the elevated levels of activity in the ribosomal protein kinase (S6K1)–ribosomal protein S6 signaling cascade, which regulates protein translation and ribosomal biogenesis.[137–139] Interestingly, conditional deletion of *Pten* in primary oocytes and beyond, using transgenic *Cre* expression driven by the *Zp3* promoter, had no effect on folliculogenesis or fertility.[140] These results argue that PTEN–PI3K–AKT signaling is stage dependent and confined to the primordial follicle pool. The nature of the external growth factors and stimuli within the ovary that activate the PTEN–PI3K–AKT signaling is currently unknown. A number of inhibitors and activators of this pathway are known and have been used to activate oocytes in human ovaries as a proof of principle that manipulation of the PTEN–PI3K–AKT pathway may facilitate generation of a large number of mature eggs for in vitro fertilization.[141,142] Despite promising results in rodents, little is known regarding whether the same pathways operate in humans or other mammals.

Premature loss of the ovarian reserve as well as elevated levels of serum FSH and LH in $Foxo3^{-/-}$ mice are reminiscent of premature ovarian failure (POF) in women.[135] Because the majority of POF cases are idiopathic, it is possible that in some instances, a similar mechanism may be operating through *Foxo3*. However, exon and flanking splice site sequencing of a large sample of POF or idiopathic primary amenorrhea failed to identify causal mutations,[136] arguing that *FOXO3* is not a major genetic determinant of human premature ovarian insufficiency.

Mammalian target of rapamycin complex 1 (mTORC1) is a key mediator of cell growth, and growth factor receptors activate mTORC1 by signaling through the PI3K–AKT and RAS–ERK mitogen-activated protein kinase (MAPK) pathways. mTORC1 promotes protein translation and cell growth largely through activation of S6K1 (by phosphorylation of threonine 389) and through phosphorylation and inactivation of eukaryotic translation initiation factor 4E (eIF4E)-binding proteins. S6K1 is responsible for phosphorylation and activation of ribosomal protein rpS6, which leads to enhanced protein translation and ribosome biogenesis. Tuberous sclerosis complex1 (TSC1) represses mTORC1 functions, and conditional inactivation of *Tsc1* in oocytes of primordial follicles causes massive PFA by enhancing mTORC1 activity.[143] These data also indicate the importance of the mTORC1 pathway in primordial follicle activation. The phenotype of the *Tsc1* conditional knockout ovaries is highly reminiscent of the *Pten* oocyte conditional knockout phenotype.

Kit Ligand (*Kitl*) and Kit Receptor (*Kit*)

Kit encodes a transmembrane receptor with tyrosine kinase activity and belongs to a family that includes colony-stimulating factor 1 (CSF1) and platelet-derived growth factors A and B (PDGFA and PDGFB). *White spotting* (*W*) and *Steel* (*Sl*) loci in the mouse encode KIT and its ligand, KITL, respectively.[144–147] KIT–KITL signaling plays important roles in hematopoiesis, gametogenesis, and melanogenesis.[148,149]

The first insight into the role of KITL and its receptor, KIT, came from the studies of mice spontaneously mutated at the *Kitl* and *Kit* loci.[150,151] There are more than 60 spontaneous mutations in the *Kitl* and *Kit* loci, and, depending on the severity of the loss of function, these mice show mild to severe defects in pigmentation, hematopoiesis, and reproduction. The mutant alleles are semidominant: some are lethal when homozygous, and others are viable. *Kit* expression can be detected as early as 7.5 days post conception and is high in proliferating germ cells.[144] *Kit* ceases to be expressed when oogonia enter meiosis, around E13.5, and is re-expressed in newborn ovaries. A similar pattern of expression is observed for *Kitl*.[145] *Kitl* is expressed in newborn ovaries in pregranulosa cells and continues to be expressed in granulosa cells throughout folliculogenesis.[152–154] *Kit* is expressed in primordial follicles and other oocytes throughout follicular growth.[152,155] KIT has also been detected in thecal cells, and KITL–KIT interactions can stimulate thecal cell growth and androstenedione production in the absence of gonadotropins.[156] Both in vivo and in vitro data provide evidence that KITL and KIT interactions play a significant role in the primordial to primary follicle transition.[157,158] The Sl^d allele causes infertility due to a complete absence of germ cells in the ovary.[159] The Sl^t allele, however, causes infertility due to the early growth arrest of ovarian follicles.[158] Folliculogenesis in mice that carry the Sl^t allele is arrested early, with many primordial follicles present and few growing follicles.[158]

Neutralizing antibodies against KIT have been used to study the functions of KIT in early folliculogenesis.[160] Mice injected with an anti-KIT neutralizing antibody at birth have blocked follicle development at the primordial follicle stage. Incorporation of BrdU into granulosa cells is diminished in these ovaries, indicating that DNA synthesis is compromised. If antibodies are injected starting at day 2, when primordial follicles are already apparent in mouse ovaries, primary follicular development is only slightly perturbed, and these mice have a diminished number of antral follicles with decreased proliferation and DNA synthesis in granulosa cells. Therefore, it appears that functional KIT is important at the very early stages of folliculogenesis as well as at the later antral stage. Because primordial follicle formation is not disturbed in any of these experiments, the conclusion can be made that KIT is not essential for the initial step of germ cell cyst breakdown into individual primordial follicles.

KIT is an attractive candidate for the transmembrane receptor tyrosine kinase that regulates PI3K activity. As discussed in this chapter, the PI3K–AKT-signaling pathway is an important regulator of primordial follicle activation; however, the nature of receptors and signals that converge upon it are unknown. KIT activates PI3K through a direct interaction with an SH2 domain on the p85 regulatory subunit of PI3K. This interaction is entirely dependent on the KIT tyrosine residue 719, which undergoes autophosphorylation following ligand binding. Mutation of this tyrosine residue prevents binding of KIT to p85 and thus completely abrogates KIT signaling via PI3K.[161] "Knockin" mice expressing KIT with a loss-of function mutation in tyrosine residue 719 (Kit^{Y719F}) are fertile and undergo primordial follicle activation. However, mice homozygous for the congenital Kit^{Y719F} mutation had accelerated primordial follicle depletion, and accumulated morphologically abnormal primary and secondary follicles.[162] Thus, KIT appears to be important in the maintenance of the primordial follicle reserve and in the primary to secondary follicle transition, but not in the primordial follicle activation.[162] These conclusions were based solely on analyzing the congenital Kit^{Y719F} mutation, and the phenotype of stage specific activation of the Kit^{Y719F} mutation is unknown.

Anti-Müllerian Hormone (*Amh*)

The dimeric glycoprotein anti-Müllerian hormone (AMH) is a member of the transforming growth factor beta (TGFβ) superfamily of growth and differentiation factors (see the section Regulation of Folliculogenesis by TGFβ Family Signaling in the Ovary). During embryogenesis, AMH induces degeneration of Müllerian (female) ducts during male fetal sex differentiation.[163] Postnatally in ovaries, AMH and its receptor AMHR2 are expressed in granulosa cells of primary and growing follicles in rodents and humans, but their expression is lost in FSH-responsive antral and preovulatory follicles.[164–167] AMH expression, however, is maintained in cumulus cells.[168] AMH appears to be the only natural ligand of the AMHR2 receptor, as *Amh* and *Amhr2* mutants phenocopy each other.[169] Although *Amh* null mouse females are fertile with normal litter sizes,[163] a careful histologic analysis of ovaries from 25-day-old, 4-month-old, and 13-month-old mice revealed significantly fewer primordial follicles in 4- and 13-month-old *Amh* null ovaries.[170] AMH therefore appears to inhibit the growth of primordial follicles, and absence of AMH leads to a faster depletion of primordial follicles. AMH also inhibits growth of primordial follicles in cultures of human ovarian cortical tissue biopsies.[171] In mice, experiments show that AMH attenuates the effects of FSH, and the absence of AMH potentiates the proliferative effects of FSH in the sexually mature ovary (4- and 13-month-old ovary), leading to greater recruitment of primordial follicles.[172] In vitro studies using 2-day postnatal mouse ovaries cultured in the presence and absence of AMH show that fewer growing follicles are found in ovaries treated with AMH.[173]

AMH is detectable in serum, and because of its expression pattern in growing preantral follicles, AMH

was proposed to be a measure of the ovarian reserve and ovarian function.[174,175] Serum AMH levels positively correlate with the size of the primordial follicle pool.[176] There is an interdependent relationship between the size of the resting and growing follicle pools,[177] and because AMH is expressed within growing follicles, AMH is still an indirect measure of ovarian reserves. AMH levels in women decline with age and ovarian dysfunction.[178–180] The proposed clinical uses of AMH include assessing the ovarian reserve prior to assisted reproduction, predicting gonadal function after cancer therapy or surgery, predicting the age at menopause, and diagnosing and measuring granulosa cell tumor recurrences.[181,182] AMH is a more sensitive marker of the ovarian reserve than the previously used marker, FSH, which has been a standard for clinical care.[183] However, the need remains for a better biomarker to directly measure the remaining oocyte pool and to reliably predict reproductive life span and ovarian failure. Such biomarkers are greatly needed to identify women at risk for ovarian failure, and to identify women who can benefit from advanced fertility preservation technologies prior to oocyte loss.[184]

Nerve Growth Factor and Receptors

Nerve growth factor (NGF) belongs to a family of proteins known as neurotrophins.[185] Neurotrophins are required for the survival and differentiation of neurons within both the central and peripheral nervous systems. In the rat ovary, *Ngf* and its two receptors, *Ngfr* and *Ntrk1* (also called *TrkA*), are expressed before primordial follicle formation, suggesting that NGF plays a role in early folliculogenesis.[186–188] Seven-day-old *Ngf* null mice have a drastic reduction in the number of primary and secondary follicles as compared to control mice.[189,190] Although the number of primordial follicles is equivalent, the number of oocytes within the germ cell cysts increases in the *Ngf* null ovaries. NGF may play a role in differentiation of flat pregranulosa cells into the cuboidal cells that characterize primary follicles. A reduction in the number of secondary follicles also could indicate that NGF functionally interacts with growth differentiation factor 9 (GDF9) and/or KITL to induce proliferation and differentiation of somatic cells surrounding the oocyte. The number of proliferating cell nuclear antigen (PCNA)-positive cells is distinctly lower in *Ngf* null ovaries, suggesting that somatic cells in *Ngf* null mice have reduced ovarian mitotic activity.

The lack of NGFR does not affect primordial, primary, or secondary follicle formation, as null mice are fertile.[191] Unfortunately, NTRK1 deficiency causes death at or shortly after birth.[192,193] The NTRK2 receptor (also called TrkB) is also expressed in primordial and growing oocytes, while ligands that bind NTRK2 (i.e., neurotrophin 4/5(NT5) and brain-derived neurotrophic factor (BDNF)) are expressed in rodent and human primordial oocytes and granulosa cells.[194,195] Both NTRK1 and NTRK2 receptors are necessary for the timely assembly of primordial follicles and for sustaining early follicular development.[89] *Ntrk2* deletion in mice does not affect the number of primordial follicles; however, the number of secondary follicles is significantly lower in 7-day ovaries.[195] Double mutants of *Nt5* and *Bdnf* also show significantly lower levels of secondary follicles.[195] As measured by PCNA, granulosa cell proliferation is diminished in NTRK2 null animals, while apoptosis is not increased. Quantitative real-time polymerase chain raction comparing *Ntrk2* null mice with wild-type mice did not identify mRNA differences between the growth factor genes *Gdf9* and *Kitl*, or their receptors *Bmpr2* (a receptor for BMPs and GDFs) and *Kit* (the receptor for KITL). On the other hand, the amount of FSH receptor mRNA was significantly reduced in *Ntrk2* null ovaries as compared to wild-type ovaries, and thus NTRK2 receptors appear to facilitate follicle development by inducing the formation of functional FSHR. FSH levels are somewhat diminished in NTRK2 null mice but not significantly different from those of heterozygous animals. NTRK2 deficiency is perinatal lethal, and NTRK2 null ovaries, when transplanted under the kidney capsule of adult female C57BL/6J mice, rapidly lose oocytes and arrest follicular development. Therefore, it appears that NTRK2, and its ligands NT5 and BDNF, act in similar fashion to KITL and KIT to regulate early survival of female germ cells. In vitro studies using a tyrosine kinase inhibitor, K252a, to block all NTRK receptors in the mouse newborn ovaries and human fetal ovaries show that NTRK receptors are important for germ cell survival.[196] In addition, studies using postnatal rat ovaries have shown that exogenous administration of neurotrophin NT3 to cultures promoted primordial to primary follicle transition, while BDNF only showed effects at high doses and nerve growth factor had no effect.[197] In total, these data indicate that neurotrophins provide support to nonneuronal targets and play an important role in intraovarian regulation of early folliculogenesis along with GDF9 and KITL.

Forkhead box L2 (*Foxl2*)

Blepharophimosis ptosis and epicanthus inversus syndrome can occur with (BPES I) or without ovarian failure (BPES II). Nonsense mutations and duplications in *FOXL2*, a member of the forkhead–hepatocyte nuclear factor 3 (FKH–HNF3) gene family of transcription factors, cause BPES.[198,199] The phenotype of BPES I women with ovarian failure ranges from ovaries that contain large numbers of primordial follicles to streak ovaries that have no germ cells as detected at the time of laparoscopy and during the examination of ovarian biopsies.[200,201] In humans, *FOXL2* is preferentially expressed in the ovary.

In situ hybridization of mouse ovaries indicates that *Foxl2* is expressed postnatally in the pregranulosa cells that surround primordial follicles.[198] Ovaries from *Foxl2*$^{-/-}$ mice contain oocytes surrounded by flat granulosa cells that grow slowly compared to wild-type ovaries.[202,203] Expression of the oocyte-specific genes *Figla*, *Gdf9*, and *Kit* is not affected by the lack of FOXL2, and ovarian morphology as well as oocyte count are also unaffected at birth. Granulosa cells in *Foxl2*$^{-/-}$ ovaries fail to complete the transition from squamous to cuboidal shape. In addition, *Foxl2*$^{-/-}$ follicles undergo massive activation followed by oocyte atresia and premature follicle loss.

A different phenotype develops when *Foxl2* is conditionally deleted from adult mouse ovaries. In this case, conditional deletion of *Foxl2* causes transdifferentiation of granulosa cells into Sertoli-like cells, and induces expression of male-specific genes, including SRY-box containing gene 9 (*Sox9*); nuclear receptor subfamily 0, group B, member 1(*Nr0b1/Dax1*); desert hedgehog (*Dhh*); doublesex and mab-3 related transcription factor 1 (*Dmrt1*); and GATA-binding protein 1 (*Gata1*).[204] Moreover, Sertoli-like cells within the mutant ovaries produce testosterone. FOXL2 and ESR1 cooperatively repress *Sox9* expression.[204] Thus, FOXL2 plays a critical role in actively maintaining the identity of granulosa cells and the ovary as a whole. FOXL2 appears to be a stage-specific regulator of sex determination, as the global gene knockout (i.e., as opposed to the conditional knockout) does not exhibit the same phenotype.

Several studies have identified additional downstream targets of FOXL2.[205] The steroidogenic acute regulatory (*Star*) gene contains multiple putative forkhead DNA consensus sites, and FOXL2 binds these sites directly.[206] FOXL2 acts as a repressor of *STAR*, and dominant negative mutations within FOXL2 most likely interfere with repressor activity of the wild-type protein and cause a de-repression of *STAR*.[206] Wild-type FOXL2 also represses cytochrome P450 cholesterol side chain cleavage enzyme (*CYP11A1*), aromatase (*CYP19a1*), and cyclin D2 (*CCND2*),[207] all known to be involved in granulosa cell growth and differentiation. However, de-repression of these genes in mice that lack *Foxl2* is probably not the cause of the early follicular block at the primordial or primary follicular stage, and those genes remain to be identified. Overexpression of *Foxl2* and subsequent microarray analysis showed 118 differentially expressed genes and five classes of potential transcriptional targets: chemokines, apoptosis-related genes, proteolysis, and various signal transduction components and transcription factors.[208] FOXL2 interacts with LATS1, a tumor suppressor gene in the Hippo-signaling pathway. LATS1 phosphorylates FOXL2, and this interaction enhances the ability of FOXL2 to repress *Star*.[209] Deletion of *Lats1* in mice causes soft tissue sarcomas and stromal tumors of the ovary by 3 months of age, as well as decreased female fertility.[210]

A mutation in *FOXL2* is pathognomonic for adult granulosa cell tumors (AGCTs); 97% of AGCTs harbor a somatic missense mutation in a highly conserved cysteine residue at amino acid position 134 (c.402C→G; p.C134W) in *FOXL2*.[211] The FOXL2 C134W mutation does not disrupt expression, localization, protein folding, or DNA binding,[211,212] so the role it plays in AGCT pathogenesis is currently unclear. Efforts have thus focused on finding DNA-interacting partners and identifying altered transcriptional targets. FOXL2 has known interactions with Dead (Asp–Glu–Ala–Asp) box 20 (DDX20), steroidogenic factor 1 (SF1, or NR5A1), ESR1, and Sma- and Mad-related proteins (SMAD) family member 3 (SMAD3), a TGFβ transcription factor.[204,213–218] The FOXL2 C134W mutation increases aromatase (*CYP19*) to a greater degree than the wild-type form.[205] In addition, the pathogenesis of FOXL2 in GCT may relate to its role in regulating apoptosis. Wild-type FOXL2 induces an apoptotic response in granulosa cells, but FOXL2 C134W cannot elicit the same response.[213,219] Ectopic expression of wild-type FOXL2 induces a mitochondria-mediated apoptotic cell death involving members of the BCL2 family, which is only weakly induced by FOXL2 C134W.[219] Moreover, cells that express the mutant FOXL2 are less susceptible to death receptor signaling (via tumor necrosis factor receptors 1 (TNFR1) and Fas) and serum deprivation than those expressing the wild-type form.[219] This may in part relate to disrupted protein–protein interactions of the mutant FOXL2, which does not induce apoptosis when co-expressed with CXXC finger 5 (CXXC5); glucocorticoid modulatory element–binding protein 1 (GMEB1); nuclear receptor subfamily 2, group C, member 1 (NR2C1); and nuclear antigen Sp100 (SP100), all of which have been shown to interact with wild-type FOXL2 to increase apoptosis.[220]

REGULATION OF FOLLICULOGENESIS BY TGFβ FAMILY SIGNALING IN THE OVARY

Proteins that comprise the TGFβ family form the largest structurally related group of growth factors. The 33 mammalian members include TGFβ, for which the family is named, as well as the bone morphogenetic proteins (BMPs), growth factors and GDFs, activins, inhibins, and AMH. These are multifunctional proteins that control cell proliferation and differentiation in many cell types. In the ovary, TGFβ family ligands are expressed from almost all cell types of the ovarian follicle, including two, *Gdf9* and *Bmp15*, which are exclusively expressed from oocytes. Members of the TGFβ family are secreted as dimeric proteins that are processed from larger pre-pro-precursor forms. The pro region is cleaved by a specific protease in the intracellular or extracellular

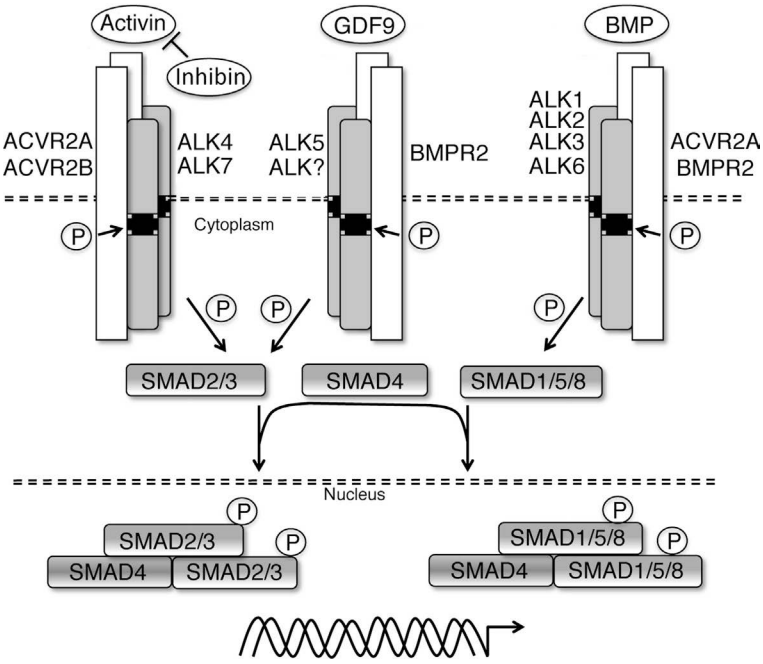

FIGURE 21.5 Summary of the core signaling pathway used by ligands of the TGFβ family. The TGFβ family signals through a membrane-bound receptor complex consisting of two type II and two type I receptors, which activate the intracellular SMAD transcription factors. Additional co-receptors and regulatory proteins are not shown. Activin signaling can be antagonized by inhibin (see the text for discussion). Activin signals through activin receptor type IIA (ACVR2A) or IIB (ACVR2B). BMPs may also signal through ACVR2A, but they also use the bone morphogenetic protein receptor type II (BMPR2). BMPR2 also binds GDF9. Once the ligand is bound, the type II receptor phosphorylates (P) and activates the type I receptor (ALK4 or ALK7 for activin, ALK5 for GDF9, and ALK1, ALK2, ALK3, or ALK6 for the BMPs). ALK5 has been shown to be a type I receptor for GDF9 in vitro but not in vivo; therefore, additional type I receptors (indicated by "ALK?") are likely also involved. Activated type I receptors then phosphorylate a restricted set of SMADs at a C-terminal motif. ALK4/5/7 phosphorylate SMAD2 and SMAD3, while ALK1/2/3/6 phosphorylate SMAD1/5/8. The receptor-restricted SMADs then form a trimeric complex with SMAD4, accumulate in the nucleus, and mediate gene transcription along with additional co-factors.

compartment to generate the bioactive mature domain. They bind to heteromeric cell surface receptor complexes that contain two types of receptors: type II and type I serine–threonine kinase receptors (Figure 21.5). In some instances, an additional, type III receptor facilitates ligand binding. Activation of the receptor complex results in phosphorylation of the intracellular SMAD transcription factors. Mouse models for the ligands, receptors, and signaling SMADs have elucidated the complex role of this protein family in follicle development, and often resulted in phenotypes that would not have been predicted from other types of analyses. This section is a summary of several of these models.

Inhibins

While the gonadotropins, FSH and LH, govern the final stages of follicular development and ovulation (see the section Key Roles of the Hypothalamus and Pituitary), the ovary participates in the endocrine control of gonadotrope function by the production of steroid and nonsteroid hormones in the hypothalamic–pituitary–gonadal axis. Some of the best-characterized nonsteroid hormones that regulate pituitary function are the activins and the inhibins (Figure 21.5).[221] Inhibin was first described as a proteinaceous substance from the testes that could inhibit FSH secretion.[222] Inhibin purification led to the discovery of activin, a protein that stimulates the release of pituitary FSH.[223,224] In addition, the ovary produces follistatin, a negative regulator of activin and BMP function.[225–227] Inhibin and activin share a common β subunit; activins are ββ dimers (primarily activin A (βA:βA), activin B (βB:βB), and activin AB (βA:βB)), while inhibins are αβ heterodimers (inhibin A (α:βA) or inhibin B (α:βB)). Genetic disruptions in inhibins, activins, and follistatin have demonstrated that these proteins have important autocrine, paracrine, as well as endocrine roles in the reproductive axis.

Inhibins are produced locally in many tissues, including the brain, pituitary, placenta, and adrenal gland,[228–233] but the major site of inhibin production is the gonads.[234–236] Castration of male or female rats results in the loss of circulating inhibin and the subsequent rise in pituitary FSH.[237,238] Serum inhibin is a major factor contributing to the rise in FSH, as demonstrated by the increase in FSH during passive immunization of rats with anti-inhibin antisera.[239–243] Inhibin was also hypothesized to have a paracrine role in the ovary since it was demonstrated that, in vitro, inhibins modulate thecal cell androgen production, follicular growth, and oocyte maturation.[244–248] In general, inhibin functions as an antagonist of activin, as no unique inhibin-binding receptor or signal transduction pathway has ever been conclusively demonstrated (Figure 21.5).[249,250] Experiments have shown that inhibin can block BMP signaling,[251] although inhibin suppression of BMP signaling in the ovary is less well defined than its action as an antagonist of activins.

In female rodents, inhibin A and inhibin B are produced from granulosa cells. In humans, inhibin α is also detected in thecal cells and corpora lutea.[252,253] While very little is understood about the biological differences between inhibin A and B isoforms, in the rat and mouse their production varies with follicle size and cycle stage.[237,254,255] Inhibin B is likely the product of small developing follicles,[256] and in rats, inhibin B is inversely correlated with FSH levels throughout the cycle and

TABLE 21.2 Phenotype Analysis of Female Double Mutants With *Inha*$^{-/-}$

Mouse Model	Female Phenotype	Survival Relative to *Inha*$^{-/-}$	References
Inha$^{-/-}$	Sex cord stromal tumors with cancer cachexia; death by 19 weeks of age		41
Activin signaling			
Inha$^{-/-}$ *Acvr2*$^{-/-}$	Tumors develop, elevated activin, but no evidence of cachexia	Increased	306
Inha$^{-/-}$ *MT-Fst*	Similar tumor development with less cachexia	Increased	492
Inha$^{-/-}$ *Smad3*$^{-/-}$	Delayed tumor development and delayed cachexia	Increased	310
Inha$^{-/-}$ *Smad2*^{cKO} *Amhr2cre*	Tumor development and cachexia similar to *Inha*$^{-/-}$	Unchanged	311
Cell cycle regulators			
Inha$^{-/-}$ *Ccnd2*$^{-/-}$	Slower tumor progression but elevated FSH	Increased	316
Inha$^{-/-}$ *Cdkn1b*$^{-/-}$	Early tumor development in females with increased serum activin and death by 8 weeks of age	Decreased	318
Inha$^{-/-}$ *Rb*^{cKO} *Amhr2cre*	Similar tumor histology, although increased mitosis; slightly increased death	Decreased	493
Estrogen signaling			
Inha$^{-/-}$ *Esr1*$^{-/-}$	Tumor development with a more pronounced cachexia	Decreased	494
Inha$^{-/-}$ *Esr2*$^{-/-}$	Similar to *Inha*$^{-/-}$	Similar	494
Inha$^{-/-}$ *Esr1*$^{-/-}$ *Esr2*$^{-/-}$	Aggressive tumor development and rapid death by 9 weeks	Decreased	494
Gonadotropin signaling			
Inha$^{-/-}$ *Fshb*$^{-/-}$	Slow-growing tumors with suppressed activin and estradiol and little cachexia	Increased	313
Inha$^{-/-}$ *Gnrh*$^{hpg/hpg}$	Pretumor foci without full tumor development	Increased	314
Inha$^{-/-}$ *Lhb*$^{-/-}$	Delayed tumor progression with lower FSH and estradiol	Increased	495
Other growth factors			
Inha$^{-/-}$ *Gdf9*$^{-/-}$	Adult tumor development similar	Unchanged	417

increases in response to FSH stimulation, remaining high through midcycle and declining following the primary gonadotropin surge.[237] In contrast, inhibin A levels are low following the primary gonadotropin surge, but expression is stimulated following the secondary FSH surge and slowly rises during early cycle stages.[237] Inhibin A, unlike inhibin B, correlates with estradiol production and the development of preovulatory follicles.[255] The highly regulated production of inhibin during the estrous cycle suggests that its regulation and function are critical to female fertility. It is not surprising, then, that genetic mutations in all three inhibin subunit genes (*Inha*, *Inhba*, and *Inhbb*)[41,257–259] show reproductive defects. In addition, the creation of an inhibin α (*Inha*) null mouse led to the unexpected discovery of inhibin α as a tumor suppressor gene.[41]

Mouse Models of Inhibin Action

Inha was the first of the three inhibin–activin genes to be knocked out in mice[41] (Table 21.2). Because the inhibin α subunit was targeted, activin (i.e., the βA and βB subunit) expression is left "intact." While mice null for inhibin α are viable during embryonic and prepubertal development, all homozygous mice develop tumors within the ovary and die from a cancerous, cachexia-like wasting syndrome by 12–17 weeks of age (Figure 21.6).[41,300] Tumors are mixed granulosa–Sertoli cell tumors. In adult mice, FSH levels are elevated two- to threefold, and activin expressed in the serum is 20-fold higher in the null mice when compared to wild-type mice. At 4 weeks of age, prior to gross tumor formation, granulosa cells appear abnormal but display hyperplasia. *Inha* null mice are infertile, and when immature mice are superovulated, they produce fewer eggs than wild-type mice.[301] Follicle development is abnormal in prepubertal *Inha* null mice. Ovaries from immature *Inha* null mice show precocious follicle development as seen by the appearance of large, multilayered follicles by 12 days of age.[302] Gonadectomy does not rescue the lethality of the *Inha* mutation because these mice also develop adrenal tumors.[300] Thus, the phenotype of *Inha* null mice is complex, and the contributions of elevated serum FSH and activins have been further analyzed.

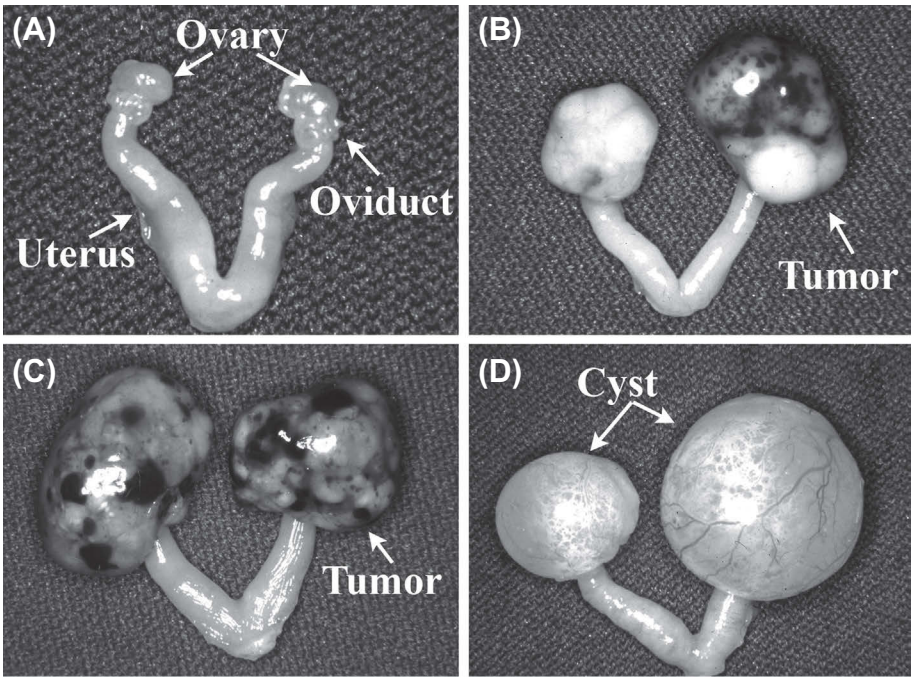

FIGURE 21.6 Gross anatomy of ovarian tumor and cyst formation in several mouse models. *Panel (A)*: Reproductive tract of an adult female mouse. *Panel (B)*: Female *Inha*$^{-/-}$ mice develop ovarian tumors with 100% penetrance. *Panel (C)*: Double-knockout *Inha*$^{-/-}$ *Acvr2*$^{-/-}$ mice develop tumors similar to those with *Inha*$^{-/-}$ despite the lack of activin signaling through ACVR2. *Panel (D)*: Bilateral fluid-filled cysts in a *Gdf9*$^{-/-}$*Bmp15*$^{-/-}$ double-knockout ovary.

Activin is a potent growth factor, and rodents treated in the short term with recombinant activin exhibit a wasting syndrome.[303,304] Thus, it was hypothesized that superphysiologic levels of activin cause cachexia.[300,305,306] Because activin partially signals through the activin type II receptor (ACVR2),[307] knockouts for the *Acvr2* gene (as discussed in this chapter)[257] were crossed to *Inha* mutant mice to generate double homozygotes (*Inha*$^{-/-}$ and *Acvr2*$^{-/-}$).[306] These mice are deficient in activin signaling through ACVR2 as well as FSH synthesis because mice null for *Acvr2* have reduced FSH levels due to the lack of activin signaling in the pituitary.[257] The double-homozygous mutant mice develop gonadal tumors similar to those in *Inha*$^{-/-}$ mice and reinforce the hypothesis that inhibin α is a tumor suppressor gene (Figure 21.6). However, despite elevated peripheral activin levels, double-homozygous mutant mice do not develop the wasting syndrome seen in the *Inha*$^{-/-}$ mice, demonstrating that early death, but not tumor formation, is due to activin signaling. However, another high-affinity receptor for activin, ACVR2B, is expressed in granulosa cells,[308] and it is unclear if additional activin signaling through this receptor also contributes to the tumor phenotype. Knockout mice for *Acvr2b* have been generated, but homozygous mice die shortly after birth due to cardiac defects,[309] leaving questions of additional activin signaling in the ovary currently unanswered. Activin signals through the SMAD transcription factors, SMAD2 and SMAD3, and female mice null for both *Inha* and *Smad3* have delayed tumor development and cancer cachexia.[310] In contrast, conditional loss of SMAD2 has very little effect on tumor development in *Inha*$^{-/-}$ females, indicating that SMAD2 is not required for gonadal tumor development in *Inha* null female mice.[311] The difference in these phenotypes may be due to the known functional redundancy between SMAD2 and SMAD3 in granulosa cells,[312] with SMAD3 transducing the essential signals leading to tumorigenesis.[311]

The contribution of elevated FSH to tumor formation in *Inha*$^{-/-}$ mice has also been examined. Elevated FSH by itself does not cause tumor development but results in the formation of hemorrhagic cysts in the ovaries, as demonstrated by overexpression of FSH in transgenic mice.[313] Eventually, these mice die due to enlarged cystic ovaries and urinary bladder obstructions.[313] Crosses of hypogonadal (*Gnrh*^{hpg}) mice, which lack hypothalamic gonadotropin-releasing hormone 1 (GnRH1), to *Inha* mice to generate *Gnrh1*$^{hpg/hpg}$ *Inha*$^{-/-}$ mice prevent the wasting syndrome, but in females this does not prevent premalignant ovarian tumor foci.[314] Granulosa cells from *Inha* null mice initiate an abnormal gene expression program in response to gonadotropin stimulation.[315] Instead of inducing antral follicle formation and cumulus expansion, exogenous gonadotropin treatment causes excessive proliferation of the granulosa cells, which results in loss of oocytes and the secretion of a large quantity of extracellular matrix.[315] The gene expression pattern in *Inha* null cells includes changes in extracellular matrix and cell adhesion genes, as well as genes encoding steroidogenic enzymes and regulators of the cell cycle.

A clear demonstration that FSH modulates granulosa cell tumor progression, but not tumor formation, was shown by generating *Inha*$^{-/-}$ *Fshb*$^{-/-}$ mice.[313] While these mice live longer than *Inha*$^{-/-}$ females,

double-homozygous female mice die by 39 weeks, in contrast to males, which survived to 1 year. Tumors from these mice grow slower and are less invasive. In addition, they do not display the high levels of activin and estradiol as do the *Inha*$^{-/-}$ single-mutant mice. Double mutants of *Inha* and both α and β forms of the estrogen receptor (or all three genes) in females do not affect tumor formation, indicating that estrogen signaling does not contribute to the tumor pathogenesis.[316] These data demonstrate that while FSH and activin signaling modulate the tumor phenotype, they do not directly contribute to the tumor formation. Additional and as-of-yet undescribed mechanisms for the tumor suppressor function of inhibin in sex cord–stromal tumors must be operating.

Because tumor suppressor genes function through an antiproliferative signal, the cell cycle regulatory genes, p27 and cyclin D2, were examined in the *Inha*$^{-/-}$ background. Cyclins, cyclin-dependent kinases (CDKs), and cyclin-dependent kinase inhibitors (CKIs) regulate cell cycle progression. Overexpression of cyclins and CDKs, or underexpression of CKIs, results in uncontrolled cell proliferation. Cyclin D and p27 are involved in the G1-S phase transition; cyclin D2–CDK promotes S phase commitment, while p27 inhibits it.[317] Cyclin D2 has been shown to be FSH responsive and cyclin D2 knockout mice are infertile due to a lack of granulosa cell proliferation,[262] and cyclin D2 is upregulated in *Inha*$^{-/-}$ mice.[318] Double-knockout mice of cyclin D2 and inhibin α have delayed ovarian tumorigenesis but eventually develop tumors and die by 29 weeks of age, very similar to *Inha*$^{-/-}$ *Fshb*$^{-/-}$ mice.[313,316] However, prior to advanced tumor stages, in the absence of inhibin, more advanced preantral follicles are seen than in the cyclin D2 knockout alone.[316] p27 is a CKI that, when mutated in mice, results in increased body size, hyperplasia of multiple organs, cancer, and infertility in female mice due to granulosa cell defects.[319–321] Double-homozygous mutants of p27 and inhibin α have an enhanced *Inha*$^{-/-}$ phenotype and die by 8 weeks of age.[318] It is clear from these studies that while modifiers of the *Inha*$^{-/-}$ phenotype have been discovered, the tumor-suppressing activity of inhibin is still largely a mystery.

Inhibin Mutations and Human Disease

Genetic mutations in the inhibin α gene associate with primary ovarian insufficiency (POI), also known as premature ovarian failure (POF). POI is usually without known cause (idiopathic) and is characterized by amenorrhea, hypoestrogenism, and elevated levels of gonadotropins in women under the age of 40. Because high levels of serum gonadotropins might indicate a disruption in the negative feedback from the gonads, inhibin represents a good candidate gene. Several studies of POI patients have identified mutations in the inhibin α gene. In one study of 43 POF patients from New Zealand and Slovenia, three women showed an G→A missense mutation at nucleotide 769, which would cause an alanine-to-threonine amino acid substitution in the coding region of inhibin α; only one out of 150 control subjects were found to carry this same mutation.[322] In a second study of a cohort of Italian women with ovarian failure, a significant increase was seen in the number of females with this same mutation in both POI (7/157) and primary-amenorrhea (3/12) but not in control (0/100) and early-menopause (0/36) patients.[323] This mutation has subsequently been verified in populations from India, Korea, and Argentina, but is not significantly different from the control frequency in a German cohort or a subsequent POI population from Italy.[324] Functional analysis showed that the mutation did not interfere with dimeric inhibin production, but instead expression of the mutant INHA subunit reduced the bioactivity of inhibin B compared to that of the wild type.[325] Additional studies have looked for links between inhibin and female reproductive disease, but currently no other mutations in inhibin α have been described. Upregulated inhibin A levels have been used as a marker for granulosa cell tumors and mucinous carcinomas,[326–330] but unlike the mouse model, loss of inhibin α has not been associated with granulosa cell tumors. This has led to the suggestion that granulosa cell tumors may acquire a resistance to the actions of inhibin.[301] In contrast, the absence of inhibin α expression and a loss of heterozygosity are associated with some forms of prostate cancer.[331,332]

PCOS is another ovarian pathology with many potential etiologies. PCOS patients show infertility, disrupted menstrual cycles, hyperandrogenism, hyperinsulinemia, and high levels of serum LH. Superficially, the phenotype of constitutive overexpression of inhibin α in mice resembles PCOS, but cyst morphology suggests that the mouse model and the human disease are different pathologies.[333] In addition, while some studies have shown higher levels of inhibin in cystic follicles of PCOS patients, other studies have not supported this.[334–338] Clearly, the etiology of PCOS is complex and multifactorial, and multiple perturbations in the reproductive axis are present. A genome-wide association study (GWAS) among Han Chinese women with PCOS identified eight highly significant loci.[339] Among these are three novel loci that contain LH–choriogonadotropin receptor (*LHCGR*), thyroid adenoma-associated gene (*THADA*), and differentially expressed in normal and neoplastic cells (DENN)–MAPK activating death domain (MADD) containing 1A (*DENND1A*). *THADA* and *DENND1A* loci associations have been replicated in some of the European cohorts.[340,341] Little is known about the roles of *THADA* and *DENND1A* in ovarian biology, and GWAS studies may have identified novel pathways relevant to

ovarian biology. Whether these particular loci or neighboring genes are the true causes of PCOS remains to be functionally proven.

Activins

Activins are dimers of the inhibin β subunit. There are four isoforms of the mammalian β subunit: β_A, β_B, β_C, and β_E. Biological activity of activin A (β_A:β_A) and activin B (β_B:β_B) homodimers has been well described,[342–344] but less is known about activin AB (β_A:β_B) heterodimers.[345] The β_C and β_D isoforms are liver specific, but knockout mice show no apparent defects, and therefore their biological significance is unclear.[346,347] Activins signal through a heteromeric assembly of membrane-bound serine–threonine kinase receptors that include a type II ligand-binding receptor and a type I signaling receptor.[307] Two type II receptors named ACVR2 and ACVR2B are the predominant activin-binding receptors.[41,348,349] The type II receptors complex with one of two type I receptors called activin receptor-like kinases (ALKs), primarily through ALK4 but also with ALK7,[350–352] which results in the phosphorylation and activation of the transcription factors SMAD2 and SMAD3.[353] SMAD2 and SMAD3 then mediate gene transcription with interactions with transcriptional co-activators and co-repressors.[354]

The roles of activin in the reproductive axis are diverse. A local feedback mechanism operates in the pituitary to activate synthesis and release of FSH.[355–359] Within the ovary, activin has been proposed to act on multiple cell compartments. Activin subunits are expressed in granulosa cells of rats, mice, and humans.[234,360–362] In vitro, activin induces granulosa cell proliferation and FSH receptor expression in immature follicles, and increases FSH-dependent aromatase activity.[166,363–366] In more mature follicles, activin can inhibit terminal differentiation by inhibiting premature luteinization through suppression of progesterone production.[366–368] In thecal cells, activin opposes inhibin and decreases androgen production.[244,369] Oocytes also respond to activin, and activin can promote oocyte maturation in primates and humans.[247,370] Thus, the potential paracrine effects of activin in the ovary are wide-ranging.

To understand activin's effects in vivo, knockout mice for activin β_A (*Inhba*) and β_B (*Inhbb*) were generated.[257,258] Mice homozygous for β_B are viable and give birth to live pups but are unable to nurse their offspring due to defects in milk production.[258] In contrast, *Inhba* null mice die shortly after birth with craniofacial defects, including cleft palate; loss of whiskers, upper and lower incisors, and lower molars; and defects in the retina.[257,371–373] The perinatal lethality is attributed to the inability of homozygous pups to suckle. The perinatal lethality and craniofacial defects can be rescued by "knocking in" the activin β_B subunit gene into the β_A locus, suggesting that activin A and activin B have redundant functions in craniofacial development.[259] Conditional deletion of the activin βA subunit in granulosa cells results in subfertility and the development of follicles containing multiple oocytes.[83] When the conditional deletion of activin βA is generated in mice that are homozygous null for the activin βB subunit, infertility results.[83] The most striking defect in activin-deficient ovaries is the progressive accumulation of corpora lutea, which causes a dramatic increase in overall size and a significant increase in serum progesterone levels. Corpora lutea in activin-deficient ovaries are unable to undergo structural regression, although the role of activins in luteolysis is not fully characterized. Connective tissue growth factor (*Ctgf*), a matricellular protein belonging to the CCN family with roles in cell adhesion, migration, proliferation, and angiogenesis,[374] is downregulated in activin-deficient ovaries,[83] and mice deficient in *Ctgf* show a similar, although milder, phenotype.[375,376] An accumulation of CLs is also seen in mice conditionally null in granulosa cells for *Tsc1*,[377] a negative regulator of mTORC1, and in granulosa cells deficient in *Pten*. How these pathways intersect to control corpus luteum regression is not known. However, unlike the activin-deficient mouse model, the *Tsc1* cKO and the *Pten* cKO are fertile, with enhanced ovulation and no changes in serum progesterone.[378] Because the activin-deficient ovary secretes high levels of progesterone but is also defective in structural regression of CLs, this indicates that the functional (endocrine) activity of CLs can be uncoupled from the structural luteolysis, and that activin signaling may lie upstream of the PI3K pathway during this process.

A null allele for one of the activin receptors, ACVR2, has also been generated.[257] Twenty percent of the homozygous null mice die perinatally with primary defects in the mandible and secondary defects in the palate. Surviving female homozygous *Acvr2* null mice display disrupted pituitary function related to the suppression of FSH. Ovaries from the surviving homozygous null females are small, with no corpora lutea and minimal antral follicle development. Transplants of $Acvr2^{-/-}$ ovaries to ovariectomized immunocompatible donor females showed normal follicular development and restored fertility to the host mice.[296] An additional activin receptor, ACVR2B, binds activin with a higher affinity than ACVR2,[349] and this receptor is expressed in mammalian ovaries.[308,361,379] The contribution of ACVR2B to activin signaling in the ovary remains to be explored since these null mice mostly die.

Activin signaling, like TGFβ signaling, results in the phosphorylation and activation of SMAD2 and SMAD3.[380,381] Null alleles for many of the Smad genes have been generated, but given the significance of TGFβ–activin- and BMP-signaling pathways in the formation of embryonic and extraembryonic tissues,

many of the knockouts are embryonic lethal. *Smad2* null embryos fail to gastrulate, are defective in mesoderm formation, display anteroposterior axis defects, and die at E6.5 through E8.5. Mice with congenital deficiency of *Smad3* are viable[382,383] and show reduced fertility.[384,385] Single conditional knockout of either *Smad2* or *Smad3* has no effect on female fertility,[312] but double conditional knockouts in granulosa cells of the ovary (*Smad2–Smad3* double knockout) are infertile by 5 months of age.[312] The phenotype of *Smad2–Smad3* double conditional knockout is very similar to that of the granulosa cell conditional knockout of *Smad4*, the common SMAD.[386] Both of these mouse models have ovaries with decreased numbers of antral follicles, oocytes trapped within luteinizing follicles, reduced ovulation, and impaired cumulus expansion. The cumulus expansion defects are likely attributable to loss of GDF9 signaling from the oocyte to the cumulus cells, as this is the pathway that GDF9 utilizes.[387,388] In addition, it has been shown that oocyte-secreted factors are required to specify the cumulus cell lineage and prevent premature luteinization of granulosa cells,[389] and these data support a role for GDF9 signaling through SMAD2/3 as a primary mediator of this activity. As TGFβ, activin, and GDF9 transmit signals through SMAD2 and SMAD3, the challenge will be to understand the molecular output of each of these ligands in normal ovarian physiology at the different stages of follicle development and how this is altered in the pathological state.

Follistatin

Follistatin was first identified from gonadal extracts as an FSH-modulatory factor.[226,390,391] Follistatin is a single polypeptide protein that is not structurally related to the TGFβ superfamily but acts as a binding protein and functional antagonist of activin.[379,392,393] In the ovary, granulosa cells and luteal cells are the main sites of production,[362,394,395] but this is stage dependent. Follistatin is not expressed in primordial and primary follicles, but is expressed from antral stages.[362,395]

Both loss-of-function and gain-of-function mutations have been generated for the follistatin gene.[396,397] Follistatin null mice die within hours after birth; however, these mice are a resource for understanding prenatal ovarian development since follistatin activity is required for embryonic ovarian development.[264] *Fst* null ovaries, similar to *Wnt4* null XX gonads, lose 90% of their germ cells at E16.5 and have almost none by birth. When both *Inhbb* and *Fst* are deleted together, normal ovary development is restored.[398] Follistatin is also known to block BMP signaling, and BMP2 is expressed in the developing ovary, although its role has not been fully established.[399] Conditional deletion of follistatin in the granulosa cells of postnatal mice results in fertility defects that include reduced litter size and number, ovulation and fertilization defects, and elevated levels of FSH and LH with reduced levels of testosterone.[265] In addition, the ovaries contain Sertoli-like structures also seen in inhibin α null mice, ERα and ERβ knockouts, and FSHβ, follistatin, and AMH transgenic mice.[41,397,400,401] Granulosa cell function appears generally disrupted, possibly because of unrestricted activity of activin. In humans, it is known that serum generally contains high levels of follistatin-bound, and therefore inactive, activin,[255] and follicular fluid shows high levels of unbound follistatin.[402] Disruptions in the balance of follistatin to activin would lead to more activin signaling, and potentially the disease state seen in follistatin-null ovaries. Transgenic mice that overexpress follistatin are viable but show reproductive defects.[397] In transgenic lines with the highest expression of follistatin, females were infertile. In the transgenic lines with less follistatin expression, females developed infertility within several months. Histologically, ovaries appeared similar to FSHβ and *Acvr2* null mice, with a follicle block prior to the antral stage and an increase in follicular atresia. The serum profiles confirmed that FSH was suppressed, and estradiol was undetectable. It is unclear whether all of the ovarian pathologies can be attributed to diminished pituitary function or whether there is a local abrogation of activin (and other TGFβ family) signaling in the ovary as well (Figure 21.5).

BMP Signaling SMADs

The BMPs signal through the BMP type I receptors, ACVRL1 (ALK1), ACVR1A (ALK2), BMPR1A (ALK3), and BMPR1B (ALK6), which phosphorylate SMAD1, SMAD5, and SMAD8. Mice conditionally null for these SMADs have been generated using *Amhr2cre* transgenic mice.[403] Similar to single deletions of *Smad2* or *Smad3*, single deletion of *Smad1* or *Smad5* has no effect on female fecundity. In addition, *Smad8* homozygous null mice are viable and fertile.[403,404] Generating a conditional *Smad1* or *Smad5* deletion in the *Smad8*$^{-/-}$ background does not affect the ability of female mice to reproduce. However, a combined deletion of *Smad1* and *Smad5* in granulosa cells initially results in a reduction in litter sizes and infertility at 4–6 months of age. The infertility at this age is likely caused by the development of granulosa cell tumors, which occurs in 100% of the *Smad1/Smad5* double-knockout mice. Conditional deletion of the BMP receptors, *Bmpr1a* and *Bmpr1b*, also causes granulosa cell tumor formation, although at later ages than with the *Smad1/Smad5* double knockout.[405] Granulosa cell tumors are identifiable histologically in ovaries in the *Smad1/Smad5* double conditional knockouts by 8 weeks of age, and by 8 months of age, peritoneal implants are found

in a majority of knockouts. The endocrine profile as well as tumor histology indicate that the *Smad1/Smad5* double-knockout tumors are similar to the juvenile form of granulosa cell tumors (JGCT).[406] The etiology of human JGCTs is unknown and, unlike the adult form (AGCT), does not contain a *FOXL2* C134W mutation. However, aggressive forms of JGCT are associated with decreased or absent levels of *FOXL2*.[407] *Smad1/Smad5* double-knockout tumors also show a significant reduction in levels of *Foxl2*, and genes differentially expressed in the *Smad1/Smad5* double-knockout tumors are enriched for FOXL2-binding sites within their promoters.[408] These data suggest that FOXL2 could play a key role in the pathogenesis of both forms of granulosa cell tumors.

Oocyte-specific TGFβ Members

Oocyte–granulosa cell interactions are paramount for the growth of oocytes and proliferation and differentiation of granulosa cells.[9] Currently, oocytes are ascribed a dominant role in directing folliculogenesis.[409,410] The hypothesis that oocytes control the rate of follicular development was tested by isolating oocytes from the secondary follicles of 12-day-old mice and combining these oocytes with the somatic cells isolated from newborn mouse ovaries. When these re-aggregates were implanted beneath the renal capsules of ovariectomized host females, antral follicles were visible in 9 days, indicating that the rate of follicular development was accelerated by almost twofold.[410] As a control, oocytes from primordial follicles of newborn mice were reaggregated with the same-age somatic cells from newborn mice, and after 9 days, only secondary follicles were observed with an absence of antral follicles. The granulosa cells from the reaggregated ovaries underwent cumulus expansion when exposed to FSH in vitro, and recovered oocytes were competent to undergo fertilization and embryonic development. These experiments indicate that a 12-day oocyte is able to accelerate somatic cell differentiation and proliferation with resulting accelerated production of antral follicles. Cumulus cells are directly coupled to the oocyte via gap junctions[411,412] and provide nutrient support for the oocytes, which are deficient in some cellular processes such as amino acids uptake, glycolysis, and cholesterol metabolism.[413] Oocyte–granulosa cell communication requires that oocytes secrete factors that have an autocrine or paracrine function, and/or express receptors on their cell surface for detecting granulosa cell–derived paracrine factors. GDF9 and BMP play dominant roles in mediating this interaction.

Growth Differentiation Factor 9

GDF9 is an oocyte-specific member of the TGFβ superfamily.[414] In mice, *Gdf9* is preferentially expressed in oocytes within the ovary as early Type 2 and Type 3a follicles, and its transcripts are found abundantly in unfertilized eggs.[414,415] *Gdf9* null mice form primary follicles with large oocytes that are greater than 70 μm in diameter and that are surrounded by a single layer of cuboidal granulosa cells (Figure 21.3).[254,266,416] Fully grown oocytes isolated from *Gdf9* null mice can progress to advanced stages of differentiation that are equivalent to sizes found in antral follicles of control mice.[416] In vitro maturation of oocytes from *Gdf9* null mice revealed that most of the oocytes are capable of resuming meiosis. Among the characteristic ultrastructural features of oocytes from *Gdf9* null mice are perinuclear organelle aggregation, unusual peripheral Golgi complexes, and a failure to form cortical granules.[416]

Gdf9 null mice showed convincingly for the first time that factors secreted by oocytes are critical for granulosa cell proliferation and follicular progression. The knockout model also demonstrated that GDF9 is not critical in the transition from primordial to primary follicles but plays a crucial role in the growth of follicles beyond the primary follicular stage. Molecular analysis of the *Gdf9* knockout revealed a lack of thecal cell markers (17α-hydroxylase, LH receptor, and KIT).[254] While thecal cell *Kit* expression is absent in these mice, *Kit* expression in the oocyte is unchanged. *Kitl* and inhibin α levels are upregulated in follicles that lack GDF9, indicating that GDF9 may negatively regulate these growth factors. In vitro studies corroborate the suppression of *Kitl* expression in granulosa cells of preantral and mural granulosa cells by recombinant GDF9.[154] The increase in oocyte size in the *Gdf9* null ovaries has been attributed to the upregulation of KITL leading to greater signaling through oocyte-expressed KIT.

A direct role for inhibin in the pathogenesis of the *Gdf9* knockout phenotype has also been described. Granulosa cells of arrested *Gdf9*$^{-/-}$ primary follicles expressed abnormally high levels of inhibin α.[254] *Inha* is also abnormally expressed in steroidogenic follicular nests and in follicles containing degenerating oocytes. To determine if the elevated inhibin levels were partly responsible for the nonproliferative state of granulosa cells in *Gdf9* null ovaries, crosses of *Inha* mutant mice to *Gdf9* mutant mice were generated. Unlike *Gdf9*$^{-/-}$ ovaries, double-mutant *Inha*$^{-/-}$ *Gdf9*$^{-/-}$ follicles become multilaminar and acquire a morphologically distinct thecal cell layer (Figure 21.3).[417] Thus, by decreasing inhibin α expression, granulosa cell proliferation and thecal cell recruitment were recovered. The increased proliferation of granulosa cells is not due to increases in FSH, because serum levels of FSH are lower in prepubertal *Inha*$^{-/-}$ *Gdf9*$^{-/-}$ mice compared to *Gdf9*$^{-/-}$ ovaries, and are similar to WT levels.[418] Serum levels of LH have not been measured in *Inha*$^{-/-}$ *Gdf9*$^{-/-}$ mice.[417]

Even though thecal cells develop in *Inha*$^{-/-}$ *Gdf9*$^{-/-}$ ovaries, they appear to be nonfunctional and do not

express markers associated with their differentiation, including LH receptor, *Kit*, and the steroidogenic enzyme 17alpha-hydroxylase (*Cyp17*). One possibility is that the elimination of inhibin α in double-null ovaries relieves the repression of granulosa cell growth and thecal cell recruitment by allowing the formation of activin dimers. Because activins are known regulators of preantral follicle growth and differentiation and have stage-specific effects on different classes of follicles, activins (or BMPs) may promote granulosa cell proliferation in the small preantral follicles.[363,364,419] However, follicles in *Inha*$^{-/-}$/*Gdf9*$^{-/-}$ double knockouts arrest again at the 5b stage, and thus additional proteins, including GDF9, may be necessary again at this stage for subsequent development. Because the inhibins and activins are secreted ligands and are functional antagonists, it is likely that their production as well as access to the cell signaling machinery are under strict molecular regulation.

Because *Gdf9* knockout ovaries arrest very early in postnatal folliculogenesis, studies looking for effects of GDF9 at later stages have had to rely on in vitro studies. These have shown that GDF9 signaling is critical in antral follicle growth and ovulation.[420–423] During ovulation, GDF9 induces cumulus cell expansion, an inflammation-like process that involves the mucification of the cells surrounding the oocyte. This process binds the cumulus cells to the oocyte, protects the oocyte during follicular extrusion, and assists in sperm binding and fertilization. Cumulus cell expansion partly involves the production of hyaluronic acid (HA), prostaglandin E2 (PGE2), pentraxin 3 (PTX3), and tumor necrosis factor alpha induced protein 6 (TNFαIP6),[283,284,424] all of which have been demonstrated to be regulated by GDF9 in vitro.[283,423,425] The role of GDF9 in preovulatory granulosa cells has been studied by isolating mural granulosa cells from large antral follicles, culturing them with and without recombinant GDF9, and comparing gene expression using oligonucleotide array hybridization with microarray chips. Genes noted to be highly induced in granulosa cells treated with recombinant GDF9 include *Ptx3*, *Tnfip6*, and gremlin.[283,426]

Ptx3 knockout female mice are subfertile and have defects in ovulation.[283,427] Superovulated *Ptx3* null mice release fewer oocytes than controls. Surprisingly, many oocytes isolated from *Ptx3* null mice were denuded of cumulus granulosa cells, whereas oocytes from control mice retained their cumulus cells. Ovulation induction followed by fertilization revealed that only 1.3% of oocytes fertilized in *Ptx3* knockout, while 55% fertilized in heterozygous controls. It is therefore likely that disruption in the cumulus mass surrounding the oocytes causes reduced fertilization and the subfertility in *Ptx3* knockout mice.

The importance of cumulus cell expansion in fertility has also been demonstrated by the knockout mouse model for tumor necrosis factor alpha induced protein 6 (*Tnfaip6*).[284] Female *Tnfaip6*$^{-/-}$ mice are infertile.[284] The cumulus cell complexes from these mice fail to expand due to the lack of hyaluronan and heavy-chain inter-α-trypsin inhibitor family complexes. Fertility can be restored by treating cumulus cell–oocyte complexes in vitro with recombinant TNFαIP6, which has been shown to catalyze the transfer of heavy chains to hyaluronan in cell-free systems.[284] Thus, knockout mice for two of the genes demonstrated to be downstream of GDF9 signaling, *Ptx3* and *Tnfaip6*, have severe defects in cumulus expansion that render the mice sterile.

The signaling receptor complex for GDF9 has not been fully elucidated. Blocking signaling from the TGFβ type I receptor (TGFBR1, also called ALK5) or the BMP type II receptor (BMPR2) inhibits GDF9 activity in mouse or rat granulosa cells.[428–430] However, mice with a conditional null mutation in *Tgfbr1* in the reproductive tract, while sterile, have minimal ovarian defects.[431] Cumulus cells from *Tgfbr1* conditional knockout mice undergo cumulus expansion in vitro and in vivo, and show normal regulation of GDF9 target genes. In addition, careful analysis shows that *Tgfbr1* expression in the mouse ovary is predominantly localized to thecal cells, corpora lutea, and oocytes, with minimal to background expression in granulosa cells.[431] These data suggest that GDF9 may signal via additional receptors. Small molecule inhibitors that block ALK4, ALK5, and ALK7 signaling also inhibit GDF9-induced phosphorylation of SMAD2/3 and gene expression.[428,429] Therefore, ALK4 and/or ALK7 may be the physiologic receptors for GDF9 in granulosa cells (Figure 21.5).

Mice lacking ALK4 die prior to gastrulation of embryos,[432] and the role of ALK4 in granulosa cells has not been determined. However, *Alk7* knockout mice are viable.[433] In the ovary, *Alk7* is expressed in steroidogenic factor1 (*SF1*, also called *Nr5a1*)–positive granulosa cells.[434] *Alk7* is also expressed in gonadotropes of the pituitary as well as some neurons of the neuroendocrine hypothalamus.[434] Female mice null for *Alk7* (*Alk7*$^{-/-}$) have a delayed reproductive cycle, although the phenotype is complicated due to the expression pattern in multiple reproductive tissues. However, there are more degenerating follicles in juvenile *Alk7*$^{-/-}$ ovaries, at a time when follicle growth is thought to be mainly independent of pituitary gonadotropins. In addition, superovulating juvenile mice with exogenous gonadotrophins produce fewer cumulus–oocyte complexes in *Alk7*$^{-/-}$ females than their wild-type controls, indicating an impaired ovulatory response. These data suggest that there may be defects in intraovarian signaling due to loss of ALK7. However, neither *Alk7*$^{-/-}$ nor *Alk5* cKO phenocopies *Gdf9* knockout ovaries, indicating either redundancy between receptors or that *Alk4* may be the predominant signaling receptor for GDF9 in mice.

TABLE 21.3 Sheep Mutations Causing Ovarian Defects

Mutation (Strain)	Gene	Type of Mutation	Region of Mutation	Possible Mechanism of Disruption
$FecX^{I}$ (Inverdale)	*BMP15*	Substitution D→V	Mature	Protein dimerization[17]
$FecX^{H}$ (Hanna)	*BMP15*	Substitution (premature stop)	Mature	Truncated protein product[17]
$FecX^{B}$	*BMP15*	Substitution S→I		Residue may be involved in type II receptor binding[18]
$FecX^{L}$	*BMP15*	Substitution C→Y		Prevents protein processing and disrupts secretion of precursor and mature[19]
$FecX^{G}$	*BMP15*	Substitution (premature stop)	Pre-pro	Premature termination in pre-pro region[18]
$FecG^{H}$	*GDF9*	Substitution S→F	Mature	Possibly interrupts type I receptor binding[18]
FecB (Booroola)	*BMPR1B*	Substitution Q→R	Kinase domain	Kinase activity[20]

There are species-specific differences in the signaling of recombinant GDF9. Mouse GDF9 is secreted in an active form, while human GDF9 is not.[387] Human GDF9 is secreted in the latent form in association with its pro-domain, and removal of the pro-domain will activate it.[435] Site-directed mutagenesis that introduces mouse residues into the pro-domains and mature domains of human GDF9 identified Gly^{391} as the residue that confers latency to human GDF9.[435] This residue lies in the mature domain of GDF9, which is 90% identical in mouse and human GDF9. Gly^{391} is found in all species except mouse, rat, hamster, galago, and possum, where it is found as an arginine.[435] This amino acid is located in the prehelix loop that forms the predicted type I receptor-binding pocket.[436,437] Arg at this residue increases affinity of GDF9 for its receptor.[435] Regulation of active and latent forms of GDF9 may contribute to the differences seen in follicle development and ovulation rates across species. Additionally, both mouse and human GDF9 form heterodimers with BMP15, and the mouse GDF9:BMP15 heterodimer has greater biopotency than homodimeric GDF9[438] (see the section Bone Morphogenetic Protein 15 (*Bmp15*)).

Bone Morphogenetic Protein 15 (Bmp15)

BMPs form the largest subgroup of the TGFβ superfamily of growth and differentiation factors,[297] and BMP15 was cloned using degenerative oligonucleotides to identify novel members of the BMP family.[439] BMP15 is expressed in the ovary, and by in situ hybridization, *Bmp15* appears to be exclusive to oocytes as early as Type 3a but also within oocytes of more advanced follicular stages.[415,439] Mouse and human BMP15 share 76% amino acid identity, and both map to the X chromosome.

Bmp15 null female mice are subfertile on certain genetic backgrounds and have few ovarian histopathologies.[260] Rather, these mice have defects in ovulation and fertilization rates. Because GDF9 and BMP15 expression patterns overlap, it was hypothesized that GDF9 and BMP15 have redundant functions or, alternatively, may function as heterodimers.[260] To address these questions, double-mutant mice were generated. $Bmp15^{-/-}$ $Gdf9^{+/-}$ ovaries have more defects than $Bmp15^{-/-}$ mutants alone, and double-homozygous $Bmp15^{-/-}$ $Gdf9^{-/-}$ resemble $Gdf9^{-/-}$ mutants and display oocyte loss and large fluid-filled cysts (Figure 21.6). Subsequently, it was shown that homodimeric mouse BMP15 is essentially inactive during in vitro assays that assess the process of cumulus expansion.[438] In contrast, heterodimers of mouse GDF9 with mouse BMP15 are 10- to 30-fold more potent than homodimeric mouse GDF9.[438] Thus, the phenotype of $Bmp15^{-/-}$ $Gdf9^{+/-}$ is likely due to loss of the GDF9:BMP15 heterodimer, as well as a decrease in homodimeric GDF9.[438]

In contrast to mouse, BMP15 is important for ovarian function in sheep. This was initially discovered in the study of Inverdale and Hanna sheep. Naturally occurring X-linked mutations cause increased ovulation in the heterozygote animals but primary ovarian failure in homozygote animals[440,441] (Table 21.3). Linkage mapping of the Inverdale mutation was used to identify *BMP15* as one of the candidate genes. *BMP15* gene sequence comparison between Inverdale and wild-type sheep identified a substitution of a highly conserved hydrophobic valine residue of the mature protein with aspartic acid.[17] Mutational analysis of the Hanna sheep identified a nonsense mutation in the *BMP15* coding region.[17,450] Ovarian follicles in sheep homozygous for the *BMP15* mutations do not normally grow beyond the primary follicle stage.[450–452] The ovarian pathology in the *BMP15* sheep mutants resembles the ovarian pathology observed in *Gdf9* null mice. Based on the data that in humans and mice the GDF9:BMP15 heterodimer is the most bioactive form and that only one homodimer is active (i.e., GDF9 in mice and BMP15 in humans),[438] the phenotypes of mutant sheep likely reflect loss of the GDF9:BMP15 heterodimer or the BMP15 homodimer. The differences in use of a GDF9 homodimer (in mice) versus a BMP15 homodimer (in sheep or human) may reflect a fundamental biological difference that remains to be characterized between polyovulatory (i.e., mice and rats) and monoovulatory animals (i.e., sheep, humans, and primates).

Mutations in human *BMP15* mutations have been discovered in a family that had ovarian dysgenesis (ovaries characterized by a lack of germ cells).[453] Two female siblings of an Italian family had primary amenorrhea, ovaries that lacked follicles, a normal 46 XX karyotype, and no evidence of autoimmune disease or consanguinity. A search for candidate genes revealed a mutation in the human *BMP15* gene that involved an A-to-G transition at nucleotide 704, changing a tyrosine at position 235 to a cysteine. The tyrosine residue is highly conserved in human, sheep, mouse, rat, and pig and is located in exon 2 in the propeptide region of the protein. The mutant Y235C protein is unable to stimulate incorporation of ^{3}H-thymidine into human granulosa cells, and it also inhibits wild-type BMP15's ability to stimulate ^{3}H-thymidine incorporation into granulosa cells. In addition, human granulosa cell proliferation is enhanced in the presence of wild-type BMP15, but is unaffected by mutant protein.[453] These data support a hypothesis that secretion of unprocessed monomer or dimeric mutant products causes dominant negative interactions with the wild-type protein, thereby resulting in an early block in folliculogenesis. In addition, it is unclear whether the Y235C mutation affects human BMP15 interactions with GDF9. Polymorphisms in human *BMP15* have also been associated with higher response to ovarian stimulation[454] as well as age at menopause.[455] Variants of BMP15 are also found in patients with premature ovarian failure.[456,457]

BMP Receptor Type IB Receptor (*Bmpr1b* and *Alk6*)

Receptor complexes for the TGFβ family members consist of two closely related transmembrane serine–threonine kinases, the type I and type II receptors. Seven type I and five type II receptors have been identified in mammals. Upon binding the dimeric ligands, the receptor complexes phosphorylate intracellular signaling molecules called SMADs, which subsequently accumulate to the nucleus and regulate transcription of target genes.[297,458,459] Studies on BMP15 signaling using rat primary granulosa cells from early antral follicles and a human granulosa cell line COV434 indicate that BMP15 binds with the highest affinity to ALK6 receptors on the surface of the granulosa cells.[460] Modeled after signaling by other BMP ligands, it has been proposed that the BMP15–ALK6 complex then recruits the type II receptor BMPR2, resulting in signal transduction (SMAD1/5/8 phosphorylation).[460] Human BMP15 also signals using the type I BMP receptor, *BMPR1B* (also called *ALK6*).[461] Consistent with these studies, purified recombinant BMP15 activates the SMAD1/5/8-signaling pathway and regulates cumulus expansion genes (*Ptx3*, *Has2*, *Tnfaip6*, and *Ptgs2*), inhibitor SMADs (*Smad6* and *Smad7*), and BMP antagonists (*Grem1* and *Fst*).[462] BMPR1B is also required for signaling by mouse and human GDF9:BMP15 heterodimers.[438]

The Q249R (glutamine→arginine) mutation in the regulatory serine–threonine kinase domain of the ALK6 (also called BMPR1B) receptor was identified in Booroola ewes and causes increased fertility[20,450,463] (Table 21.3). Females with one copy of this mutation have an ovulation rate of three or four, while those with two copies have an ovulation rate of between five and 14. Animals that carry both the Inverdale and Booroola mutation (i.e., the *BMP15* and *ALK6* mutation, respectively) have greater fertility, further corroborating an interaction between BMP15 and this receptor.[450] BMPR1B is expressed in primordial follicles, and careful examination of ovaries in BMPR1B mutant sheep suggests that BMPR1B affects follicle development from the onset of follicular formation.[464]

A mouse knockout of *Alk6* has been generated, and these mice show infertility, with effects both in the ovary and in the endometrium.[261] In mice, ALK6 is expressed in oocytes of maturing (Type 6) follicles and in oocytes and granulosa cells of antral follicles. No *Alk6* transcripts are detected in granulosa cells of resting, primordial, developing (Type 1–5b), or atretic follicles, corporal lutea, or theca, or in the uterine endometrium.[261] However, histological and transmission electron microscopy studies of *Alk6* null ovaries demonstrate no obvious differences between null and wild-type ovaries. Similar to *Bmp15* knockout mice, *Alk6* knockout mice show defects in cumulus expansion and fertilization in vivo. The mechanism for defective cumulus expansion is not clear, but neither FSH receptor mRNA levels nor prostaglandin E2 receptor mRNA levels are affected. Paradoxically, cyclooxygenase 2 (*Ptgs2*) mRNA levels are elevated in *Alk6* null mice, and this observation has not been explained. Just like the BMP15 ligand, the *Alk6* receptor knockout in mice has a phenotype quite different from that observed in sheep. However, it has not been fully demonstrated what type of mutation (inactivating or activating) the Booroola mutation represents and how changes in the receptor results in altered follicle development and ovulation.

TRANSCRIPTION FACTORS IN PREANTRAL-STAGE FOLLICULOGENESIS

TAF4B RNA Polymerase II and TATA Box Binding Protein (TBP)-Associated Factor (*Taf4b*)

A transcription factor that has been shown to have a critical role in later stages of folliculogenesis is TAF4B (also known as $TAF_{II}105$). TAF4B is a cell type–specific subunit of the core promoter transcription factor,

TFIID.[295] TFIID contains TBP (the TATA-binding protein) and several TAFs (TBP-associated factors).[465] TAF4B is preferentially and highly expressed in germ cells at E14.5 and postnatal day 2 (PD2).[466] After PD2, its expression in germ cells continues until PD25, when it starts to diminish. TAF4B is also expressed in granulosa cells of the adult ovary.[467] The *Taf4b*$^{-/-}$ ovaries are very small and lack functional follicles in the adult mice. *Taf4b*$^{-/-}$ ovaries have a reduced number of oocytes at PD3 and an increased proportion of atretic follicles. *Taf4b*$^{-/-}$ ovaries have fewer primordial follicles as compared with wild type by PD8.[466] In addition, *Taf4b*$^{-/-}$ mice show a premature reproductive senescence.[468] Induced ovulation in *Taf4b*$^{-/-}$ mice results in the release of abnormal oocytes surrounded by an abnormally low number of cumulus cells. Proliferation of granulosa cells is reduced, and the rates of germinal vesicle breakdown (GVBD) and polar body extrusion are reduced in mutant mice. Few oocytes are fertilized, and embryo development does not proceed beyond the two-cell stage.[466] Thus, TAF4B is essential for follicle formation, follicular development, and fertilization. Importantly, the AKT–FOXO3-signaling pathway is downregulated in *Taf4b*$^{-/-}$ adult ovaries.[468] Conditional deletion of *Taf4b* specifically in germ cells and granulosa cells will help to determine the separate contributions of *Taf4b*-deficient soma or germline to ovarian failure.

TATA-Binding Protein 2 (*Tbp2*)

TBP2 (also known as TBPL2 or TRF3) is a germ cell–specific transcription factor that binds to the TATA box of promoters, interacts with transcription factors IIA and IIB (TFIIA and TFIIB), and mediates RNA polymerase II transcription initiation in vitro. In the ovary, the expression of TBP2 has been detected exclusively in the oocytes throughout folliculogenesis.[469] *Tbp2*$^{-/-}$ mice are infertile due to a defect in folliculogenesis. Adult *Tbp2*$^{-/-}$ ovaries have an increased number of primordial and primary follicles, but a diminished number of secondary follicles, the majority of which are morphologically abnormal. In addition, a gene expression profiling study revealed downregulation of *Gdf9*, *Bmp15*, and *Zp3* and upregulation of *Oct4* and *Nobox* in *Tbp2*$^{-/-}$ ovaries.[470] The mechanisms that underlie TBP2 action remain to be elucidated.

Forkhead (*Foxo*) Transcription Factors

The forkhead transcription factors (FOXO) have roles in cell cycle arrest and apoptosis, and, as demonstrated by the embryonic lethality of a *Foxo1* knockout, these proteins are important in embryogenesis.[471] There are four FOXO family members—FOXO1, FOXO3, FOXO4, and FOXO6—although expression of the latter is predominantly restricted to the brain.[472] In contrast to *Foxo1*, the mouse knockout of *Foxo3* is viable but shows some hematologic defects in addition to an age-dependent infertility[135] (see the section PI3K–AKT and Tsc1–mTORC1 Signaling Pathways). *Foxo4* null mice are also viable and fertile, with no indication of reproductive dysfunction.[471] *Foxo1* is preferentially expressed in granulosa cells of growing follicles. Expression of a constitutively active FOXO1 in granulosa cells suppresses expression of key genes that control proliferation: cyclin D2 (*Ccnd2*), aromatase (*Cyp19a1*), FSH receptor (*Fshr*), and *Lhcgr*.[473] FOXO1 also negatively regulates genes involved in sterol and lipid biosynthesis,[474] and thus it is possible that defects in FSH, IGF1, or insulin signaling impact FOXO function, and thus underlie infertility in some human diseases such as diabetes.[475]

Conditional deletion of both *Foxo1* and *Foxo3* using either *Amhr2cre* or *Cyp19cre* results in female sterility.[285] Part of the phenotype arises because of undetectable levels of FSH; loss of FSH results from suppression of *Fshb* gene expression along with several of its transcriptional regulators in the pituitary. Gonadotropin stimulation can induce ovulation in younger mice (i.e., at 25 days of age), but ovulation is impaired by 2 months of age, indicating that gonadotropin loss cannot fully explain the phenotype. Ovariectomy in mutant mice results in an increase in *Fshb* expression and serum FSH levels, suggesting that the *Foxo1/Foxo3* double-knockout ovary produces an inhibitory substance that acts on the pituitary. Serum levels of estradiol and testosterone are similar to those of controls, and serum levels of inhibin A or inhibin B, or granulosa cell expression of *Inha*, *Inhba*, or *Inhbb*, are unchanged. This has led to the hypothesis that the ovary-derived FSH inhibitor substance is a potentially novel, nonclassical endocrine factor.[285] *Foxo1/Foxo3* null granulosa cells show a disrupted gene expression pattern characterized by a loss of growth-promoting and apoptosis-related genes.[320] Several growth-promoting genes are co-regulated by activin and FOXO1/3, while the apoptosis genes are co-regulated by BMP2 and FOXO1/3, suggesting that FOXO1 and FOXO3 interact with both SMAD pathways to exert control over granulosa cell growth and differentiation.[320]

GATA Transcription Factors

There are six members of the GATA family of transcription factors, GATA1–6, which regulate development and cell specification for variety of tissues. GATA factors bind to a consensus site, (A/T)GATA(A/G), from which their name is derived. *Gata4* and *Gata6* are highly expressed in granulosa cells and regulate the expression of genes involved in follicle development and steroid synthesis.[476–478] In the postnatal ovary, *Gata4* expression commences in granulosa cells during the transition from primordial to primary follicle,[477,479] while *Gata6* expression is delayed until adulthood.[477]

Deletion of *Gata6* in granulosa cells has no effect on female reproduction, although conditional deletion of *Gata4* causes subfertility.[480] Co-deletion of *Gata4* and *Gata6* causes irregular estrous cycles, a lack of follicular development, and an increase in atretic follicles.[480] This is due in part to loss of expression of *Fshr*, which is undetectable in ovaries of *Gata4* single conditional knockouts and *Gata4/Gata6* double conditional knockouts, as well as genes downstream of FSH signaling and markers of granulosa cell differentiation, such as *Lhcgr* and *Cyp19a1*.[480] Loss of *Fshr* can be attributed to loss of GATA4, as GATA4 directly binds and stimulates expression from the *Fshr* promoter.[480] Currently, there are no data supporting GATA4 regulation of *FSHR* in humans, although this may be a mechanism that could account for the low levels of FSHR in women with poor ovarian response to gonadotropin stimulation who are undergoing infertility treatments.[480]

ANTRAL FOLLICLE DEVELOPMENT

Key Roles of the Hypothalamus and Pituitary

Formation of the antrum heralds the final phases of folliculogenesis and the transition from intraovarian to extraovarian regulation. The hypothalamic–pituitary–gonadal (HPG) axis in females coordinates follicle maturation with sexual behavior and the physiologic preparation for pregnancy. Prior to a mature HPG axis, follicles are recruited to grow from primordial follicles, but cannot form antral follicles without sufficient gonadotropin stimulation. Spontaneous and targeted mutations in mice, as well as genetic disorders in humans, affecting pituitary production of various peptides and hormones have provided important insight into the hormonal regulation of folliculogenesis. This section will focus on the role of pituitary hormones in ovarian function.[481]

Follicle-Stimulating Hormone

The pituitary glycoprotein FSH plays an essential role in reproduction through interaction with gonadal FSH receptors. FSH is a dimeric glycoprotein composed of a unique β subunit complexed with a common α subunit that is shared with thyroid-stimulating hormone, LH, and chorionic gonadotropin. FSH binds to the FSH receptor and activates cell signals that lead to germ cell maturation and follicular growth.[482] Male mice deficient in FSH through deletion of the *Fshb* gene are fertile, but with decreased sperm concentration, sperm motility, and testes size. In contrast, females lacking FSH are infertile, with small uteri and ovaries, elevated LH, and lack of estrous cycles[22] (Figure 21.3(B)). Ovarian histology at 6 weeks revealed the presence of primordial, primary, and multilayered preantral follicles. However, no antral follicles are formed, and the knockout mice do not ovulate. Subsequently, it was shown that during the initial wave of postnatal follicle growth, there are fewer growing follicles in $Fshb^{-/-}$ mice, and FSH accelerates some functions necessary for oocyte development, such as the acquisition of developmental competence.[483]

Multiple genes are abnormally regulated in the FSHβ knockout mice.[484] *Fshr* is upregulated in FSHβ null mice almost twofold as compared to wild type. *Lhcgr* is expressed almost fivefold less in FSHβ null ovaries, consistent with the lack of corpora lutea and late-stage follicles. Serum- and glucocorticoid-inducible kinase (SGK), which is induced in preovulatory granulosa cells by FSH and is thought to play a role in the transition of the proliferative to terminal luteal stage,[485–487] is also downregulated in FSHβ knockout mice. As expected, *P450scc* mRNA levels are downregulated almost sixfold, also likely due to the lack of corpora lutea. Aromatase (CYP19), which converts androgens to estrogens,[488,489] is decreased sixfold in the FSHβ null mice.

FSH promotes granulosa cell proliferation, and FSHβ knockouts arrest at the preantral stages of follicular development. Cyclin D2 null[262] and *Igf1* null female mice[268,269] are also infertile and demonstrate an arrest at the same preantral follicle stages as FSHβ null ovaries. Interestingly, the lack of granulosa cell proliferation in FSHβ null mice is not due to lack of cyclin D2 since it continues to be expressed,[484] and the RNA levels of the cyclin-dependent kinases *Cdk2* and *Cdk4* are not affected in the FSHβ null ovaries. Although *Igf1* mRNA levels are not altered in FSHβ null ovaries, FSH receptor mRNA is depressed in *Igf1* null mice.[268] Thus, one of the major defects in the *Igf1* null ovaries is due to defective FSH signaling.

The phenotype of FSHβ null mice can be rescued by transgenes expressing a human FSHβ subunit gene under its own promoter, as well as constitutive and ectopic expression of human FSHβ under the control of the metallothionein-I promoter.[490] Complete restoration of fertility and folliculogenesis was accomplished when the human FSHβ gene was expressed, indicating that FSH function in mammals is evolutionarily conserved. In humans, inactivating mutations in the FSHβ gene exist, and several women with these mutations have been identified[443,444,491] (Table 21.4). A 27-year-old woman with primary amenorrhea, absent breast development, low FSH, and high LH was found to be homozygous for a two base pair (bp) deletion at codon 61 (Val61X). This frameshift mutation was predicted to change amino acids 61 to 86, followed by a stop codon resulting in truncated FSHβ protein lacking amino acids 86–111.[443] A second patient who was 15 years old had absent breast development, primary amenorrhea, low FSH, undetectable estradiol, and elevated LH. She was a compound heterozygote for two different FSHβ gene mutations, Val61X, and a Cys51Gly missense mutation. Both the Val61X and the Cys51Gly

TABLE 21.4 Human Mutations Causing Reproductive Axis Defects in Females

Gene	Mutation	Type of Mutation	Clinical Presentation	References
CYP19	$Arg^{435}Cys$ $Cys^{437}Tyr$	Loss-of-function (compound)	Primary amenorrhea and cystic ovaries	442
FSHB	Frameshift Val^{61} $Cys^{51}Gly$	Loss-of-function	Primary amenorrhea and infertility	443,444
FSHR	$Ala^{189}Val$	Loss-of-function	Defects in follicle development	21,445
GNRHR	$Gln^{106}Arg$ $Arg^{262}Gln$	Hypomorphic	Primary amenorrhea, and low estrogens, FSH, and LH	446
LHR	$Cys^{554}X$ $Ala^{593}Pro$	Truncation Inactivating	Primary amenorrhea Secondary amenorrhea	447 448
LEPR	Mutation in splice donor site exon 16	Truncation	Primary amenorrhea	449

mutations resulted in unmeasurable immunoreactive and bioactive FSH levels.[444] The mouse FSHβ mutant mimics well the phenotype observed in women with the FSHβ mutations and shows the essential role that FSH plays in fertility of mice and humans. Women with FSH mutations gave further insight into the roles of FSH in androgen production. Although LH levels were high in one patient, she had normal levels of testosterone and no evidence of hirsutism. These findings argue that FSH is important in LH-induced androgen production in the theca, either by inducing inhibin and LH receptors, and or through other growth factors.[491] It is clear that studying animals and humans with induced and naturally occurring mutations in particular genes yields useful information with regard to hormonal function during folliculogenesis.

Follicle-Stimulating Hormone Receptor

FSHRs are transmembrane G (guanine nucleotide-binding) protein–coupled receptors. Members of this family have an extracellular hormone-binding domain, a seven transmembrane spanning region, and an intracellular region that couples to G proteins. Two different groups have generated FSHR-deficient mice by deleting the first exon and portions of the promoter and first intron.[23,24] Female mice lacking FSHRs are infertile. The ovaries and uteri of mutant mice are smaller and thinner in both studies, while in one study, vaginas were imperforate. A lack of biologically active estrogen is the probable cause for small uteri and imperforate vaginas. Primordial, primary, and secondary follicles are present, but large antral follicles and corpora lutea are absent, which accounts for the smaller size of the mutant ovaries. The lack of FSHR does not affect growth of early follicles, consistent with the FSHβ knockout[22] and the hypothesis that gonadotropins are not essential for the early stages of folliculogenesis. FSH and LH levels are higher in null mice as compared to control mice. Similar to FSHβ knockout mice,[22] continuous exposure of ovarian stroma to elevated LH levels most likely accounts for the observed hypertrophy of the interstitial tissue as well as numerous small lipid droplets.

Studies on female mice older than 8 weeks revealed formation of tumor-like structures and cysts by 12 months of age in FSHR null mice.[496] The histology of these tumors showed an equal admixture of tubular structures and vacuolated plump stromal cells reminiscent of Sertoli and Leydig cells, respectively. It is likely that elevated levels of gonadotropins are responsible for the tumor-like defects similar to those in mice that overexpress FSH, LH, or human choriogonadotropin (hCG).[313,497–499] However, there is no convincing evidence that inactivating mutations in FSHR cause tumors in primates and humans, and searches for such mutations in granulosa cell tumors have been unproductive.

The controversial role of FSHR in the primordial-to-primary follicle transition was also addressed using *Fshr* knockouts. *Fshr* mRNA is expressed in 1-day-old mice—a time when many germ cell cysts are present and primordial follicles form. Histomorphometric analysis, which measures follicle numbers by different types at 2, 10, and 24 days, showed the presence of primary and secondary follicles in 2-day-old knockout mice, while secondary follicles were not yet formed in the wild-type controls. There was a subsequent decrease in the total number of primordial, primary, and preantral follicles in the *Fshr* knockouts as compared to the wild type. The differences were modest but statistically significant. Based on these data, the lack of FSHR accelerates very early stages of follicular growth, which subsequently blocks at the preantral stage. Also, it is possible to conclude that FSHR plays a small but significant enough role in early follicular growth, and it is possible that the accelerated follicle recruitment in early ovaries leads to premature ovarian failure observed in 1-year-old female mice.

Only a few mutations in the *FSHR* gene in humans have been identified.[500] A Finish population-based study identified patients having XX gonadal dysgenesis (46,XX women with primary or secondary amenorrhea and

serum FSH > 40 MIU/ml).[21] These 75 cases comprised 57 sporadic cases and 18 cases having affected relatives (seven different families). Most cases were found in north-central Finland, a sparsely populated part of the country. The overall frequency of the disorder in Finland was one per 8300 live-born females, a relatively high incidence attributed to the founder effect. An alanine→valine mutation at position 189 of FSHR segregated with the phenotype. The effect of this mutation on FSHR expression and activity has been studied in vitro. The mutant receptor mutation is inactivating, with impaired trafficking and folding that ultimately result in the decreased ability of the receptor to bind FSH.[500,501] Compound heterozygous mutations in the FSHR gene were identified in a group of French women who had primary or secondary amenorrhea and follicle development up to the secondary stage.[502] These inactivating mutations are in the extracellular domain and affect ligand binding and receptor trafficking.[500]

Luteinizing Hormone

Similar to FSH, LH is a glycoprotein hormone with a common α subunit but a unique hormone-specific β subunit, and it is synthesized in the pituitary gonadotropes. Recently, a mouse model for the loss of LHβ has been generated.[271] *Lhβ* null mice are viable, but males and females are infertile. Female mice are hypogonadal with decreased serum levels of estradiol and progesterone. Histologic analysis of the ovaries demonstrates that large antral follicles and corpora lutea are absent. Primary and secondary follicles with intact thecal cell layers develop, and thecal cell markers *Lhcgr*, *Bmp4*, and *Cyp17a1* are expressed, indicating that LH is not necessary for initial differentiation of the thecal cell layer.[271] However, low serum steroid levels and the decreased expression of the steroidogenic enzymes *Cyp11a1*, *Cyp19a1*, and *Cyp17a1* demonstrate that LH is necessary for steroid production.[271] Similar to the FSHβ knockout mouse model, the phenotype of LHβ null mice can be rescued by treatment with exogenous gonadotropins, suggesting that the block in development is not irreversible.

Mutations in *LHB* in humans are rare, but the first was identified in a male with a family history of male infertility.[503] Female members of that family do not appear to be affected. Subsequently, two other mutations have been described.[504,505] In one of the families, a female with hypogonadism related to LH deficiency was the first woman shown to harbor an inactivating mutation in *LHB*.[504] She had normal pubertal development but secondary amenorrhea and infertility.[504]

LH Receptor (Lhcgr)

Like FSHRs, LH receptors are single-chain transmembrane glycoproteins that belong to the G protein–coupled receptor family. Although LH and FSH are not thought to play major roles in early folliculogenesis, truncated LH receptor mRNA is expressed in newborn ovaries[506] at the time when germ cell cysts are present and primordial follicles are formed. Full-length transcripts are not detectable until postnatal day 5, but based on all LHβ and LH receptor mutations, these transcripts are unlikely to play major roles in early follicular formation and function. To study the effects of LH receptor deletions on ovarian development and folliculogenesis, two knockouts have been generated. In the first knockout, exon 1 and the 5′ flanking region containing multiple transcription initiation sites and the promoter region were deleted.[272] Both male and female null mice were sterile. At 60 days, null females had markedly smaller ovaries (more than 50% less than the weight of wild-type ovaries) with pale and thin uteri and delayed vaginal opening. Preantral and antral follicles, but not preovulatory follicles or corpora lutea, were present in females lacking LH receptor. As compared to FSHR knockouts, LH receptor knockouts have more advanced follicular structures, but ovaries in both knockouts continue to be quite small. Like the LHβ knockout, this argues that LH receptor is not required for early gonadal and reproductive tract differentiation. Serum LH levels are high in LH receptor knockouts, which could be due to loss of estradiol negative feedback and/or loss of negative LH feedback on its own secretion through decreased hypothalamic GnRH levels in both sexes. Moderate elevation in serum FSH levels could be due to decreased gonadal inhibin secretion. FSHR and progesterone receptor mRNA levels were not significantly affected by the lack of LH receptor. Estrogen receptor α (*Esr1*) and *Star* mRNA levels are decreased in the LH receptor knockouts, and estrogen receptor β (*Esr2*) mRNA levels are increased in both ovaries and testes. Because LH maintains *Esr1* and *Star*, and inhibits *Esr2* mRNA levels, these results in the LH receptor knockout are consistent with those of previous studies. Hormone replacement therapy with estrogen and progesterone did not restore fertility or reverse ovarian morphology, which argues that other downstream targets of LHCGR are involved in stimulating follicular growth beyond the antral stage.

Another LH receptor knockout was generated by deleting exon 11 of the LH receptor, which encodes the transmembrane and cytoplasmic receptor domains and part of the 3′ extracellular domain, thus preventing the formation of full-length functional LH receptor capable of anchoring to the plasma membrane and of signal transduction.[273] These *Lhcgr* knockout mice did not synthesize any mRNA encoding the full-length functional LH receptor, and the phenotype was remarkably similar to the *Lhcgr* knockout (described here) in which the promoter and first exon were removed.

In humans, mutations in the LH receptor cause gonadal dysgenesis. Sultan et al.[507] tabulated the 46,XY cases, with nine having female phenotype and two having

micropenis. All 46,XX cases occurred in sibships in which an affected 46,XY male had Leydig cell hypoplasia. Latronico et al.[447] reported primary amenorrhea in a 22-year-old woman. In that family, three males and one female had a homozygous nonsense (stop) mutation at codon 554 (C554X). The newly produced stop codon resulted in a truncated protein having five rather than seven transmembrane domains. The affected female had breast development but only a single episode of menstrual bleeding at age 20 years. The mutation reduced signal transduction activity of the LH receptor gene. In a 46,XX case reported by Toledo et al.,[448] secondary amenorrhea occurred. The mutation was from alanine to proline at position 593.

LH signaling upregulates the expression of epidermal growth factor (EGF)-like family members during cumulus expansion at ovulation.[508,509] LH receptor, expressed on mural granulosa cells, responds to the peri-ovulatory LH surge by increasing epiregulin (*Ereg*), amphiregulin (*Areg*), and betacellulin (*Btc*) in a protein kinase A–dependent manner. Receptors for the EGF-like proteins are found in cumulus cells, and activated EGF receptor tyrosine kinase receptors, signaling through the MAPK pathway, induce expression of cumulus expansion genes *Has2*, *Ptgs2*, and *Tnfaip6*.[510] *Areg*$^{-/-}$ and *Ereg*$^{-/-}$ mice have cumulus expansion defects.[508] Furthermore, depletion of *Erk1/2* in granulosa cells causes infertility due to a failure of ovulation.[511] Cumulus oocyte complexes from mice with deletion of *Erk1/2* fail to expand, and oocytes do not resume meiosis. In addition, granulosa cells fail to terminally differentiate, and they continue to proliferate and generate estradiol.

Steroid-Signaling Pathways

Estrogen Signaling

Estrogen receptors α and β, encoded by *Esr1* and *Esr2*, respectively, are expressed in many organs, including the hypothalamus, anterior pituitary, mammary gland, uterus, and ovary.[400] Estradiol stimulates granulosa cell growth, increases synthesis of granulosa cell insulin-like growth factor 1 (IGF1), maintains FSH receptors, induces LH receptor, augments aromatase activity and subsequent estradiol production, and attenuates granulosa cell apoptosis. Estrogens bind specialized receptors that are nuclear transcription factors that bind DNA and activate transcription of other genes. Estrogen receptors are members of the steroid receptor superfamily of ligand-activated transcription factors. Estrogen receptors and their ligand, 17β-estradiol, play critical roles in the development of female secondary sexual characteristics as well as in the female reproductive tract, and they maintain pregnancy.

Esr2 is expressed at higher levels in granulosa cells of growing follicles as compared to *Esr1*, while *Esr1* is expressed in thecal cells and interstitial cells.[512] The mRNAs corresponding to *Esr1* and *Esr2* are first expressed around day 8 in the rat ovary, which at this age contains primordial, primary, and some secondary follicles. Although estrogen is thought to act mainly on antral follicles, earlier expression of mRNA of the receptors in growing follicles may indicate additional functions or possibly estrogen-independent activity of the receptors.

Initial observations in mice null for *Esr1* showed that ovaries from these mutant mice contained cystic and hemorrhagic follicles, and hypoplastic uteri.[275] The mouse model also indicated that another estrogen receptor existed, because estradiol binding in uterine preparation from mutant animals still occurred. This estrogen receptor turned out to be *Esr2*.[513–515] *Esr2* knockout mice are viable but subfertile.[276] Litter sizes in *Esr2* knockouts are 35% of wild type. Superovulation experiments showed a dramatic reduction in the number of oocytes ovulated per female, with only six oocytes per female in the knockouts as compared to 52 in the control mice. Other observed effects included decreases in the cellular mass of the cumulus cells, and an inadequate response to exogenous hormones. The endogenous gonadotropin levels were normal in these mice.

To study the effects of a complete lack of ERα and ERβ on reproduction, double knockouts have been generated.[400,516] The double-mutant ovaries have a more severe phenotype that is distinct from either single-receptor knockout. Unlike prepubertal wild-type ovaries that show mostly secondary follicles, prepubertal double-knockout mice possess adult-like follicles with defined antra and theca, characteristic of hypergonadotropic–precocious maturation of the ovary. Serum LH levels are higher in double knockouts as compared to *Esr1* knockout alone, which suggest that both estrogen receptors are required for estradiol-mediated regulation of LH secretion in the hypothalamic–hypophyseal axis. In 10-week- to 7-month-old ovaries, primordial and growing follicles are visible, but corpora lutea are lacking. Interestingly, follicles are replaced by structures that resemble seminiferous tubules of the testis in the double-knockout ovaries, some with degenerating oocytes, granulosa cells, and Sertoli-like cells (Figure 21.3(D),(G)). Sertoli cell differentiation markers, *Amh* and *Sox9*, are elevated in the *Esr1* and *Esr2* double knockouts. As discussed here, FOXL2 and ESR1 cooperatively repress *Sox9* expression, and conditional knockout of *Foxl2* also causes transdifferentiation in mice. Aromatase knockout ovaries (discussed further in this chapter) also develop Sertoli and Leydig-like cells at puberty, which argues that the lack of estrogens causes transdifferentiation of the granulosa cells.

The aromatase knockout (ArKO) gives additional insight into the effect of a complete lack of estrogen on follicular development.[274] Aromatase is a cytochrome P450 enzyme that catalyzes the formation of aromatic C18 estrogens from C19 androgens. Because aromatase

is required for the synthesis of estradiol, a knockout renders the mice fully estrogen deficient. Knockout mice are phenotypically normal but infertile. Testosterone levels are almost 10 times the wild-type levels. LH levels are elevated two- to 10-fold, and FSH levels are also elevated three- to fourfold, over the wild-type levels. These findings corroborate previous studies that showed the importance of estrogen in the negative-feedback regulation of gonadotropins, and the high testosterone levels presumably reflect LH-stimulated androgen production by the thecal cells. Ovaries from 12- to 14-week-old mice show the presence of many antral follicles, with hypertrophied stromal cells and atretic follicles. No corpora lutea are visible; therefore, no ovulation occurs. Another knockout was generated, and analysis of these mice showed that at 8 months of age, hemorrhagic cysts were observed, with mast cell infiltration and reduced number of follicles with widespread follicular atresia.[517] Although the aromatase knockout mice could not be superovulated, oocytes recovered from ArKO mice can be matured in vitro, with successful fertilization and early embryo development.[518] Estrogen appears to play an important role in feedback regulation of gonadotropin secretion and continued follicle growth but does not interfere with oocyte growth.

The role of estrogen metabolism is also important in humans. Ito et al.[442] reported a mutation in the *CYP19* (P450 aromatase) gene in an 18-year-old 46,XX woman with primary amenorrhea and cystic ovaries. The patient was a compound heterozygote for two different point mutations in exon 10. The mutant protein had no activity. Most of the mutations in humans involve amino acid substitutions as well as premature stop codons. It is a rare disorder, with only a few women reported.

Deficiency of the aromatase enzyme is more often associated with genital ambiguity. Shozu et al.[519] detected placental aromatase deficiency manifesting as maternal virilization during the third trimester. The 46,XX infant was born with genital ambiguity (female pseudohermaphroditism). Adrenal enzyme defects were not evident. The molecular basis of the mutation was an 87 bp insert in exon 6 of the aromatase gene, altering the splice junction site to produce a novel protein with 29 additional amino acids. Aromatase mutations in 46,XX female infants associate with genital ambiguity[442] or clitoromegaly.[520] In the later cases, clitoral enlargement occurred at puberty, but breast development did not. Multiple ovarian follicular cysts were evident. FSH was elevated, while estrone and estradiol were low. Estrogen and progesterone therapy resulted in a growth spurt, decreased FSH, decreased androstenedione, decreased testosterone, breast development, menarche, and fewer follicular cysts.

Androgen Signaling

Androgens play a central role in the development of male sex organs and secondary sexual characteristics, but less is known about androgen's role in the development and function of normal ovaries. Androgens are thought to mainly signal via the androgen receptors (ARs). ARs are highly expressed in the granulosa cells of rodent ovaries.[521] AR is encoded on the X chromosome and activates gene expression as a result of multiple events, including ligand binding, homodimerization, nuclear translocation, DNA binding, and complex formation with co-regulators and general transcription factors.[522] AR mouse knockouts are subfertile and have normal initial primordial follicle population, with disruption of later stages in folliculogenesis and eventual premature ovarian failure.[277,278] AR deficiency in the ovary reduces the number of corpora lutea, increases granulosa cell apoptosis, and decreases *Kitl* production. Selective ablation of ARs in the granulosa cells or oocytes suggests that the observed ovarian pathology is due to AR actions in the granulosa cells and not the oocytes.[279,280] Overall, these results suggest that AR has an important role in postnatal ovarian development and folliculogenesis, and future studies will be important to delineate the specific targets of AR action within the ovary.

Hyperandrogenism in women is associated with the relatively common PCOS, a syndrome where many arrested small antral follicles give rise to the cystic appearance of the ovary.[523] Androgens increase the growth of immature follicles in the primate ovary, and treatments with testosterone and its active form, dihydrotestosterone, caused significant, three- to fourfold increase in IGF-I mRNA concentration in granulosa, thecal, and interstitial ovarian compartments.[524] Both testosterone and dihydrotestosterone induced significant increases in the amount of IGF-I receptor mRNA, most notably in thecal cells and less markedly in granulosa and interstitium of the primate ovaries. These data suggest that IGF-I and its cognate receptor may mediate androgen-induced ovarian follicular and thecal–interstitial growth. Moreover, additional studies showed that androgen treatment significantly increased granulosa cell *FSHR* mRNA. These data suggest that androgens promote follicular growth and estrogen biosynthesis in part via enhancement of the FSH effect, which partially explains the enhanced responsiveness to gonadotropin stimulation noted in women with POCS.[525] Hyperandrogenism and its effects on ovarian development and PCOS are subjects of intense studies, with data suggesting that prenatal exposure to testosterone in humans, primates, and sheep can lead to multifollicular ovaries reminiscent of the PCOS-like phenotype.[526–529]

Gap Junction Membrane Channel Proteins and Connexins

Connexins are a family of at least 15 proteins that form intercellular membrane channels of gap junctions

and allow diffusional movement of ions, metabolites, and potential signaling molecules. These structural molecules play important roles in bidirectional communication between the oocyte and granulosa cells. Several connexins have been shown to be important during folliculogenesis. *Gja4* (connexin 37) is expressed in primary and growing follicles. Ovaries from *Gja4* knockouts at 2 weeks of age contain many developing preantral follicles, but not larger antral follicles.[281] Therefore, histologically, it appears that lack of *Gja4* blocks preantral-to-antral follicle development. Electron microscopy studies showed that while the *Gja4* knockouts contained adherens junctions, they lacked the gap junctions present on wild-type ovaries. In addition, despite the lack of Graafian follicles, the null ovaries displayed smaller structures resembling corpora lutea that were 5–10 times more abundant. Luteinization usually occurs following ovulation when oocyte–granulosa cell communication is lost. The premature luteinization observed in *Gja4* null animals suggests that junctional communication is a major mechanism regulating corpus luteum formation. It can be hypothesized that an inhibitory signal is transferred through gap junctions from the oocyte to the granulosa cells that result in the prevention of luteinization until after ovulation has occurred. An additional phenotype of the *Gja4* knockout was that the average oocyte size was smaller. While 52.8% of oocytes from $Gja4^{+/-}$ mice resumed meiosis, with 26.8% of these proceeding to metaphase of meiosis I and 26% to metaphase of meiosis II, only 2.2% of $Gja4^{-/-}$ mice resumed meiosis in culture, with the vast majority (86.3%) retaining germinal vesicles. All of these observations clearly indicate that *Gja4* is essential at the time of the preantral-to-antral follicle transition, and is most likely involved in the transport of metabolites necessary and critical for this transition.

GJA1 (connexin 43) protein is the most abundant connexin in the ovary, and is expressed as early as E14.5. GJA1 gap junctions interconnect granulosa cells that surround the oocyte as well as oocytes and granulosa cells. *Gja1* knockouts die at birth due to cardiovascular failure.[530] Ovaries in null mice, retrieved at the time of birth, are unusually small, apparently due to a deficiency of germ cells.[282] The germ cell deficiency was traced back as far as E11.5 of gestation, implying that it arises during early stages of germline development. To study postnatal folliculogenesis in $Gja1^{-/-}$ mice, fetal and neonatal ovaries have been grafted into the kidney capsule of an ovariectomized and immunocompromised $prkdc^{scid/scid}$ mouse.[531] The grafts were removed after 1–3 weeks, and ovarian histology was examined. After 3 weeks of development in kidney grafts, wild-type ovaries contain a range of follicles from primordial through primary, secondary, and tertiary stages. In contrast, *Gja1* null ovaries did not proceed beyond primary follicles. Mutant granulosa cells did not proliferate in grafts after 1, 2, and 3 weeks. Oocyte growth was also slower in mutants as compared to wild-type animals. Oocytes recovered from *Gja1* mutants could not be fertilized. Electron microscopy also showed that these oocytes are structurally abnormal as compared to the wild type.

Gja1 appears to be regulated by the gonadotropins, FSH and LH. Because *Gja1* is more abundant in large antral follicles as compared to small antral and preantral follicles, some have hypothesized that FSH induces GJA1 synthesis. Interestingly, *Gja1* mRNA levels drop after a preovulatory surge of LH.[532,533] Further corroboration came from experiments utilizing exogenous administration of gonadotropins. Injection of FSH into hypophysectomized rats promoted an increase in the ovarian gap junctional membrane, while hCG, which induces ovulation, led to a significant reduction in the quantity of gap junctions in granulosa cells.[534,535] In vitro administration of FSH and LH to granulosa cell lines corroborated in vivo observations.[536,537] It has been suggested that effects of the gonadotropins on *Gja1* are mediated through steroid hormones.[538] Other signaling pathways may also regulate *Gja1*, including the BMP–Smad and the MAPK–Ras pathways, which have been shown to regulate *Gja1* promoter in vitro.[539,540]

The gap–junctional coupling mediated by GJA1 channels plays indispensable roles in both germline development and postnatal folliculogenesis. The different ovarian phenotypes resulting from the *Gja4* and *Gja1* knockouts possibly indicate that gap-junctional coupling between the oocyte and its cumulus granulosa cells serves a distinct role from that between the granulosa cells themselves, possibly involving different molecules. However, ectopic expression of *Gja1* in *Gja4* null oocytes fully restores fertility to *Gja4* null mice, indicating that both connexins are equally able to couple oocytes to somatic cells.[541] GJB1is also expressed in the ovary, but knockout is not infertile,[542] meaning that redundant gap junction components exist.

Additional cellular connections may be important for antrum formation and granulosa cell–oocyte interactions. Intercellular communications between granulosa cells and oocytes are thought to be mediated by transzonal projections (TZPs) between the oocyte and granulosa cells.[92] Microtubule TZPs (MT-TZPs), in particular, appear to be involved in this communication.[92] The MT-TZP connections between the oocyte and granulosa cells have been hypothesized to be under the control of FSH.[543] FSH priming of wild-type and $Fsh^{-/-}$ mice results in alterations in the MT-TZP, such that the TZPs are retracted from the oocyte,[543] and enhances oocyte chromatin remodeling, apical centrosome positioning, and acquisition of meiotic competence.[543] From these experiments, it can be hypothesized that without FSH priming, granulosa cells have a stable interaction with the oocyte, allowing

for the paracrine exchange of factors. Once MT-TZPs are retracted, oocyte development is accelerated.

METABOLIC INFLUENCES ON FOLLICLE DEVELOPMENT

Globally, there are several peptide growth factors that may influence ovarian function due to their endocrine (metabolic) effects on the HPG axis.[298] In this section, we will briefly discuss intraovarian effects of leptin, growth hormone, and IGF1.

Leptin

The *Lep* gene (also known as the *ob* (obesity) gene) encodes leptin. Leptin is mainly secreted by adipose tissue, and much attention has been devoted to studying its effect on the central nervous system to diminish appetite and to increase energy consumption. In the murine ovary, leptin is expressed in oocytes, granulosa cells, and theca cells. Using immunohistochemistry, it appears that the highest expression of leptin is confined to the oocyte, while the leptin receptor has highest expression in theca cells.[544] Leptin administration does not affect spontaneous or induced maturation of either isolated denuded oocytes or cumulus–oocyte complexes, but it does significantly increase the rate of meiotic resumption in preovulatory follicle-enclosed oocytes.[544] This effect does not appear to be mediated by activation of phosphodiesterase 3B and subsequent cyclic adenosine monophosphate reduction.[544] It is possible that leptin induces secretion of a factor important in meiotic resumption.[544] In humans, leptin is immunolocalized to oocytes in primordial and primary follicles as well as granulosa cells surrounding the oocytes.[545] In preantral follicles, leptin is preferentially expressed in granulosa cells, and in antral follicles, leptin is preferentially expressed in the thecal layer. Rat studies also document expression of leptin and its receptor in granulosa, theca cells, and oocytes.[546] Interestingly, although leptin protein is detected in oocytes, the leptin mRNA is not detected in oocytes.

ob/ob mice, which are deficient in leptin, are morbidly obese and have a subnormal number of ovarian follicles.[270] Weight loss in *ob/ob* mice does not restore fertility, but *ob/ob* ovaries do produce viable eggs when transplanted into wild-type mice, indicating that nongonadal leptin can restore fertility.[547] Ovaries from *ob/ob* mice contain antral follicles with pronounced apoptosis of granulosa cells. The infertility in these mice is partially due to an altered pattern of GnRH secretion leading to decreased secretion of LH and FSH. Leptin replacement increases FSH and LH levels in *ob/ob* mice and restores fertility.[547] In addition, administration of gonadotropins also restores fertility, indicating that the absence of leptin influences the hypothalamus and pituitary. Because FSH and LH levels are only partially suppressed in *ob/ob* mice, and some direct effects of leptin on the gonads are likely also important, it is unclear whether the increased apoptosis of granulosa cells that is observed in the *ob/ob* preantral and antral follicles is due to low FSH and LH levels, a result of absent leptin signaling in the ovary, or a combination of both.

Leptin shows an important link between metabolism and reproduction. Leptin may serve as a metabolic signal informing the reproductive system that sufficient fat stores are available to meet the caloric demands of reproduction. Metabolic stresses such as food restriction and severe exercise will lead to low circulating levels of leptin and lower fertility. Ovarian transplantation experiments using leptin receptor–deficient mice (*db/db* animals) suggest that reproductive dysfunction is not due to impaired intraovarian signaling.[548] It appears that the majority of leptin actions on reproduction are mediated via the hypothalamus.

Leptin deficiency in humans is rare and causes morbid obesity. Follicular development and function as well as gonadotropin secretion in these children are unknown. Women with mutations in the leptin receptor are also obese and have primary amenorrhea.[449] FSH and LH levels are low to normal, and estradiol concentration is low. Consistent with the *db/db* mouse studies,[548] these findings reflect hypogonadism of central origin that is likely responsible for the observed infertility.

However, in vitro studies have supported that leptin has some local effects in the ovary. Leptin can suppress ovarian steroid synthesis in primary rat granulosa cells and in rat and human granulosa cell lines.[549] Other studies have shown that leptin inhibits IGF1 synergistic effects with FSH on estradiol production by rat granulosa cells as well as inhibiting aromatase activity.[550] In contrast, it has been reported that leptin increases aromatase mRNA and protein expression in human granulosa cells.[551] The use of supraphysiologic amounts of leptin may account for different results, and further studies may be helpful, such as conditional knockouts of leptin or its receptor in the ovary, to study the local effects of leptin in ovarian physiology and settle the hypothalamic versus intraovarian causes of reproductive dysfunction. In the rat, leptin mRNA increases threefold within 2 h of hCG administration, and leptin receptor mRNA levels also increase dramatically 9 h after hCG administration.[546] These results suggest that expression of leptin and its receptor peaks at the time of ovulation, but their specific functions are unknown. Lastly, leptin is also expressed in human vasculature as well as ovarian endothelial cells, and it promotes angiogenesis. Leptin-induced angiogenesis may be important in folliculogenesis.[552] The data discussed here suggest that leptin exerts its major effect via the hypothalamus, with intraovarian effects playing a minor role.

Growth Hormone

Growth hormone (GH) is secreted by the anterior pituitary and can act directly via binding to the growth hormone receptor (GHR) to effect growth and the onset of sexual maturity.[553] In addition, GH controls the synthesis of IGF1, which additionally affects ovarian function. GH receptor is expressed in multiple tissues, including the ovary. GHR RNA is located in the granulosa cells of antral follicles and the corpus luteum but is not detected in preantral follicles, theca, oocytes, or stroma.[554]

In horses and rodents, GHR RNA is detectable in oocytes, cumulus cells, and granulosa cells throughout folliculogenesis.[555,556] GH augments cumulus expansion and improves nuclear maturation in equine cumulus cell–oocyte complexes.[555] In the cow, GH receptor protein and RNA are detected as early as primordial oocytes and granulosa cells of primary follicles and more advanced follicles.[557] Mice that lack GHR are subfertile dwarfs[558–560] with delayed age of conception, low plasma IGF1, and elevated plasma GH levels.[561] A subset of GHR knockout females is infertile, although the reason for why some are subfertile while others are infertile is unknown, but it may have to do with epigenetic effects.[558] Treatment with recombinant human IGF1 normalizes the age of puberty.[561] However, IGF1 does not restore the ovulation rate in GHR knockout mice, and therefore growth hormone may affect follicular growth independent of IGF1.[562] More detailed analysis of ovaries from GHR knockouts revealed significantly smaller numbers of preovulatory follicles and corpora lutea, decreased plasma estradiol levels, and an increased number of apoptotic follicles.[558] It appears that *Ghr* deficiency in mice does not affect primordial follicle endowment but, rather, their recruitment into growing follicles.[563] Women with mutations in *GHR* (Laron syndrome) can become pregnant despite low levels of IGF1.[564–566] It is unclear whether these women are subfertile, as seen in *Ghr* knockout mice.

Female mice that overexpress bovine GH under the control of the strong phosphoenolpyruvate carboxykinase (PEPCK) promoter have a 60% rate of infertility, an increase in the numbers of preovulatory follicles and corpora lutea, and decreased granulosa cell apoptosis in developing follicles.[567,568] The mechanism of GH suppression of apoptosis may involve production of local as well as systemic IGF1[569,570] or poorly understood GH effects that are independent of the IGF1. Female mice that overexpress human GH using the metallothionein I (MT) promoter have a 20% rate of infertility. A similar rate of infertility is observed for bovine GH driven by the metallothionein I promoter.[570] Because PEPCK is a stronger promoter than MT, excess GH is disruptive to reproduction. Infertility in these transgenic mice may be due to inadequate luteal function since progesterone treatment led to pregnancy. In addition, GH-induced overstimulation of the tuberoinfundibular dopaminergic neurons and suppression of endogenous prolactin release could have adversely affected luteal function.[571] Acromegaly, a condition that causes excessive growth due to excess GH secretion, often leads to infertility. Because acromegaly is often caused by adenomas (benign tumors of the pituitary), infertility may be due to hyperprolactinemia as well as gonadotropin dysregulation; this is because the expanding tumor may affect other cell types in the pituitary. Analysis of 196 women with acromegaly revealed that 60% had amenorrhea, and of these 45% had elevated prolactin levels. FSH and LH levels in these women were not reported, but it is possible that GH hypersecretion alone can lead to infertility.[572] Other studies on women with acromegaly have suggested that all three mechanisms may operate: lactogenic effects of prolactin and GH on the gonadotropic axis, gonadotropin dysregulation due to tumor mass effects, and direct GH action on the ovary.[573]

IGF-signaling System

The IGF family has long been recognized as having an important role in female reproduction. The signaling components include two ligands (IGF1 and IGF2), two receptors (IGF1R and IGF2R), and a number of binding proteins (IGFBPs) that regulate IGF action.[574] Of these, IGF1 is known to be important and required for sexual development and reproduction.[575,576] *Igf1* is expressed in granulosa cells of growing follicles, and one receptor, *Igf1r*, is expressed in both growing and atretic follicles.[577,578] In mice, IGF1 levels are low at the primary follicle stage, but increase during preantral and antral follicle growth.[578] The expression of *Igf1* may be under local paracrine control, as microarray analysis has shown that recombinant mouse GDF9 can upregulate expression of the *Igf1* gene.[283,426] *Igf2* expression in the ovary is lower than *Igf1*,[578] and the *Igf1* knockout mouse has reproductive defects, while the *Igf2* null mouse has normal fertility.[269,579] Most *Igf1* null mice die perinatally, but those that survive to adulthood are infertile.[269] Ovaries from *Igf1* knockout mice do not contain antral follicles and cannot be ovulated. As mentioned in this chapter, this defect may be partially due to decreased FSH receptor expression and aromatase expression[268] because *Igf1* knockout mice phenocopy FSHβ and FSHR null mice.

CONCLUSION

Over the past decade, numerous mouse models have been generated that demonstrate the importance of multiple signaling systems in follicle development. These mice provide a basis for understanding mammalian infertility and disease as well as revealing new candidate genes for mutational analysis. Conditional knockouts

using the Cre–LoxP system allow specific cell-type deletions, thereby circumventing problems associated with global knockouts such as embryonic lethality and phenotypes associated with multiple tissue disruption. Combined, these models hold the promise of a comprehensive understanding of the physiology and pathology of human fertility.

Human GWASs on the aspects of reproductive biology, such as the age of menopause and menarche, have additionally opened new vistas into pathways that may play a role in ovarian biology and that should be functionally addressed by basic biologists.[15,580] For example, menopause GWASs discovered 17 loci, with an excess of them associating with DNA repair and immune pathways.[15,581] A number of these loci have not been previously linked to ovarian biology and may represent novel and fruitful pathways for further investigation. A nonsynonymous single nucleotide polymorphism in a minichromosome maintenance deficient 8 gene (*MCM8*) shows the strongest association with menopause, and its corresponding knockout mice have disrupted ovarian development and genome instability due to impaired homologous recombination.[582] Whether all of the associated loci have relevance to ovarian biology remains to be determined via functional studies in the future.

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CHAPTER

22

Ovulation

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INTRODUCTION

Ovulation of an oocyte capable of being fertilized is a primary function of the female gonad, the ovary (Figure 22.1). Ovulation *is a unique biological process* that is so precisely regulated in mammals that less than 1% of all oocytes present at birth are ever released from the surface of the ovary.[1,2] Most ovarian follicles succumb to atresia wherein granulosa cells undergo apoptosis (programmed cell death), oocytes die, and theca cells become part of the ovarian stroma. Thus, ovulation is the ultimate consequence of a follicle being in the right place at the right time. The ovulatory process is orchestrated, in part, by hormonal regulatory loops that control the ovarian–hypothalamic–pituitary axis and by intraovarian regulatory connections operating between theca–granulosa–cumulus cells and the oocyte.[3] Although ovulation is often referred to as "rupture" of the ovulatory follicle, the process is not explosive. Rather, motion pictures of the release of the oocyte surrounded by cumulus cells showed early on that ovulation is a slow extrusion process, a "rather gentle occurrence",[4] and likely occurs by degradation of the follicle wall.[5]

The dramatic events of ovulation are preceded by (1) the formation and development of ovarian follicles, (2) the hormonal-mediated differentiation of granulosa cells and theca cells to functional endocrine tissues, and (3) the acquisition of oocyte competence for meiotic progression and fertilization[6,7] (see Chapters 1, 2 and 21). Thus, at the ovarian level, successful ovulation depends upon specific cellular and molecular events that occur sequentially and precisely during follicular development.

Some of the hormonal, cellular, and molecular events controlling ovulation have been described in detail in previous reviews[1,7] and in earlier editions of the present book.[3,8] However, a brief overview will outline some of the highlights that preceded, paved the way, and provided the impetus for the studies reviewed in this chapter.

LH Receptors

Specifically, studies in the 1960s and 1970s elegantly used radio-immunoassays to quantify the levels of FSH and LH during reproductive cycles in many species and documented that elevated levels of LH are released during the preovulatory surge and initiate ovulation[9,10] (Chapters 26–29). At this time, radio-receptor assays were also developed and showed that not only do hormone levels in serum change dramatically during the cycle but the levels of receptors for these hormones in target tissues are highly regulated. Importantly, the acquisition of LH receptors in granulosa cells of preovulatory follicles is coordinated precisely with the LH surge, ensuring that only the preovulatory follicle can respond.[2,11] These studies of the LH receptor were subsequently integrated by those highlighting the roles of prostaglandins (PGs) and progesterone in ovulating follicles.

Prostaglandins

Studies in the 1980s and 1990s also began to document evidence that an ovulating follicle exhibited physical characteristics of inflammation such as hyperemia and edema.[12] In addition, the ovulating follicles exhibited biochemical features of inflammation including the production of prostaglandins (PGs) PGE1/2 and PGF2α.[13–17] Moreover, some studies indicated that ovulation could

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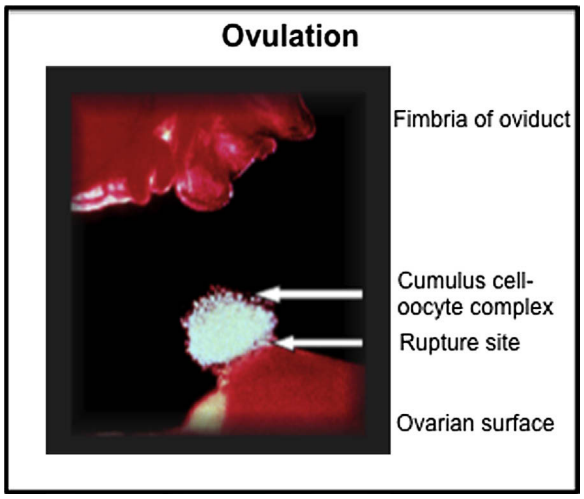

FIGURE 22.1 Moment of rupture of a rabbit follicle. This figure is reproduced in color in the color plate section. Ovulation of a fertilizable oocyte involves disintegration of the ovarian surface epithelium and extrusion of the expanded cumulus cell–oocyte complex (COC) through the ovulation pore. This is followed by retrieval of the COC by the ciliated cells of the oviduct and its subsequent transport down the oviduct where fertilization occurs. (See text.) *Source: Ref. 3.*

be blocked by the nonsteroidal inhibitor of PG biosynthesis, indomethacin.[18] Based on the accumulating evidence in his own laboratory and that of others, Dr Lawrence Espey was the first to describe ovulation as an inflammatory-like response in a key paper in 1980.[12] Since then many studies have supported and expanded this insightful hypothesis and will be highlighted briefly.

First, two immune-distinct forms and molecular weight variants (69 and 72 kDa) of the prostaglandin synthase (PGS), the rate-limiting step in PG biosynthesis, were identified in rat ovarian tissues.[19] These results indicated for the first time that there was not just one enzyme but two enzymes controlling PG biosynthesis. Furthermore, the 72-kDa form was induced specifically in granulosa cells of ovulating follicles by LH whereas the 69-kDa form was constitutively expressed in theca cells and other tissues.[20] Purification of the 72-kDa inducible form of PGS and amino-terminal sequence results documented that the inducible form was distinct from the constitutive form.[21] Ultimately, the genes encoding each distinct protein were cloned and are now known as prostaglandin synthase1, PTGS1 (also known as cyclooxygenase 1; COX-1) and PTGS2 (cyclooxygenase 2; COX-2).[22] Promoter analyses revealed that the *Ptgs2* gene was regulated by several factors including AP1 factors and C/EBPβ.[23] Lastly, targeted disruption of *Ptgs2*[24] or its receptor *Ptger2*[25,26] in mice led to anovulation and infertility, providing conclusive evidence that PGs play critical roles in the ovulation process. However, luteinization occurred in these mutant mice, as indicated by the presence of oocytes entrapped in corpora lutea. This result supported previous data that the role of PGs in ovulation was separable from that of factors controlling luteinization. Based on the knockout mice and in vitro studies we know that (1) PGs are essential for cumulus cell–oocyte complex (COC) expansion, the generation of a hyaluronan-rich matrix, and the stabilization of this matrix by a scaffolding of hyaluronan-binding proteins; and (2) proper COC expansion (also known as mucification) and the composition of the matrix is critical for release of COCs from the surface of the ovary.

Progesterone

Studies from the 1970s to the 1990s also began to document that the ovulation process was dependent on the increased production of the steroid hormone progesterone that occurs following LH stimulation of preovulatory follicles.[27,28] The progesterone synthesis inhibitor epostane blocked ovulation,[29] but this effect could be reversed by exogenous progesterone.[30] A functional role for progesterone in granulosa cells was first established when induction of the progesterone receptor (PGR) in these cells by LH was reported.[31] The essential roles of progesterone and its nuclear receptor were unequivocally documented by targeted disruption of the PGR in mice. The progesterone receptor knockout (PRKO) mice fail to ovulate, contain corpora lutea with entrapped oocytes, and are completely infertile.[32] Molecular analyses comparing control and mutant follicles have documented that progesterone regulates the expression of several genes that are critical for ovulation, including the EGF-like factor amphiregulin (*Areg*), the protease a disintegrin and metalloproteinase with thrombospondin-like motifs-1 (*Adamts1*),[33] and cGMP-dependent protein kinase 2 (*Prkg2*)[34] (discussed later). Overexpression of PGR-A and -B in granulosa cells in culture further documents that PGR-A is more critical than PGR-B and confirms the direct regulation of specific target genes in the absence of LH.[35] Additional studies show that PGR regulates the ADAMTS1 gene and other genes by acting as a co-regulatory factor that binds transcription factors such as SP1.[36] Thus, the role of PGRs in the ovulation process appears to be distinct from that of PGs.

EGF-Like Factors

More recently, the roles of the EGF-like factors, RAS and MAP kinases (ERK1/2), in ovulation have taken center stage.[7] As will be discussed in more detail in the review, LH induces the EGF-like factors that then activate the EGF receptor, RAS, and ERK1/2. Targeted disruption of the EGF receptors or ERK1/2 in granulosa cells completely blocks ovulation, oocyte maturation,

cumulus expansion, and luteinization. These results link the LH-induced cAMP signaling pathway to the EGF receptor tyrosine kinase pathway that has been implicated in inducing *Ptgs2* and other factors.[37]

The Oocyte

The critical role of the oocyte and oocyte-derived factors in controlling cumulus cell functions and ovulation will also be highlighted in the review.

The goal of this chapter is to highlight studies that have been published primarily since 2005 and that have made major impacts on our understanding of ovulation and the complexity of events that occur at the cellular and molecular levels during this process. In particular, this review will focus on new insights into how the LH surge regulates (1) granulosa cell functions, (2) the granulosa cell–cumulus cell–oocyte complex (COC) connection, and (3) ultimately, the cumulus cell–oocyte connections that are obligatory for ovulation. Of particular relevance, recent studies have unveiled how specific genes regulate the unique fates of granulosa cells and cumulus cells in response to the ovulatory LH signal. Granulosa cells eventually become endocrine, progesterone-producing luteal cells. Cumulus cells, on the other hand, provide a conveyance mechanism and protective system for oocytes during their release from the ovary and fertilization.[7]

The hormonal and molecular regulators of ovulation appear to be highly conserved in mammals, including the primates. Therefore our increased understanding of the underlying molecular mechanisms that control ovulation, using primarily knockout mouse models, has significant implications for understanding what controls ovulation in women, how in vitro fertilization (IVF) protocols might be improved, and how infertility-related diseases, such as polycystic ovarian syndrome (PCOS), obesity, and endometriosis, alter ovarian functions and ovulation.[38,39]

OVULATION: A PERFORMANCE PLAYED BY MANY ACTORS

Successful ovulation in response to the LH surge involves multiple cell types. These include the theca cells, granulosa cells, and cumulus cells within the preovulatory follicle itself, the surrounding stroma tissue, and the ovarian surface epithelium (OSE).[3] In essence, the follicle is a multilayered sphere, each layer being separated from the other by a specialized extracellular matrix (Figure 22.2). The theca cell layer is the outmost

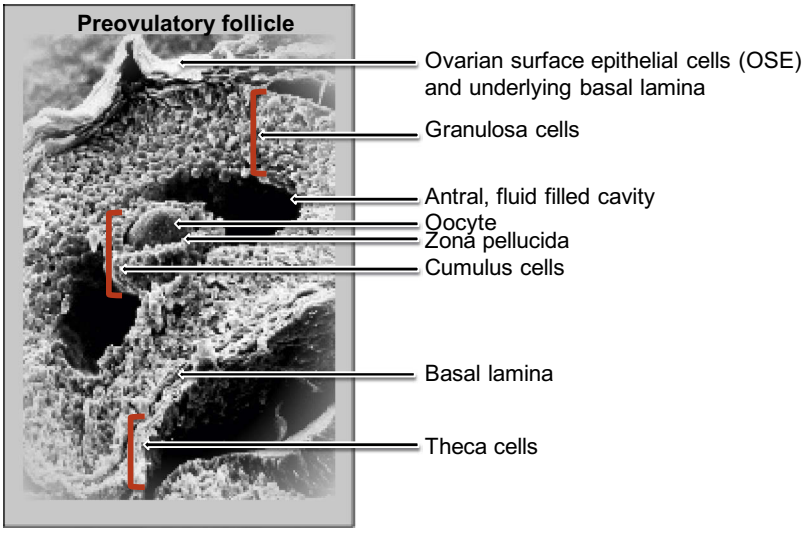

FIGURE 22.2 Cell types and organization within the preovulatory follicle. The preovulatory, antral follicle is composed of several cell types. The ovarian surface epithelium is composed of mesoepithelial cells that face the peritoneal cavity and are separated from the ovarian stroma by a basal lamina. The theca cells are the outermost layer of the follicle and produce androgens. The theca cell layer is separated from the granulosa cells by another extracellular basal lamina. The granulosa cells of preovulatory follicles express receptors for FSH and LH and aromatase (*Cyp19a1*) that converts theca cell-derived androgens to estradiol. The antral cavity is filled with fluid that contains serum components as well as factors secreted from the granulosa cells, cumulus cells, and the oocyte. The cumulus cells are connected directly to the oocyte by gap junctions (Connexin37; GJA4) that permit the transfer of small molecules, such as cGMP, from the cumulus cells to the oocyte. Factors that are produced by oocytes, such as GDP9, BMP15, and FGF8, are secreted from the oocyte into the antral fluid and modify cumulus and granulosa cell functions. The extracellular matrix between the oocyte and the cumulus cells is the zonal pellucida that is composed of specific proteins involved in follicle formation as well as fertilization. The cumulus cell–oocyte complex (COC) is a specific niche in the preovulatory follicle. (See text.)

layer of cells in the preovulatory follicle and is composed of the theca externa and theca interna. The theca externa contains fibroblasts that generate extracellular collagen to provide a semirigid, supportive structure around the follicle. The theca externa is also endowed with an elaborate vascular network that supplies nutrients and oxygen to the follicle. The granulosa cell layer is separated from the theca layer by a thin collagen/laminin-rich basal lamina. This matrix provides an attachment surface for the outermost layer of granulosa cells, the mural granulosa cells. The remaining granulosa cells form a syncytium in which they are interconnected by specialized gap junctions, composed primarily of connexin 43 (GJA1).[40] The cumulus cells, the inner layer of somatic cells, surround the oocyte, forming the cumulus cell–oocyte complex (COC). The cumulus cells are separated from the oocyte by the zona pellucida, a matrix of specific glycosylated zona proteins that serve to protect the oocyte and to provide structure and function during fertilization. The zona also allows for the attachment of cumulus cells and their connections with the oocyte via gap junctions, comprised exclusively of connexin 37 (GJA4), in the COCs that are critical for oocyte development.[41,42] The OSE is derived from cells of a distinct origin from that of the mesenchymal cells or germ cells within the ovary.[43] These OSE cells lay external to a basal lamina and thus face outward toward the peritoneal cavity. The role of these mesoepithelial cells in ovarian function and ovulation is not entirely clear but appears not to be essential for ovulation.[44,45] However, they respond to hormones and can lead to OSE cell cancer, often referred to as "ovarian cancer".[43]

In order for the oocyte and its surrounding somatic cumulus cells, the COCs, to be released from the surface of the ovary and hence ovulated, specific, highly organized, and sequential morphological, biochemical, and molecular changes occur within the preovulatory follicles in response to the LH surge. The major morphological changes that occur in follicles at the time of ovulation have been described in detail previously[3] and therefore will not be elaborated here. However, it is important to note that there are five key events that ultimately permit ovulation to be successful. These are (1) COC expansion, (2) breakdown of gap junctions, (3) resumption of meiosis in the oocyte, (4) release of the COC complex from the surface of the ovary, and (5) luteinization (Figures 22.1, 22.3, 22.4 and 22.5).

COC Expansion

In brief, the compact COC complex present in the preovulatory follicle undergoes the process of expansion also known as mucification (Figure 22.3). In this process, the cumulus cells make an extracellular hyaluronan-rich matrix, migrate outward from the oocyte to fill the antral cavity, and become dissociated from each other. The process of COC expansion and matrix formation is obligatory for successful extrusion of the oocyte from the surface of the ovary, as reviewed recently and outlined briefly below.[1,7,46]

Although the precise mechanisms by which extrusion of the COCs from the ovary is accomplished remain to be determined, the process of COC expansion is dependent on LH induction of selected genes (Figure 22.4(A); step (1), highlighted in red). Specifically, the LH surge rapidly induces expression of hyaluronan synthase 2 (*Has2*), which generates long polymers of hyaluronan. These polymers form the backbone of the COC matrix that is stabilized by a scaffold of hyaluronan-binding proteins and proteoglycans (Figure 22.4(B)).

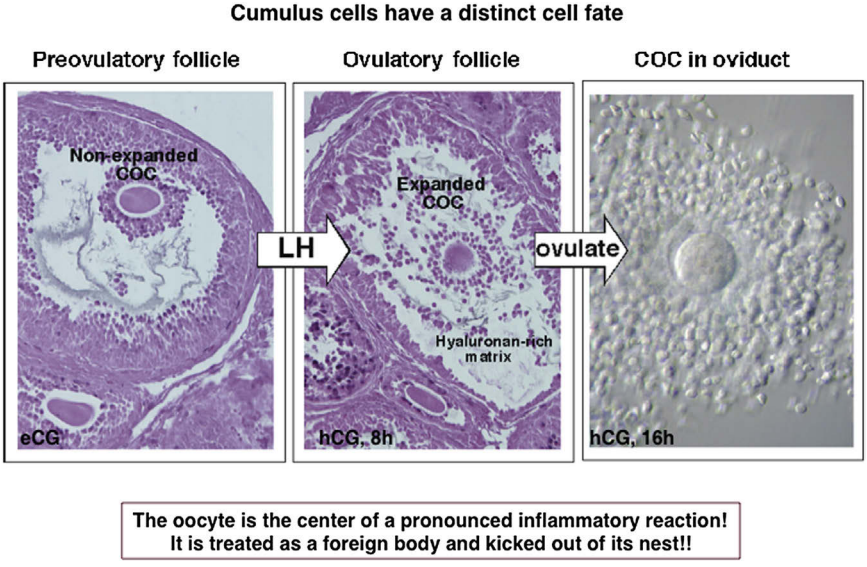

FIGURE 22.3 The LH surge induces expansion of the cumulus cell-oocyte complex (COC). This figure is reproduced in color in the color plate section. Expansion is dependent on the biosynthesis of long polymers of hyaluronan by HAS2. The hyaluronan polymers are stabilized by specific hyaluronan-binding factors including the proteoglycan versican and inflammatory-related factors such as TNFAIP6, IαI, and PTX3. COC expansion is obligatory for release of the enclosed oocyte from the surface of the ovary into the oviduct. (See text.)

LH also rapidly induces expression of the epidermal growth factor-like factors (EGF-like factors), amphiregulin (*Areg*), epiregulin (*Ereg*), and betacellulin (*Btc*). These factors bind the EGF receptor, leading to the induction of prostaglandin synthase 2 (*Ptgs2*) and increased intrafollicular PGs, principally PGE1/2. The EGF-like factors regulate the expression of genes involved in the stabilization of the COC matrix: tumor necrosis factor alpha-induced protein 6 (*Tnfaip6*), pentraxin 3 (*Ptx3*), and the proteoglycan versican (*Vcan*). TNFAIP6 delivers a serum factor IαI to the hyaluronan backbone. PTX3 binds exclusively to TNFAIP6. Versican binds hyaluronan and is the preferred substrate for the protease ADAMTS1. The scaffolding of proteins and the proteoglycan, versican, that bind hyaluronan, act to stabilize the matrix that surrounds the oocyte (Figure 22.4(B)). This matrix is essential for extrusion of the oocyte from the surface of the ovary and its subsequent transit down the oviduct (Figure 22.1).

Gap Junctions and Meiosis

Downregulation and disruption of gap junction proteins facilitate the resumption of meiosis in oocytes by regulating intraoocyte levels of cGMP and cAMP (Figure 22.4(A); steps (2) and (3)) and will be reviewed below.

Ovulation

The LH surge also induces expression of the PGR that, as mentioned, preferentially impacts the process of follicle "rupture" and extrusion of the oocyte more than it impacts COC expansion (Figure 22.4; step (4), highlighted in green). The induction of PGR facilitates the induction of other specific genes encoding the protease ADMATS1, a factor involved in protein secretion, synaptosome-associated factor 25 (SNAP25), cGMP-dependent protein kinase 2 (PRKG2), and endothelin 2.

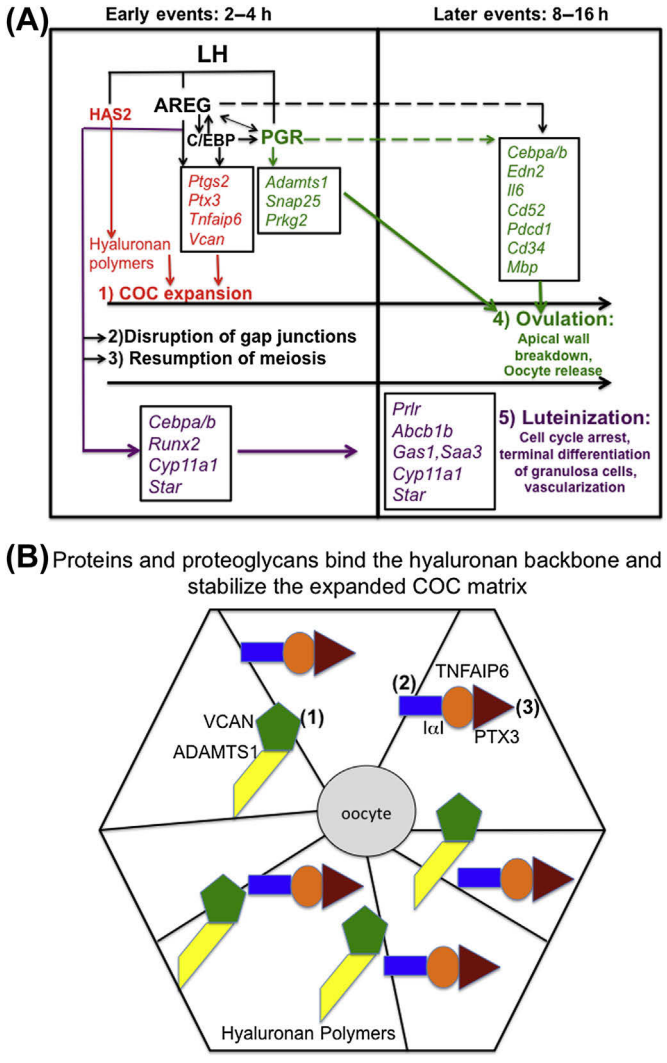

FIGURE 22.4 (A) Early and later events induced by the LH surge. Successful ovulation depends on several sequential events. (1) COC expansion must occur. This is regulated by LH induction of EGF-like factors (AREG), activation of C/EBPα/β, and the induction of specific matrix-stabilizing factors. (2) Gap junctions between granulosa cells and cumulus cells and between the cumulus cells and the oocyte must be disrupted to reduce intraoocyte levels of cGMP. (3) Meiosis is resumed in the oocytes. (4) Release of the oocyte from the surface of the ovary ("ovulation per se") is dependent on proteolytic activity and cytokines. (5) Luteinization is the process by which the granulosa cells terminally differentiate to nondividing luteal cells and begin to produce copious quantities of progesterone. This is dependent on the neovascularization of the avascular granulosa cell layer and occurs following ovulation. (B) Components that control COC expansion. COC expansion is dependent on the production of a hyaluronan-rich extracellular matrix. The long polymers of hyaluronan are stabilized by (1) the binding of the proteoglycan versican (VCAN) and associated ADAMTS1, (2) the TNFAIP6-dependent delivery of IaI, and (3) the binding of PTX3 to TNFAIP6. This scaffold of factors prevents the breakdown of hyaluronan.

Genes that are induced at later times after the LH surge and closer to the time of actual extrusion of the oocyte from the surface of the ovary include cytokines and chemokines as well as other genes whose functions remain to be defined.

At the ovarian surface, the morphological events associated with ovulation involve the dissolution of collagen fibers in the theca layer and the consequent thinning of the surrounding stromal tissue. The vascular network in the theca cells of ovulating follicles becomes highly hyperemic and dilated, indicative of the inflammatory-like activities that are occurring in the somatic cells. The PGs, cytokines, and chemokines that are being released alter vascular functions and appear to recruit immune cells. Ultimately, the basal lamina of the OSE that separates the apex of the follicle from the peritoneal cavity (human and other species) or bursa (mouse, rat) loosens and permits the release of the expanded COCs. The COCs are then picked up by the ciliated cells of the fimbria and transported to the oviduct, where fertilization occurs. Successful release of the oocyte must occur before luteinization; otherwise the oocytes are trapped with the corpora lutea.

Luteinization

The LH surge and the EGF-like factors also induce genes that regulate the process of luteinization (Figure 22.4(A); step (5), highlighted in purple) and terminate the expression of genes regulated by the FSH program. The genes associated with luteinization include the induction and activation of transcription factors C/EBPα/β and RUNX2, steroidogenic enzymes (*Cyp11a1* and *Star*), as well as the prolactin receptor (*Prlr*) and others whose functions remain to be determined. Luteinization occurs following ovulation and is associated with (1) the marked neovascularization of the granulosa cell layer, (2) terminal differentiation of the granulosa cells to nondividing luteal cells, and (3) enhanced steroidogenesis related to increased mitochondrial growth and activity. This rapid and profound development of the vascular network in the newly developed corpus luteum is essential for the delivery of progesterone and other factors needed to support uterine development and implantation.

SETTING THE STAGE: GRANULOSA CELL FUNCTIONS IN PREOVULATORY FOLLICLES

It is now axiomatic that only preovulatory follicles are capable of ovulating in response to the ovulatory surge of pituitary LH. This has been experimentally established in most (all) mammals studied by the evidence showing that granulosa cells in preovulatory follicles selectively express elevated levels of LH receptors that bind LH or hCG.[1,7,47] Thus, preovulatory follicles are the only follicles in the ovary that actually "see" and respond to the LH surge (Figure 22.5). The induction of LH receptor (designated the LH and chorionic gonadotropin receptor, LHCGR) protein and mRNA in granulosa cells of preovulatory follicles depends on (1) the increased sensitivity of granulosa cells to FSH[48,49]; (2) increased expression of *Cyp19a1* (aromatase), production of estradiol, and enhanced activation of the estradiol receptor β (ESR2)[50]; and (3) expression and/or action of activin, a member of the TGFβ family that enhances FSH-induced transcription of the *Lhcgr* gene in granulosa cells but may suppress *Lhcgr* expression in luteal cells.[51,52] In addition to the induction of the LH receptor, granulosa cells of preovulatory follicles produce elevated levels of cAMP in response to LH (and FSH), thereby enhancing activation of the protein kinase A signaling cascade, the canonical pathway downstream of the LH receptor and adenylyl cyclase.[49] This enhanced production of cAMP appears to be critical for initiating the processes of ovulation and luteinization. This is supported by evidence that high levels of FSH or LH or the pharmacological agent forskolin that activate adenylyl cyclase, do not induce ovulation-related genes

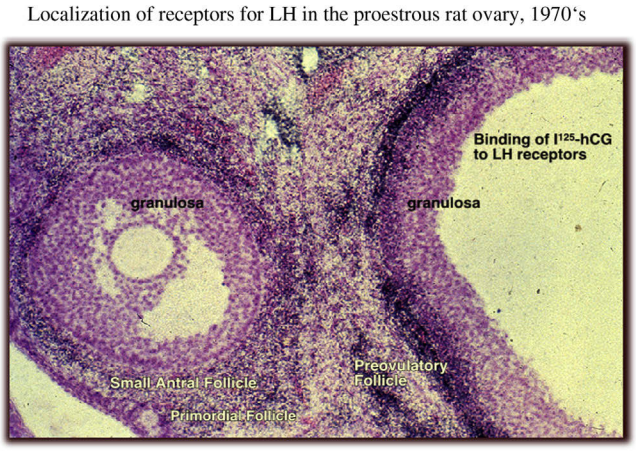

FIGURE 22.5 Induction of LH receptors (LHR; LHCGR) in granulosa cells is the key distinguishing hallmark of preovulatory follicles. This figure is reproduced in color in the color plate section. The presence of LHR in granulosa cells of preovulatory follicles permits them (but not small follicles lacking LHR in granulosa cells) to respond to the LH surge and initiate the molecular programs required for ovulation, COC expansion, resumption of meiosis, and luteinization. This image shows the uptake of radiolabeled hCG (LH) into the theca cells and mural granulosa cells of a preovulatory follicle (right) but only in theca cells of small follicles (left). Primordial follicles do not exhibit detectable uptake of the hormone. Uptake reflects the presence of receptors.

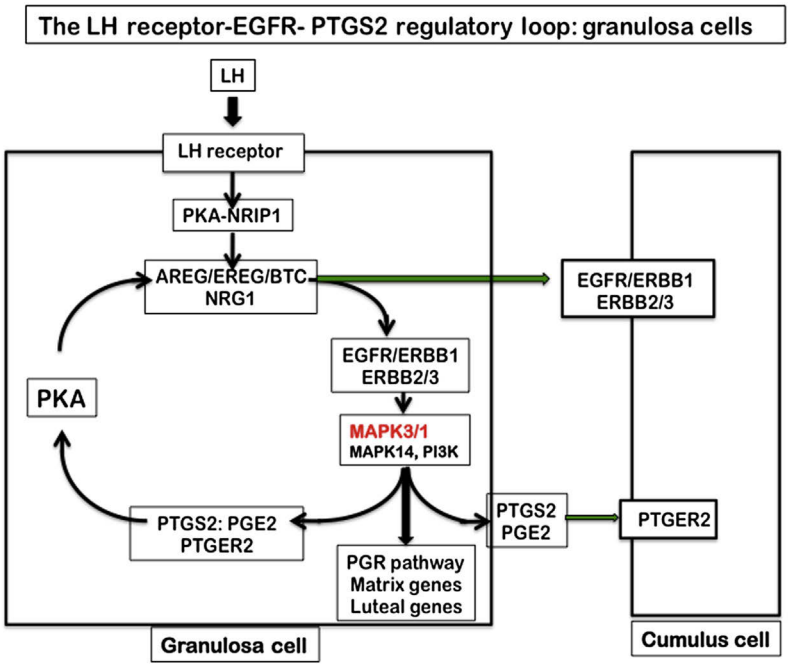

FIGURE 22.6 The LH-EGFR-PTGS2 regulatory loop in granulosa cells. The LH receptor induces expression of the EGF-like factors (*Areg*, *Ereg*, *Btc*, and *Nrg1*) that bind the EGFRs (ERBB1 or ERBB2/3) to activate the MAPK3/1 and PI3K pathways, respectively. Activation of MAPK3/1 leads to (1) the induction of PTGS2, (2) the production of PGE2, (3) activation of the PTGER2 (EP2) receptor, (4) activation of PKA, and (5) further expression of the EGF-like factors. The EGF-like factors and PGE2 produced by this regulatory loop also act by paracrine mechanisms to stimulate similar pathways in cumulus cells (See text and Figure 22.7).

in granulosa cells or cumulus cells of preantral or small antral follicles.[53] Conversely, depletion of phosphodiesterase 4B in granulosa cells and cumulus cells leads to premature elevation of cAMP and luteinization but not ovulation,[54,55] indicating that the level and timing of cAMP production are of the essence in permitting ovulation to occur.

ON-STAGE ACT I: LINKING THE LH RECEPTOR TO OTHER SIGNALING PATHWAYS IN GRANULOSA CELLS

The LH–EGF Receptor Connection

The LH surge not only activates the adenylyl cyclase, cAMP, protein kinase A pathway in granulosa cells but also leads to the activation of other critical signaling cascades (Figure 22.6). Major milestones in our understanding of the molecular events controlling ovulation came when (1) expression of the EGF-like factors (amphiregulin, AREG; epiregulin, EREG, and betacellulin, BTC) and type III neuregulin 1 (NRG1) was shown to be rapidly induced by LH in vivo and in follicle, granulosa cell, and COC cultures[56–58]; (2) expression of hypomorphic alleles or conditional disruption of the EGF receptor (*Erbb1*) pathway in granulosa cells impaired ovulation, oocyte maturation, and luteinization[46,59]; and (3) conditional disruption of the mitogen-activated protein kinases (MAPK3/1) (also known as ERK1/2) blocked ovulation, oocyte resumption of meiosis, and luteinization.[60] The induction of EGF-like factors has been verified in other species, including humans, bovine, porcine, and equine follicles.[61–63] In addition, mice null for the transcriptional co-regulator molecule nuclear receptor interacting protein 1, *Nrip1* (also known as RIP140), are infertile, fail to ovulate, and express low levels of EGF-like factor (*Areg*, *Ereg* and *Btc*) mRNAs.[64] These data suggested that RIP140/NRIP1 might act upstream to regulate the expression of the *Areg* gene.[65] Indeed, recent studies have shown that NRIP1 binds to the *Areg* promoter and enhances forskolin-mediated activation of its promoter activity.[65] The transcriptional effects of NRIP1 are dependent on the recruitment and binding of CREB/JUN factors to a CREB response element in the *Areg* promoter. Importantly, AREG can restore COC expansion in *Nrip1* null mice in vivo but does not restore ovulation, indicating that NRIP1 regulates other factors required for follicle rupture.[65]

Collectively, the data from these mouse models, as well as results from numerous pharmacological studies using selective inhibitors of receptor-kinase (EGFR, MAPK3/1, protein kinase A, PKA) pathways, allow us to link activation of the LH receptor, cAMP, and PKA pathway directly to the induction of the EGF-like factors AREG, EREG, and BTC. These factors, in turn, bind the EGF receptor (ERBB1) and ultimately activate the RAS and MAPK3/1 pathway. The sustained activation of the MAPK3/1 for at least 4 h is critical for COC expansion and expression of *Ptgs2*[66] and may involve the generation of reactive oxygen species that occurs during inflammatory responses.[67] This prolonged activation also appears to be

dependent, at least in part, on the delayed increase in the MAPK3/1-specific dual phosphatase (DUSP6/MKP3).[68] In cumulus cells, the effects of the EGF-like factors are enhanced by oocyte-derived factors (growth differentiation factor 9, GDF9 and bone morphogenic protein 15, BMP15) that increase the expression of the EGF receptor (ERBB1) by a SMAD2/3-dependent pathway.[69]

Another EGF-like factor, type III NRG1, is also induced by LH/hCG, binds to and activates other EGF receptors (ERBB3/ERBB2), and preferentially stimulates the PI3K pathway rather than the MAPK3/1 pathway.[58] However, when NRG1 is combined with AREG, the phosphorylation and activation of MAKP3/1 in granulosa cells is markedly enhanced above that with AREG alone, indicating that these pathways together control distinct events during ovulation. Defining how LH induces the EGF receptor pathways and how they interact has been a major leap forward in our understanding of the molecular and biochemical events that initiate the first steps leading to successful ovulation (Figure 22.6).

Targets of the LH–EGF Receptor Pathway

The LH surge leads to the expression of specific genes that are associated with ovulation, COC expansion, oocyte maturation, and luteinization; and suppresses many genes mediating FSH action in preovulatory follicles (Figure 22.7). Genes associated with ovulation and COC expansion are induced rapidly but are expressed transiently. In the mouse, the expression of many critical genes peaks at 2–4 h and declines by 8 h after the administration of an ovulatory dose of hCG; others are induced between 8 and 16 h.[70] Genes that initiate luteinization occur early, but those that control the formation of the corpus luteum, its vascularization, and its maintenance occur later and are sustained.[71] Once these LH-induced ovulation and luteinization programs are set in motion and the follicle program is turned off, these molecular events cannot be reversed. Thus, in a sense the LH surge is a unique hit-and-run reaction.

Some of the downstream targets of LH and EGF receptor signaling pathways have been identified by gene expression profiles obtained from the *Nrip1*,[64,72] *Mapk3/1*,[60] and *Cebpa/b* mutant mouse models[71]; gene profiling of ovulating follicles by differential display technology[73,74]; gene profiling of mouse COCs during the ovulation process[70]; a rat genome database[75]; a primate genomic database[76]; and the relative expression levels of mRNAs in granulosa cells of the *Nrip1*, *Erbb1*, *Mapk3/1*, and *Cebpa/b* mutant models compared to wild-type controls. Genes whose expression is reduced by disrupting *Nrip1*, *Erbb1*, or *Mapk3/1* include *Pgr*, *Ptgs2*, *Has2*, *Tnfaip6*, *Adamts1*, *Ptx3*, *Prkg2*, *Runx1*, *Runx2*, *Snap25*, and *Nrg1*. Some of these genes, such as *Areg*, *Ereg*, *Btc*, *Adamts1*, and *Snap25*, are also specific targets of *Pgr* as documented in the *Pgr*-null mouse model where ovulation is blocked but luteinization proceeds.[33,35,57,77] Disruption of *Adamts1* also reduces fertility[78,79] (discussed below). By contrast, the expression of versican (*Vcan*), a major extracellular matrix proteoglycan that binds hyaluronan and is the preferred substrate of ADAMTS1, is reduced in the *Nrip1*-null mice but not

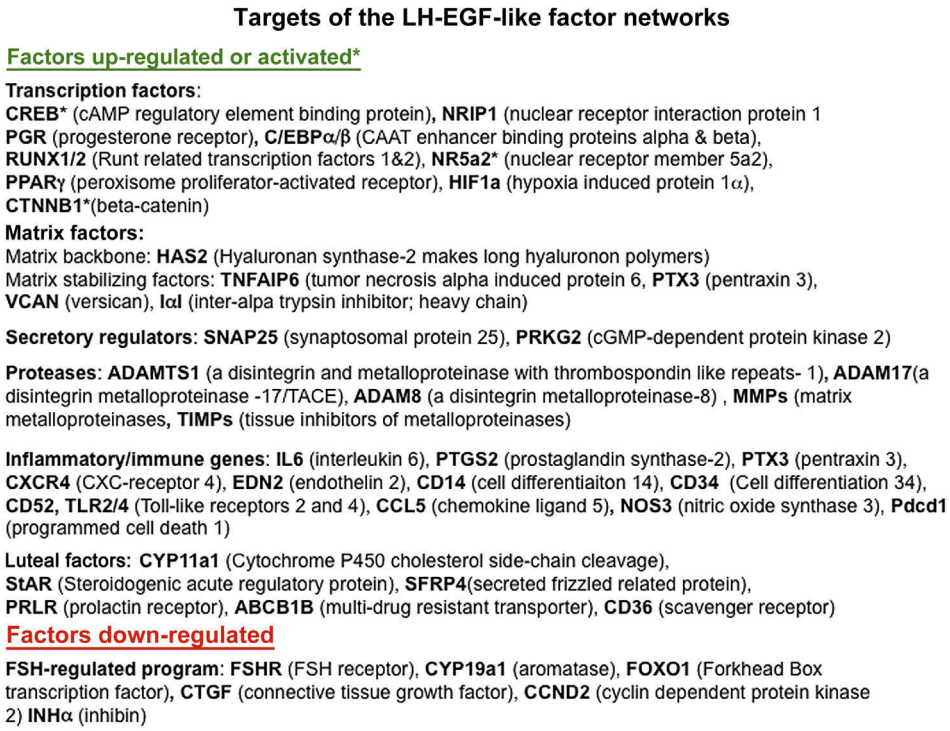

FIGURE 22.7 **Targets of the LH and EGF-like factors in ovulation.** Genes that are induced in granulosa cells and cumulus in response to the LH surge include (1) transcription factors (see also Figure 22.11); (2) components of the COC matrix; (3) factors controlling secretion of peptides, such as cytokines and chemokines; (4) proteases involved in follicle rupture; (5) inflammatory/immune factors that impact COC expansion, ovulation, and fertilization; and (6) genes that maintain luteal cell functions. Factors that are downregulated in granulosa cells by the LH surge are components of the FSH response network that control granulosa cell proliferation and differentiation (including the induction of the LHR) preceding the LH surge.

in the *Pgr* or *Mapk3/1*-null mice.[64] Thus, NRIP1 appears to regulate the expression of *Vcan* and likely additional genes by mechanisms other than, or in addition to, its role in the induction of *Areg*. This may help explain why AREG can restore COC expansion, but cannot induce ovulation, in the *Nrip1* mutant mice in vivo.[65]

Recent studies indicate further that *Nrip1* expression is low in ovaries of immature mice but is increased in response to eCG and is localized selectively to cumulus cells and the inner layer of granulosa cells. This increase likely facilitates the induction of *Areg* in COCs of ovulatory follicles. Conversely, low levels of *Nrip1* in cumulus cells of small antral follicles may be controlled by oocyte-derived factors GDF9 and FGF8, thereby reducing their competence to express *Areg* prematurely.[80] However, this remains to be verified.

The Central Role of ERK1/2 (MAPK3/1)

The expression of the *Lhcgr* gene is not altered in granulosa cells of *Pgr, Nrip1, Erbb1, Mapk3/1*, or *Cebpa/b* mutant mouse models, indicating that it is upstream of these genes. Many genes that are expressed at high levels in granulosa cells of preovulatory follicles and that are normally downregulated as a consequence of the LH surge, remain high in the *Mapk3/1*-mutant mice.[60] These include *Fshr, Cyp19a1, Foxo1, Ctgf, Ccnd2*, and *Inhba*, as well as *Lhcgr* itself.[81] Thus, activation of the MAPK3/1 pathway by LH induction of the EGF-like factors is essential not only *for turning on* genes that regulate ovulation and luteinization but also *for turning off* genes that establish and maintain the preovulatory granulosa cell phenotype. Importantly, this appears to involve activation of C/EBPα/β.[60,81] It is impressive to observe that healthy preovulatory follicles remain present as long as 48 h after administration of hCG to eCG-primed *Mapk3/1*-mutant mice, whereas corpora lutea are fully formed in the wild-type control mice similarly treated. Thus, activation of MAPK3/1 is critical for both ovulation and the terminal differentiation of granulosa cells. It is also important to note that disruption of *Mapk3/1* in the pituitary causes infertility in female (but not male) mice by blocking the LH surge but not basal levels of LH.[82] Thus, the MAPK3/1 pathways in the pituitary and ovary are critical and tightly integrated to control ovulation.

The Multiple Roles of RAS

The selective timing of the RAS/MAPK3/1 activation by LH and EGF-like factors in granulosa cells of preovulatory follicles, but not by FSH in small follicles, appears to be functionally critical for the timely progression of follicular development in vivo. Specifically, when a mutant active form of RAS ($KRAS^{G12D}$) is expressed prematurely in granulosa cells of small follicles, follicle growth is totally derailed. Granulosa cells cease dividing, do not undergo apoptosis, and fail to differentiate. As a consequence, the ovary becomes filled with abnormal follicle-like structures that are devoid of oocytes.[68,83] Thus, activation of the RAS/MAPK3/1 pathway is highly deleterious, if activated prematurely. This illustrates how elegantly this signaling cascade must be orchestrated and controlled to ensure ovulation occurs only in preovulatory follicles and that follicular growth is not disrupted.

ON-STAGE ACT II: SIGNAL CONNECTIONS BETWEEN GRANULOSA CELLS AND THE CUMULUS CELL–OOCYTE COMPLEX DURING OVULATION

COC expansion and the composition of the expanded matrix are critical for successful ovulation (Figures 22.3 and 22.4). Targeted disruption of matrix-related genes *Ptgs2, Ep2, Has2, Adamts1, Tnfaip6, Ptx3*, and *IaI* in mice or in culture impairs COC expansion and the mice are infertile or subfertile.[24,78,79,84–89] If the COC matrix is not made appropriately and lacks the hyaluronan backbone (made by HAS-2) or is not stabilized by the scaffolding of proteins that bind the hyaluronan polymers directly (IαI heavy chain and versican) or to each other (IαI-TNAIP6-PTX3), COCs are not released, and as a consequence the oocytes are trapped inside the nonovulated, luteinized follicles.

The EGF Receptor Loop in COCs

One question that has plagued reproductive endocrinologists for many years is how the LH surge can mediate cumulus cell–oocyte complex expansion. This query was posed because, in most species studied (including humans),[47] LH receptors are highly and selectively expressed in the outer layer of mural granulosa cells but are either absent or low in cumulus cells (Figure 22.8). This perplexing puzzle was resolved relatively quickly after reports by Espey et al.[73], Conti et al.[56], and Shimada et al.[57] showed that the LH surge or exogenous hCG induces the EGF-like factors, *Areg, Ereg*, and *Btc* in granulosa cells of ovulatory follicles. These in vivo observations supported previous in vitro studies showing that EGF itself could impact cumulus cell functions and oocyte maturation in several species. However, the physiological relevance of EGF was difficult to explain because this ligand is not expressed in granulosa cells.

The induction of the EGF-like factors by LH provided the first impressive physiologically relevant evidence showing that endogenous ligands for the EGF receptors were induced and expressed in ovulating follicles.

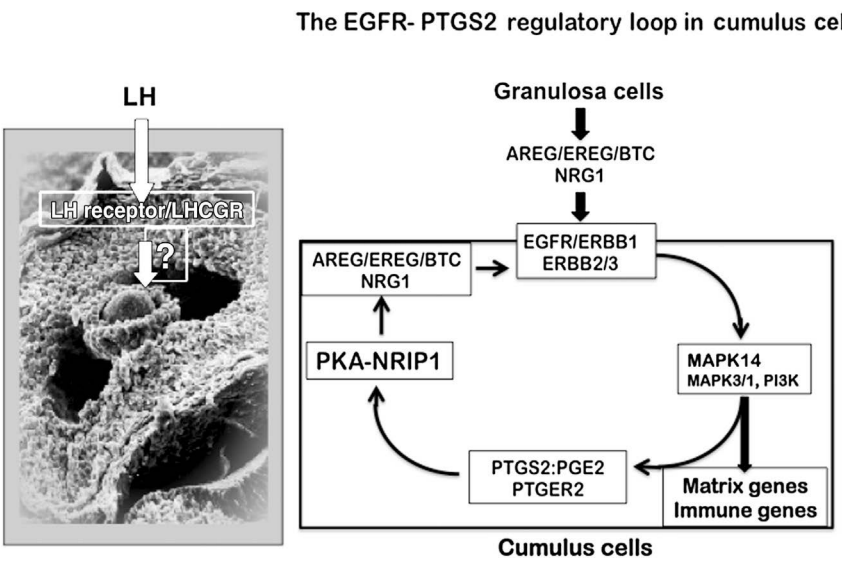

FIGURE 22.8 The EGFR-PTGS2 regulatory loop in cumulus cells. Once the EGF-like factors are secreted from the granulosa cells in response to the LH surge, they bind to EGF receptors present on the surface of cumulus cells and initiate an autocrine regulatory loop in the cumulus cells. The EGF-receptors activate MAPK14 (and MAPK3/1), leading to the induction of PTGS2 and the production of PGs (PGE2). PGE2 binds the PG receptor PTGER2 (EP2) and stimulates the production of cAMP, which activates protein kinase A (PKA). This in turn leads to the induction of the EGF-like factors within the COC. NRG1 activates the PI3K pathway in cumulus cells that impact the resumption of meiosis (See text).

Cleavage enzymes, such as TACE/ADAM17, that activate the EGF-like factors are also induced and critical for EGF-like factor functions.[90] The rapid induction of AREG, EREG, and BTC as well as type III NRG1 by LH (or by its analog hCG) functionally links activation of the LH receptor in granulosa cells to LH-mediated actions in cumulus cells where LH receptor levels are low[47,70] (Figure 22.8). When these EGF-like factors are added to follicles and COCs in culture in the absence of LH, they stimulate COC expansion and thereby convincingly bypass the need for LH or other cAMP-inducing agents.[57,91,92] Data from several laboratories show that AREG stimulates COC expansion in cultures of preovulatory follicles and in intact COCs by inducing genes known to be obligatory for the COC expansion process and ovulation.[46,57]

The EGF-Like Factor PTGS2 Regulatory Loop

Genes that are induced by the EGF-like factor AREG include *Ptgs2*, *Tnfaip6*, *Adamts1*, *Ptx3*, and *Pgr*. Furthermore, mice expressing a hypomorphic allele for the EGF receptor are subfertile, and mice in which the *Erbb1* gene is disrupted selectively in granulosa cells using *Cyp19*-Cre mice are infertile.[46,59] Both of these *Erbb1*-mutant mouse models exhibit impaired COC expansion and oocyte maturation. Type III NRG1 alone does not induce COC expansion, likely because it does not activate MAPK3/1.[58] However, when AREG and NRG1 are combined, expansion is enhanced, and the expression of *Tnfaip6* is significantly increased. Conditional disruption of *Nrg1* also impairs fertility by altering the onset of oocyte maturation and reducing embryo development, but the phenotype is less severe than that of *Erbb1*-mutant mice (Shimada, unpublished data).

Of further critical importance to successful ovulation is the establishment of an autocrine regulatory loop between AREG and PTGS2 in COCs (Figure 22.8). AREG induces *Ptgs2*, leading to increased production of PGs.[57] PGE2, by binding to its cognate EP2 G-protein coupled receptor, activates adenylyl cyclase and cAMP production, leading to transactivation of *Areg*, *Ereg*, and *Btc*.[57,93] This regulatory loop allows the COCs to function independently of LH and the LH receptor to control genes obligatory for matrix formation and matrix stabilization such as *Has2*, *Tnfaip6*, *Ptx3*, and *IaI*.[57,87,88,94] This regulatory loop in COCs also appears to be dependent, in part, on the oocyte and oocyte-derived factors such as GDF9 and BMP15 and the SMAD2/3 pathway.[69,95] This same autocrine regulatory loop also may be operative in granulosa cells, where activin acting via SMAD2/3 may substitute for GDF9 and BMP15, thereby maintaining AREG production in these cells where genes controlling luteinization are preferentially induced.

PGs (PGE2) can also directly impact mouse and human oocytes by binding to EP2 (PTGER2) and EP4 (PTGER4) receptors present in these germ cells, increasing cAMP levels and delaying oocyte maturation.[96,97] Although PTGS2 (COX-2) inhibitors have been found to block ovulation in rats, results of their effectiveness for contraceptive use have been variable and timing is critical.[98,99]

The MAPK14-PTGS2 Connection in COCs

Another kinase, *Mapk14* (p38MAPKα) (Figure 22.8), plays a modulatory role in regulating COC expansion.[100] Targeted disruption of *Mapk14* in granulosa and cumulus cells does not impair fertility, but COCs lacking *Mapk14* fail to expand in culture in the presence of PGE2 or FSH, and expression of *Areg*, *Ereg*, *Ptgs2*, *Tnfaip6*, and *Ptx3*

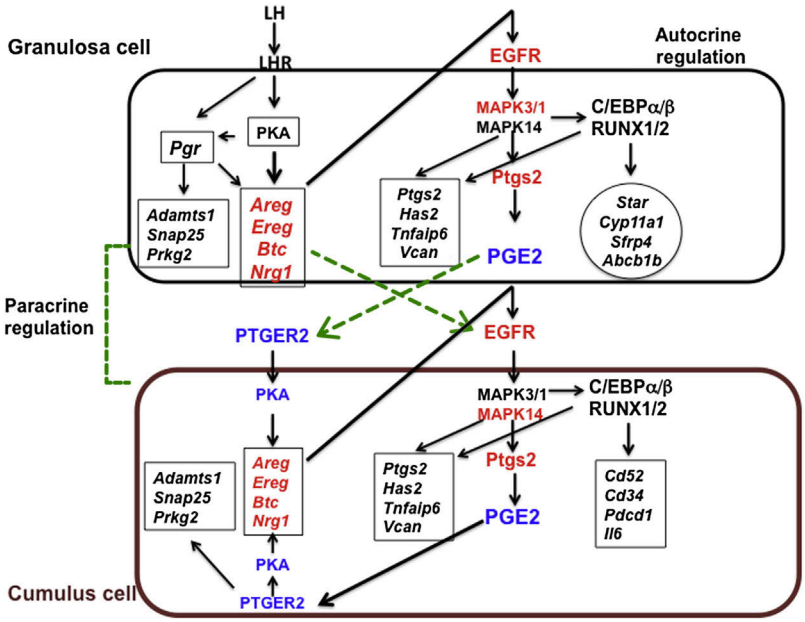

FIGURE 22.9 Schematic of the autocrine and paracrine networks that are initiated by the LH surge and coordinate the functions of granulosa cells and cumulus cells. The LH surge activates its cognate receptor leading to the production of cAMP and activation of PKA. This leads to the induction of specific genes including PGR and the EGF-like factors. The EGF-like factors activate their receptors and MAPK3/1 leading to the induction of matrix and inflammatory genes including PTGS2. The PGE2 that is produced following the induction of PTGS2 by the MAPK3/1 pathway and the EGF-like factors then act in a paracrine manner (dashed arrows) to stimulate an autocrine regulatory loop in cumulus cells that leads to the induction of matrix and immune-related genes.

mRNAs is markedly reduced. Expansion can be restored in the *Mapk14*-mutant COCs by adding AREG. Therefore, MAPK14 is required for mediating PGE2 and FSH induction of *Areg*, and hence genes that are downstream of AREG, in cumulus cells.[100] Confirming the importance of MAPK14, MAPK14 inhibitors block the induction of COC expansion by PGE2 in wild-type COCs.

On the other hand, *Areg* expression is *increased* in granulosa cells lacking *Mapk14*, suggesting that in these cells MAPK14 may suppress the cAMP-PKA pathway.[100] Thus, granulosa cell production of AREG appears to compensate in vivo for the lack of AREG production in the *Mapk14*-mutant cumulus cells.

The autocrine and paracrine signaling networks that are induced by the LH surge and control both granulosa cell and cumulus cell functions are summarized in Figures 22.8 and 22.9.

Nongenomic Processes that Control COC Expansion

COC expansion involves the migratory activity of cumulus cells.[101] This activity is mediated by nongenomic events that are regulated by (1) the EGF receptor-MAPK3/1 pathway and (2)[101] an EGF receptor-calcium dependent mechanism. This has been elegantly demonstrated in studies that examined the mechanisms by which cumulus cells detach from each other and migrate within the matrix and away from the oocyte.[102] Specifically, the EGF receptor-MAPK3/1 pathway and EGF receptor-calcium pathways act coordinately to activate specific proteases known as calpains. These proteases disrupt cell–cell adhesion by cleaving and degrading specific components of focal adhesion complexes, such as paxillin and talin 1. Inhibition of calpain activity significantly reduces COC expansion by impairing cumulus cell migration in vivo and in culture. Thus, cumulus cells, like cancer cells, exhibit migratory behavior that is facilitated by the calpain-mediated loss of cell–cell adhesion.

Other nongenomic events also occur during ovulation. These include the disruption of functional gap junctions between granulosa cells and between cumulus cells and the oocyte.[103] The inactivation of gap junctions is mediated, at least in part, as a consequence of MAPK-mediated phosphorylation of connexin 43 in granulosa cells and connexin 37 in cumulus cells.[46,59,104] This prevents the transfer of substances such as cGMP from cumulus cells to the oocyte. This transport system is critical because cGMP prevents oocyte maturation by inhibiting the specific phosphodiesterase PDE3A, thereby keeping oocyte levels of cAMP elevated.[104–107] As a consequence, oocytes remain arrested in meiotic prophase I.

The cGMP Regulatory Pathway in COCs

The cGMP present in oocytes is generated by the combined functions of granulosa cells and cumulus cells (Figure 22.10). This pathway is comprised of the natriuretic peptide precursor C (NPPC) and the guanylyl cyclase receptor 2 (NPR2). NPPC that is produced and secreted by granulosa cells binds NPR2 that is present primarily in cumulus cells.[108–110] The cGMP that is produced in cumulus cells is then transferred to oocytes. The LH surge not only disrupts gap junctions but also reduces the expression of *Nppc*[111] and NPR2,[109] leading to decreased activation of guanylyl cyclases (NPR2) and reduced levels of cGMP in cumulus cells and the

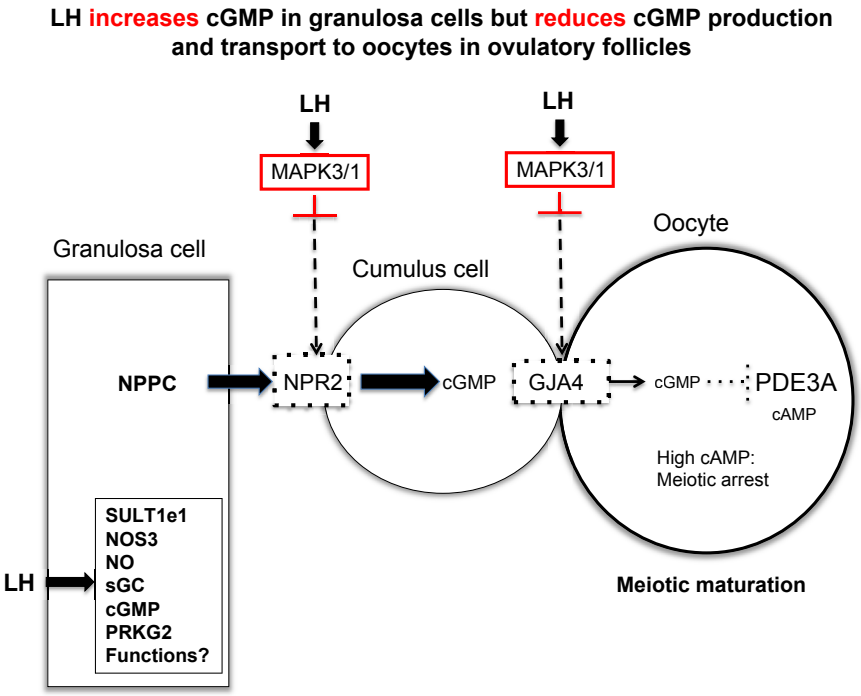

FIGURE 22.10 Regulation of cGMP production in granulosa cells and cumulus cells. In preovulatory follicles, the natriuretic peptide precursor C (NPPC) activates the natriuretic receptor 2 (a receptor linked guanylyl cyclase), leading to increased cGMP production in cumulus cells. cGMP produced by cumulus cells is transported by GAP junctions (GJA4) to the oocyte, where it inhibits the phosphodiesterase 3A, leading to high intraoocyte levels of cAMP. High cAMP prevents the resumption of meiosis. In response to the LH surge and activation of MAPK3/1, expression of NPR2 is reduced and GAP junctions are phosphorylated and inactivated. As a consequence, cGMP levels in the oocyte decrease, PDE3A activity increases, cAMP levels decline, and meiosis resumes. By contrast, the LH surge increases cGMP in granulosa cells by inducing nitric oxide synthase 3, production of nitric oxide (NO), and activation of soluble guanylyl cyclases in the granulosa cells. The coordinate induction of cGMP-dependent protein kinase 2 (PRKG2) suggests that this pathway controls specific functions in granulosa cells.

oocyte.[112] Reduced levels of cGMP in oocytes leads to increased activity of PDE3A and lower levels of cAMP. This then permits the resumption of meiosis of ovulatory follicles (Figure 22.10) (See Chapters 1, 2).

In granulosa cells, another pathway can generate cGMP (Figure 22.10). This pathway is comprised of nitric oxide synthase 3 (NOS-3) that is induced by the LH surge and generates nitric oxides that activate soluble guanylyl cyclases and produce cGMP[104] (see below). cGMP-dependent protein kinase II (cGKII or PRKG2) is also induced by LH in a PGR-dependent manner.[34] Thus, PRKG2 may play a critical role in granulosa cells of ovulatory follicles by mediating the effects of cGMP; however, the precise functions of PRKG2 in ovulatory granulosa cells remain to be resolved.

New Roles for Progesterone

Nongenomic actions of progesterone may also impact somatic cell and oocyte functions during ovulation and oocyte maturation, and these effects of progesterone may be mediated by specific membrane-related receptors that have been identified and localized to preovulatory follicles.[113–115] However, the exact functions of these proteins remain to be determined.

ON-STAGE ACT III: THE OOCYTE REIGNS AT CENTER STAGE

The oocyte itself impacts the function of the surrounding somatic cells during all stages of follicular development and thus is the central player in the follicle. By releasing GDF9, the oocyte in primary follicles organizes the stromal precursor cells to form the theca cell layer.[116,117] In preovulatory follicles, GDF9, BMP15, and FGF8 modulate the functions of cumulus cells by suppressing the expression of the LH receptor and steroidogenesis.[118] However, GDF9 and BMP15 are also essential for facilitating proper formation and stabilization of the matrix during COC expansion. Specifically, GDF9 and BMP15 modulate the expression of *Ptgs2*[95] as well as the innate immune-like factor *Ptx3* that binds and stabilizes TNFAIP6.[88,119] The oocyte also regulates the metabolic functions of cumulus cells to ensure that proper nutrients are supplied to the oocyte.[118,120] Thus, the oocyte is not a passive passenger in COCs prior to or during transport to the oviduct. Rather the oocyte is an active player that helps to direct its own fate throughout follicular development.[121]

ON-STAGE ACT IV: KEY TRANSCRIPTION FACTORS THAT IMPACT OVULATION

Ovulation involves dramatic changes in granulosa cell and cumulus cell functions. Therefore, specific transcription factors are likely to be the targets of the hormone, cytokine, and kinase-activated pathways that coordinate the critical events controlling ovulation (Figures 22.9 and 22.11). As already discussed, the nuclear PGR and the receptor co-regulator NRIP1 play essential roles in ovulation; their disruption causes infertility and ovulation failure.[33,65] *Pgr* is induced by the LH surge by

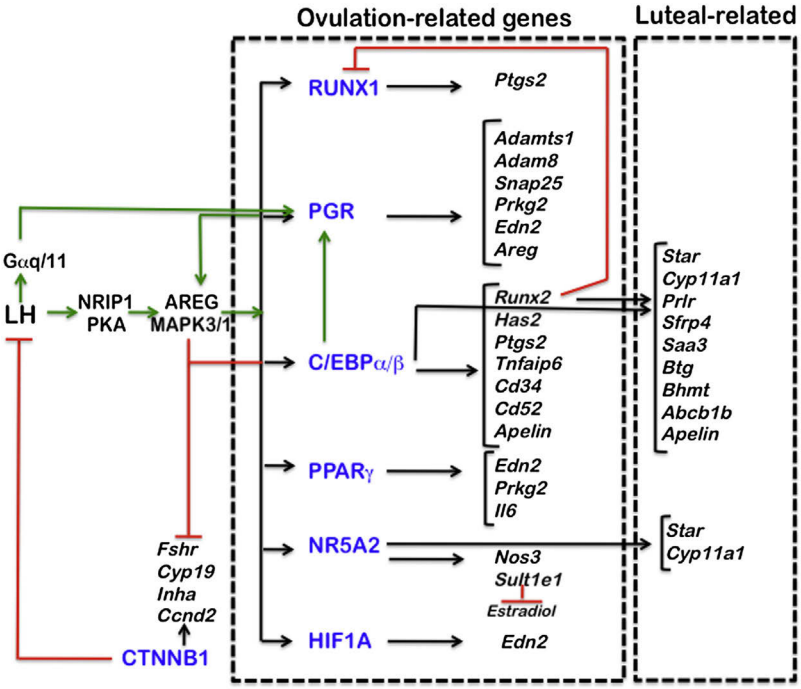

FIGURE 22.11 Transcription factors that mediate LH actions in ovulation and luteinization. The LH surge induces and activates (*) specific transcription factors in granulosa cells. These include (1) RUNX1, which impacts the expression of *Ptgs2*; (2) the nuclear progesterone receptor (PGR), which regulates the expression of the EGF-like factor *Areg*, proteases (*Adamts1, Adam8*), secretory factors (*Snap25, Prkg2*), and endothelin 2 (*Edn2*); (3) C/EBPa/b, which enhances the expression of matrix factors (*Has2, Tnfaip6*), inflammatory factors (*Il6, Cd34, Cd52*), and factors related to luteinization (*Runx2, Star, Prlr, Cyp11a1, Sfrp4*), and suppresses genes mediating the FSH program; (4) RUNX2, which suppresses RUNX1 and regulates genes associated with luteinization, and PPARγ, which regulates genes overlapping with those regulated by PGR; (5) NR5A2, which regulates cGMP production in granulosa cells, and estradiol levels, and also impacts genes associated with luteinization; (6) CTNNB1, which facilitates expression of *Fshr* and aromatase but blocks expression of the LH receptor; and (7) HIF1α, which regulates genes associated with vascularization.

the activation and binding of SP1 to the *Pgr* promoter.[122] Recent studies also indicate that LH induction of PGR requires not only the PKA pathway but also the activation of Gαq/11 pathway.[123] These results support the synergistic actions of forskolin (PKA) and PMA (PKC) in the induction of *Pgr*,[122,124] *Adamts1*,[36] and *Prkg2*.[34] Thus, the LH surge appears to activate not only Gαs and adenylyl cyclase but also Gαq/11 and the inositol phosphate-PKC pathway.

PGR regulates specific target genes, most notable of which are *Adamts1*,[33,36] *cathepsin l*,[33,125] *Adam8*,[126] *Snap25*,[77] *Prkg2*,[127] *Pparg*,[127] and *Edn2*.[128] PGR regulates these genes by interacting with other transcription factors. For example, PGR interacts with C/EBPβ, NF1, and SP1/SP3 to activate the *Adamts1* promoter,[36] and interacts with SP1 to regulate the *Snap25* promoter.[77] In this context, PGR acts more like a transcriptional co-regulatory factor than a transcription factor binding to PGR consensus DNA response elements. NRIP1 appears to be an essential co-activator of the *Areg* gene by binding to CREB/JUN factors on the *Areg* promoter.[65] One would therefore predict that targeted disruption of CREB and specific members of the AP1 family using *Pgr-Cre* mice would also exert detrimental effects on the induction of *Areg* and hence the ovulation process.

C/EBPα and C/EBPβ

Several other transcription factors impact ovulation. Notable among these are the CCAAT enhancer-binding proteins alpha and beta (C/EBPα/β) that are phosphorylated and activated by MAPK3/1.[71,129] Sterneck et al. showed originally (1997) that mice null for *Cebpb* are infertile and do not ovulate.[130] However, these mice suffer from many other maladies. Therefore, the *Cebpb* and *Cebpa* genes were conditionally disrupted in granulosa and cumulus cells using *Cyp19-Cre* mice.[71] The *Cebpa/b* double mutant mice are infertile and anovulatory, whereas either mutant alone is fertile or subfertile. Gene profiling of granulosa cells obtained from the *Cebpa/b* mutant mice revealed a novel set of genes that are mostly downstream of MAPK3/1 and are involved in ovulation and luteinization.[71] Genes that may control the neovascularization of the ruptured, ovulated follicle appear to be major targets, along with *Runx2*, specific transporters, the prolactin receptor *Prlr*, and the acute-phase marker serum amyloid A, *Saa3*.[131] The betaine–homocysteine S-methyltransferase *(Bhmt)* gene was highly downregulated in the *Cebpa/b*-mutant mice, suggesting that methionine metabolism might be critical for ovulation or luteal cell function. Although pharmacological disruption of BHMT reduced progesterone biosynthesis in luteal cells of wild-type mice, the *Bhmt* null mice are fertile.[132] Thus, the functional significance of this gene, and other novel C/EBPα/β-regulated genes remains to be determined.

Induction and activation of C/EBPα/β are also critical for mediating LH/ERK1/2-mediated suppression of genes regulating the FSH-driven program. For example, C/EBP activation suppresses expression of genes encoding *Fshr, Ccnd2, Cyp19a1*, and *Inha*.[60,81]

RUNX1 and RUNX2

The Runt-related transcription factors 1 and 2 (*Runx1* and *Runx2*) are induced rapidly in granulosa cells and

cumulus cells prior to ovulation and luteinization.[70,75,133] Although[134] targeted disruption of these genes in mice has not yet been reported, siRNA for *Runx2* transfected into rat granulosa cells causes the levels of *Runx1* and *Ptgs2* to increase.[135] Because RUNX1 increases the expression of *Ptgs2*, *Ptgs2* appears to be a specific target of RUNX1 but not RUNX2 in vitro. Conversely, RUNX2 regulates the expression of *Runx1* and other genes, some of which are also targets of C/EBPα/β.[136] RUNX2 also induces BTG, a gene that may regulate granulosa cell proliferation during the transition to nondividing luteal cells. That *Runx2* is reduced in the *Cebpb*-null mice and *Cebpa/b*-mutant mice[71] indicates that C/EBPα/β regulate the expression of the *Runx2* gene and may coordinate with RUNX2 to control expression of luteal-related specific genes such as *Abcb1b, Btg*, and *Spp1*.The importance of these genes in ovulation and luteinization remain to be determined.

The Orphan Nuclear Receptor NR5A2

Targeted disruption of the nuclear receptor *Nr5a2* gene also impairs ovulation and luteinization.[137] NR5A2 (also known as liver receptor homolog1, LRH1; Steroidogenic factor 2, SF2; Fushi tarazu F1, FTF) is a close relative of NR5a1 (also known as Steroidogenic factor 1, SF1). NR5A2 is expressed at elevated levels in granulosa cells of growing and antral follicles,[138] whereas NR5A1 is preferentially expressed in theca and stroma cells as well as luteal cells.[138] That disruption of *Nr5a2* impairs ovulation and luteinization more than follicular growth is somewhat surprising and indicates that the functions of NR5A2 in preovulatory follicles are distinctly different from those in growing follicles or the embryonic gonad.[139] Because NR5A2, like C/EBPβ, is phosphorylated by MAPK3/1[140] and MAPK14,[138] the functional activity of NR5A2 may be altered such that it is recruited to, and activates, different target genes in preovulatory granulosa cells following the LH surge. Ovulatory follicles in *Nr5a2*-mutant mice exposed to LH/hCG exhibit impaired expression of matrix-related genes *Ptgs2, Tnafip6*, and *Cd44*.

In addition, NR5A2 regulates transcription of nitric oxide synthase 3 (*Nos3*), which produces nitric oxide (NO). NO activates soluble guanylyl cyclase, leading to increased levels of cGMP. The expression of NOS3 and NO appear to negatively regulate expression of aromatase (*Cyp19a1*) and reduce estradiol production in ovulating follicles.[137] The mechanisms by which NO blocks *Cyp19a1* expression are not known, but may involve NO activation of soluble guanylyl cyclase, cGMP, and PRKG2, which represent a functional signaling pathway in ovulatory follicles.[34,104] The loss of *Nos3* in the *Nr5a2*-conditional knockout mice is also associated with reduced expression of estrogen-specific sulfotransferase (*Sult1e1*), which inactivates estradiol.[137] These combined events lead to prolonged, elevated levels of estradiol and appear to contribute to the impaired ovulation in the *Nr5a2*-mutant mice.[137] The critical role of reducing estradiol in ovulating follicles is supported by the evidence that total loss of *Sult1e1* in mice leads to elevated levels of estradiol, reduces expression of *Ptgs2* in cumulus cells, reduces COC expansion, and impairs ovulation.[141] Estradiol also promotes the expression of NRP2, which leads to increased cGMP, thereby blocking oocyte maturation.[142]

NR5A2 is also critical for the induction of luteal-specific genes, including *Cyp11a1* and *Star*, that control progesterone biosynthesis.[137]

Peroxisome Proliferator-Activated Receptor Gamma

Peroxisome proliferator-activated receptor gamma (*Pparg*) appears to be a target of the PGR in ovulatory follicles. Conditional disruption of the *Pparg* gene in granulosa cells of preovulatory follicles in vivo using *Pgr-Cre* impairs fertility, reduces ovulation, and lowers the expression of specific genes (*Edn2, Prkg2*, and *Il6*) that are also targets of PGR.[143] Of note, however, PPARγ does not regulate the expression of *Adamts1*, a highly specific target of PGR in vivo and in culture. Thus, PGR and PPARγ share some overlapping and coordinate functions as well as some distinct functions in ovulating follicles. The precise mechanisms by which PPARγ regulates ovulation remain to be more clearly documented.

Hypoxia-Related Events

Hypoxia is presumed to occur at the time of ovulation, leading to the increased expression and activation of the transcription factors HIF1α and HIF1β.[143,144] These factors rapidly and transiently induce the expression of specific genes including endothelin 2 (*Edn2*). EDN2 is presumed to bind one of the endothelin receptors EDNR1 or EDNR2 present in theca cells to stimulate smooth muscle contractions, leading to release of the expanded COCs.[128,145] Although EDNR1 and EDNR2 are expressed in granulosa cells, EDNR2 is not required for ovulation. Rather, disruption of the EDNR2 genes leads to increased numbers of corpora lutea.[146] However, disruption of *Edn2* does impair ovulation.[147]

The WNT/FZD/CTNNB1 Pathway

The canonical WNT/Frizzled (FZD) pathway activates beta catenin (CTNNB1) and modulates embryonic gonadal sex differentiation, follicle growth, and ovulation.[148] These results indicate that the expression and regulation of WNT ligands, FZD receptors, and

CTNNB1 are precisely controlled in ovarian follicles at multiple stages of development. Of relevance to ovulation, mice expressing a constitutively active form of CTNNB1 lacking exon 3 (*Ctnnb1*^{ex3}) are subfertile.[149,150] In preovulatory follicles of these *Ctnnb1*^{ex3}-mutant mice, FSH-mediated expression of selected target genes, *Fshr, Cyp19a1*, and *Ccnd2*, is enhanced, indicating that CTNNB1 promotes the preovulatory granulosa cell phenotype. Despite these positive actions of CTNNB1^{ex3}, the *Ctnnb1*^{ex3}-mutant mice exhibit ovulatory defects that are associated with reduced expression of the LH-induced, ovulatory-related genes *Areg, Ptgs2, Tnfaip6*, and *Ptx3* as well as *Lhcgr*.[149] The mutant stable CTNNB1 appears to act, in part, by reducing the binding of CREB to the *Areg* promoter. However, the mechanism by which this is mediated remains to be determined. By contrast, conditional disruption of the *Ctnnb1* gene in granulosa cells does not impair fertility despite the fact that expression of *Cyp19a1* is reduced and of *Lhcgr* is enhanced.[150]

ON STAGE ACT V: KEY PROTEASES IN OVULATION

Ovulation involves and requires the breakdown of the basal lamina separating mural granulosa cells from theca cells as well as the dissolution of the basement membrane underlying the OSE.[3,8] This "thinning" of the ovulatory follicle wall and the surface epithelium ultimately permits the extrusion of the COCs through the ovulation pore. This latter event appears to depend, in part, on EDN2 and smooth muscle contractions within the theca layer[128] (see above). The former events appear to involve various proteases and tissue protease inhibitors, the list of which is long and the functions of which are complex.[151] Despite much research, data from knockout mice lacking various proteases have shed little insight into which ones might be the key players close to or at the time ovulation. However, protease inhibitors can block ovulation, as shown recently in primates.[152] An outstanding and comprehensive review has covered this topic recently; therefore only one protease will be discussed here.[151,153]

As indicated above, *Adamts1* is rapidly and markedly induced in granulosa cells of preovulatory follicles by the LH surge and in a PGR-dependent manner. Not only does the temporal expression of ADAMTS1[36,154] and its control by PGR[33] and possibly CTGF[155] suggest its key role in ovulation, but also the functional activities of ADAMTS1[156,157] indicate that it is a key protease related to ovulation. Mice null for *Adamts1*, as well as mice in which *Adamts1* has been conditionally disrupted in granulosa cells, are subfertile.[79] The proteoglycan versican is one of the preferred substrates for ADAMTS1 and is a key component of the extracellular matrix in expanded COCs and in the follicle wall. In the absence of ADAMTS1, both the invasion of the theca cell layer into the granulosa cell layer as well as the structure of the COC matrix are abnormal. How these defects ultimately cause subfertility and reduce release of COCs remains to be more clearly defined.[158]

ON STAGE ACT VI: CUMULUS CELLS HAVE IMMUNE-LIKE FUNCTIONS AND A DISTINCT FATE

Cumulus cells are distinct from granulosa cells in many ways. As indicated, they are in close physical contact with the oocyte and thereby are exposed to high levels of oocyte-derived regulatory factors that control their function. The oocyte-derived factors, such as GDF9, BMP15, and FGF8, act singly or in combination to suppress cumulus-cell expression of the LH receptor (*Lhcgr*) and steroidogenic genes and promote the expression of other genes in cumulus cells.[120,159] Cumulus cells also have a distinct fate (Figure 22.3). During COC expansion, they are embedded within the expanded extracellular matrix, produce many cytokines and inflammatory factors, and are released from the ovary along with the oocyte at the time of ovulation.[70,160] Importantly, factors within the matrix also control cumulus-cell functions.[161,162] Thus, each COC is a highly specialized microenvironment with inflammatory-like responses that control the formation and stabilization of the matrix.[1,7,163] Hence, during ovulation, the oocyte is the center of a pronounced inflammatory reaction and is essentially treated as a foreign body and kicked out of the nest (Figures 22.3 and 22.12).

The Expression and Functions of Cytokines

In cumulus cells, AREG induction of *Ptgs2* and the production of PGs represent key mediators of inflammation. AREG also leads to the induction of genes (*Alcam, Il6, Cd34, Cd52, Pdcd1*) that are associated with inflammation.[70] In addition to IL6, other cytokines (*Ccl2, Ccl4, Ccl5*) are expressed in cumulus cells, raising the possibility that cytokines and their receptors play distinct roles in the functions and fate of COCs prior to and after ovulation.[164] IL6 alone can stimulate COC expansion and induce genes required for this process by activating the MAPK3/1 pathway and JAK/STAT pathways.[165] IL6 also enhances embryo development.[165] However, *Il6*-null mice are fertile, indicating that other cytokines being released from COCs may act in a redundant manner during ovulation to facilitate COC expansion and expulsion as well as to recruit immune cells to the ruptured ovulation pore. *Cd52* is expressed in ovulating mouse and human COCs, but the functions remain to

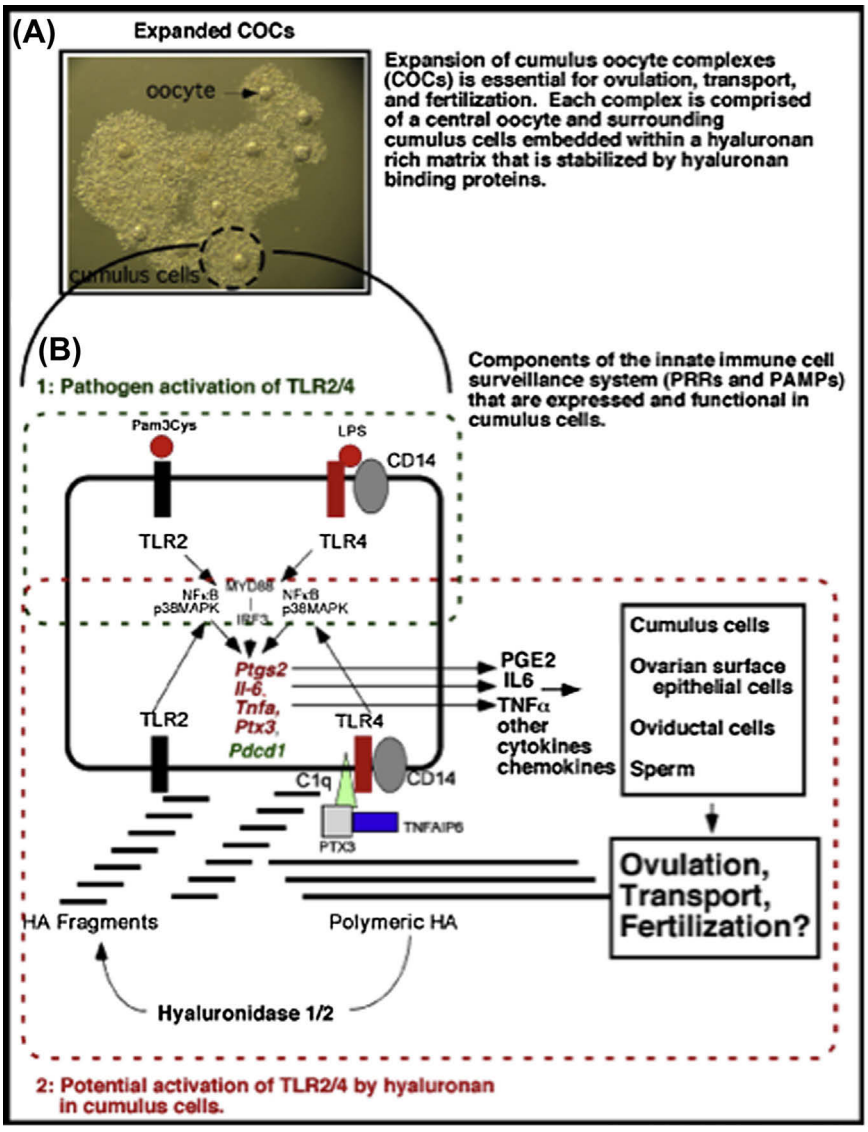

FIGURE 22.12 Schematic of TLR pathway activation and functions in cumulus cells during COC expansion, ovulation, transport and fertilization. This figure is reproduced in color in the color plate section. (A) In response to the ovulatory surge of LH, cumulus cell–oocyte complexes (COCs) undergo expansion. This process requires the synthesis of a hyaluronan (HA)-rich matrix and factors that bind HA to stabilize the matrix. This process is critical for ovulation. (B) Cumulus cells express members of the Toll-like receptor (TLR) superfamily and can respond to specific ligands (1: pathogen-derived or 2: matrix-derived), leading to the induction of inflammation- and innate immune-related genes. These include: *Ptgs2, Il6, Tnfaip6, Tnfa*, and *Pdcd1*. Prostaglandins (PGE2) synthesized by PTGS2, IL6, and TNFα, as well as other cytokines and chemokines, are released from cumulus cells and can impact the function not only of the cumulus cells but also oviductal cells during transport and sperm during fertilization. The degradation of polymeric HA by hyaluronidases is presumed to lead to the generation of HA fragments that activate TLR2 and TLR4. *Source: Ref.160.*

be determined.[166,167] *Pdcd1*-null mice are fertile (Liu and Richards, unpublished observations).[168] Targeted disruption of genes encoding other cytokines is needed to further define their roles in ovulation.

The Role of Immune-Related Factors

Components of the innate immune system are an evolutionarily conserved receptor recognition system that includes the pattern-recognition receptors (*Cq1a, Cd14, Trl4, Tlr2*). In immune cells these receptors are used to discriminate self from nonself and bind such factors as bacterial lipopolysaccharide, LPS/Pam3Cys.[169] These receptors are also expressed in nonimmune cells including cumulus cells.[70,134,170] In cumulus cells they are functional and bind LPS as well as fragments of hyaluronan[91,169] (Figure 22.11). Specifically, LPS itself induces marked induction of *Il6* and *Ptgs2* in cumulus cells. Furthermore, fragments of hyaluronan that are generated by hyaluronidase that is released from cumulus cells or sperm also bind to these receptors and increase *Il6* and *Ptgs2*.[91,164] Thus, cumulus cells have the potential to monitor their environment within the follicle and the oviduct.[160]

Once the COCs are in the oviduct, cytokines released from the cumulus cells may impact the function of oviductal cells. For example, COCs interact with the ciliated cells of the oviduct during transport and cluster in a region where the oviduct is "ballooned" and hence appears to be responding to the presence of the COCs. Although the extent to which factors from cumulus cells regulate these events is not entirely clear, transport and oviductal swelling are not observed in *Ptx3*-null mice in which the matrix disappears rapidly following ovulation.[88,119] The Toll-like receptors may also be involved in fertilization.[164] Specifically, IVF assays have shown that

sperm induce cumulus cells to release specific cytokines and chemokines. Certain chemokines then bind chemokine receptors on sperm and enhance fertilization. These events are dramatically reduced in the presence of TLR2- and TLR4-blocking antibodies.[164] Importantly, recent studies show that not only cytokines and chemokines but also antibacterial factors are released from the ovulated COCs and activate sperm,[171,172] providing evidence that the inflammatory and immune factors may participate in the fertilization process as well.

Recent studies have also proposed that there is a pituitary–ovary–spleen axis associated with ovulation that promotes the trafficking of immune cells to the ovulating follicle.[173] Because many cytokines and chemokines are made in response to the LH surge, they likely recruit immune cells that then further contribute to the inflammatory-like functions in the ovulatory follicle.

OFF-STAGE AND BEHIND THE SCENES: FOLLICLE ATRESIA AND GRANULOSA CELL APOPTOSIS

As mentioned in the introduction to this chapter, one of the most amazing aspects about ovarian follicular development in mammals is that less than 1% of the follicles present in the ovary at birth ovulate successfully. This means that 99% of all growing follicles are eliminated from the ovary by the process of atresia in which granulosa cells undergo apoptosis (programmed cell death). Thus, apoptosis profoundly regulates the number of follicles ovulating and the number of eggs that can be fertilized. That so many follicles undergo atresia underscores the critical importance of this process and apoptosis in controlling the growing follicle pool; hence the number of follicles capable of ovulating.[174]

Recent studies indicate that the forkhead boxO transcription factors FOXO1 and FOXO3 are key mediators of metabolism and tissue homeostasis in all vertebrates.[175,176] In the mouse and human ovary, *Foxo1* and *Foxo3* are expressed at high levels in granulosa cells and luteal cells, respectively[177,178]; appear to regulate granulosa cell metabolism, proliferation, and apoptosis[179–181]; and can functionally interact with steroid hormone receptors.[182] Conditional depletion of *Foxo1* and *Foxo3* in murine granulosa cells using *Amhr2-Cre* or *Cyp19-Cre* mice reveals that these two factors control critical genes and metabolic pathways in ovarian granulosa cells by interacting with the activin and BMP2 pathways.[183] Whereas activin and FOXO1 are expressed in granulosa cells of growing follicles, BMP2 and higher levels of FOXO1 are selectively expressed in granulosa cells of follicles undergoing atresia. By interacting selectively with the activin pathway, FOXO1/3 control genes involved in follicle growth. These include *Ctgf*, *Lox*, *Irs1*, *NrOb1*, and *Amh*. These interactions of activin and FOXO1/3 occur independently of FSH. In addition, activin enhances FSH induction of *Cyp19a1* and *Ccnd2* but impairs FSH-mediated induction of *Lhcgr*. By interacting with the BMP2 pathway, FOXO1/3 induce genes controlling responses to metabolic stress, granulosa cell apoptosis, and follicle death, including *Jnk3*, Bim(*Bcl2l11*), and caspase 3. Thus, it is proposed that a switch from growth to apoptosis in granulosa cells is mediated by FOXO1/3 swapping its interactions from activins to BMP2. What controls this switch remains to be determined but is obviously of utmost importance in the fate of growing follicles.

FOXO1/3 also regulate pituitary expression of *Fshb* by controlling a regulatory loop between the ovary and the pituitary.[183] In the *Foxo1/3*-mutant mice, serum levels of FSH and pituitary expression of the *Fshb* gene are severely suppressed even though serum levels and tissue expression of inhibin mRNAs remain within the normal range. Serum FSH and pituitary expression of *Fshb* can be restored in the mutant mice by removing the ovaries, indicating that the inhibitory factor is an ovarian-derived substance. This regulatory loop therefore also controls the growing follicle pool and ultimately the number that can ovulate.

FOXO1/3 are targets of the PTEN-PI3K-AKT pathway, and when phosphorylated by AKT are generally excluded from the nucleus and degraded. Targeted disruption of *Pten* in granulosa cells increases the number of ovulating follicles and the presence of corpora lutea,[184] perhaps related to decreased activity of FOXO1 but increased levels of FOXO3. Likewise, targeted disruption of another component of the PI3K pathway, tuberous sclerosis complex 1 (*Tsc1*), also leads to increased accumulation of corpora lutea.[185] Although the mechanisms are not known they may involve changes in the activity of mammalian target of rapamycin complex 1 (mTORC1), target of TSC1 and also possibly FOXO1/3.

TRANSLATIONAL CLINICAL CONSIDERATIONS

Many similar signaling cascades and molecular mechanisms control ovulation in mammals. Therefore, the transfer of information obtained from studies in mouse, pig, bovine, and primate models to clinical studies in women is relatively direct. However, understanding the genetic and molecular basis for infertility and anovulation in women is much more difficult to determine. Moreover, ovarian studies in women are particularly challenging because of the lack of available tissue for mechanistic and molecular analyses.

Incidence and Causes of Anovulatory Infertility

Infertility affects at least 10–15% of women in the United States during their reproductive years, and in many cases the underlying mechanisms of infertility remain poorly understood. Endometriosis is often associated with infertility. Although the underlying causes of ovarian dysfunction in this disease are not clearly defined, excess cytokines produced as a consequence of chronic inflammation that is characteristic of endometriosis may be contributing factors. Clearly, cytokines such as IL6 can impact granulosa cell functions, stimulate COC expansion, and modify oocyte quality.[165] Therefore, excess IL6 and other cytokines may interfere with the normal functions of the ovary.

PCOS at the Ovarian Level

PCOS is the most common endocrine defect and cause of *anovulatory infertility* in women in their reproductive years. PCOS afflicts more than 5–10% of women in the United States, costs more than $4 million per year to manage, and is becoming increasingly prevalent. A PubMed search of "ovulation" lists scores of papers that are predominantly related to current clinical studies describing ways to improve treatment of infertile women, especially those with anovulatory PCOS. The number of basic research papers on ovulation pales in comparison. Although some molecular aspects of theca cell androgen production have been determined in PCOS, our understanding of the molecular consequences of PCOS in granulosa cells and cumulus cells is less well defined.

Although elevated androgens (hyperandrogenemia) are one defining characteristic of PCOS, it is also frequently associated with metabolic defects related to insulin resistance and a pro-inflammatory state that may contribute to insulin resistance. Thus, understanding the consequences of endocrine, cytokine, and metabolic defects at *the ovarian level* are critical for improving ovulation, IVF procedures, and oocyte quality in PCOS patients.[38,39] PCOS is often, but not always, associated with obesity, suggesting that cytokines or other factors coming from adipose tissue likely impact the hypothalamic–pituitary–ovarian axis. Two major cytokines derived from adipose tissue are leptin and adiponectin. Whereas leptin regulates appetite, adiponectin has been shown to alter steroidogenesis, genes involved in ovulation, and those involved in apoptosis.[186–189] That adiponectin enhances oocyte and embryo development in mice indicates that this may be a "good" cytokine.[189] Furthermore, expression of adiponectin receptors is correlated positively with oocyte maturation in samples obtained from IVF patients.[189] That levels of adiponectin are reduced in PCOS patients may indicate that it plays some role in optimal ovulation and oocyte quality.[190] Other cytokines released from adipose tissue or immune cells include CRP and TNF alpha, which cause pro-inflammatory reactions in cells.

Ovaries in women with anovulatory PCOS contain numerous small antral follicles that are characterized by high levels of androgens. However, these follicles also produce estradiol. Successful ovulation can be achieved frequently in PCOS patients by decreasing estradiol or its actions and by judiciously increasing endogenous or exogenous FSH. These results indicate that anovulation is related not only to ovarian problems but also to hypothalamus–pituitary events. Ovarian hyperstimulation[191] syndrome (OHSS), in which the ovary produces excessive amounts of estradiol and progesterone and ovarian blood vessels become highly leaky, is frequently associated with hormonal stimulation of IVF patients and complicates IVF outcomes.[191] Therefore it is important to understand what factors contribute to PCOS and to OHSS, including what functions are altered in PCOS ovaries, including the ovarian vasculature[39,191] (see Chapter 29).

Restoring Fertility with IVM, IVF, and ICSI

IVF and intracytoplasmic sperm injection (ICSI) are the most frequent methods used for treating infertile couples. However, the effectiveness of IVF/ICSI procedures is compromised by the transfer of multiple embryos and multiple births. Now that we know many of the genes regulating COC expansion, these genes might be used as markers to identify healthy versus compromised oocytes during IVF.[192] In vitro maturation (IVM) of oocytes provides promise as an alternative to IVF, but oocyte quality remains an issue,[193–198] and better techniques need to be developed in the years ahead.

LH Receptor Mutants and Infertility

Although rare, women with LH receptor insufficiency/mutations exhibit impaired ovulation.[199,200] Based on current information, these defects might be overcome by treating these patients with FSH and EGF-like factors to bypass the need for LH receptor activation.

CONCLUSION

Ovulation is a complex process essential for the reproductive success of every species. Although we have learned a great deal about how LH initiates this process in mice and other mammals, there are still many blank spaces on the canvas and in the script. We know some of the transcription factors that are essential for ovulation, but the regulatory networks that they control remain incompletely defined.

World population continues to soar, and therefore the need to regulate population growth remains critical in many areas of the globe. Conversely, rescuing fertility has a major impact on allowing infertile couples to have the children they desire. Therefore, understanding what regulates ovulation and oocyte quality is of prime importance for the development of new contraceptive approaches as well as for designing improved procedures for IVF of an oocyte and IVM of follicles and oocytes. Although the transfer of some of our current knowledge to clinical issues is occurring, much still remains to be applied to IVF and especially IVM where conditions are less than optimal.

New Contraceptives?

Although the pharmaceutical industry has almost virtually closed the door on research related to reproductive hormones and ovarian function and contraceptive development, the wealth of new knowledge that is now available may one day provide alternative avenues for controlling (blocking or enhancing) ovulation, as well as for improving IVM of follicles and oocytes. Perhaps even microRNAs could be used effectively.

A Role for MicroRNAs?

MicroRNAs (miRNAs) have emerged as a powerful post-transcriptional gene regulatory mechanism for globally changing cell functions. Specifically, long primary microRNA transcripts are processed to mature miRNAs, ~22 nucleotides in length, by specific ribonuclease-splicing enzymes. Drosha along with its RNA binding cofactor (DGCR8) cleaves primary transcripts to precursor transcripts in the nucleus. Once in the cytoplasm, Dicer1 cleaves pre-miRNAs to mature miRNAs. The miRNAs then bind and act on specific sequences to regulate messenger RNA (mRNAs) cleavage and translational repression. Our understanding of which microRNAs control ovulation (or other ovarian functions) remains in its infancy.[201–203] However, mice in which *Dicer1* has been conditionally disrupted in granulosa cells using *Amhr2-Cre* are infertile.[204] The mice exhibit reduced ovulation rates and impaired oocyte and embryo development[204] associated with altered expression of specific genes and miRNA-503.[205] Based on the prediction that Dicer1 regulates the formation of all mature miRNAs in a cell, it is remarkable that the effect of disrupting *Dicer1* is not more severe. This may indicate that miRNAs do not globally affect the granulosa cell mRNA landscape at all stages of follicle development or other ribonucleases that are critical and serve as alternative miRNA regulators. Perhaps when the mRNAs and genes that are altered in the *Dicer1*-mutant granulosa cells are identified, the roles of Dicer1 will be more completely understood in this context. Even though miRNAs that are induced by the LH surge (miR-21, -132, and -212) have been identified, their specific functions and the genes they control during ovulation remain to be clearly defined.[202] Therefore, more studies are needed to determine the roles of miRNAs in ovulation and their possible use as contraceptives.

Identifying Gene Markers of Healthy Oocytes

Many genes that are critical for the function of COCs and oocyte maturation are now known. Some of these may be useful for improving IVM of oocytes, a procedure that currently remains suboptimal[193–206] and for determining the identity of "good" versus "not so good" oocytes used for IVF and intra-cytoplasmic sperm injection (ICSI) procedures.[74,141,206,207] For example, the key roles played by the EGF-like factors in ovulation, as demonstrated in mutant mouse models, is supported by the increased levels of AREG and EREG in follicular fluid of human, porcine, bovine, and primate (Rhesus macaque) ovulatory follicles. Furthermore, when small follicles of the macaque are cultured with gonadotropins to stimulate in vitro follicle and oocyte maturation, the addition of AREG at low levels enhances oocyte maturation and embryo development.[208] Although the effectiveness of AREG in the maturation of human oocytes for IVF/ICSI procedures has not yet been thoroughly analyzed, it would likely enhance human oocyte maturation as well.[209] One of the major obstacles is optimizing the culture conditions that foster oocyte quality, including metabolic factors such as pyruvate and glutamine (not present in some studies)[209] as well as cytokines. For example, secreted factors, including IL6, might also eventually serve to improve IVF/ICSI procedures, if given at the right time and dose.

Small, immature ovarian follicles can now be grown in culture to the preovulatory stage; oocytes from these follicles can be retrieved and fertilized; and the embryos can develop into normal offspring.[210,211] These major advances provide another system in which to analyze the mechanisms of ovulation strictly associated with the follicle in a defined culture system that is devoid of surrounding stromal tissue and the OSE. This approach is being developed to provide a way to restore fertility in women who are infertile due to cytotoxic drugs used for cancer therapies. Implants of ovarian sections have also been effective in restoring fertility in women. In light of these developments, the concept of "oncofertility" has been coined and is being avidly pursued.[212]

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CHAPTER

23

Structure, Function, and Regulation of the Corpus Luteum

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INTRODUCTION

"Although much is known, much still remains to be discovered, and much of what has been discovered needs to be uncovered." R.V. Short[1]

The corpus luteum is an endocrine gland within the adult ovary that forms from the follicle wall after ovulation.[2,3] It is associated with four unique features. The first is its ephemeral nature, as the corpus luteum exists for a limited lifespan, which in many species depends upon the fate of the oocyte released by the antecedent ovulatory follicle. Second, its primary, if not sole essential, function is to synthesize and secrete the steroid hormone, progesterone. Progesterone has numerous biological effects on target tissues,[3] but particularly acts in the reproductive tract to permit implantation of the early embryo in the uterine endometrium and to support a maternal environment that sustains intrauterine pregnancy. Thus, the third feature of the corpus luteum is its physiologic connection to viviparity.[2] All mammals, many reptiles, and a few fishes that produce live young exhibit corpora lutea, although the essential connection between the corpus luteum and viviparity is less obvious in nonmammalian species. In some of these species, luteal function may be related to final oocyte maturation, egg movement in the oviduct, or oviposition, since the corpus luteum exists in some oviparous reptiles, amphibians, and birds as well.[4] The latter serves as a preface to the fourth key feature of the corpus luteum, i.e., the tremendous species diversity in the mechanisms that evolved to control the structure and function of the corpus luteum. Although the mammalian corpus luteum seems similar to the steroidogenic adrenal cortex[2] and develops from the steroidogenic ovarian follicle, the latter endocrine tissues display relatively minor differences in regulation of development and function across mammalian species. In contrast, the differences in endocrine and local control of the development, function, and lifespan of the corpus luteum are remarkable. Limitations prevent addressing many individual species in this chapter, but the reader is encouraged to peruse other comparative reviews to marvel at the challenges facing ovarian biologists and their attempts to promote or control fertility in domestic and wild animals, endangered species, and human beings. This review will focus on selected species of traditional laboratory (rodents and rabbits) and domesticated (sheep, cow, and horse) animals, plus primates (Old World macaques and humans), where the majority of research activity occurred over two decades since the publication of the chapter on the corpus luteum by Niswender and Nett[3] in the second edition of *The Physiology of Reproduction*.

Historical Perspective

An interesting reference on the discovery and study of the corpus luteum was provided by R.V. Short in 1977.[1] Existence of the corpus luteum as a structure within the ovary was noted by several renowned anatomists in the sixteenth and seventeenth centuries, such as Fabricius,[5] who provided the first illustrated accounts of the comparative anatomy of the female reproductive tract, including ovaries in the pregnant pig containing "numerous little glands". Although Regnier de Graaf is chiefly remembered for his description of the antral Graafian follicle, he provided the first detailed descriptions of the "globular bodies" (corpora lutea; Figure 23.1) that formed in

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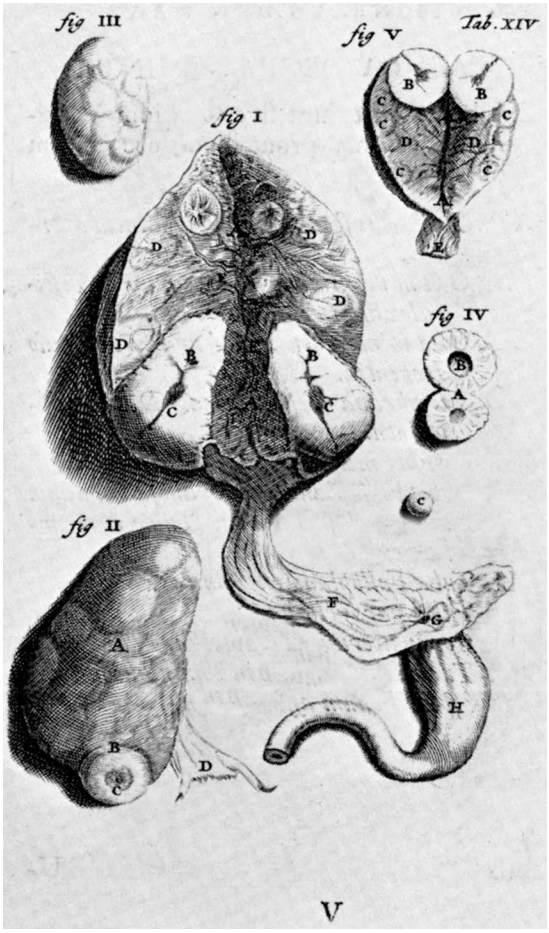

FIGURE 23.1 Early (1672 AD) drawing of sheep ovaries by Regnier de Graaf. The ovary (center, top right) is bisected to display the presumptive corpus luteum, as well as other tissue compartments. *Source: From Ref. 7 as reported by R. Short,[1] with permission.*

place of the "ova" (follicles), which gave an indication of the number of fetuses present[6] (as translated by Jocelyn and Setchell). His appreciation of the diversity of colors (pink, red, purple, and yellow) associated with the gland in various species did not lead him to name the structure the "corpus luteum" (yellow body), which is attributed to Malpighi's observations of cow ovaries. Nevertheless, Malpighi speculated correctly on the corpus luteum's "glandular nature" and stated that its structure closely resembled the suprarenal (adrenal) gland.[7] During this era, the ovaries and their structures were described, but their functions and regulation were not appreciated.

Although de Graaf and the Scottish anatomists William and John Hunter[8] recognized the positive correlation between the number of corpora lutea and the number of offspring arising from pregnancy, and clinicians such as Pott[9] recognized that ovariectomy resulted in cessation of menstruation in women, the importance of these relationships remained unclear.[10] Many scientists remained thoroughly indoctrinated in the prevailing views of Aristotle's "seed and soil" concept that the male provided the "seed" and the female played a passive role by providing the "soil" in which the seed could grow (as recounted by Thompson).[11] The soil was the uterine fluids, and women menstruated because their nature resulted in periodic repletion with excess blood that was removed by menstruation. There was some belief that the ovarian follicle was the egg, but it was not until the 1800s that the egg was first described within the mammalian follicle[12] and that the processes of ovulation and formation of the corpus luteum from the follicle wall were identified.[13] Nevertheless, the general opinion was that corpora lutea were just scar tissues formed after ovulation. Only after Knauer[14] definitively proved that the ovary was a gland of "internal secretion", through classic ablation replacement studies in various mammals, did investigators such as Beard[15] and Prenant[16] hypothesize that the corpus luteum had specific functions in uterine development and gestation due to its secretion of one or more products into the bloodstream. Studies by Fraenkel[17] and Magnus[18] confirmed the endocrine actions of the corpus luteum, but the search to isolate the luteal hormones only began in earnest after suitable bioassays, such as the maternal deciduoma response,[19] were devised. In 1929, Corner and Allen[20] prepared an alcoholic extract from pig corpora lutea that mimicked luteal actions in the uterus and maintained pregnancy in ovariectomized animals. Hisaw[21] also established that similar luteal extracts produced the full secretory-phase development of uterine endometrium in a primate, the rhesus monkey. By then, it was recognized that menstruation in primates did not correspond to the period of "sexual excitement" or estrus in other species (i.e., it was not the time of ovulation and optimal fertility).[22] Rather, it marked the end of the cycle and the absence of ovulation—a wiser basis for the "rhythm method" of contraception.

The modern era of research began in 1934 with the isolation and characterization of the steroid progesterone from luteal extracts by four independent groups.[23–26] Investigators quickly established that this progestin (see Parkes, Ref. 27, for an explanation of how the name was agreed upon) mimicked many of the roles of the corpus luteum. With the development of reliable radioimmunoassays[28] to replace bioassays, attention turned to understanding the processes controlling the functional lifespan of the corpus luteum in various species. After the discovery that hypophysectomy eliminated cyclic ovarian function in adult mammals, particular attention focused on identifying the pituitary gonadotropins[29] and established the vital roles of luteinizing hormone (LH) and prolactin (PRL) as "luteotropic hormones" in the development and steroidogenic function of the corpus luteum in various species.[30] Also, the report that hysterectomy prolonged

the lifespan of the corpus luteum led to investigations into the role of the uterus,[31] and the subsequent discovery that the arachidonate metabolite prostaglandin (PG)-$F_{2\alpha}$ is the uterine "luteolytic hormone" terminating the lifespan of the corpus luteum in nonfecund ovarian cycles of various species.[32]

The discovery of the luteal hormone, progesterone, also had pronounced effects on clinical approaches to assure sufficient luteal support during pregnancy, to control fertility, and to prevent undesirable effects of menopause in women. Although major ovarian pathologies (e.g., ovarian cancers and polycystic ovarian diseases) do not emanate from the corpus luteum, there are situations of deficient luteal function in natural ovarian cycles (short or insufficient luteal phases) or controlled ovarian stimulation cycles (short luteal phases) that may be associated with subfertility.[33] Therefore, a synthetic progestin or, more recently, progesterone supplements can be administered to replace or assist in luteal functions during the menstrual cycle.[34] Also, after the discovery that exogenous progesterone could inhibit ovulation in mammals,[35] researchers formulated the idea that administration of a combination of estrogen and progestational agents would block ovulation and thus prevent conception in women while preserving the semblance of normal menstrual cycles in women. This work was facilitated by the success of chemists (e.g., R. Marker, see Ref. 36) in generating inexpensive, abundant supplies of steroid analogs from plant sapogenins, which are steroids that structurally resemble cholesterol. The basic[37] and clinical testing by G. Pincus, M-C. Chang, J. Rock, and E. Rice-Way proved that the combinational steroid "pill" provided virtually complete protection from pregnancy. The oral contraceptive pill introduced in 1960 had a profound impact on every aspect of society, and it gave women for the first time the opportunity to control their reproductive status. Likewise, the discovery of molecules with antiprogestin activity (e.g., RU486 at Rossel Uclaf) demonstrated the molecular basis of luteal ablation on uterine function and pregnancy, resulting in the availability of a nonsurgical protocol for pregnancy termination or contragestation.[38] The use of progestins, antiprogestins, and, more recently, selective progestin receptor modulators (which may have agonist or antagonist actions at specific target tissues[39]) continues to be of great interest for such purposes as birth control, treatment of disorders of the reproductive tract (e.g., persistent or irregular menstruation), and cancer therapy.

As discussed in this chapter, recent advances in cell and molecular biology are contributing greatly to our understanding of the mechanisms of action of luteotropic and luteolytic factors, the intracellular pathways controlling steroidogenesis, and the cell-to-cell interactions that are critical for the development, function, and normal lifespan of the corpus luteum. Unlike in earlier eras, investigators of the corpus luteum are faced with a tremendous increase in scientific information on a daily basis. In this century of information overload and short information "sound bites" or "abstracts", the scholar is urged to rediscover the major breakthroughs, prevailing controversies, and dogmas of eras that provided the groundwork of current concepts that have yet to stand the test of time. This review attempts to build on prior chapters[3,40] and coalesce ideas from recent reviews of leaders in this field, as referenced in the following sections and cited in the Acknowledgments section.

CLASSIFICATION OF TYPES OF CORPORA LUTEA

Due to its transient nature, one can typically discern a developing phase, a functional phase, and a regressing phase in the lifespan of the corpus luteum. These phases are usually associated with increasing levels, elevated levels, and then declining levels of progesterone in the circulating blood.[2] As adapted from Rothchild and others,[2,41] mammals can be divided into three major categories (Figure 23.2) based on how long the corpus luteum functions after ovulation in the nonfecund ovarian cycle and how the luteal lifespan is affected by pregnancy onset and gestation.

Long-Lived Corpora Lutea

There are some monestrous species (i.e., animals with estrous cycles that occur only at long intervals, such as every 6–12 months) in which a fully functional corpus luteum develops after ovulation and its functional lifespan does not differ remarkably regardless of whether the corpus luteum originates during a fertile or nonfertile cycle.[2] This category, which includes mammalian carnivores (e.g., dogs, wolves, foxes, cats, ferrets, and skunks[42,43]) and other animals such as the roe deer, armadillo, and marsupials,[44–46] contains corpora lutea that last from less than 2 weeks to as long as 6 months. In the domesticated dog,[47,48] for example, the length of the luteal phase in pregnant and nonpregnant animals is ~65 days. At the end of pregnancy, which typically occurs in each cycle of monestrous animals, there is rapid luteolysis. However, if pregnancy does not occur, the loss of luteal structure and function is very protracted; the mechanisms of this are unclear. Many marsupials can be included in this category because, even though the luteal lifespan may last only about 14 days, it is not altered by the occurrence or lack of pregnancy. This category of corpora lutea also illustrates the evolution of mechanisms whereby the corpus luteum's lifespan can be modified by the addition of a *quiescent* interval after ovulation

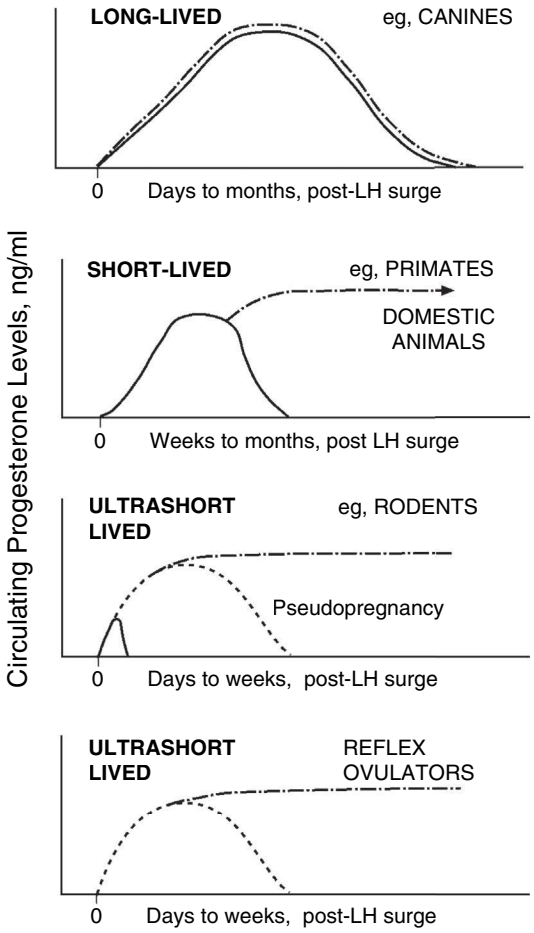

FIGURE 23.2 Types of mammalian corpora lutea based on their functional lifespan (long, short, or ultrashort) during the nonfecund ovarian cycle (solid lines). The effects of nonfertile mating on ultrashort-lived corpora lutea (pseudopregnancy; dotted line) and of pregnancy in all three categories (dashed lines) are demonstrated. Reflex ovulators, such as rabbits and ferrets, can also be categorized as having ultrashort-lived corpora lutea since they have no luteal phase due to the absence of an LH surge unless mating occurs.

before luteal function begins. This can be related to seasonal cues (e.g., in the skunk and badger[49]) or both lactation and season (e.g., in the wallaby[50]).

Short-Lived Corpora Lutea

In many polyestrous species (i.e., animals with frequent ovarian cycles), the corpus luteum develops and functions for a finite interval during the ovarian cycle, but the luteal lifespan is markedly increased if pregnancy ensues. This category includes the primate species of monkeys, great apes, and humans,[51–53] wherein a functional corpus luteum forms rapidly after ovulation and functions for a sufficient interval (~2 weeks) to permit timely movement of the early embryo through the oviduct and to prepare the uterus for implantation. If pregnancy does not occur, the corpus luteum regresses after 2 weeks. However, if pregnancy occurs, the functional lifespan of the corpus luteum is extended for a finite interval[54] until its critical activities (i.e., progesterone production) are assumed by the developing placenta. The time of the luteal–placental shift, after which the corpus luteum is no longer essential for pregnancy, varies between primates (3 and 7 weeks of gestation in rhesus macaques and women, respectively). It is not clear if the processes of luteal regression around the luteal–placental shift are comparable to those during luteolysis in the nonfecund cycle. However, there is intriguing evidence that after a quiescent interval at midgestation, the corpus luteum in primates rejuvenates by term pregnancy and is again a progesterone-secreting gland,[55] but its importance as an endocrine gland is unclear considering the massive steroidogenic activity of the term placenta.

Many of the ungulates, such as the domesticated farm animals, also display short-lived corpora lutea. For example, in sheep and cattle, the corpus luteum forms rapidly after ovulation and functions until day 17 and 21 post estrus, respectively.[56,57] Again, if pregnancy does not occur, the corpus luteum undergoes rapid luteolysis; however, if intrauterine gestation begins, the functional corpus luteum is sustained for several months until the luteal–placental shift or even until the end of pregnancy (e.g., in the pig[58]). However, there are important differences between primates and ungulates with short-lived corpora lutea in the activities and processes controlling the length of the ovarian cycle and the luteal phase, plus maternal–ovarian recognition of pregnancy. First, the ungulate corpus luteum permits waves of antral follicle growth during the luteal phase,[59] and hence the interval of the follicular phase after luteolysis to development of the next ovulatory follicle is very short (2–3 days). In contrast, in many primates, the ensuing follicular phase is relative long (~2 weeks) due to the "dominance" of the corpus luteum, which prevents further development of small antral follicles until after luteolysis.[60,61] Second, luteolysis in ungulates requires the presence of a nonfecund uterus, leading to the concept that in the absence of implantation, a uterine luteolytic signal promotes the regression of the corpus luteum of the cycle.[62] In contrast, hysterectomy does not alter the functional lifespan of the corpus luteum in primates exhibiting menstrual cycles (e.g., macaques and women[63,64]), indicating the absence of a uterine luteolytic signal. Third, whereas ungulates evolved a mechanism that apparently blocks the synthesis and/or secretion of the uterine luteolytic signal in early pregnancy, higher primates utilize an embryonic hormone that acts directly on the corpus luteum to rescue it from impending regression and promote luteal structure and function in early pregnancy.[65] Again, there are exceptions or additions to these concepts. For example, some primates (e.g., New World monkeys and Prosimians[66]) do not menstruate and may produce a uterine luteolytic signal.[67] The horse also produces a

fetal hormone after the first month of gestation that may stimulate the function of existing corpora lutea, but also induces ovulation of large antral follicles and the formation of "secondary" corpora lutea that assist in progesterone secretion for 5 months until the luteal–placental shift.[3]

Ultrashort-Lived Corpora Lutea

Species with this feature[2] do not form a functional corpus luteum (e.g., polyestrus rodents and perhaps some insectivores) or indeed any corpus luteum during the ovarian cycle unless mating results in ovulation (reflex ovulators, e.g., rabbit[68]) and/or pseudopregnancy or pregnancy (e.g., rat and mouse). In rodents such as the laboratory rat or mouse,[69,70] the estrous cycle lasts 4–5 days and lacks a true luteal phase. After ovulation, the ruptured follicle takes on some of the characteristics of a corpus luteum, but the luteal gland is not well developed and is considered nonfunctional since it does not secrete sufficient progesterone to stimulate a uterine response (i.e., decidualization) to implantation cues. Progesterone produced within the corpus luteum is rapidly metabolized to 20α-hydroxyprogesterone, an inactive progestin that will not support pregnancy. Mating or a facsimile (cervical stimulation) during estrus that does not result in pregnancy results in development of functional corpora lutea that secrete progesterone for 12–14 days. This interval of luteal function, known as pseudopregnancy, is an unusual artifact since mating in the natural environment typically results in pregnancy. However, pseudopregnancy is a valuable means of distinguishing mechanisms initiated by the neuroendocrine reflex arc in response to cervical stimulation that promote luteal development in animals with ultrashort-lived corpora lutea. If mating initiates pregnancy, continued progesterone secretion by the corpus luteum is required throughout gestation. Continued luteal structure and function are achieved by a combination of maternal (uterine decidual) and fetal (trophoblast) hormones or hormonal precursors that act on or are converted to essential factors by the rodent corpus luteum of pregnancy.[71] Regression of the corpus luteum at the end of gestation, and perhaps at the end of pseudopregnancy, is controlled by a uterine luteolytic factor.[69]

Thus, mammalian species have developed diverse means (Figure 23.2) to ensure that ovarian cyclicity returns in the event of a nonfecund cycle (to restore fertility potential), yet provide adequate progesterone support if pregnancy ensues (to support intrauterine gestation until delivery of offspring). The simplest case is in animals with a "long-lived" corpus luteum, where gestation lasts no longer than the normal lifespan of the corpus luteum in the ovarian cycle. However, many animals have evolved means for keeping the functional lifespan of the corpus luteum "short" (i.e., less than necessary for successful pregnancy) in the ovarian cycle to increase reproductive efficiency. Therefore, processes evolved for preventing the development of a functional corpus luteum (an "ultrashort" lifespan), for uterine control of luteolysis in the nonfecund cycle (a "short" lifespan), and for uterine–placental extension of luteal function in the event of pregnancy initiation. Detailed discussion of these processes and the factors that control luteal structure and function are included in the following sections.

LUTEINIZATION

The corpus luteum forms from the cells comprising the wall of the ovulatory follicle. Luteal formation (i.e., luteinization) begins prior to ovulation but proceeds in earnest after follicle rupture and continues for a species-specific number of days until the fully formed corpus luteum is present. As reviewed by Murphy,[72] luteinization is a remarkable event involving cell proliferation, cell differentiation, and tissue remodeling that is unparalleled in the adult mammal. It is composed of two major processes: (A) eventual cessation of proliferation plus rapid hypertrophy and differentiation of the steroidogenic cells of the follicle into the luteal cells of the corpus luteum; and (B) the rapid growth of blood vessels (i.e., angiogenesis) and, in some species, the lymphatics (i.e., lymphangiogenesis)[73] into the previously avascular granulosa layer of the follicle to form the extensive microvascular bed of the corpus luteum (Figure 23.3). Although luteinization is often referred to as "terminal differentiation" of the follicle, it should be recognized that in many species, from rodents to primates, further differentiation may occur in luteal tissue in response to pregnancy. The angiogenic process in the corpus luteum is remarkable, even in comparison to the rapid vascular development in tumors or the placenta, and offers a unique model for studying microvessel development, maintenance, and regression in normal adult tissue. As adapted from Murphy[72] and others,[3,74,75] this section will discuss the processes and regulation of luteinization resulting in a fully developed, progesterone-secreting corpus luteum.

Morphologic Events

A common event during collapse of the follicle antrum at ovulation is the infolding of the follicle wall, with breakdown of the basement membrane separating the granulosa and theca layers of the follicle. This is followed by invasion of microvascular cells into the avascular granulosa layer, as well as other cells associated with the vasculature (e.g., perivasculature support cells, or pericytes,[77] and migrating cells of the immune system). In a few species, such as the dog,[78] this process starts

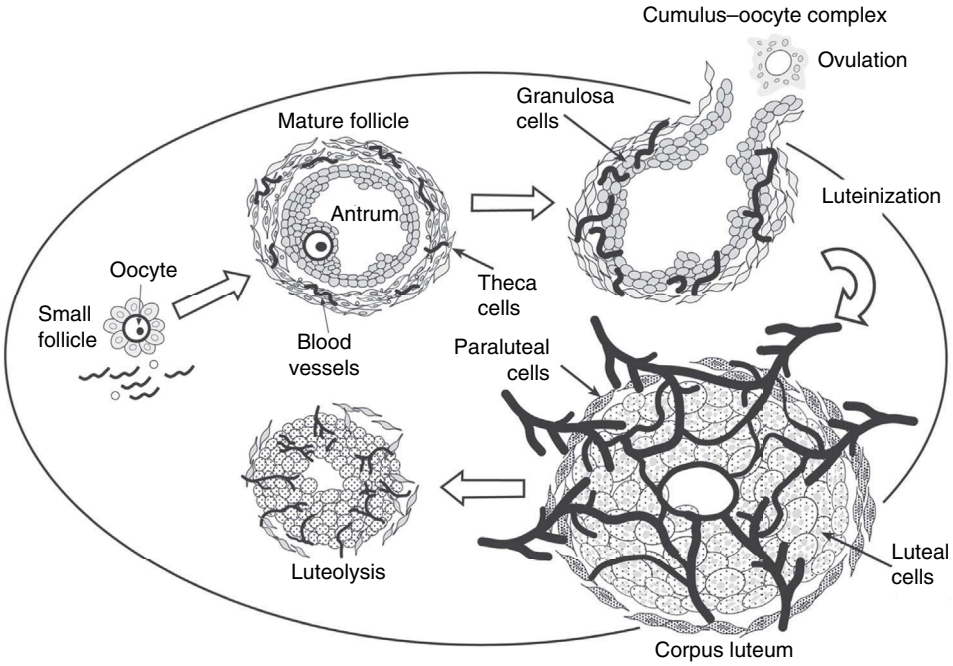

FIGURE 23.3 Development of the corpus luteum from the mature (Graafian) follicle. Folliculogenesis results in a large antral follicle in which vascularization (black blood vessels) is limited to the outer somatic (theca) cell layers. A basement membrane separates the theca from the avascular somatic (granulosa) cell layer, which encloses the gametogenic (oocyte) cell and a fluid-filled antrum. Near the time of ovulation, the basement membrane breaks down and angiogenesis occurs in the granulosa layer. Granulosa cells hypertrophy, and in many species luteinized granulosa and theca cells intermingle within the luteal tissue. In other species (e.g., primates), some compartmentalization remains as theca-derived paraluteal cells are found around the periphery and along infoldings in the corpus luteum. At luteolysis (see the Section Luteolysis), both the luteal cells and microvasculature degenerate. *Source: From Ref. 76, with permission.*

well before ovulation. But in most mammals, it follows rupture of the follicle. The follicular infolding and vascular invasion also transport theca cells toward the center of the follicle cavity. In many species, including rodents and various domestic animals, theca-derived luteal cells appear to disperse throughout the parenchyma of the corpus luteum.[75] Thus, unlike in the antecedent follicle, there is no apparent compartmentalization of theca- and granulosa-derived cells in the corpus luteum of these species. However, in some primate species, such as Old World macaques and humans, areas of the theca-derived compartment remain discrete from the remainder (including granulosa-derived) of the luteal tissue throughout its lifespan, even into pregnancy.[79] The fate of the theca interna versus theca externa is controversial; they may degenerate or be absorbed into the surrounding stroma, become incorporated into the luteal parenchyma as thecal–luteal cells, and/or remain as paraluteal cells clustered along the periphery or along infoldings of the luteal gland. The fate of thecal cells may depend upon their differentiated state or proximity to luteinizing granulosa cells at ovulation. Certainly, their destruction is documented in some species, such as the horse,[80] and there is speculation that they degenerate and are absent from the corpus luteum of a New World monkey, the marmoset.[81]

In addition to the dramatic increase in vascularity, the large increase in mass of the corpus luteum is due to hypertrophy of the luteinizing granulosa cells.[82] The granulosa cell with a high nuclear–cytoplasmic ratio is converted into a luteal cell with a low nuclear–cytoplasmic ratio, at a volume increase of up to 10-fold.[83] Even before ovulation, there are significant cellular changes as the nuclear chromatin disperses and the nucleolus forms concomitant with the increase in number of polyribosomes.[82,84] Intracellular connections (e.g., gap junctions) between granulosa cells (as well as theca cells) disappear, presumably as a prelude to tissue remodeling.[85] The amount of smooth endoplasmic reticulum increases and the characteristics of the mitochondria change, as they become rounded and their cristae convert from lamellar to primarily tubular form. The luteal cells may also contain well-developed Golgi complexes, as well as lipid droplets and protein-secretory granules. These changes are consistent with a cell that is very active in steroidogenesis, as well as peptide–protein secretion. However, again the characteristics (e.g., lipid droplets and protein-secretory granules) and the stage at which they develop (e.g., early after ovulation in domestic animals or primates versus midpregnancy in rodents[82]) vary between species.

It appears that marked changes in the cytoskeleton play an important role in optimizing cell size as well as

steroidogenic function.[75] Limited research to date suggests that luteinizing granulosa cells transiently alter their expression of microtubule components (e.g., tubulin) around ovulation, as well as acquire the ability to express smooth muscle actin, cytokeratin, vimentin, and desmin.[86,87] Again, there are likely differences between granulosa- and theca-derived luteal cells, as theca-derived cells only express large amounts of vimentin, at least in some species.[86]

Also, it is likely that changes in the extracellular matrix (ECM) and cell–ECM interactions are critical in the tissue remodeling and function within the developing corpus luteum. Prior to ovulation, theca cells are separated from the granulosa cells via a basal lamina composed of collagen type IV; laminin α, β, and γ chains; nidogens; and the proteoglycan perlecan.[88,89] Aggregates of a novel basal lamina–like matrix, named "focimatrix" for focal intraepithelial matrix,[90] are scattered in between granulosa cells. After ovulation, focimatrix and the follicular basal lamina undergo degradation.[91] In the nascent corpora lutea, a basal lamina of similar composition restricted to capillary or subendothelial basal laminas appears as new blood vessels develop. In the bovine corpus luteum, the type IV collagen in the basement membrane of the follicle is replaced by the fibrillar (type I) collagen, which remarkably comprises one-sixth of the mass of luteal tissue.[92] Not only are there qualitative and quantitative changes in the ECM during luteal development, but also cell surface–associated glycoproteins, such as the integrins, expressed on resident luteal cells appear to be critical in the developing corpus luteum. For example, there is speculation that removal of alpha 6, beta 1 integrin-mediated ECM binding following ovulation permits functional luteinization.[93] Restoration or expansion of other cell–ECM interactions may be critical as well (e.g., integrin beta 3 expression is upregulated in the mouse corpus luteum).[94] In human luteinized granulosa cells, alpha 5, beta 1 and alpha 5, beta 3 integrin expression facilitated cell adhesion to fibronectin as well as cellular migration, and prevented apoptosis, which in turn may promote the motility and survival of granulosa cells during luteal development.[95] Fibronectin also induces the expression of secreted protein, acidic, cysteine-rich (SPARC) in bovine luteal cells, a proteolytic fragment of which promotes endothelial network organization and progesterone synthesis.[96] Creative approaches using in vivo and in vitro models remain essential for dissecting the roles and interactions between cell populations and the ECM during the lifespan of the corpus luteum.

A number of proteases are implicated in luteal remodeling in various species,[97] including serine proteases (e.g., the plasminogen–plasmin system[98]), the matrix metalloproteinases (MMPs) and their endogenous tissue inhibitors (TIMPs) (for a review, see Ref. 99), as well as those proteases belonging to the **a** **d**isintegrin **a**nd **m**etalloproteinase with **t**hrombo**s**pondin-type repeats family (ADAMTS).[100] The roles of the plasminogen (PLG)–plasmin system and metalloproteinases in luteal development are uncertain, however, plasminogen null-mutant mice ($Plg^{tm1Dco}/Plg^{tm1Dco}$) treated with the broad-spectrum metalloproteinase inhibitor Galardin formed functional corpora lutea following the establishment of pseudopregnancy.[101] While the corpus luteum appeared normal morphologically, plasminogen deficiency was associated with a 50% reduction in the level of circulating progesterone. Injection of Galardin into the macaque follicle at the time that animals received an ovulatory stimulus (e.g., human chorionic gonadotropin (hCG)) prevented ovulation but did not affect subsequent levels of circulating progesterone or luteal lifespan.[102] Such findings indicate that protease activity after an ovulatory stimulus, while necessary for follicle rupture, is not required for luteinization and luteal development in primates. Moreover, additional complexity is evident from rodent studies demonstrating that TIMPs, endogenous MMP inhibitors, may serve to regulate luteal development and function through both MMP-dependent and independent mechanisms.[103] Thus, although there are reports of marked differences in protease expression between cell types comprising the corpus luteum and between species,[104–106] the exact roles that ECM remodeling and proteolytic activity play in luteal development remain uncertain and warrant further study. Recent genomic studies determined that the mouse and human genomes contain 628 and 553 protease-encoding genes, respectively.[107] Although limited information exists with regard to their biochemical and cellular activities, additional studies are needed to characterize the repertoire of ECM and ECM-remodeling proteases and their potential functional activity in the developing corpus luteum.

Regulation and Role of Luteal Neovascularization

The changes in vascularity associated with the development of a functional corpus luteum are remarkable (for a review, see Ref. 108). Prior to ovulation, the primary follicular layer destined to form the corpus luteum (i.e., the granulosum) is avascular, whereas after luteal development each steroidogenic cell is adjacent to a microvascular element[109] and cells associated with the microvasculature comprise about 50% of the total cell population.[110] However, the precise mechanisms whereby angiogenesis[111] and possibly vasculogenesis (from local[112] or circulating[113] vascular progenitor cells) form the dense network of capillaries in the developing corpus luteum are not well defined. There is evidence of early migration of microvascular cells into the luteinizing granulosa cell layer,[79] but much of the capillary

expansion appears caused by endothelial cell proliferation. Cell proliferation is greatest in the corpus luteum during early development, and evidence suggests that the vast majority (up to 95%) of the dividing cells are vascular endothelial cells.[109] The interval of angiogenesis varies between species, but in primates the capillaries reach the central cavity by day 4 and venules appear along the cavity border that lead back through the luteal tissue to large veins outside the corpus luteum by day 6 of the luteal phase.[79] Nevertheless, there is evidence for some continued microvascular cell proliferation after luteal development. For example, the vascular space with the corpus luteum of domestic animals and primates increases from the early to midluteal phase, and the number of nonsteroidogenic cells (i.e., primarily associated with the microvasculature) increases to the late luteal phase.[110] This may be associated with further maturation of the microvessels (as they acquire endothelial support cells, or pericytes[77,114]), but this important feature has received limited attention to date.[108]

Since the discovery that follicular fluid and ovarian extracts contained "angiogenic activity",[115] investigators have strived to identify the factors that control vascular events (e.g., endothelial cell proliferation) in the developing corpus luteum. Many of the earlier candidates (e.g., fibroblast growth factor 2 (FGF2), also referred to as basic FGF) were pleiotrophic factors that stimulated proliferation or migration of various cell types. Although some of these factors, including FGF2, may[108] or may not[75] have a role in luteal angiogenesis, major progress occurred when researchers determined that newly discovered endothelial-specific growth factors[116] associated with vasculogenesis in the developing embryo and angiogenesis in pathologic conditions (e.g., tumor growth) were also present in the ovary. Ongoing studies support the hypotheses that such factors play key roles in physiologic angiogenesis in the corpus luteum, that their regulation may be different from that observed in other tissues, and that cell–cell interactions via these factors are critical for the normal development, function, and perhaps lifespan of the corpus luteum.

Vascular Endothelial Growth Factor (VEGF)

VEGF,[116] also discovered as a vascular permeability factor (VPF), specifically acts on vascular endothelial cells to promote proliferation, migration, tube formation, and vessel permeability. Five molecular forms of VEGF (with 121, 145, 165, 189, and 205 amino acids) can be produced by alternative splicing, and differ in solubility and heparin-binding ability.[116] Recent evidence from other tissues indicates that the isoforms may have different physiologic functions. Also, the VEGF family continues to expand to include newly discovered, structurally related proteins that share homology with VEGF (now called VEGFA), including VEGFB, VEGFC, and c-fos-induced growth factor (FIGF, previously known as VEGFD)[116] that may well be expressed in the ovary.[117] In addition, researchers have identified high-affinity receptors that include kinase insert domain receptor (KDR, also known as VEGFR), fms-related tyrosine kinase 1 (FLT1, also known as VEGFR2), and fms-related tyrosine kinase 4 (FLT4, also known as VEGFR3), as well as proposed co-receptors (neuropilin 1 and 2) on endothelial cells that mediate or facilitate VEGF action.[118,119]

Collective evidence suggests that all the necessary components of the VEGFR system are present and dynamically expressed in the corpus luteum of several species (for reviews, see Refs 120,121). Although primarily based on mRNA data, luteinizing granulosa cells appear to express high levels of *VEGFA*,[117] with *VEGFA* expression remaining high in the corpus luteum during luteal development and in the developed corpus luteum.[108] Experiments localized VEGF mRNA and protein to the steroidogenic luteal cells[122] and, in one report, to endothelial support cells.[77] Also, FLT1 and KDR were localized to microvascular cells and, intriguingly in a few reports, to luteal cells.[123,124] There are few studies to date on specific cell types[125,126] to directly evaluate the production, regulation, and actions of VEGFA isoforms, VEGF types, or VEGFRs and co-receptors in the corpus luteum. Limited evidence, however, suggests that one isoform (VEGFA 165b[127]) may be anti-angiogenic in some species.

Limited studies suggest that regulation of VEGF expression in the ovulatory follicle and corpus luteum differs from that in many tissues. It is generally believed that a decline in local oxygen concentrations (hypoxia) is a primary stimulator of VEGF production and angiogenesis in normal and pathologic conditions.[128] Indeed, a similar scenario was proposed for the follicle.[129] However, Martinez-Chequer and colleagues[130] observed that hypoxic conditions did not increase VEGFA production by nonluteinizing granulosa cells from the preovulatory follicle or luteinizing granulosa cells from the periovulatory follicle in Old World monkeys. Rather, evidence from several species indicates that gonadotropin, notably the midcycle LH surge, promotes VEGF production[130] via transcriptional and/or posttranscriptional[131] actions in the luteinizing granulosa layer and developing corpus luteum. In addition, local factors such as insulin-like growth factors 1 and 2 (IGF1 and IGF2) may modulate or synergize with LH to control VEGF expression in luteinizing granulosa cells.[130] However, reports suggest that LH action on luteal tissue is less or not important in controlling VEGF production per se (except as a general luteotropin for maintaining luteal structure and function), and that local regulators including hypoxia predominate.[132,133]

A critical role for VEGFA in luteal angiogenesis and corpus luteum development and function is now established.[108,134] Since homozygous and heterozygous

null mutations for *Vegfa* (*Vegfa*^{tm1Gn}/*Vegfa*^{tm1Gn}) were embryonically lethal in mice (due to vascular malformations[135,136]), more classical ablation approaches were required, including protocols that prevented potential antecedent effects during follicle development. Administration of VEGFA antagonists (e.g., VEGF antibody and soluble VEGFR chimeras) to mice or monkeys either at the time of ovulation (i.e., during luteinization) or at midluteal phase (i.e., after luteal development) markedly inhibited subsequent luteal structure and function.[134,137] The suppression of luteal endothelial cell proliferation, endothelial cell area, and circulating progesterone levels, plus evidence that direct injection of VEGFA antagonist into the periovulatory follicle blocks its rupture and impairs luteal architecture,[138] suggests that VEGFA: (1) acts primarily through local effects in the follicle and corpus luteum, not via indirect actions on other tissues controlling ovarian function (e.g., via the hypothalamus–pituitary to alter gonadotropin secretion[139]); (2) is essential for ovulation of the mature follicle and normal development of the corpus luteum; and (3) remains important in the developed corpus luteum to maintain luteal structure and function. Nevertheless, there are few studies to date on the mechanisms of action or direct effects of VEGFA, or other VEGF family members, on luteal cell types. There is one report that VEGF stimulates proliferation of endothelial cells isolated from the monkey corpus luteum.[140]

Other Angiogenic and/or Angiolytic Factors

The angiopoietins (ANGPTs), unlike VEGFs, are not endothelial cell mitogens, but nevertheless are essential for normal vascular development.[141] Four different ANGPTs were identified in the mouse or human, and they all bind to the receptor tyrosine kinase, endothelial (TEK, also known as Tie-2), as either an agonist (ANGPT1, plus ANGPT4 in the mouse) or antagonist (ANGPT2, plus ANGPT3 in humans). An evolving model proposes that interactions between VEGF and ANGPT agonists and antagonists are critical for vessel development and maturation during embryogenesis and tumorigenesis.[142] Whereas VEGFA promotes angiogenesis to the stage of capillary tube formation, the local tissue ratio of ANGPT (1:2) appears critical for capillary growth, maturation, or degeneration. ANGPT2 may loosen cells from the ECM, allowing VEGFA to stimulate endothelial cell events, whereas ANGPT1 recruits periendothelial cells (i.e., pericytes) to stabilize and mature capillaries. In the relative absence of VEGF or other angiogenic factors, ANGPT2 may destabilize vessels and cause capillary degeneration, especially of those not supported by pericytes.

One can hypothesize that a similar scenario is important for vascular events occurring during the development, maintenance, and regression of the corpus luteum. Both ANGPT1 and ANGPT2, plus *TEK* mRNA or TEK protein, were detected in the luteal and endothelial cells, respectively, of several species. Limited reports on (1) the different temporal expression patterns for ANGPT1/2 and VEGFA during the luteal lifespan,[122,143] and (2) the preponderance of immature vessels (not supported by pericytes) in the ovine corpus luteum,[114] are consistent with this model. However, further research is needed on the cellular sites of ANGPT expression and action in the corpus luteum. Preliminary evidence that intrafollicular injection of the antagonist ANGPT2, but not the agonist ANGPT1, destroys the periovulatory follicle and resets the ovarian cycle in monkeys portends a vital role for ANGPTs in follicle–luteal structure and function.[144] Likewise, little is known about the regulation of ANGPT expression, except that the ovulatory gonadotropin surge stimulates ANGPT1 (but not ANGPT2) expression in luteinizing granulosa cells,[131] and chorionic gonadotropin (CG) exposure and early pregnancy promote ANGPT expression in the corpus luteum[143] of primate species. However, given the in vivo approach of these studies and the delayed response (36 h) of the ovulatory follicle,[131] it remains to be determined if these are direct or indirect effects of gonadotropin.

To date, VEGFA and its hypothesized interactions with ANGPTs have received the most attention regarding the development and maintenance of the vasculature in the corpus luteum. But future research must also resolve the role of other emerging factors that may synergize with VEGFA either to promote neovascularization or maturation of blood vessels, or to serve as a check on VEGFA action. One such factor is prokineticin 1 (PROK1), also named endocrine gland (EG)-VEGF due to having similar biologic actions as VEGF,[145] which is highly expressed in steroidogenic tissues, including the corpus luteum.[146] However, the different expression patterns for VEGFA and PROK1, which is molecularly distinct from VEGF and a member of the AVIT family, in the primate[147] and bovine[148] corpus luteum suggest complementary or diverse actions. Likewise, it appears that members of the SLIT-ROBO ligand–receptor family involved in angiogenesis[149] are expressed in the corpus luteum.[150] The concept of negative-feedback regulation of VEGF was advanced following the recent discovery of vasohibins (VASHs[151]) and the subsequent hypothesis that VASH1 is a negative regulator of VEGF's angiogenic action in the bovine corpus luteum.[152] Also, the discovery that the delta-like ligand 4 (DLL4)–Notch receptor system negatively regulates VEGF-mediated angiogenesis led to experiments in monkeys showing that DLL4 neutralization during luteal development increased angiogenesis but markedly suppressed the steroidogenic function and lifespan of the corpus luteum.[153] Studies on these and other potential regulators (e.g., the apelin–apelin receptor system[154]) are warranted. A scenario seems to be emerging that proposes a dynamic balance between factors that promote the development, maturation and function, and degeneration of the microvasculature that

is critical for the normal functional lifespan of the corpus luteum (Figure 23.4).

Finally, with the validation of specific markers for lymphatic cells, investigations recently characterized the robust lymphatic system in the corpus luteum, especially in larger animals such as primates[73] and domestic animals.[155] Moreover, these reports indicate the lymphangiogenic VEGFC and FIGF and their receptor FLT4 are dynamically expressed in these corpora lutea. The lymphatic vasculature plays a number of important roles, including maintaining fluid balance and transporting immune cells, in the body. Understanding the roles of the "second circulatory system—the lymphatics" in the ovulatory follicle[156] and corpus luteum will also be critical to systems analyses of ovarian function.

Differentiation of Steroid-Secreting Cells

Suppression of Steroidogenic Cell Proliferation

It is proposed that further differentiation of follicular steroidogenic (i.e., theca and granulosa) cells into primarily progesterone-secreting (i.e., luteal) cells is associated with cessation of proliferation.[72] Based on limited studies in a few species, primarily during superovulation protocols wherein the LH surge is replaced with a bolus of the LH-like hormone hCG, this appears to be the case. In rodents, cell mitotic activity generally ceased by 7 h after administration of the hCG bolus, and before the occurrence of ovulation at 12 h.[157] However, cell proliferation may continue in the cumulus cell compartment[158] and in follicles of species with a long LH surge-to-ovulation interval. Chaffin and colleagues propose that a secondary rise in cell proliferation occurs after the gonadotropin surge,[159] and numbers of dividing cells do not decline until 24 h after the hCG bolus[160] in monkeys. A small percentage (10%) of cells continued cell cycle activity up to the time of ovulation (36 h post hCG[161]). Indeed, there is evidence that proliferation of granulosa–luteal cells persists through ovulation[72] and in the developed corpus luteum.[109,162,163] However, the percentage of dividing cells is small, may decline with advancing age of the corpus luteum, and remains to be shown to have biologic relevance. For example, in primates there is little evidence of increased proliferation of steroidogenic, or even nonsteroidogenic (microvascular[164]), cells in species where the luteal lifespan is prolonged in actual or simulated pregnancy.[109,165]

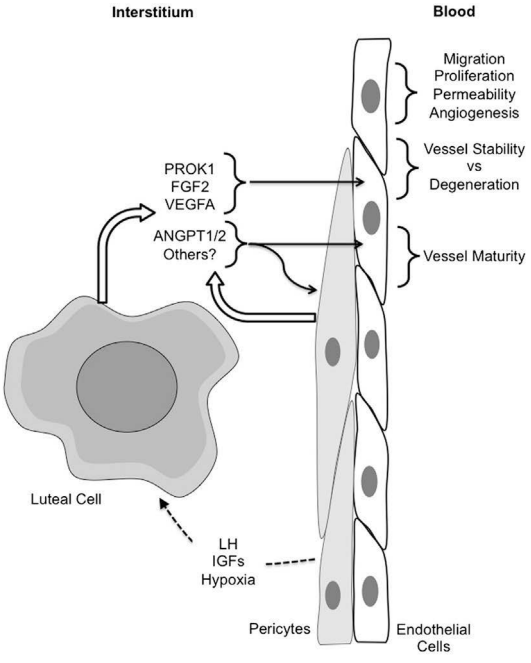

FIGURE 23.4 Schematic of the proposed interactions between luteal cells, capillary endothelial cells, and (in some species) periendothelial support cells (pericytes) for the production and action of angiogenic factors that control the development, maintenance, and regression of the microvasculature in the corpus luteum during the ovarian cycle. White arrows denote synthesis and release of vascular endothelial growth factors (VEGFs), prokineticin 1 (PROK1), basic fibroblast growth factor (FGF2), and/or the angiopoietins (ANGPT1 and 2). The dotted arrows indicate known regulators of VEGF–PROK1–ANGPT expression. The black arrows denote sites of action leading to the indicated effects on the microvasculature. See the text for further details. *Source: Adapted from Stouffer,[74] with permission.*

Considerable information has accrued in understanding how follicle–luteal cell progression through the cell cycle, or its cessation, is controlled by the intracellular proteins called cyclins,[72] their interaction with specific cyclin-dependent kinases (CDKs, e.g., CDK2), and inhibition of cyclin–CDK activity by cyclin-dependent kinase inhibitors (CDKNs). Studies in rodents indicate that cyclins D2 and E, plus Cdk2, which promote cell passage through the G1 and G1-S transition phases of the cell cycle, are downregulated in granulosa cells following the ovulatory hCG bolus.[72,166] Cyclin D2 is rapidly suppressed, whereas cyclin E does not change until 48 h post hCG. Although the mechanisms regulating cyclin expression require further investigation, there is some evidence for dephosphorylation of proteins (i.e., retinoblastoma proteins (RB)[72]) during luteinization in mice and women that could suppress cyclin D and E activity. The importance of the cyclin D system in controlling follicle growth is supported by evidence that granulosa cells from cyclin D2 null-mutant mice ($Ccnd2^{tm1Wbg}/Ccnd2^{tm1Wbg}$) are unable to proliferate, but they do luteinize following a gonadotropin bolus.[167] However Cdk4 null-mutant mice ($Cdk4^{tm1Kiyo}/Cdk4^{tm1Kiyo}$) demonstrated normal follicular development and granulosa cells exited from the cell cycle after ovulation, suggesting that another Cdk (possibly Cdk6) complexed with cyclin D2 regulates proliferation.[168] Again, there may be important species differences at this control point since Chaffin et al.[161] did not observe a decrease in cyclin D2 or E mRNA expression in the granulosa cells of monkey preovulatory follicles during the decline in cell proliferation initiated by an hCG bolus. Rather, the levels of cyclin B1 mRNA are reduced

in macaque granulosa cells within 12 h of exposure to a gonadotropin bolus. Also, phosphorylated RBs are present in the developing corpus luteum in primates.[169]

Unlike cyclin expression or activity, evidence suggests that the expression of CDK inhibitors is more consistent among species during luteinization. A similar profile of a transient increase in cyclin-dependent kinase inhibitor 1A (CDKN1A, or CKIp21), followed by a later rise in cyclin-dependent kinase inhibitor 1B (CDKN1B, or CDKp27), occurs in granulosa cells of rodents[170] and primates[161] after an ovulatory hCG bolus. The early rise in CDKN1A is associated with the marked decline in cell mitosis, but the later rise in CDKN1B may be critical for sustained cell cycle arrest. Studies in *Cdkn1b* null-mutant mice (*Cdkn1b*^{tm1Ako}/*Cdkn1b*^{tm1Ako}) indicate that follicles grow normally, but luteal cells do not stop proliferating following an ovulatory stimulus.[171] Subsequent generation of the *Cdkn1a*$^{-/-}$, *Cdkn1b*$^{-/-}$ double null-mutant mouse provided evidence that both cyclin-dependent kinase inhibitors (CKIs) cooperate to control exit of granulosa cells from the cell cycle.[172] Interestingly, despite continued proliferation, they differentiated into luteinized cells. Further studies are needed to understand the dynamics of cyclin–CDK–CDKN pathways in the follicle and corpus luteum, and their roles in controlling cell proliferation and differentiation. It is also likely that other pathways, such as the MYC–MAX–MXD1 family of transcription factors and TP53 (i.e., p53)-regulated $Mg2^{+}$–$Mn2^{+}$-dependent protein phosphatase 1D (PPM1D), also regulate cell proliferation[159] via or independent of the cyclin system.

To date, little is known about the gonadotropin actions that inhibit follicle cell proliferation. Gonadotropin surge levels could act directly or via LH–CG-stimulated intermediates, such as local growth factors or steroids (e.g., progesterone[173]), to control early and late (periovulatory) events in the cell cycle. Likewise, there is little information on proliferation or its control in luteinizing theca cells.[72]

Enhancement of Progesterone Production

One of the hallmarks of luteinization is the switch from the principal steroid product of the maturing follicle (i.e., estrogens) to that of the developing and mature corpus luteum (i.e., progesterone). This is both a qualitative and quantitative change, as the mammalian corpus luteum produces up to 100-fold greater amounts of steroid than the follicle. Thus, circulating levels of estradiol originating from the preovulatory follicle reach the picomolar range, whereas progesterone levels produced by the corpus luteum can reach the nanomolar range. The human corpus luteum, for example, is estimated to secrete up to 40 mg of progesterone per day during the menstrual cycle.[174] To achieve such steroidogenic activity, it appears that at least two major pathways are enhanced in luteal cells: (1) cell-specific amplification of steps in the steroidogenic pathway converting cholesterol to progesterone; and (2) increased ability to sequester bloodborne sources of cholesterol and to store cholesterol-rich lipid deposits.[175]

Circulating progesterone levels increase within the first half-hour to 2 h in several species, including primates and rodents, after exposure to a gonadotropin surge or bolus.[158,176] This suggests that a basal level of LH-sensitive progestogenic activity can be in place, but is relatively inactive until the LH surge. Indeed, nonluteinized granulosa cells from monkey preovulatory follicles appear to possess substantial levels of the enzyme 3 beta-hydroxysteroid dehydrogenase (HSD3B1 in nonprimate species; HSD3B2 in primates), since they readily convert pregnenolone to progesterone.[176] However, these cells cannot readily convert cholesterol analogs or cholesterol sources such as low-density lipoprotein into progesterone. Evidence from some species suggests that luteinization is associated with a marked increase in expression and/or activity of the cytochrome P450 side-chain cleavage enzyme ($P450_{scc}$ or CYP11A1) responsible for converting cholesterol to pregnenolone in the inner mitochondrial membrane, but there may be a divergence in CYP11A1 expression and enzyme activity as there are reports that *CYP11A1* mRNA initially declines following the onset of the LH surge.[177] Since CYP11A1 is required for androgen synthesis in the theca layer of the follicle,[178] its enhancement during luteinization may be primarily in the granulosa cells. Moreover, a further increase in HSD3B expression, and a decrease in 17α-hydroxylase, 17-20 lyase ($P450_{17\alpha}$ or CYP17A1, which catalyzes androgen production from progesterone), and aromatase ($P450_{arom}$ or CYP19A1, which converts androgen to estrogen) may facilitate the cellular emphasis on progestin production. Detailed genomic studies revealed that steroidogenic gene expression in the macaque follicle shortly after ovulation and through the initial stages of the corpus luteum is, in fact, associated with a preferential upregulation of key progestogenic enzymes (e.g., *CYP11A1* and *HSD3B2*) and reduced expression of enzymes involved in androgen (e.g., *CYP17A1*) and estradiol (e.g., *CYP19A1*) synthesis.[179,180]

Since initial steroidogenesis (i.e., CYP11A1 activity) occurs inside the mitochondria, the mechanism by which cholesterol is transported from outside to the inner mitochondrial membrane has been an area of important investigation.[181,182] Although recognized for years as the rate-limiting step in steroidogenesis, and the site of acute regulation via synthesis of a short-lived protein, it wasn't until the mid-1990s that this protein was identified, characterized, and named steroidogenic acute regulatory protein (STAR).[183] Mutations of the *Star* gene in mice (*Star*^{tm1Klp}/*Star*^{tm1Klp})[184] or that occur spontaneously in humans[182] are characterized by the inability

to synthesize adequate amounts of steroids. However, placental steroidogenesis in some species (e.g., humans) does not appear to involve STAR, but possibly a related protein, STARD3,[185] that possesses STAR-related lipid-transfer (START) domains. The critical role of STAR in cholesterol transport in adrenal and gonadal cells is established, but the mechanism of action awaits resolution. Although targeted to the mitochondrial membrane by specific amino acid sequences, STAR does not appear to require a receptor-type molecule for cholesterol transfer once it reaches the mitochondria. STAR has been postulated to act as an intermitochondrial shuttle[186] that interacts with other proteins on the outer mitochondrial membrane such as the translocator protein (TSPO; also known as the peripheral benzodiazepine receptor), voltage-dependent anion channel 1, and phosphate carrier protein. Each molecule of STAR transfers hundreds of molecules of cholesterol before undergoing a cleavage or inactivation event.[186] Notably, the expression of STAR is markedly upregulated in the granulosa layer during luteinization in many species.[187,188]

In several species, there is a transient peak in STAR expression occurring early after the LH–CG bolus, followed by a decline,[180,187–189] before returning to high levels in the corpus luteum. Biphasic patterns were also reported for expression of the steroidogenic enzymes CYP11A1 and HSD3B2.[180,188] These patterns could be related to transient downregulation of LH–CG receptors or desensitization of the receptor-stimulated pathways,[3] which is known to occur in rodents at ovulation,[190] but this awaits investigation in other species. Alternatively, the renewed expression could reflect the end of tissue-remodeling activities essential for follicle rupture and the emergence of steroidogenic activities in the developing corpus luteum. Nonetheless, high levels of expression of STAR, CYP11A1, and HSD3B2 immediately after follicle rupture ensure uninterrupted and increasing levels of progesterone synthesis through the initial period of luteal development in primates.[179,180] Such a similar pattern of expression for STAR, CYP11A1, and HSD3B2 could stem from shared (NR5A1–NR5A2 and GATA4–GATA6) and gene-specific transcription factors (e.g., NE5A1–NR5A2 and GATA4–GATA6).[191] Finally, the renewal of expression of the steroidogenic enzymes (CYP17A1,[190,192] or CYP19A1[179,193,194] after the development of the corpus luteum in various species, e.g., rodents and primates) likely reflects the unique ability of their corpus luteum to also synthesize androgens and/or estrogens. It is intriguing that the compartmentalization of the luteal tissue in Old World monkeys, baboons, and women retains the distribution of androgen- and estrogen-producing enzymes observed in the follicle. Immunocytochemical localization of CYP17A1 to the paraluteal and thecal–luteal cells and CYP19A1 to the granulosa luteal cells implies the continuation of a two-cell model for estrogen production in the corpus luteum of higher primates.[195,196]

To produce large quantities of progesterone, a major increase in availability of the steroid precursor, cholesterol, is required. The process of de novo synthesis of cholesterol by cells is well established.[175] However, de novo synthesis by luteal cells appears to be a minor source for cholesterol steroidogenesis, since expression of 3-hydroxy-3 methylglutaryl CoA (HMG–CoA) reductase (the enzyme catalyzing the rate limiting in cholesterol synthesis from two carbon precursors) appears constant in the corpus luteum.[197] Moreover, pharmacologic blockage of this enzyme does not alter circulating progesterone levels in women[198] or progesterone secretion by human luteinized granulosa cells.[199] Rather, most species use bloodborne sources of cholesterol esters, either in the form of high- or low-density lipoproteins (HDLs or LDLs), for luteal steroidogenesis. Notably, marked increases in the expression of receptors for and binding of HDL (scavenger receptor B1, SCARB1) and/or LDL (LDLR) occur during luteinization[200–204]; that may actually precede cell exposure to circulating substrate after ovulation. The relative contribution of the two lipoproteins varies between species, with the rodent corpus luteum relying primarily on HDL and the primate corpus luteum requiring LDL.[205] The preferential use of one lipoprotein type over another can occur, again depending on the species, despite the fact that both SCARB1 and LDLR are coexpressed in the corpus luteum. In luteinizing macaque granulosa cells, LDL primarily serves to support progesterone synthesis,[200,206] although both SCARB1 and LDLR expression increase after gonadotropin stimulation.[200] Further confirmation of the preference for LDL as a source of cholesterol in the corpus luteum comes from studies whereby patients that lack circulating LDL due to phenotypic abetalipoproteinemia have reduced levels of luteal progesterone synthesis.[207,208] Although receptor-dependent lipoprotein uptake is critical for sustained steroidogenesis, sufficient intracellular quantities of stored cholesterol esters exist to support the initial rapid increase in progesterone synthesis post LH and hCG (i.e., within the first few minutes to hours).[200]

Despite clear evidence demonstrating that LDL is the preferential source of cholesterol esters for primate luteal steroidogenesis, recent studies support a critical role for SCARB1 as a regulator of cholesterol uptake and progesterone synthesis within the primate corpus luteum. Using the human granulosa cell line HGL5, which can be induced to synthesize progesterone in response to increased intracellular cyclic adenosine monophosphate (cAMP) levels, a small interfering RNA (siRNA)-mediated reduction in SCARB1 levels significantly reduced the expression of CYP11A1, HSD3B2, and STAR.[209] Also, several *SCARB1* gene variants that alter function have been identified, and certain alleles are

associated with differences in circulating lipid levels, especially in women younger than 50 years of age.[210] One such SCARB1 variant (small nucleotide polymorphism, or SNP rs4238001) results in a glycine-to-serine substitution at position 2, which in turn leads to an increased rate of SCARB1 turnover and reduced cell surface expression.[211] This receptor variant was associated with lower follicular progesterone levels in women undergoing infertility treatments than those who possessed the wild-type allele. Of nine women possessing this variant, none had a viable pregnancy after embryo transfer, which is in contrast to the 30% pregnancy rate in control women without that particular variant. These studies parallel the findings from mice lacking SCARB1 that exhibited a 50% reduction in circulating progesterone levels relative to wild-type animals.[212] Although the molecular mechanisms require characterization, these studies suggest that SCARB1 action extends beyond its capacity to serve solely as a cholesterol–HDL uptake protein.

Relative to the mechanisms involving cholesterol uptake in luteal cells, its intracellular movement and processing are not as well characterized. Current evidence suggests that apolipoprotein A1 (APOA1) binds to SCARB1 and facilitates transfer from HDL-derived cholesterol ester.[213] Also, the Niemann Pick C1 protein (NPC1), which mediates transfer of LDL-derived cholesterol from the endosome to the Golgi apparatus,[214] is upregulated during luteinization.[215] Deficiencies in these components in rodents (e.g., APOA1[216]) or women (NPC1[217]) will diminish circulating progesterone levels and impair fertility. In human luteinizing granulosa cells, disruption of the NPC1-dependent cholesterol transport pathway blocks progesterone synthesis that is induced in response to LDL supplementation.[218] Reduced progesterone production by these cells was associated with excessive free cholesterol accumulation in large granules, suggesting a block in the movement of cholesterol from the lysosome to smaller storage vesicles or mitochondria.[218] However, when mice bearing a spontaneous mutation in the *Npc1* gene (*Npc1*^{m1N}/*Npc1*^{m1N}) receive exogenous gonadotropins, or their ovaries are transplanted into wild-type recipients, follicles ovulate and form corpora lutea.[219] Further investigation of *Npc1* mutant mice revealed that the reproductive defect likely stems from abnormal pituitary function. Moreover, *Npc1* null-mutant mice (*Npc1*^{tm1Mbjg}/*Npc1*^{tm1Mbjg}) that were unable to utilize LDL cholesterol as a substrate for steroid synthesis and that possessed preantral and antral follicles with highly vacuolated granulosa cells still generated circulating progesterone concentrations that were similar to those in wild-type mice.[220] Thus, it is likely that redundant pathways (e.g., Niemann Pick C2) are critical in allowing for continued luteal steroid synthesis when spontaneous or engineered mutants arise in individual genes encoding NPC1 or other cholesterol handling and transport proteins.

Following uptake from LDL or HDL or de novo synthesis, reserves of cholesterol available for steroidogenesis are stored in lipid droplets in the form of cholesterol esters. The synthesis of esterified cholesterol is dependent on the action of acyl coenzyme A–cholesterol acyl transferase (ACAT1) in the rough endoplasmic reticulum.[175] Free cholesterol must then be liberated by the actions of one or more lipase enzymes. Thus, another mechanism whereby luteal cells can acutely increase the availability of substrate for steroidogenesis is by hydrolysis of intracellular stores of cholesterol esterase in lipid droplets. The enzymes catalyzing this process belong to the lipase family, with hormone-sensitive lipase (LIPE) and lysosomal acid lipase (LIPA; also referred to as cholesterol esterase) being expressed in the corpus luteum of different species at luteinization.[221,222] Moreover, hepatic lipase (*Lipc*) null mutants (*Lipc*^{tm1Unc}/*Lipc*^{tm1Unc}) produce significantly less progesterone than their wild-type counterparts following the administration of an ovulatory bolus of hCG.[223] As the lipase family is composed of 12 different members, a complete characterization of which enzymes are critical for supplying the cholesterol needed for steroid production in the developing corpus luteum remains to be determined. Once free cholesterol is available, it is then transported intracellularly via sterol carrier proteins (e.g., SCP2 in rodents[224]) and cytoskeletal elements such as vimentin.[225] Figure 23.5 provides a schematic of progesterone biosynthesis in a "generic luteal cell", as adapted from Niswender, Ho, and colleagues.[182,226] However, there are many molecular and cellular events in this complex pathway, plus their regulation, which remain poorly understood and display species diversity.

It is likely that other mechanisms ensure that an adequate supply of cholesterol is available for steroid synthesis within the corpus luteum. The aforementioned description of cholesterol uptake and release from cellular stores is just one aspect of this complex system. Recent data from primates strongly suggest that increased steroid production by luteinized granulosa cells is also dependent on a reduction in the active efflux of cholesterol out of the cell, otherwise known as reverse cholesterol transport (RCT). RCT is mediated primarily by the cholesterol transport proteins such as ATP-binding cassette transporter 1 (ABCA1), which shuttles intracellular cholesterol to extracellular HDL lipoprotein acceptors. Expression of ABCA1 and the lipoprotein acceptor APOE are upregulated by the nuclear receptors NR1H3 and NR1H2 (also known as liver x receptor (LXR) alpha and beta, respectively), which are critical for maintaining cellular cholesterol homeostasis.[227] In luteinized human granulosa cells, which express both isoforms, treatment with synthetic agonists that activate both receptor subtypes increases the expression of the RCT system.[228] Consequently, increased RCT activity markedly reduced granulosa cell cholesterol levels and their progesterone-synthesizing capacity. In rhesus

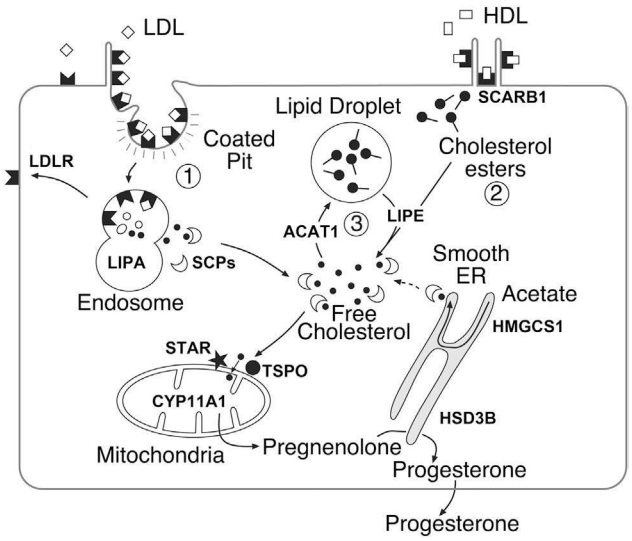

FIGURE 23.5 Simplified illustration of the three major sources of intracellular, unesterified (i.e., "free") cholesterol for progesterone synthesis in luteal cells. (1) The LDL-receptor mediated pathway leading to internalization of coated pits, and fusion with lysosomes to form endosomes, where lysosomal acid lipase (LIPA) generates free cholesterol (·). (2) The HDL receptor (SCARB1) pathway wherein hormone-sensitive lipase (LIPE) generates free cholesterol. (3) The storage and turnover of free cholesterol in lipid droplets via ACAT1-catalyzed cholesterol ester formation and LIPE-catalyzed ester hydrolysis. Intracellular transport of hydrophobic free cholesterol appears actively directed by various proteins, including NPC1 protein and sterol carrier proteins (e.g., SCP2). Cholesterol transport to the inner mitochondrial membrane involves the steroidogenic acute regulatory protein (STAR), possibly in close association with TSPO–peripheral-type benzodiazepine receptor and an endogenous ligand (e.g., diazepam binding inhibitor (DBI)). Cholesterol is enzymatically catalyzed to pregnenolone by the CPY11A1 complex. After moving to the smooth endoplasmic reticulum (ER), pregnenolone is converted to progesterone by 3β-hydroxysteroid dehydrogenase-Δ^{5-4} isomerase (HSD3B). Progesterone then exits the cell. Except under certain pathologic conditions where it may serve as a compensatory pathway,[194] de novo synthesis of cholesterol from acetyl CoA–acetate by 3-hydroxy-3 methylglutaryl CoA (HMG–CoA) synthase and reductase (dotted line) appears minimal in luteal cells.

macaque granulosa cells obtained from animals undergoing a controlled ovarian stimulation protocol, NR1H3 and NR1H2 as well as ABCA1 expression decreased within 3 h of an ovulatory stimulus. Incubating these cells with an LXR agonist also significantly reduced their ability to produce progesterone[229] due to increased RCT activity and direct suppression of the expression of key steroidogenic enzymes (e.g., STAR, CYP11A1, HSD3B2, and SCARB1). Whether the RCT system and its regulation is a common feature in controlling the corpus luteum in other species awaits investigation.

Steroid- and/or Peptide-Secreting Cells

Although the primary hormone produced by the corpus luteum is the steroid progesterone, it is clear that luteal cells synthesize and secrete many peptides–proteins that can serve in a species-dependent manner as local (paracrine or autocrine) or endocrine factors. Three types of hormones have been identified: oxytocin, relaxin, and inhibin–activin.

As reviewed by Sherwood and Fields[230] and Ivell,[231] following discovery of a "contractin factor" in cow luteal extracts that stimulated uterine contractions, 70 years passed before it was identified as molecularly homologous to oxytocin in the posterior pituitary.[232] Subsequent studies confirmed that oxytocin is synthesized in large luteal cells in much the same way as pituitary cells; first as a precursor protein that contains the nonapeptide sequence for oxytocin plus its carrier protein neurophysin I, followed by packaging of the prohormone into secretory granules[233] where it undergoes a series of enzymatic steps to release oxytocin.[231] The factors promoting luteal oxytocin mRNA expression, protein synthesis, and secretion are not well understood.[230,231] In the cow, oxytocin–neurophysin mRNA increases markedly in luteinizing granulosa cells after ovulation, but production of luteal oxytocin is markedly delayed and does not peak until well into the luteal phase (days 8–12). The delay appears due to a lag in the availability of peptidyl glycine α-amidating monooxygenase, which is the terminal enzyme in post-translational processing. Moreover, oxytocin secretion and action in the late luteal phase may occur when its mRNA levels are reduced or virtually absent; this paradox can be explained by posttranscriptional regulation and oxytocin stores in granules that are released in response to a secretagogue (e.g., a $PGF_{2\alpha}$ pulse). In domestic animals, such as cows, sheep, goats, and pigs, there is evidence that luteal oxytocin plays a role in luteal regression at the end of the nonfertile cycle[41] (see the section Luteolytic Factors on $PGF_{2\alpha}$). The release of oxytocin at midcycle may upregulate its own receptors in the uterus, and the subsequent pulses of Ca^{2+}-dependent oxytocin release provide a positive-feedback loop for promoting $PGF_{2\alpha}$ release from the uterus at luteolysis.

Luteal oxytocin is detectable in other species, including primates, but at 1000-fold lower levels; more sensitive techniques (e.g., real-time polymerase chain reaction) are required to detect oxytocin mRNA, and oxytocin concentrations in ovarian venous blood are typically not high enough to have an endocrine effect on nonovarian tissues.[231] A physiologic role for oxytocin in such species is perhaps limited to a local action, especially at luteinization. In many species,[234] including primates,[235] there is evidence that the LH surge promotes expression and production of oxytocin, and its receptor,[231,235] in luteinizing cells of the ovulatory follicle. Generally, data from a variety of species support a pro-luteinization action of oxytocin (e.g., to promote progesterone production and secretion).[235,236] Ivell[231] speculates that the paracrine luteotropic action of oxytocin during development of

the corpus luteum occurs in various species, only to be usurped by an endocrine role as a luteolytic factor during regression of the corpus luteum in domestic animals. Further studies are needed to establish the physiologic importance of oxytocin in periovulatory events and luteinization.

Although reports[237,238] suggest that relaxin (RLN) or a relaxin-like factor (RLF, or INSL3) are expressed by the ovulatory luteinizing follicle, and could play a role in ovulation,[239] relaxin has generally been considered a hormone of pregnancy and parturition.[230] Although discovered by F. Hisaw in 1926,[240] until recently the molecular nature of relaxin family members and their receptors proved elusive (for a review, see Ref. 241). Three genes encoding relaxins were identified in humans, which belong to the superfamily of structurally related hormones, including insulin, IGFs, insulin-like peptides (INSLs), and relaxin-like factor (RLF or INSL-3). RLN1 is typically produced and secreted by rodents (rat and mouse) and domestic animals (pig), whereas RLN2 is found in the circulation of primates (human). RLN3 is expressed in the brain, but its physiologic significance remains undefined. Recently, orphan receptors belonging to the leucine-rich guanine nucleotide-binding (G-protein) coupled receptors were identified as RLN family peptide (RXFP) receptors.[242]

The corpus luteum of many species produces and secretes RLN in a stage-dependent manner.[241] In species such as the rat, mouse, pig, and human, the corpus luteum is the sole source of RLN throughout pregnancy. However, the dynamics of RLN production and resultant levels or patterns in the circulation during pregnancy vary between species.[241] In the pig, *RLN* mRNA and protein increase at midluteal phase, particularly in the fertile cycle. The protein then progressively accumulates in membrane-bound cytoplasmic granules in large luteal cells throughout gestation, until about 2 days before delivery when degranulation occurs, luteal RLN levels markedly decline, and circulating RLN levels surge 12–24 h before delivery. A similar prepartum surge occurs during late gestation in rats and coincides with the decline in progesterone levels that is required for onset of parturition. In contrast, in primates, including women, the levels of RLN are detectable but much lower in the corpus luteum, and it does not appear this protein accumulates in membrane-bound granules. Also, circulating RLN levels are higher during early pregnancy, compared to the second or third trimester, and there is no evidence of a prepartum surge.[243,244] In nonprimates,[241] data suggest that RLN serves as a hormone during pregnancy to promote growth of the lower reproductive tract (cervix and vagina); facilitate rapid, successful delivery (cervical softening); and contribute to development of mammary gland alveoli (pig) or nipples (rat). In primates, limited studies have not demonstrated a physiologic role for RLN, but abnormal patterns of circulating RLN have been associated with early pregnancy loss in women.[245] Nevertheless, there is reason to suspect that it does not play a critical role in women since sole use of sequential estrogen and progesterone treatment in assisted reproductive technology protocols to mimic ovarian function results in normal gestations with timely delivery of offspring and successful lactation.[65] This does not, however, rule out a local ovarian action,[246] or actions on the embryo[247] in nonhuman primates. Clearly, the parturitional and lactational activities of RLN vary between species, as effects of relaxin deficiency are more dramatic in rats or pigs[241] than mice (i.e., $Rln^{tm1Wint}/Rln^{tm1Wint}$ or $Rxfp1^{tm1Jgo}/Rxfp1^{tm1Jgo}$ mutants).[248] An extreme example is the sheep corpus luteum, which does not produce RLN.[249]

Inhibin and activin are members of the transforming growth factor beta (TGFβ) superfamily of ligands initially identified based on their ability to inhibit and stimulate pituitary FSH, respectively.[250] The sequential expression of inhibin B (αβB) and inhibin A (αβA) as antral follicle growth proceeds is well documented.[251] Although inhibin production typically ends following the LH surge in many species, the corpus luteum of Old World monkeys[252] and women[253] continues to synthesize and secrete significant amounts of inhibin A and its α subunit. Investigators have localized the inhibin subunits primarily to granulosa luteal, but not theca luteal, cells[254]; these may also produce the binding proteins follistatin and follistatin-related gene products,[255] which neutralize inhibin–activin activity. However, follistatin is highly expressed in luteal tissue of other species (e.g., rat[256]) that do not produce inhibin–activin. Thus, these proteins may bind other factors, such as bone morphogenic proteins (BMPs), which could have local actions during luteinization and luteolysis.[256] Although the major source of inhibins and activins after early pregnancy in primates is the fetal–placental unit,[257] the corpus luteum secretes appreciable amounts of inhibin A in early gestation.[258] However, an endocrine role for luteal inhibin or activin remains unclear.[250] Immunoneutralization studies employing inhibin antisera suggest that testicular inhibin B plays a critical role in regulating circulating FSH levels in male primates,[259] but such studies failed to establish a similar role for ovarian inhibin A in females during the luteal phase of the menstrual cycle.[260] Proposed local actions to modulate luteal structure and function await delineation.[250]

LUTEOLYSIS

The corpus luteum typically ceases function and structurally involutes at one of three stages: (1) near the end of a nonfecund ovarian cycle; (2) at the point in

gestation where its progestogenic function is no longer needed to maintain intrauterine pregnancy, or if the corpus luteum is required throughout pregnancy; and then (3) at term delivery of the offspring. This loss of luteal structure–function is often referred to as luteolysis. As elegantly reviewed by Davis and Rueda,[261] the area is not without controversy as some investigators have used the terminology of functional luteolysis versus structural luteolysis and debated whether these events are independent, dependent, or interdependent. This issue is again related to species differences, such that in some species (e.g., the hamster[262]) the corpus luteum undergoes loss of function and essentially involutes within one ovarian cycle, whereas in others (e.g., the mouse and rat) the corpus luteum no longer functions but three or more generations of corpora lutea may be evident in the ovary during the cycle.[263] Likewise, the corpus luteum of primates may cease function 3–4 days before menstruation at the end of the cycle,[51] but residual luteal mass is evident into the next follicular phase and can even respond in a limited manner to exogenous gonadotropin (LH/CG) treatment.[264] Another level of complexity may be added when comparing luteolysis at the end of the ovarian cycle versus that in pregnancy. There are reports that structural involution can occur at a slower rate following pregnancy compared to the end of the ovarian cycle.[265] Such differences may be due to exposure to different milieus of luteotropic or luteolytic factors (see the section Regulation of Luteal Structure and Function) and/or reliance on different cell processes for destruction.[266] For example, although there is little evidence for a significant role of prolactin in promoting the structure and function of the primate corpus luteum during the menstrual cycle, this hormone may delay the luteolytic process in the "rejuvenated corpus luteum" after parturition, especially during lactation.[51] To date, for various reasons, many researchers investigated the process of luteolysis near the end of the nonfecund cycle, and often used the model of $PGF_{2\alpha}$-induced luteolysis once this prostaglandin was established as the uterine luteolytic factor in rodents and domestic animals. The hallmark of early luteal regression is declining progesterone production, clearly demarcated by the fall of circulating blood levels of this steroid hormone.[267] In species not utilizing a uterine luteolytic ($PGF_{2\alpha}$) process, however, the mechanisms and cellular events involved in loss of luteal function and structure remain poorly understood.

With considerable interest in recent years in the events that control cell death during tissue development and homeostasis, investigators began to apply advances in this field to studies on germ cell depletion, follicle atresia, and luteolysis in the ovary. While six different forms of cell death have been described,[268] the majority of studies conducted to date have focused primarily on the type of programmed cell death termed apoptosis.[269] Apoptosis describes the coordinated collapse of the cell, protein degradation, and DNA fragmentation, followed by rapid loss of dead cell fragments (e.g., by engulfment by immune cells).[270,271] Key characteristics include cell shrinkage (not swelling, as noted in necrosis), cytoplasmic "blebbing", chromatin condensation, and internucleosomal DNA cleavage that yields a "ladder" of 180 bp incremental sizes of genomic DNA that can be discerned by gel electrophoresis.

Increasing evidence supports the theory that apoptosis is an important mechanism controlling involution of the corpus luteum. Apoptotic indices, either morphologic or biochemical, were observed during structural involution of the corpus luteum in rodents[270,271] domestic animals,[272–274] and primates.[275–277] There is increasing evidence that the pro-apoptotic members of the death receptor family and their ligands are involved in luteal regression, including early disruption of progesterone production and structural involution. Notably, luteal expression of tumor necrosis factor (TNF) receptor superfamily (TNFRSF) members and corresponding ligands, which also include FAS ligand (FASL) and its receptor FAS, is associated with luteolysis in several species.[275,278,279] TNF can cause death of endothelial cells derived from the corpus luteum[280,281] and augment interferon gamma-induced death of luteal cells[282] from domestic animals (see the section Luteolytic Factors). Moreover, pseudopregnant mice treated with a TNF-neutralizing antibody and *Tnfrsf1a* null-mutant animals were refractory to $PGF_{2\alpha}$-mediated suppression of luteal progesterone production and luteal regression.[279] The pro-apoptotic actions of TNF appear to be dependent on its ability to elicit the synthesis of ceramide through the action of acid sphingomyelinase.[279] Evidence for a possible role of this superfamily in luteolysis is further derived from *Tnfrsf1a*-deficient mice ($Tnfrsf1a^{tm1Imx}/Tnfrsf1a^{tm1Imx}$), who fail to progress through the luteal (diestrus) phase of the cycle.[283] Exposure to FASL or FAS-activating antibody causes apoptosis of luteal cells in several species,[284,285] as well as luteinized granulosa cells from women.[286] The functional importance of the FASL–FAS system during luteolysis in the mouse is supported by evidence that: (1) FAS-activating antibody causes premature luteal regression[287]; whereas (2) mutants lacking FASL ($Fas^{gld/gld}$ mice) or FAS ($Fas^{lpr/lpr}$ mice) have irregular ovarian cycles attributed to defects, including aberrant luteolysis.[287] While such evidence supports a role for the TNFRSF family in regulating apoptosis, other pathways involved in determining luteal cell fate are likely involved as well. For example, it was recently discovered that expression of select SLIT proteins (e.g., SLIT2 and SLIT3) and an associated receptor (e.g., ROBO2), which promotes apoptosis and inhibits cell migration,[149] peak in the human corpus luteum during the period of luteal regression. Upon luteal rescue with exogenous

hCG administration, SLIT2, SLIT3, and ROBO2 expression was significantly lower, approaching early luteal and midluteal phase levels. By treating primary cultures of luteal cells with a recombinant ROBO1–Fc chimera that inhibits SLIT–ROBO signaling, cellular migration was increased while apoptosis decreased significantly,[150] suggesting an important role for this autocrine–paracrine pathway in primate luteolysis.

There is considerable evidence that members of the B-cell chronic lymphocytic leukemia (CLL)–lymphoma 2 (BCL2) family of apoptosis-regulatory proteins are present in the mammalian ovary, including the corpus luteum. It is possible that the prevailing theory[268] suggesting the balance between pro-apoptotic (e.g., BCL2-associated X protein, or BAX; and BCL2-antagonist/killer 1, or BAK1) and anti-apoptotic (e.g., BCL2; and BCL2L1, commonly known as BCL-X) factors, perhaps modulated by stimulus-sensitive BH3-only proteins (e.g., BNIP3, BAD), which controls cell death, also applies to follicle and corpus luteum turnover. The evidence is strongest for follicle atresia, where BAX protein levels are elevated[288] and *Bax*-deficient mice ($Bax^{tm1Sjk}/Bax^{tm1Sjk}$) accumulate abnormal follicles as their granulosa cells atrophy but presumably fail to undergo apoptosis during attempted atresia.[289] However, there is less evidence on the dynamics and role of BCL-like proteins in the corpus luteum; indeed, whether the ratio of pro (e.g., BAX)-to-anti (e.g., BCL2) apoptotic proteins changes in association with apoptosis in the primate corpus luteum during the menstrual cycle[290,291] or early pregnancy[292] is controversial. In human corpora lutea that were undergoing regression, as determined by declining levels of circulating progesterone, the antiapoptotic protein BCL2 was significantly lower, which correlated with increased cellular apoptosis. In contrast, expression of the pro-apoptotic proteins BAX and BAK1 remained constant, supporting the concept that the ratio of pro- and anti-apoptotic factors is critical in determining luteal cell survival.[277]

Although some progress has occurred in evaluating local death ligands and apoptotic regulatory proteins in the corpus luteum, there is little information on the molecular pathways that execute apoptosis and degrade cellular targets. In mammals, at least 13 different **c**ysteine **as**partate-specific prote**ases** (caspases or *CASP* genes) have been identified that serve as either "initiator" caspases (i.e., activate other caspases or initiate early mitochondrial changes) or "effector" caspases (hydrolyze a broad spectrum of proteins, including protein kinases, cytoskeletal components, and inhibitors of DNAses). Initial reports identified several caspases in luteal tissue,[293,294] and limited evidence supports a key role for the effector CASP3 in luteolysis. Notably, the uterine luteolytic factor, $PGF_{2\alpha}$, activates CASP3 during luteal regression in rodents[295] and sheep.[296] Also, *Casp3* null mice ($Casp3^{tm1Flv}/Casp3^{tm1Flv}$) exhibit delayed onset of luteal involution in gonadotropin-stimulated ovarian cycles.[297] However, there may be CASP3-independent mechanisms that are dependent upon the stage of the corpus luteum.[298] A complex interplay between inhibitors and activators of caspase activity also appears critical. The CASP8 and FADD-like apoptosis regulator protein (CFLAR, also known as cFLIP), which prevents CASP8 activation, is downregulated during structural luteal regression.[299]

While evidence to date supports a role for apoptosis in luteolysis, numerous issues remain to be resolved. There is evidence that pharmacologic methods of induced luteolysis (e.g., GnRH antagonist–induced LH deprivation versus $PGF_{2\alpha}$-induced luteolysis) can result in different patterns of cell death.[300] Studies are needed to compare processes of cell death during luteolysis in the ovarian cycle, pseudopregnancy, and pregnancy. Likewise, the physiologic stimuli and cellular pathways that result in cell death in the corpus luteum await clear definition. Extensive research in invertebrate and vertebrate systems established that deprivation of critical survival-promoting "growth factors" or expression of "death ligands" is used in normal tissue turnover to eliminate cells by apoptosis.[268] One can hypothesize that removal of or reduced sensitivity to luteotropic factors and/or additional or increased sensitivity to luteolytic factors promotes apoptosis in the corpus luteum. However, whether these factors act directly or indirectly (e.g., via regulation of local death ligands[268]) and how cellular processes are temporarily activated are not clear.

It also remains unclear whether the cell death during luteolysis occurs solely via apoptosis. For example, there is some controversy as to whether luteal cells in primates are dying by apoptosis[163,276] or by autophagy.[300,301] Autophagy is the process whereby cytoplasmic macromolecules and organelles (e.g., mitochondria and endoplasmic reticulum) are enveloped within vesicles (autophagosomes) that subsequently fuse with lysosomes, resulting in the degradation of their contents.[302] Certain characteristics of primate luteal cells during regression are indicative of autophagy rather than apoptosis, including cytoplasmic vacuolization and the appearance of prominent lysosomes in the absence of nuclear degeneration.[300,301] Nonetheless, recent evidence suggests that the relationship between autophagy and cell viability is more complex. While under certain circumstances autophagy serves to initiate cell death independently from caspase activation and apoptosis, it can also facilitate cell viability by allowing for the recycling of damaged or nonfunctional macromolecules or organelles.[303] Reportedly, both apoptotic cell death and autophagy occur in the human corpus luteum following natural and induced (GnRH antagonist) luteolysis, whereas hCG supplementation blocked apoptosis but was without any effect on autophagy.[277] The expression of

Beclin 1 (BECN1), a key upstream initiator of autophagosome formation, is highest in the human corpus luteum during its development, peak function, and during early pregnancy, whereas BECN1 expression was absent during luteolysis.[304] Thus, the significant levels of BECN1 and autophagosomes during periods of peak progesterone production[277,304] support a role for autophagy not necessarily in cell death, but in maintaining luteal cell viability in primates. In contrast, markers of autophagy peak in the rat corpus luteum only as it undergoes spontaneous or $PGF_{2\alpha}$-induced luteolysis during pseudopregnancy.[305] Moreover, pharmacologic inhibition of autophagosome degradation increased rat luteal cell death in vitro, while in contrast, blocking autophagosome formation inhibited luteal cell death. Whether autophagy promotes luteal cell death or survival may be yet another example of a species-dependent process. Further research is needed to identify the forms of cell death and their regulation during luteal regression in a species-specific manner.

REGULATION OF LUTEAL STRUCTURE AND FUNCTION

It is well established that the ovulatory LH surge initiates many of the early events in luteinization; indeed, the LH-induced early rise in progesterone synthesis appears essential for a number of hormonal as well as local actions of progesterone ranging from controlling the strength and duration of the LH surge[306] to ovulation,[307] respectively. However, the luteinizing ovulating follicle rapidly becomes dependent on additional luteotropic support for development and maintenance of luteal structure and function, as evidenced by: (1) the undeveloped corpus luteum and ultrashort luteal phase following the LH surge in unmated, nonfecund rodents; and (2) the ability of LH antagonists to quickly suppress luteal development and function within 24 h after the LH surge in species with a short luteal phase (e.g., primates).[308] A widely accepted concept is that the functional lifespan of the corpus luteum after the LH surge is dependent upon a balanced, and often sequential, exposure to factors that either promote (luteotropins) or inhibit (luteolysins) luteal structure and function.[309] Again, species have evolved various mechanisms for controlling the corpus luteum. Thus, luteotropic or luteolytic factors may originate from within the ovary or corpus luteum, as well as from classical or nonclassical endocrine glands, such as the pituitary, uterus, and placenta.

Luteotropic Factors

The primary luteotropic hormones vary between species, but generally include PRL-[310] or LH-like[311] hormones from the pituitary or uterine–placental unit. However, vital complementary roles may be played by local factors such as ovary- or luteal-derived steroids or growth factors.

Prolactin-Like Luteotropic Hormones

As recently reviewed by Stocco, Telleria, and Gibori,[75] either PRL secreted by the anterior pituitary or PRL-like hormones from the uterine decidua and placenta are the essential luteotropins in rodents, such as the rat, depending upon the stage of the corpus luteum following ovulation (Figure 23.6). Their critical role is evident from *Prl* ($Prl^{tm1Hmn}/Prl^{tm1Hmn}$) or Prl receptor ($Prlr^{tm1Cnp}/Prlr^{tm1Cnp}$) null-mutant mice, in which ovulation and early luteinization occur, but progesterone levels are subnormal and the corpora lutea regress rapidly, resulting in sterility.[312,313] However, the defect in uterine decidualization, implantation, and early embryonic development

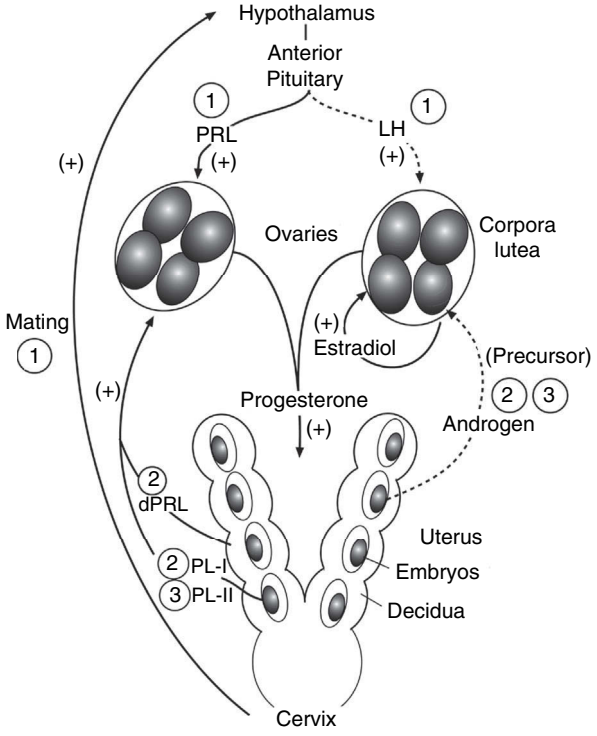

FIGURE 23.6 Diagram of the neuroendocrine, endocrine, and local mechanisms promoting luteal structure–function during early (1), mid- (2), and late (3) pregnancy in the rat. Three different sources of prolactin (PRL)-like hormones provide luteotropic support as gestation proceeds: (a) a neuroendocrine reflex arc activated by mating results in PRL release from the anterior pituitary; (b) by the second week of pregnancy, decidual prolactin (dPRL) can provide support; and (c) by mid- to late pregnancy, placental lactogens (PRL3D1, or PL-I; and then PRL3B1, or PL-II) are the primary luteotropins. Also, in early pregnancy, LH promotes production of the local luteotropin, estradiol. By mid- to late pregnancy, LH is no longer required because androgen from the placenta serves as a precursor for luteal estrogen production. Prolactin-like hormones and estradiol combine to maintain the functional corpus luteum throughout pregnancy, which is essential since the rat placenta is not a significant source of progesterone.

can be overcome by exogenous progesterone. Progesterone replacement does not, however, prevent fetal death at midpregnancy, suggesting additional vital roles for PRL within the uterus and placenta. Mating at estrus (i.e., stimulation of the cervix) activates a neuroendocrine loop that results in PRL secretion from the pituitary in two daily surges (one nocturnal, one diurnal) for 10–12 days.[70] It is experimentally established that pituitary PRL secreted during the first week of pregnancy or pseudopregnancy is solely capable of supporting luteal development and sustaining normal levels of progesterone secretion.[75] However, by day 7, pituitary PRL is no longer essential due to the expression of PRL-like luteotropins by the decidua and placenta.[75] From days 7 to 11, this activity appears to be a "decidual luteotropin", which according to recent data is identical in sequence and biologic activity to pituitary PRL.[314] Although capable of compensating for pituitary PRL, its role is probably comparable for decidual-derived PRL in primates (i.e., as a local "growth factor").[315] Nevertheless, by day 12, as pituitary PRL surges end and decidual PRL production declines, the pituitary is no longer essential for pregnancy maintenance.[75] Remarkably, the corpora lutea of pregnancy increase in mass, vascularity, and steroidogenic capacity due to secretion of other PRL-like factors, termed chorionic somatomammotropin hormones and known as prolactin family 3, subfamily d, member 1 (*Prl3d1*; commonly known as placental lactogen I), and prolactin family 3, subfamily b, member 1 (*Prl3b1*; commonly known as placental lactogen II).[316] Rat PRL3D1 is a glycosylated, 36–40 kDa protein produced by trophoblast giant cells that is only detectable in the circulation at midpregnancy. Rat PRL3B1 is a nonglycosylated, 25 kDa protein with a much shorter half-life that is also produced by the trophoblast giant cells. Onset of PRL3B1 precedes the decline in PRL3D1 expression in these cells, and circulating PRL3B1 levels rise from midpregnancy until parturition. It is now believed that both rat and mouse PRL3D1, as well as PRL3B1, bind to the PRL receptor and are sequentially responsible for maintenance of luteal structure and function in late pregnancy (as reviewed by Refs 75,317).

Thus, in rodents, such as the rat and mouse, PRL or PL binding to PRL receptors promotes luteal structure and function. Experiments in hypophysectomized rats suggest that between days 6 and 11 of pregnancy, LH also acts in concert with PRL to promote progesterone secretion.[318] However, subsequent studies suggest that the actual luteotropin is locally synthesized estradiol,[319] which results from LH-stimulated production of androgen by the ovary and corpus luteum that is aromatized to estradiol.[75] This absolute requirement for estradiol develops after the first week of pregnancy and it remains until parturition. But by midpregnancy, pituitary LH is no longer required because placental androgen production by trophoblast cells increases markedly and serves as a substrate for estrogen production by the ovary.[320] Although the rodent decidua and placenta produce PRL-like hormones, much like in primates,[315] there is no conclusive evidence that the former produces LH-like hormones, analogous to CG in monkeys or women.

The mechanisms of PRL action in the rodent corpus luteum are an area of active investigation.[321] The expression of species-specific numbers of long and short isoforms of the PRL receptor (PRLR-L and -S), is induced by luteinization,[321] and PRL itself may stimulate its own receptor's expression.[322] Until recently, PRL regulation of the rodent corpus luteum was thought to primarily involve PRLR-L.[321] But recent experiments on mice expressing only PRLR-L suggest that the short from is also required; critical defects in tissue matrix (collagen IV), angiogenic factors (VEGF), and microvascular endothelial cell organization were associated with the lack of luteinization.[323] Thus, as in the follicle,[321] PRLR-S as well as PRLR-L may impact the functional lifespan of the rodent corpus luteum.

Although the PRLR isoforms have identical extracellular domains, differences in the lengths and sequences of their intracellular domains lead to different signaling properties.[321] PRL-receptor signaling activates a number of signal transduction pathways, but most information to date originates from studies on PRLR-L activation of the Janus kinase 2 (JAK2)–signal transducer and activator of transcription 5A (STAT5) signal transduction system.[321] The importance of this pathway was established from dual *Stat5a* (*Stat5a*^{tm1Jni}/*Stat5a*^{tm1Jni}) and *Stat5b* (*Stat5b*^{tm1Jni}/*Stat5b*^{tm1Jni}) null-mutant mice that exhibit inadequate luteal function and infertility.[324] Several kinase pathways activated by PRL in other tissues warrant further evaluation (e.g., phosphatidylinositide 3-kinase (PI3)–protein kinase B (AKT), mitogen-activated protein kinase, C-Src,[75] and especially PKCdelta, which appears essential for PRL-stimulated relaxin expression in the rat corpus luteum).[325] PRL signaling through the short form of PRLR is poorly understood, but it is hypothesized to act as an inhibitor of PRLR-L function or to have other isoform-specific actions.[321]

PRL directly stimulates progesterone production in luteal cells by several mechanisms (for a review, see Ref. 75). First, PRL promotes steroidogenesis by upregulating enzymes involved in progesterone (CYP11A and HSD3B) and estradiol (CYP19A1) synthesis, as well as preventing progesterone degradation by inhibiting 20α-HSD activity through the reduced expression of the *AKR1C3* gene that encodes this enzyme.[326,327] Since the enzymes involved in progesterone production (i.e., CYP11A and HSD3B) are generally expressed in excess and are not rate limiting, the primary action of PRL appears to be total suppression of *AKR1C3* mRNA–20α-HSD activity, thereby preventing progesterone catabolism to the inactive progestin,

20α-dihydroprogesterone. Another enzyme that is regulated, notably by stabilization without affecting mRNA transcription,[328] is 17βHSD-7 (HSD17B7), which is involved in estradiol synthesis. This enzyme isoform was originally identified in the rodent corpus luteum at midpregnancy and term pregnancy as prolactin receptor–associated protein (PRAP) because of its association with the short form of PRLR.[329] A second and probably lesser mechanism of PRL action is to promote cholesterol availability from extracellular and intracellular stores, while promoting intraluteal cholesterol stores.[330] PRL increases the expression of HDL receptor (SCARB1), hormone-sensitive lipase, and sterol carrier protein 2 (SCP2),[331] and many of these effects appear mediated at the transcriptional level. Microarray experiments also identified new gene targets for PRL, which differ from those of LH, in the rodent corpus luteum.[331,332]

Less is known of the mechanisms where PRL regulates other luteal activities, thereby maintaining luteal structure and function. Since PRL promotes luteal expression of LH receptors,[333] enzymes involved in estradiol synthesis (as discussed here), and both ESR1 (ERα) and ESR2 (ERβ),[75] some of the effects are likely indirect and mediated by LH (e.g., STAR expression) or estradiol (e.g., angiogenesis[75]). Alternatively, some PRL effects may be direct, such as stimulation of proteases or their endogenous inhibitors (e.g., α2-macroglobulin[334]) or enzymes that scavenge oxygen-free radicals (e.g., superoxide dismutase[75]). Such actions may be important for stage-specific tissue remodeling and stability during luteal development and function, respectively. PRL may also influence cell hypertrophy either directly (e.g., via the induction of eukaryotic elongation factor 2 (EEF2), which promotes protein translation events[335]) or indirectly (e.g., via local synthesis and action of growth factors, such as IGF1, on large luteal cells[336]). However, much remains to be understood regarding the actions of PRL and PL in the corpus luteum at the physiologically relevant stages during the luteal lifespan of rodent species, as well as in other species where the corpus luteum is PRL dependent and sensitive.

Luteinizing Hormone (LH)-Like Luteotropins

Despite considerable controversy that extended into the 1980s, it is now generally accepted that following ovulation, the corpus luteum of many species, including domestic animals (e.g., sheep, cow, and horse[3]) and primates (monkeys to humans[337]), is primarily dependent upon LH from the anterior pituitary for the development and maintenance of luteal structure and function. Hypophysectomy,[338] administration of LH antisera,[339] or suppression of endogenous LH secretion[308] inhibits the corpus luteum, whereas co-administration of LH with such treatments can restore the normal functional lifespan of the corpus luteum during the nonfecund ovarian cycle. LH depletion and neutralization beginning within 1–2 days of ovulation can prevent the normal development of the corpus luteum, whereas treatment at midluteal phase will cause premature regression of the developed corpus luteum (for a review, see Ref. 307).

Nevertheless, there are likely differences in LH support of the corpus luteum between primate and nonprimate species, including acute regulation of progesterone secretion. In monkeys and women, pulses of secreted LH are entrained to increase progesterone secretion by midluteal phase, with steroid levels at baseline in the lengthening intervals between LH pulses by the late-luteal phase of the nonfecund cycle.[340] This, plus evidence that GnRH antagonist treatment rapidly and completely suppresses circulating progesterone levels (and causes early menstruation[308]), demonstrates the absolute requirement of primate luteal cells for LH exposure to maintain and stimulate progesterone secretion. In contrast, Diaz and colleagues[341] review evidence from cows and sheep that LH pulses are not entrained to progesterone secretion and that GnRH antagonist treatment only modestly (50%) decreases circulating progesterone levels in a stage-specific manner. Such findings suggest that a portion of luteal function in these domestic animals is not dependent on LH and is consistent with the concept that the developed corpus luteum contains a cell type (i.e., large luteal cells) that constitutively produces progesterone. The purported role of "constitutive" versus "LH-dependent" stimulation of luteal progesterone production between cell types during the luteal lifespan and between species warrants further evaluation (see the section Small versus Large Luteal Cells).

Despite this "constitutive activity", the corpus luteum in many domestic animals requires continual LH support until luteal function is no longer required to maintain pregnancy.[3] This interval in gestation may be relatively short (30 days in sheep), long (200 days in cow), or until term delivery (115 days in pig). In contrast, in many primate species, pituitary LH secretion is only required until late in the luteal phase of the fecund cycle. Thereafter, the LH-like hormone CG is secreted by trophoblast cells of the developing placenta, which extends the functional lifespan of the corpus luteum in early pregnancy.[54] CG is synthesized for a species-specific interval during gestation in primates, including throughout pregnancy in women.[65] The primary, although not sole (e.g., local uterine–placental[342]), action of CG is to sustain luteal structure and function for several days to weeks in early pregnancy until progesterone production is pronounced in the placenta. After this time (i.e., the luteal-placental shift), the ovary and corpus luteum is not required to maintain pregnancy in primates. Domestic animals do not typically produce a placental LH-like hormone, but the horse is a unique example. Although not likely the initial sustainer of luteal function in early pregnancy (see the section Small versus Large Luteal Cells), the mare placenta

begins to secrete large quantities of an LH-like hormone, originally termed pregnant mare serum gonadotropin (PMSG) and now referred to as equine CG (eCG).[3] The eCG induces ovulation of additional follicles that grow in intervals during early pregnancy and promotes the function of these secondary corpora lutea.[343] The corpora lutea secrete progesterone until eCG disappears from the circulation at 150 days of gestation, but luteal-derived progesterone is only essential until about day 70. Thereafter, progesterone support from the fetal–placental unit is sufficient to sustain pregnancy in mares.[344]

Pituitary LH, placental CG, and equine CG are members of the glycoprotein family of hormones that includes the pituitary follicle-stimulating hormone (FSH) and thyroid-stimulating hormone (TSH). Containing noncovalently linked α and β subunits with distinct glycosylation patterns (see a review in Ref. 311), the luteotropic LH–CG moieties bind with high affinity to one receptor (i.e., the LH–CG receptor), but not the FSH or TSH receptors. However, there is species specificity of the receptor, as primate LH–CG receptors (like primate GH receptors) have a much higher affinity for human LH or hCG than nonprimate gonadotropins.[345] Since the cloning of the LH–CG receptor,[346] considerable information has accrued on this member of the seven-transmembrane domain, rhodopsin-like, G protein–coupled receptor family.[347] LHCGR expression and hormone binding correlate with luteal function and circulating progesterone levels, as activity increases during luteal development and decreases during luteal regression in various species.[3] However, whether LHCGR levels directly influence luteal structure and function is less clear, in part because *Lhcgr* null-mutant mice (*Lhcgr*^{tm1Hht}/*Lhcgr*^{tm1Hht}),[348,349] as well as inactivating *LHCGR* gene mutations in women,[350] cause follicular defects and anovulation, which preclude analysis of luteal function. Also, the observation that few of the LHCGR need to be occupied for maximal steroidogenesis in the corpus luteum led to the concept of "spare receptors" for this luteotropin (see Ref. 3 for a review). Whether these receptors are really spare is unclear since signal transduction (e.g., cAMP production) continues to increase even though acute steroid production is maximal. There is speculation that this level of receptor occupancy and cAMP signaling is important for other aspects of luteal structure and function, especially in conditions of higher luteotropic support such as CG exposure in pregnancy.

G protein–coupled signaling though the LHCGR receptor involves activation of adenylate cyclase, production of cAMP, and activation of cAMP-dependent protein kinases (C kinases), which can have profound acute (steroidogenic) and chronic (structure–function) effects on the corpus luteum (see Refs 72,347 and Chapter 20 for reviews of signal transduction pathways). Although LH–CG can activate cAMP-independent pathways (e.g., phospholipase C–mediated synthesis of inositol-1,4,5 triphosphate (IP_3) and diacyl glycerol (DAG), leading to activation of protein kinase C (PKC) and intracellular calcium fluxes),[351,352] many of the effects of LH on steroidogenesis can be mimicked by exogenous cAMP and appear similar to those described here for PRL in rodent luteal cells (i.e., enhancement of lipoprotein (LDL) receptors and uptake, metabolism of stored cholesterol esters to free cholesterol, and promotion or maintenance of key enzymes such as CYP11A1 and HSD3B). However, there are two major differences between PRL–placental lactogen and LH–CG action in luteal cells of relevant species: (1) LH–CG does not regulate circulating progesterone levels by inhibiting its rapid metabolism by 20α-HSD; but, rather; (2) LH–CG action to acutely promote progesterone secretion involves rapid increases in STAR activity (via PKA-mediated phosphorylation), followed by a transcriptional effect to promote STAR expression. This mechanism, which remains an area of intense investigation, may be a critical site of LH–CG action in luteal cells of various species. Notably, STAR expression as well as that of key steroidogenic enzymes is sustained during prolongation of primate luteal function by CG treatment mimicking pregnancy initiation.[353,354]

There is much less known regarding the physiologic actions of LH via cAMP-independent pathways[347] or the nonsteroidogenic actions of LH to maintain luteal structure and function. Recent evidence suggests that the cAMP–PKA pathway is associated with morphological changes and regulation of apoptosis, whereas additional pathways (e.g., AKT- and ERK1/2) are also involved in LH–CG-regulated cell proliferation, differentiation, and survival.[355] Other pathways, including cAMP–PKA-dependent regulation of the pathways GSL3β–β-catenin[356] and MTOR–S6K1,[357] appear critical for various LH–CG actions. LH regulates a number of processes and proteins in luteal cells, including proteases and their inhibitors (i.e., MMPs, ADAMTS, and TIMPs[99,100,106]), cell–cell communication via gap junctions,[358] pro- and anti-apoptotic factors (e.g., MCL1[359]), and expression of other local (e.g., angiogenic[360]) or hormonal (e.g., relaxin[361]) factors. Investigators using differential display techniques or cDNA microarrays[354,362,363] are discovering novel or underappreciated LH-regulated pathways, such as the corticotropin receptor–binding protein (CRF–urocortin-R_1–R_2-BP) system in the monkey and human[364] corpus luteum, which are dynamically expressed and may regulate luteal structure and function.[365] Rigorous investigations of these genes and gene families and their proteins in the corpus luteum will increase our understanding of the complex systems controlling luteal structure, function, and lifespan.

Other Luteotropic Factors

There is growing evidence that the primary luteotropic hormones, PRL or LH, exert their actions, at least in part, through local synthesis and secretion of tropic

factors. These factors include steroids, peptide–proteins, and arachidonate metabolites.

Estradiol is an essential luteotropin for the maintenance of luteal structure and function in the rabbit (for a review, see Ref. 366), and also at a specific interval during pregnancy (days 7–11) in the rat.[75] Indeed, the corpus luteum of many species contains the classical nuclear estrogen receptor ESR1 and/or the recently discovered ESR2 (i.e., ERβ) form.[75] Experiments in gene-mutant mice suggest that estradiol can act via either ESR1 or ESR2 to influence luteal structure and function, but one cannot rule out effects predetermined by loss of estrogen action in the antecedent follicle.[367] Also, one cannot rule out the local actions of high estrogen levels on putative membrane receptors.[368,369] Estrogen's actions within the corpus luteum are not well defined, but there are limited reports that it enhances STAR expression and mitochondrial cholesterol transport in rabbit luteal tissue,[370] and SCARB1 expression, sterol content, and cholesterol transport (SCP2) capacity in rat luteal tissue.[75,371] One can hypothesize that estrogen synergizes with LH or PRL action to promote luteal structure and function. For example, LH-stimulated estrogen increases eukaryotic translation elongation factor 2 (EEF2) expression in rat luteal cells, and EEF2 is then activated by PRL, leading to a significant increase in luteal cell protein content.[335,372] Estrogen may also regulate luteal angiogenesis, notably at midpregnancy in the rat.[75] There is evidence for ER expression in endothelial cells[373] and estrogen regulation of angiogenic factors and their receptors,[123,374] such as that found in other vascular beds.

Since Rothchild[2] first proposed that progesterone itself is a local luteotropin in the mammalian corpus luteum, evidence (for a review, see Ref. 307) has accumulated for LH-induced transient expression of progesterone receptors (PGRA and PGRB) in the ovulatory, luteinizing follicle of many species, and continued PGR expression in the developed corpus luteum of many species with short- and long-luteal phases (from dogs to cows and primates). Studies in null-mutant mice established an essential role of *Pgra* ($Pgr^{tm1Omc}/Pgr^{tm1Omc}$), but not *Pgrb* ($Pgr^{tm2Omc}/Pgr^{t21Omc}$), in ovulation, but progesterone's role during luteinization appeared minimal.[375] Nevertheless, there are reports of progestin and antiprogestin effects in the rodent corpus luteum despite the absence of nuclear PGR.[376] Progesterone inhibited the expression of AKR1C3–20α-HSD (and hence suppressed progesterone metabolism) in rat luteal cells, and evidence was presented for this action via progestin binding to the glucocorticoid receptor.[377] Progestins may also have actions via plasma membrane receptors proposed to exist in granulosa and luteal cells[378] that prevent apoptosis and hence promote survival, but do not regulate progesterone secretion.[379] However, in species other than rodents, there are data suggesting that progesterone acts as a local luteotropin to promote luteal development and sustain luteal structure and function during the ovarian cycle and perhaps in pregnancy (for a review, see Ref. 307). In vitro and in vivo studies employing antiprogestins (e.g., RU486[380]) or progesterone synthesis inhibition and replacement (e.g., a HSD3B inhibitor with and without a progestin, R5020[381]) demonstrate a critical role for progesterone in controlling luteal structure and function as well as ovulation. Specific progesterone actions to control luteal tissue remodeling (e.g., protease expression[106]), health (suppression of apoptosis[380]), and sensitivity to other local factors (e.g., via ESR expression[382]) have been reported. Again, one can hypothesize that LH effects that are independent of progestin action and those that are mediated by LH-stimulated progesterone action synergize to control the development or structure–function of the corpus luteum in a species-dependent manner (Figure 23.7). Recent genome-wide analyses of the effects of LH ablation–replacement versus steroid ablation and progestin replacement in the corpus luteum (e.g., in primates[363]) are providing valuable databases for clarifying the roles of gonadotropins and steroids in luteal development and function. Further studies are needed to discern the role of progesterone and its potential receptor–signal transduction pathways in controlling the functional lifespan of the corpus luteum in species with short versus ultrashort luteal phases.

Direct effects of growth hormone (GH) on the ovarian follicle and corpus luteum were reported (see a review in Ref. 383), and there is some evidence that GH may be required for optimal luteal development. For example, both LH and GH were needed to obtain normal luteal weight and development in hypophysectomized ewes, although LH alone supported normal progesterone levels in the circulation.[226] Although there is some evidence for receptors for the metabolic hormones GH and insulin in luteal tissues, there is speculation that many of their effects pertain to GH-induced elevations in circulating IGF1 or IGF2, or local IGF production in response to luteotropic hormones.[384,385] There is evidence that IGF1 or IGF2 stimulates progesterone secretion by luteinizing granulosa cells or luteal cells from various species,[130,385,386] but the cellular mechanisms of IGF action in luteal cells have received little attention.[385,387] In the human, LH stimulation of STAR expression is enhanced by IGFs.[388] There are limited reports of IGF stimulating peptidergic activity (oxytocin production), production of angiogenic factors (VEGF), or expression of luteotropin receptors (e.g., ERβ in rat luteal cells), often in synergy with LH or PRL action. Significant advances have occurred in our understanding of gonadotropin control and dynamic expression of components of the IGF-R-BP (insulin-like growth factor binding protein 3) system within the follicle, and its integral role in influencing steroidogenesis and growth versus atresia of the maturing antral follicle.[389] It

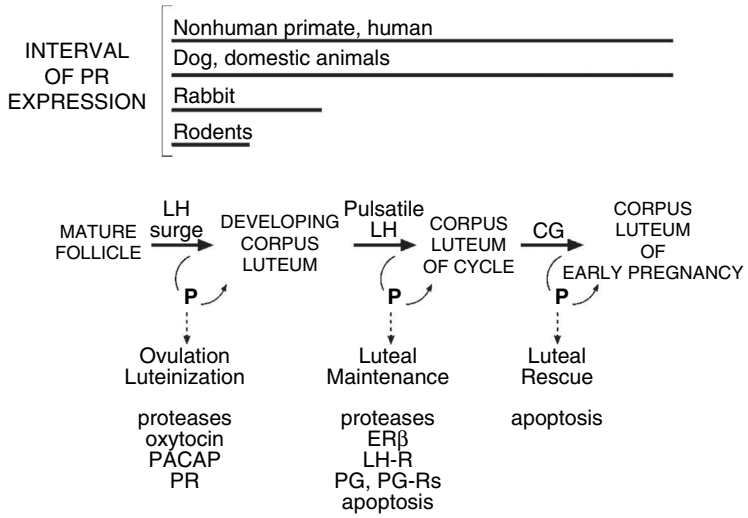

FIGURE 23.7 Schematic of the duration of "classical" progesterone receptor (PGR) expression in the corpus luteum of various species during its functional lifespan in the ovarian cycle and early pregnancy (top), with the hypothesis that LH–CG-stimulated synthesis of progesterone (P) generates a critical luteotropic factor essential for ovulation (in all species examined to date), development of the corpus luteum (except in rodents), maintenance of the corpus luteum during the ovarian cycle (in cows and primates), and extension of luteal structure and function in pregnancy (in cows and primates). Some of the gene products and processes identified as P-regulated pathways are listed. Growing evidence supports the concept of P as a local luteotropin.[2] Proteases include ADAMTS1, cathepsin L (rodent), and MMP1 (nonhuman primate). ADCYAP1: adenylate cyclase activating polypeptide 1 (pituitary). See text and other reviews[307] for further details.

is possible that similar dynamics of the IGF system play a role in the development and functional lifespan of the corpus luteum.[384] Further studies are needed to discern similarities and differences in the regulation and role of the species-specific IGF (e.g., IGF1 in rodents and IGF2 in primates)-R-BP system in the corpus luteum.[389]

Although investigations on eicosanoids have focused primarily on the luteolytic action of prostaglandin (PG) $F_{2\alpha}$ (see the section Vascular Endothelial Growth Factor), there is growing evidence that other PGs and arachidonate metabolites can have luteotropic or luteoprotective effects on the corpus luteum of various species. Notably, PGs of the E, I, and D series stimulate cAMP and progesterone by luteal preparations in vitro,[390,391] and can prevent spontaneous and/or $PGF_{2\alpha}$-induced luteolysis during local intrauterine or intraluteal administration in monkeys[392] and domestic animals (see Ref. 393). The concept of a balance between luteotropic versus luteolytic PGs controlling luteal development, structure and function, and lifespan[309] is supported by recent evidence from the bovine[393] and primate[394] corpus luteum such that components involved in PGE synthesis, transport and signaling (e.g., PGE synthase, PG transporter, and PGE receptor subtypes 2 and 3), are activated during luteal development and maintenance, whereas the PGF system (PGF receptor, or PTGFR) is activated at luteal regression. These data may explain the puzzling observation that infusions of a PG synthesis inhibitor can decrease circulating progesterone levels.[395,396] Further studies are needed on the putative luteotropic role of PGs (e.g., PGI[397] as well as PGE), the role on other arachidonate metabolites,[398,399] and their relationship to the luteolysin $PGF_{2\alpha}$ in controlling luteal structure and function. To date, the best evidence for a physiologic role of luteotropic PGs is the purported role of PGE_2 during early pregnancy in domestic animals (after interferon tau (IFNτ) declines and luteal resistance to $PGF_{2\alpha}$ is lost[400]; see the section Domestic Animals).

Luteolytic Factors

As elegantly reviewed by Niswender[3] and McCracken,[41] the concept of physiologically important luteolytic factors (i.e., those that promote the loss of function and structural involution of the corpus luteum) began with the observation by Loeb in 1923[31] that removal of the uterus (i.e., hysterectomy) lengthened the lifespan of the corpus luteum in guinea pigs. Subsequently, investigators reported that the timely demise of the corpus luteum was also delayed following hysterectomy in many species during pseudopregnancy (e.g., rat,[401] mice,[402] and rabbit[403]), or in other species that develop a functional corpus luteum during the nonfecund ovarian cycle (e.g., cow and sheep,[404] pig,[405] and horse[406]). There is variation among such species in the length of time that corpora lutea survive after hysterectomy, but luteal tissue will generally retain both structure and function for at least the duration of normal pregnancy. Nevertheless, the extended luteal function may be at a lower level than that achieved earlier in pseudopregnancy (rabbit) or the luteal phase of the estrus cycle (horse). In many, but not all,[3] such species, the luteolytic effect of the uterus

appears to be exerted locally. Removal of one of two uterine horns in many domestic[407] and laboratory[408] animals does not prevent timely luteal regression in the ovary adjacent to the intact horn, but prevents luteolysis on the other ovary. Therefore, it appears unlikely that the luteolytic factor is transported from the uterus to the ovary through the systemic circulation. Studies by Ginther[409] and others[41] suggest that the uterine luteolytic factor passes directly from the uterine vein into the ovarian artery in sheep and cattle, not through direct connections but a countercurrent mechanism that permits transfer between regions of extensive contact between these two vessels. Throughout the broad ligament, the vessels have thinner walls where they are in contact, they share a common tunica adventitia, and the ovarian artery is very tortuous on the uterine vein—all features consistent with venoarterial exchange.[409] However, in some species such as primates (Old World monkeys[63] and humans[64]) and dogs,[410] hysterectomy does not alter the normal lifespan of the corpus luteum. In such species, as well as those where the uterine luteolytic effect is not exerted locally, there is less contact between the uterine vein and ovarian artery.[411,412] Nevertheless, once the concept developed of a uterine luteolytic signal near the end of the nonfecund ovarian cycle, pseudopregnancy, or parturition in many species, investigators found it very difficult to elucidate the bioactive agent. It was not until the late 1970s, after considerable controversy, that there was general agreement that the uterine luteolytic factor was not a protein, peptide, or steroid, but rather $PGF_{2\alpha}$.[32]

Prostaglandin (PG) $F_{2\alpha}$

As reviewed by Sirois et al.,[413] prostaglandins (PGs) are lipid molecules that belong to the eicosanoid family, which also includes prostacyclin, epoxyeicosatrienoic acids, thromboxanes, leukotrienes, and lipoxins. They are derived from 20-carbon (C) polyunsaturated fatty acids, typically arachidonic acid. The various classes of PGs are synthesized through the intermediate PGH_2, which is generated via the action of prostaglandin synthase (PTGS) activity. There are two PTGS enzymes (PTGS1 and PTGS2), with the PTGS2 enzyme being the predominant isoform responsible for PG synthesis in the ovary. $PGF_{2\alpha}$ synthesis can occur via different routes, including: (1) direct conversion from PGH_2; (2) through the 9-keto reduction of PGE_2; and (3) through PGD_2 via 11-keto reductase activity. The enzymes responsible for the formation of $PGF_{2\alpha}$ from such varied substrates belong to the aldo-keto reductase (AKR) superfamily, primarily the 1C and 1B group.[414] AKR1B5, which is highly expressed in the bovine uterus, appears to be a major source of $PGF_{2\alpha}$ synthesis.[415] However, the level of contribution by other AKR family members is unclear, as their role in PG metabolism in the corpus luteum has not been studied in detail.

A major breakthrough occurred with the discovery that $PGF_{2\alpha}$, but not other PGs, caused premature luteolysis in pseudopregnant rats.[416] This observation was followed by evidence that: (1) exogenous $PGF_{2\alpha}$ had luteolytic effects in every species tested that depended on uterine control of the corpus luteum, except only to a limited extent in pigs[417]; and (2) $PGF_{2\alpha}$ synthesis and secretion are elevated in endometrial tissue during luteal regression,[418,419] plus pulses of $PGF_{2\alpha}$ release into the uterine vein (often detectable in the circulation) increase in frequency and amplitude during the onset of luteolysis in several species.[41,267] Critical evidence for a causal role of endogenous $PGF_{2\alpha}$ was derived from studies wherein nonsteroidal anti-inflammatory drugs that inhibit PG synthesis, such as indomethacin, or active or passive immunization to $PGF_{2\alpha}$, blocked spontaneous luteolysis during the estrous cycle and prolonged pseudopregnancy and pregnancy in domestic animals and rodents.[420,421] Evidence that null-mutant mice lacking the $PGF_{2\alpha}$ receptor ($Ptgfr^{tm1Aic}/Ptgfr^{tm1Aic}$)[422,423] do not display a timely preparturitional decline in circulating progesterone and fail to deliver, plus that this can be overcome by ovariectomy, supports a critical role for $PGF_{2\alpha}$ in signaling luteolysis.

The molecular and cellular mechanisms involved in controlling the synthesis and release of uterine $PGF_{2\alpha}$ during luteolysis remain to be fully defined. The endocrine action of $PGF_{2\alpha}$ between the uterus and the ovary, however, is clearer. As posited by McCracken[41] from studies in sheep (Figure 23.8), the following model emerged whereby changes in steroid sensitivity toward the end of the luteal phase: (1) stimulate the hypothalamic oxytocin pulse generator to increase the frequency of oxytocin release from the posterior pituitary; and (2) upregulate oxytocin receptors in the uterine endometrium. These combined effects lead to the release of low (subluteolytic) pulses of $PGF_{2\alpha}$ from the uterus, which are sufficient to cause release of oxytocin stores from the corpus luteum via $PGF_{2\alpha}$–PTGFR signal transduction. The supplemental release of luteal oxytocin amplifies the release of uterine $PGF_{2\alpha}$, resulting in large (luteolytic) pulses of $PGF_{2\alpha}$ that cause luteal regression. Moreover, Wiltbank and colleagues[424] proposed another amplification system in the corpus luteum, wherein small amounts of uterine $PGF_{2\alpha}$ stimulate intraluteal production of $PGF_{2\alpha}$ via induction of PTGS2. The positive-feedback loops between the uterus and corpus luteum, and within the corpus luteum, lead to sufficient $PGF_{2\alpha}$ action causing luteolysis. A similar amplification loop was also reported to be active in the corpus luteum of pseudopregnant rabbits.[425] It is noteworthy that luteal tissue in domestic animals acquires the ability for $PGF_{2\alpha}$-induced PGF synthesis at the stage when the corpus luteum first develops the capacity for $PGF_{2\alpha}$-mediated luteal regression (day 7 in cows and day 13 in pigs[424,426]).

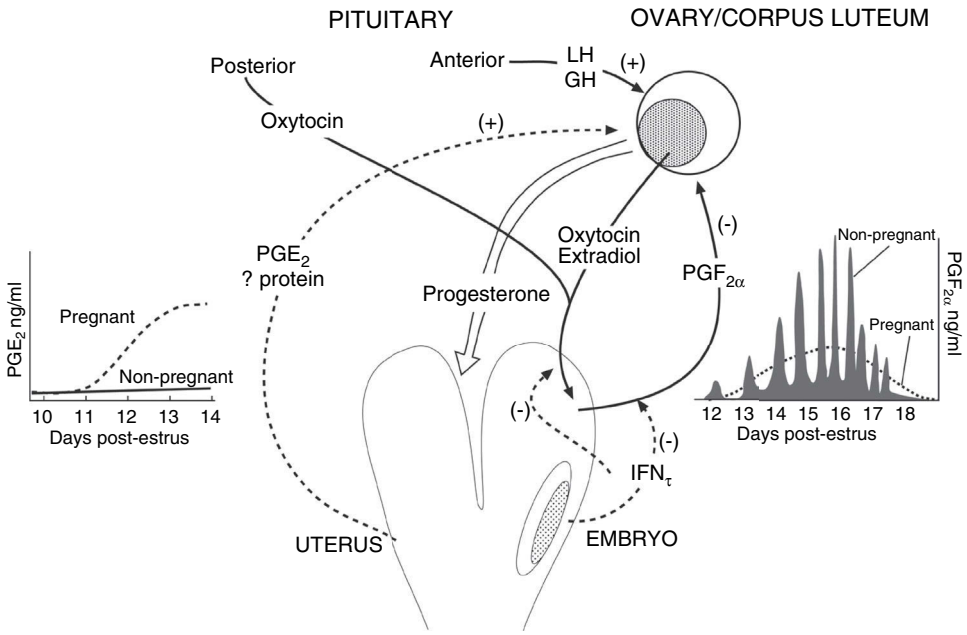

FIGURE 23.8 Model of the neuroendocrine, endocrine, and local mechanisms controlling the functional lifespan of the corpus luteum during the estrous cycle (solid lines) and during early pregnancy (dotted lines) in ruminants, such as sheep. During the cycle, the corpus luteum is controlled by luteotropic factors (luteinizing hormone (LH) and growth hormone (GH)) from the anterior pituitary and a uterine luteolytic factor ($PGF_{2\alpha}$) whose timely pulsatile release (right inset) is influenced by pituitary (oxytocin) and ovarian (steroids and oxytocin) hormones. Following implantation, embryo-derived interferon tau (IFNτ) prevents the pulsatile release of $PGF_{2\alpha}$, thereby preventing luteolysis in the fertile cycle. Also, secretion of PGE_2 (left inset) or unknown proteins from the uterus may promote luteal function during pregnancy. Hence, the corpus luteum continues to produce progesterone, which sustains intrauterine pregnancy. *Source: Adapted from Refs 3,428, with permission.*

The potential for $PGF_{2\alpha}$ to promote luteolysis is dependent on the responsiveness of several genes that are critical effectors of luteal function and luteolysis after a certain point in the luteal phase, including genes encoding angiogenic (i.e., *VEGF*, *VASHs*, and *FGF2*) and vasoactive factors (i.e., endothelin 1, angiotensin-converting enzyme, and endothelial nitric oxide synthase; discussed further in this chapter).[427] Since many other substances, including gonadotropins, progesterone, and cytokines, are proposed modulators of luteal or uterine $PGF_{2\alpha}$ production,[41,424] the precise manner in which the luteolytic signal is controlled remains incompletely defined.[424]

PTGFR, the receptor for $PGF_{2\alpha}$, is a G protein–coupled cell surface receptor with seven transmembrane domains.[413] It is one of the group 2 contractile PG receptors, which also includes PGE (PTGER1) and thromboxane A_2 receptors, thought to signal through $G_{\alpha q}$ and the phospholipase C pathway to increase intracellular Ca^{2+} levels. Although many studies in the 1970s reported specific $PGF_{2\alpha}$ ligand binding to luteal tissue preparations,[41] there have been relatively few studies on the expression and activity of PG receptor subtypes in luteal tissue since their discovery. The regulation of PTGFR expression in the corpus luteum through the luteal phase is species dependent,[261,394] but is generally present and capable of inducing luteolysis late in the ovarian cycle. There is evidence that PTGFR is expressed in the corpus luteum of many species,[429,430] including those lacking a uterine luteolytic mechanism.[394] Regarding the latter, a comprehensive analysis of the rhesus macaque transcriptome at different stages of the natural luteal phase revealed that PTGFR expression is dynamically regulated, wherein its expression is minimal during luteal development and the interval of peak progesterone synthesis, but then increases dramatically just prior to and during the period of luteolysis.[179,394] The level of change in mRNA expression in the macaque corpus luteum was greater for *PTGFR* than for any other gene. Likewise, rescue of rhesus macaque luteal function through the administration of increasing daily doses of hCG (i.e., in a simulated early-pregnancy model) prevented the spike in *PTGFR* mRNA levels that were routinely observed prior to and during luteolysis (NCBI Geo Accession GSE25335[354]). Collectively, these findings support the need for additional studies to establish the role that PTGFR signaling plays in the internal "self-destruct" mechanism proposed for primate luteolysis.[431]

The number of reports linking the Ca^{2+}–protein kinase C pathway to $PGF_{2\alpha}$ action in the corpus luteum is growing,[432] particularly in relation to antisteroidogenic effects and cell death.[261,433] Through the PKC-dependent activation of RAF1, $PGF_{2\alpha}$ stimulates the extracellular signal-regulated kinase (ERK) family of MAPKs,[434] which

in turn results in the phosphorylation and activation of ribosomal protein S6 kinase 1 (RPS6K1) signaling in steroidogenic luteal cells.[435] Inhibition of the mammalian target of rapamycin (MTOR) with rapamycin prevented $PGF_{2\alpha}$ stimulation of RPS6K1 and downstream mechanisms involved in cellular protein synthesis.[435] Other reports suggest that Ca^{2+}-dependent $PGF_{2\alpha}$ signaling via ERK interferes with gonadotropin upregulation of STAR expression and progesterone secretion through the activation of the transcription factor Yin Yang 1 (YY1), which inhibits STAR transcription through the recruitment of histone deacetylase 1.[436] $PGF_{2\alpha}$-dependent activation of ERK signaling was also reported to diminish the responsiveness of bovine luteal cells to IGF1 through the phosphorylation of insulin receptor substrate 1 (IRS1), a key intermediate in the insulin–IGF1 pathway,[387] and to increase the expression of the nuclear receptor NR4A1 (Nurr77), a transcription factor that is critical for increasing 20α-hydroxysteroid hydrogenase activity attributed to AKR1C3.[437] There is also evidence that $PGF_{2\alpha}$ increases expression of SOCS3, a member of the suppressor of cytokine signaling family, which can limit PRL–PL action by inhibiting the JAK2–STAT5 pathway.[438]

While the mechanisms responsible for PGFTR signaling are becoming more defined, the effector mechanisms of PTGFR stimulation in regulating luteolysis remain unclear, with several avenues proposed that range from reducing luteal blood flow to blocking the action of luteotropic factors, inhibiting steroidogenesis, and promoting cell destruction and tissue dissolution. Again, however, the effects may vary between species and differ between in vivo (e.g., intact tissue) and in vitro (e.g., dispersed, cultured cells) experimental systems. Based on its potent vasoconstrictor activity in various vascular beds, and evidence that exogenous $PGF_{2\alpha}$ can markedly decrease luteal blood flow in sheep,[439] it was originally proposed that one of the early, important actions of the luteolytic $PGF_{2\alpha}$ signal was to limit the influx of bloodborne nutrients and the like to the luteal tissue and the efflux of metabolic byproducts, thereby causing tissue damage and dissolution. However, other investigators did not observe early changes in total or regional (luteal) ovarian blood flow during spontaneous or $PGF_{2\alpha}$-induced luteolysis in rodents,[440] rabbits,[366] or domestic animals[41] when progesterone levels were first declining (up to 50%). Moreover, when blood flow is assessed soon (i.e., within an hour) after administration of a $PGF_{2\alpha}$ bolus or a $PGF_{2\alpha}$ agonist, an increase in blood flow was reported.[441] Studies were recently performed that allowed for a detailed characterization of the endogenous $PGF_{2\alpha}$ pulses that occur before, during, and following luteolysis in domesticated animal species.[442,443] More rigorous studies providing exogenous $PGF_{2\alpha}$ that mimic such endogenous pulses near the end of the luteal phase, however, had no effect on acute changes on luteal blood flow, suggesting that a single, large bolus of $PGF_{2\alpha}$ may elicit nonphysiological effects. There is little doubt that later decreases in blood flow, potentially initiated through the loss of capillaries and small vessels,[444] are associated with continued reduction in luteal function and tissue destruction. However, in vivo and in vitro evidence that $PGF_{2\alpha}$ can suppress progesterone production and antagonize luteotropic hormone action preceding the later reduction of blood flow points to other major actions in luteal tissue.

Current evidence suggests that $PGF_{2\alpha}$ induction of luteolysis can also occur via multiple, nonvascular effects. $PGF_{2\alpha}$ increases the percentage of steroidogenic cells progressing through the cell cycle, which is a prerequisite for the induction of apoptosis.[445] $PGF_{2\alpha}$ can also rapidly decrease luteal progesterone synthesis by inhibiting the intracellular transport (e.g., by decreasing the mRNA levels and activity of SCP2 in rodents[446]) and entrance of cholesterol into mitochondria (e.g., by decreasing the activity and expression of STAR in domestic animals and rodents[436,447]). It now appears that previous observations that $PGF_{2\alpha}$ inhibited lipoprotein-supported progesterone production by luteal cells[440] were not due primarily to lesions in cholesterol uptake or storage,[448] but rather to lesions in cholesterol delivery to CYP11A1.[440] Reports that transport-independent hydroxylated cholesterol substrates enhance steroid production by luteal tissue during spontaneous or $PGF_{2\alpha}$-induced regression support this concept. Nevertheless, there is evidence that $PGF_{2\alpha}$ causes a similar reduction in mRNA expression of some steroidogenic enzymes (e.g., *HSD3B* in the corpus luteum of sheep[449]). Moreover, in the rat corpus luteum, $PGF_{2\alpha}$ rapidly induces the transcription of the progesterone-degrading enzyme *AKR1C3*/20α–HSD.[75]

Although the ability of $PGF_{2\alpha}$ to serve as the luteolytic signal and cause of early loss of luteal function in many species is generally accepted, $PGF_{2\alpha}$ effects on individual cell types and their interactions with one another leading to the loss of luteal structure and cell death are less clear. The separation of actions on function and structure is obvious from in vitro studies where addition of $PGF_{2\alpha}$ suppresses steroidogenesis without reducing cell viability.[450] Morphometric and biochemical studies of cellular constituents also suggest that cell–cell interactions are important in luteal regression.[451] When steroidogenic cells (i.e., large and small luteal cells) isolated from the bovine corpus luteum are incubated without other luteal cell types (i.e., immune and endothelial cells), $PGF_{2\alpha}$ failed to inhibit progesterone production.[452] When endothelial and immune cells are included, $PGF_{2\alpha}$ reduced progesterone production and induced the synthesis of nitric oxide (NO) and other arachidonic acid–derived mediators (e.g., leukotrienes). In addition to the evidence suggesting that the "large" luteal cells are the predominant target for $PGF_{2\alpha}$,[453] there are reports that the

numbers of "small" luteal cells and microvascular cells decrease before any loss of large cells at luteolysis.[454] Evidence of apoptosis in small luteal cells and endothelial cells before large cells[62,433,455] supports this concept.

While the direct actions of $PGF_{2\alpha}$ on subsequent changes in luteal structure and function, such as induction of cell-signaling and effector pathways (e.g., PKC, death receptor family members, caspases, etc.), is of considerable interest in terms of mechanisms of $PGF_{2\alpha}$ action, it is likely that $PGF_{2\alpha}$ promotes the production of local luteolytic factors that also contribute to the functional and structural demise of the corpus luteum. The following are examples that serve as potential intraluteal mediators of a luteolytic program.

Vasoactive Factors: Endothelin and Nitric Oxide

Research conducted mostly over the past two decades, involving primarily domesticated animal species, supports an essential role for vasoactive substances in luteolysis. As reviewed by Miyamoto et al.,[456] several vasoactive pathways, including endothelin–endothelin receptor and nitric oxide systems, appear to be critical intraluteal factors involved in luteolysis. Moreover, endothelin and nitric oxide serve as effectors of the luteolytic program induced by uterine $PGF_{2\alpha}$.[427,457]

Endothelins (EDNs) are a peptide family consisting of three different members (EDN1, EDN2, and EDN3) and sarafotoxins, which were originally defined by their vasoconstrictive actions.[458] However, EDN1 has also emerged as a local modulator in regulating luteolysis, whereas EDN2 is implicated in control of ovulation and luteinization.[459,460] Research by Milvae, Meidan, and colleagues[460,461] suggests that EDN1 is an important factor controlling luteolysis in domesticated animal species. There is evidence that endothelial cells within luteal tissue are the primary sites of *EDN1* mRNA expression. However, *EDN1* is transcribed as a "prepro EDN1" molecule, and the active peptide is ultimately formed from the intermediate "big ET1" by the action of endothelin-converting enzyme (ECE1) on endothelial cells and luteal cells. Several reports indicate that the EDN1 receptor (EDNRA and EDNRB) system is present in the corpus luteum,[462] with the expression and differential regulation of each receptor subtype through the luteal phase being species dependent. At the time of luteal regression, the ovine and porcine corpus luteum expresses predominantly EDNRA,[463,464] whereas the pseudopregnant rabbit corpus luteum possesses both EDNRA and EDNRB.[465] EDNRA expression is consistently observed in luteal endothelial cells, whereas expression in both small and large luteal cells or only in small luteal cells is species dependent.[463,464] Thus, the microvasculature synthesizes precursor and big EDN1, but both luteal and endothelial cells can produce bioactive EDN1 and transduce EDN1 signals via receptor-mediated pathways. Moreover, Meidan and Levy[460] proposed that increased expression and activity of the EDN1 synthetic (ECE)–EDNRA system at the time of luteal regression explains the sensitivity of the corpus luteum to the luteolytic actions of $PGF_{2\alpha}$ during the latter half of the luteal phase. From a functional standpoint, there are reports that addition of EDN1 inhibits basal and/or LH-stimulated progesterone production by mixed or purified preparations of cells from the corpus luteum.[462] Notably, preincubation with an EDNRA antagonist not only prevented the effects of exogenous EDN1 but also blocked the antisteroidogenic effect of $PGF_{2\alpha}$ in luteal tissue slices.[466] In sheep receiving intraluteal EDNRA antagonist infusion via osmotic minipump, a luteolytic bolus of $PGF_{2\alpha}$ induces an acute (<24 h) reduction in serum progesterone concentrations that then recover to levels equal to those observed in saline-treated control animals.[463] Thus, inhibition of EDNRA-mediated signaling had no effect on the short-term actions of $PGF_{2\alpha}$, but inhibited its long-term luteolytic activity. Intraluteal infusion of an EDNRB selective antagonist, however, was unable to block $PGF_{2\alpha}$-mediated reduction in luteal progesterone synthesis. In vivo administration of the same EDNRA antagonist in cattle also prevented a drop in circulating progesterone levels and delayed functional luteolysis,[467] further supporting a role for EDN1 and EDNRA in regulating ruminant luteal lifespan.

Nitric oxide (NO) is another potent vasoregulatory substance that recently emerged as an important luteolytic factor. NO can be synthesized by three different nitric oxide synthase (NOS) enzymes (neuronal NOS1, inducible NOS2, and endothelial NOS3) that utilize L-arginine as a substrate.[468] Both NOS1 and NOS3 genes are viewed as constitutive, resulting in a level of NO synthesis that regulates vascular tone. In contrast, NOS2 is expressed in many cell types following an inflammatory stimulus in response to cytokines or prostaglandins, leading to the generation of large amounts of NO.[468] As reviewed by Shirasuna,[469] NO synthesis and participation in luteolysis were reported in rat, rabbit, cow, sheep, and human. In general, intraluteal NO is synthesized by inducible NOS2 and/or endothelial NOS3, depending on the species being studied. For example, NOS2 and NOS3 protein levels in the bovine and pseudopregnant rabbit corpus luteum increased from the early to late luteal phases, with expression being localized to both endothelial cells and luteal cells.[470] In the human and equine corpus luteum, however, NOS3 expression increased at the end of the luteal phase, while inducible NOS2 remained relatively constant.[471,472] *NOS2* and *NOS3* mRNA levels declined in the pseudopregnant rabbit corpus luteum toward the end of the luteal phase, whereas NOS3, but not NOS2, protein levels increased two fold through this interval.[473] Subsequent analysis of NOS3 expression revealed a heterogeneous pattern of

expression in bovine luteal tissue that appears when the corpus luteum becomes sensitive to the luteolytic action of $PGF_{2\alpha}$. In the early luteal phase, NOS3 was homogeneously distributed throughout the corpus luteum but became localized primarily to the periphery at the mid- to late luteal phases.[474] The redistribution of NOS3 to the periphery may be responsible for the increased localized blood flow in the same region noted shortly (<1 h) following the administration of a luteolytic bolus of $PGF_{2\alpha}$.[469] However, whether such an NO-dependent increase in peripheral luteal blood flow occurs during spontaneous luteolysis or when $PGF_{2\alpha}$ is administered to reflect its endogenous pulsatility remains to be determined.

$PGF_{2\alpha}$ is a key inducer of NO production in the corpus luteum of several species,[475–477] where resident endothelial cells appear to serve as the major site of synthesis.[457,469,478] The ability to recapitulate in vitro the induction of NOS activity following $PGF_{2\alpha}$-induced luteolysis in vivo is dependent on the presence of multiple cell types. Co-cultures of bovine aortic endothelial cells, smooth muscle cells, and luteinized granulosa cells synthesized significantly higher levels of NO in response to $PGF_{2\alpha}$ relative to those cultures that did not include luteinized granulosa cells.[476] Moreover, both NO and $PGF_{2\alpha}$ production in cultured luteal cells is induced in response to EDN1 stimulation, suggesting that an amplification loop exists between these three luteolytic factors.[479] Further support for a positive-feedback loop comes from rodent studies whereby the induction or inhibition of NO production resulted in increased or diminished synthesis of $PGF_{2\alpha}$, respectively.[475] As NO synthesis is known to occur in response to an inflammatory stimulus, it is not surprising that pro-inflammatory cytokines (e.g., TNF and IFNγ)[480,481] and arachidonic acid–derivied leukotrienes (e.g., leukotriene C_4)[482] also induce luteal NOS expression and NO production. NO synthesis increases in response to both TNF and IFNγ in isolated bovine endothelial cells, which, in turn, could be blocked by also including progesterone.[481] TNF- and IFN-mediated increases in NO production are associated with elevated *NOS2* mRNA levels.

Compounds that block NOS activity (or, in contrast, act as NO donors to increase NO concentrations in vitro or in vivo) have been valuable in terms of defining the functional aspect of NO involvement in luteolysis. Administration of an NO synthesis inhibitor during spontaneous and $PGF_{2\alpha}$-mediated luteolysis extended the functional interval or progesterone- and oxytocin-producing potential of the bovine corpus luteum.[470,483,484] The ability of a systemic dose of TNF, one that increases luteal $PGF_{2\alpha}$ synthesis, to reduce circulating progesterone levels and estrus cycle length can be inhibited by the co-administration of an NOS inhibitor.[485] As opposed to inhibiting NOS activity, injection of an NO donor into the bovine corpus luteum increased luteal blood flow in its periphery and reduced circulating progesterone levels as well as luteal phase length.[484] In cultured luteal cells, increased levels of NO through the addition of an NO donor resulted in elevated levels of intracellular Ca^{2+} as well as stimulated *FAS*, *BAX*, and *CASP3* mRNA levels.[486] A corresponding increase in CASP3 activity and apoptosis was observed following luteal cell culture with elevated NO levels, suggesting a role for NO in structural regression. Moreover, a similar increase in luteal cell apoptosis was observed in slices of human corpus luteum cultured in the presence of the exogenous NO substrate L-arginine.[487] While the majority of evidence to date supports a role for NO in both functional and structural luteolysis, conflicting reports by Weems and colleagues suggest a luteotropic role for NO. When an NOS inhibitor was infused via an intrauterine route ipsilateral to the ovine ovary possessing the corpus luteum, there was no delay in the loss in progesterone-producing potential or luteal weight during the ovine estrous cycle.[488] Moreover, the NOS inhibitor reduced the capacity of the uterus to convert PGE_2 to $PGF_{2\alpha}$, leading the authors to speculate that NO may be luteotropic in sheep.[489]

Further studies are warranted to determine if these conflicting results are due to differences between species, the route of administration, or the timing of administration. Moreover, little is known regarding the signaling mechanism through which NO affects luteal regression. In other cell types, NO functions by diffusing through target cell membranes, binding and activating soluble guanylate cyclases (e.g., GUCY1A2, GUCY1A3, and GUCY1B3), which in turn leads to elevated cyclic guanosine monophosphate (cGMP) levels. Increased levels of intracellular cGMP subsequently regulate phosphodiesterases, ion-gated channels, or cGMP-dependent protein kinases.[490] It is currently unclear which, if any, of these NO targets are activated in responsive luteal cells.

Collectively, data suggest that the EDN1 and NO systems, through their regulation by luteotropic and luteolytic hormones, play a local inhibitory role in the corpus luteum (Figure 23.9) and mediate, at least in part, $PGF_{2\alpha}$ actions during luteal regression.[460,462] However, key experiments remain to be performed, including investigations discerning whether modulating endogenous EDN1 and NO synthesis or action alters the function or lifespan of the corpus luteum during the natural cycle. Although limited evidence suggests that the EDN1–NO system is present in other species, including rodents and primates,[471,487,491–493] further studies need to address its importance beyond domestic animals. Finally, clarification is needed on the relative importance of $PGF_{2\alpha}$ action on luteal versus endothelial cells, and on the local amplification of $PGF_{2\alpha}$[341] versus EDN1 and/or NO[460] in the cascade of events leading to loss of luteal structure and function during luteolysis (Figure 23.9).

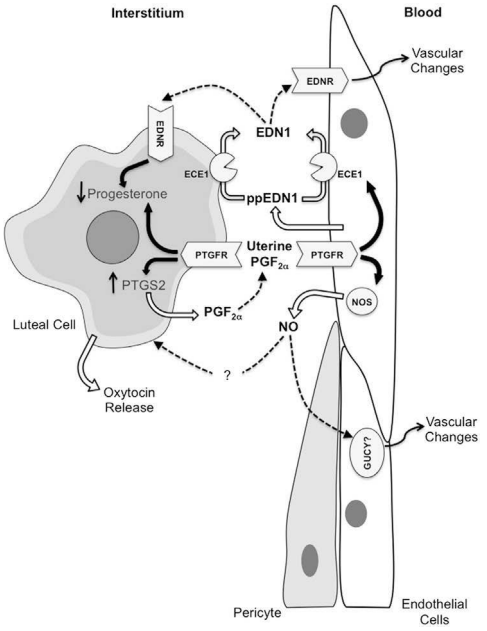

FIGURE 23.9 Diagram of proposed interactions between luteal cells and microvascular cells for the production and actions of local endothelin (EDN), nitric oxide (NO), as well as $PGF_{2\alpha}$ in response to the uterine luteolysin $PGF_{2\alpha}$. It is hypothesized that the initial $PGF_{2\alpha}$ signal is amplified or mediated by positive-feedback loops to increase intraluteal levels of $PGF_{2\alpha}$,[341] EDN1,[494] and NO,[457,469,478] which then alters luteal and endothelial cell function and causes tissue regression. Heavy black arrows: cellular responses to uterine $PGF_{2\alpha}$ through the activation of the $PGF_{2\alpha}$ receptor (PTGFR), including induction of the enzymes EDN-converting enzyme 1 (ECE1), nitric oxide synthase (NOS), and PG synthase 2 (PTGS2); white arrows: uterine $PGF_{2\alpha}$–induced luteal synthesis of preproEDN1, NO, $PGF_{2\alpha}$, and oxytocin; dotted lines: action of EDN1 and NO on luteal and endothelial cells (EDNR: EDN receptor; GUCY: guanylate cyclase, NO receptor); thin black arrows: possible luteolysis-promoting effects. *Source: From Ref. 74, with permission.*

Immunomodulators: Cytokines and Chemokines

Increasing attention has been directed at the cells of the immune system that, whether residing throughout or attracted at specific stages, comprise a significant portion of the luteal tissue (for reviews, see Refs 495,496). All types of leukocytes, with the exception of B cells, appear present with their relative numbers changing during the lifespan of the corpus luteum, sometimes in a species-specific manner. For example, neutrophilic granulocytes are at their highest density during luteal development in the rat (i.e., early pregnancy or pseudopregnancy), with considerable numbers also present at luteal regression.[497] In contrast, few neutrophils were detected in the pig corpus luteum,[498] and although they are present in the human corpus luteum, no differences in density were noted at luteolysis.[499] Eosinophilic granulocytes are relatively sparse in the rat corpus luteum,[497] yet infiltrate the ovine corpus luteum in large numbers after ovulation and are suspected to have a luteolytic role in sheep.[500] Likewise, the numbers of lymphocytic subtypes in luteal tissue, such as cytotoxic T cells ($CD8^+$), helper T cells ($CD4^+$), and natural killer (NK) cells, vary markedly between species. T cell subset distribution in the corpus luteum throughout its lifespan is best characterized in cattle. By using quantitative flow cytometry, Poole and Pate[501] observed a greater percentage of $CD8^+$ T cells than $CD4^+$ T cells in the corpus luteum, opposite of what is found in peripheral blood. Following the administration of a luteolytic $PGF_{2\alpha}$ dose, a significant increase in a subset of $CD8^+$ T cells ($CD8\alpha\alpha^+$) that possess greater immunoregulatory activity than cytotoxic potential was observed. The proportion of $CD8^+$ cells increased in the corpus luteum of early pregnancy relative to the corpus luteum of nonpregnant animals.[501] Moreover, the number of regulatory T cells (T_{reg} and forkhead/winged helix transcription factor 3 expressing cells ($FOXP3^+$) cells) that secrete immunosuppressive TGFβ1 dramatically declined following $PGF_{2\alpha}$ induction of luteolysis. In the human corpus luteum, B cells and NK cells were sparse to nondetectable,[499,502] although $CD4^+$ cells were abundant in several species, especially at luteolysis.[497,503] Macrophages are the cell type most consistently detected in the corpus luteum; these cells reportedly comprise more than 10% of all cells in human luteal tissue.[502] Moreover, an increase in macrophage density was detected in a number of species during luteal regression.[495,504]

In general, the changes in leukocyte cell density during the early and late luteal phases correlate with the tissue reorganization occurring during development and dissolution of the corpus luteum, respectively. Investigators related these events to an acute inflammatory response and tissue repair during ovulation and luteinization,[505] and an immune reaction and tissue elimination at luteolysis.[506,507] Major histocompatibility complex (MHC) class II proteins are expressed on the surface of bovine[508] and primate[509] luteal cells, which can be further enhanced by $PGF_{2\alpha}$.[508] In the rhesus macaque corpus luteum, levels of mRNA encoding both MHC class I and class II proteins increase significantly at the time of functional and structural regression.[179] Indeed, the increased expression of MHC class I and class II mRNAs and proteins is consistent with the induction of an immune response during luteolysis. However, limited evidence to date supports the physiologic relevance of local immune cells in controlling luteal function or lifespan, and MHC-II molecules could serve in alternate roles (e.g., as receptors triggering apoptosis).[510] This is due in part to difficulty in specifically modulating leukocyte numbers or action in the corpus luteum in vivo. One early approach to suppress macrophages and T lymphocytes with glucocorticoid administration yielded equivocal results in rabbits and rats.[511,512] Such approaches may be confounded by direct actions of glucocorticoids in luteal tissue via receptor-mediated pathways.[513] In an opposite approach, Bagavant and colleagues[514] noted that an exaggerated pro-inflammatory response and elevated cytokine levels in mouse ovaries

elicited through T helper cells were compatible with normal ovarian cyclicity, ovulation, and fertility.

Nevertheless, it is well established that leukocytes express a cell-specific array of cytokines, enzymes, and reactive oxygen species that promote the destruction of cells and ECM during tissue remodeling.[500,515] Alternatively, cytokines may act to stimulate or inhibit steroid synthesis, at least as demonstrated in numerous in vitro experiments.[516,517] Also, macrophages reportedly serve to eliminate dying luteal cells or cell fragments (apoptotic bodies) by phagocytosis.[518] Brannstrom and Friden[500] provide a useful schematic of potential luteotropic versus luteolytic actions of cytokines on cell types of the corpus luteum.

Numerous studies established that the cytokine TNF, a 17 kDa polypeptide, is produced in the ovary[519] and can have luteotropic or luteolytic effects depending upon the species, stage of the luteal phase, and local milieu.[261] Although perhaps produced by nonimmune cells, including endothelial and luteal cells, detailed studies in the pig[520] indicated that the luteal macrophage population secretes up to 100-fold greater quantities of bioactive TNF than other luteal cell types. In the bovine corpus luteum, TNFα and its receptors, TNFRSF1A (TNFR-I) and TNFRSF1B (TNFR-II), all increase during luteolysis.[521] TNF mRNA and protein, as localized by in situ hybridization and immunohistochemistry, respectively, are expressed in large and small steroidogenic cells as well as immune cells in the bovine corpus luteum.[278] TNFRSF1A and TNFRSF1B expression was also noted in the steroidogenic and immune cells, as well as endothelial cells, suggesting that TNF acts as a paracrine–autocrine mediator of luteolysis. In primate species, *TNF* gene expression peaks in the rhesus macaque corpus luteum during the period of functional regression,[275] which also is the interval of the luteal phase when isolated human luteal cells produce the most TNF.[522] Reports suggest that TNF inhibits luteal cell response to luteotropins, as judged from progesterone production in vitro, by the mid- to late luteal phase in rodents, domestic animals, and primates.[261] Moreover, TNF reportedly induces the death of microvascular endothelial cells from the bovine corpus luteum in vitro,[280] causes regression of blood vessels in the rabbit corpus luteum,[523] and augments IFNγ-induced death of luteal cells, including isolated endothelial cells.[282,524] Experiments on TNFR null mice ($Tnfrsf1b^{tm1Imx}/Tnfrsf1b^{tm1Imx}$)[283] suggest that *Tnfrsf1b* (TNFRII; lacks a death domain and is generally thought to mediate TNF's tropic functions, e.g., cell proliferation) deletion does not markedly alter ovarian function or reproductive capacity. However, *Tnfrsf1a* (TNFRI; possesses a canonical death domain) deletion caused numerous female reproductive defects, including early onset of puberty and premature cessation of ovarian cyclicity, with those that continued to cycle having prolonged diestrus intervals (see the section Luteolysis for an overview of TNFR-related family members and involvement in apoptosis). The prolonged diestrus may indicate a role of the TNFRSF1A system in luteolysis, but other defects in the antecedent follicle or hypothalamic–pituitary function cannot be ruled out. Nevertheless, evidence that exogenous TNF can cause functional as well as structural luteolysis[523,525] led investigators to propose that TNF interacts with $PGF_{2\alpha}$, as well as with EDN1 and NO,[472,526] to promote regression of the corpus luteum. Although TNF's signal transduction pathways in the corpus luteum remain to be resolved,[261,352] its effects include the suppression of luteotropic action and steroidogenesis.[283]

Chemokines, defined as a family of small cytokines with two adjacent cysteine residues (the C–C motif family), two cysteines with an intervening amino acid (the C–X–C motif family), or those that possess a single cysteine (the C motif family), exhibit selective chemoattractant and activating properties on leukocyte populations and are differentially distributed within the ovary.[527] For example, evidence suggests that interleukin 8 (IL8) promotes neutrophil accumulation and activation (e.g., elastase activity) and plays a critical role in gonadotropin-induced ovulation[528] and perhaps luteal development (e.g., angiogenesis).[529] The chemokine receiving the most attention to date, however, is chemokine (C–C motif) ligand 2 (CCL2, commonly known as monocyte chemoattractant protein 1, or MCP1), which attracts and activates T lymphocyte subtypes and monocytes–macrophages.[530] Increased *CCL2* mRNA and protein expression occurs in the corpus luteum of rodents, domestic animals, and primates during luteolysis.[531–534] Also, luteolytic agents such as $PGF_{2\alpha}$ increase CCL2 expression in luteal tissue either directly or indirectly via production of other cytokines.[535] $PGF_{2\alpha}$-mediated upregulation of CCL2 expression occurs in corpora lutea obtained at the end of the luteal phase[536,537] or in functional corpora wherein progesterone synthesis was inhibited.[537] The cellular sources of CCL2 are unclear, and there are conflicting reports regarding the ability of luteal cells to synthesize CCL2.[538,539] Isolated luteal endothelial cells cultured in the presence of peripheral blood mononuclear cells expressed significantly higher levels of CCL2 than when each cell type was cultured by itself.[540] Although $PGF_{2\alpha}$ did not affect the level of CCL2 expression in individual cell cultures or cocultures, these findings support a synergistic role of both cell types in the synthesis of CCL2 in the corpus luteum. Nevertheless, the data are consistent with the hypothesis that CCL2 promotes the influx of macrophages that occurs in aging luteal tissue and that these cells then play a role in the structural, if not earlier functional, demise of the corpus luteum. As proposed by Penny,[535] chemokines such as CCL2 may prove to be a paracrine link between hormonal and cellular events within the corpus luteum involving

steroidogenic luteal, microvascular endothelial, and immune cell types. While the chemokine CCL2 has received the most attention with regard to its potential role in luteolysis, there are in total 24 CC, 13 CXC, and 2C motif-containing family members that have yet to be studied in detail regarding their role in luteal biology, including luteolysis. Luo and colleagues recently analyzed the expression of the chemokines IL8, CXCL2, CCL2, CCL8, CCL4, and CCL11, as well as the chemokine receptors CCR1, CCR2, CXCR2, and CCR5 in the early- and late-stage ($PGF_{2\alpha}$-sensitive) porcine corpus luteum.[537] $PGF_{2\alpha}$ increased mRNA levels for all but *CXCL2* and *CCL11* genes in the late- but not early-stage corpus luteum. Thus, a careful examination of recently obtained genomic databases is warranted to fully characterize the scope of chemokine involvement in luteolysis in different species.[179,541–543]

Transforming growth factor β1 (TGFB1) is a multifunctional secreted protein that generally regulates immune function,[544] as well as cell survival and migration, through the Sma- and Mad-related proteins (SMAD) signaling pathway.[545] In addition to being synthesized by follicular cells,[546] luteal cell cultures produce high levels of TGFB1.[547] The transcription factor early growth response gene 1 (*EGR1*) mRNA and protein were induced in response to $PGF_{2\alpha}$ in bovine luteal cells in vivo and in vitro, which led to EGR1 binding to the TGFB1 promoter. $PGF_{2\alpha}$-induced EGR1 activation was associated with increased *TGFB1* mRNA and protein expression, which could be blocked by adenoviral-mediated expression of an EGR1 binding protein (NAB2) that inhibits its transcriptional activity. Moreover, luteal cells treated with TGFB1 displayed reduced progesterone secretion.[548] Through the classical SMAD signaling pathway, TGFB1 disrupted the potential of isolated bovine microvascular endothelial cells to migrate and form capillary-like structures when cultured on Matrigel.[549] Moreover, TGFB1 caused loss of cell–cell contacts and increased the permeability of confluent cells. Treatment of fibroblasts isolated from bovine corpora lutea with TGFB1 also induces laminin, collagen I, and MMP1 expression.[550] Collectively, these results suggest that TGFB1 promotes structural regression of the corpus luteum by destabilizing blood vessels, as well as by inducing fibroblast-mediated remodeling of the ECM. However, in other species such as the rhesus macaque, expression of TGFB1 and its receptor is highest in the corpus luteum of early pregnancy, suggesting a role in supporting luteal function.[551] Thus, further studies are needed to clarify the role that TGFB1 plays in luteolysis of other species.

Extension of Luteal Function in Early Pregnancy

Mammals have developed diverse means for ensuring that ovarian cyclicity returns in the event of a nonfecund cycle, yet providing adequate progesterone support if pregnancy ensues. In the simplest case (e.g., many carnivores and marsupials), gestation lasts as long as or is shorter than the normal lifespan of the corpus luteum in the ovarian cycle (see the section Long-Lived Corpora Lutea). In these animals with "long-lived corpora lutea", there is no need for the conceptus or mother to alter luteal function because the developed corpus luteum is destined to provide the necessary progestational support. However, most animals have evolved a means for keeping the functional lifespan of the corpus luteum "short" (i.e., less than necessary for successful pregnancy) in the nonfecund cycle to increase reproductive efficiency. The most extreme cases (see the section Ultrashort-Lived Corpora Lutea) are the reflex ovulators (e.g., the rabbit), which do not even ovulate unless mating occurs, and the spontaneous ovulators that do not form fully developed, functional corpora lutea unless mating occurs (e.g., mouse and rat). Earlier in this review, emphasis was placed on the complex and sequential roles of maternal and decidual PRL, placental PL, and ovarian–placental steroids (i.e., estrogen) in promoting the functional lifespan of the rodent corpus luteum during pseudopregnancy and/or pregnancy. However, other animals that develop a functional corpus luteum during the ovarian cycle evoked processes for extending luteal function if pregnancy ensues, by circumventing the luteolytic signal at the end of the cycle and/or by providing additional luteotropic support to maintain luteal structure and function. The mechanism whereby the developing conceptus (implanting blastocyst) extends luteal function is the critical event in "maternal recognition of pregnancy".[54,65] The corpus luteum may be a critical source of progesterone throughout pregnancy, or become unnecessary after the "luteal–placental shift" when the placenta replaces the corpus luteum as the primary source of progesterone. Domestic animals (notably the ruminants, such as sheep and cattle) and primates with short-lived corpora lutea provide excellent models for illustrating different approaches to extending luteal function in early pregnancy.

Domestic Animals

It is generally regarded that uterine exposure to progesterone during the 10–12 days following ovulation not only prepares the endometrium for implantation but also primes the mechanisms for production of the uterine luteolytic signal, $PGF_{2\alpha}$, if pregnancy does not occur. The proposed interaction between the posterior pituitary, uterus, and ovary to control the release and action of oxytocin and $PGF_{2\alpha}$ was alluded to earlier (see the section Luteolytic Factors). It is now well established that extension of the functional lifespan of the corpus luteum in the fertile cycle is due to production of an antiluteolytic factor from the developing conceptus. In sheep,

embryos transferred to synchronized recipients as late as day 12 post estrus will prevent timely luteolysis.[552] Hence, the critical period for maternal recognition of pregnancy in the sheep is days 12–13 of the cycle.[3] Evidence indicates that the conceptus secretes antiluteolytic activity, which is not an LH- or PRL-like luteotropin,[553] beginning around day 10, increasing through day 16, and becoming nondetectable by days 21–22. An important advance was the discovery by Bazer, Roberts, and colleagues[554,555] that the antiluteolytic substance in ewes is IFNτ produced by mononuclear cells in the embryonic trophectoderm.

Ovine IFNτ was originally called trophoblastin[556] or trophoblast protein 1 (TP1) because it is the first major protein secreted by the trophectoderm of the peri-implantational conceptus. This protein was subsequently identified as a type 1 IFN with potent antiviral, antiproliferative, and immunomodulatory activities.[554] There is a high sequence homology of IFNτ across ruminant species, where it apparently has a unique role in pregnancy initiation. There are multiple IFNτ isoforms that arise from multiple genes; however, their functional significance remains unclear since all IFNτ isoforms tested to date extend luteal function when injected into the uterine lumen at the appropriate time in ewes, cows, and goats.

The mechanisms of paracrine action of IFNτ to prolong the function of the corpus luteum is an area of intense research (Figure 23.8). It is well established that the high-amplitude pulses of luteolytic $PGF_{2\alpha}$ coming from the uterus in nonpregnant ewes are absent in fertile cycles.[41] IFNτ appears to act locally to prevent and/or alter the pattern of $PGF_{2\alpha}$ secretion.[557] Although basal secretion of uterine $PGF_{2\alpha}$ is higher in early pregnancy, the high-amplitude, episodic pulses of $PGF_{2\alpha}$ release do not occur. There is also evidence from some species that $PGF_{2\alpha}$ secretion may be directed into the uterine lumen rather than the bloodstream. IFNτ appears to interact with type 1 IFN receptors in the endometrium to block expression of estrogen and oxytocin receptors, and this action prevents estrogen and oxytocin responses and may be the primary event preventing uterine-induced luteolysis.[558] Although the primary action of IFNτ to prevent luteal regression may be at the uterus, there is evidence of an endocrine action to promote luteal resistance to $PGF_{2\alpha}$ and prolong the function lifespan of the corpus luteum.[559] Recent reports identified IFNτ-stimulated genes[560] and IFNτ-promoted lymphangiogenesis[561] in luteal tissue.

Since intrauterine, as well as venous, infusions of IFNτ alone can extend the lifespan of the ruminant corpus luteum,[560,562] some investigators believe that this is the only pregnancy recognition factor produced by the conceptus. However, others noted that the sensitivity of the corpus luteum to the luteolytic action of $PGF_{2\alpha}$ is reduced in early pregnancy.[563] Since the uterine endometrium secretes another PG, PGE_2, in greater amounts during early pregnancy[564] and PGE_2 infusion delays luteolysis,[565] it was suggested that this PG suppresses the luteolytic actions of $PGF_{2\alpha}$.[400] Further studies are needed to establish the potential endocrine roles of IFNτ versus PGE, perhaps via IFNτ promotion of intraluteal PGE and PGE receptor (PTGER) synthesis,[566] to prevent $PGF_{2\alpha}$ action in the corpus luteum and hence promote establishment of pregnancy. Again, species differences in maternal recognition of pregnancy exist even between domestic animals. Although the critical role for IFNτ appears valid for sheep, cows, and goats, the mechanisms are clearly different in pigs and horses.[567,568] The corpus luteum in the pig is required throughout pregnancy,[58] and it is hypothesized that PGE and/or estrogens (not IFNτ) secreted by the porcine conceptus prevent the luteolytic actions of uterine $PGF_{2\alpha}$.[568] A similar mechanism may occur in the mare, except the fetal trophoblast also begins to secrete an additional luteotropin (eCG; see the section Luteinizing Hormone (LH)-Like Luteotropins) that sustains the function of secondary corpora lutea until the time of the luteal–placental shift around day 150 of gestation.[344]

Primates

In primates, such as Old World monkeys and women, it is clear that the demise of the corpus luteum near the end of the nonfecund menstrual cycle is not due to a uterine luteolytic factor. Nevertheless, the factors responsible for luteolysis in primates remain an enigma. As reviewed by Zeleznik and Benyo,[337] research has focused on either a relative loss of luteotropic support (LH) or intraovarian luteolytic factors (estrogen or $PGF_{2\alpha}$). Although the frequency of LH pulses declines by midluteal phase,[340] subsequent studies demonstrated that this apparent reduction in luteotropic support does not cause luteolysis since the length of the luteal lifespan was unaltered if pulse frequency was not allowed to decline.[569] Nevertheless, considerable evidence suggests that luteal cell sensitivity to LH, including LH receptors or receptor isoforms,[570–572] LH-stimulated cAMP production, and cAMP signaling,[573] declines as the lifespan of the primate corpus luteum progresses; and incremental increases in LH are needed to sustain the normal functional lifespan of the corpus luteum in the menstrual cycle.[574] However, the intracellular mechanisms controlling LH sensitivity and its role in onset of luteolysis are unclear.

The lack of a uterine or pituitary role in signaling luteolysis led investigators to propose "self-destruct" mechanisms[51] whereby local factors that are synthesized in the ovary or corpus luteum lead to loss of luteal structure and function. Based on the ability of the primate corpus luteum to synthesize estrogen, plus reports that systemic or local delivery of estradiol induced premature

luteolysis, it was proposed that estrogen was the local luteolytic factor.[51] However, further studies investigating endogenous[575] and exogenous[576] estrogen actions did not support its physiologic or local luteolytic role. Difficulty in detecting appreciable levels of the classical estrogen receptor, ESR1, in primate luteal tissue further reduced enthusiasm for this scenario. However, the discovery of the β isoform of ER (ESR2) in primate luteal tissue[577,578] plus evidence that progesterone downregulates luteal ESR2 expression, which is highest at luteolysis in the monkey,[382] suggest that the issue of local estrogen action in the primate corpus luteum warrants reevaluation. Because primate luteal tissue synthesizes PGs,[579,580] a self-destruct scenario involving $PGF_{2\alpha}$ was also envisioned. Early in vivo studies yielded variable results, in part due to rapid metabolism, stage-specific sensitivity of the corpus luteum, or high levels of endogenous PGs in luteal tissue. A recent comprehensive analysis of individual mRNAs or proteins involved in PG synthesis, metabolism, and signaling[394] indicates that PGE_2 synthesizing (e.g., microsomal PGE_2 synthase 1) and signaling (PGE_2 receptor 3) peak during development and maintenance of the primate corpus luteum, whereas $PGF_{2\alpha}$ signaling (PTGFR) increases during functional luteolysis. The data support the concept that there is a shift from luteotropic (PGE) to luteolytic (PGF) PG action near the onset of luteal regression. Intraluteal administration of $PGF_{2\alpha}$ analogs induces premature luteolysis in monkeys and women.[392,581] Evidence of $PGF_{2\alpha}$ binding and PTGFR receptors in luteal tissue, plus antigonadotropic effects of $PGF_{2\alpha}$ on primate luteal cells in vitro,[582] support a direct action of this PG in the corpus luteum of the menstrual cycle. Nevertheless, without specific agonists and antagonists of individual PGs, it has been difficult to substantiate a physiologic role for endogenous $PGF_{2\alpha}$ in primate luteolysis. Following recent advances in our understanding of PG metabolism and action,[413] and the production of more selective inhibitors of PG synthesis and PG receptors, further studies are warranted to define the putative roles of luteotropic (PGE and PGI) versus luteolytic (PGF) PGs, and other arachidonate products, in the primate corpus luteum.

The causes of luteolysis in primates remain unclear, but it is generally believed that secretion of another LH-like hormone, CG, by the implanting blastocyst and the syncytiotrophoblast cells of the placenta is responsible for "rescuing" the primate corpus luteum near the end of the fertile cycle and extending luteal function in early pregnancy.[54,65] Administration of hCG to mimic the patterns and levels of endogenous CG elicits changes in the corpus luteum that are characteristic of maternal recognition of pregnancy: (1) the functional luteal lifespan is prolonged, and timely menstruation does not occur; (2) circulating levels of progesterone increase transiently and then decline as the time of the luteal–placental shift approaches; and (3) nonsteroidogenic activities, including relaxin and inhibin A production, also increase. Notably, passive or active immunization of nonhuman primates and women against CG produces infertility despite normal ovulatory menstrual cycles.[583,584] Thus, CG independent of any other embryonic factors appears capable of rescuing the corpus luteum of the menstrual cycle and converting it into that of early pregnancy.

Although CG secretion appears characteristic of pregnancy in all primates, the patterns and peak levels vary among humans, apes, and Old World and New World monkeys.[65] Peak CG concentrations circulating in women are 100–1000 higher than in baboons, macaques, or marmosets. Although CG is first detected in utero–ovarian venous and then peripheral blood around the time of implantation in all primates, the duration of placental production varies. In humans and apes, CG levels peak in the first trimester, then decline but remain at substantial levels throughout gestation. However, in baboons and macaques, CG levels decline to near or below the limits of detection by midpregnancy. In marmosets and squirrel monkeys, CG levels do not peak until midpregnancy and decline just before parturition. In addition, the forms of CG secreted by the implanting blastocyst and developing placenta may vary. There is evidence from assisted reproductive technology (ART) protocols in women that free βhCG was first detectable about 2 days before intact α-βhCG,[585] and that by the later stages of the first trimester and again at term pregnancy there is a relative excess of free αhCG when CG levels are declining. Research on the control of CG expression and secretion is limited by the difficulty of obtaining early primate embryos or placental tissue.[54] However, such studies are needed to understand the control of pregnancy initiation and early pregnancy loss in women.

CG is an LH-like glycoprotein hormone that shares the same α subunit (glycoprotein hormones, alpha polypeptide (CGA)) with pituitary LH (and FSH and TSH[311]). However, chorionic gonadotropin, beta polypeptide (*CGB*) genes are distinct from the LH beta gene (*LHB*), and it is proposed that the primate *CGB* gene evolved from an ancestral *LHB*-like gene through a single base pair (bp) deletion in exon III and a 2 bp insertion that extended the translated region of the C-terminal region. Thus, human LHB and CGB demonstrate remarkable similarity (85% homology) in the first 114 amino acids, but CGβ is unique from other glycoprotein hormone β subunits due to its 24–31 amino acid extension at the C-terminus. Also, their glycosylation with N-linked complex carbohydrates and sulfation vary between pituitary gonadotrophs and placental trophoblast cells. Consequently, hCGA and hCGB (but not LH subunits) contain terminal sialic acid residues, and are not sulfated like oligosaccharide termini on LH. These differences markedly influence the molecule's size and charge, as well as circulating half-life and bioactivity.

Again, for ethical and practical reasons, there have been few studies on the cellular and molecular changes during CG rescue of the human corpus luteum in early pregnancy. However, in every primate species studied,[54] evidence supports the concept that pituitary LH and placental CG share a common receptor that regulates luteal cells at least in part through stimulation of adenylate cyclase, to form cAMP and activate cAMP-dependent pathways (e.g., C kinases). Models simulating early pregnancy in monkeys and women via exogenous CG exposure provide some insight.[54,586] Morphologically,[587] there are rapid changes in granulosa luteal cells suggestive of rapid use of steroid precursors (fewer lipid droplets) and increased production of secretory proteins (more membrane-bound granules). In contrast, the theca luteal cells display changes suggestive of increased steroid precursor accumulation (more lipid droplets) and steroidogenesis (more agranular endoplasmic reticulum). However, the increased mass and steroidogenic (both progesterone and estrogen) activity are not apparently accompanied by a renewal of angiogenesis in the corpus luteum. There is little evidence that the marked decline in microvascular cell proliferation that occurs as the corpus luteum ages in the menstrual cycle is altered by CG exposure.[109,165] However, this does not rule out CG effects on the luteal vasculature either directly or via vascular-specific factors (e.g., ANGPT1 or ANGPT2[164]) to influence vessel structure and function (e.g., vascular permeability).

CG exposure results in divergent changes in luteal expression of mRNAs and proteins involved in steroid and peptide hormone production. In the monkey corpus luteum,[588] 7 days of CG exposure transiently stimulates progesterone production by enhancing the uptake and availability of cholesterol stores (e.g., LDL) for steroidogenesis, not by stimulating key enzymatic steps (e.g., CYP11A1 or HSD3B2). In contrast, CG markedly increases the expression and/or enzyme activity for androgen (CYP17A1) and estrogen (CYP19A1) production in primate luteal tissue.[588,589] Remarkably, although relaxin secretion by the primate corpus luteum increases in early pregnancy, it remains unclear whether CG directly or acutely exerts transcriptional or posttranscriptional control over relaxin expression from the *H2* (not H1) gene.[54] In a detailed analysis of changes in the macaque luteal transcriptome following 1–9 days of hCG exposure simulating maternal recognition of pregnancy,[354] levels of more than 1000 gene products (mRNAs) changed significantly compared to pretreatment control tissues. Further studies on such databases (transcriptomes and proteomes) are needed to clarify the direct versus indirect effects of CG exposure that transiently prevent luteolysis and extend the structure and function of the primate corpus luteum in early pregnancy. Consideration of the direct versus indirect effects of CG action must take into account the dynamics of LHCGR–effector systems in primate luteal tissue. Unlike in rodent systems, where a large bolus of LH or CG results in "downregulation" of gonadotropin receptors, the scenario in primate luteal tissue to high, rising levels of CG appears different. Although many of the LHCGRs became occupied, there was a remarkable constancy in the total receptor population as the duration and level of CG exposure increased.[54,590] Nevertheless, homologous desensitization of adenylate cyclase became apparent, as cAMP production in response to LH or CG (but not PGE_2 or forskolin) became severely impaired.[591,592] It is possible that uncoupling of LH–CG receptor–signal transduction via cAMP is important for the transient progestational response of the corpus luteum to continued CG exposure in early pregnancy. However, the continued response to local luteotropic factors, notably PGE_2[592] or progesterone,[593] may promote luteal structure and function.

Given that LH continues to circulate in the late luteal phase of the menstrual cycle, it remains unclear why or how another LH-like hormone (i.e., CG) rescues the primate corpus luteum in early pregnancy (for a review, see Ref. 54). Based on studies on cells and membranes from nonprimate species, it was initially proposed that there were differences in receptor movement or turnover following binding of human CG versus ovine LH that prolong CG action (e.g., progesterone production) by luteal cells. That LH–CG receptors in monkeys and women demonstrate a unique specificity for primate gonadotropins[345] emphasizes the need for further studies in relevant species.

Although initial studies employing macaque granulosa cells did not support the concept of different kinetics for hLH versus hCG promotion of progesterone production,[594] recent reports using human cell systems suggest that LH and CG differ in their potency and chronic ability to stimulate cAMP and progesterone secretion[355] and the activation of signaling pathways.[355,595] For example, hCG appears more potent in cAMP production (and hence steroidogenesis), whereas hLH is more potent in ERK and AKT activation to promote cell proliferation or survival during luteal development. However, Zeleznik[596] presented data suggesting that administration of increasing amounts of recombinant hLH, as well as hCG, around the expected time of implantation increased progesterone levels and prevented timely luteolysis during the menstrual cycle in macaque monkeys. These data are consistent with the concept that CG's ability to rescue the primate corpus luteum is due to qualitative and quantitative differences in the circulating gonadotropin support between LH (intermittent pulses with intervals of gonadotropin deprivation) and CG (continuous, increasing levels) near the end of the nonfecund versus fertile cycle. Further studies are needed to discern how CG prevents timely luteolysis in the fertile cycle and whether luteal regression after the

luteal–placental shift involves similar processes as in the nonfecund cycle.

LUTEAL CELL TYPES AND THEIR REGULATION

Small versus Large Luteal Cells

Many years ago, investigators proposed that the corpus luteum consisted of at least two types of luteal cells based on differences in size, morphology, and staining properties.[597] In domestic animals, the "large" luteal cells contain more smooth and rough endoplasmic reticulum, mitochondria, extensive Golgi apparati, and secretory granules than "small" luteal cells—features that are consistent with greater steroid and peptide–protein hormone production by large cells (for a review, see Ref. 3). The often-stated opinion is that the large cells are derived from the granulosa layer, whereas the small cells originate from the theca layer of the luteinizing follicle. This concept has not been definitively tested, although Meidan and colleagues[598] report that pharmacologically induced luteinization of granulosa and theca cells in vitro results in many of the characteristics of large and small luteal cells, respectively. However, limited evidence suggests that this concept is an oversimplification. Using monoclonal antibodies (Abs) to granulosa and theca cell surface antigens, Alila and Hansel[599] determined that small luteal cells bound theca-specific Abs, whereas large luteal cells bound either granulosa-specific or theca-specific Abs. Moreover, the percentage of large luteal cells that bound granulosa-specific Abs declined, whereas those that bound theca-specific Abs increased, as the bovine corpus luteum aged. This evidence suggests that small luteal cells become large luteal cells as the lifespan of the corpus luteum progresses. But whether this scenario: (1) implies further cell differentiation as well as hypertrophy, within discrete large cell subtypes; or (2) is conceptually relevant to other species, including primates with compartmentalized theca–luteal and granulosa–luteal cells in luteal tissue, is not clear.

Studies in several species indicate that the number and size of luteal cells change during the lifespan of the corpus luteum. In general, the numbers and sizes of steroidogenic luteal cells increase as the corpus luteum develops (e.g., during the early to mid-luteal phases in domestic animals and primates; for a review, see Ref. 600), whereas their numbers and size decline during luteal regression near the end of the cycle. It is likely that increases in luteal cell number and size along with vascular development (increased endothelial cells; see the section Luteinization) influence corpus luteum activity after ovulation, as these events parallel the rise in circulating progesterone levels. Moreover, the removal of granulosa cells from the preovulatory follicle markedly reduced luteal mass and serum progesterone levels (but not luteal lifespan) in domestic animals[601] and primates.[602] These studies established the critical importance of granulosa-derived luteal cells in the progestogenic function of the corpus luteum during the ovarian cycle.

However, it appears that marked changes in function occur in luteal cells before changes in cell number or size at luteolysis. Moreover, morphometric evaluations of cellular constituents in a limited number of species suggest that cellular destruction does not occur simultaneously in all cell types during luteal regression. For example, small luteal cells and endothelial cells were the first cell types to decrease in the sheep corpus luteum, followed by the loss of large luteal cells.[454] However, because luteolysis also involves a decline in cell size (e.g., as a result of loss of lipid droplets or apoptosis), it is difficult to establish whether large luteal cells actually disappear or no longer meet the size criterion. Again, differences appear evident in species with compartmentalized luteal tissue. In primates, notably the human, it was suggested that luteolytic changes first occur in the granulosa-derived luteal tissue. Sasano and Suzuki[603] observed that the number of granulosa luteal cells decreased, whereas the paraluteal region of theca luteal cells remains prominent and steroidogenically active in the corpus luteum near the end of the menstrual cycle.

Function and Regulation of Luteal Cell Types

Despite the caveats of identifying luteal cells on the basis of size, which clearly varies between species and during the lifespan of the corpus luteum (for a review, see Ref. 600), considerable progress in characterizing luteal cell types was made by Niswender,[454] Hansel,[604] and others[341,494] when they separated cells from luteal tissue of domestic animals based on differences in cell size and density. Consistent with fine structural analyses of cellular organelles, the large luteal cells (≥20 and 30 μm diameter in sheep and cows, respectively) secrete 10- to 30-fold more progesterone than small luteal cells (<20 and 17 μm diameter), but the presence of cholesterol precursor (e.g., LDL) is required to sustain this difference during longer term incubation in vitro. The higher progestogenic activity of large luteal cells in sheep is associated with greater expression of CYP11A1 and HSD3B enzymes, and mitochondrial cholesterol transport is elevated. The mechanisms responsible for maintaining this high steroidogenic activity are not well defined; Bogan and Niswender[605] suggest that it is due to tonically active protein kinase A and phosphorylated STAR in ovine large luteal cells. Other reports indicate that the large luteal cells of various species are the primary, although not necessarily exclusive, producer of relaxin and/or oxytocin.[230] This research suggests that large luteal cells can produce more than one hormone (e.g., they are dual secretors of steroid

and peptide–protein hormones). Taylor and colleagues[606] identified four subpopulations of large luteal cells in the pig by combining chemical and hemolytic plaque assays to detect steroid and relaxin production.

Small and large luteal cell preparations that differ in steroidogenic activity were also prepared from the corpus luteum of other species (e.g., rodents, rabbits, and primates; for a review, see Ref. 600). In monkeys, the 30- to 40-fold greater level of basal progesterone production by large luteal cells compared to small luteal cells is similar to that observed in other species. However, small three- to fourfold differences were generally observed between small and large cells from the human corpus luteum.[607] A critical unanswered question is how these large and small luteal cell preparations in primates compare to the compartmentalized luteal and paraluteal cells observed in situ.[195,196] Evidence indicates that basal estrogen production by all luteal cells is low, with only the large cells having the capacity to convert exogenous androgen to estrogen. Thus, the large cells contain aromatase (CYP19A1), which is also localized to the true luteal cells in the tissue parenchyma. One might expect the small luteal cells, which in other species are thought to be of thecal origin, to correspond to the exteriorized paraluteal cells that contain $P450_{C17}$ (CYP17A1) for androgen synthesis. However, androgen synthesis was 10-fold greater in large versus small cells from the monkey corpus luteum,[608] with reports on human luteal cells yielding conflicting data.[609,610] These data and histologic observations[587] suggest that size alone cannot distinguish between luteal and paraluteal cells in the primate corpus luteum. Rather, the large cell population may be composed of highly steroidogenic (both progestogenic and either androgenic or estrogenic) cells of both granulosa and theca origin (as proposed from the original work of Alila and Hansen in sheep[599]). The origin and ultimate fate (e.g., to differentiate further into large cells as the corpus luteum ages) of small luteal cells in the primate corpus luteum remain speculative.

There are also marked differences between small and large luteal cells in their responsiveness and receptor levels for luteotropic and luteolytic factors.[611] For example, ovine large cells are minimally responsive to gonadotropin (LH), cAMP, or other activators of the protein kinase A pathway, whereas small cells respond with up to a 10-fold increase in progesterone production.[341] Related observations and calculations led to the proposal that in sheep, the chronically elevated steroidogenic activity of large cells is "constitutive" (i.e., minimally regulated by ongoing hormone exposure) and responsible for 80% or more of the secreted progesterone. LH exposure may increase progesterone levels somewhat through acute actions on small luteal cells, but may also sustain levels through the conversion of small "less active" to large "high steroidogenic" luteal cells.[612] However, $PGF_{2\alpha}$ appears to have early luteolytic actions through the large luteal cells. In domestic animals, such as sheep and cows, large luteal cells express much greater levels of PTGFR receptors than small luteal cells.[341] It appears that $PGF_{2\alpha}$ activates the phosphatidyl inositol (PI) pathway, with both branches of PI signaling (i.e., elevated intracellular CA^{2+} levels and protein kinase C (PKC) activation) occurring primarily in large cells.[341] A scenario is proposed by Hoyer[433] and Niswender[611] that uterine $PGF_{2\alpha}$ reduces progesterone secretion and increases oxytocin and $PGF_{2\alpha}$ production by large luteal cells. The decline in intraluteal P levels permits oxytocin action on small luteal cells to increase intracellular Ca^{++} levels and subsequent apoptosis. Luteal-derived $PGF_{2\alpha}$ also activates Ca^{++} channels in large luteal cells that may result in apoptosis. Thus, the interplay between uterine $PGF_{2\alpha}$ and locally produced oxytocin and $PGF_{2\alpha}$ on different luteal cell types may be critical for timely functional and structural luteolysis.[611,613]

Although the sheep and cow are excellent large-animal models for preparations of luteal cells, important species differences in the hormone responsiveness of luteal cell types are likely with rodents and primates. For example, although small luteal cells typically respond to LH-like hormones, there are reports that large luteal cells also respond (e.g., in rats[614] and women[615]) or are more responsive (e.g., in rhesus monkeys[616]) than small luteal cells. This issue requires further investigation. Some differences may be technique related, for example the human corpus luteum is difficult to disperse into cell preparations, such that the prevalent use of harsh proteases (e.g., trypsin) likely alters cell responsiveness or causes cell damage. This may explain why steroid production in vitro by large versus small cells from women is reportedly much less than that of other species. However, Brannian and colleagues[617] used similar techniques to isolate cell types from the sheep and monkey corpus luteum and confirmed species differences. As expected, small cells from the ovine corpus luteum were much more responsive to LH than large cells; however, the opposite was observed for the monkey—large luteal cells were more responsive to LH. This difference, if validated (detailed studies on LH–CG receptors and gonadotropin-activated signaling pathways in primate luteal cell types are limited), could relate to the apparent differences in the luteotropic roles of LH-like hormones in ruminants versus primates. Although elimination of endogenous LH support (using a GnRH antagonist) only modestly reduces (up to 50%) circulating progesterone levels in cows[618] or sheep[619] depending on the stage of the luteal phase, it causes complete and rapid suppression of progesterone levels and early luteolysis at all stages of the luteal phase in primates.[574,620] Likewise, the entrainment of LH pulses to progesterone secretion by the midluteal phase of the menstrual cycle, with steroid levels at baseline in the lengthening intervals between LH pulses by the mid- to late luteal phase,[340] demonstrates: (1) the continued absolute requirement of primate luteal

cells for LH-like hormone; and (2) the absence of any luteal cell type that constitutively produces progesterone. These findings would suggest that both small and large luteal cells in the primate corpus luteum respond to LH and CG during the menstrual cycle and at recognition of early pregnancy, respectively.

The effects of other luteotropic or luteolytic factors, and the possible dynamics of cell responsiveness or receptor signaling during the luteal lifespan, have received less attention. It was proposed that LH-stimulated estradiol production in the rabbit corpus luteum leads to estrogen action on both small and large cells to promote progesterone production.[621] There are limited reports of different steroidogenic responses of small and large luteal cells from rodent corpora lutea to PRL,[622] notably PRAP (HSD17B-7)[75]; it is exclusively expressed in large luteal cells. Large luteal cells in sheep have greater numbers of GH receptors than small cells,[341] whereas small, but not large, luteal cells from women responded to GH with enhanced progesterone production.[607] There is also evidence that luteal cell responsiveness to LH, but not the local luteotropin PGE_2, decreases in large (as well as small) cells as the monkey corpus luteum ages.[616] Whether or how such changes assist in regulating the functional lifespan of the corpus luteum in various species requires further investigation. After considerable effort to isolate and characterize luteal cell types in the 1980s through the mid-1990s, this research area received less attention in recent years in part because of: (1) the vagaries in arbitrarily defining cell types based on a variable (i.e., size or diameter), which changes during the lifespan of the corpus luteum and in response to luteotropic or luteolytic factors; and (2) the lack of established markers (e.g., cell surface proteins) to identify and separate luteal cell types. Nevertheless, new information on differences between "small and large"[181] or granulosa- versus theca-derived[623,624] luteal cells, as well as endothelial, immune, and stromal-like cells,[385,625] continues to increase our understanding of the cell heterogeneity within the corpus luteum. As global gene and epigenetic[626] protein expression or novel tissue micromanipulative techniques (e.g., laser capture microdissection) are applied to the corpus luteum, a better perspective will be obtained on the origins, dynamics, and interactions between luteal cell subpopulations that are important for the functional lifespan of the corpus luteum during the ovarian cycle and pregnancy (Figure 23.10).

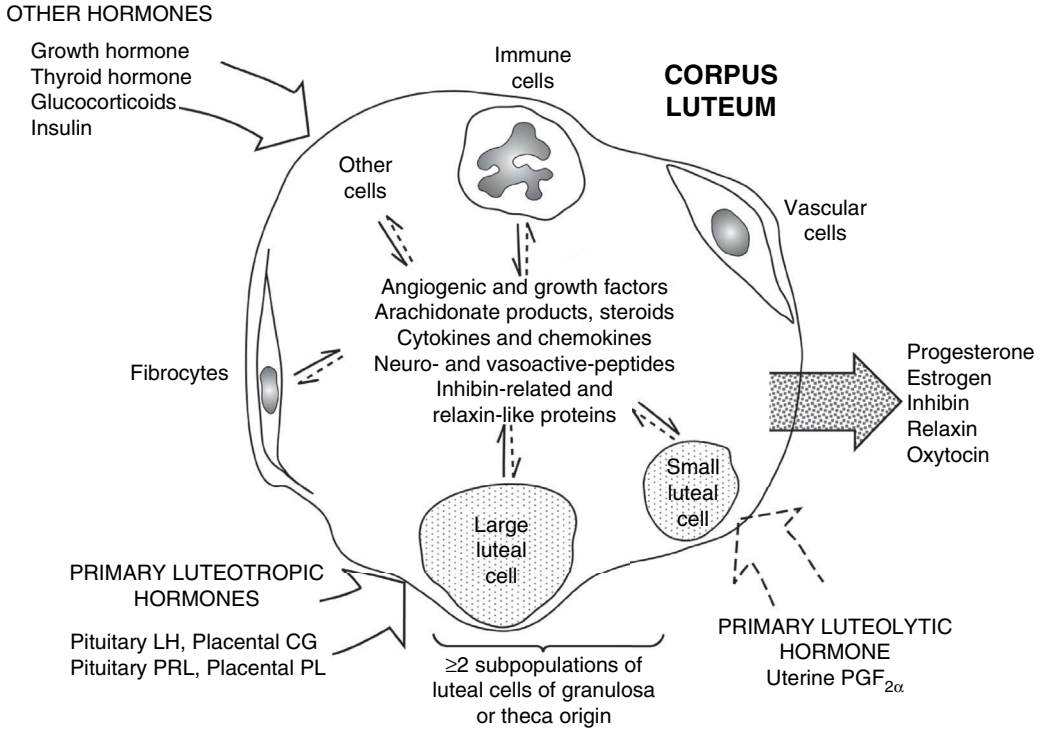

FIGURE 23.10 Conceptual summary of the multiple cell types in the corpus luteum, the luteotropic and luteolytic hormones regulating luteal structure and function, and the categories of products that may serve as autocrine or paracrine regulators of the corpus luteum as well as secreted hormones. Solid open arrows denote hormones from the pituitary, placenta, and other endocrine glands that promote the development, function, and/or lifespan of the corpus luteum, whereas the dotted open arrow depicts the uterine luteolysin $PGF_{2\alpha}$. Line arrows denoted secretion (solid) by or action (dotted) on cell types within the corpus luteum. The gray open arrow denotes hormone secretion from the corpus luteum. As evident from the text, this composite corpus luteum does not exist in nature, as species evolved mechanisms relying on various hormones and local factors for their specific niche in reproduction. *Source: Adapted from Ref. 76.*

CONCLUSION

The past two decades provided major increases in our knowledge of the processes that promote luteal development, the cellular components and control of steroidogenesis, and the mechanisms involved in extending luteal function in early pregnancy (Figure 23.10). Likewise, information has accrued on the cellular actions of luteotropic hormones and luteolytic $PGF_{2\alpha}$, and the possible role of local factors in controlling luteal structure and function. Nevertheless, many issues need to be resolved at the systemic, cellular, and molecular levels to understand the activities of the corpus luteum and its regulation by endocrine and local factors. The identification of genes that are exclusively or preferentially expressed in ovarian compartments at specific stages of the reproductive cycle,[627] and the initial results from global gene microarray analyses,[628] portend rapid advances in identifying novel or underappreciated processes in the corpus luteum. Because of the corpus luteum's origin from the follicle, traditional gene deletion techniques may be difficult to interpret; however, advances in acute, conditional, and tissue-specific gene knockout approaches, as well as "knockdown" approaches using antisense oligonucleotides or siRNAs, should provide valuable tools for analyzing the roles of genes and gene products in the corpus luteum. Likewise, the advent of basic and clinical proteomics will impact ovarian biology in the near future. Recognizing the myriad of species differences in the control of the corpus luteum, a comparative database will be essential for future applications to improve or control fertility for such purposes as preserving endangered species, controlling species with burgeoning populations, improving the efficiency or quality of food (meat) production, and treating ovarian disorders in women's health. The ovarian biologist will continue to find a plethora of intriguing problems and hypotheses for testing to understand this unique gland, the corpus luteum, which is essential for intrauterine pregnancy.

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CHAPTER

24

Human Oviduct and Endometrium: Changes over the Menstrual Cycle

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INTRODUCTION

Driven by ovarian steroid hormones, the oviducts and uterine endometrium undergo a series of biochemical and histological changes to facilitate the establishment and maintenance of a pregnancy. An increase in cilia and secretory function in the fallopian tube epithelium occurs in response to estradiol production from the maturing ovarian follicles and enables pickup of the oocyte from the ovary, travel of sperm to the oocyte, an environment conducive for fertilization and early embryo development, and transportation of the fertilized embryo to the uterus. The rise in progesterone from the newly formed corpus luteum signals the regression and atrophy of the oviductal epithelium. Responding to these same signals of estradiol followed by progesterone, the endometrium undergoes a period of cellular proliferation followed by differentiation in preparation for embryo implantation. In the absence of a pregnancy, progesterone falls and much of the endometrium is shed. A rise in estradiol from the next cohort of developing follicles starts the cycle anew, with the oviduct and endometrium once again responding to these signals in a coordinated fashion in order to achieve pregnancy.

In this chapter, we will review these changes to the oviduct and endometrium throughout the menstrual cycle. We will trace the development of our understanding of fallopian tube and uterine biology from the early nonhuman histological studies through the latest genetic and molecular advances. In the second edition of this text (published in 1994),[1] histological analysis—most often in the nonhuman primate—dominated the description of changes to the oviduct and endometrium over the menstrual cycle, while the roles of the ovarian steroid hormones in regulating reproductive organ function were studied using binding and immunoassays to detail hormone receptor quantity and location in the various compartments over the course of the menstrual cycle. In the third edition (2006),[2] the authors explored in great depth the emerging gene-profiling experiments of the endometrium over the menstrual cycle. In the current edition, emphasis will be placed on studies in humans or involving human tissue, although we will explore in detail how modern transgenic studies, primarily in the mouse, have advanced our understanding of this dynamic system. We will review what is thought to underlie normal oviductal and endometrial function and explore some of what is known about their dysfunction in diseases such as endometriosis and endometrial hyperplasia and malignancy. Finally, with an eye to the future, we anticipate the ability to correct defects in tubal and endometrial function, whether to relieve symptoms, treat and prevent cancer, or improve outcomes through assisted reproduction.

ANATOMY, EMBRYOLOGY, AND HISTOLOGY: AN OVERVIEW

The paired oviducts, each approximately 13 cm in length, are contiguous with the endometrial cavity and terminate in communication with the peritoneal cavity. The oviducts are divided into four anatomic regions:

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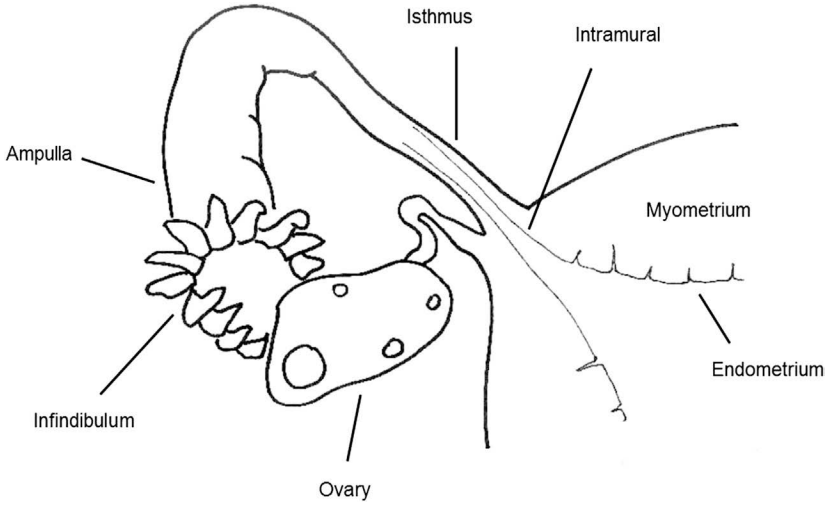

FIGURE 24.1 Anatomic regions of the human fallopian tube.

intramural, passing through the uterine wall; *isthmus*, the narrow proximal one-third of the tube; *ampulla*, the widening distal two-thirds of the tube; and *infundibulum*, the large terminus of the tube, open to the peritoneum and containing the tubal fimbriae (Figure 24.1).[2] In cross-section, one can appreciate the three distinct tubal layers of the inner *mucosa*, middle *muscularis*, and outer *serosa* (Figure 24.2), which are part of the visceral peritoneum that drapes over the pelvic organs and makes up the mesosalpinx. The mucosa is composed of longitudinal folds, which become more prominent and branch toward the infundibulum. The mucosa is lined by a single layer of epithelium of two cell types: ciliated and secretory (nonciliated). These epithelial cells are responsive to ovarian steroid hormones and, as such, undergo changes over the menstrual cycle that we can interpret in histological and biochemical terms. These cyclic changes will be addressed later in the chapter. Vascularized loose connective tissue makes up the lamina propria and supports the epithelium. The mucosa is made up of smooth muscle and connective tissue in an inner circumferential and outer longitudinal arrangement.

The uterus is pear shaped, with an average weight of approximately 50–100 g in the premenopausal adult, and is made up of the following regions: the *corpus* (the body), *fundus* (the top portion), and *isthmus* (the lower segment, contiguous with the cervix) (Figure 24.3, upper panel). The uterus can be divided into three distinct layers: an inner endometrium, myometrium, and outer serosa. The myometrium makes up the majority of the uterus and is composed mostly of smooth muscle along with vascular tissue and some supporting stroma. The outer serosa is contiguous with the visceral peritoneum, which drapes the uterus and oviducts in the pelvis. The endometrium is a complex collection of cell types and is lined by a single layer of columnar epithelium (luminal epithelium). This epithelium lines the uterine glands (glandular epithelium) as well. The glands are branched, tubular structures that extend through the stroma and can traverse the entire endometrial thickness to the myometrium underneath. The stroma is composed of connective tissue and extracellular matrix; and, as we will see, the various responses of the different uterine compartments, including the epithelium and myometrium, to circulating ovarian hormones are mostly coordinated through the stroma.

The human endometrium can be further divided into four zones that were originally used by Bartelmez (referenced in 2) to describe the endometrium of the rhesus macaque (Zone I, composed of the luminal epithelium and adjacent stroma, through Zone IV adjacent to the myometrium). More commonly, we can discuss the endometrium in reference to the "functionalis" (Zones I and II) and "basalis" (Zones III and IV) (Figure 24.3, lower panel). The functionalis is the portion of the endometrium that responds to the ovarian hormones, undergoes dramatic changes in cell appearance and function over the menstrual cycle, is the site of embryo implantation, and is shed each month in the absence of a pregnancy. The underlying basalis is not shed but participates in the regeneration of the functionalis after menses.

The anatomy of the uterus and fallopian tubes was first described by Johannes Müller in 1825.[3] The uterus and fallopian tubes originate from the Müllerian or paramesonephric ducts composed of coelemic epithelium, and their development occurs during weeks 9–14 of fetal life.[4] The formation of these ducts is a process of initiation, invagination, and elongation, the molecular cues for which have been shown to involve members of the LIM (**L**in-11, **I**slet-1, and **M**ec-3), WNT (**W**ingless and I**nt**egrase), and PAX (**Pa**ired bo**x**) protein families.[5–7] The cranial portions of the ducts, open to the peritoneum, become the oviducts, while the caudal portions of the ducts migrate medially and fuse to become the uterovaginal primordium. The lower portion of this primordium becomes the vagina and

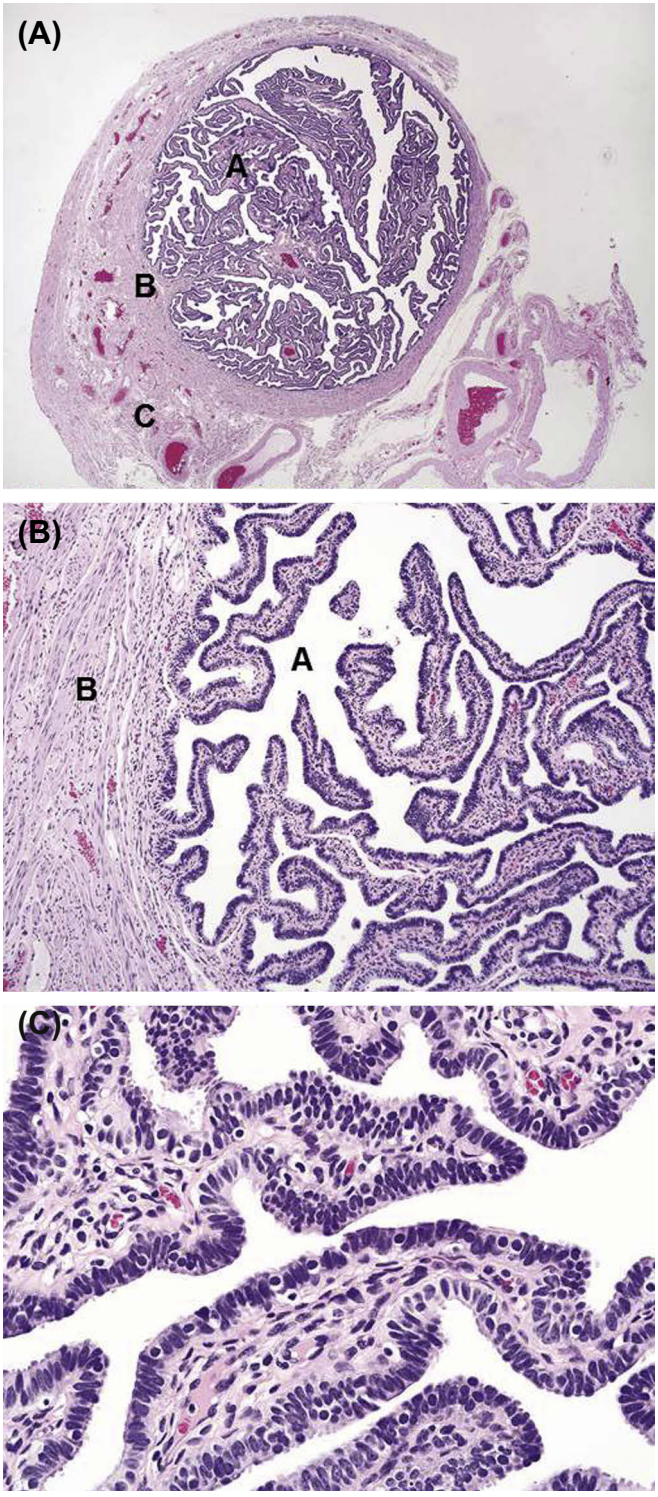

FIGURE 24.2 Cross-section of human fallopian tube, ampulla. This figure is reproduced in color in the color plate section. Inner mucosa (A) is arranged in longitudinal folds within the middle muscularis (B) and outer serosa (C). The mucosa is lined by a single layer of epithelium (lower panel) composed of ciliated and secretory (nonciliated) cell types. Hematoxylin and eosin (H & E) stain, magnification 20–400×. *Source: Images courtesy of Ramya Massand and Jason Moss.*

cervix, and the upper portion becomes the uterine glands and epithelium. The endometrial stroma derives from the adjacent mesenchyme.[2] After fusion of the two ducts to form the future uterus, there is regression in an area of the midline to create the uterine cavity. Failure to achieve proper paramesonephric duct formation, fusion, or regression leads to a spectrum of clinically relevant malformations of unicornuate uterus (hemi-uterus), uterus didelphys (double uterus), or uterine septum, respectively. Evolutionarily, the development of a single uterine cavity in humans can be compared to most nonprimate mammals, and even some primates, in which the uterus does not undergo extensive midline fusion and remodeling and thus remains didelphic.

By midgestation, smooth muscle is apparent in the future myometrium, and pouches (future glands) form from the epithelium into the underlying mesenchyme. By month 7 of gestation, glands are apparent and the endometrium responds to the steroid hormones produced by the placenta.[4] The endometrium is hypertrophic at birth and can even be shed as withdrawal from placental hormones occurs. At birth, the lower uterus and cervix make up two-thirds of the uterus, with the smaller fundus comprising the upper one-third. These proportions remain intact as the uterus grows through childhood in proportion to the rest of the body. At puberty, the endometrium and myometrium both respond to circulating ovarian steroid hormones. The uterus assumes the adult phenotype in which the corpus is larger than the cervix and the endometrium changes and is shed with each menstrual cycle.

OVIDUCT

For much of the past 450 years since Gabriel Fallopio first described the tube that now bears his name, the oviduct has been viewed as a passive conduit for the gametes. Modern investigation, however, describes a much more involved role for the oviduct in reproduction. The concept of the oviduct as an active participant in the establishment of a pregnancy likely arose with histological investigation of the nonhuman primate oviduct; much of this work was done by Brenner and colleagues and was discussed in detail in earlier editions of this text.[1,3] The tubal epithelium undergoes morphologic changes over the course of the menstrual cycle, including increases in cilia and cell height that peak at the time of ovulation. With the rise in progesterone following ovulation, the tubal epithelium loses cilia and atrophies. In many species, the utero-tubal junction is a barrier to limit the number of sperm gaining access to the oviduct. In humans, the tube may act as a sperm reservoir as it does in many other species and perhaps even actively coordinates sequential release of smaller numbers of sperm to continue the path to the oocyte,[8,9] while postovulatory oviductal fluid plays a role in sperm activation.[10] Oocyte pickup from the ovary and

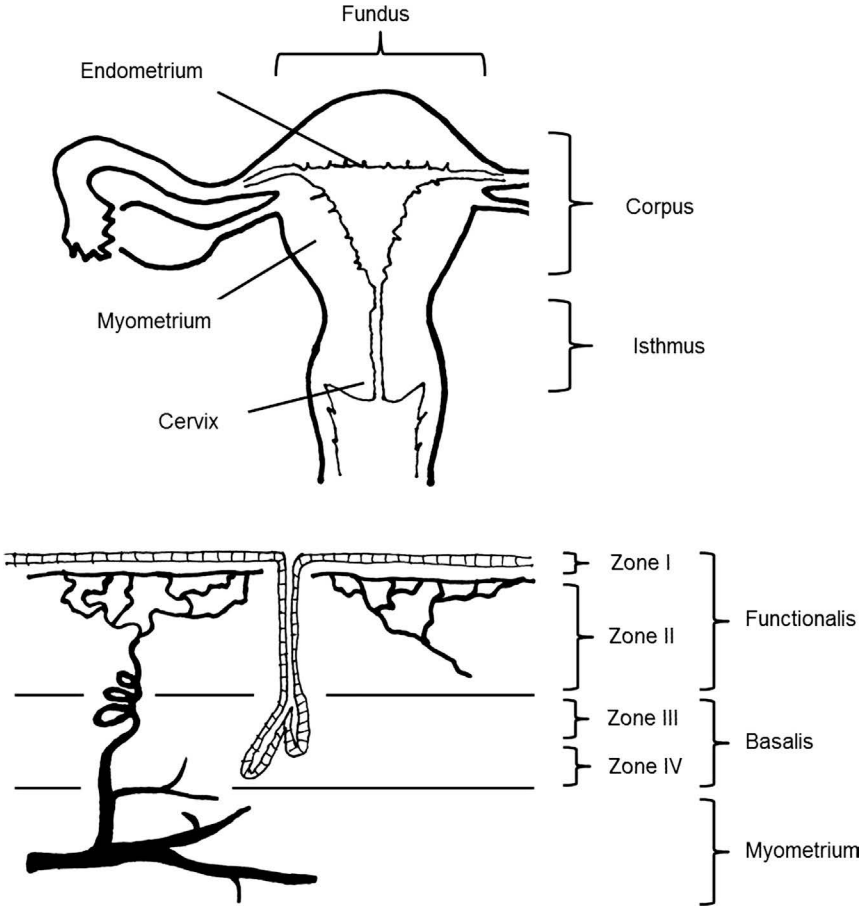

FIGURE 24.3 **Anatomic regions of the human uterus (upper). Cross-section of human endometrium (lower).**

transport of the embryo to the uterus depend on proper function of tubal cilia and smooth muscle in addition to important tubal interactions with the embryo. Additionally, co-culture of gametes and embryos with tubal epithelium has been shown to improve fertilization rates and certain developmental parameters of the embryo, while abnormalities in in vitro embryo development suggest an absence of exogenous components that would be encountered in vivo.[2,10] Taken together, these findings suggest that the structure of the oviduct and substances secreted by certain epithelial cells play important roles in gamete function. In this section, we will discuss what is known of the morphologic and biochemical changes to the oviduct over the course of the menstrual cycle. Central to these changes are the ovarian steroid hormones, which act directly on the oviduct and lead to communication between the cells and tissue types of the tube. Similar to the endometrium, we will see that the effect of the steroid hormones on the epithelium is dependent on signal transduction via the underlying stroma.

Oviduct Histology

The human oviduct is composed of smooth muscle lined internally by a single layer of epithelium. The epithelium is made up of three cell types: ciliated cells, secretory cells, and a third cell type of either an undifferentiated state or an altered, functionally depleted form of ciliated or secretory cells (intercalated or peg cells).[2,11] The ratio of ciliated to secretory cells varies by location through the tube, generally increasing from proximal to distal. The narrow isthmus contains the fewest ciliated cells, while, in the distal infundibulum, the majority of the cells are ciliated.[2,12,13] As early as 1928, researchers reported changes to the tubal epithelium over the menstrual cycle with enlarging cells and increasing cilia at midcycle.[2,14] The growth of these cells throughout the follicular phase was demonstrated to occur under the influence of estradiol while progesterone could be shown to inhibit it. Such hormonal control of the epithelium was supported by a series of clinical studies in women.[2] Cilia could be induced to grow in postmenopausal women with the addition of estrogen[15] or in patients immediately postpartum with the addition of a synthetic estrogen. Such changes could be abolished with the addition of progesterone.[16]

Much of our understanding of tubal histology arises from study of the nonhuman primate, a review of which can be found in great detail in earlier editions of this text.[2,3] These changes have been correlated with serum

estradiol and progesterone levels as well as with concurrent endometrial and ovarian histology. From their work in nonhuman primates, Brenner and colleagues described eight stages of the oviductal epithelium during the menstrual cycle in consideration of multiple morphologic characteristics, including the height of the cells, mitotic activity, the degree of ciliation, the appearance of glycogen and granules in the secretory cells, and the presence of macrophages.

Estradiol and Progesterone

The consistent morphologic changes to the oviduct over the menstrual cycle and the reproducibility of these findings with the administration of exogenous hormones suggest tight control by the ovarian steroid hormones estradiol and progesterone. Using autoradiographic techniques, studies in the 1970s and 1980s investigated the presence and binding capacities of estrogen receptor (ESR) and progesterone receptor (PGR) in the different regions of the oviduct, between nuclear and cytoplasmic fractions, and across the menstrual cycle. The presence of bound ESR and PGR was found to vary across tubal regions, with the highest binding in the ampulla.[17] For estradiol, greater nuclear binding was found as serum estradiol levels increased through the follicular phase of the cycle. Similarly, this nuclear binding decreased as serum progesterone levels rose after ovulation.[18] For progesterone, cytosolic fractions were always found to be greater than nuclear ones, and these findings were consistent across tubal regions. The ratio of nuclear to cytoplasmic binding, however, varied through the menstrual cycle, with an increasing ratio (relatively more nuclear PGR) mirroring serum progesterone levels in the luteal phase.[19]

Throughout the reproductive tract, estradiol stimulates the production of both ESR and PGR, a finding conserved across multiple species, including humans, nonhuman primates, chickens, and mice.[2] Receptor expression in both the cytosol and nucleus increases over the follicular phase and then decreases as progesterone levels rise after ovulation. These findings have been shown most convincingly in the nonhuman primate, where exogenously administered estradiol has been shown to upregulate ESR in the oviductal epithelium while treatment with progesterone decreased the receptor number. The receptor number increased again upon the withdrawal of progesterone. Furthermore, less binding of estradiol to its receptor was found in the presence of progesterone despite continued high levels of estradiol.[20] Measuring these receptors in naturally cycling animals confirmed this finding that progesterone suppresses the amount of ESR in the fallopian tube.[21]

Similar findings arose from advances in immunohistochemistry that enabled further examination of ESR and PGR in the oviduct through the menstrual cycle. In the luteal phase of human subjects, ESR and PGR were observed in both epithelial and stromal compartments of the oviduct, with higher PGR levels in the stroma of isthmic cells compared to other tubal regions. Upon administration of the antiprogestin mifepristone (RU486), PGR increased in both epithelial and stromal cells while ESR increased in the epithelial compartment.[22] Brenner and colleagues also showed increased nuclear ESR staining of the stromal, smooth muscle, and epithelial cells with administration of estradiol, a finding that was reversed with administration of progesterone. PGR increased in response to estradiol treatment in all of the cell types but was suppressed to a greater degree in the epithelium than in the stroma upon treatment with progesterone. The persistence of PGR in the stroma points to this compartment as central in the coordination of downstream responses in the oviduct to progesterone.

Regarding the two isoforms of the progesterone receptor, PGRA and PGRB, very little is known about the relative contributions of each in the oviductal response to progesterone. The two isoforms are encoded by the same gene under the control of different promoters and result in proteins of different size (PGRA, 94 kDa; and PGRB, 116 kDa), with PGRB containing an additional 164-amino-acid sequence at the N terminus.[23–26] Ratios of the different isoforms appear to vary among the different reproductive tissues and in relation to the context of hormonal environment and development.[27–29] For example, in the mouse, ablation of both PGR isoforms (knockout, or PRKO) leads to an infertile animal with multiple reproductive abnormalities, including failure to ovulate, implantation defects, uterine abnormalities, and behavioral disruption.[30,31] Knockout of only the A- or B-isoform has improved understanding of how each contributes to reproductive function, although this knowledge primarily involves ovarian and endometrial function. PGRA is required for both ovulation and the changes to the uterus required for implantation and pregnancy. When the A-isoform is knocked out, PGRB supports epithelial proliferation rather than limiting it.[30] Specific to the oviduct, little is known of the relative contribution of the two PGR isoforms to tubal function, although both are expressed.

The two estrogen receptors, alpha (ESR1) and beta (ESR2), have been detected in the epithelium of the oviduct but recent studies have shown differences in cellular location. In the human, ESR2 expression is mostly limited to the nuclei of the epithelium, with some immunoreactivity in the cytoplasm of these cells as well as in the nuclei of neighboring connective tissue cells. ESR1 was present in all of the oviductal compartments but always limited to the nuclei.[32] The role of each isoform in the function of estrogen in the oviduct remains largely unknown.

Oviductal Fluid and the Facilitation of Gamete Transport

The fluid of the tubal lumen is a product of epithelial secretory cells as well as transudate via the underlying vasculature. Oviductal fluid increases in amount and viscosity through the follicular phase of the cycle under the influence of ovarian estradiol, and bulk flow of the luminal fluid during this time is toward the abdominal cavity.[10] As has been shown in several species, the volume of fluid decreases around ovulation and the overall flow reverses, passing through a relaxed isthmus and into the uterus. The viscosity of the tubal fluid likely has implications for sperm movement, with entirely different patterns of flagellar beating noted using in vitro viscosity gradients and in the mouse oviduct in vivo.[33–35] Aquaporins may provide one means of such water regulation in the fallopian tube.[10,36] The composition of tubal fluid has also been shown to vary over the cycle as well as between regions of the oviduct. The regional differences in composition might be facilitated by macromolecules and the epithelial architecture of folds and grooves, which become more prominent through the follicular phase.[37]

The temporal differences in the consistency and composition of tubal fluid strongly suggest control by the ovarian steroid hormones, estrogen and progesterone; however, in the majority of cases, the mechanism of hormonal control is unknown. Oviductin, an oviduct-specific glycoprotein highly conserved among mammals, may also be regulated by luteinizing hormone (LH), the major ovulatory signal in these species. While oviductin production can be restored in ovariectomized mice with the addition of estrogen (and suppressed again with progesterone), LH has been shown to increase the half-life of the protein by decreasing its degradation.[38] Oviductin in humans has been correlated with serum estradiol and LH levels, and the expression has been shown to vary in different parts of the tube (it is higher in the ampulla and fimbriae), again suggesting both tight control by the ovarian steroid hormones and the different roles played by the various tubal regions.[39] Oviductin has been shown in humans to bind to the zona pellucida and sperm and can improve sperm binding to the zona.[40]

In humans, as in cows, sheep, rabbits, primates, dogs, and cats, semen is ejected into the anterior vagina during coitus. This stands in contrast to horses and many rodents in which semen is deposited directly into the uterus. Thus, in humans, sperm must first gain entry to the oviduct via the ostium at the utero-tubal junction. As it turns out, gaining access to the tube is not a trivial feat, and various knockout models in the mouse are infertile due to an inability of sperm to negotiate this first step. Interestingly, though several knockout models exist that show this same phenotype, the models share a commonality of lacking a particular protein, ADAM3 (a disintegrin and metallopeptidase domain 3), which is likely critical to facilitate entry into the tube.[9] Once in the isthmus, sperm bind to the ciliated epithelial cells, a process likely mediated by carbohydrate residues in the epithelial cells and lectin-like residues in the sperm head,[41] though the actual residues and proteins of this interaction vary among species. The isthmus serves as a reservoir for sperm with sequential release of small numbers of sperm, possibly limiting the number of sperm with access to the egg in the critical fertilization window and, as such, decreasing the incidence of polyspermy.[10] The release of sperm from the isthmus is temporally modulated and has been shown to increase in the periovulatory period.[42] These changes do not appear to be related to the number of carbohydrate-binding sites in the epithelium but are likely controlled by one or more of several other processes, such as sperm motility and binding. Sperm gain a hyperactivation status and thus may escape tubal binding. Oviductal fluid glycosidases and frucosidases have been shown to vary temporally through the cycle and may play a role in sperm release. Finally, evidence exists that a known spermadhesin, AWN (sperm-associated AWN protein), which exists in the sperm head and binds to carbohydrate residues in the oviduct, is actually secreted by oviductal epithelial cells in swine and thus may compete with sperm head binding to the epithelial cells.[43] Progressive waves of oviductal contraction also play a role in sperm transit through the tube. Such myometrial activity is modulated by a ratio of estradiol to progesterone, peaking in cyclic animals close to the time of ovulation.[10]

Capacitation is the process by which sperm acquire the ability to fertilize an egg, a process uncovered independently by Austin and Chang over 60 years ago.[44,45] Since capacitation occurs in the oviduct, the ability to achieve capacitation in vitro has been possible using media mimicking oviductal fluid. Such media require bicarbonate, calcium, energy sources, and serum albumin. Additionally, sperm gain a status of hyperactivation in the oviduct, and calcium flux is central to the acquisition of hyperactivation. Changes in sperm calcium levels have been observed with progesterone treatment, and changes in oviductal pH have been shown during peri-estrus.[9] Overall, increasing evidence suggests that changes in the oviduct specific to the time around ovulation are critical for sperm function.

The oviduct is also critical for regulating oocyte pickup and transport of the embryo to the uterus. Cilia covering the surface cells of the infundibulum beat in the direction of the ostium and induce a current of peritoneal fluid into the tube. Progesterone receptor has been localized in the lower half of motile cilia in oviductal epithelial cells and may regulate beat frequency.[46] Rhythmic contractions of the oviduct also play a role in

oocyte transport. Slow-wave impulses transmitted via interstitial cells of Cajal associated with the oviductal smooth muscle cells are not neuronally mediated, but rather originate from these cells of Cajal.[47] Passage of the oocyte–cumulus complex to the site of fertilization at the isthmic–ampulla junction is relatively rapid (9–15 min in rabbits and 30–45 min in pig), while a slow, controlled transit of the embryo to the uterus (2–7 days) follows fertilization.[10] Part of the regulation of this transit may involve the role of nitric oxide, known to affect oviductal motility and embryo transport.[48,49] Nitric oxide synthase has been shown to be upregulated by estradiol and suggests a mechanism by which ovarian steroid hormones regulate oviductal tone and thus gamete movement in the tube.[49,50]

Certainly, the fallopian tube will receive a great deal of attention in coming years as researchers seek to expand upon this understanding of the fallopian tube as an active contributor to reproduction. While we have an appreciation for tubal responsiveness to the ovarian steroid hormones, unraveling the estradiol and progesterone signaling pathways orchestrating tubal physiology will require a good deal more investigation. Furthermore, recent evidence that a great number of what previously were thought to be cancers of ovarian origin might originate from the fallopian tube will, no doubt, encourage immediate and accelerated research on this dynamic organ.[51–53]

ENDOMETRIUM

The human endometrium is a dynamic structure that cycles through periods of growth and differentiation to facilitate embryo implantation. In the absence of pregnancy, the endometrium breaks down and then regenerates in preparation for the next ovulation. The cues controlling this remarkable plasticity are estradiol and progesterone, which act both directly and through downstream signaling pathways to affect the changes in the endometrium required for implantation and pregnancy.

Much of our understanding of endometrial biology derives from a direct investigation of human endometrium and cell cultures obtained from endometrial biopsy specimens. Human cells, specifically the endometrial stromal cells, can be isolated, cultured, and studied in vitro. Upon treatment with a simple combination of hormones (cyclic adenosine monophosphate (cAMP), estradiol, and progesterone or another progestin such as medroxyprogesterone acetate) mimicking the in vivo environment following ovulation, these cells will undergo decidualization—the phenotypic and biochemical alteration heralded by the rise in luteal-phase progesterone that is required for the establishment and maintenance of a pregnancy. In this section, we will review the endometrium and the changes it undergoes through the menstrual cycle. Our grasp of this fascinating regenerative tissue arises from a combination of in vitro studies of human endometrium together with a system of powerful mouse genetics that has allowed molecular dissection of the various compartments of the endometrium and the molecular pathways underlying its complex function. Genome-wide approaches to characterize genetic transcript across the menstrual cycle and in disease states such as endometriosis have begun to reveal additional candidate genes involved in endometrial function.[54–58]

Histology, Structure, and Molecular Phenotype

Histology and Structure

Histological changes to the uterine endometrium over the menstrual cycle were reported over 100 years ago by Hitschmann and Adler,[2] findings that revolutionized the understanding of uterine biology, which until that point had been predicated on a theory that the physiological and pathological changes to the endometrium represented a spectrum of "chronic endometritis."[59] In the years following, studies in both humans and other primates began to explore the changes observed in this tissue over the menstrual cycle and correlate these findings with changes observed in the ovary. From this early work arose the central dogma of endometrial biology: the ovarian hormones, estradiol and progesterone, control the growth, differentiation, apoptosis, and functioning of the endometrium.[2,60,61] Noyes, Hertig, and Rock cataloged the histological stages of the endometrium based on an "ideal" 28-day cycle using nearly 8000 endometrial biopsies.[62] From this work and others, the human endometrium was described in its relation to the ovarian steroid hormones and the menstrual cycle.

The first half of the menstrual cycle is referred to as the follicular or proliferative phase, depending on whether the focus lies on the ovary or endometrium, respectively. During the proliferative phase, and under the influence of estradiol, the endometrium thickens from several millimeters to approximately 12–14 mm. High mitotic indices are apparent in the epithelium, stroma, and vascular epithelium as DNA synthesis increases, especially toward the end of the proliferative phase under the influence of the highest levels of circulating estradiol. Peak mitotic activity mirrors peak estradiol levels and maximum estrogen receptor expression around days 8–10 of the menstrual cycle.[63] The straight endometrial glands become larger and more tortuous, the vascular network expands, and the stroma proliferates (Figure 24.4).

During the luteal or secretory phase (again, ovarian or endometrial focus), the endometrium comes under the control of progesterone. For the first 36–48 h after the rise in serum progesterone, there is little observable

FIGURE 24.4 Human endometrium from proliferative (upper), early-secretory (middle), and late-secretory phases (lower). This figure is reproduced in color in the color plate section. Note the wide spacing between simple-appearing glands (proliferative), apically displaced nuclei in the glandular epithelium due to glycogen accumulation (middle), and tortuous glands with secretions and a decidualized stroma with abundant pale cytoplasm (lower). H&E stain, magnification 100–200× (Images courtesy of Ramya Massand and Jason Moss).

difference in the histology of the endometrium. After this point, however, dramatic changes ensue. Differences are first apparent in the epithelium, where glycogen begins to accumulate near the basalis and the nuclei begin to appear displaced upward by the glycogen granules.[2] By days 19–20 of a 28-day cycle, few vacuoles remain as the contents have been expelled into the glandular lumen. The amount of glandular fluid, a product of these epithelial cells and transudate from the endometrial vasculature, peaks at this time and is temporally aligned with embryo implantation. A defined "window of receptivity" exists in which the endometrium is capable of supporting an embryo, and it occurs 6–10 days after the LH surge.[64] Historically, timing of the implantation window was suggested by a classic 1956 study examining early-pregnancy specimens obtained at hysterectomy. In secretory-phase specimens, embryos found before cycle day 20 were free-floating, while those found after day 20 had attached or invaded the endometrium.[65] Later, the window of receptivity was found to coincide with the appearance of epithelial pinopodes, protrusions of the endometrial epithelium important for embryo attachment and water transport. The discrete implantation window is supported by clinical observations of high continuing pregnancy rates (84%) for embryos implanting postovulation days 8–10, versus low rates (18%) when implantation (as determined by chorionic gonadotropin detected in the urine) occurs more than 11 days after ovulation.[66] Similarly, in assisted reproductive cycles, pregnancy rates are 40.5% when embryo transfers are performed between cycle days 17 and 19, compared to 0% after day 20.[2,67]

By the onset of the implantation window, changes to the endometrial stroma are readily apparent, with increased mitoses, edema, and endothelial cell proliferation in the now-coiling spiral arterioles.[62] This edema and permeability precede the decidualization of the stroma in which the spindle-shaped stromal fibroblasts undergo transformation to a round, epithelial-like phenotype (Figure 24.5).[2,68] These cells are characterized classically by their appearance, the production of secreted products such as insulin-like growth factor binding protein 1 (IGFBP1) and prolactin (PRL), and characteristics of both epithelial cells and mesenchymal cells. Decidualized stromal cells have a lamin-rich basement membrane and intercellular adherens junctions (epithelial-like) but stain with immunohistochemical antigens that are classic for mesenchyme, such as vimentin and desmin, and not with certain antigens specific for epithelium, such as epithelial membrane antigens or cytokeratins.[2]

The decidualization of the stroma begins near the spiral arterioles and capillaries and then spreads, encompassing the entirety of the compartment before menses ensues. By the end of the implantation window, lymphocyte infiltration is visible in the stroma; and, immediately

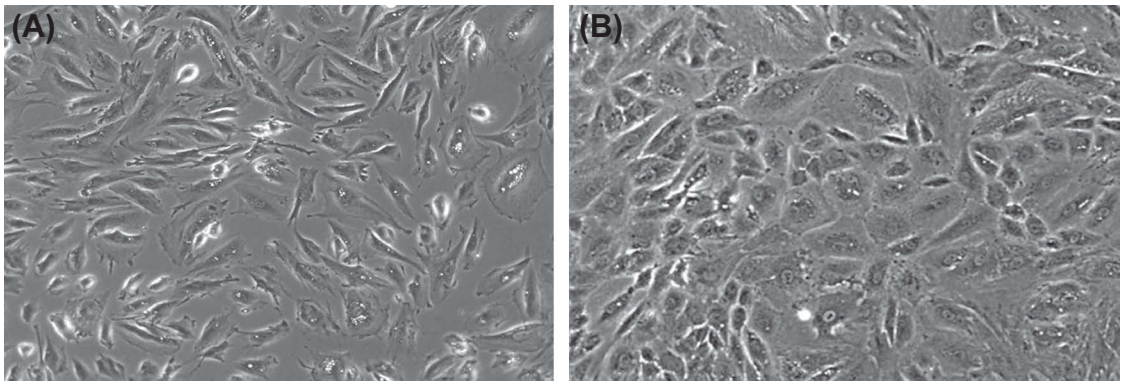

FIGURE 24.5 Human endometrial stromal cells in vitro before (A) and after 6 days of treatment with estradiol, medroxyprogesterone acetate, and cAMP (B). Note the plump, rounded cell morphology with enlarged nuclei in the decidualized cells.

preceding the menses, there is an influx of polymorphonuclear leukocytes (PMNs).[62] These immune cells are thought to be involved in establishing the protected relationship of the conceptus within the maternal immune system. The same cells likely also function to mediate the process of endometrial shedding that occurs in the absence of a pregnancy. In the absence of embryo implantation and the presence of human chorionic gonadotropin (hCG) from the trophoblast to maintain the corpus luteum, estradiol and progesterone levels fall. In this setting, the endometrium undergoes apoptosis, vascular basement membrane breakdown, bleeding, vasospasm, and desquamation, which result in the menses.[2] The vasomotor response to the fall in steroid hormones is dramatic and detailed in early studies of the nonhuman primate.[69,70] The spiral arteries lose height, blood flow in both the arterial and venous beds slows, and vasospasm of the spiral arterioles ensues in a rhythmic pattern of increasing intensity and duration, ultimately resulting in the blanching of the endometrium. Ischemia and capillary leak follow with leukocyte infiltration visible, first surrounding the vessels and then extending throughout the stroma. Prostaglandin (PGF2α, PGE2, and PGI2) production is maximal at the time of menstruation, and these factors are thought to underlie much of the vascular response of the spiral vessels. Enzymes (including matrix metalloproteinases (MMPs)) induced with the fall in progesterone and mediators of progesterone action (such as transforming growth factor beta (TGFβ)) catalyze the breakdown of transmembrane proteins and other components of the extracellular matrix. The cytokine tumor necrosis factor alpha (TNFα) also is thought to play a major role in menstruation through disruption of cell-to-cell communications and induction of apoptosis.[71] Vascular endothelial growth factors (VEGFs) have been implicated in both menstruation and the neovascularization of the proceeding cycle. Bleeding ensues as blood vessels are compromised and the surrounding tissue weakens, and the flow of menstrual blood is influenced by multiple factors, including clotting and fibrinolytic factors and the contraction of myofibroblasts surrounding the spiral arteries.[72] Ultimately, the shrinking functionalis splits from the underlying basalis and is shed. The proliferative phase begins again, the first few days of which are actually independent of the ovarian steroids as these levels are still low. Repopulation of the functionalis is thought to occur, in part, by stem cells from the basalis.[73] As estradiol levels rise, the proliferation and thickening of the endometrium follow once more.

Genomic Profiling of the Human Endometrium

Recent work has subjected the human endometrium to global gene expression profiling across the menstrual cycle.[54–58] Oligonucleotide microarrays and cDNA hybridization with polymerase chain reaction (PCR) techniques have been used to compare secretory endometrium to proliferative-phase endometrium. In addition, various points in the secretory endometrium phase (i.e., early versus mid versus late secretory) have been queried. Investigators have sought to describe the differentially regulated genes in terms of the functions of various gene groups through the menstrual phases, describing lists of genes consistent with much of what is known or suspected regarding the biology of the secretory endometrium. When comparing implantation window secretory endometrium to proliferative-phase endometrium, many of the differentially regulated genes are involved in cell cycle regulation, extracellular matrix synthesis, cell adhesion, immune modulation, and secretion.[55] Several studies have described large-scale gene expression profiles of human endometrium; however, significant variations exist in sampling, study design, and bioinformatics analysis, making comparisons difficult. Recently, work has been undertaken to compile data from these various studies,[74,75] including the creation of a database of nearly 20,000 genes expressed in the human endometrium, 179 of which have been consistently shown to exhibit differential regulation in the implantation window.[76]

Other studies have examined the promoter regions of these differentially regulated genes for the presence of progesterone response elements (PREs) or estrogen response elements (EREs) as evidence for direct regulation by the ovarian steroid hormone receptors.[55] The candidate genes uncovered through such expression profiling are currently under investigation to better understand their roles in both normal endometrial function and endometrial disease states, such as endometriosis, polyps, hyperplasia, and malignancy. Easy accessibility to such ever-increasing high-content data will help to facilitate the exploration of candidate genes and pathways involved in endometrial function and dysregulated under certain conditions or disease states. These gene lists will be supplemented by new techniques—for example, deep-sequencing transcriptome analysis (e.g., RNA sequencing)—which may overcome some intrinsic biases to array-based technologies and allow for the analysis of molecular isoforms in endometrial function.

Steroid Hormone Signaling and the Mediators of Compartmental Cross-Talk

The pattern of endometrial growth, differentiation, regression, and regeneration over the menstrual cycle is coordinated through actions of estradiol and progesterone. While these hormones act directly on the various compartments of the endometrium, strong evidence supports additional paracrine interactions between the epithelium and stroma to orchestrate proper functioning. Much of our understanding of this endometrial biology arises from studies using the transgenic mouse. While gene profiling has begun to detail regulatory pathways of the human endometrium at a transcriptional level during the menstrual cycle and in certain disease states, transgenic mouse models have been used to dissect these pathways at a functional level. Figure 24.6 outlines the regulation of endometrial function in the mouse in the peri-implantation period, briefly introduced here and discussed in greater detail later in the chapter. From day 0 to day 1.5 of the mouse estrous cycle, estrogen signaling predominates. Epithelial proliferation is driven indirectly via the paracrine action of growth factors produced under the control of stromal ESR, while estrogen regulation of target genes such as mucins and ion channels responsible for edema is regulated directly by epithelial ESR. At day 2.5 of pregnancy, the rise in progesterone coincident with luteal development acts predominantly through PGR in the uterine epithelium, inducing factors such as Indian hedgehog (IHH) that act in a paracrine fashion to prepare the underlying uterine stroma. As pregnancy progresses (Figure 24.6: Day 3.5), waning levels of PGR in the epithelium act to inhibit epithelial proliferation via the induction of Krüppel-like factor 15 (KLF15). Meanwhile, increasing levels of PGR in the stroma promote proliferation of said compartment while simultaneously inhibiting epithelial proliferation by inducing HAND2 (human heart– and neural crest derivatives–expressed 2). Embryo attachment is mediated both by leukemia inhibitory factor (LIF) and by sodium ion channels serving the dual purpose of luminal closing and signal transduction (Figure 24.6: Day 4.5). Implantation of the blastocyst triggers decidualization of the endometrial stroma, which is propagated through progesterone action and its downstream signaling paradigm (Figure 24.6: Decidua).

In the remainder of this section, we will review what is known of these complex signaling networks, both direct and cross-compartmental, that coordinate the changes of the endometrium over the menstrual cycle—changes that enable the establishment and maintenance of a pregnancy.

Estrogen Receptor Signaling

Estrogen receptor is critical for regulating the growth and integrity of the endometrium and for coordinating embryo receptivity. Acting via its receptors, estrogen regulates endometrial proliferation and inhibits apoptosis. In humans, lack of estrogen—such as that which occurs after menopause—leads to an absence of endometrial proliferation with resulting atrophy. On the other hand, estrogen-induced proliferation that goes unchecked by the differentiating effects of progesterone—as occurs in anovulatory states like polycystic ovary syndrome (PCOS)—increases the risk for endometrial hyperplasia and malignancy. The high levels of estradiol that result from the multifollicular development of ovarian hyperstimulation in assisted reproductive techniques (ARTs) for couples struggling with subfertility may have a detrimental effect on endometrial function, with resulting impacts on both the chance for and outcomes in a resulting pregnancy. Finally, the endometrium that exists *ex utero* in endometriosis is understood to display altered estrogen signaling, which likely contributes to its pathogenesis. Thus, understanding estrogen signaling—including its control over proliferation, implantation, and progesterone sensitivity—is critical as we strive to correct some of the most common and morbid diseases of the endometrium.

The estrogen receptor exists in two forms, alpha (ESR1) and beta (ESR2), transcribed from unique genes and each with different and specific roles in reproduction. Both ESR1 and ESR2 are expressed in the glandular epithelium and stroma of the human endometrium. Expression levels are high during the proliferative phase and decline during the secretory phase.[77,78] Studies in the mouse have determined that the alpha receptor is the critical estrogen receptor controlling estrogen action in the endometrium. In human endometriosis, however, the ratio of ESR1 to ESR2 has been shown to be altered,[79] a finding that might help to explain the particular growth characteristics, altered progesterone

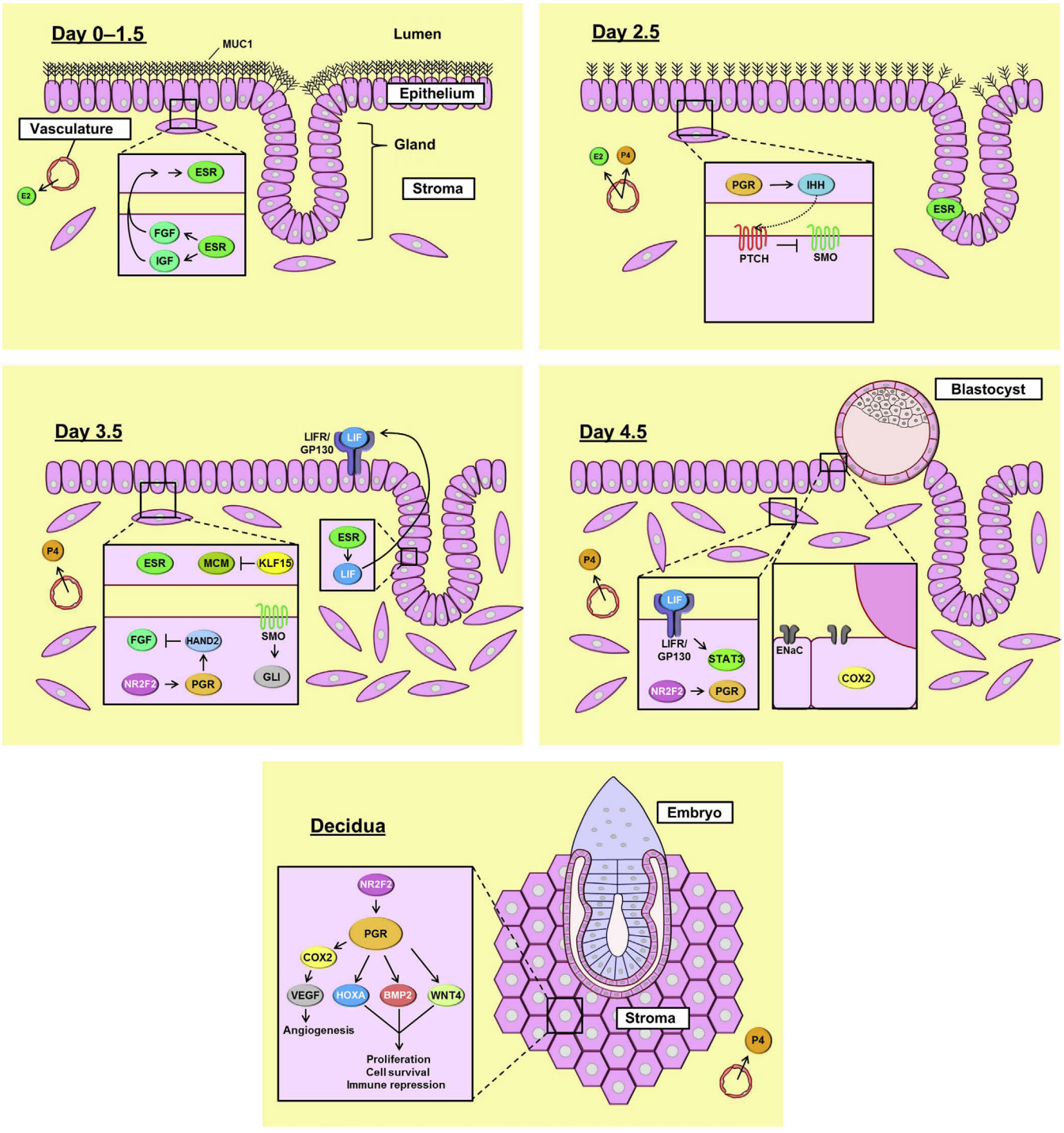

FIGURE 24.6 Estrogen and progesterone receptor signaling pathways in the murine endometrium. The early postovulatory phase (Days 0–1.5) is dominated by follicular estradiol (E2) acting via estrogen receptor (ESR) and driving epithelial proliferation (via stromal ESR and downstream growth factors) and the expression of epithelial ESR target genes such as *Muc1*. At Day 2.5, the epithelium is still proliferating, and progesterone receptor (PGR) is expressed in the uterine epithelium. Indian hedgehog (*Ihh*), a major PGR target gene, is expressed in the uterine epithelium but acts in paracrine fashion in the stroma, where it binds its receptor (PTCH), which then relieves smoothened (SMO) from repression. By Day 3.5, PGR, in part regulated by NR2F2 (COUP-TFII), is acting to inhibit proliferation of the epithelium, accomplished by both epithelial expression of KLF15 and stromal activation of HAND2, while inducing proliferation of the underlying stroma. *Lif*, an ESR target gene necessary for blastocyst implantation, is expressed in the glandular epithelium. LIF secreted from the glandular epithelium acts via its receptor, LIFR/GP130, in the luminal epithelium. With invasion of the blastocyst through the epithelium at Day 4.5, LIF expression shifts into stromal cells surrounding the implanting blastocyst and acts through the transcription factor, STAT3. COX2, a key enzyme in prostaglandin synthesis, is induced through a pathway involving the epithelial sodium channel, ENaC, and emerges as one of the first markers of stromal decidualization. In the stroma, expression of key regulators of decidualization increase robustly from basal levels observed at the time of implantation, concurrent with the wave of stromal cell differentiation (Decidua).

signaling, and inflammation that have been associated with endometriosis.

ESR1 has garnered the bulk of attention when studying the mouse uterus as *Esr2*-null mice demonstrate a very mild uterine phenotype compared to those lacking *Esr1*.[80,81] In the mouse, ESR1 exhibits a dynamic expression pattern in the various epithelial compartments, through the progression of pregnancy, and upon steroid treatment.[82–84] ESR1 is expressed in both the epithelium and stroma and promotes proliferation under the influence of estradiol, as has been discussed in this chapter. With ovulation and the rise in progesterone, PGR expression is induced in the luminal epithelium. Just prior to the window of receptivity, LIF is induced by estradiol in the glandular epithelium,[85,86] while PGR expression is extinguished in the epithelium.[83] Expression of LIF and downregulation of PGR in the epithelium are both critical for embryo implantation. Progesterone then acts via stromal PGR to regulate decidualization.

Steroid hormone control of ESR1 and PGR expression was shown using ovariectomized mice under the influence of exogenous steroids.[84] Without steroids, ESR1 was observed at high levels in both the epithelium and stroma while PGR expression was highest in the luminal and glandular epithelium. Upon administration of estradiol, ESR1 was upregulated in the luminal epithelium but inhibited within the stroma. PGR was observed in all compartments without hormone, but upon the administration of estrogen, PGR expression was lost in the luminal epithelium. Thus, estrogen was shown to drive PGR expression from the epithelium to the stroma while inducing expression of ESR1 in the epithelium.[82] Induction of ESR1 in the glandular epithelium was shown to be under the control of progesterone, and progesterone was shown to decrease the global expression of its own receptor.

Estrogen Receptor—Compartmental Cross-Talk

While estrogen and progesterone acting through their nuclear receptors can have a direct effect on the endometrial compartment in which the receptors are expressed, it is now clear that the action of these steroid hormones in regulating the function of the endometrium occurs via paracrine interactions between the epithelium and stroma. Endometrial epithelial cells do not respond to estrogen when cultured in vitro, which suggests the requirement of the underlying stroma to support their growth. Such epithelial–stromal interaction had previously been uncovered in the mouse mammary gland and testis where androgen action promoting development required androgen action through its receptor in the underlying stroma.[87–89] The generation of mutant mice with specific ablation of these steroid hormone receptors in either the whole uterus or specifically in the epithelium has confirmed the cell-specific roles of estrogen and progesterone in the uterus.

The paracrine regulation of the endometrial epithelium by estradiol was initially suggested by tissue reconstitution xenograft studies combining epithelial and stromal cells isolated from wild-type and *Esr1*-null mice.[90] These studies showed that estradiol could stimulate epithelial growth only when ESR1 was expressed in the underlying stroma, while ESR1 expression in the epithelium did not affect growth. A system of conditional knockout of *Esr1* only in the uterine epithelium confirmed this observation; while estradiol is indeed required for growth of the epithelium, its action does not occur directly through its receptor in the epithelium but rather through the ESR1-mediated production of growth factors such as insulin-like growth factor 1 (IGF1) and CCAT–enhancer binding protein beta (CEBPB) that originates in the stroma.[91]

Progesterone Receptor Signaling

PGR is likewise expressed in both uterine epithelium and stroma. Several studies have examined the presence of the progesterone receptor isoforms, PGRA and PGRB, in the various compartments of the human endometrium throughout the menstrual cycle. In the proliferative phase, PGRA and PGRB are present in both the glandular epithelium and stroma. PGR increases in both compartments throughout the proliferative phase, though PGRA is always present in greater quantity than PGRB. After ovulation, PGRA and PGRB are initially present in the glandular epithelium before levels of both isoforms decline. Levels of PGRB fall rapidly in the glandular epithelium but have been shown to rise again in the midsecretory phase. PGRA shows a more constant, steady decline in the epithelium throughout the secretory phase. In the stroma, PGRA and PGRB persist throughout the secretory phase.[27,92–94]

The relative importance of PGRA and PGRB in endometrial function has been elucidated through study of receptor-specific conditional knockout mice. Global knockout of *Pgr* (PRKO) in the mouse resulted in anovulation and an inability to respond to exogenous gonadotropins.[30] These mice were shown to be insensitive to the inhibitory effect of progesterone on estrogen-induced epithelial cell proliferation. A study in which chimeric uteri were created using the four possible combinations of wild-type and PRKO epithelium and stroma likewise confirmed the ability of progesterone to inhibit the proliferation of epithelial cells only when PGR was expressed in the stroma.[95] In other words, progesterone inhibited E2-induced DNA synthesis only in the two recombinants expressing PGR in the stroma (wild-type stroma combined with either wild-type or PRKO epithelium). However, a genetic model lacking *Pgr* in the adult epithelium demonstrated that epithelial PGR is also required to inhibit epithelial proliferation, the mechanisms of which will be discussed in this chapter.[96]

Using *Pgr-A* (PRAKO) and *Pgr-B* (PRBKO)-specific null mice, it was observed that PGRA is the obligatory receptor for fertility as these mice failed to ovulate normally and implantation–decidualization was prevented. These findings were not apparent in mice lacking *Pgr-B*.[97]

Other isoforms of the progesterone receptor likely exist (e.g., PGRC, PGRM, PGRS, and PGRT),[98] though clear roles for their function have not been shown. Some evidence exists that these isoforms may antagonize PGRA and PGRB by sequestering progesterone. In many mammals, progesterone levels fall immediately preceding parturition, supporting a role for progesterone in pregnancy maintenance. In humans, however, serum progesterone levels remain high through birth. Much effort is currently underway to characterize the changes to the uterus with the initiation of labor, with attention paid to reconciling differences between humans and other mammals regarding progesterone levels. Current theories include a role of PGRC in parturition,[99] isoform switching between PGRA and PGRB, and increased metabolism of progesterone,[100] among others.

In addition to acting via its nuclear receptors, progesterone can exert its effects via rapid cytoplasmic signaling. Classically, progesterone is understood to act via its nuclear receptors (as explained here). In this setting, ligand-bound progesterone receptor interacts directly with *cis*-acting progesterone response elements in the promoter regions of target genes.[101] PGRB can also interact with the Src tyrosine kinases at the plasma membrane, transducing a signal via the Ras–MAPK (mitogen-activated protein kinase) pathway.[102] These pathways of rapid cell signaling are important in proliferation, differentiation, angiogenesis, and decidualization.[103,104] Membrane-bound progesterone receptors distinct from the nuclear receptors have also been implicated in such rapid signaling involving calcium, MAPK cascades, and cAMP.[105,106]

Progesterone Receptor—Endometrial Compartment Cross-Talk

As with the estrogen receptors, PGR is involved in paracrine cross-talk between the stromal and epithelial compartments. While only a few studies using human tissue have investigated epithelium–stroma cross-talk in the endometrium, this work appears to support more extensive studies in the mouse. In vitro co-culture studies of human endometrium have shown that stromal cells play an important role in the epithelial response to estrogen and progesterone.[87,107,108] For example, epithelial proliferation was induced by estradiol and inhibited by medroxyprogesterone acetate only in the presence of stromal cells. Furthermore, PGR was not detected in the epithelium, suggesting that the action of progesterone to mediate epithelial function occurs via stromal PGR. IHH is expressed in the human endometrium and peaks during the secretory phase,[109] as is GLI family zinc finger 1 (GLI1), a downstream effector of IHH signaling. IHH has been shown to depend on the presence of progesterone,[110] and dysregulation of IHH has been shown in samples of endometriosis, a disease in which progesterone signaling is likely altered.[58]

Studies in the mouse using tissue recombination and compartmental-specific gene deletions have identified progesterone-regulated cross-talk both from epithelium to stroma and from stroma to epithelium. PGR cross-talk was initially studied by xenograft reconstitution experiments using various combinations of wild-type and PRKO epithelial and stromal cells. This analysis showed that PGR in the stroma was critical for regulation of epithelial-specific *Ihh* as well as the ability of progesterone to inhibit the epithelial cell proliferation induced by estradiol stimulation. Tissue reconstitution experiments indicated that stromal PGR is critical both for regulating progesterone-stimulated epithelial gene expression and for inhibiting estrogen-induced epithelial proliferation. However, unlike studies with ESR in which in vivo analysis or epithelial-specific ablation of *Esr* confirmed the importance of stromal ESR1 in the regulation of epithelial cell proliferation, in vivo conditional ablation of *Pgr* in the epithelium did not corroborate these stromal findings.[96]

Through studies of epithelial-specific ablation of *Pgr*, it was demonstrated that the progesterone-responsive gene *Ihh* could be directly regulated by epithelial PGR after progesterone stimulation. Also, this analysis demonstrated that epithelial PGR is directly responsible for the inhibition of estrogen-induced epithelial cell proliferation. Additionally, a microarray analysis of mouse endometrial epithelium during the window of receptivity[111] revealed induction of a transcription factor, KLF15, which was then shown, in the presence of progesterone, to inhibit epithelial proliferation by regulating minichromosome maintenance 2 (MCM2), an essential component in DNA synthesis initiation.[112] Understanding the discrepancy between the tissue recombination experiments and the in vivo compartmental-specific analysis can be accomplished by understanding the timing and nature of the paracrine cross-talk between these compartments.

During the preimplantation period in the mouse, PGR is expressed predominantly in the epithelial compartment of the endometrium, and this epithelial PGR is required for the inhibition of epithelial cell proliferation,[96] which likely occurs via KLF15. Interestingly, PGR transduces a signal from the epithelium to the stroma via the transmembrane receptor patched homolog 1 (PTCH1), which acts by inhibiting another transmembrane receptor, smoothened (SMO). Thus, binding of hedgehog to the PTCH1 receptor results in an active smoothened signal (Figure 24.6: Day 2.5). This signal activates transcription of the glioma-associated oncogene (*Gli*) family

of transcription factors and the orphan nuclear receptor chicken ovalbumin upstream transcription factor II (*Coup-TfII*), also known as nuclear receptor subfamily 2, group F, member 2 (*Nr2f2*).[113] IHH is expressed in the uterine epithelium just prior to the window of receptivity in the mouse, and the expression of NR2F2 target genes follows in the stroma.[114] These transcription factors have been shown to activate downstream targets of the hedgehog ligands by binding directly to response elements in the upstream promoters of the target genes.[82]

IHH and NR2F2 are fundamental to development, as the ablation of either gene in the mouse results in an embryonic lethal phenotype.[115,116] Mice heterozygous for *Nr2f2* have reproductive defects, including an inability to undergo the changes necessary for embryo implantation.[117] With such severe phenotypes in the global knockout of these genes, an understanding of the interplay between these factors has benefited from the use of conditional knockout studies in the mouse. Using a Cre recombinase system, selective knockout of these genes was possible in tissues expressing PGR (Pgr^{cre}). Both *Ihh* and *Nr2f2* conditional knockouts resulted in a similar phenotype in which embryos were unable to attach to the uterine lumen[118,119] and failed to undergo decidualization. The combined defects of attachment and decidualization again support the IHH–NR2F2 axis acting in both epithelial and stromal compartments to mediate proper endometrial function, and these factors have been shown to be important in the steroid hormone control of the endometrium. Estrogen promotes epithelial proliferation and the expression of factors on the epithelium, such as the large glycoprotein MUC1, during the proliferative phase of the cycle. Halting epithelial proliferation and downregulation of MUC1 after ovulation are critical steps in facilitating embryo attachment to the epithelium. Ablation of *Nr2f2* results in a failure to stop estrogen-related proliferation in the epithelium, and, similarly, MUC1 is not downregulated.[96] Furthermore, when these *Nr2f2*-ablated mice were treated with an ESR1 inhibitor, embryo attachment and decidualization were rescued.[120] The mechanism by which NR2F2 inhibits estrogen signaling may include HAND2, a PGR target shown to be important in downregulating the growth factor ligands that are critical for estrogen-driven epithelial proliferation.

The mechanism of progesterone inhibition of epithelial proliferation has been explained, at least in part, by studies investigating the role of HAND2. HAND2 is a basic helix–loop–helix transcription factor and a direct progesterone target that is upregulated in the stroma upon treatment with progesterone. In turn, HAND2 inhibits the expression of several fibroblast growth factor ligands, molecules that act as mediators to induce epithelial proliferation in response to estrogen.[121] This temporal progression of growth factor signaling indicates that PGR initially acts directly in the epithelial compartment to regulate gene expression and inhibit estrogen-induced cell proliferation. As pregnancy progresses and PGR is lost from the epithelium, stromal PGR then regulates epithelial cell function (Figure 24.6).

We have seen that dissemination of the progesterone signal to promote decidualization in the stroma and halt proliferation in the epithelium occurs via PGR through the aforementioned signaling pathways. Certainly, we have just begun to understand how progesterone acts to remodel the endometrium. Recent and current studies adopting a genome-wide assessment of progesterone binding and gene transcription will undoubtedly provide additional targets to investigate further both the mechanisms by which progesterone coordinates its effects on the endometrium and what, exactly, these changes entail. Excellent and detailed reviews of progesterone signaling and implantation are available for those interested in further information.[87,122]

Decidualization: Restructuring the Endometrium

While we have reviewed some of what is known regarding how steroid hormone signaling drives the endometrial transition, there is considerable information describing the changes of decidualization, even if the signaling pathways underlying these changes remain largely unknown. Ultimately, the function of the endometrium is singular: to serve as the interface between the mother and fetus. The endometrium allows attachment of the embryo and facilitates its invasion deeper into the lining. The highly invasive trophoblast is, at the same time, limited and nurtured by the endometrium. Again, while the signaling mechanisms remain, for the most part, poorly understood, it is clear that the rise in postovulatory progesterone initiates a cascade of events to allow the attachment and invasion of the embryo. Progesterone induces secretion in the endometrial glands and inhibits growth in the epithelium. Locally produced chemokines attract specialized uterine natural killer cells (uNKs, $CD56^{bright}$–$CD16^{-}$), which express immunomodulatory molecules and have been shown to play an important role in producing cytokines and angiogenic factors that reorganize the maternal spiral arteries.[123–125] Furthermore, progesterone has been shown to alter the contractile activity of junctional myocytes, a process likely important in allowing apposition of the blastocyst to the luminal epithelium.[123,126]

The most apparent—and thus the most studied—change to the human endometrium prompted by the rise in progesterone after ovulation is the structural and biochemical transformation of the endometrial stromal cells. Interestingly in humans, unlike in many mammals, decidualization occurs independent of pregnancy. The cues and functional ability to decidualize are inherent to

the cells themselves and depend on signaling that originated with progesterone from the corpus luteum.

Stromal Cell Decidualization

One reason why stromal cell decidualization is relatively well understood in comparison to changes involving other cell types (immune, vascular, etc.) occurring in the endometrium is the ability to isolate and grow these stromal cells in culture and then to recapitulate their transformation in vitro. The phenotypic and biochemical changes to stromal cells undergoing decidualization have been nicely reviewed.[123]

At a microscopic level, the elongated fibroblasts transform to plump, rounded cells (Figure 24.5). The nucleus becomes enlarged and rounded, and there is expansion of the rough endoplasmic reticulum and Golgi apparatus. Glycogen and lipid droplets accumulate. Processes appear on the cell surface and protrude into the extracellular matrix, impinging on neighboring cells. Extracellular matrix proteins such as type IV collagen, laminin, fibronectin, heparin sulfate proteoglycans, and decorin are produced. Part of the stromal cell transformation is acquisition of high secretory capacity, and secreted products from these cells (IGFBP1 and PRL) have become standard markers for the decidualization process. These cells also produce cytokines, growth factors, and signaling molecules thought to act in local autocrine or paracrine fashion—there is no evidence that these proteins are secreted into the circulation—to propagate the signals and processes of decidualization. Human endometrial stromal cells undergoing decidualization in vitro have been subjected to microarray and other transcriptome analyses that reveal enrichment pathways involved in cellular adhesion, organization of the cytoskeleton and extracellular matrix, signal transduction, cell cycle progression, and apoptosis, among others.[57,64,78,127,128] A major current goal is determining how these transcriptional networks translate into the biology of decidualization. This will be accomplished, no doubt, through continued study in humans (e.g., interrogation of biopsy specimens, and primary cell culture), other mammals (e.g., conditional knockout studies), and combinations of the two (e.g., human tissue xenografted into other animals).

Mediators and Products of Decidualized Cells

While endometrial stromal cells will respond in vitro to progesterone alone, the change is slow and not consistent with the temporal patterns in vivo. The addition of cAMP, however, drastically alters the rate of decidualization. Indeed, stromal cells will respond to cAMP alone, though this response is brief. The role of cAMP in the decidualization process has been explained as that of a progesterone sensitizer.[129] cAMP is stimulated by a variety of local and endocrine factors (prostaglandins, relaxin, and corticotropin-releasing hormone), and the production of cAMP is robust enough to be detected between proliferative and secretory cells. After pregnancy, the endometrium is exposed to vast amounts of hCG, which signals primarily through cAMP.[123] Exactly how cAMP and progesterone act in relation to one another is not well understood, though it is thought to involve recruitment of coregulators (see Chapter 9). cAMP has been shown to induce the transcription factors specificity protein 1 (SP1), Forkhead box O1 (FOXO1), signal transducer and activator of transcription 3 and 5 (STAT3/5), and CEBPβ (CCAAT/enhancer binding protein beta), all of which are known to function with progesterone receptor to affect decidualization.[129] Also, cAMP may be indirectly involved in the activity of PGR through alterations in enzymes that modify the attachment or removal of small ubiquitin modifier (SUMO)—a small peptide that changes nuclear localization patterns, associations, and activities of the transcription factors it binds. Thus, decreasing the amount of bound SUMO translates into a more active PGR.[130,131]

The prolactin secreted from the decidualized endometrium is structurally and functionally identical to prolactin from pituitary prolactin, though the pituitary prolactin secretagogues (dopamine, bromocriptine, and thyrotrophin-releasing hormone) have no effect on endometrial prolactin production. Prolactin from the endometrium can be stimulated by IGF1, relaxin, and insulin, each acting through its native receptor. A prolactin-releasing factor has been isolated from placenta, and an inhibitor of this releasing factor has been purified from decidua. Since prolactin can exert an overall inhibitory effect on decidualization, there may be a balance in the control of its production from the trophoblast and endometrium in early pregnancy.

Interestingly, certain factors at first expressed in the epithelial compartment during the proliferative phase of the menstrual cycle (superoxide dismutase 2 (SOD2), growth arrest and DNA-damage-inducible 45 (GADD45), CEBPβ, and FOXO1) are induced in the stroma during decidualization. Similarly, factors expressed in the decidualized stroma (prolactin, IGFBP1, and pregnancy-associated plasma protein A (PAPPA)) are eventually produced at high levels by the placenta during pregnancy.[123,132]

Since decidualization of the endometrial stroma in humans occurs in the absence of pregnancy and it is possible to recapitulate decidualization in vitro, investigators have begun to study the decidualization process in great detail, utilizing samples from human subjects. Clearly, however, study of a single cell type in the artificial in vitro environment cannot begin to explain the complexities of interactions between different cell types, compartments of the endometrium, or the endometrium and embryo. Here, too, additional animal model systems

have been used to understand this complex biology. For example, the cross-talk between epithelial and stromal compartments of the endometrium that has been shown to be critical to downregulation of estrogenic effects in the epithelium, as we saw in this chapter, is also important for promoting decidualization in the stroma. The conditional knockout mouse has enabled a better understanding of the mediators linking progesterone to the changes in the uterine stroma required to support a pregnancy.

Expression of key regulators of decidualization increases robustly from basal levels observed at the time of implantation (Figure 24.6: Decidua). COX2 (transcribed from the gene *Ptgs2*), a key regulator of prostaglandin synthesis, is one of the first markers observed. *Ptgs2*-null mice display multiple fertility defects, including impaired ovulation and fertilization as well as defective implantation and decidualization.[133] As COX2 is an early, rate-limiting step in the production of prostaglandins, it was not clear which prostaglandins were relevant to implantation and decidualization. Further work on COX2 revealed that COX2-derived prostacyclin (PGI_2) may be the primary prostaglandin involved in implantation and decidualization, and that the effects of PGI_2 are likely mediated through the nuclear receptor, peroxisome proliferator-activated receptor delta.[134]

Recently, regulation of COX2 has been shown to originate from blastocyst attachment to the endometrial epithelium. The process of blastocyst attachment has long been known to involve the movement of fluid out of the endometrial cavity, leading to a collapsed endometrium around the blastocyst.[135,136] Study of such fluid and electrolyte transport led to work on the voltage-gated sodium channel (the epithelial sodium channel, or ENaC), which now has been shown to be critical in transmitting a signal from the trophoblast to the endometrial stroma. Trypsin, a serine protease released from the trophoblast, leads to epithelial cell depolarization and the activation of L-type sodium channels. The resulting influx of calcium ions leads to the release of prostaglandin E2 (PGE_2) via COX2 upregulation that may occur via the cAMP-responsive transcription factor, cAMP response element-binding protein (CREB). This epithelial-derived PGE_2, triggered by the blastocyst, then functions in the stroma to regulate decidualization.[137] Further work is needed to better understand how interactions between the attaching blastocyst and endometrial epithelium affect the decidualization process and the requisite preparation of the stroma for embryo invasion.

The homeobox transcription factor, HOXA10, is also critical for decidualization of the endometrial stroma. *Hoxa* genes are essential in the mouse for endometrial development and implantation. *Hoxa10*-null mice display severely compromised decidualization,[138] and part of the effect of ablating *Hoxa10* involves altered function of cyclin D3 (CCND3), as replacement of cyclin D3 rescues much of decidualization and placentation, though the mice remain infertile.[139] In women with infertility, altered HOXA10 expression levels have been found in the endometrial stroma during the window of receptivity.[140]

Members of the bone morphogenetic protein (BMP) and wingless-related MMTV integration site (WNT) families are critical to these changes in early pregnancy.[82] BMP2, for example, is highly expressed in the stroma in the peri-implantation period and is thought to be involved with promoting decidualization after embryo implantation, a hypothesis supported by rescue of impaired decidualization in conditionally ablated *Bmp2* mice through the administration of recombinant BMP2.[141] WNT4 has been suggested to lie downstream of BMP2,[142] with this signaling axis originating via epithelial IHH and stromal NR2F2 (Figure 24.6: Decidua). Of note, BMP2 is also expressed at high levels in human endometrial stroma, suggesting a similar role in decidualization and conservation among species.[142]

Clearly, a better understanding of the complex biology of decidualization will come with continued investigation into multiple model systems, including cultures of human cells, transgenic mice, and combinations of the two.

Remodeling the Vasculature, Extracellular Matrix, and Immune System

Vascular remodeling is critical in a system such as the endometrium in which rapid, cyclic growth and regression occur, and the endometrium is a rich source of angiogenic factors in both pregnant and nonpregnant states. VEGFs, specific for endothelial growth, are expressed in the endometrium, with peak expression correlating to maximum angiogenesis during the secretory phase.[132] Certain members of the VEGF family are stimulated by estrogen in the proliferative phase and are sensitive—and upregulated—in the hypoxia that occurs with myometrial contraction and vascular interruption at the end of a menstrual cycle. Prompted by hypoxia and rising estradiol production from the new developing follicles, VEGFs once again promote the vascularization required to re-epithelialize and develop the endometrium. Angiopoietins and members of the fibroblast growth factor family are also involved in the growth of endothelial and endometrial stromal cells and act to maintain the endometrium by inhibiting apoptosis and stabilizing blood vessels. Further balance exists between potent vasoconstrictors, such as endothelin 1, and vasodilators such as nitric oxide and prostacyclins, though the exact control over these relationships remains poorly understood.

In the setting of an invading trophoblast, other local factors are involved in moderating the vasculature. Once the trophoblast has begun to invade the endometrium, spiral arterioles are plugged into the region surrounding

the embryo, creating a region with low oxygen tension that has been interpreted to be necessary for the continued growth and invasion of the pregnancy. This hypoxia comes at an expense, however, as the potential for damage from reactive oxygen species increases. Part of the decidualization process is an induction of both enzymes that neutralize reactive oxygen species (glutathione, monoamine oxidases, peroxidases, S-transferases, superoxide dismutases, and thioredoxin) and processes to repair the damage they cause (GADD45α).[123,143,144]

An additional requirement for trophoblast invasion is remodeling of the endometrial extracellular matrix. Extracellular matrix proteins are broken down by MMPs, a large family of proteolytic enzymes, several of which are expressed by the invading trophoblast. The action of MMPs is opposed by inhibitors of metalloproteinases (TIMPs), factors that are expressed by both the stromal cells and the trophoblast itself. Expression of TIMP3, for example, can be induced in stromal cells by embryo coculture.[145] The TGFβ family is also important in the regulation of MMPs. Multiple members of the TGFβ family are differentially regulated (most are downregulated) with decidualization and may be directly regulated by PGR. Interestingly, with the fall in progesterone at the end of the menstrual cycle, many of these factors have been shown to be upregulated and involved in the breakdown of the endometrium preceding menses. Thus, progesterone may play a direct role in the modulation of the TGFβ pathways, while additional fine-tuning occurs in the presence of the invading trophoblast. Similarly, plasmin and its various modulators play important roles in the structural reorganization of the stroma.

Immune factors also play an important role in the establishment and maintenance of pregnancy. Indeed, the invading trophoblast and embryo must be both tolerated by the maternal immune system and protected from pathogens. The specialized uterine natural killer (uNK) cells are recruited to the decidua by locally produced cytokines (CCL4 and CXCL9/10).[123] uNK cells express certain immunomodulatory molecules (galectin 1 and glycoledin A), which have been shown to inhibit T-cell activation, proliferation, and survival and attenuate the production of proinflammatory cytokines.[146] Other factors from the decidua have also been shown to modulate T-cell-mediated inflammation and are known to be involved in the control of apoptosis.

CONCLUSION

Originating from studies on the nonhuman primate and currently utilizing state-of-the-science techniques of genome-wide interrogation and transgenic animals, investigators have sought to understand the remarkable changes to the fallopian tube and endometrium over the menstrual cycle. By nature, in the case of the endometrium, rapid cycling of proliferation, differentiation, and regression requires a coordination of diverse cellular processes including cytoskeletal organization, cell cycle progression, cellular secretion, angiogenesis, and apoptosis. As the endometrium undergoes decidualization regardless of pregnancy in humans, the ultimate control of this process originates with the ovarian steroid hormones, estradiol and progesterone. Acting both directly and via multiple downstream signaling pathways, estradiol and progesterone coordinate the patterns of growth and differentiation that can be described histologically, genetically, and biochemically. When the regular, cyclic production of progesterone is lost—for example, with anovulatory conditions like PCOS—so too are lost the various stages of differentiation. Proliferation continues unchecked, and the risk of hyperplasia and malignancy increases. Endometriosis, a disease where endometrium exists outside the uterus, has been explained to a degree by progesterone resistance. The ectopic foci of endometrium contain glands and stroma, as with endometrium in its native state, but display altered patterns of gene expression and a defective decidual response to progesterone.[147] Critical to developing endometriosis therapies, however, is an understanding of the pathogenesis of the disease, knowledge that has remained largely elusive since Sampson described his hypothesis in the 1920s,[148] and whether differences in fundamental steroid hormone signaling underlie the ability for the endometrial tissue to persist and grow *ex utero*.

While evidence suggests that the endometrium is altered in hyperstimulated ART cycles, very limited data exist describing consistent—and thus fixable—defects in the receptive endometrium. While some advocate freezing all embryos of an in vitro fertilization cycle, with subsequent transfer of frozen-thawed embryos in subsequent hormonally prepared cycles meant to better recapitulate the endometrium of a natural cycle, the hyperstimulated endometrium remains receptive (even if altered), and most pregnancies still result from transfer of fresh embryos in the hyperstimulated cycle. Perhaps even more interesting, much attention is now focused on the earliest communication between the embryo and endometrium in the establishment of proper placentation and whether an abnormal endometrium might be responsible for outcomes such as growth restriction, preterm birth, and preeclampsia, which are associated with the patient's underlying infertility, the ART process itself, or both.

In the case of the fallopian tube, what was once considered a passive conduit for sperm and embryo transport is now better understood as a complex and dynamic system that actively participates in the movement of the gametes and contributes to the earliest steps of embryonic development. Dysfunction of the tube can prohibit

pregnancy altogether or result in life-threatening ectopic pregnancy. Recent work suggests that certain categories of malignancy whose origin has been thought to be the ovary may well originate from the fallopian tube.

The current application of genome-wide strategies to query the transcriptome and binding sites of the steroid hormone receptors across the human menstrual cycle and under specific treatment or disease states will, no doubt, provide new insights into the biology of the endometrium and fallopian tube. Powerful transgenic animal systems can be used to ablate or overexpress candidate genes in specific tissues or under certain conditions, thus fine-tuning our understanding of how these molecules regulate oviductal and endometrial function. Certainly, the development of new diagnostics and therapeutics for a host of endometrial and fallopian tube disorders will take advantage of our ever-sharper understanding of estrogen, progesterone, and the downstream molecular signaling underlying the cyclic physiology of these remarkable organs.

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CHAPTER

25

Steroid Receptors in the Uterus and Ovary

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INTRODUCTION

The ovarian-derived sex steroid hormones dictate the uterine estrous or menstrual cycle in mammals and are therefore essential to the establishment and maintenance of pregnancy. The uterine response to the preovulatory rise in circulating estradiol (E2) is required to prepare the tissue for the forthcoming rise in progesterone (P) that accompanies ovulation and is critical to embryo implantation. This physiological coordination between the ovary and uterus is common to the large majority of mammals studied and the spectrum of actions and effects of the sex steroids in uterine tissue are mediated by their cognate NRs. Estrogen was first described almost 100 years ago as a substance that induced estrus. We now know from many studies over the past 50 plus years that its physiological activity is broader than simply inducing a secretory response in the reproductive tract—estrogen also affects multiple organs that previously were not considered estrogen responsive. The mechanistic theory for explaining the biological actions of steroid hormones came in 1960 when Elwood Jensen and colleagues first described the uptake and retention of labeled E2 in certain tissues that, because of this retention, became referred to as estrogen target tissues. The ovary and uterus were amongst the tissues showing this property. The hormone tissue retention concept was then shown to be reflective of other steroid hormones, exemplified by progesterone, glucocorticoid, and thyroid hormones. The Jensen and Gorski laboratories were then the first to show that the tissue retention of estrogen was due to the presence of specific proteins contained within those tissues, which became referred to as estrogen receptors (ER). Numerous research groups followed with studies showing that the receptor proteins were hormone specific and their presence was linked to some biological activity in certain tissues. Subsequent development some 20 years later of the first antibodies to the ER allowed for more direct detection and qualification of the protein levels in tissues under different physiological states or disease conditions. Although initially the dramatic physiological effects of estrogens on the mammalian uterus were appreciated and well described, later investigations began to elucidate the precise mechanisms and signaling pathways involved. Our understanding of steroid hormone action in the uterus has been greatly advanced by utilization of new technologies with the generation of knockout and knock-in models, including receptor or coactivator null, tissue and cell selective null, and mutant knock-in models in mice. These models are also used in combination with microarray, RNA-seq, and ChIP-seq methods that allow for comprehensive mapping of interaction of nuclear receptors (NRs) with chromatin and modulation of genomic response to steroids in uterine

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tissues. Together, these models and techniques have led to better understanding of the molecular details of steroid receptor roles in biological processes.

Receptor-mediated actions of progestins, androgens, and estrogens are central to reproduction. Perhaps unique among the various categories of signaling pathways, the sex steroid hormones and their cognate receptors are surely mentioned, if not discussed in detail, in every chapter of this volume. The sex steroid hormones are integrated into every aspect of mammalian reproductive physiology in both sexes, including sexual maturation and development, gametogenesis, hypothalamic–pituitary control of gonadal function, sexual and maternal behavior, pregnancy, and lactation. Disruption of the signaling pathways for any one of the three gonadal steroids leads to reduced fecundity, if not infertility, due to aberrations in multiple organ systems. Therefore, a thorough understanding of the sex steroid receptors in terms of their expression in reproductive and endocrine tissues, their mechanism of action, their role in reproductive processes, and their interaction with nonsteroidal signaling pathways is instrumental to our ability to manage infertility and reproductive disease.

This chapter principally covers the signaling pathways for progestins, androgens, glucocorticoids, and estrogens, and the role of each in ovarian and uterine function in mammals. Because of the importance of the sex steroids and the likelihood that each is discussed in several other chapters of this volume, it is appropriate to first discuss the structure, mechanism of action, and signaling pathways for each of the sex steroid receptors. Sections then follow on the role of sex steroids in ovarian and uterine function. We chose to divide each of these sections according to the sex steroid receptors: the estrogen receptors (ERs), progesterone receptors (PRs), androgen receptors (ARs), and glucocorticoid receptors (GRs). The section Sex Steroid Receptors and Ovarian Function discusses what is currently known concerning each particular sex steroid signaling pathway in terms of its expression pattern and distribution within the ovary, and its intraovarian role in granulosa cell proliferation and differentiation, thecal cell function, and ovulation. Because a separate chapter (Chapter 23) is dedicated to the corpus luteum, the role of the sex steroid receptors in luteal function is not covered in detail. The section Sex Steroid Receptors in Uterine Function discusses what is currently known concerning each sex steroid signaling pathway in terms of its expression pattern and distribution within the uterus and its role in uterine cell proliferation and differentiation. New since the previous editions of this volume is the development of tissue selective null or "knock-in" mouse models for each of the sex steroids. The study of each of these models has already and will continue to make enormous contributions to our understanding of the reproductive role of each receptor signaling pathway in toto. Therefore, we include a detailed description of the ovarian and uterine phenotypes for each of these models in their respective sections.

THE STEROID RECEPTORS

The steroid receptors are part of the family of nuclear receptors (NRs) that is ubiquitous throughout the animal kingdom. The members of the NR family fulfill a plethora of functions and are integral to the development and maintenance of multiple physiological systems. Continued discovery of new NRs has led to a considerable expansion of the NR family and demanded a system of categorization. NRs have been assigned to three classes.[1–3] Class I includes the steroid receptors (ER, PR, AR, GR), which interact with high specificity to their steroid ligand as well as to DNA motifs comprised of inverted-repeat hexanucleotides separated by a 3-base pair (bp) spacer. The Nuclear Receptors Nomenclature Committee has developed an NR nomenclature scheme that is based largely on sequence homology and evolutionary comparisons and divides the over 60 NRs into seven subfamilies and a varied number of groups within each subfamily.[4] The ERs (ERα and ERβ) are the sole members of the NR3A subgroup, ERα and ERβ being NR3A1 and NR3A2, respectively.[4] The PR and AR are the third and fourth members of the NR3C subgroup that also includes the GR and the mineralocorticoid receptor.[4] The official gene names for these steroid receptors are *ESR1* and *ESR2* for ERα and ERβ, respectively, *PGR* for progesterone receptor, *AR* for androgen receptor, and *GCR* for glucocorticoid receptor. The general properties of the sex steroid receptor family and those mechanisms of receptor action that are most relevant to ovarian and reproductive tract function are outlined following.

Genes, mRNA, and Regulation

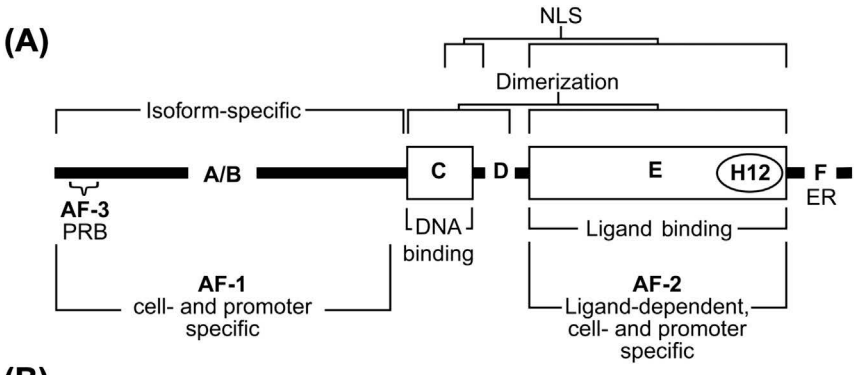
The genes encoding the sex steroid receptors exhibit a highly conserved structural organization. The human PR (*PGR* or *NR3C3*), AR (*AR* or *NR3C4*), ERα (*ESR1* or *NR3A1*), ERβ (*ESR2* or *NR3A2*), and GR (GCR or NR3C1) genes are each composed of eight coding exons, and vary in length from 40 kb (ERβ) to >140 kb (ERα).[5–8] In each case, the N-terminal domain (NTD) of the receptor is usually encoded by a single exon, the two zinc fingers of the DNA binding domain, each encoded by separate exons (2 and 3), and the ligand binding domain (LBD), encoded by exons 4–8 (Figure 25.1).

Estrogen Receptor

The human *ESR1* (ERα) cDNA was first cloned in 1985[9–11] and has since been isolated from numerous additional species.[12,13] A second ER gene, termed *ESR2* (ERβ), was discovered in 1996 in rat[14] and human[15] tissues and has since been cloned from many species.[13] Unlike the A and B forms of PR and AR, ERα and ERβ are not isoforms but rather distinct receptors encoded by two genes on different chromosomes. The ERα proteins are 595 and 599 amino acids in length in human and mice, respectively, with an approximate molecular weight of 66 kDa (Figure 25.1).[8,16–18] Multiple promoter and regulatory regions in the 5′-untranslated sequences of the human and rat *ESR1* gene have been described, yet all possess the same single open reading frame.[8] Numerous naturally occurring variants of the *ESR1* mRNA in normal and neoplastic tissues of several species have been described, but the existence of corresponding proteins remains controversial.[18]

The promoter region of the *ESR1* gene has been relatively well characterized and indicates a complex regulation of expression.[8,19–21] Cicatiello et al.[22] demonstrated that the proximal 0.4 kb of the mouse *Esr1* promoter is sufficient to provide for widespread but specific expression of a reporter construct in vivo. Regulatory elements that may provide for AP-1, Sp1, and ER autoregulation of the human *ESR1* promoter have been described.[23,24] Decreased *ESR1* expression is linked to receptor-mediated actions of vitamin D[25] and increased intracellular cAMP or mitogen-activated protein kinase activity.[23] Increased methylation of the *ESR1* promoter is implicated in reducing ER levels, especially in tumorigenic tissues.[23]

The *ESR2* genes of multiple species yield numerous transcripts that range from 1 to >9 kb in length, in contrast to the single predominant transcript of ~7 kb transcribed from the *ESR1* gene.[18] Initial descriptions of human and rodent ERβ projected a protein of 485 amino acids. However, it is now apparent that translation of the *ESR2* mRNA initiates upstream of these original open reading frames and yields a receptor of 549 amino acids in rodents and 530 amino acids in humans, each with an approximate molecular weight of 60–63 kDa (Figure 25.1).[8] Therefore, ERβ is slightly smaller than ERα, and most of this difference lies within the N-terminus. A number of



(B)

Comparison of human steroid receptor domain structures

NR	Gene symbol	Systemic name	A/B; N-terminal domain (NTD)	C; DNA binding domain (DBD)	D; hinge domain	E; DNA binding domain (LBD)	F domain	Endogenous ligand	Synthetic or selective ligand
ERα	Esr1	Nr3a1	1–184	185–250	251–310	311–551	552–595	Estradiol	PPT
ERβ	Esr2	Nr3a2	1–148	149–214	215–303	304–499	500–530	Estradiol	DPN
AR	AR	Nr3c4	1–558	559–631	632–689	690–919		Testosterone	DHT, R1881
PRA	Pgr	Nr3c3	165–551	551–632	632–687	687–933		Progesterone	R5020
PRB	Pgr	Nr3c3	1–551	551–632	632–687	687–933		Progesterone	R5020
GR	Gcr	Nr3c1	1–420	421–486	487–527	528–777		Hydrocortisone	Dexamethasone

FIGURE 25.1 The steroid family of receptors and endogenous ligands. (A) Schematic illustration of the structural organization of the sex steroid nuclear receptors. The more highly conserved C and E domains are depicted as *open boxes* and the less well-conserved A/B, D, and F domains as *filled bars*. The F domain is unique to the estrogen receptors (ER). The functions of the modular domains are indicated as are the two known transcriptional activation function domains, AF-1 and AF-2 (as well as AF-3 found only in PRB). The AF-1 domain is harbored in the A/B region and exhibits constitutive activity in vitro, whereas the AF-2 domain lies with the ligand binding (E) domain and is critical to ligand-induced receptor activation. Both domains synergize during ligand-activated receptor actions. NLS, nuclear localization signal. (B) Comparison of the human steroid receptors, the androgen receptor (AR), estrogen receptors α (ERα) and β (ERβ), progesterone receptor B (PRB) and A (PRA) and the glucocorticoid receptor (GR). The amino acids that compose each domain of the human forms are indicated. For PRA, the numbers refer to the amino acid residues in reference to PRB.

variant transcripts of the *ESR2* gene have been described; however, unlike ERα, there is growing evidence that some of these variants may coexist with the wild-type (WT) receptor form in certain tissues (Figure 25.2). Portions of the mouse *Esr2* gene have been characterized and possess potential binding sites for numerous transcription factors[8,26] and the initiation of transcription from at least two distinct untranslated exons.[27]

Progesterone Receptor

Two distinct promoters in the *PGR* gene provide for the generation of two major PR isoforms, PRB (114 kDa) and PRA (94 kDa), in most species except rabbit, which possess PRB only. The two PR forms are identical in all regions except the NTD, which is truncated by 128–164 amino acids in PRA, depending on the species (Figure 25.1). Both PR isoforms are expressed at relatively equal levels in tissues, although differences in the ratio have been noted in certain tissues. A third isoform, termed PR-C, an N-terminally truncated form that lacks the full A/B domain and first zinc finger of the C domain, has been described, but its expression as a protein is controversial.[29,30] The level of PR expression is modulated by ER-mediated effects of E2 in certain but not all tissues, and this is especially true in the female reproductive tract.[31–34] Several naturally existing *PGR* variant transcripts harboring exon deletions or distinct 5′-untranslated sequences have been described but are not well characterized in terms of protein expression or functionality.[27,30,35]

Androgen Receptor

The *AR* gene is located on the X chromosome, and therefore genetic males possess only a single copy.[7,36,37] Two distinct start sites in the *AR* gene are used to produce two isoforms, AR-B (110 kDa) and AR-A (87 kDa), which differ only in the N-terminus (Figure 25.1).[12,38] However, in contrast to the PR, the two known AR isoforms exhibit only subtle functional differences. A unique feature of the *AR* gene relative to

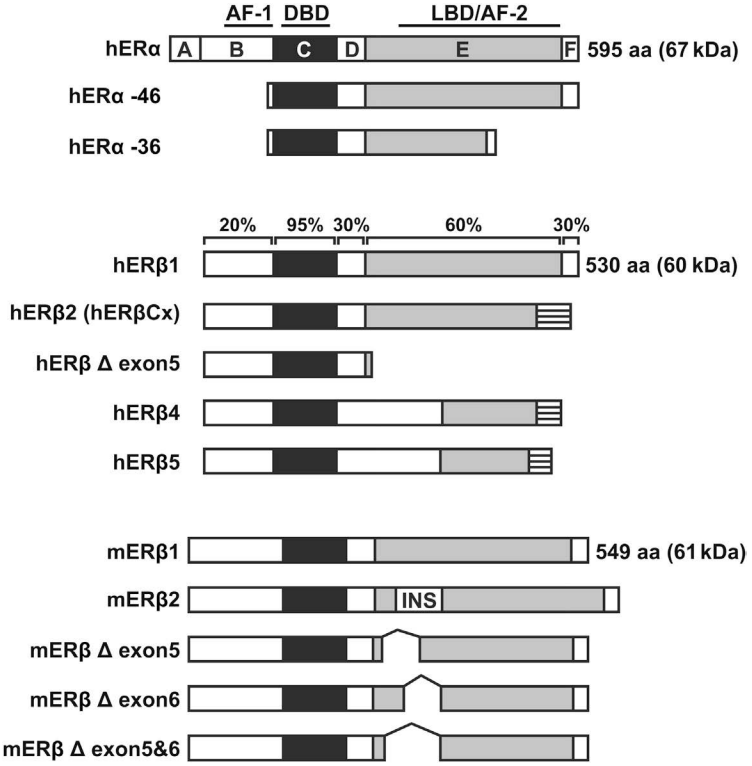


FIGURE 25.2 Human (h) and mouse (m) estrogen receptor variant transcripts. ERs are shown, with the general modular structure of steroid hormone receptors illustrating the N-terminal (A/B) domain, DNA binding (C) domain, hinge (D) region, ligand binding (E) domain, and C-terminal (F) domain (see Figure 25.1 for details). *Top*: hERα full-length and truncated variants (hERα-46 and hERα-36), lacking N-terminal AF-1. *Bottom*: Full-length hERβ (or ERβ1) with % sequences similarity in comparison to ERα indicated above it. Reported hERβ isoforms are shown beneath. hERβ2 is also called ERβCx, and encodes an alternate C-terminal region, depicted by the striped fill, also seen in hERβ4 and 5. hERβcx is unable to bind E2 but acts as a dominant negative receptor when heterodimerized with ERα in vitro.[28] hERβΔexon5 is produced by deletion of exon 5, and produced a truncated protein due to a frame shift leading to translational termination, and has been found in other mammals.[18,28] mERβ2 contains a 54-bp insert (INS) that codes for an additional 18 amino acids in the ligand binding (E) domain and has only been found in rodents. mERβ2 exhibits an approximate 35-fold decreased affinity for E2 relative to WT mERβ and a significantly reduced transactivational activity in vitro.[18] mERβΔexon5, mERβΔexon6, and mERβΔexon5&6 lack parts of the LBD. *Source: Redrawn with permission based on a figure from Ref. 18.*

its sex steroid receptor counterparts is the presence of polymorphic repeats of glutamine and glycine in the NTD, the former of which has been linked to certain chronic diseases and cancer in humans.[39] There are binding sites for a wide array of transcription factors in the *AR* promoter, including Sp1, cAMP-response element binding (CREB) protein, and c-*myc*, suggesting complex tissue-specific regulation. Direct androgen-mediated autoregulation of *AR* expression has also been shown.[39]

Glucocorticoid Receptor

Although not historically recognized for its roles in uterus and ovary, GR is ubiquitously expressed, and more recent studies have demonstrated important interactions between GR and sex steroid receptor mediated signaling.[40,41] GR isoforms, are produced by alternate splicing leading to different c-terminal amino acids. GRα is the primary isoform, whereas GRβ is unable to bind GR ligands and can behave as a dominant negative inhibitor of GRα activity.[42]

Receptor Structure

Common to all members of the NR family is a modular structure of domains, each of which harbors an autonomous function that is critical to total receptor action.[1,2,12,43–47] The sex steroid receptors are composed of five functional modules, an N-terminal domain (NTD) or A/B domain, the DNA binding (C) domain, a hinge (D) region, and an LBD (E) (Figure 25.1). The ERs also possess a unique C-terminal F domain of unknown function. Our understanding of the NR functional domains and their importance to overall receptor activity is largely derived from the in vitro study of artificially generated mutant receptors and more recently from X-ray crystallography studies.

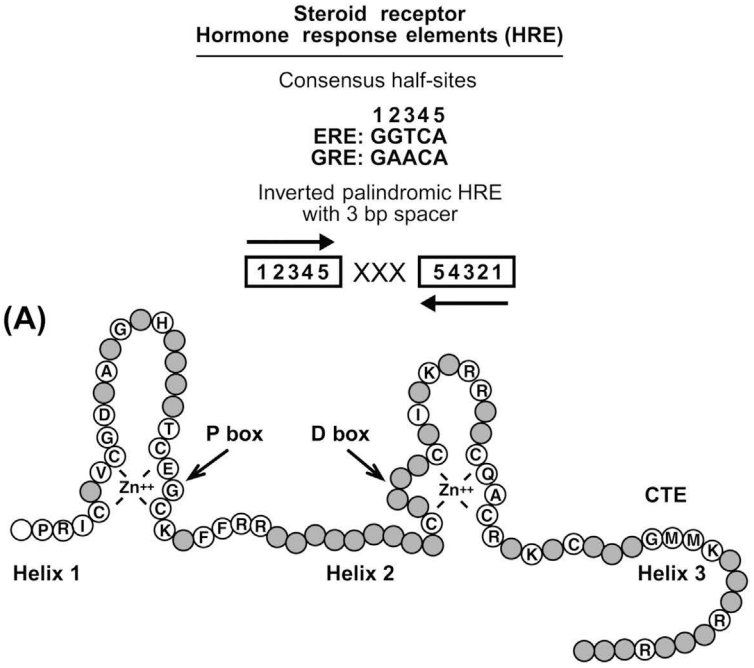
NTD or A/B Domain

The NTD or A/B domains of the NR family members greatly vary in length and share little homology among the steroid receptors, although some structural features are conserved (Figure 25.1). Crystallography studies of the steroid receptor NTD have been largely unsuccessful because this portion of the receptor fluctuates in aqueous solutions. However, evidence suggests that intramolecular interactions between the A/B and other receptor domains are likely to induce a more structured NTD.[1,37,45] Current models of NR signaling incorporate the flexibility of intrinsically disordered (ID) regions of the receptor, including the NTD, into a mechanism of allosteric interaction and coordination of ligands, DNA motifs, and NR domain functions.[1,45,48] The NTD of each of the sex steroid receptors harbors the transcriptional activation function-1 (AF-1) domain and provides for cell and promoter-specific activity of the receptor as well as a site for interaction with co-receptor proteins (Table 25.1). The AF-1 domain alone can confer constitutive transcriptional (i.e., ligand-independent) activity when linked to a heterologous NR DNA binding domain in vitro, but this function is largely overcome in the context of the whole receptor.[12] Posttranslational modifications to the A/B domain can dramatically affect the overall behavior of the receptor and are thought to be an important mechanism for the modulation of AF-1 functions.[17] Phosphorylation of the A/B domain is the most well-characterized posttranslational modification and occurs in all three sex steroid receptors via the actions of multiple intracellular signaling pathways, including mitogen-activated protein kinase pathways,[49] the cAMP/protein kinase A (PKA) pathway, and cyclin-dependent kinases.[12,37,39,43,50]

The extended N-terminal sequences that are unique to PRB (Figure 25.1) provide a third transcriptional AF-3 domain in this isoform that is lacking in PRA. This may allow for PRB-specific expression of certain P regulated

TABLE 25.1 Steroid Receptor Co-Regulator Complexes

Complex	Functions	Comments	References
SRC1, SRC2, SRC3	Interact with Helix12 of agonist-bound NR, interact with SWI/SNF, histone modifiers		51,52
Mediator	"Bridges" NR and transcriptional "machinery" (RNA Pol II) to control transcription	Made up of >20 subunits, MED 1-31, arranged in 3 modules (head, middle, tail)	53,54
SWI/SNF	Regulate access to enhancer sequences via chromatin remodeling, ATPase activity	Made up of 9+ subunits, examples include BRG1, BRM, BAF subunits	55
Histone Modifiers	Modify histones to increase or decrease transcription	Acetyltransferase (HAT; e.g., p300/CBP), deacetylase (HDAC; e.g., NCoR), Methyl transferase (e.g., PMRT/CARM), de-methylase	56,57
26S Proteasome	"Clears" transcriptional modulatory proteins to facilitate subsequent transcription, transcriptional termination	Structure made up of 20S catalytic core particles (CP), 19S regulatory particles (RP)	58,59



(B) Comparison of amino acids in P box region:

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ERα,ERβ:    CEGCKAFFKRSIQ
NERKI ERα:  CAACKAFFKRSIQ
EAAE ERα:   CEGCAAFFAESIQ
GR:         CGSCKVFFKRAVE
AR:         CGSCKVFFKRAAE
PR:         CGSCKVFFKRAME
```

Comparison of amino acids in CTE:

```
ERα: GMMKGGIRKDRRGGRMLKH
ERβ: GMVKCGSRRERCGYRLVRR
GR:  GMNLEARKTKK--KIKGIQ
AR:  GMTLGARKLKKLGNLKLQE
PR:  GMVLGGRKFKKFNKVRVVR
```

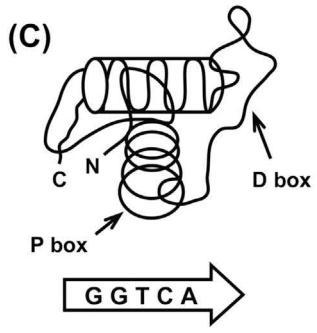


FIGURE 25.3 The nuclear receptor response elements and primary structure of the DNA binding domain. *Top*: Shown is the canonical core hormone response element (HRE) for the estrogen receptors (ERE) and glucocorticoid receptors (GRE). These sequences are located in the regulatory regions of sex steroid target genes and provide a site for receptor binding and transactivational activity. A full HRE consists of two core palindromic sequences arranged as an inverted repeat, always separated by three bp (denoted by XXX). Note that a change from GT to AA at positions two and three of the core sequence modifies the ERE to a GRE. *Bottom* (A) The DNA binding domain (C) of the sex steroid receptors is highly conserved and composed of two zinc fingers and a C-terminal extension (CTE). The ERα DBD is shown. Each zinc finger is composed of four conserved cysteine residues that coordinate to chelate a single zinc ion. Helix 1 of the first zinc finger contains the P-box residues involved in the discrimination of the HRE. Residues in the second zinc finger (helix 2) form the D box that provides a dimerization interface. The CTE is critical

genes.[29,50,60] In general, PRB is a stronger activator of transcription versus PRA when acting on a hormone response element (HRE)–driven gene construct in vitro.[60] Furthermore, antagonist-bound PRB acts as a strong transcriptional activator in certain cell and promoter contexts, whereas antagonist-bound PRA is inactive.[29]

The NTD of the AR is especially autologous in vitro and is likely more important to overall AR transactivation than the C-terminal AF-2 domain (described later).[1,37–39] Furthermore, the first 30 residues of the AR NTD are highly conserved and important for interactions with the LBD that provide for agonist-induced stabilization of the receptor.[37] Unique to the AR among the sex steroid receptors are a series of amino acid repeat sequences in the NTD, most notably the polymorphic tracts of glutamine and glycine, of which certain lengths are thought to be linked to various human diseases or cancer.[37,38]

The greatest structural disparities between ERα and ERβ lie within the A/B domain, which is approximately 30 amino acids shorter in ERβ and exhibits only >20% homology.[8,61,62] This divergence likely accounts for the many functional differences that have been revealed from comparative studies of the two ER forms.[8,17,18,62] In general, ERβ tends to be a less effective transcriptional activator compared with ERα when acting in a classic estrogen response element (ERE)–driven mechanism in vitro.[18] An ERα/ERβ chimera in which the A/B domain of ERβ has been replaced with that of ERα is able to activate transcription much better than the native ERβ.[61] Furthermore, certain antagonists (e.g., tamoxifen) that exhibit some agonist-like properties through AF-1 when bound to ERα exhibit no such activity with ERβ.[61,63]

DNA Binding or C Domain

The C domain of the NR family members is that portion of the receptor that specifically functions to recognize and bind to the *cis*-acting enhancer sequences, or HREs, that are located within the regulatory regions of target genes (Figure 25.3). It is the most highly conserved (55–80%) region among the NR family members.[2,46,64] The C domains of ERα and ERβ are practically identical (>95% homology) in most species and are therefore expected to exhibit a similar affinity for the same HREs.[62] The functionality of the C domain is provided by a motif of two zinc fingers, each composed of four cysteine residues that chelate a single Zn^2 ion, and are always encoded by separate exons within the gene (Figure 25.3). Crystallography studies indicate a highly conserved structure consisting of dual α-helices positioned perpendicular to each other[1,45,46] (Figure 25.3). Amino acids in the C-terminal "knuckle" of the first zinc finger form the "P box" (proximal box) of the DNA binding domain and confer sequence specificity to the receptor; hence, the proximal zinc finger is often referred as forming the "recognition helix" (Figure 25.3).[64] Amino acids at the N-terminal "knuckle" of the second zinc finger form the "D-box" (distal box) and are more specifically involved in differentiating the "spacer" sequence within the HRE as well as providing an interface for receptor dimerization (Figure 25.3).

The consensus HREs that sex steroid receptors bind are composed of two 6-bp palindromic sequences arranged as an inverted repeat and always separated by a 3-bp spacer (Figure 25.3). Because the P-box residues are identical among the AR, PR, and GR, these receptors bind a common consensus HRE consisting of two 6-bp palindromic half-sites (5′-PuG[G/A]ACA) arranged as an inverted repeat and always separated by a 3-bp spacer (Figure 25.3). The consensus ERE bears the same arrangement but is composed of a unique palindromic half-site (5′-PuGGTCA) (Figure 25.3). The inverted-repeat arrangement of the palindromic sequences is unique to sex steroid receptor HREs and dictates that the receptors homodimerize in a "head-to-head" position when bound to DNA. Evidence indicates that the AR can uniquely dimerize in a "head-to-tail" fashion on an HRE of the monomer 5′-GGTTCT. More recent structural analysis has revealed the importance of the 10-30 amino acid carboxy terminal extension (CTE) of the DBD in DNA interaction.[1,45,46,48] Although this region is variable between steroid receptors, it is crucial for DNA binding, particularly for sequence selectivity of DNA binding, by extending the interaction surfaces between the receptor and the DNA.

Hinge Region or D Domain

Historically, the D domain was thought to primarily serve to connect the more highly conserved C and E domains of the receptor.[12] The previously described CTE extends into the hinge region, which also harbors a nuclear localization signal, and influences cellular compartmentalization of receptors, as well as sites of posttranslation modifications.[48] Current mechanisms suggest this nonconserved and intrinsically disordered (ID) domain is important for intramolecular allosteric

for monomeric DNA binding. Diagram includes amino acids found in ERα. (B) Amino acid sequences in the vicinity of the P box and CTE regions of the steroid receptors are compared. The underlined residues in the P-box sequences are critical for HRE selectivity. Amino acid substitutions made within the P box to create the NERKI and the EAAE DNA binding–deficient ERα mouse lines are also shown. The EAAE mutations also includes an amino acid change in the "tip" of the first finger that isn't in the P box. (C) Shown is a ribbon diagram of the DNA binding (C) domain illustrating the putative arrangement of helix 1 and helix 2 crossing at right angles to form the core of the DNA binding domain that recognizes a hemi-site of the response element. *Source: (A) reproduced with permission from Ref. 43 and (C) reproduced with permission from Ref. 65.*

interactions involving the N-terminal and ligand binding domain (LBD)[48] (described earlier in the section NTD or A/B Domain). This type of flexible structural interaction works to allow rapid response to diverse modulators governing changes in biological environments.[48]

LBD or E Domain

The LBD or E domain of the steroid receptors is a highly structured multifunctional region that primarily serves to specifically bind ligand and provide for hormone-dependent transcriptional activity.[12] An AF-2 domain located in the C-terminus of the E domain mediates this latter function. The AF-2 domain is subject to posttranslational modifications[12] and is an especially strong activator of transcription in the ER and PR but is markedly weaker in the AR, where it is more involved in interactions with residues in the NTD.[37,38] Also harbored within the E domain is a strong receptor dimerization interface, sites for interaction with heat shock proteins, and nuclear localization signals.[12,48] Although there is minimal homology in the primary sequence of the LBD for the sex steroid receptors, comparative studies of the crystal structures of liganded and unliganded LBDs indicate a highly conserved structural arrangement. These structural studies indicate that the LBD is composed of 11 α-helices (H1, and H3–H12) arranged in a three-layer α-helical sandwich to create a hydrophobic ligand binding pocket near the C-terminus of the receptor.[48] The resulting shape and volume of the ligand binding pocket is larger than necessary to accommodate the corresponding ligand, suggesting that key interactions between the ligand and specific receptor residues are more critical to conferring ligand specificity.[61] Receptor binding to an agonist ligand leads to rearrangement of the LBD such that H11 is repositioned and H12 swings back toward the core of domain to form a "lid" over the binding pocket. This agonist-induced repositioning of H12 leads to the formation of a hydrophobic cleft, or "NR box", by helices 3, 4, and 5 on the receptor surface, constituting the AF-2, which serves to recruit co-activators (Table 25.1) to the receptor complex. In contrast, receptor antagonists are unable to induce a similar repositioning of H12, leading to a receptor formation that is incompatible with co-activator recruitment and is therefore less likely to activate transcription. The LBDs of PRB and PRA are identical and provide for high affinity binding to P.[12,50] The LBD of the AR in humans, rats, and mice is identical and provides for high affinity binding of two endogenous androgens, T and 5α-hydroxyT (DHT), the latter of which binds with much greater affinity.[38,66] Numerous high affinity synthetic ligands for PR and AR have been developed, including the agonists R5020 and R1181, respectively (Table 25.2).[12] The LBDs of ERα and ERβ exhibit less than 60% homology but bind the endogenous ligand, E2, with similar affinity (ERα, 0.1 nM; ERβ, 0.4 nM).[8,12,17] Both ER forms also bind the synthetic estrogen diethylstilbestrol (DES) with relatively equal affinity.[67] However, given the divergence in homology, it is not surprising that ERα and ERβ exhibit measurable differences in their affinity for other endogenous steroids and xenoestrogens.[8,17] Natural and synthetic steroidal and nonsteroidal ER agonists and antagonists have been described, some of which show specificity for one or the other ER subtype (Table 25.2), illustrating differences between the LBDs of ERα and ERβ, and provide for powerful pharmacological tools to discern the overall function of each ER (Table 25.2). The most widely used ER subtype selective ligands currently in use are propylpyrazole (PPT), an ERα selective agonist, and diarylpropionitrate (DPN), an agonist showing preference, but not exclusive selectivity, towards ERβ.[8]

F Domain

Among the sex steroid receptors, only ERs possess a well-defined F domain (Figure 25.1). This region is relatively unstructured and harbors little known function, although some data indicate a role in co-activator recruitment, dimerization, and receptor stability.[61,62,68–70]

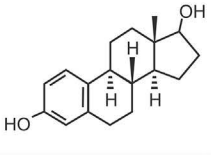
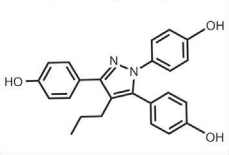
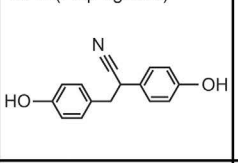
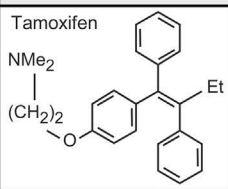
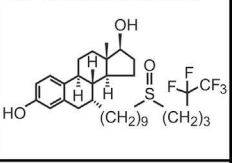
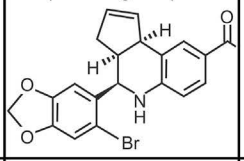
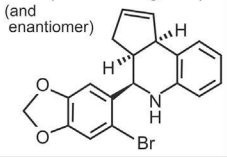
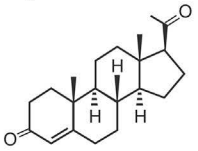
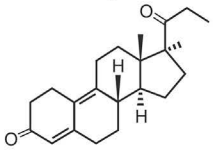
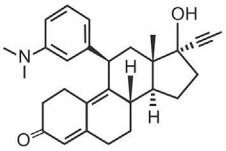
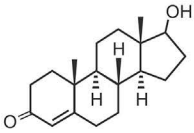
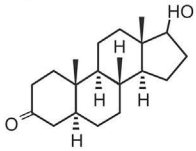
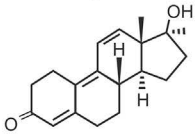
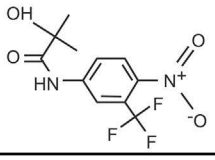
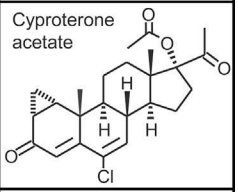
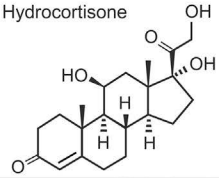
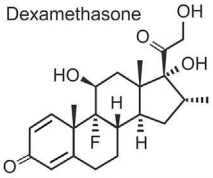
Co-Regulatory Complexes

All steroid receptors interact with co-regulatory molecules, co-activators, and co-repressors.[51,71] The primary co-activator interaction for steroid receptors (SR) is with p160/SRC (steroid receptor co-activator) 1, 2, and 3.[52,72,73] SRC1 (NCOA1), SRC2 (GRIP1, TIF2), and SRC3 (pCIP, RAC3, ACTR, TRAM, A1B1) interact with helix 12 of SRs via "LXXLL" motifs in their nuclear receptor interacting domain, which are leucine rich regions with "X" designating any amino acid.[52] SRCs also contain activation domains that recruit secondary molecules such as p300, and a bHLH-PAS motif within the N-terminal region, which can interact with other transcription factors.[52] The elegant complexity of co-activator composition and function has been increasingly revealed. Steroid receptors and SRCs function as a nexus interacting with massive multimeric complexes, such as the SWI/SNF chromatin remodeler, mediator complex, or proteasomes (Table 25.1).[73] These interactions facilitate coordination of the specific functions necessary to allow appropriate gene and cell selective access to chromatin, via modifications of histones or members of co-regulatory complexes.[74] In this way, co-activators dynamically mediate and coordinate processes necessary to accomplish transcription, including initiation, elongation, termination, and clearing or turnover of the transcriptional modulators.

Receptor Mechanisms of Action

Our understanding of the mechanisms by which steroid hormones and their cognate intracellular receptors influence cell function and behavior has expanded

TABLE 25.2 Structures of Endogenous and Synthetic Agonists and Antagonists of Steroid Receptors

	Agonists			Antagonists	
ER	Estradiol 	PPT (ERα agonist) 	DPN (ERβ agonist) 	Tamoxifen 	ICI 182780 Fulvestrant 
GPER			G1 (GPER agonist) 	G15 (GPER antagonist) (and enantiomer) 	
PR	Progesterone 	R5020 Promgesterone 		RU486 Mifepristone 	
AR	Testosterone 	Dihydrotestosterone 	R1881 Methyltrienolone 	Hydroxyflutamide 	Cyproterone acetate 
GR	Hydrocortisone 	Dexamethasone 			

profoundly since 1988 when Clark and Markaverich reviewed the field for the inaugural edition of this volume. Much of that earlier chapter remains contemporary in reference to general receptor biochemistry and ligand-dependent activation, which is now referred to as the "classical" or ligand-dependent direct-DNA binding model of receptor function (Figure 25.4). In the years since, numerous discoveries have been made that illuminate the complexity of sex steroid receptor signaling in cells and tissues, such as the discovery of additional receptor forms and variants (e.g., ERβ) and high-resolution crystallography of receptor domains. The entrée into the "omics" era has facilitated massive expansion of study of transcriptional regulation and chromatin remodeling. In addition, several alternative receptor signaling mechanisms that diverge from the classic model have become apparent, including "tethering" of the sex steroid receptor to heterologous DNA-bound transcription factors to provide for steroid regulation of genes that lack HRE sequences within their promoter (Figure 25.4); plasma membrane steroid signaling, often referred to as "nongenomic" steroid actions; and ligand-independent "cross talk" with intracellular and second messenger systems that provide for sex steroid receptor activation in the absence of the cognate steroid ligand (Figure 25.4). The existence of multiple receptor-mediated signaling pathways likely accounts for the refined control and plasticity of tissue responses to sex steroids. These modes of sex steroid receptor action as currently understood are discussed following.

Ligand-Dependent Actions: Direct or Classical

The classic model of steroid receptor action states that the receptor resides in the nucleus or cytoplasm but is sequestered in a multiprotein inhibitory complex in the absence of hormone (Figure 25.4). The lipophilic steroid ligands are able to freely diffuse across the plasma and nuclear membranes and bind their cognate receptor. The binding of ligand results in a

conformational change in the receptor, transforming it to an "activated" state that is now available for homodimerization, increased phosphorylation, and binding to an HRE within target gene promoters, and interaction with chromatin and transcriptional mediators. NRs seem to be preferentially recruited to open regions of chromatin.[75] Studies using MCF7 breast cancer cells indicate that FoxA1 acts as a pioneering factor, providing accessible regions in the chromatin that recruit ERα.[76–79] The ligand-HRE-bound receptor complex then engages co-activator molecules as described before,[52] leading to modulation of transcription rates of responding genes. This classic steroid receptor mechanism is dependent on the functions of both AF-1 and AF-2 domains of the receptor, which synergize via the recruitment of co-activator proteins, most notably the p160 family members.[52] Depending on the cell and target gene promoter context, the DNA-bound receptor complex may positively or negatively affect expression of the downstream target gene. Initially, study of NR-mediated gene regulation was carried out on a gene-by-gene basis using a handful of known hormone regulated transcripts, such as the ER target genes PS2, lactotransferrin, and PR or the AR target gene prostate specific antigen. Now, after numerous comprehensive analyses of hormonally regulated transcriptional profiles, using microarray and more recently RNA-seq, thousands of NR targets have been found in various cell lines and tissues.

In general, PRB can better or uniquely activate transcription versus PRA when acting on progesterone response element–regulated genes in various cell types in vitro due to the presence of a third AF-3 in the NTD of PRB.[50,60] Furthermore, PRA can repress the transcriptional activities of PRB in certain cell and promoter contexts,[50] suggesting that PRA may modulate P responsiveness in certain tissues. Interestingly, PRA can also repress the actions of other heterologous NRs, including the AR, ER, and GR.[12,50,60] The variant, PR-C, is primarily cytosolic and lacks the A/B domain and the 5′ portion of the C domain, and has been shown to bind P and enhance the actions of PRB and PRA,[80] but the presence of encoded protein and a potential biological role remains controversial.[30,81] Some evidence for a role in P signal dampening during labor has been described for PR-C.[82] When acting via a classic ERE-driven mechanism in vitro, ERα homodimers and ERα/ERβ heterodimers tend to be stronger activators of transcription compared with ERβ homodimers.[18,61] Corroborating in vivo evidence of differential regulation and heterodimer formation by the ER subtypes has been difficult to generate. However, a microarray study by Lindberg et al.[83] using bone tissue from ovariectomized mice found that ERβ generally inhibits ERα-mediated gene expression, but in the absence of ERα, ERβ can partially provide some E2-stimulated gene expression. No evidence for ERα or ERβ preferential regulation of specific transcripts or promoters has been forthcoming; rather, it seems the

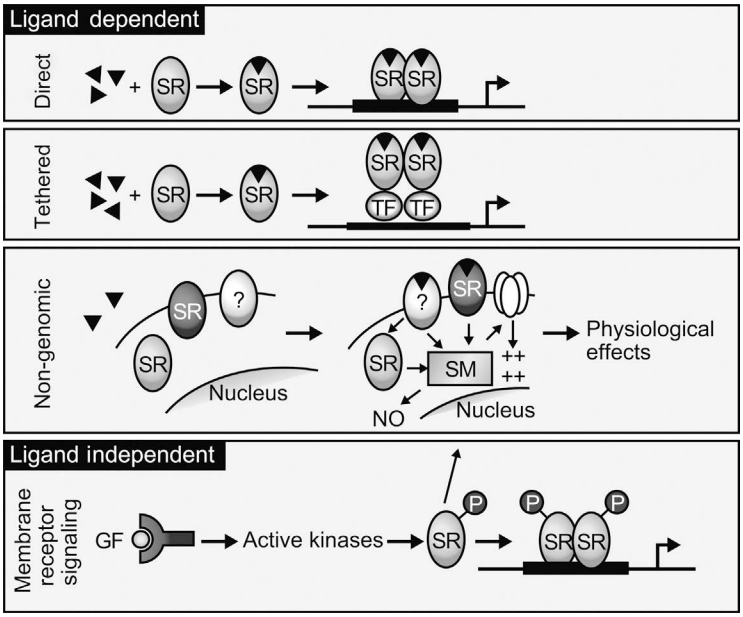


FIGURE 25.4 Ligand-dependent and ligand-independent nuclear receptor mechanisms. The direct "classic" model of sex steroid receptor (SR) action involves direct interaction between SR bound to hormone (triangles) and HRE; the tethered pathway utilizes indirect "tethering" of SR to genes via interactions with other transcription factors (TF). "Nongenomic" signaling is initiated by membrane-localized receptors modulating extranuclear second messenger (SM) signaling pathways. Ligand-independent responses occur as a result of transduction of membrane receptor signaling, such as growth factors, to nuclear SR. *Source: Adapted with permission from Ref. 18.*

important factor is which receptors are expressed in a responding tissue.

Indirect/Tethered Actions (HRE Independent)

Ligand-activated steroid receptors can stimulate the expression of genes that lack a conspicuous HRE within their promoter. This has been especially demonstrated for ERα[84–86] but is also known to occur for PR.[87] This mechanism of HRE-independent steroid receptor activation is postulated to involve a "tethering" of the ligand-activated receptor to transcription factors that are directly bound to DNA via their respective response elements (Figure 25.4), This mechanism involves a mediator component (e.g., p160) between ER and AP-1 versus direct interaction.[85] Interestingly, ERβ is unable to enhance the actions of a DNA-bound AP-1 complex when bound to E2 but can do so in the presence of certain selective estrogen receptor modulators (SERMs) such as tamoxifen, raloxifene, and ICI 164,384.[18] A similar HRE-independent mechanism of sex steroid receptor regulation has been documented for genes that possess a GC-rich region or Sp1 binding site within the promoter, upon which the actions of a bound Sp1 complex can be enhanced by ERα.[84] These mechanisms have primarily been demonstrated using in vitro cell culture models.

Nongenomic Actions

All models of sex steroid receptor action described thus far influence cellular phenotypes by acting in the nucleus and modulating target gene expression. The required gene transcription and mRNA translation is a relatively slow process; microarray studies demonstrate that initial transcriptional responses occur beginning about 60 min after administering hormones. Therefore, the numerous examples described to date in which steroids affect cellular dynamics within seconds to minutes of exposure have remained difficult to explain within the context of the previous models. Rapid cellular changes such as increased intracellular calcium or cAMP or activation of kinase signaling cascades have been attributed to sex steroid exposure of varied cell types and tissues.[88,89] Because these steroid effects do not involve direct steroid receptor activation of gene transcription, they are often collectively referred to as representing "nongenomic" pathways of steroid action. Indeed, several of the documented effects lack a conspicuous nuclear component or have even been demonstrated to occur in enucleated cells, yet others can ultimately affect gene expression. Therefore, rather than nongenomic, some have proposed these types of steroid actions to be loosely categorized as plasma membrane–associated steroid signaling events. Although there are numerous examples of putative membrane-associated steroid signaling effects in reproductive tissues and processes, a thorough description of these mechanisms is beyond the scope of this chapter.

An obstacle to our better understanding of membrane-associated effects of steroids is the lack of data on the nature of the cell-surface steroid "receptors". Questions remain concerning whether the membrane-associated receptors are identical or variant forms of the sex steroid NR or instead distinct receptors altogether. P is known to rapidly induce maturation in *Xenopus* oocytes that are arrested in the G2 phase of meiosis I,[90] without requiring protein synthesis. Most evidence indicates this process begins at plasma membrane progesterone binding sites that are unique from the classic nuclear forms of PR, called progesterone receptor membrane component 1 (PGRMC1),[91] although cDNA homologs of the PR were cloned from *Xenopus* and found to be associated with P-induced oocyte maturation.[89] In other studies, P is able to inhibit apoptosis of rat granulosa cells in vitro via a membrane-bound receptor that is immunoreactive to antisera directed against the LBD of the classic PR.[89]

Rapid effects of E2 have been described in a vast array of tissues, including a rapid activation of endothelial nitric oxide synthase in endothelial cells, potentiation of currents induced by the ion channel agonist kainite in hippocampal neurons, and influx of calcium in uterine endometrial cells.[88,90] The evidence of an association between ERα and plasma membrane–bound components has increased, supporting the view that this mechanism may indeed account for certain rapid effects of E2.[92,93] In this sense, the various nongenomic actions of E2 are often categorized according to their susceptibility to inhibition by classical ER antagonists (e.g., ICI type compounds).[92] E2-induced activation of membrane ion channels, endothelial nitric oxide synthase, and mitogen-activated protein kinase kinase are all inhibited by ER antagonists and therefore likely involve membrane-associated ERα or ERβ.[92] In contrast, examples of E2-induced PKA and protein kinase C (PKC) activation are not inhibited by ICI compounds and are therefore not likely to involve the nuclear ER form.[92] A recent study utilized a mutated form of the mouse ERα engineered to remain sequestered outside the nucleus (ERαH2NES), regardless of estrogen ligand. This form of ER did not mediate transcriptional responses but maintained estrogen-induced MAPK phosphorylation.[94] Targeting steroid receptors to the membrane involves palmitoylation, which is facilitated by HSP27.[88] The palmitoylation promotes interaction with caveolin-1, which then results in localization of the receptor in membrane caveolin rafts. Another potential mediator of rapid membrane localized hormone response is the G protein-coupled receptor GPER (originally referred to as GPR30), which is activated by E2.[95] GPER-null mice lack reproductive phenotypes,[96] although effects on the degrees of uterine responses elicited by E2 have been observed with G15, a GPER selective antagonist, suggesting a potential role for GPER in modulating

ERα-mediated outcomes.[97] A multiprotein complex of extracellular signal–related kinases, the inner plasma membrane component of tyrosine kinase receptors (c-Src), and the nuclear sex steroid receptors[92,98–101] has also been described as a mediator of rapid response to E. A different mechanism speculates that androgens or E2 may act as extracellular signaling hormones via binding to sex hormone binding globulin that is already bound to the cell membrane via a G protein–coupled-like sex hormone binding globulin cell-surface receptor.[102]

Ligand-Independent Actions: Membrane Receptor Cross Talk

We now have ample evidence that the sex steroid receptors can be activated via intracellular second messenger and signaling pathways, allowing for the induction of sex steroid target genes in the absence of steroid ligand, or the enhancement of steroid hormone signaling (Figure 25.4).[29,103–105] Polypeptide growth factors are able to activate ERα-mediated gene expression via mitogen-activated protein kinase activation of ERα in the absence of E2 (Figure 25.4). Similarly, interleukin-6 stimulation of cells leads to increased AR-mediated gene expression,[38,106] and cyclin-dependent kinases have been shown to activate PR-regulated gene expression.[29] The intracellular signaling molecule cAMP, a common second messenger of G protein–coupled receptors and an activator of the PKA pathway, can stimulate increased PR-, AR-, and ER-mediated transcription of target genes in the absence of steroid ligand.[29,103] A well-characterized example of ligand-independent cAMP activation of PR occurs after dopamine stimulation of dopaminergic neurons[107] and is the mechanism by which sexual behavior is induced in female rodents. Likewise, growth factors are able to mimic the effects of E2 in the rodent uterus via E2 independent activation of ERα.[109,110] Most interesting are recent studies indicating that the MAP kinase protein ERK is co-recruited to chromatin with ERα.[111] Ligand-independent activation of sex steroid receptors is believed to rely largely on cellular kinase pathways that alter the phosphorylation state of the receptor and/or its associated proteins (e.g., co-activators, heat shock proteins) (Figure 25.4). As in the classic ligand-dependent mechanism described earlier, specific receptor domains are critical to ligand-independent activation as well. ER activation by peptide growth factor–signaling pathways appears to be more dependent on AF-1 functions, whereas the effects of increased intracellular cAMP are postulated to depend on the AF-2 domain and do not require a functional AF-1.[103]

Receptor Antagonists

Structural analyses of the sex steroid receptors provide insight into the agonist/antagonist actions of endogenous or synthetic ligands (Table 25.2). Certain ligands possess "mixed" agonist/antagonist activity that is dependent on the receptor, cell, and promoter context, leading to the more descriptive terms of SERMs, selective PR modulator, and selective AR modulator. As described in the subsection Receptor Mechanisms of Action of the section Steroid Receptors, sex steroid receptor agonists interact with the LBD to generate an ordered array of 11 α-helices that is most conducive to receptor interaction with co-activator proteins and/or promotes disassociation of co-repressor proteins. In turn, antagonist ligands generally fail to generate the necessary changes in receptor structure that induce full transcriptional activity.[48,112–114]

Antiprogestins have a number of applications in reproductive medicine, including use as contraceptives due to their ability to prevent implantation or ovulation.[115] The currently available PR antagonists are all competitive inhibitors of P binding to the PR LBD.[115] Type I antiprogestins, such as ZK 98299 (onapristone), bind PR but fail to induce receptor phosphorylation and induce weak receptor binding to HREs.[115,116] In contrast, type II antiprogestins, such as RU 486 (mifepristone, Table 25.2), bind PR and promote receptor phosphorylation, homodimerization, and binding of the receptor complex to an HRE, but are unable to activate transcription.[50] Crystallography data indicate that RU 486 binding to PR induces an arrangement of helix 12 that is not conducive to binding of steroid receptor co-activators and may even promote recruitment of co-repressors such as NCoR.[50] Type II antiprogestins do possess some PR agonist activity when acting in the context of the cAMP/PKA signaling pathway in certain cell types.[50]

The field of antiestrogens and SERMS has been an area of intense research because the potential clinical applications include treatments for fertility, cardiovascular disease, osteoporosis, cognitive function, postmenopausal symptoms, and breast and gynecological cancers.[18] The best-characterized antiestrogens are competitive inhibitors of E2 binding to ER and have been classified into two major groups. The type I class of ER antagonists includes the triphenylethlylene compounds, tamoxifen (Table 25.2), hydroxy-tamoxifen, and raloxifene and are characterized by mixed agonist/antagonist activity depending on the receptor, cell, and promoter context.[18] Upon binding the ER, these compounds selectively inhibit AF-2 function but leave AF-1–mediated receptor functions intact, thus explaining the selective agonist activity. In contrast, the type II compounds are considered pure antagonists, the most well characterized being ICI 182,780 (Table 25.2), which is a 7α-substituted derivative of E2. This compound binds the ER but possesses extended side chains that prevent co-activator association with the NR box of helix 12 and may even promote interaction with co-repressors as well as increased receptor degradation,[18] resulting in

decreased ER protein. Compounds of this type have now been designated as selective estrogen receptor degraders (SERDs). ER-selective nonsteroidal ligands exploit differences between the LBDs of ERα and ERβ and provide powerful pharmacological tools to discern the overall function of each ER (Table 25.2).

Clinical use of antiandrogens has focused largely on the treatment of prostate cancer.[38,117] The best-characterized AR antagonists include the steroidal compound cyproterone acetate (Table 25.2) and nonsteroidal compounds hydroxyflutamide (Table 25.2), casodex, and nilutamide. Hydroxyflutamide is considered a pure AR antagonist that competitively binds AR and reduces AR transcriptional activity by preventing interaction with co-activators.[117,118]

SEX STEROID RECEPTORS IN UTERINE FUNCTION

Estrogen Receptor Signaling in Uterine Function

The ovarian-derived sex steroid hormones dictate the uterine estrous or menstrual cycle in mammals and are therefore essential to the establishment and maintenance of pregnancy. The uterine response to the preovulatory rise in circulating E2 is required to prepare the tissue for the forthcoming rise in P that accompanies ovulation and is critical to embryo implantation. This physiological coordination between the ovary and uterus is common to the large majority of mammals studied, and the spectrum of actions and effects of the sex steroids in uterine tissue are mediated by their cognate NRs. Estrogen was first described almost 100 years ago as a substance that induced estrus.[119] Although initially the dramatic physiological effects of estrogens on the mammalian uterus have been appreciated and well described, later investigations begun to elucidate the precise mechanisms and signaling pathways involved in estrogen responses. Our understanding of steroid hormone action in the uterus has been greatly advanced by utilization of new technologies with the generation of knockout and knock-in models, including receptor or co-activator null, tissue and cell selective null, and mutant knock-in models in mice. These models are also used in combination with microarray, RNA-seq, and ChIP-seq methods that allow for comprehensive mapping of interaction of NRs with chromatin and modulation of genomic response to steroids in uterine tissues. Together, these models and techniques have led to better understanding of the molecular details of steroid receptor roles in biological processes. The following sections discuss what is currently known and hypothesized about sex steroid receptor actions in the mammalian uterus.

ER Expression in the Uterus

RODENTS

ERα is present in the female reproductive tract of rodents throughout late fetal and neonatal development, puberty, and adulthood.[120,121] In mice, ERα transcript has been detected in the blastocyst, but immunoreactivity first appears in the mesenchymal cells of the fetal uterus as early as gestational day 15,[120,121] whereas epithelial expression is delayed until the late fetal period but increases substantially on postnatal day 4 and peaks on day 16.[122,123] The level of ERα transcripts in the murine uterus during neonatal development closely mirrors the patterns indicated by earlier immunohistochemical studies.[124] There is reportedly no ERβ immunoreactivity in the neonatal mouse uterus, although transcripts are detected at a modest level.[124] In adult mice, ERα transcripts and immunoreactivity are quite high in uterine epithelial cells before ovulation[125] but decrease during pregnancy, whereas expression in the stroma continues.[126] In contrast, ERβ expression is relatively low in uteri of virgin and pregnant mice.[124,127,128] Adult rats exhibit a similar uterine ER expression pattern such that ERα transcripts and immunoreactivity are easily detectable in the epithelium, stoma, and myometrium, and levels peak during the proestrous period of increased circulating E2 levels.[129] The evidence of ERβ expression in the rat uterus is conflicting; whereas ERβ expression has been reported to be in approximately 40% of rat uterine epithelial and stromal cells regardless of the estrous cycle stage,[129] others report a total lack of detectable ERβ.[130] These observations are plagued by the inconsistent quality of antibodies against ERβ. In ovariectomized mice, ERα and ERβ proteins were detected in the epithelial, stromal, and myometrial cells,[131–133] indicating that removal of ovarian hormones alters the ER expression pattern. Although ERβ protein is present in the mouse uterus, there is overwhelming functional evidence that ERα is responsible for mediating the many effects of estrogens in the uterus. As described following, only ERα-null female mice exhibit a severely attenuated response to acute and chronic estrogen (e.g., E2 or DES) treatment in terms of uterine growth and altered gene expression, as well as exhibit impaired embryo implantation.[110,134,135] Furthermore, categorical studies have shown that the toxic effects of neonatal estrogen exposure in the neonatal female mouse uterus are clearly mediated by ERα.[136,137]

DOMESTIC ANIMALS

In the neonatal ovine uterus, ERα mRNA and protein levels are highest in the developing glandular structures; detectable but lower in the luminal epithelia, stroma, and vascular endothelial cells; and absent in the myometrium.[138,139] In the adult ewe, ERα expression is detected

in all uterine cell types and is highest on day 1 of the 16- to 18-day cycle, concurrent with peak plasma E2 levels.[138,140] During days 1–6, when P levels rise and E2 declines, ERα expression decreases accordingly and does not begin to rise again until the end of the cycle nears on days 11–15.[138,140] A similar pattern of uterine ERα expression is reported during the 21-day bovine cycle.[141] In situ hybridization and immunohistochemical analyses indicate maximum ERα levels on days 1–3, especially in the glandular and stromal cells; these levels generally decrease by day 6, although cells comprising the deep glands maintain ERα expression.[142] The observed cyclical changes in ERα expression in ovine and bovine uteri are consistent with steroid regulation of receptor levels. Indeed, E2 treatment of ovariectomized cows leads to increased ERα expression in the glands, stroma, and luminal epithelia, whereas P alone or in combination with E2 elicits a decrease in ERα levels.[142]

PRIMATES

Early investigations in human uterine tissue found that levels of ER immunoreactivity or high-affinity E2 binding vary during the menstrual cycle such that peak expression occurs in the mid- to late proliferative phase and then decreases at ovulation and commencement of the secretory phase.[143–145] This peak in uterine ER levels at the time of rising plasma E2 levels is similar to several other species and suggests that expression is autoregulated. However, maintenance of uterine ER expression in postmenopausal women suggests that nonsteroidal regulatory factors are also involved.[145] The ovulatory decline in ERα levels among all uterine cell types is concurrent with the rise in circulating P levels and suggests that PR-mediated P actions may act to downregulate ER expression. Such PR-mediated decrease in ERα has been reported in experimental systems as well.[146] A distinct gradient of ER levels among the functional components of the human uterus was also reported such that levels are highest in the fundus and progressively decrease in those tissues closer to the cervix.[147,148]

The discovery of ERβ forced a reevaluation of uterine ER expression because the early studies discussed previously did not use methods that adequately distinguished between the two ER forms. More recent studies definitively show that ERα is the predominant form in the human uterus and accounts for the increased receptor levels that occur during the proliferative phase.[149] ERα transcripts and protein are especially high in glandular epithelial and stromal cells during the proliferative phase and exhibit a dramatic decrease among uterine tissues upon entering the secretory phase of the cycle.[149] The levels of ERα transcripts and immunoreactivity in the uterine myometrium are much lower and exhibit little change during the menstrual cycle.[149] Still, comparatively higher ERα levels are described in the subendometrial myometrium during the proliferative phase and are postulated to be involved in uterine peristaltic activity.[149]

ERβ expression in the human uterus is much lower relative to ERα but is generally present in the same cell types and exhibits comparative changes during the menstrual cycle.[149,150] There is one report of increased ERβ immunoreactivity in the uterine stroma during the secretory phase.[150] ERβ immunoreactivity is especially detectable in vascular smooth muscle cells and increases therein during the secretory phase, suggesting a role for this receptor form in vascularization.[150] The human-specific ERβ isoform, ERβcx, also called ERβ2 (Figure 25.2), is also detected in the uterus and is present along with full-length ERβ (ERβ1) in the functional and basal layer endometrial glands.[151] Interestingly, ERβcx immunoreactivity decreases in the basal layer glandular cells during the mid-secretory phase, whereas little change occurs in the level of full-length ERβ,[151] suggesting that differential expression of ERβ isoforms over the course of the menstrual cycle may modify the cellular responses to E2.

Nonhuman primates exhibit uterine ER expression patterns similar to those described in humans. ERα immunoreactivity in baboon uteri is highest in endometrial glandular, stroma, and myometrial smooth muscle cells during the proliferative phase and decreases in all cell types upon commencement of the secretory phase.[152] A similar ERα expression pattern in epithelial, stromal, and myometrial cells is reported in the cynomolgus monkey uterus.[153] ERα immunoreactivity is also reported in the vasculature, including the spinal arteries, of the baboon uterus.[152] Ovariectomy followed by exogenous E2 or E2 plus P treatments in baboons leads to changes in uterine ER expression that mirror the menstrual cycle, supporting the direct role of ovarian steroids in the regulation of uterine ER levels.[152] Considerable levels of ERβ transcripts are also detected in the epithelial, stromal, and myometrial cells of cynomolgus monkey uteri.[154] In contrast, ERβ immunoreactivity is reportedly low in normal uterine tissue from baboons, although higher levels are observed in diseased glands associated with endometriosis.[155]

Uterine Phenotypes in Mouse Models of Disrupted Estrogen Signaling

The murine models of disrupted estrogen signaling have proven invaluable to experimental investigation of estrogen actions in the uterus and the contribution of each ER form to these functions (Table 25.3). In addition to the ER-null models are two independently derived lines of mice that lack the capacity to synthesize E2 due to disruption of the *Cyp19* gene.[131,156] Following, we will describe how these different mouse models have helped to delineate the role of ER in the uterus.

TABLE 25.3 Uterine Phenotypes in Mice Null or Mutated for Sex Steroid Receptors and Signaling

Mutated or Null for Sex Steroid Receptors and Signaling	Uterine Phenotypes	References
Esr1$^{-/-}$ (homozygous null alleles for ERα: αERKO and Ex3αERKO)	Normal uterine development but exhibits hypoplastic uteri Insensitive to the proliferative and differentiating effects of endogenous growth factors and exogenous E2 Implantation defect *Exhibit decidualization Infertile	135,157–161
NERKI$^{+/-}$ (one mutated allele of two-point mutation in ERα DBD and one WT allele)	Normal uterine development but exhibits hyperplastic uteri Hypersensitive to estrogen Infertile	132
KIKO (ER$^{AA/-}$) (one mutated allele of two-point mutation in DNA binding domain of ERα and one ERαKO allele)	Normal uterine development Insensitive to the proliferative effects of exogenous E2 treatment Infertile	162,163
ERα$^{EAAE/EAAE}$ (homozygous animal of 4-point mutation of DBD ERα)	Normal uterine development but exhibits hypoplastic uteri Loss of E2-induced uterine transcripts Infertile	164
ERαAF-1^0 (deletion of amino acids 2–128 on ERα)	Normal uterine development and architecture Blunted E2 response Infertile	165,166
ERαAF-2^0 (deletion of amino acids 543–549 on ERα)	Normal uterine development but exhibits hypoplastic uteri Insensitive to E2 treatment Infertile	167
ENERKI (ERα^{G525L}) (homozygous animal of 1-point mutation in LBD of ERα)	Normal uterine development but exhibits hypoplastic uteri Insensitive to E2 treatment IGF1 induced slight uterine epithelial proliferation compared to control littermates (nonhomogenous pattern) Infertile	168
AF2ER$^{KI/KI}$ (homozygous knock-in of 2-point mutation in LBD of ERα)	Normal uterine development but exhibits hypoplastic uteri Insensitive to E2 treatment ER antagonists and partial agonist (ICI 182,780 and TAM) induced uterine epithelial proliferation Growth factor did not induce the uterine epithelial cell proliferation Infertile	169
Wnt7a^{Cre+};*Esr1*$^{f/f}$ (uterine epithelial cell–specific deletion of ERα)	Normal uterine development Sensitive to E2- and growth factor–induced epithelial cell proliferation Selective loss of E2-target gene response Implantation defect Infertile	170
Esr2$^{-/-}$ (homozygous null alleles for ERβ: βERKO, Ex3βERKO, and **ERβ$_{ST}^{L-/L-}$)	Exhibit grossly normal uterine development and function Sensitive to E2 treatment Some *Esr2*$^{-/-}$ lines reported elevated uterine epithelial proliferation after E treatment compared to WT Some are completely sterile (due to ovarian phenotype)	158,171–173
αβERKO (homozygous null for both ERα and ERβ)	Normal uterine development but exhibit hypoplastic uteri, similar to those of Esr1$^{-/-}$ Insensitive to E2 Infertile	158,174
Cyp19a1$^{-/-}$ (homozygous null aromatase: ArKO)	Normal uterine development but exhibits hypoplastic uteri Sensitive to E2-induced epithelial cell proliferation Infertile	131,156,175
Pgr$^{-/-}$ (homozygous null alleles for PRA and PRB: PRKO)	Exhibit grossly normal uterine development Implantation and decidualization defects Exhibit epithelial hyperplasia in response to E2 Loss of P-induced proliferative switch in uterine epithelial and stromal cells Infertile	176–178

(Continued)

TABLE 25.3 Uterine Phenotypes in Mice Null or Mutated for Sex Steroid Receptors and Signaling—cont'd

Mutated or Null for Sex Steroid Receptors and Signaling	Uterine Phenotypes	References
Wnt7a^{Cre+};*Pgr*$^{f/-}$(uterine epithelial cell–specific deletion of PR)	Normal uterine development Loss of P-induced uterine epithelial P target gene transcription Abnormal proliferative switch of uterine epithelial and stromal cells Implantation and decidualization defect Infertile	179
PRKO and *PR*$^{d/d}$ (homozygous null alleles for PR)	Normal uterine development Abnormal epithelial proliferation in response to P Implantation and decidualization defect Infertile	176
PRA$^{-/-}$ (homozygous null alleles for PRA: PRAKO)	Normal uterine development Resemble PRKO phenotypes Infertile	180
PRB$^{-/-}$ (homozygous null alleles for PRB: PRBKO)	Normal uterine development Normal implantation and decidualization responses Do not exhibit epithelial hyperplasia in response to E2 Normal fertility	181,182
AR$^{-/-}$ (homozygous null alleles for AR: ARKO)	Normal uterine development, function, and maintenance of pregnancy Slightly longer uterine horns compared to controls Slightly reduced uterine circumference Reduced uterine hypertrophic response to gonadotropins Increase placenta size (placentamegaly) Subfertile	183,184
AR$^{-/-}$ in-frame deletion of exon 3, loss of DNA binding activity	No overt uterine phenotype Subfertile	185
SPARKI (homozygous knock-in of mutated DBD of AR)	Normal female fertility, no uterine phenotype reported	186

** Ex3αERKO females have a similar uterine phenotype to the original αERKO except for exhibiting a decidualization defect, which may be due to the splice variants in the original αERKO that retains ER activities.*
*** ERβ$_{ST}^{L-/L-}$ females are the only line of ERβ knockout animals that are reported to be completely sterile.*

MICE LACKING ERα

Unlike the AR, which is mutated in the naturally occurring Tfm rat and mouse, no known mutations of the ERα have been reported, thus the deletion of ERα was thought to be lethal. Similarly, only one male patient and one female patient with ERα mutation have been discovered.[187,188] The male patient's mutation results in severe truncation of the ERα protein due to a stop codon in the A/B domain. The female patient has a point mutation in her ERα LBD that decreases activity by reducing E binding more than 200-fold. ERα-null mice were the first experimentally generated steroid-receptor-null animal, reported in 1993, and preceded the discovery of ERβ in 1996. There are currently numerous reported lines of ERα-null mice and additional lines of mice with mutations in functional domains of ERα. Three separate lines of ERα-null mice were generated: the αERKO, first described by Lubahn et al. in 1993,[157] the ERαKO (or Ex3αERKO), described by Dupont et al. in 2000[158] and by Hewitt in 2010,[159] and ERα$^{-/-}$, described by Antonson et al. in 2012.[160] Homologous recombination was employed to either disrupt (αERKO) or completely excise (ERαKO and ERα$^{-/-}$) exon 3[158,160] of the murine *Esr1* (ERα) gene. The uterine estrogenic response in αERKO females differs from the latter two lines, as αERKO animals have a low level of truncated ERα protein produced from a splice variant, which preserves some residual biological functions,[189] but all ERα-null female mice are infertile. Recently, an ERα-null rat has been derived using zinc finger nuclease (ZFN) genome editing. All phenotypes examined thus far were previously seen in the ERα-null mice, including infertility due to hypoplastic uteri, polycystic ovaries, and ovulation defects.[190] Interestingly, the recently described female patient with homozygous ERα mutation also has cystic ovaries and a small uterus despite elevated circulating E.[188]

MICE LACKING ERβ

The first description of ERβ-null mice followed the discovery of ERβ by only two years. Prior to generation of the ERβ-null model, very little was known as to the role of ERβ in biological processes, making it difficult to predict possible phenotypes. Therefore, the ERβ-null mice have served a tremendous role in providing insight

into the importance of the newly discovered ER form to female fertility, and studies to date indicate ERβ plays a particularly important role in ovarian function. Four different lines of ERβ-null mice have been described. The βERKO was first described by Krege et al. in 1998,[171] and the ERβKO, or Ex3βERKO, was described by Dupont et al. in 2000.[158] Homologous recombination was employed in both lines to disrupt exon 3[158] of the murine *Esr2* (ERβ) gene. As described to date, the reproductive, endocrine, and ovarian phenotypes of both lines are indistinguishable, with both exhibiting female subfertility. Then, in 2002, Shughrue et al. reported the third line of ERβKO animals, however, no uterine or ovarian phenotypes were reported.[191] Recently, $ERβKO_{ST}^{L-/L-}$ animals, which contain *LoxP* sites flanking exon 3 of *Esr2*, were generated using the *Cre/loxP* recombination system.[172] Interestingly, female mice from this recently described $ERβKO_{ST}^{L-/L-}$ colony were reported to be sterile due to an ovarian defect.

MICE LACKING ER α AND β

Much insight into the physiological roles of E2 has been gained from the study of mice lacking one or the other known ER forms. Still, conclusions drawn from these models are confounded by possible compensatory mechanisms provided by the opposite ER in each respective ERKO model. Furthermore, mice lacking only one ER form will lose possible ERα/ERβ heterodimer-mediated actions.[192,193] Therefore, compound ER-null mice (i.e., αβERKO) represent an opportunity to elucidate potential compensatory and cooperative actions of ERα and ERβ. The two reported lines of compound ER-null mice are the αβERKO, described by Couse et al. in 1999,[174] and the ERαβKO, described by Dupont et al. in 2000.[158] Both were generated by crossbreeding animals heterozygous for the respective individual ER-null mice and, as described to date, exhibit comparable reproductive, endocrine, and ovarian phenotypes as discussed in the subsection Mice Lacking ER α and β within the section Ovarian Phenotypes in Mouse Models of Disrupted Estrogen Signaling of this chapter. Shughrue et al. generated an additional ERαβKO line in 2002, however, their reproductive function was not described, as the study focused on ER-mediated estrogen responses in the brain.[191]

MICE LACKING CYP19

Estrogens are produced by aromatase cytochrome P450, the product of *Cyp19* gene. Female mice with disruption of circulating estrogen production exhibit altered female reproduction.[131,156,175] There are three animal models of Cyp19-null (called ArKO). Fisher et al. reported the first mouse line in 1998, which disrupted exon 9 of *Cyp19* gene, as the region is a highly conserved region among all aromatase.[156] Later, in 1998, Honda et al. reported a mouse line with targeted disruption of exons 1 and 2 of the *Cyp19* gene.[175] Toda et al. generated the most recent mouse line of Cyp19-null in 2001 with a targeted disruption of exon 9 of the *Cyp19* gene.[131] These ArKO female phenotypes are indistinguishable.[131,156,175] Briefly, these mice are infertile due to ovarian dysfunction marked by cystic follicles and a failure to respond to exogenous gonadotropins. This phenotype is described in detail in the subsection Mice lacking Cyp19 within the section Ovarian Phenotypes in Mouse Models of Disrupted Estrogen Signaling of this chapter.

UTERINE PHENOTYPES IN GLOBAL ER- OR CYP19-NULL MICE

Females within each respective model exhibit a similar phenotypic syndrome. Female mice lacking ERα or aromatase are infertile due to dysfunction of numerous physiological systems, including the ovary (see the section Estrogen Receptor Signaling in Ovarian Function) and uterus, whereas ERβ-null females exhibit reduced or lost fecundity that is largely attributable to ovarian dysfunction. A level of caution is warranted when making phenotypic comparisons between the ER-null and Cyp19-null models because sensitivity to maternally derived estrogens may provide a more normal developmental environment during gestation in Cyp19-null mice, and sensitivity to dietary estrogens during adulthood is able to abate several phenotypes in Cyp19-null mice.[194] The reported uterine phenotypes are summarized in Table 25.3.

All lines of ER-null females exhibit uteri that possess the expected tissue compartments, myometrium, endometrial stroma, and epithelium[134,159,174] (Figure 25.5). However, in females lacking functional ERα or Cyp19, uteri are overtly hypoplastic and exhibit severely reduced weights relative to WT littermates,[131,134,156,195] whereas ERβ-null females possess uteri that are grossly normal and responsive to ovarian-derived steroids[134] (Figure 25.5). The uterine endometrium of ERα-null females is severely hypotrophic, poorly organized, and possesses a paucity of glandular structures (Figure 25.5(B)).[159,196] The luminal and glandular epithelial cells in ERα-null uteri are severely immature with fewer glands and consistently exhibit a cuboidal morphology, versus the tall columnar morphology and basal location of the nucleus of an "estrogenized" epithelium in WT uteri (Figure 25.5(B)). Therefore, fetal, neonatal, and perinatal development of the female reproductive tract in mice is largely independent of ERα- and ERβ-mediated actions, but estrogen responsiveness and sexual maturation of the adult uterus are ablated after the loss of functional ERα. The totality of the ERα-null phenotype and lack of any overt uterine abnormalities in ERβ-null females suggest that ERβ has little function in mediating estrogen actions in the uterus. Moreover, ERαβ-null also demonstrated a similar uterine phenotype as ERα-null (Figure 25.5).[197]

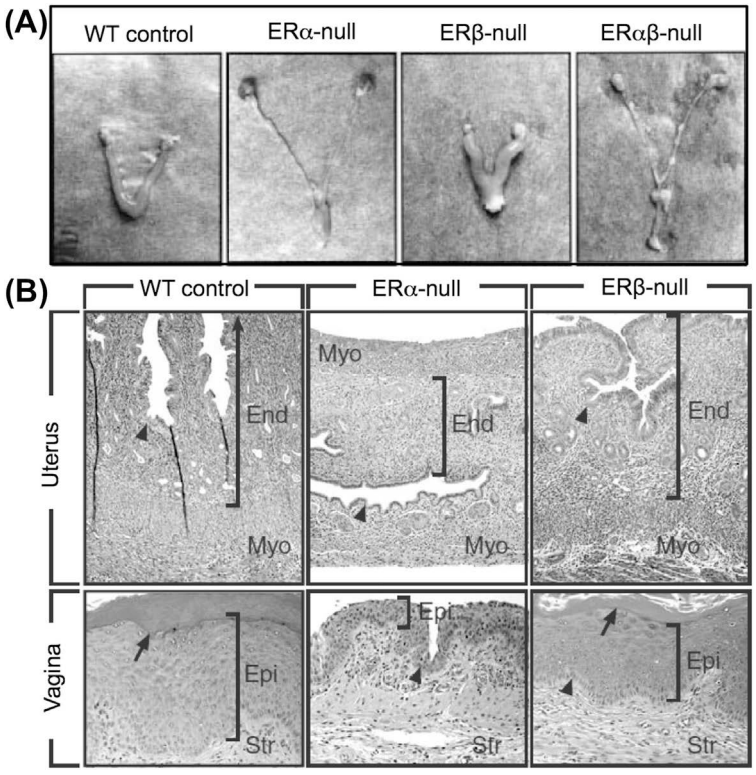


FIGURE 25.5 Gross uterine morphology of adult female estrogen receptor knockout mouse models. (A) Female reproductive phenotype of wild-type (WT) control, ERα-null, ERβ-null, and ERαβ-null. (B) Histology of uterine and vaginal tissue of WT, ERα-null and ERβ-null females. *Top*: Uterine cross-sections from representative adult WT, ERα-null and ERβ-null adult females illustrating the presence of a normal uterine architecture in all. The WT uterine section illustrates a normal myometrium (Myo), endometrial stroma (End), and columnar luminal epithelium (arrowhead). The ERα-null uterine section illustrates the characteristic hypoplasia of each anatomical compartment, a slightly disorganized endometrial stroma, and cuboidal luminal (arrowhead) and glandular epithelium. The ERβ-null uterine section is indistinguishable from that of the WT. *Bottom*: Vaginal cross-sections from tissue adult WT, ERα-null, and ERβ-null female mice. The WT and ERβ-null vaginal tissues illustrate a normal stroma (Str) and hypertrophied epithelium (Epi) showing estrogen-induced stratification and cornification (arrow). In contrast, the ERα-null vaginal tissue indicates complete estrogen insensitivity, as illustrated by a thin epithelium (Epi) and total lack of cornification. *Source: Reproduced with permission from Refs 197 and 134.*

Weihua et al. reported that ERβ-null females exhibited a slightly aberrant uterine growth response after estrogen replacement; however, the uterine bioassay was conducted in immature intact, not ovariectomized adult, animals.[133] In addition, Wada-Hiraike et al. showed that in immature females, loss of ERβ leads to increased uterine epithelial proliferation induced by E2 compared to WT uteri.[173] Although ERβ-null females are subfertile, when pregnancies are established they are sustained to term,[171] indicating uterine competence. More recent findings suggest that loss of ERβ leads to complete sterility due to a defect in ovarian function,[158,172] described in more detail in the subsection Mice Lacking ERβ of the section Ovarian Phenotypes in Mouse Models of Disrupted Estrogen Signaling of this chapter.

MICE WITH UTERINE-SPECIFIC DELETION OF ERα

Currently, only a single mouse model with tissue-specific deletion of ERα in the uterus has been published. Our laboratory has described selective deletion of ERα, using the *Cre/loxP* recombination system, by crossing *Wnt7a*$^{Cre+}$[198] with *Esr1*$^{f/f}$ animals[159] to generate mice with deletion of ERα in uterine epithelial cells (*Wnt7a*$^{Cre+}$;*Esr1*$^{f/f}$). The expression of ERα in the uterine luminal and glandular epithelium of these animals was ablated, while the ERα expression in the stromal cells and other uterine cells remains intact.[170] The epithelial ERα was ablated not only in the uterus in this mouse line[170] but also in the oviduct (unpublished data). As expected, based on findings in the global ERα knockouts, loss of uterine epithelial ERα has no effect on female reproductive tract development. Uterine histological analysis showed a similar uterine morphology as wild-type control.[170] The *Wnt7a*$^{Cre+}$;*Esr1*$^{f/f}$ uteri are sensitive to 24 h treatment of E2, as the uterine epithelial proliferation is preserved (Figure 25.6). However, *Wnt7a*$^{Cre+}$;*Esr1*$^{f/f}$ uteri lack a complete uterine response to E2, apparent following a 3-day uterine bioassay, which demonstrated a blunted growth response and increased apoptotic activity in *Wnt7a*$^{Cre+}$;*Esr1*$^{f/f}$ compared to the

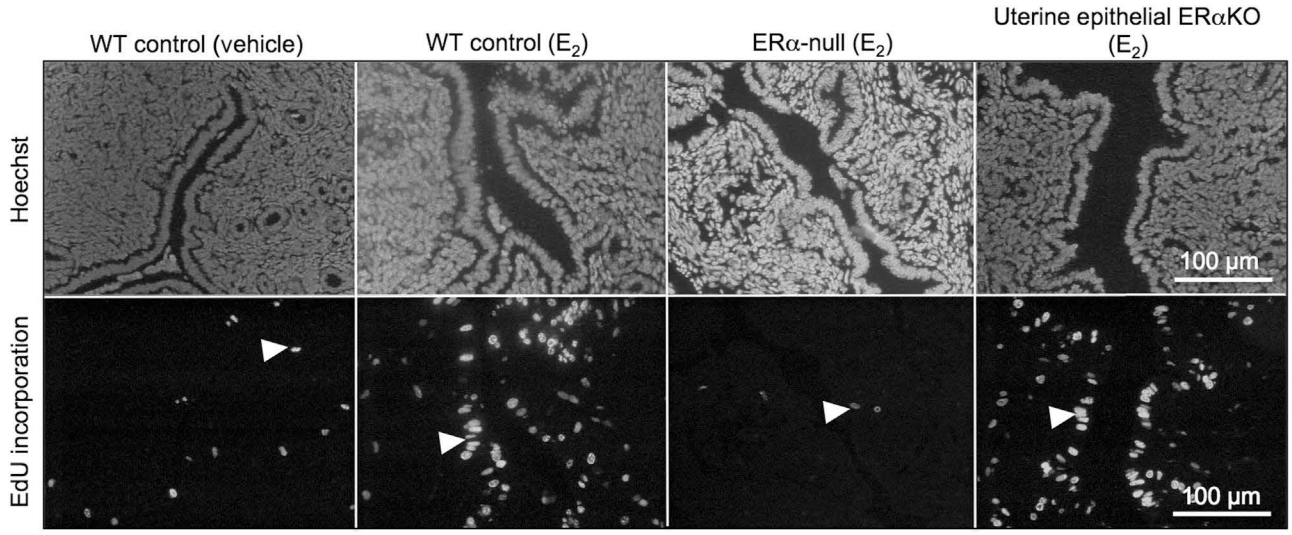


FIGURE 25.6 Estrogen-induced epithelial DNA synthesis in the uterus of adult ovariectomized mice is ERα dependent but occurs independent of epithelial ERα. Shown are cross-sections of uterine tissues from ovariectomized WT control, ERα-null, or uterine epithelial ERαKO mice treated for 24 h with vehicle or E2. DNA synthesis as indicated by nuclear EdU incorporation is detected in the luminal epithelia (arrowheads) of vehicle-treated WT uteri, and this is dramatically increased after E2 treatment of WT or epithelial ERαKO uteri. In contrast, ERα-null uteri exhibit a basal level of DNA synthesis that is unchanged after E2 treatment, indicating that E2-induced proliferation of the uterine epithelium is dependent on functional ERα (Scale bar = 100 μM). Hoeschst was used as a counterstain to visualize the tissue. *Source: Adapted with permission from Ref. 170*

control uteri. Additionally, a lack of ERα expression in the uterine epithelial cells contributes to complete infertility, partly due to an implantation defect.[170] This suggests that uterine epithelial ERα is dispensable for early uterine proliferative responses but crucial for complete biological responses induced by E2, as well as for embryo implantation.

MICE WITH MUTATED DNA BINDING DOMAINS OF ERα

The generation of mouse lines with mutation in the specific domains of ERα allows us to dissect the biological functionality of individual domains of the receptor in vivo. To date, there are two mouse lines with mutations that are designed to disrupt the DNA binding function of the ERα that have been "knocked-in" (KI) at the ERα gene locus. The first line was generated by replacing critical P-box amino acids E207 and G208 with alanines (Figure 25.3(B)). This line was named "Nongenomic ER knock-in" (NERKI), as these mutations were intended to restrict ERα signaling to the nongenomic and tethered mechanisms (Figure 25.4). Female NERKI$^{+/-}$ animals that have one mutated allele and one WT allele[132] were infertile, exhibiting a highly novel hyperplastic uterine phenotype, so NERKI$^{+/-}$ males were crossed with ERα-null heterozygous (WT/KO) females to produce mice with one NERKI mutated allele and one deleted *Esr1* allele, called ERα KIKO or ERα$^{AA/-}$ as described by O'Brien et al. in 2006.[162] The second line of DNA binding domain knock-in animals possessed mutation of four amino acids in the first zinc finger of the *Esr1* gene, substituting Y at position 201 with E, and in the critical P box (Figure 25.3(B)), K at position 210 with A, K at position 214 with A, and R at position 215 with E (Figure 25.3(B)) as described by Ahlbory-Dieker et al. in 2009 (called ERα$^{EAAE/EAAE}$).[164]

The NERKI$^{+/-}$ females have normal uterine development but exhibit hyperplastic uteri and are hypersensitive to estrogen.[132] This suggests that disruption of the ERα binding to ERE while maintaining the presence of a normal WT allele leads to an aberrant overstimulated uterine response to estrogen. Interestingly, these NERKI$^{+/-}$ are infertile and exhibit a uterine abnormality of enlarged hyperplastic endometrial glands despite possessing normal levels of circulating sex steroids.[132]

ERα$^{AA/-}$ females have normal uterine development. Initially, O'Brien et al. reported that ERα$^{AA/-}$ females, with mutation of the DNA binding domain, maintained proliferative responses—induced by E2.[162] However, in subsequent studies, no uterine proliferation was observed.[163,199] Ahlbory-Dieker et al. showed that, unlike the NERKI$^{+/-}$, females heterozygous for the ERα^{EAAE} mutation are fertile. The homozygous ERα$^{EAAE/EAAE}$ females have normal reproductive tract development, but uteri are severely hypoplastic (Figure 25.7(A)), similar to global ERα-null uteri. Additionally, ERα$^{EAAE/EAAE}$ uteri do not respond to E treatment, as the estrogen-regulated genes (such as *Pgr*, *Cdkn1a*, and *Igf1*) failed to be upregulated in ERα$^{EAAE/EAAE}$ compared to control uteri. The females from these two mouse lines with point mutations in the DNA binding domain of ERα are infertile. Thus the physiological function of the DNA binding domain of ERα is crucial for female reproduction.

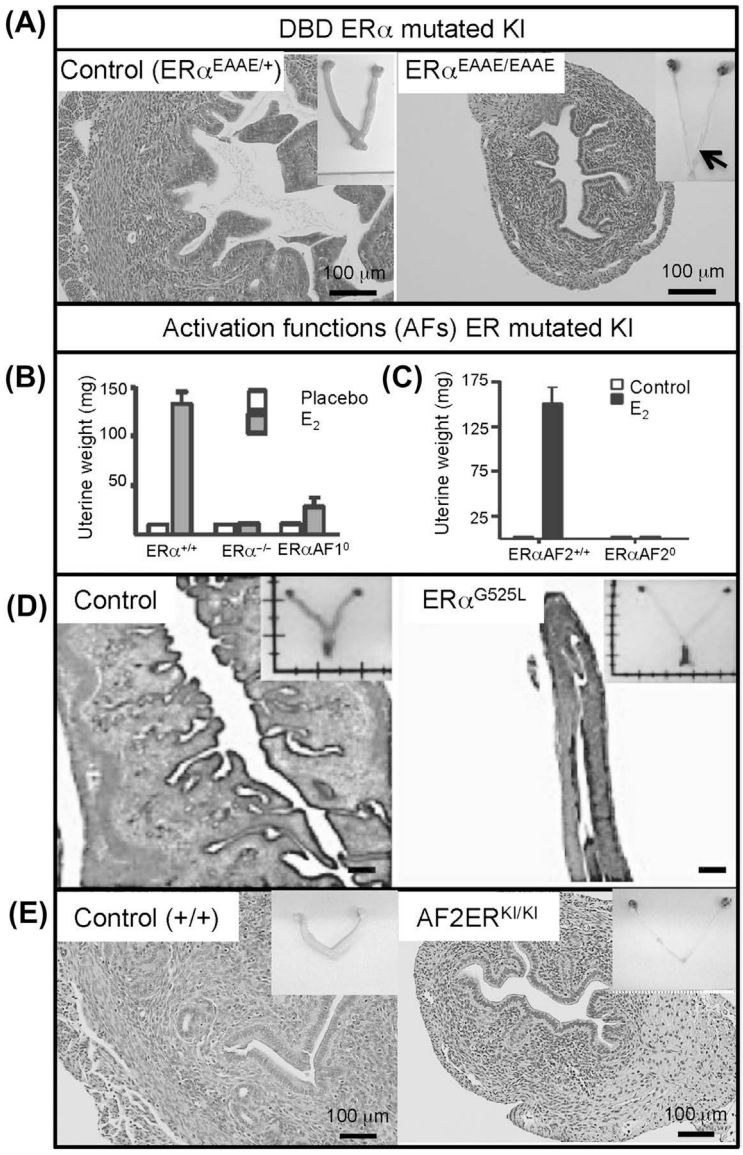


FIGURE 25.7 Gross uterine morphology and histological analysis of mice with knock-in of mutated ERα. (A) Female homozygous ERα$^{EAAE/EAAE}$ mice develop hypoplastic uteri (indicated by arrow) compared to heterozygous control (ERα$^{EAAE/+}$). (B) Uterine weight of ERα$^{+/+}$, ERα$^{-/-}$, and ERαAF-1^0 or (C) ERαAF-2$^{+/+}$ and ERαAF-2^0 mice treated with placebo control or E2 (80 μg/kg per day for 2 weeks). (D) Adult reproductive tract histology from WT control and ERα^{G525L} mice. H&E staining demonstrated that ERα^{G525L} animals exhibit hypoplastic uteri compared to WT control uteri. Insets depict gross reproductive tract morphology. Scale bar = 100 μm. (E) H&E staining of uterine tissues from WT control (+/+) and AF2ER$^{KI/KI}$ mice. Insets depict gross reproductive tract morphology. Scale bar: 100 μm. *Source: Reproduced and modified with permission from Refs. 164,165,167–169.*

MICE WITH MUTATED AF-1 OR AF-2 DOMAINS OF ERα

As discussed in the section Receptor Structure, AF-1 and AF-2 are important for ER transcriptional activity (Figure 25.1). A single mouse line with amino acids 2–128 deleted from exon 1 of *Esr1*, which removes the AF domain, called ERαAF-1^0, was described by Billon-Gales et al. in 2009.[165] There are three reported mouse lines with mutation in AF-2 domain of ERα. In the first line, the animals have a single point mutation in ERα of G at position 525 to L in the ligand binding domain (LBD), and this model was described by Sinkevicius et al. in 2008 (called "Estrogen-nonresponsive ERα Knock-in or ENERKI" or ERα^{G525L}).[168] Billon-Gales et al. generated a second line of LBD mutation animals in 2011, with amino acids 543–549 deleted from the LBD of ERα, removing helix 12 and thus AF-2 functionality (called ERαAF-2^0).[167] One month after the second line was published, Arao et al. reported the third line of animals with mutations within LDB of ERα[169] with two point mutations of L at position 543 and 544 to A (called AF2ER$^{KI/KI}$ animals). All females from the ERαAF-1^0, ERα^{G525L}, ERαAF-2^0, and AF2ER$^{KI/KI}$ mouse lines are sterile.[165,167–169]

ERαAF-1^0 females exhibited minimal uterine wet weight gain compared to ER$^{+/+}$ uteri after treatment with E2 pellets for two consecutive weeks (Figure 25.7(B)), while ERαAF-2^0 females did not respond (Figure 25.7(C)).[165–167] This indicates that the ERαAF-2 functional domain contributes to minimal uterine weight increase induced by E2 in the absence of AF-1. Both lines of AF-2-mutated animals (ERα^{G525L} and AF2ER$^{KI/KI}$) display severely hypoplastic uteri (Figure 25.7(D,E)) and lack uterine growth response to E2 treatment.[167–169] Interestingly, uterine wet weight can be increased by using the synthetic ERα agonist PPT (Table 25.2) in ERα^{G525L}

or by using the ER antagonists ICI 182,780 or tamoxifen (Table 25.2) in AF2ER$^{KI/KI}$ females.[168,169] The ability of the antagonists to mediate responses seems to be due to a unique conformation of the LBD of the AF2ER that leads to AF-1-dependent transcriptional activity.[169,200] Arao et al. also demonstrated that the uterine response to ICI or tamoxifen includes increased DNA synthesis in the uterine epithelial cells of AF2ER$^{KI/KI}$. The growth factor IGF1 (insulin-like growth factor 1) induced minimal uterine epithelial proliferation in ERα^{G525L} and was ineffective in AF2ER$^{KI/KI}$ uteri.[168,169] Together, these findings indicated that both AF-1 and AF-2 activation domains of ERα contribute to a normal regulation of uterine growth and reproductive functions. As the AF domains mediate ER-co-regulator interaction (Table 25.1), this emphasizes the importance of effective ERα co-activator protein recruitment for successful uterine E2 response. Similarly, mice lacking sufficient SRC-1 co-activator (*Src1*$^{-/-}$) exhibit measurably diminished uterine response to E2.[201]

Uterine Phenotypes in Mouse Models with Mutations in Transcriptional Proteins that Affect ER Function

MICE WITH UTERINE-SPECIFIC DELETION OF COUP-TFII

Chicken ovalbumin upstream promoter transcription factor II (COUP-TFII), encoded from the *Nr2f2* gene, is a transcription factor, belonging to nuclear receptor superfamily. COUP-TFII is expressed in uterine stromal cells and is crucial for female reproduction.[202–204] The tissue-specific deletion of COUP-TFII (*COUP-TFII*$^{d/d}$) in the uterus using *Pgr*$^{Cre+}$ crossed with *COUP-TFII*$^{f/f}$ animals demonstrated that *COUP-TFII*$^{d/d}$ females exhibited normal uterine development but were completely infertile, in part, due to lack of implantation.[202] Kurihara et al. also showed an enhanced estrogenic response in the epithelial cells of *COUP-TFII*$^{d/d}$ uteri, as E-responsive genes lactotransferrin (*Ltf*) and mucin 1 (*Muc1*) were elevated during the receptive window, which suggested that COUP-TFII modulates uterine epithelial ERα activity during pregnancy.[202] Inhibition of ERα activity using ER antagonist (ICI 182,789) partially rescued implantation in *COUP-TFII*$^{d/d}$ females and corrected the expression level of *Ltf* and *Muc1*.[203] These results suggest COUP-TFII plays an essential role in regulating uterine ERα activity during early pregnancy in rodents.

MICE WITH UTERINE-SPECIFIC DELETION OF REA

Repressor of estrogen receptor activity (REA) is an ER co-regulator that represses ER activity both in vitro and in vivo.[205–209] In Chinese hamster ovarian (CHO) cells, REA is shown to be a direct co-regulator of ERα and ERβ, but not PR or RAR.[205] In 2005, Park et al. generated a mouse line with a targeted deletion of REA exon sequence encoding amino acids 12–201 (heterozygous REA or REA$^{+/-}$).[208] Gross uterine morphology of REA$^{+/-}$ appeared normal compared to control littermates. However, the REA$^{+/-}$ females exhibited hypersensitivity to E2 treatment, as uterine weight and luminal epithelial cell height and proliferation were increased compared to E2-treated control uteri. In addition, E2-induced uterine genes (such as *C3* and *Ltf*) were also elevated in E2-treated REA$^{+/-}$ compared to E2-treated control uteri.[208]

Due to the embryonic lethality of REA$^{-/-}$,[208] uterine deletion of REA was generated using *Pgr*$^{Cre+}$ crossed with *REA*$^{f/f}$, producing *REA*$^{f/d}$ (heterozygous deletion) and *REA*$^{d/d}$ (homozygous deletion) animal models. Uterine epithelial cell hyperproliferation after E2 treatment was seen in *REA*$^{f/d}$, similar to the E2 response of REA$^{+/-}$ uteri.[207] The enhanced uterine weight increase by E2 in *REA*$^{f/d}$ females is in part due to increased levels of the aquaporin water transport gene (*Aqp4*). The levels of *Aqp3* and *Aqp5* induced by E2 treatment were similar between control and REA$^{f/d}$ uteri, but higher levels of *Aqp4* were reached in *REA*$^{f/d}$ than control uteri after E treatment.[207] *REA*$^{f/d}$ females were subfertile, while *REA*$^{d/d}$ females were completely infertile. No ovulation defect was found in *REA*$^{d/d}$ females, rather *REA*$^{d/d}$ females showed altered uterine growth and maturation, as well as implantation, decidualization, and placentation defects.[207] Aberrant apoptosis is observed in *REA*$^{d/d}$ uteri, as reflected by drastic increases in both TUNEL and active caspase-3 levels in *REA*$^{d/d}$ uteri compared to wild-type uteri.[207] These findings suggest that REA regulates proper ER responsiveness in vivo and is crucial for ER-mediated female uterine function.

MICE WITH UTERINE-SPECIFIC DELETION OF MSX1 AND MSX2

Mammalian homeobox genes, *Msx1* and *Msx2*, are crucial for organogenesis and embryo development. Nallasamy et al. reported that the selective ablation of either *Msx1* (*Pgr*$^{Cre+}$;*Msx1*$^{f/f}$, called *Msx1*$^{d/d}$) or *Msx2* (*Pgr*$^{Cre+}$;*Msx2*$^{f/f}$, called *Msx2*$^{d/d}$) in the uterus lead to subfertility in females, however, deletion of both *Msx1* and *Msx2* in the uterus (*Msx1*$^{d/d}$*Msx2*$^{d/d}$), resulted in complete infertility.[210] This indicates that *Msx1* and *Msx2* functions can partially compensate for each other. The female infertility in *Msx1*$^{d/d}$*Msx2*$^{d/d}$ females is in part due to an implantation defect.[210] Deletion of *Msx1* and *Msx2* in the uterus causes uterine hypersensitivity to estrogen due to an increase in uterine ERα activity. Increased ER phosphorylation in uterine epithelial and subepithelial stromal cells, resulting from high fibroblast growth factor (FGF) expression, were observed.[210] Female mice with uterine *Msx1* and *Msx2* deletion exhibited not only an altered ERα activity but also showed aberrant expression of Wnt/β-catenin signaling molecules.[210] This indicates that homeobox genes *Msx1* and *Msx2* in the uterus are mediators of physiological ERα activity, which subsequently play pivotal roles in establishing successful pregnancy.

MICE WITH UTERINE-SPECIFIC DELETION OF NCOA6

Nuclear receptor co-activator-6 (NCOA-6) is an ERα co-activator. Kawagoe et al. reported that NCOA-6 regulates uterine ER activity by using selective deletion of *Ncoa6* in the uterus (Pgr^{Cre+};$Ncoa6^{f/f}$, called $Ncoa6^{d/d}$).[211] Ovulation, fertilization rates, and blastocyst development are all normal in $Ncoa6^{d/d}$ females; however, embryo implantation fails. The implantation defect is in part due to an increased level of the ERα co-activator, SRC3, in the uterine epithelial cells of $Ncoa6^{d/d}$, which causes elevated uterine epithelial ERα activity compared to wild-type uteri. Moreover, a diminished PR activity, particularly during the implantation period, was observed. Implantation was partially rescued and the level of MUC1 was corrected in uterine luminal epithelia by treating $Ncoa6^{d/d}$ females with the ER antagonist ICI 182 780.[211] These findings suggest that loss of uterine NCOA-6 causes implantation failure due to an aberrant ER-estrogen hypersensitivity.

MICE WITH UTERINE-SPECIFIC DELETION OF MIG6

Mitogen-inducible gene 6 (Mig-6, encoded from the *Errfi1* gene) is a downstream target of P-PR and SRC1 action in the uterus.[108] However, uterine-specific deletion of Mig-6 ($Mig\text{-}6^{d/d}$ or Pgr^{Cre+};$Mig\text{-}6^{f/f}$) demonstrated altered ERα signaling resulting in uterine hyperplasia.[108] $Mig\text{-}6^{d/d}$ uteri are enlarged, with weights approximately 3-fold more than wild-type ($Mig\text{-}6^{f/f}$) uteri. Immunohistochemical analysis demonstrated that $Mig\text{-}6^{d/d}$ uteri exhibit hyperproliferation of the endometrium, with increased phospho-H3 staining, as a result of increased ERα expression and phosphorylation.[108] The ERα targeted genes, lactoferrin (*Ltf*), chloride channel calcium–activated 3 (*Clca3*), and complement component 3 (*C3*) were also elevated in $Mig\text{-}6^{d/d}$ uteri in the presence of both E and P, however, the PR-targeted genes amphiregulin (*Areg*) and follistatin (*Fst*) remained comparable to wild-type uteri. Ovariectomized $Mig\text{-}6^{d/d}$ females that were treated with E for 3 months exhibited endometrial cancer. However, co-treatment with E and P attenuated the pathology in the uterus of E treated $Mig\text{-}6^{d/d}$, although the uterine hyperplasia was still observed in $Mig\text{-}6^{d/d}$ females. Additionally, the expression level of MIG-6 in women with endometrioid carcinoma was elevated compared to normal endometrium.[108] These findings together suggest that Mig-6, as a tumor suppressor in both rodents and human, plays a crucial role in uterine growth in response to E in part by mediating the protective action of P.[108]

MICE WITH UTERINE-SPECIFIC DELETION OF HAND2

Heart and neural crest derivatives expressed 2 (Hand2) is regulated by P in the uterus, as protein expression of HAND2 is induced in the uterine stromal cells after P treatment.[212] PRKO uteri showed loss of Hand2 expression, indicating the direct regulation of Hand2 by PR.[212] Selective ablation of Hand2 in the uterus ($Hand2^{d/d}$) using the Pgr^{Cre+};$Hand2^{f/f}$ animal model demonstrated an elevated uterine ER activity.[212] Loss of uterine Hand2 expression in the stromal cells leads to aberrant fibroblast growth factor receptor (FGFR) signaling, which subsequently induced ERK signaling and ERα activity in the uterine epithelium (Figure 25.8), resulting in complete infertility. The infertility in $Hand2^{d/d}$ females is in part due to an implantation defect as a result of enhanced uterine epithelial ERα

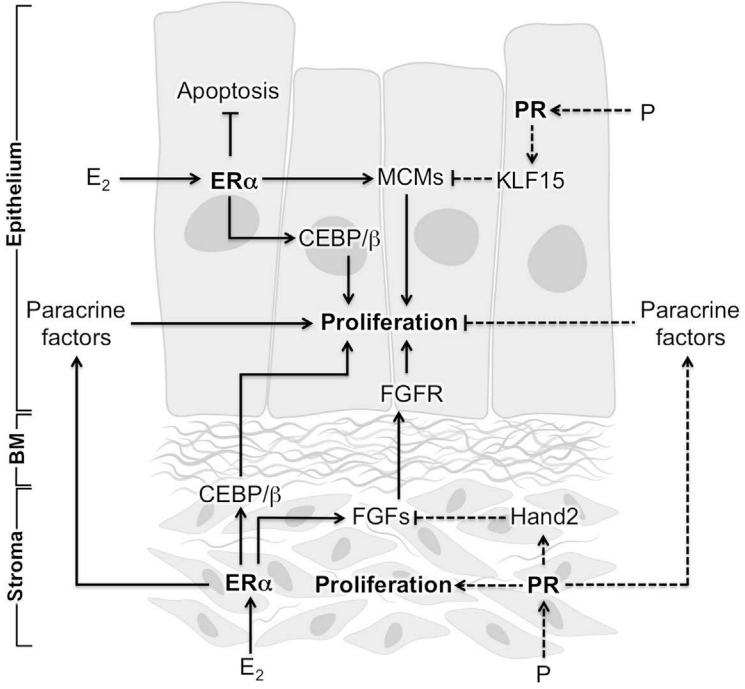


FIGURE 25.8 Schematic representation of paracrine and autocrine mechanisms of uterine proliferation in response to hormones. E2 stimulation of proliferation of the uterine epithelium requires the presence of functional ERα in the underlying stroma independent from epithelium, indicating that E2/ERα actions in the stroma induce the secretion of paracrine factors that then act on the epithelium to stimulate proliferation. Progesterone (P) acts through epithelial and stromal PRs to inhibit the proliferative response of the epithelium to E, while inducing proliferation of the underlying stroma. BM, basement membrane. (For detail see text.)

activity during the implantation period.[212] This suggests that *Hand2* expression in the uterus, orchestrated by an action of PR, mediates a proper physiological ERα activity (Figure 25.8) in response to ovarian hormones, leading to successful pregnancy establishment.

Vaginal and Cervical Phenotypes in Mouse Models of Disrupted Estrogen Signaling

The vaginal mucosa is composed of an epithelium and underlying stroma, possesses significant levels of ER,[213] and is highly sensitive to estrogens.[214] Endogenous or exogenous estrogens induce stromal differentiation, epithelial proliferation, and epithelial keratin synthesis to produce the stratified layer of cornified cells that lines the vaginal lumen.[214–216] These changes in the vaginal mucosa are used experimentally to estimate the level of circulating gonadal steroids and approximate the estrous cycle stage. Despite exposure to elevated endogenous E2 levels, the vaginal tissue of ERα-null females lacks any indications of estrogenization (Figure 25.5(B)).[134] Exogenous administration of E2 or DES also elicits no discernible vaginal response in ERα-null females.[196] In contrast, the vaginal mucosa of ERβ-null females undergoes the normal cyclic changes as dictated by ovarian sex steroids (Figure 25.5(B)). An effect on the vaginal mucosa similar to that observed in ERα-null females is produced in rodents after prolonged ovariectomy or exposure to ER antagonists.[217–219] Therefore, estrogenization of the vaginal mucosa in rodents, which is critical to mating, is ERα dependent. Using a human papilloma virus (HPV) transgenic model of cervical cancer, Chung et al. demonstrated that estrogen promotes development of HPV-induced cervical cancer through ERα, as αERKO HPV–transgenic animals were protected from developing disease.[220,221]

Progesterone Receptor Expression in the Uterus

In the murine uterus, PR transcript and protein levels are low on days 1–2 of pregnancy with the highest expression occurring in the epithelia and subepithelial stroma.[126] PR levels rise in these same tissues on days 3 and 4 of pregnancy, leading up to implantation.[126,222] Receptor-mediated E2 actions are a primary regulator of PR expression in the rodent uterus, but these actions are complex and specific to the different uterine compartments. Ovariectomy leads to increased PR levels in the uterine luminal epithelium that can be reduced upon exogenous E2 treatments, indicating E2 acts to repress PR expression in this uterine compartment.[128,223] Recombination experiments of stromal and epithelial tissues similar to those described earlier, as well as studies using epithelial selective ERα deletion, indicate this to be a paracrine-mediated effect that requires functional ERα in the stroma.[125,170] Simultaneous with causing decreased PR expression in the epithelium, E2 treatments lead to increased PR levels in the uterine stroma of ovariectomized mice.[128,223] This is an ERα dependent regulation, as PR is found only in epithelial cells, regardless of E2 treatment in αERKO. Additionally, epithelial ERα is dispensable for this response as increased PR expression in the stromal cells is preserved in the absence of epithelial ERα.[170] These observations have been largely confirmed in studies using a PR-lacZ reporter mouse that represents *Pgr* gene promoter activity as β-galactosidase activity in situ.[224] In these studies, β-galactosidase activity representative of PR expression is observed in luminal and glandular epithelium after ovariectomy and decreased after estrogen treatment.[224] P treatments also lead to decreased PR-lacZ expression in all cell types.[224]

In the adult ovine and bovine uterus, PR expression is highest during the early proliferative phase and present in all uterine cell types but is most abundant in the stroma and myometrium.[138,140–142] PR levels decline as the cycle progresses to the secretory phase, initially in the stroma and myometrial cells.[138,140–142]

In the human uterus, PR immunoreactivity is increased in all cell types during the proliferative phase but measurably lower than ER levels.[143,145] During the secretory phase, PR levels are maintained in the stroma and myometrium but decrease in the glandular epithelium such that levels are eventually undetectable.[143,145] Studies using PR isoform selective antibodies have shown that the decrease in PR levels that occurs in the glandular epithelium during the mid-secretory phase is largely representative of PRA, leading to an increased PRB/PRA ratio in these cells.[225] In stroma, where fewer cells expressed PR relative to glandular epithelium, PRA is more abundant.[225] During the late secretory phase, PRA levels in the stroma decrease but remain detectable, whereas stromal PRB expression decreases occur earlier and are significantly diminished by the late secretory phase.[225] The predominance of PRA in the glandular epithelium during the early secretory phase may serve to inhibit further estrogen-mediated proliferation, as the uteri in PRA-null mice exhibit an exaggerated proliferative response to estrogens.[181] As P levels rise during the mid-secretory phase, PRB is concentrated in nuclear foci, suggesting active transcriptional activity.[226] A similar finding of focal-concentrated PRB is reported in endometrial cancer cells in postmenopausal women, suggesting that inappropriate PRB activity may be associated with uterine cancer.[226] The pattern of PR expression in uteri of nonhuman primates is similar to that described in humans. In female baboons, PR levels are increased during the proliferative phase and then decrease accordingly during the secretory phase.[152]

Uterine Phenotypes in Mouse Models with Disrupted Progesterone Signaling

MICE LACKING PR

There are several reported lines of PR-null mice. Mice lacking both nuclear PR isoforms (PRKO) were first

described by Lydon et al. in 1995 and were generated via targeted disruption of exon 1 of the murine *Pgr* gene.[176] In 2006, Hashimoto-Partyka et al. generated the PR ($Pgr^{f/f}$)-targeted mutation in exon 1 of PR using *EIIa*-Cre promoter, which expressed Cre recombinase in the preimplantation embryo in order to create a deletion of exon 1 and loss of PR protein.[177] In 2010, Fernandez-Valdivia et al. described a mouse line that had a deletion of PR exon 2 and loss of PR protein using $Pgr^{f/f}$ and *ZP3*-Cre promoter expressing mice (called $PR^{d/d}$).[178]

Mice lacking only PRA (PRAKO)[180] or PRB (PRBKO)[182,227] were generated via a *Cre/loxP*-based gene targeting strategy that mutated the respective start codons for each isoform while preserving the translational reading frame of the other. Comparative studies in all models have greatly furthered our understanding of the divergent functions for the two PR isoforms. Recently, Franco et al. generated an epithelial cell specific deletion of both PRA and PRB in the uterus (called *Wnt7a-Cre*$^{+}PR^{f/-}$) using *Wnt7a*-Cre expressing promoter crossed with $Pgr^{f/-}$ animals.[179]

PR-null mice exhibit normally developed uteri that possess the expected uterine architecture (Table 25.3).[181,182] During the secretory phase of the murine estrous cycle, decreasing circulating levels of E2 concurrent with rising P cause a shift in uterine proliferation from the epithelial to stroma cells.[228] Estrogen, through ERα, is known to stimulate uterine epithelial cell proliferation in adult mouse uteri,[125,159,229] and P exhibits the antiproliferative effect on the action of E2 (Figure 25.8).[230,231] Therefore, PR-mediated P actions negatively modulate E2-induced proliferation of the uterine epithelium.[228] In addition, ovariectomized PR-null females treated with daily injections of E2 and P for 3 weeks exhibit abnormally large fluid-filled uteri that are characterized by a thickened uterine wall due to extensive extracellular edema, hyperproliferation of the glandular epithelia resulting in a disordered cellular arrangement, and acute inflammation of the endometrium.[176] The hyperestrogenic response in PR-null uteri is similar to that observed in ovariectomized mice after prolonged exposure to unopposed estrogens[229] and provides strong support of the modulatory actions of PR in the uterus.[176] Similar experiments in isoform-specific PR-null mice indicate this phenotype is reproduced in PRA-null mice only, whereas PRB-null mice exhibit a normal uterine response, indicating that PRA is primarily responsible for negatively modulating the uterine response to E2.[180–182]

Uterine Phenotypes in Mouse Model with Mutations in Transcriptional Proteins That Mediate PR Function

MICE WITH UTERINE-SPECIFIC DELETION OF P160 (SRCs)

The SRCs mediate P-dependent action in the uterus.[232] Immunohistochemical analysis in the uteri of pregnant mice demonstrated the spatiotemporal expression pattern of SRC1 and SRC2 in the luminal epithelial, stromal as well as secondary decidual zone. The uterine expression pattern of SRC1 and SRC2 was similar to the pattern of PR expression during pregnancy.[232] In uteri from ovariectomized animals, the majority of SRC1 and SRC2 was expressed in the luminal and glandular epithelium, much like PR expression.[232] Both E and P, either alone or administered together, increased the expression of SRC1 and SRC2 in the uterine stromal cells.[232]

There are several reports describing SRC-null mice and resulting effects on female reproductive functions.[201,232–234] Global disruption of the *Src1* gene demonstrated that both males and females were fertile, however, females exhibited a blunted response to ovarian hormones.[201] Treatment of $Src1^{-/-}$ females with E2 for 3 consecutive days resulted in less uterine weight increase than in WT, similarly, artificial induction of decidualization resulted in less stromal differentiation of $Src1^{-/-}$ uteri.[201] Due to a lethality of *Src2* global knockout,[235] uterine-specific SRC2 deletion ($Src2^{d/d}$) was generated using Pgr^{Cre+} crossed with $Src2^{f/f}$ animals.[233] $Src2^{d/d}$ females were infertile, in part, due to an implantation and decidualization defect.[232,233,236] E2-induced uterine epithelial cell proliferation was preserved in $Src2^{d/d}$ uteri.[233] The decidualization markers such as *Bmp2*, *Ptgs2* (Cox-2), and *Fst* were significantly decreased in $Src2^{d/d}$ when compared to wild-type uteri.[233] A complete absence of decidual response was observed in $Src2^{d/d}$ uteri in a $Src1^{-/-}$ background.[233] However, $Src3^{-/-}$ females had no overt uterine phenotype.[232] These findings indicate that of the three SRCs, SRC2 is most crucial for P-dependent uterine function.

MICE WITH UTERINE-SPECIFIC DELETION OF COUP-TFII

COUP-TFII regulates uterine ER function during early pregnancy as mentioned earlier. Additionally, the $COUP\text{-}TFII^{d/d}$ animal model also demonstrated that COUP-TFII is a downstream effector for PR-mediated decidualization.[202] Loss of uterine COUP-TFII resulted in a defect in decidualization, as reflected by lack of expression of decidual cell markers (WNT4 and BMP2).[203] Treatment of $COUP\text{-}TFII^{d/d}$ females with ICI 182,780 restores the expression of WNT4 and BMP2 in the decidual cells.[203] Together, the findings from uterine-specific deletion of COUP-TFII suggest that COUP-TFII regulates P signaling during implantation and decidualization by controlling uterine ER activity.

MICE WITH UTERINE-SPECIFIC DELETION OF REA

As discussed earlier, REA is crucial for uterine responses to ovarian hormones and establishment of pregnancy. Using $REA^{d/d}$ animals also demonstrated that loss of uterine REA led to aberrant PR function, which resulted in a decidualization defect.[207] The decidual cell

markers, *Wnt4* and *Bmp2*, were not increased in $REA^{d/d}$ uteri after artificial decidualization. The defect in decidualization is due to loss of PR expression after uterine REA ablation.[207] These findings indicate that uterine REA expression is not only crucial for ER activity but also for PR function to establish and maintain pregnancy.

MICE WITH UTERINE-SPECIFIC DELETION OF FKBPs

FK506 Binding Proteins 4 (FKBP4; encoded by *Fkbp52* gene) and 5 (FKBP5; encoded by *Fkbp51* gene) are immunophilin family co-chaperones that interact with PR in the absence of the ligands.[237] *Fkbp4* is expressed in the luminal and glandular epithelial cells during implantation, whereas a minimal level was detected in the uterine stromal cells, similar to PR expression.[237] During the decidual response, *Fkbp4* was highly expressed in both primary and secondary decidual zones. Although the expression of *Fkbp5* also showed a similar pattern, the level of expression was much lower than that of *Fkbp4*.[237] The global knockout of FKBP5 was fertile and showed no impairment of uterine functions.[237,238] However, the global knockout of FKBP4 caused complete female infertility,[237,238] due to implantation and decidualization defects,[237,238] suggesting that FKBP4 not only binds to PR but is crucial for PR action during pregnancy.

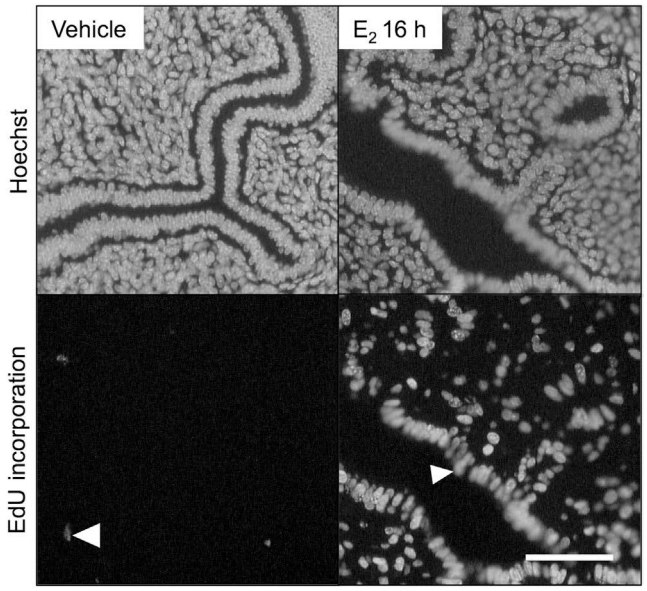


FIGURE 25.9 Estrogenic response in immature uteri in mice (21-day-old). DNA synthesis as indicated by nuclear EdU incorporation is detected in vehicle-treated uterine epithelial cells (arrowhead). However, DNA synthesis is dramatically increased in the luminal and glandular epithelia as well as stromal cells in the uteri 16 h after E2 treatment. Hoescht was used as a counterstain to visualize the tissue. Scale bar = 100 μm. Arrowhead indicates EdU positive epithelial cell.

Uterine Response to Estradiol and Progesterone

Changes in Physiology

Estrogens stimulate a complex process of epithelial proliferation and differentiation in the sexually mature uterus that leads to the formation of a multilayered secretory endometrium. This effect is critical to provide a suitable intrauterine environment for the establishment and maintenance of pregnancy. Early studies in neonatal and prepubertal rodents found that both the uterine stroma and epithelium proliferate in response to estrogens (Figure 25.9);[239] however, estrogen-induced mitogenesis in uteri of sexually mature rodents is limited to the epithelium.[229] Therefore, sexual maturation of the rodent uterus is not simply marked by the presence of ER or an estrogen response but is rather the acquired capacity to undergo synchronized phases of proliferation and differentiation as dictated by the ovarian-derived sex steroids. The lack of estrogen-induced uterine epithelial proliferation in ERα-null uteri indicates the essential role of ERα in this process (Figure 25.6). Although ERα is present in both epithelial and stromal compartments, tissue recombination studies and study of the uterine epithelial-specific ERα null indicate the proliferative epithelial response to estrogens is indirect and dependent on stromal ERα actions (Figure 25.6 and a working model in Figure 25.8).[170,240]

Treatment of ovariectomized mice with estrogens (e.g., E2 or DES) has long served as an experimental model to mimic the uterine events that occur during the estrous phase of the rodent cycle or immediately after the preovulatory E2 surge. Morphological and biochemical changes occur in the rodent uterus after estrogen stimulation following an established biphasic temporal pattern[241,242] (Table 25.4). Estrogen-stimulated changes in the rodent uterus that occur early, within the first 6 h after treatment, include increases in nuclear ER occupancy, water imbibition, vascular permeability and hyperemia, prostaglandin release, glucose metabolism, eosinophil infiltration, gene expression (e.g., c-*fos*), and lipid and protein synthesis (Table 25.4). Recent ERα ChIP-Seq profiles from uterine tissues showed that the receptor preoccupies chromatin sites and that E2 treatment increases ERα recruitment.[243] These processes are then accompanied by a delayed response that peaks after 24–72 h and includes dramatic increases in RNA and DNA synthesis, epithelial proliferation, and differentiation toward a more columnar secretory phenotype, dramatic increases in uterine weight, and continued gene expression (e.g., lactotransferrin) (Table 25.4). Ovariectomized mice exhibit a three- to four-fold increase in uterine weight after three daily treatments with E2 or DES, whereas no such response is observed in the uteri of ERα-null females.[157,159,244] The early phase effects of water imbibition and hyperemia as well as the late-phase effects of increased DNA synthesis and epithelial proliferation are absent in ERα-null uteri[159,189,196] (Figure 25.6). Interestingly, females that are heterozygous for the *Esr1* gene

TABLE 25.4 Biphasic Response of the Rodent Uterus to Estrogens

EARLY UTEROTROPIC RESPONSES (WITHIN 6 H)
Nuclear localization of estrogen receptor/recruitment to chromatin
Activation of receptor-tyrosine kinase pathways
Changes in gene expression (induction/repression of "early" genes)
Increased vascular permeability
Increased protein synthesis
Water imbibition
Hyperemia
Eosinophil infiltration
Albumin accumulation
Increased electrolytes
Lysozyme labilization
Increased cyclic nucleotides, prostaglandins, and associated enzyme activity
Increased glucose metabolism and associated enzyme activity
Calcium influx
Increased lipid synthesis
LATE UTEROTROPIC RESPONSES (WITHIN 24 H)
Second peak of nuclear localization of estrogen receptor
Changes in gene expression (induction/repression of "late" genes)
Increased protein synthesis
Increased DNA synthesis
Epithelial proliferation in "waves"
Cellular hypertrophy
Overall increase in uterine dry weight

disruption possess approximately one-half the normal level of ERα in the uterus, but their response to estrogen treatment is comparable with wild-type females. The total lack of response to estrogens in ERα-null uteri as well as a lack of late biological response in epithelial ERα knockout uteri provide strong evidence that ERα is required to mediate the full biochemical and biological uterine response to estrogens.[159,170]

Molecular Mechanisms of Estrogen-Induced Uterine Proliferation

Numerous studies have elucidated the molecular mechanisms of E2-induced uterine epithelial cell proliferative responses in animal models. For example, the transcription factor CCAAT enhancer binding protein beta (C/EBPβ) is involved in hormone-induced uterine proliferation.[245] Maximum uterine expression of C/EBPβ is induced 1 h after E2 treatment in both epithelial and stromal cells.[245,246] ICI 182,786 (ER antagonist) strongly inhibited E2-induced *Cebpb* transcript in the uterus, suggesting an ER-dependent expression of C/EBPβ.[247] In addition, loss of epithelial ERα in the uterus did not alter E2-induced *Cebpb* expression, indicating that *Cebpb* expression is independent of epithelial ER.[170] This points to the action of estrogen through ERα as the major mediator of C/EBPβ expression in the uterus. Indeed, the deletion of C/EBPβ (C/EBPβ$^{-/-}$) leads to a lack of the E-induced uterine proliferative response[245] as reflected by the absence of mitotic activity, S-phase activity, and an increase in apoptotic activity in the uterine epithelial cells.[246] In addition to a blunted uterine growth response to hormones, the C/EBPβ$^{-/-}$ females also exhibit complete infertility[247] due to implantation and decidualization defects.[245]

Pan et al. demonstrated that the uterine expression of minichromosome maintenance proteins (MCMs), a complex required for DNA synthesis initiation, is induced after E2 treatment, specifically MCM2 and MCM3[248] (Figure 25.8). MCM2 activity is crucial and required for DNA synthesis in the uterine epithelial cells.[249] The DNA replication of uterine epithelial cells induced by E2 is attenuated by P action, which will be discussed further in a later section.

Estrogen–Growth Factor Cross Talk in the Uterus

The autocrine and paracrine actions of polypeptide growth factors are an integral component of the uterine response to estrogens. The uterine response to E2 is modulated by stromal factors, such as IGF1, that are induced by E2 and then impact epithelial responses.[250] *Igf1* transcript is increased with concomitant decrease of *Igfbp3*[242] and activation of the IGF1 receptor and downstream effectors following E2 treatment.[251] *Igf1* transcript is increased in both stromal and epithelial compartments of the uterus by E2, with greater signal apparent in the stroma (Figure 25.10(A)).[252] Igf1 has been demonstrated to play an essential role in the uterine growth response, as *Igf1* null mice lack a full uterine proliferative response, and more specifically, lack G2/M progression of the epithelial cells following E2 stimulation.[253] Additionally, transgenic mice overexpressing *Igfbp1*, which sequesters, and therefore decreases, the amount of available IGF1, have an attenuated uterine response to E2.[254] Uterine response is restored by transplanting Igf1KO uterine tissue into a WT host,[255] which demonstrated the paracrine effect of the host Igf1. Further, E2 treatment results in the activation of downstream mediators of Igf1 signaling, including the Igf1 receptor, IRS1,[251] AKT, and inhibition of GSK3β,[252] leading to nuclear translocation of CCND1 and epithelial proliferation. Additionally, picropodophyllin (PPP), an IGF1R inhibitor, blunted the proliferative effect of E2. This effect of PPP on E2-stimulated uterine proliferation could be reversed, however, by

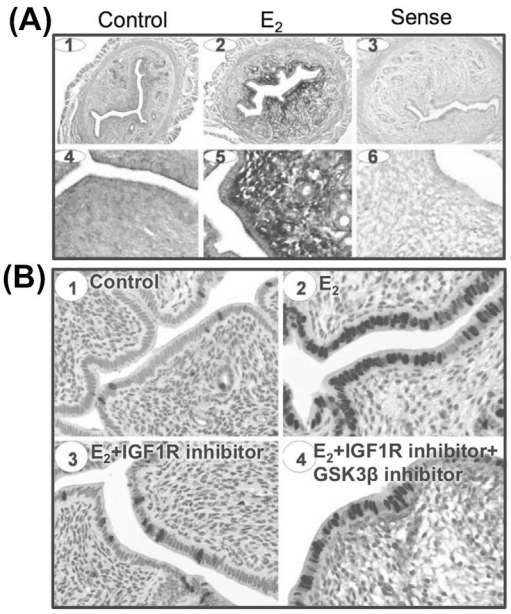


FIGURE 25.10 E2 treatment stimulates uterine epithelial proliferation through IGF1 signaling. (A) In situ hybridization of transverse sections of uteri of control (1 and 4) and E2-treated (2, 3, 5, and 6) mice probed by using antisense (1, 2, 4, and 5) or sense (3 and 6) probes. Dark precipitate represents the hybridization signal and indicates an upregulation of *Igf1* after E2 treatment principally in the stroma but also in the luminal and glandular epithelium. (Magnification: 1–3, 10×; 4–6, 40×.) (B) E2 signals through IGF1 and GSK3*β* to induce DNA synthesis in the uterine epithelium. BrdU incorporation in uterine section from ovariectomized mice 15 h after the following treatments: (1) control, (2) E2 (subcutaneous, s.c.), (3) E2 (s.c.) + PPP (picropodophyllin; IGF1R inhibitor) given intraluminally, and (4) E2 (s.c.) + PPP + SB415286 (GSK3*β* inhibitor) given intraluminally. *Source: Reproduced and modified with permission from Ref. 252. Copyright (2007) National Academy of Sciences, USA.*

co-treatment with PPP and the GSK3β inhibitor, SB415286 (Figure 25.10(B)).[252] These findings elegantly illustrate a direct role for IGF1R-initiated signaling through AKT and GSK3β on uterine response to E2.

In addition, IGF1 or EGF treatment of ovariectomized wild-type mice elicits a pattern of global gene expression similar to that induced by E2, although some estrogen-specific genes are revealed.[256] IGF1 treatment was shown to increase the expression of an ERE-driven luciferase reporter gene in transgenic mice, providing the first in vivo evidence of E2-independent ER activation by growth factor.[257] ER-null mice provide an excellent in vivo model for the study of ER–growth factor "cross talk" mechanisms in the uterus. ERα-null uteri express wild-type levels of functional EGFR but are unresponsive to the mitogenic actions of EGF, confirming the interaction of these two signaling systems.[258] However, not all EGF responses are lacking in the uteri of αERKO females, as upregulation of the c-*fos* gene by this growth factor remains intact. Similar studies have demonstrated that the uterine response to IGF1 is compromised in ERα-null females.[259] Cunha and colleagues[125,250,255] used the ERα-null mice in a series of tissue recombination experiments to further demonstrate interaction between ER and growth factor signaling in the murine reproductive tract. In these studies, uterine stoma and epithelium are enzymatically disassociated from prepubertal mice and recombined with corresponding tissue from animals of different treatments or genotypes. The resulting chimeric stromal-epithelial unit is then implanted under the kidney capsule of an ovariectomized nude mouse that is then treated with various hormonal combinations. A caveat to these studies is that neonatal tissues are used and may not accurately reflect the uterine physiology of sexually mature females. Nonetheless, these methods have been effectively used to demonstrate that estrogen-induced proliferation of uterine and vaginal epithelium requires functional ERα in the underlying stroma only,[250] whereas estrogen-induced increases in secretory products (e.g., LTF, complement C3, keratins) in the uterine or vaginal epithelium requires functional ERα in both uterine compartments. These findings have now been confirmed in sexually mature intact uterine tissue using uterine epithelial cell–selective ERα-null mice.[170] In addition, treatment of uterine epithelial ERα-null females with IGF1 or EGF mimics the uterine epithelial cell DNA synthesis stimulated by E2 (Figure 25.11). These studies strongly support a paracrine mechanism of estrogen-mediated epithelial proliferation that requires ERα in the stroma (working model in Figures 25.8 and 25.11). Interestingly, microarray studies indicate that the growth factor–induced genomic response is intact in ERα-null uteri, although epithelial proliferation is absent,[256] suggesting a greater complexity to the estrogen–growth factor signaling "cross talk" than was originally perceived. GF signaling is thought to mainly utilize AF-1 via MAPK phosphorylation sites in the N-terminus. Thus is was expected that IGF1 or EGF treatment of AF2ER$^{KI/KI}$ mice would lead to uterine growth, however, no response was seen,[169] indicating functional AF-2 is also involved in this mechanism.

Antiproliferative Functions of PR in the Endometrium

Tong and Pollard demonstrated that P inhibits E-induced uterine proliferation via the inhibition of DNA synthesis and PCNA expression (a component of DNA polymerase gamma, which is required for S-phase entry).[228] Additionally, Ray and Pollard demonstrated that Krupple-like transcription factor 4 (KLF4) expression is induced whereas KLF15 is suppressed by E2 treatment in the uterine epithelial cells.[249] Conversely, co-treatment with P and E decreased KLF4 expression and increased KLF15 in the uterus.[249] In addition, overexpression of uterine KLF15 using an adenovirus leads to a lack of E2-induced uterine epithelial proliferative

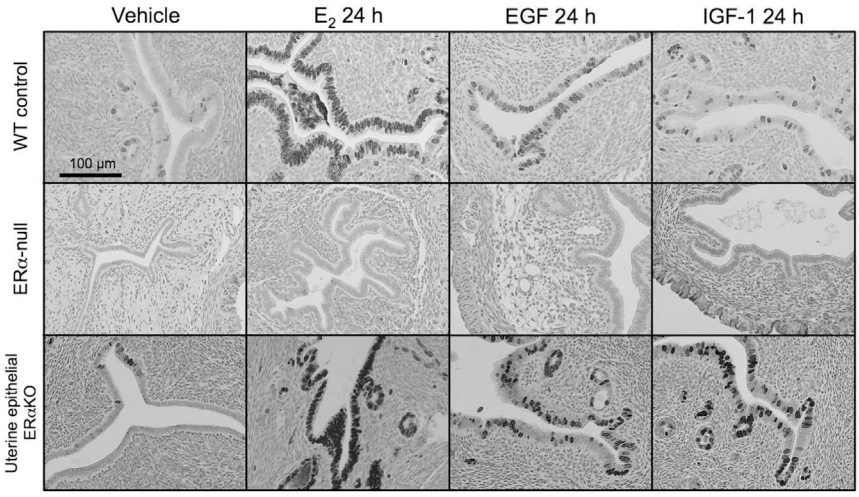


FIGURE 25.11 Growth factors are able to mimic the proliferative effects of E2 in the mouse uterus. Shown are uterine cross-sections from ovariectomized WT, ERα-null, and uterine epithelial ERα-knockout (uterine epithelial ERαKO) mice treated for 24 h with vehicle, E2, epidermal growth factor (EGF), or insulin-like growth factor 1 (IGF1). Uterine tissue was then subjected to immunohistochemistry for the antigen Ki67, a marker of cell proliferation. As expected, E2 treatment induces a dramatic increase in Ki67 immunoreactivity in uterine luminal and glandular epithelium, and EGF and IGF1 mimic this effect in the absence of endogenous E2. *Source: Reproduced and modified with permission from Refs 159,170.*

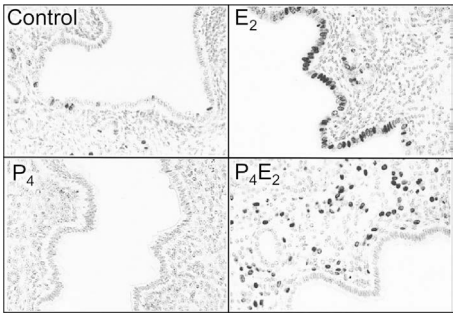


FIGURE 25.12 Progesterone inhibits E2-induced uterine epithelial cell proliferation and stimulates stromal cell proliferation. BrdU incorporation in the uterine tissues from ovariectomized mice treated with control (no treatment), E2 (15 h), P (daily for 4 days), and PE2 (P for 4 days and E2 on the fourth day for 15 h). *Source: Reproduced and modified with permission from Ref. 228.*

response due to the loss of MCM2 expression,[249] demonstrating that KLF15, through the action of P, inhibits E2-induced uterine epithelial DNA synthesis by inhibition of MCM2 expression. Moreover, P also diminished E2 induction of Cyclin D1 nuclear translocalization and expression of Cyclin A, which consequently decreased the phosphorylation of pRb and p107.[228,260,261] Together, these findings described the mechanism by which P inhibits cell cycle activity induced by E2 in the uterine cells (see Figure 25.8).

P not only inhibits the uterine epithelial proliferation but also contributes to an induction of stromal cell proliferation in the presence of E2. Recombination experiments using uterine tissues from PR-null mice demonstrate that the antiproliferative functions of P in the uterine epithelium are paracrine mediated and require PRA in the stroma[125,262] (Figure 25.12). However, selective deletion of PR in the uterine epithelial cells (*Wnt7a-Cre*$^{+}$*;PR*$^{f/-}$ animal model) demonstrated that epithelial PR expression is dispensable for E2-induced uterine weight increase and induction of stromal cell proliferation but required for the P-mediated inhibition of epithelial cell proliferative response induced by E2.[179,228] *Wnt7a-Cre*$^{+}$*PR*$^{f/-}$ animals exhibit an aberrant uterine expression of MCM3 and Cyclin D1,[179] which are important mediators of E2-induced uterine epithelial proliferative responses.

Moreover, P treatment also inhibits the pro-inflammatory activity induced by estrogen.[263] The inflammatory response observed in PR-null uteri after prolonged estrogen/P treatment is extensive and consists of "marked" infiltration of polymorphonuclear leukocytes into the endometrial stroma, mucosal epithelium, and uterine luminal fluid.[176] In vitro studies have shown that P inhibits the expression of chemotactic cytokines for neutrophil and lymphocyte infiltration[264,265] and may reduce prostaglandin E levels in uterine decidual and chorionic tissue during early pregnancy.[266] Therefore, PR-mediated P actions may be critical to suppressing the uterine inflammatory response that may accompany embryo implantation.[176,267] The uterine levels of macrophages, neutrophils, and *Ltf* are increased in both WT and PRKO uteri after unopposed E treatment. The pro-inflammatory activity is decreased by co-treatment of E with P in WT, but not in PRKO uteri,[223,263] thus PR function is crucial to control uterine inflammatory response during the ovarian cycle.

Roles of PR in the Rodent Uterus during Pregnancy

PR-mediated P actions in the uterus are critical to preparing the uterine endometrium for pregnancy. Embryo implantation is a highly complex process that requires synchronized cooperation between the blastocyst and uterine endometrium (see Chapter 38). Circulating E2 levels peak at ovulation and elicit a cascade of proliferation and differentiation in the luminal and glandular epithelium of the uterus, including induction of PR expression in the endometrial stroma and myometrium.[223] Postovulatory increases in circulating P then

cause decidualization, a complex process that involves massive proliferation and differentiation of the endometrial stroma along with localized increases in vascular permeability and edema.[34,268,269] This process involves the synthesis and interaction of numerous hormones and signaling pathways, including prolactin, cytokines, prostaglandins, and extracellular matrix components.[268,269] The result is a remarkable swelling of the uterine stroma that is thought to be necessary for implantation by forcing the uterus to close down on the blastocyst.[268,269] The final stage of apposition is a grasping of the blastocyst and ultimate attachment to the uterine wall, a process thought to be dependent on secondary rises in ovarian-derived E2.[268,269] Therefore, preparation of the uterus for blastocyst implantation is dependent on the multifunctional and sometimes opposing effects of E2 and P. In addition, PR-null models suggest that PRA is crucial not only for implantation but also the decidualization process as female mice lacking only PRA fail to exhibit a uterine decidual response, illustrating the importance of PRA for establishing successful pregnancy.[176,178,180] Recently, Franco et al. demonstrated that the epithelial PR is responsible for establishing successful pregnancy as the deletion of uterine epithelial PR contributes to an impaired embryo attachment, implantation, and decidual response.[179] Details of implantation and decidualization mechanisms are discussed in Chapter 38.

Maintenance of Progesterone Action in the Absence of ER

The *Pgr* gene is a well-described target of estrogen-induced expression via the classic model of ER action, especially in the uterus.[32,33] The lack of E2-induced increases in *Pgr* expression in ERα-null uteri confirms the regulatory dependence on ERα action.[189] Therefore, it was hypothesized that disruption of the ERα gene may subsequently result in abnormally low levels of PR in ERα-null uteri and thereby render this tissue refractory to P as well. However, assays for *Pgr* expression and P binding in ERα-null uteri indicate PR levels are only reduced by approximately half.[110,161,189] Furthermore, a greater proportion of PR is nuclear localized in ERα-null uteri (≈25%) relative to wild-type (≈5%).[161] Western blots indicate no difference in the relative levels of PRA and PRB between genotypes, with PRA consistently present in greater amounts in both.[161] Therefore, a loss of ERα action in the uterus has not led to a complete lack of PR or to altered and preferential transcription from one of the two *Pgr* gene promoters. Furthermore, several P-dependent actions are preserved in ERα-null uteri, including P-induced expression of amphiregulin and calcitonin and the uterine decidual response,[161] but not embryo attachment or implanation.[135]

The postovulatory nadir in circulating E2 levels has long been known to be critical to uterine decidualization in mice.[270] Studies have shown that the ER antagonist ICI 182,780 prevents artificially induced uterine decidualization in wild-type mice, indicating involvement of ER signaling.[161] Therefore, it is surprising that ERα-null mice exhibit a uterine response when exposed to an artificial model of hormonal and mechanical induction of uterine decidualization.[161,271] However, this may partly be due to the residual ERα protein produced by the splice variant found in the uterus of the αERKO line made by targeted disruption.[189] Nevertheless, these findings suggest that an altered "organization" of the uterine tissue or compensatory pathways might provide for hormonally driven uterine decidualization in ERα-null mice. Alternatively, some genes detected at the time of implantation, including the gap junction protein connexin 26[272] and the cytokine leukemia inhibitory factor,[135] are regulated by dual pathways. One involves estrogen-stimulated ERα, and the ability to induce via this mechanism is lost in the αERKO. The second pathway is initiated by decidualization-associated signals and is ERα independent and retained in the αERKO. This indicates a redundancy of regulatory mechanisms that allows retention of gene regulation in the αERKO and accounts for the ERα-independent uterine decidualization response observed in ERα-null mice. Recent studies demonstrated that de novo E2 synthesis occurs locally during decidualization, especially on day 6 and 7 of pregnancy in mice,[273] indicating an important role for local E2 synthesis for establishment of successful pregnancy.

Androgen Receptor Signaling in Uterine Function

AR Expression in the Uterus

AR is present in uterine tissues of multiple species,[130,274–278] although the function of androgen signaling in the uterus remains unclear. In rodents, ARs are present in all uterine cell types but most highly expressed in the myometrium, where expression may be positively regulated by estrogens.[276,279] In humans, ARs are also detected in the myometrial and endometrial uterine tissues, and levels increase during the proliferative phase.[280]

MICE LACKING AR

There are several reported lines of AR-null as well as mice with knock-in of mutated forms of AR (reviewed in Refs 281,282). *Tfm* mice were first described in 1970 and are a naturally existing androgen-resistant mutant[283] with an inactivating mutation of the *Ar* gene.[284,285] Comparable inactivation mutations of the *AR* gene and resulting phenotypes are well described in rats and humans.[286] Because the *Ar* gene is located on the X chromosome and *Tfm* males are infertile, it is impossible to breed for XX female mice that are homozygous for the

mutation. Lyon and Glenister overcame this challenge more than 30 years ago by utilizing embryo aggregation to generate a limited number of *Tfm* chimeric males that were fertile and carried germ cells harboring the *Ar* mutation.[287] More recently, AR-null female mice were generated via a *Cre/loxP* targeting scheme that allows for tissue and temporal specific disruption of the *Ar* gene and therefore the generation of fertile male carriers of the targeted *Ar* allele.[185,288–290] Exon 2 of the murine *Ar* gene was targeted for deletion in nearly all lines that have been described in the literature.[183,184,289,290] Additionally, one line of mice with in-frame deletion of exon 3 of the *Ar* gene has been generated, however, the uterine phenotype of this line was has not been described.[185] The exon 2 AR-null females have normal uterine development, although the uterine circumference is smaller than that of wild-type uteri (Table 25.3).[183,184] Hu et al. demonstrated that the AR-null uteri had increased uterine horn length, but smaller uterine diameter, as a result of decreased endometrial and myometrial areas during diestrus, when compared to wild-type uteri.[183,184] These AR-null females were subfertile, due to impaired folliculogenesis (discussed later in the section Ovarian Phenotypes in Mouse Models of Disrupted Androgen Signaling). Still, studies to date indicate that AR-null females exhibit reproductive phenotypes that are quite similar to those originally described in *Tfm/Tfm* females.

Recently, a mouse line was developed in which mutation of the AR DNA binding domain alters the ability of AR to bind to selective androgen response elements. This mouse line was generated by Schauwaers et al. with a targeted mutation of 12 amino acids in the second zinc-finger of the DNA binding domain at the exon 3 of AR (called SPARKI).[186] The heterozygous or homozygous SPARKI females do not have an overt phenotype and have normal fertility,[186] indicating selective AR DNA binding is not critical for female reproductive organ development and function.

Treatment of hypophysectomized rats with DHT is known to cause increased uterine weight in rodents, indicating that ligand-dependent AR actions can affect uterine responses.[291] Several studies also showed that DHT induced uterine weight increase in αERKO animals.[292,293] Upregulation of AR expression is observed in αERKO uteri after DHT treatment, suggesting that DHT exerts its action in the uterus through AR in the absence of ERα.[292] In addition, AR agonists increased myometrial thickness in the uteri of ovariectomized rats but inhibited estrogen-induced epithelial proliferation.[264] Similar observations were made in female mice, which exhibited not only uterine epithelial cell proliferation but also myometrial smooth muscle cell proliferation after DHT treatment.[294] AR-null female mice exhibit relatively normal uteri that are somewhat hypoplastic, although this phenotype may be more representative of decreased E2 synthesis in the ovaries rather than a role for AR in maintaining uterine weight (Table 25.3).[183] Furthermore, AR-null females are able to establish and maintain pregnancies to term.[183] Microarray studies have indicated that DHT leads to a pattern of uterine gene regulation that is remarkably similar to that elicited by estrogens but less robust,[292,293] with 86% of the DHT response consisting of a subset of estrogen responses. The fold response of the overlapping genes was in general more robust after estrogen treatment versus DHT. Thus, despite differences in biological outcomes, the global genomic patterns elicited by estrogens and nonaromatizable androgens largely overlap. Genes noted included those involved in metabolism, tissue growth and remodeling, transcription, protein synthesis and processing, and signal transduction.[293] Suppression of AR expression in human endometrial stromal cells (HESCs) using siRNA led to a decrease in decidual cell proliferation and differentiation.[295] These findings suggest that AR plays a minimal role for uterine growth and differentiation but is not essential for uterine development.

Glucocorticoid Receptor Signaling in Uterine Function

GR Expression in the Uterus

The uterus is not considered a classic target tissue of glucocorticoid action, although GRs are present throughout the cell types of the rodent uterus, including uterine natural killer cells (uNK cells).[40,296]

MICE LACKING GR

Homozygous GR-null animals generated via homologous recombination in embryonic stem cells die at birth due to respiratory failure.[297] Therefore, the generation of tissue-specific GR deletion in female reproductive tissues is crucial for studying the role of GR in reproductive functions.

Uterine Functions of GR

The limited experimental data available indicate that GR-mediated glucocorticoid actions are present in the uterus.[40] Treatment with the GR agonist, dexamethasone, did not increase the weight of immature rat uterus compared to E.[40,298] However, dexamethasone inhibits the uterine weight increase induced by estrogen in the rodent uteri.[40,298,299] Surprisingly, stimulation of the GR signaling pathway by dexamethasone treatment in immature rats elicits a pattern of gene expression that is remarkably similar to, but not as robust as, estrogen.[40] However, the molecular mechanism by which dexamethasone inhibits uterotropic action of estrogen remains unclear. A recent study reported that E-induced human uterine leiomyoma cell proliferation is suppressed by GR activation.[41] Gilz (glucocorticoid-induced

leucine zipper) is crucial for immune-related function of glucocorticoid activity.[300] Whirledge and Cidlowski also demonstrated that E suppressed *Gilz* gene expression induced by dexamethasone in human uterine epithelial (ECC1) cells.[300] This suggests that estrogen and glucocorticoid exert interplay between immune-responsive functions in the uterus.

GR activity not only exhibits inhibitory effects on estrogenic action in the uterus, but may also play important roles in the decidual response, as it is known that GR is expressed in uterine natural killer (uNK) cells,[296] and that the uNK cells play important roles for establishment and maintenance of successful pregnancy (reviewed in Ref. 301). However, the role of GR in uNK cell activity in the uterus remains unclear.

MicroRNAs in Uterine Reproductive Functions

MicroRNA (miRNA) are very small RNA molecules (18–24 nt) that are transcribed as larger primary RNAs (priRNA), either within introns of encoding transcripts or from miRNA encoding promoters. priRNA is processed to a shorter stem and loop pre-miRNA form by the activity of the DGCR8/DROSHA microprocessor complex. The pre-miRNA is then exported from the nucleus by exportin 5, to the cytoplasm where DICER cleaves the loop, leaving miRNA, which then functions to interact with mRNA, targeting it for degradation or repressing translation.[302,303] miRNA is particularly critical to female reproductive tract function, as mouse models with deletion of DICER in these tissues lose fertility. Estrogen has been demonstrated to regulate levels of miRNA in uterine tissue.[304,305]

The biological roles of Dicer are crucial for a normal development of limbs,[306] muscle,[307] and lung.[308] In addition, recent findings demonstrated that expression of Dicer is not only critical for female germ line biological functions[309] but also for embryo implantation.[310] Although miRNAs are expressed in the female reproductive tract, their precise physiological function remains unclear. To study the role of dicer and miRNAs during female reproduction, several studies used *Dicer1*$^{fl/fl}$ crossed with *Amhr2*$^{Cre/+}$ animals to generate a specific deletion of dicer in the mesenchymal layer of female reproductive tract (called *Dicer1*$^{fl/fl}$;*Amhr2*$^{Cre/+}$).[311–313] Female mice with a lack of Dicer1 expression in the mesenchyme exhibit a complete infertility.[311–313] Loss of Dicer1 has no effect on uterine development as the uterine epithelial cells, glands, stroma, and myometrium are present in *Dicer1*$^{fl/fl}$;*Amhr2*$^{Cre/+}$ females.[311,313] Uterine horns of *Dicer1*$^{fl/fl}$;*Amhr2*$^{Cre/+}$ females are shorter and hypoplastic when compared to control (*Dicer1*$^{fl/fl}$) uteri.[311,313] Additionally, the *Dicer1*$^{fl/fl}$;*Amhr2*$^{Cre/+}$ uteri respond normally to an artificial decidual stimulation.[312] An ovarian defect was observed as increased apoptosis within the granulosa cells of *Dicer1*$^{fl/fl}$;*Amhr2*$^{Cre/+}$ compared to wild-type ovaries.[312] Moreover, *Dicer1*$^{fl/fl}$;*Amhr2*$^{Cre/+}$ females exhibit an aberrant oviductal morphology with enlarged and fluid-filled cystic oviducts.[311–313] No embryos were found in *Dicer1*$^{fl/fl}$;*Amhr2*$^{Cre/+}$ uteri, but were discovered to be retained within the oviduct.[312,313] The oviducts from *Dicer1*$^{fl/fl}$;*Amhr2*$^{Cre/+}$ females have an augmented inflammatory response seen as an increase in recruitment of lymphocytes and macrophages into the oviductal tissues.[313] Moreover, *Dicer1*$^{fl/fl}$;*Amhr2*$^{Cre/+}$ females are also defective in embryo transport as transferred blue beads were mostly retained in the *Dicer1*$^{fl/fl}$;*Amhr2*$^{Cre/+}$ oviduct, whereas in WT females the beads moved through the oviduct and were found to be in the uterine lumen.[313] A lack of a proper embryo transport in *Dicer1*$^{fl/fl}$;*Amhr2*$^{Cre/+}$ appears to be due to a uterotubal junction defect, resulting in retrograde uterine flow into the oviduct.[313] Additionally, the expression of Wnt/β-catenin signaling molecules was altered in the *Dicer1*$^{fl/fl}$;*Amhr2*$^{Cre/+}$ oviduct.[311,313] A recent finding from a uterine-specific Dicer deletion using *Pgr*$^{Cre/+}$ and *Dicer*$^{fl/fl}$ animals demonstrated that the females are infertile.[314] Loss of uterine dicer leads to uterine developmental defects, including absence of glandular epithelium and increased apoptosis in the stromal cells.[314] Stromal cell proliferation could not be induced in *Dicer1*$^{fl/fl}$;*Pgr*$^{Cre/+}$ uteri treated with estrogen and P.[314] A decidualization defect was also found in *Dicer1*$^{fl/fl}$;*Pgr*$^{Cre/+}$, indicating loss of uterine response to P.[314] Additionally, loss of uterine dicer expression also leads to a dysregulation in Wnt/β-catenin signaling molecules, similar to the findings reported in *Dicer1*$^{fl/fl}$;*Amhr2*$^{Cre/+}$ females. These findings together suggest that miRNAs, produced by Dicer processing, play pivotal roles in female reproductive functions needed for establishing successful pregnancy.

Changes in Uterine Gene Expression

The dramatic physiological changes that occur in the uterus in response to steroid hormones are presumably the ultimate effects of equally dramatic changes in gene expression among the uterine cells. It is unlikely that the E2–ER complex is directly involved in mediating the whole genomic response in the uterus but more plausibly serves to stimulate a cascade of downstream signaling pathways that act to amplify the estrogen action. However, early investigations of the genomic response to estrogens in the rodent uterus discovered a handful of genes that are directly regulated via the classic ER mode of action, including *Pgr* and *Ltf*. The uteri of ERα-null females fail to exhibit estrogen-induced increases in *Pgr* and *Ltf* expression,[174] indicating the importance of ERα to this response. Furthermore, estrogen-stimulated increases in PR in the rodent uterus are localized to

the stromal and myometrial compartments, whereas increased *Ltf* is limited to luminal and glandular epithelium,[223] indicating that ERα functions are critical to induced gene expression in multiple uterine cell types. Further complexity of estrogen action in the uterus is illustrated by the simultaneous induction of PR expression in the myometrium and stroma while eliciting a decrease in PR levels in the luminal epithelium.[223] Similarly, the c-*fos* gene is rapidly induced by estrogen in the uterus, and this is ERα dependent.[159,242]

Microarray analysis of gene expression has significantly advanced understanding of genomic response of the rodent uterus to E2. Numerous studies have used microarray techniques to map the global gene expression patterns after estrogen exposure in the uterus and largely demonstrate that the biphasic uterine response to estrogens, so well characterized by physiological indicators discussed earlier (Table 25.4), is mirrored by the global changes in gene expression (Figure 25.13).[242,256,315–320] The clearly defined patterns of early and late response genes found in mouse uterine tissues are completely lacking in ERα-null uteri.[159,242] The identified genes fall into functional groupings, including signal transduction, gene transcription, metabolism, protein synthesis and processing, immune function, and cell cycle. Surprisingly, the expression levels of a striking number of genes are repressed by estrogen in the mouse uterus, and these effects were either absent in ERα-null uteri or relieved by co-treatment with ER antagonists, indicating that ERα is also actively involved in this process.[159,242] Comparative gene array analyses have also been conducted on human endometrial tissues during the proliferative and secretory phases of the menstrual cycle and have revealed gene expression patterns that are similar to those described in rodents.[321–325]

Microarray analyses have also been used to map the uterine response to P and have identified numerous target genes, including those involved in immune function, metabolism, growth factor regulation, signal transduction, and extra- and intracellular structure.[326–328] These observations have identified numerous signals and mechanisms important for normal uterine function and involved in uterine cancer (Table 25.5).[34]

Microarray approaches have been utilized to investigate the molecular responses to xenoestrogens, such as the industrial chemical bisphenol A (BPA) and the pesticide metabolite 2,2-bis(p-Hydroxyphenyl)-1,1,1-trichloroethane (HPTE). Microarray transcript profiles reveal that both xenoestrogens induce responses that are highly correlated to E2 response early (2 h), but less correlated later (24 h), similar to a pattern seen with a weak estrogen such as estriol.[336] Accordingly, BPA and HPTE, like estriol, are unable to induce uterine growth to the same degree as E2.[336]

TABLE 25.5 Steroid Receptor Uterine Target Genes from Microarray Studies

Steroid	Targets	References
P	*Ihh, Hoxa10, FoxO1, Bmp2, FKBP52, Cebpb,* Wnt pathway, *Calbindin 9k, Pparg, Lox 12/15,Mig-6, Ihh, Klk5, Klk6, Bcl2, Fst*	232,245,326,328–331
E	cell cycle regulators *CcnB1+2, Cdc2a, p21, Mad2l2, Cdc25c, Gadd45g,* DNA replication licensing MCM2, Aqp, thioredoxin pathway, *Mig-6,* Wnt signaling, *Cyr61, RAMP3, Aqp5, Bop1/Scx, Muc1, Inhbb,*	199,242,248,332–334
T	*ATM/Gadd45g pathway*	335
Dex	*Ad1, Mgla, Ykt6, Tis11, Id3, Gilz*	40,300

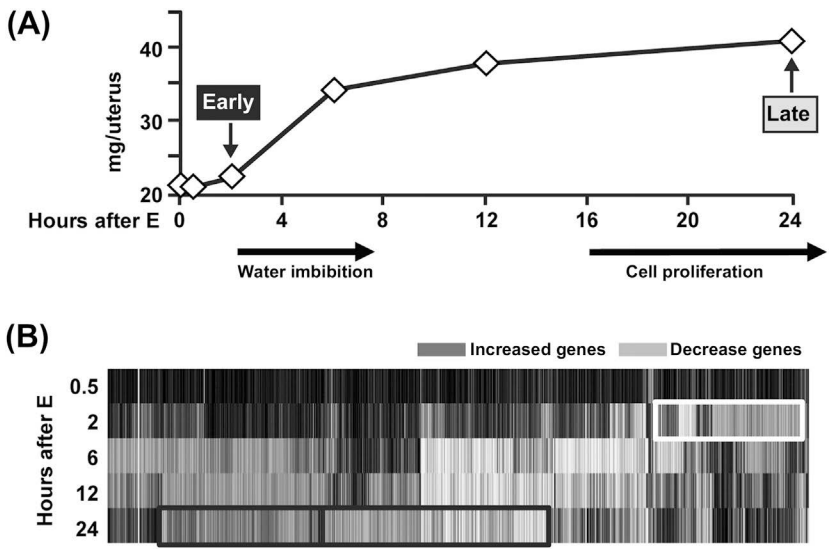


FIGURE 25.13 *Top*: Schematic representation of uterine biphasic responses that occur following a single injection of E into an ovariectomized mouse (see also Table 25.4). *Bottom*: Transcriptional profile of uterine RNA, fold changes of transcripts in comparison of vehicle-treated animals at indicated times after E injection. White and black boxes highlight transcripts that typify early and late phases, respectively.

Whole transcriptome analyses are now routinely incorporated into studies of disruptions in signaling pathways underlying uterine phenotypes of mouse models such as those described in Table 25.3. Thus, microarray comparisons have now become just one of many tools employed for investigation of uterine functions. The data are routinely deposited in the publically accessible site GEO (Gene Expression Omnibus; http://www.ncbi.nlm.nih.gov/geo/). Table 25.5 lists examples of steroid-regulated uterine transcripts discovered in microarray studies.

Chip-seq

More recently, technologies to facilitate evaluation of sites of transcription factor interaction with chromatin (by enriching a DNA binding protein, such as ERα, that has been cross-linked in situ to chromatin, with immunoprecipitation [chromatin immunoprecipitation or ChIP], followed by hybridizing the associated DNA to a chip tiled with promoter region sequences [ChIP-Chip] or by "next generation" massively parallel sequencing [ChIP-seq]) have been developed and widely utilized to study sites of nuclear receptor interaction (Figure 25.14).[75,337–341] Initial studies focused on ERα binding in MCF7 breast cancer cells, and several similar studies followed, which are summarized and compared in several review articles,[97,342–345] and reported that most sites were distal from transcriptional start sites (TSS) or were in intronic regions, rather than adjacent to TSS, as models of NR regulation of target transcripts had hypothesized. These comprehensive maps of *cis*-acting transcriptional regulators have been dubbed "cistromes". The initial ERα cistrome–associated sequences were evaluated for enrichment of transcription factor motifs, and confirmed binding to the experimentally defined "ERE" sequence. In the case of the MCF7 cells, enrichment of motifs for forkhead binding factors (Fox) was apparent. Owing to the abundant expression of the FoxA1 member of the Fox family, a potential role for FoxA1 in estrogen response was pursued with an arsenal of bioinformatic, Next Gen sequencing, and biological studies that demonstrated the ability of FoxA1 to "pioneer", and thus make accessible, regions of the chromatin that were subsequently targeted by ERα.[76]

Most ERα cistromes reported have utilized in vitro cultured cell models, although ERα profiles from mouse liver tissue have been obtained.[346] A ChIP-seq study by Hewitt et al. examining ERα binding sites in mouse uterine tissue indicated that, much like the MCF7 breast cancer study, most ERα sites were not proximal to TSS.[243] Subsequent comprehensive ChIP-Chip or ChIP-seq studies have been published for the PR in mouse uterus,[347] and T47D breast cancer and leiomyoma cells[348] and for the AR in mouse epididymis[349] skeletal muscle myoblasts,[350] ZR-75-1 breast cancer cells[351] and LnCAP prostate cancer cells.[352] GR binding has been evaluated in mouse liver tissue,[353] mouse epithelial cells,[354] and A549 lung cancer cells.[355] We have learned that NRs bind to thousands of sites within the cellular chromatin, and that not all potential HREs in every cell demonstrate cognate NR binding. Rather, it is apparent that chromatin exhibits "pre-opened" regions destined to recruit NR.[356] For ER in MCF7, FoxA1 establishes ER and AR accessible regions; for other cells or other NRs this function might be mediated by other "pioneers", such as AP1 for GR.[354] The accessible chromatin regions are co-localized within nuclear "hubs" that seem to optimize frequency of interaction with NR.[356] ChIP-seq is also used to locate other molecules involved in chromatin remodeling and transcriptional regulation, and to examine activating or repressive histone modifications or "marks". These maps of relative locations and dynamics of NR and chromatin components greatly enhance our understanding of hormone response mechanisms.[339–342,345]

SEX STEROID RECEPTORS AND OVARIAN FUNCTION

The functions of the sex steroids and their cognate receptors in ovarian function are especially complex and multifaceted. P, T, and E2 are all synthesized and secreted by the ovary during folliculogenesis and act via both extraovarian (i.e., endocrine) and intraovarian (i.e., para-/autocrine) pathways to profoundly influence all aspects of ovarian function. The endocrine actions of the sex steroids in the hypothalamic–pituitary axis are critical to the regulation of gonadotropin secretion and the ovarian cycle and are described in more detail in other chapters of this book. In turn, our appreciation of the extent and importance of the para-/autocrine actions of sex steroids within the ovary has increased substantially over the past years. Herein, we specifically review the literature concerning the expression of the sex steroid receptors (Table 25.6) in the mammalian ovary and employ the described null and mutated mouse models that have been developed as a platform to focus on recent revelations concerning the intraovarian roles of steroid signaling in ovarian function. A caveat to experimental testing of steroid action and steroid receptor studies in a tissue such as the ovary is that the organ is also producing the steroid being studied. Androgen, estrogen, and progesterone are all synthesized by the ovary; therefore, removal and replacement studies to analyze response are impossible. Thus use of mutant or null mice allows evaluation of the phenotype reflecting loss of functional interactions between steroids and their receptors.

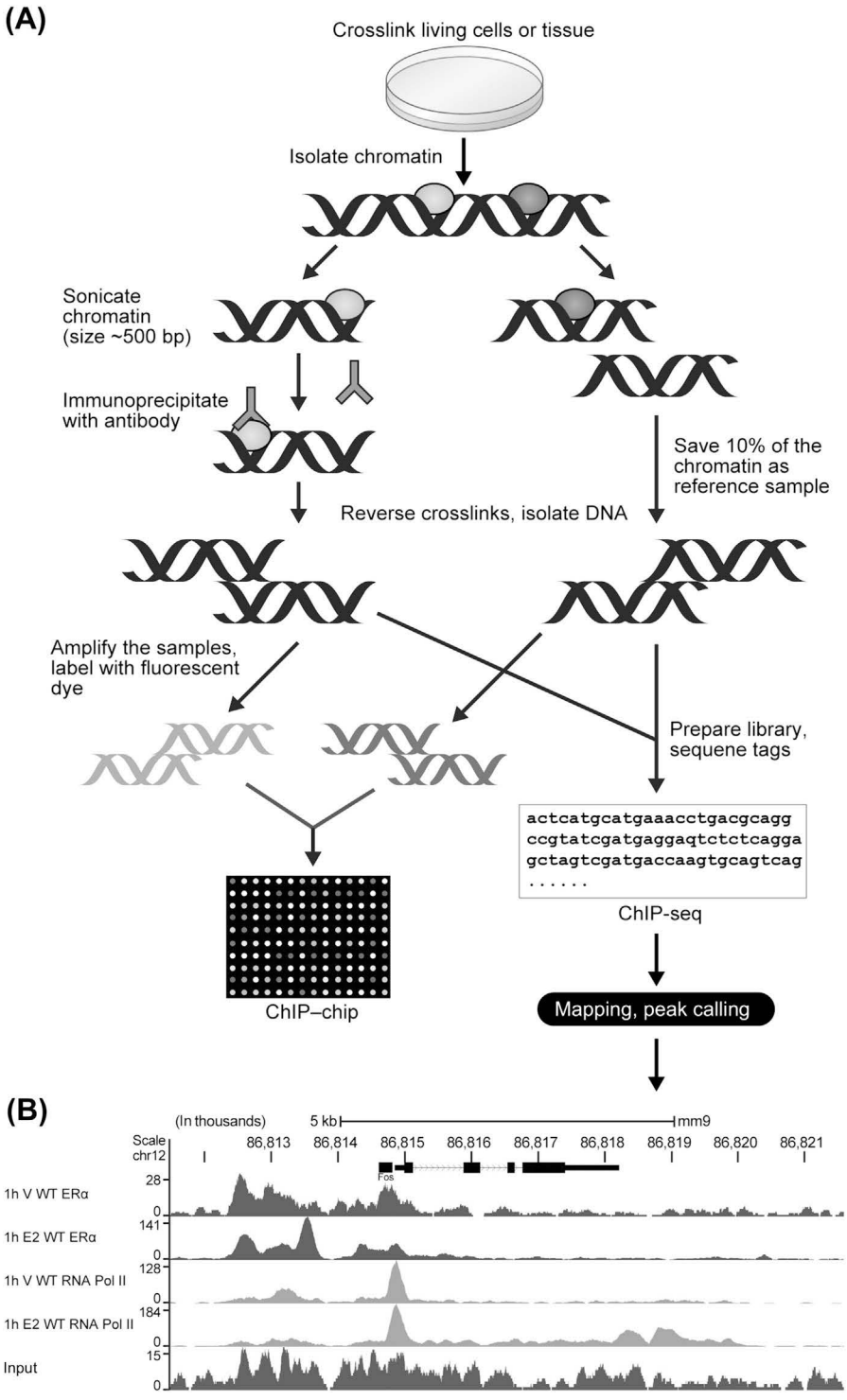


FIGURE 25.14 (A) Schematic *(Source: Modified with permission from Ref. 337)*, shows method to evaluate the "cistrome" of a steroid receptor or other chromatin interacting factors using ChIP–Chip or ChIP-seq. Cells or tissues are treated with a chemical, often formaldehyde, to cross-link DNA binding proteins to DNA. Then, chromatin (DNA and attached proteins) is isolated and fragmented with sonication or another method to break the chromatin into small pieces (approx. 500 bp). The protein of interest is immunoprecipitated with an antibody to enrich DNA fragments bound to the protein. Cross-linking is reversed to allow the enriched DNA to be purified. For ChIP–chip, DNA is amplified and labeled and hybridized to a chip spotted with promoter regions. For ChIP-seq, the DNA is sequenced using massively parallel (Deep) sequencing. The sequence "reads" are mapped onto known genomic regions, resulting in peaks indicating regions of binding of the immunoprecipitated DNA binding protein. Both methods are normalized in comparison to "input" chromatin that has not been immunoprecipitated. (B) A screenshot showing an example from ERα and RNA polymerase II cistromes in the vicinity of the Fos transcript from a mouse uterine dataset[243] is shown.

Estrogen Receptor Signaling in Ovarian Function

Estrogen Receptor Expression in the Ovary

There is a plethora of evidence for ER expression in somatic cell types of the mammalian ovary, and numerous reviews describe the importance of ERα and ERβ in ovarian function. As early as 1969, Stumpf demonstrated the specific uptake of [^{3}H]-E2 in rat ovaries using dry-mount autoradiography.[357] Richards later demonstrated that the predominance of high-affinity E2 binding sites in the rat ovary is found in the granulosa cell component and that these levels are regulated by estrogen and gonadotropins.[358,359] Successful cloning of the individual ER genes from multiple species and continued development of better ER-specific immunoglobulin allow for more detailed characterization of ERα and ERβ expression and regulation in the mammalian ovary. It should be pointed out that immunodetection and expression patterns of steroid receptors are dependent on the quality of the antibodies used. Such antibodies often vary between studies of ovarian tissue as described following and may explain both species and tissue differences.

ER expression patterns are well conserved among mammalian species, although distinct differences are apparent in late stage follicles. A high level of ERβ expression in granulosa cells of growing follicles is conserved among rodents, large animals, and primates. ERα expression is exclusive to thecal/interstitial cells in the stroma of the ovary in rats and mice (Table 25.6, mice) but this compartmental expression pattern does not hold true in hamsters, domestic animals, monkeys, or humans (Figure 25.15). In fact, current data indicate that granulosa cells of preovulatory follicles in large animals and primates express comparable levels of both ERα and ERβ, and, in humans, ERα may predominate.[360] Similarly, thecal cells in large animal and human ovaries possess both ER forms. These data force us to consider a greater potential role for ERα/ERβ heterodimers in the ovaries of large animals and primates that may not occur in rodents. The more restricted expression pattern of ERα and ERβ in rodent ovaries does not exclude possible cooperative action between the two receptors, as suggested by the unique ovarian phenotypes in compound ER-null mice compared to each single knockout ovarian phenotype (Table 25.7).[158,174] Although much has been learned concerning ER localization, it remains difficult to delineate which of the incongruent findings among species are truly representative of divergent expression patterns or are more attributable to disparities in techniques and immunoreagents. This is further confounded by evidence that levels of ER mRNA do not always correspond with the levels of immunoreactive protein within the ovarian compartments.[360] The following section reviews the literature concerning the expression patterns of ERα and ERβ in the ovaries of rodents, domestic animals, and primates, and the changes in expression patterns that occur during folliculogenesis. Altered expression has been implicated in diseases affecting ovarian function (including polycystic ovarian syndrome and ovarian cancer); however, this will not be addressed.

TABLE 25.6 Localization of Steroid Receptor Expression in the Mouse Ovary

Steroid Receptor	Granulosa	Theca	Oocyte	Stroma
ERα	+	+++	–	++
ERβ	+++	–	–	–
PRA	+++	+	–	++
PRB	+	+	–	++
AR	+++	++	+	+/–
GR	+++	++	–	ND
Aromatase	+++	–	–	–

ND: Not determined.
–: Not expressed.
+/–, +, ++, +++: Intensity of signal indicated by number of + symbols.

Estrogen Receptor α

RODENTS

ERα is localized to the ovarian interstitial/stroma, thecal cells of growing follicles, and surface epithelium in rat[129,130,274,366–372] and mouse[124,373] ovaries. This expression pattern is not evident in hamsters[374] where both receptors are detectable to varied degrees throughout the different somatic cell types of the ovary. In the fetal rat, ERα expression is detectable shortly after the first indications of gonadal differentiation.[375] Postnatal rat and mouse ovaries exhibit ERα mRNA shortly after birth, but levels remain relatively constant; whereas ERβ levels rise substantially during this period.[124,376] Immunoreactivity for ERα in neonatal rat ovaries indicates ERα expression is limited to thecal/interstitial cells and the ovarian surface epithelia and absent in granulosa cells and oocytes.[366,375] A similar pattern of ERα expression occurs in fetal hamster ovaries, which exhibit immunoreactivity as early as gestational day 14 and significant increases thereafter during the neonatal period.[377] Yang et al. report that a noticeable increase in ERα immunoreactivity occurs in the granulosa cells and oocytes of primordial follicles during neonatal days 8–15.[377]

In adult rats and mice, ERα expression continues to be exclusively localized to thecal cells of growing follicles, interstitial/stromal cells, and the ovarian surface epithelium[129,130,367,369,370,372,378,379] (Figure 25.15). Electrophoresis mobility shift assays (EMSA) of nuclear extracts from rat granulosa cells indicate that virtually all of the

TABLE 25.7 Ovarian Phenotypes in E Signaling Mutant and Null Mouse Models

Mutated or Null for Sex Steroid Signaling	Ovarian Phenotypes	Hormone Levels	References
Esr1$^{-/-}$ (homozygous null alleles for ERα animal)	Anovulatory and infertile Hemorrhagic and cystic pathology No CLs present in ovarian sections Increased expression of steroidogenic enzymes Reduced response or failure to respond to exogenous gonadotropins	Elevated T, E2 and LH Normal FSH & P	157–160
NERKI$^{+/-}$ (one mutated allele with 2-point mutation in DNA binding zinc finger of ERα and one WT allele)	Anovulatory and infertile No plugs observed in NERKI females after superovulation and natural mating Superovulation was able to partially restore ovulation in NERKI mice, however also increased hemorrhagic follicles in the ovaries	Normal LH, FSH and E2 Reduced P	132
KIKO (ER$^{AA/-}$) (one mutated allele of 2-point mutation in DNA binding domain of ERα and one ERαKO allele)	Anovulatory and infertile No CLs present in ovarian sections	Normal E2 and P	162
ENERKI (ERα^{G525L}) (homozygous animal of 1-point mutation in LBD of ERα)	Anovulatory Hemorrhagic and cystic ovarian pathology Increased number of atretic antral follicles and no CLs Hyperplastic theca cells in response to LH (data not shown)	Elevated serum E2, T and LH Normal FSH	168
ERα$^{EAAE/EAAE}$ (homozygous animal of 4-point mutation of DBD ERα)	Infertile Hemorrhagic ovaries	Not reported	164
AF2ER$^{KI/KI}$ (homozygous animal of 2-point mutation in LBD of ERα)	Anovulatory and infertile Hemorrhagic and cystic ovarian pathology No CLs present	Elevated serum LH and E2	169
Cyp17cre;ERα$^{flox/flox}$ (Theca cell specific ERα knockout)	Fertility normal in young mice, but 6 month old animals have reduced fertility and longer estrous cycle	Elevated T at both 2 & 6 months Normal FSH Decreased LH at 2 months with further decrease at 6 months	361,362
Esr2$^{-/-}$ (homozygous null alleles for ERβ: βERKO, Ex3βERKO, and **ERβ$_{ST}^{L-/L-}$)	Subfertile although some lines are infertile Reduced (or lack of) response to exogenous gonadotropins Approximately 1/5 the number of large preovulatory follicles after superovulation compared to WT mice Lack of COC expansion	Normal LH and FSH	158,171–173,363
ERαAF-1^0	Ovarian phenotype not reported	Unknown	165
ERαAF-2^0	Ovarian phenotype not reported	Unknown	167
αβERKO	Anovulatory and infertile No CLs and few large follicles Ovarian transdifferentiation to Sertoli-like cells that express Sox9 Altered expression of steroidogenic enzymes	Elevated LH and T Normal FSH and P	158,174
Cyp19a1$^{-/-}$	Anovulatory and infertile Hemorrhagic and cystic pathology No CLs present in ovarian sections Poor response to exogenous gonadotropins, although treatment with E2 in addition to gonadotropins improves ovulatory response Ovarian transdifferentiation to Sertoli-like cells that express *Sox9*	No E2 Elevated LH, FSH and T	131,156,175,194,364,365

***ERβ$_{ST}^{L-/L-}$ females are the only line of ERβ knockout animals that are sterile.*

specific ERE-bound complexes are supershifted by anti-ERβ but not anti-ERα immunoglobulin, providing further support that rat granulosa cells possess very little ERα.[367,371] Western blot analyses of whole cell or nuclear extracts from isolated rat granulosa cells do indicate a low level of ERα protein of 61 kDa,[371] and use of laser capture microdissection (LCM) shows some expression of ERα mRNA in granulosa cells (Figure 25.15).[363] Unlike ERβ, ERα expression remains relatively constant throughout the rat estrous cycle.[380] Furthermore, no change in ERα levels is detected in granulosa cells cultured in FSH/T over a period of 72 h; however, a 24-h treatment with forskolin induces a marked increase in ERα immunoreactivity in the nuclei.[371]

ERα localization in the adult hamster ovary is somewhat divergent from that in rats and mice. Yang et al. report moderate clear ERα immunoreactivity in thecal/interstitial cells, but also appreciable immunoreactive granulosa cells of small preantral follicles.[374] In the granulosa cells of antral follicles, ERα immunoreactivity is strongest among those in proximity to the forming antrum.[374] FSH treatment (twice a day for 2 consecutive days) or a single injection of E2 elicits a significant induction of ERα expression in both thecal and granulosa cells in hypophysectomized hamsters.[374] Still, Western blot analyses of whole ovarian homogenates from hamsters indicate an ERβ:ERα ratio of 14:1 during the follicular phase, indicating that ERβ predominates. However, the rapid decline in ERβ and concurrent increase in ERα that occurs just prior to and shortly after the gonadotropin surge adjusts this ratio to 2:1, suggesting that ERβ plays a more predominant role in follicular growth and ERα is more important during luteinization[374] in the hamster ovary.

DOMESTIC ANIMALS

In fetal bovine ovaries, ERα was shown to be localized in all cell types involved with follicle development, including granulosa cells, oocytes, and epithelial cells throughout development, with expression becoming

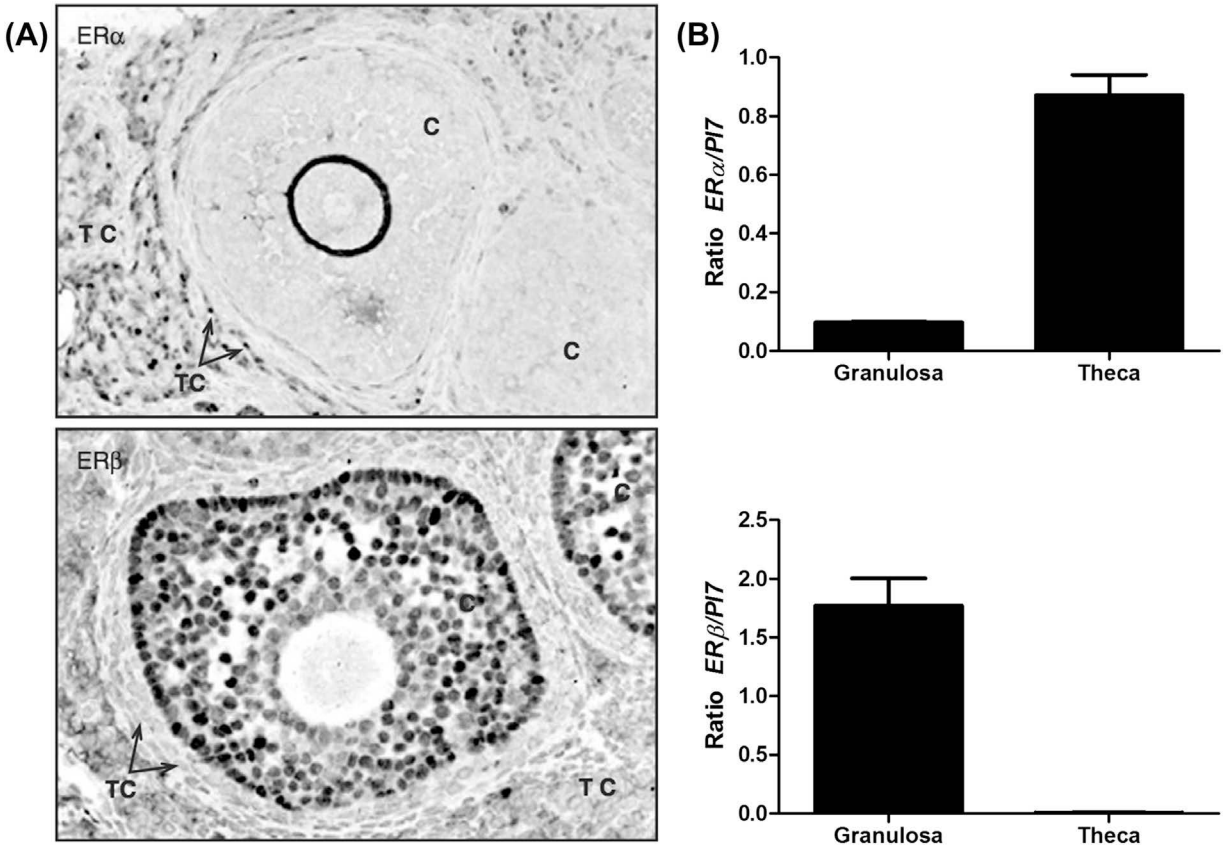


FIGURE 25.15 (A) Immunohistochemistry for ERα and ERβ in adult mouse ovary. Immunohistochemistry for ERα (*top*) indicates specific nuclear immunoreactivity in thecal/interstitial cells and thecal cells (TC) in and around a preantral follicle. Granulosa cells lack any measurable immunoreactivity for ERα. The staining around the outer surface of the oocyte is nonspecific and not representative of ERα immunoreactivity. Immunohistochemistry for ERβ (*bottom*) indicates specific nuclear immunoreactivity in the granulosa cells (GC) throughout a preantral follicle. Thecal cells (TC) lack any measurable immunoreactivity for ERβ, but the surrounding thecal/interstitial cells exhibit some cytoplasmic staining. Immunohistochemistry was done by Dr. Madhabananda Sar (see Ref. 366 for protocol). (B) Pure populations of granulosa and theca cells were isolated from large preovulatory follicles using laser capture microdissection (LCM), and RNA was reverse transcribed and real time PCR was performed using primers specific for ERα and ERβ.

more localized to thecal and some stromal cells during the later stages of development.[381] In adult bovine ovaries, ERα immunoreactivity is relatively high in thecal cells of secondary and tertiary follicles, substantially weaker in granulosa cells of tertiary follicles, and totally absent in primordial, primary, and secondary follicles.[382] In agreement, Barisha et al. found ERα transcripts are detectable in both granulosa and thecal cell fractions, but levels are substantially higher in the latter cell type.[383] Furthermore, thecal ERα expression appears to be differentially regulated as it is almost four-fold higher in follicles of 20–180 mm versus those <0.5 mm, whereas ERβ expression in thecal cells remains constant among follicles of different sizes.[383] ERα expression in granulosa cells, although much lower relative to theca, also increased with advanced follicle size.[383] In porcine ovaries, ERα immunoreactivity is detected in both theca interna and granulosa cells, but only in large follicles; and even then ERα immunoreactivity is considerably moderate relative to ERβ.[384] In ovine ovaries, ERα mRNA and immunoreactivity is detected in both cell types but predominates in granulosa cells and is particularly high in cumulus oophorus cells of small antral follicles.[385]

PRIMATES

The first reported study of ERα immunoreactivity rhesus or cynomolgus monkey ovaries found that ERα was undetectable in all ovarian cell types except the surface epithelia, regardless of menstrual stage.[386] However, a later study in baboon ovaries using the same antibody found nuclear ERα immunoreactivity in 30–40% of the granulosa cells of healthy antral follicles, and detectable but less intense staining in granulosa cells of preantral follicles.[387] This same study found the stroma, interstitial, and thecal cells to be largely unlabeled.[387] Pau et al. produced similar findings of ERα expression in the granulosa cells of rhesus monkey ovaries by in situ hybridization.[388]

High-affinity E2 binding sites in normal human ovarian tissue were first described several decades ago,[389–391] but these techniques were not able to differentiate between the two ER forms. Iwai et al. used anti-ERα antisera similar to that in the nonhuman primate studies to demonstrate immunoreactivity in the granulosa cells of antral and preovulatory follicles in human ovary but a total absence in primordial, preantral follicles, atretic follicles, and thecal/interstitial cells.[392] Pelletier and El-Alfy[393] used a different antihuman ERα antisera and reported results that are in contrast to those of Iwai et al.,[392] i.e., a total lack of ERα immunoreactivity in granulosa cells but clear nuclear staining of theca interna cells, interstitial gland cells, and ovarian surface epithelia. A possible explanation for this discrepancy may be a lack of large antral follicles in samples evaluated by Pelleteir and El-Alfy since both studies agree that ERα is not detectable in the granulosa cells of less mature follicles. In support of this explanation is a report by Taylor and Al-Azzawi in which ERα immunoreactivity is illustrated in the granulosa cells of what is clearly a large antral follicle when using antisera raised against the bovine ERα.[394] Indeed, Saunders et al. described ERα immunoreactivity that is limited to granulosa cells of large antral follicles and undetectable in smaller follicles in the human ovary.[395] Also, in agreement with Pelletier and El-Alfy,[393] Saunders et al. found ERα immunoreactivity in thecal cells of preantral and antral follicles, and ovarian surface epithelia.[395]

ERα transcripts are detectable in human nonluteinized granulosa cells[396] and granulosa-luteal cells collected at the time of oocyte retrieval during in vitro fertilization (IVF) procedures.[396,397] In fact, Jakimiuk et al. found ERα mRNA and protein levels are two- to four-fold higher in granulosa versus thecal cells of small antral follicles in human ovaries.[360] Furthermore, ERα protein levels remain elevated in granulosa cells but decrease in thecal cells of dominant follicles.[360] In luteinized human granulosa cells, Chiang et al. found ERα levels to remain relatively constant over a period of 10 days in culture but always much lower than ERα mRNA levels.[398] Recent reports in human ovarian samples collected during elective hysterectomy demonstrate that ERα is expressed in granulosa cells of antral follicles, with weak to no positive immunohistochemical staining in luteinized granulosa cells[399] supporting previous data.

Estrogen Receptor β

In the nonpregnant ovaries of most mammalian species, ERβ is clearly the predominant ER form and is most often exclusively localized to granulosa cells of follicles from the primary to preovulatory stage. This expression pattern is documented in the ovaries of rats,[14,130,366,367,370–372,380,400–402] mice,[124,373,379,402] hamsters,[374] cows,[383,403,404] sheep,[405,406] pigs,[402,407] nonhuman primates,[274,388] and humans.[393,394,402,408,409]

RODENTS

In developing rat ovaries, ERβ expression is detectable shortly after the first indications of gonadal differentiation on gestational day 14, and levels increase thereafter during prenatal development.[375] A second, more robust increase in ERβ expression occurs during days 10–15 of neonatal development in rat ovaries,[376] and immunoreactivity is largely localized to granulosa cells of growing follicles and notably absent in primordial follicles and oocytes.[366,375] Developing mouse ovaries exhibit a comparable pattern ERβ expression.[124] In contrast, ERα is limited to thecal and interstitial cells[366,375] and exhibits little change in levels with increased age[124,376] in developing rat and mouse ovaries. The hamster ovary exhibits a fairly similar ontogeny as ERβ is detectable as early

as gestational day 13 and increases thereafter to peak on postnatal day 10, throughout which ERβ immunoreactivity is predominantly localized to granulosa cells but detectable in some interstitial cells and oocytes.[377]

In the ovaries of prepubertal and adult rats and mice, ERβ immunoreactivity is nearly exclusive to the nuclei of granulosa cells of healthy follicles at all advanced stages of folliculogenesis[124,129,130,366–370,374,402] and is notably decreased or absent in atretic follicles[129,366] (Figure 25.15). Primordial follicles, thecal cells, oocytes, ovarian surface epithelium, and luteal cells lack ERβ immunoreactivity in adult rat and mouse ovaries,[129,130,366–369,374,402] although some cytoplasmic staining in the latter cell type is reported when using certain antisera.[129,366] Granulosa cell specific expression of ERβ in the rat ovary was reproduced using three separate anti-rat ERβ antisera, two raised against residues 467–485 and a third raised against residues 54–71.[369] Furthermore, independent studies using in situ hybridization produced results congruent with the immunohistochemical findings of strict localization of ERβ mRNA to granulosa cells of healthy growing follicles,[14,129,375,401] although Bao et al. reported scattered but specific hybridization for ERβ mRNA in thecal cells of healthy medium and large follicles in the rat ovary.[400]

A study by Saunders et al.[370] stands in contrast to those just discussed. In this study, strong nuclear immunoreactivity is described throughout the different somatic cell types of the rat ovary, including thecal, interstitial, and luteal cells, as well granulosa cells of maturing follicles when using an anti-rat ERβ antisera raised against residues 196–213. Yang et al. reported similar findings of ERβ immunoreactivity in thecal/interstitial cell preparations from hamster ovary using different ERβ-specific antisera.[374] Still, the results of Saunders et al. are problematic because they include reports of substantial ERβ immunoreactivity in certain reproductive tissues of the rat, such as the oviduct and uterus,[370] that are otherwise thought to possess relatively low or null levels of ERβ mRNA and immunoreactivity. Two obvious differences between the immunohistochemical study of Saunders et al.[370] and others are the aforementioned use of different antisera as well as different tissue fixative.

The previous discrepant findings notwithstanding, ERβ is clearly the predominant ER form present in granulosa cells of healthy growing follicle in the rodent ovaries. Recent isolation of pure granulosa and theca cells from mouse ovaries using laser capture microdissection demonstrates that ERβ mRNA expression is solely in granulosa cells (Figure 25.15). Any specific distribution of ERβ throughout the subpopulations of granulosa cells of growing follicles has been difficult to ascertain. In general, ERβ immunoreactivity is continuous and strong among the mural cells and relatively uniform among those layers closer to the antrum and oocyte.[366,367,369] However, not all granulosa cells of healthy growing follicles in the rodent ovary express ERβ, as negative cells are randomly distributed throughout the follicle.[366,367]

Data from Western blots of nuclear extracts from whole ovaries or isolated granulosa cells support the immunohistochemical data that ERβ is the predominant receptor form in rodent granulosa cells.[367,371] Electrophoresis mobility shift assays in which whole cell extracts from ovaries or isolated granulosa cells of rats provide further evidence of the predominance of ERβ.[367,371,410] Sharma et al. found that antisera raised against residues 54–71 of rat ERβ produces the optimum results on Western blot and detects four immunoreactive bands of 58/52 to 46/44 kDa in size, the 58 kDa protein presumably being full-length ERβ (ERβ1 in Figure 25.2).[371] Antisera raised against the AF-2 (residues 182–485) domain of rat ERβ detects a single specific protein of 60 kDa in whole-cell extracts from both rat and mouse ovaries that comigrates with the putative full-length ERβ.[367] Choi et al.[402] and Hiroi et al.[368] produced comparable findings of immunoreactive proteins at 60 and 55 kDa in rat and mouse ovarian extracts when using either a monoclonal antibody mapped to residues 272–285 of human ERβ or antisera raised against C-terminal residues 467–485 of rat ERβ, respectively. In the hamster, a single ERβ-immunoreactive protein of 54 kDa is detected.[374] To date, ERβ antibodies are not as specific as those manufactured for ERα, and produce bands in the ERβ-null tissues similar to those observed in WT tissues (unpublished data from Korach laboratory) such that further characterization of ERβ localization is challenging.

Several different ERβ isoforms are present in rodent ovaries, notably ERβ2 (Figure 25.2), ERβ-Δ3, and ERβ2-Δ3, the latter being a compound form of the two former variants. ERβ-Δ3 is an exon 3 deletion that results in a receptor lacking the C-terminal zinc-finger of the DNA binding domain and therefore unable to bind an ERE.[193] ERβ2 is an especially interesting variant because it possesses an insert of 18 amino acids in the N-terminal region of the LBD (Figure 25.2) that causes a 35-fold reduction in affinity for E2 and a 1000-fold decrease in E2-induced transactivational activity in vitro.[193] This isoform is not found in human ovary (Figure 25.2). Pettersson et al.[193] speculate that ERβ2 may be especially attuned to mediating E2 actions within healthy growing follicles where intrafollicular E2 levels greatly exceed that required to activate wild-type ER forms. Differential RT-PCR indicates a 1:1 ratio of ERβ1:ERβ2 mRNA in the ovary of adult[371,410,411] and neonatal rats,[323] while transcripts encoding ERβ1-Δ3 or ERβ2-Δ3 variants are detectable but at substantially lower levels.[410,411] Pettersen et al. demonstrated that FLAG-tagged clones of ERβ1 and ERβ2 expressed in 293T cells co-migrate at approximately 60 kDa,[411] suggesting that resolution of the two isoforms by Western blot may be difficult. However, several studies illustrate

two distinct ERβ-specific bands appear on Western blots from rat[367,368,371,410] and mouse[402] ovarian extracts. Furthermore, O'Brien et al. fractionated rat granulosa cell preparations in a 7.5% SDS-PAGE from which immunoreactive protein bands presumed to be ERβ1 (60 kDa) and ERβ2 (62 kDa) were resolved; they further remarked that an equal ratio of ERβ1:ERβ2 transcripts does not correlate to actual protein amount by Western blot, as much greater amounts of ERβ1 protein are present.[410] In retrospect, previous [^{3}H]-E2 binding data also supports a predominance of ERβ1 versus ERβ2 in rat granulosa cells. Because recombinant ERβ1 and ERβ2 differentially bind E2 with affinities of 0.14 and 5.1 nM, respectively,[411] one would expect differentiation of the two forms when assessed by Scatchard plot analysis. Therefore, the earlier studies using [^{3}H]-E2[412] and more recent studies using [^{125}I]-17α-iodovinyl-11β-methoxyE2[367] that report a single, saturable, high-affinity binding factor with a $K_d = 0.4$ nM in rat ovarian or granulosa cell extracts are congruent with ERβ1 as the predominant form present. Similar data of a single E2 binding component with a $K_d = 1$–1.4 nM in hamster ovaries is also reported.[413,414]

The majority of studies on the regulation of β expression during folliculogenesis focus on the rat ovary. While some report high but relatively constant levels of ERβ mRNA leading up to proestrus,[380] more detailed studies indicate a substantial increase in ERβ expression that peaks in medium sized (275–450 mm) follicles possessing clearly defined antrum.[400] Most studies agree on the precipitous decline in ERβ levels that occur shortly after the ovulatory gonadotropin surge and the low levels that remain during estrus in rats[380] and hamsters[374] (Figure 25.16). In retrospect, it is evident that the decreasing effect of the gonadotropin surge on ERβ levels was first indicated by Richards in 1975, in which a 75% decrease in high-affinity E2 binding sites in granulosa cells of hypophysectomized, FSH/E2-primed rats was found to occur following a single LH treatment.[358] This effect of LH on ERβ expression has been reproduced in vivo in gonadotropin-primed rats[367,380] and mice[415] in which a single hCG injection led to a rapid decrease in ERβ mRNA and protein levels of more than 50% within 6–9 h (Figure 25.16). Similarly, granulosa cells isolated from pregnant mares serum gonadotropin (PMSG)-stimulated rats and exposed to hCG in vitro exhibit a comparable loss of ERβ protein when assessed by western blot[367] or EMSA.[371]

Therefore, induction of the LH-signaling pathway in differentiated granulosa cells is the primary stimulus for rapidly decreased ERβ expression. Evidence of a direct role of LH comes from findings that only preovulatory follicles that co-express LH receptor exhibit a decline in ERβ expression; while smaller, LH-receptor negative follicles maintain ERβ expression as late as 24 h post-hCG exposure.[367,380] The inhibitory effect of LH on ERβ

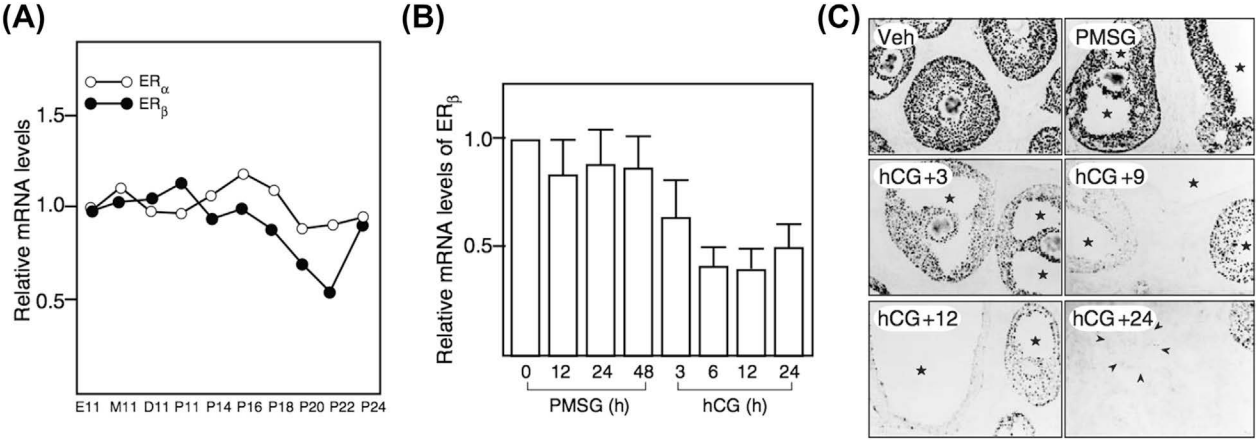


FIGURE 25.16 Regulation of ERβ expression in adult rat ovaries during the estrous cycle or after exogenous gonadotropin treatments. (A) Quantification of gene expression from Northern blot analysis (not shown) for ERα and ERβ in adult rat ovaries during the estrous cycle. Band intensities were measured on a phosphorimager and normalized to an S16 internal control for each time point. mRNA levels are shown relative to the level at E1100 (set to 1.0). Sera from animals were used to determine LH concentrations; the onset of the LH surge was observed at 1600 h of proestrous, and the peak was observed at 1800 h of proestrous. Annotation at bottom indicates the estrous cycle stage and hour of tissue collection: E, estrous; M, metestrous; D, diestrous; P, proestrous. *(Source: Reproduced with permission from Ref. 380.)* (B) Reverse transcriptase polymerase chain reaction was used to quantify ERβ mRNA levels in the ovaries of immature rats after treatment with PMSG alone or PMSG for 48 h followed by hCG. The ratio of ERβ/S16 of control rats with no hormonal treatment was set to 1.0. Shown are the mean ± SE ($n = 4$). *(Source: Reproduced with permission from Ref. 380)* (C) ERβ immunoreactivity in rat ovaries treated with vehicle (Veh), PMSG for 48 h, or PMSG for 48 h followed by hCG. ERβ immunoreactivity is detected in granulosa cells of small and large antral follicles in Veh PMSG animals. Stars indicate the location of antra in antral follicles. PMSG-treated rats were injected with an ovulatory dose of hCG and ovaries isolated after 3, 9, 12, or 24 h. The expression of ERβ protein in granulosa cells 9 h after hCG is reduced in large antral follicles (*left*), greatly reduced in preovulatory follicles (*center*), but does not change in small antral follicles (*right*). Similar expression is observed 12 h after hCG administration. One day (24 h) after hCG treatment, ERβ expression is not detected in corpora lutea (*small arrowheads*) but is highly expressed in preantral (*right*) and small antral follicles. Magnification 250×. *Source: Reproduced with permission from Ref. 367.*

expression can be reproduced by exposing differentiated granulosa cells to either forskolin or TPA,[380,410] activators of the protein kinase-A and protein kinase-C pathways, respectively, and thought to mimic the effects of LH stimulation.[416] Furthermore, forskolin or TPA treatment reproduce the same temporal pattern of decreased ERβ expression that follows LH or hCG exposure,[417] suggesting a common mechanism. Guo et al. demonstrated both activators and mimics of the LH-signaling pathway elicit the rapid decline in granulosa cell ERβ levels by decreasing the stability of ERβ transcripts rather than via a repression of gene expression.[417]

In contrast to the peri-ovulatory decrease in ERβ expression that occurs, there is little known about the positive regulation of ERβ expression in rat granulosa cells. Induction of folliculogenesis by PMSG or FSH in hypophysectomized rats leads to a significant rise in E2 binding among granulosa cells, presumably representative of ERβ,[358,418] but this is likely due more to an increased number of granulosa cell population rather than direct gonadotropin-stimulated ERβ expression. The presence of ERβ in the granulosa cells of primary follicles, considered to be insensitive to direct gonadotropin stimulation, supports a minor role for FSH in ERβ expression.[366,375] Furthermore, ERβ levels exhibit little change in immature rat or mouse ovaries 48 h after a single injection with PMSG,[367,380,415] and no reports exist of altered ERβ expression in the ovaries of mice null for FSH signaling.[419–421] Sharma et al. found that ERβ protein levels in gonadotropin-primed rat granulosa cells evaluated are highest if assessed after isolation and decrease steadily to undetectable levels within 72 h in culture, even in the presence of FSH and T.[371] However, this pattern was not totally reproduced at the level of ERβ mRNA, which drops by only 55% when cultured for the first 24 h in the absence of hormones but stabilizes upon the addition of FSH and T,[371] suggesting that FSH may be more important to the maintenance rather than stimulation of granulosa cell ERβ expression. In contrast to the previous findings, preovulatory rat granulosa cells allowed to lose ERβ expression following long-term (6 day) culture exhibit a significant rise in levels 48 h after exposure to forskolin, a direct PKA activator and thought to mimic FSH signaling.[371] Interestingly, FSH may have a greater positive influence on ERβ expression in the hamster ovary where a substantial increase in expression in preantral and antral follicles is observed in hypophysectomized females following 1–2 days of treatment with ovine FSH;[374] and ovaries of neonatal hamsters in which prenatal FSH action is inhibited exhibit significant decreases in ERβ expression.[377]

The existing evidence of E2 regulation of ERβ expression is conflicting. Treatment of hypophysectomized rats with E2 for 3–4 days results in steady and dramatic increases in E2 binding sites in granulosa cells,[358,422] but once again this is more likely due to an increased granulosa cell population. However, Tonetta et al. demonstrated that FSH-induced increases in E2 binding sites in granulosa cells of hypophysectomized rats are blocked by co-administration of an ER antagonist, suggesting an autoregulatory element for ER expression.[418] In contrast, Kim and Greenwald found little change in the number of E2 binding sites in hamster ovaries following E2 or DES treatment, although animals were exposed for only 1–2 days, and therefore significant gains in granulosa cell number may not have been achieved.[413] Drummond et al. reported that 1–4 days of treatment with DES has no discernible effect on the levels of ERβ1 or ERβ2 transcripts in immature rat ovaries.[376] Yang et al. found that hypophysectomized hamsters exhibit an almost six-fold increase in ERβ expression as detected by immunohistochemistry 24 h after a single injection of 0.1 mg E2-valerate.[374] Furthermore, rat granulosa cells isolated from E2-primed versus untreated animals exhibit a four- to six-fold higher basal estrogenic activity on an ERE-luciferase reporter construct, suggesting that individual cellular levels of ERβ are increased by E2 treatment.[371] Also, isolated rat granulosa cells exhibit an increased nuclear intensity for ERβ immunoreactivity 1.5 h after E2 exposure that is maintained for 24 h but totally lost by 48 h.[371] Still, the failure of granulosa cells to totally sustain ERβ expression when maintained in FSH and T, and therefore capable of synthesizing endogenous E2, is puzzling. It is plausible that E2 regulation of ERβ expression in granulosa cells may require the actions of ERα in thecal cells, which cannot be mimicked when granulosa cells are isolated in culture. Preservation of ERβ expression in preantral follicles of ERα-null ovaries argues against this hypothesis.[127,373] Recent work in KGN cells suggest that GIOT-4, a cofactor regulated by FSH, may act to increase ERβ expression as well as work in combination with ERβ to regulate target genes in follicles.[423] Although this work was done primarily in KGN ovarian cells, it was also reported that GIOT-4 and ERβ are co-localized in growing follicles providing support for the co-regulation. The expression of GIOT-4 has not been examined in in vitro–cultured granulosa cells. Therefore, although the signaling factors that may be most important to inducing ERβ expression remain unknown, it is clear that the level of ERβ is highly dependent upon the state of granulosa cell differentiation as cells from primary, growing, and preovulatory follicles exhibit divergent expression patterns and mechanisms of regulation.

DOMESTIC ANIMALS

Descriptions of ERβ expression in the ovaries of domestic animals are limited but generally indicate patterns that are congruent with rodent ovaries. In the fetal bovine ovary, ERβ is localized in the cell types associated with follicle growth, predominantly in pregranulosa

and granulosa cells. In early development, epithelial cells show expression; however, this becomes punctate as follicles begin to develop.[381] In adult bovine ovaries, Rosenfeld et al. found that ERβ mRNA and immunoreactivity is restricted to granulosa cells of small and large antral follicles with no detectable levels in thecal cells.[403] A follow-up study further demonstrated substantial ERβ immunoreactivity in cumulus oophorus and antral granulosa cells relative to mural granulosa, as well as mentioned significant ERβ expression in oocytes,[404] the latter finding being common only to hamsters.[374,377] In contrast to the immunohistochemical data, Walther et al.[424] and Berisha et al.[383] found ERβ transcripts are detectable in both granulosa and thecal cells. In ovine ovaries, ERβ is highly expressed in granulosa cells of growing follicles,[405,406] and levels exhibit little change over the course of the estrous cycle.[406] However, Jansen et al. report via in situ hybridization that ERβ mRNA is greatest in follicles ≤3 mm and declines in larger follicles during the early follicular phase.[405] Both reports describe low but detectable ERβ expression in thecal cells of growing follicles in the sheep ovary[405,406] as well as in ovarian surface epithelia.[406] In porcine ovaries, ERβ is almost equally expressed among the granulosa and theca interna of medium and large follicles, as well appreciable detection in the oocyte and ovarian surface epithelia.[384] Like the rodent, atretic follicles in porcine ovaries exhibit reduced ERβ expression.[384] LaVoie et al. found that ERβ mRNA levels in whole ovarian lysates exhibit little change during the porcine estrous cycle and are relatively equal when comparing follicles of 1–5 mm in size.[407]

An interesting commonality among bovine, ovine, and porcine ovaries is the presence of a truncated ERβ isoform lacking most of the ligand binding domain, termed ERβΔLBD in the cow[424] and ERβ-Δ5 in sheep and pig[406,407] (Figure 25.2). In all three species, ERβ-Δ5 is a deletion of exon 5 and encodes a receptor isoform that is truncated 15 residues into the LBD and terminates with four to nine heterologous residues depending on the species.[406,407,424] LaVoie et al.[407] and Choi et al.[402] produced Western blot evidence that ERβ-Δ5 may indeed be translated as a 35 kDa protein in porcine ovaries. Given that ERβ-Δ5 lacks the LBD, it predictably exhibits little estrogen-induced transactivational activity when overexpressed in vitro but does possess measurable ligand-independent transactivational activity that is three-fold lower than full-length ERβ, suggesting preservation of AF-1 functions.[407] ERβ-Δ5 is also able to reduce the in vitro activity of full-length ERβ by approximately 40% when present in excess.[407,424] Thus ERβ-Δ5 might modulate ERβ activity in an environment of high hormone concentrations, such as is found with estrogens in granulosa cells.

PRIMATES

In ovaries of known fertile cynomolgus monkeys, Pelletier et al. localized ERβ expression via in situ hybridization to granulosa cells of follicles at all different stages of development, as well as substantial labeling in theca interna and ovarian surface epithelia.[154] Similar findings are described by Pau et al.[388] in granulosa cells of large follicles in rhesus monkey ovary. In the baboon ovary, Pepe et al. found ERβ immunoreactivity is abundant in granulosa cells of follicles at all stages, including large antral follicles but notably lacking in thecal cells.[425] Western blots of baboon ovarian homogenates indicate two specific ERβ protein bands at 55 and 63 kDa.[425] An extensive immunohistochemical study in marmoset ovaries using antisera raised against human ERβ found extensive immunoreactivity in granulosa cells of primary through to mid- and late follicular stage follicles, including those with a large antrum, but a clear lack of staining in atretic follicles.[395] No specific pattern of ERβ immunoreactivity was apparent among subpopulations of granulosa cells within any one follicle.[395] In contrast to baboon ovaries but in agreement with cynomolgus monkey ovaries, thecal cells of large growing follicles as well as the ovarian surface epithelia in marmoset ovaries possess appreciable levels of ERβ immunoreactivity.[395]

High-affinity E2 binding sites in normal human ovarian tissue were described several decades ago.[389–391] Al-Timimi et al. found 45 of 89 normal ovaries from premenopausal women to possess detectable E2 binding sites; whereas all of the ($n = 10$) postmenopausal ovaries assayed were devoid of detectable binding.[390] Vierikko et al. reported high-affinity E2 binding in a similar percentage of premenopausal ovaries but did find 67% of postmenopausal samples were also positive, although the levels of E2 binding in both are notably lower than that for P.[391] The advent of better reagents has allowed for differentiation of the two ER forms within human ovaries. ERβ expression was first detected in human ovary by Northern blot analysis.[15,408,426,427] Follow-up studies using RNase-protection assays or RT-PCR indicate a relatively equal ratio of ERα:ERβ transcripts in mixed-cell homogenates from ovaries of pre-and postmenopausal women but exclusively ERβ transcripts in luteinized granulosa cells isolated from women undergoing IVF.[408,427] Hillier et al. report equal levels of ERα and ERβ mRNA in both nonluteinized and luteinized granulosa cells from normal human ovaries, although levels of both ER forms are reduced in the latter.[396] Another study comparing ERβ mRNA and protein levels within isolated thecal and granulosa cells of small antral follicles found mRNA levels are two-fold higher in thecal cells but protein levels are similar in both cell types.[360]

Three reports describing immunohistochemical localization of ERβ in human ovaries produced comparable findings of specific immunoreactivity among granulosa cells of follicles from primary to late antral stage, and specific, but considerably lower, levels in thecal cells of preantral and antral follicles.[393–395] Furthermore, all three demonstrate substantial ERβ immunoreactivity in human ovarian surface epithelia.[393–395] Hillier et al. also found

ERβ transcripts are detectable in primary cultures of human ovarian surface epithelial cells;[396] however, similar evaluations in cell lines derived from human ovarian surface epithelium indicate no detectable ERβ mRNA.[427]

There is currently little known about ERβ regulation in human ovaries. Jakimiuk et al. found ERβ expression is significantly decreased in thecal and granulosa cells of dominant follicles relative to those at earlier stages, yet ERβ protein levels did not mirror this difference.[360] In primary cultures of human granulosa–luteal cells, ERβ is more highly expressed relative to ERα and levels of the former increase 1.6-fold over a 10-day period during which spontaneous luteinization is believed to occur, while ERα levels remain constant throughout.[398] Treatment of human granulosa-luteal cells maintained in culture for 7 days with hCG reduces ERβ and ERα expression by almost 50% within 24 h, and this effect is mimicked by activators of protein kinase A (forskolin) or protein kinase C (TPA).[398] Recent examination of human ovaries found that ERβ1 and ERβ2 are expressed in granulosa-luteal cells as well as endothelial cells at all stages of the luteal phase. This study also used in vitro luteinized granulosa cells, treated with hCG or E2. Treatment of the luteinized granulosa cells with hCG reduced expression of ERα, ERβ1, and ERβ2 by approximately 50%, while treatment with E2 reduced expression of ERα and ERβ1 with no significant change in ERβ2,[399] suggesting that human granulosa cells may respond to LH by reducing ERβ in a manner similar to that found in rodent ovaries.

Studies to date indicate that human tissues express a unique C-terminal variant of ERβ termed $ERβ_{cx}$[426] or human ERβ2[409,428] (Figure 25.2). $ERβ_{cx}$ is a 495–amino acid–long receptor form in which the 61 C-terminal residues are replaced by a heterologous string of 26 amino acids encoded by an alternative downstream exon.[426,428] Transcripts encoding $ERβ_{cx}$ are detectable in multiple human tissues but most especially in ovary, testis, thymus, and spleen and are often at levels equal to or greater than wild-type ERβ.[426,428] Immunohistochemistry employing $ERβ_{cx}$-specific antisera indicate the variant is nuclear localized in granulosa cells of early follicles.[409] The physiological function of $ERβ_{cx}$ remains unclear. It is unable to bind E2, but in vitro studies indicate the isoform may preferentially heterodimerize with ERα and act as a dominant negative modulator of ERα action. Rodents also possess an ERβ variant (ERβ2) that poorly binds E2, but this receptor form does not inhibit wild-type ERα or ERβ activity.[193] Furthermore, categorical studies have shown that the rodent ERβ2 variant is not detected in human tissues.[409,429]

Ovarian Phenotypes in Mouse Models of Disrupted Estrogen Signaling

MICE LACKING ERα

Several lines of ERα-null mice have been characterized as listed in Table 25.7. Neonatal and prepubertal ERα-null mice exhibit relatively normal ovaries except for signs of premature folliculogenesis as indicated by the sporadic presence of large antral follicles.[158,373] Adult ERα-null females are anovulatory and hence infertile, and exhibit ovaries that possess normal pre- and small antral-stage follicles, multiple hemorrhagic cysts, absence of corpora lutea, reduced number of interstitial glandular cells,

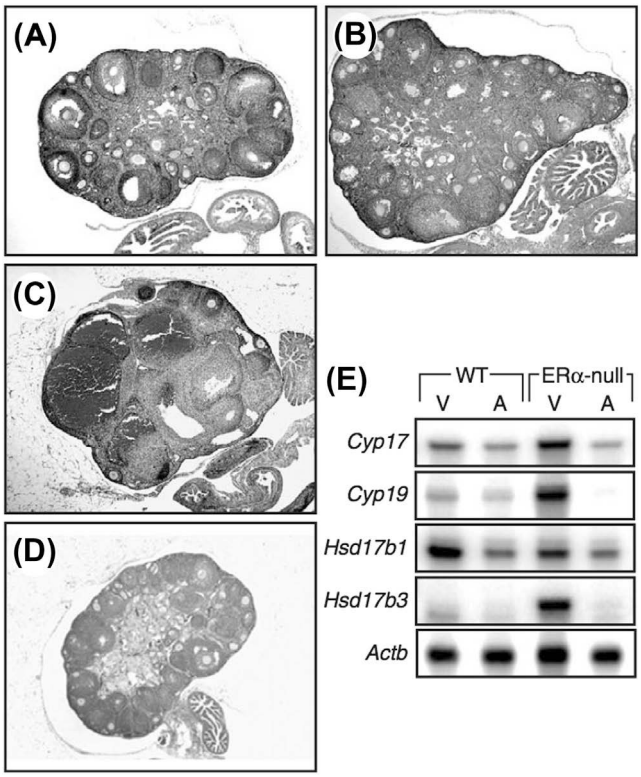


FIGURE 25.17 Ovarian phenotypes in ER-null mice. (A–C) Shown are cross-sections from representative adult ovaries of wild-type (A), ERβ-null (B), and ERα-null (C) female mice. Wild-type and ERβ-null ovaries each exhibit all stages of folliculogenesis except corpora lutea, although large antral follicles are sparse in the ERβ-null ovary. In contrast, ERα-null ovaries are characterized by several large, hemorrhagic, and cystic follicles; a sparse number of follicles at the early stages of proliferation; and a lack of corpora lutea. (D) Cross-section of a representative ERα-null ovary after prolonged treatment with a GnRH antagonist. Circulating LH levels were reduced in ERβ-null females by treatment with a GnRH antagonist (60 μg Antide) every 48 h from the age of 28–53 days. Ovaries were collected within 24 h of the final treatment. The characteristic ovarian phenotypes of ERα-null mice (shown in C) are prevented by reducing circulating LH levels, indicating these more dramatic phenotypes are secondary to the loss of ERα actions in the hypothalamic pituitary axis that are necessary to maintain proper LH levels and not due to the loss of ERα within the ovary. *(Source: Reproduced from Ref. 415.)* (E) Ribonuclease protection assays for the steroidogenic enzymes in adult wild-type (WT) and ERα-null ovaries treated either with vehicle (V) or a GnRH antagonist (A) every 48 h for 12 days (as described above). ERα-null ovaries exhibit increased expression of *Cyp17* and *Cyp19*, both of which are reduced to normal after reduction of gonadotropin levels. ERα-null ovaries also uniquely express *Hsd17b3*, a Leydig cell–specific enzyme, and this expression is dependent on gonadotropin stimulation as it is ablated after the reduction of circulating LH levels with GnRH-antagonist treatments. All samples are normalized to β-actin (*Actb*) mRNA levels. *Source: Reproduced with permission from Ref. 431.*

and mast cell infiltration in the interstitium[134,158,373,430] (Figure 25.17) (Table 25.7). The cystic and hemorrhagic follicles likely originate from antral follicles that fail to ovulate due to acyclicity (i.e., lack of an LH surge) and therefore become atretic and accumulate within the ovary. They are characterized by a mural granulosa cell layer of one to several cells thick surrounding an enormous fluid-filled antrum containing blood and immune cells; a degenerating ovum if visible at all; elevated FSH-receptor and LH-receptor expression in the granulosa cell layer, and a hypertrophied theca (Figure 25.17) that also exhibits elevated LH-receptor levels.[373,415] Therefore, ERα is not required for the recruitment and early growth of follicles or the induction of gonadotropin receptors in thecal and granulosa cells, but is vital to the later stages of folliculogenesis in the mouse ovary.[158,373]

ERα-null (αERKO) females exhibit a severely disrupted reproductive hormonal milieu, which in toto represents the cause and effect of the overt ovarian phenotypes[415,431] (Table 25.7). Plasma LH level in αERKO females is elevated three to eight-fold relative to their wild-type littermates, while FSH levels remain normal.[431] This is interesting in light of the fact that ovariectomy of normal WT mice leads to elevation of circulating FSH and suggests that the αERKO differs from ovariectomized WT females. Therefore the ovarian phenotypes of thecal hypertrophy,[373] elevated steroidogenic enzyme expression, and increased sex steroid synthesis in αERKO females are congruent with hypergonadotropic-hypergonadism.[431] A synopsis of evidence that supports acyclicity and chronically elevated LH as a primary cause of the other ovarian phenotypes in ERα-null females includes: (1) the cystic and hemorrhagic follicles appear after the onset of puberty (approximately 40 days of age),[373] (2) the cystic follicles and elevated steroidogenesis (Figure 25.17) are prevented when plasma LH levels are reduced to normal via treatments with a GnRH antagonist,[415,432] (3) transgenic mice possessing elevated LH but functional ERα exhibit a comparable ovarian phenotype[433–435] that is rescued by periodic ovulatory doses of exogenous hCG to induce luteinization,[436,437] and (4) immature αERKO females successfully ovulate, albeit with a reduced number of oocytes compared to wild-type mice, and form some corpora lutea when treated with exogenous gonadotropins prior to the onset of the cystic phenotype.[415,430,432] Others propose that aberrantly high intraovarian histamine levels due to infiltration of mast cells in the interstitium may also contribute to cyst formation in ERα-null ovaries.[158] Therefore, the previous findings indicate that a primary role of ERα in murine ovarian function may be extraovarian, i.e., as an essential mediator of the endocrine actions of E2 in the hypothalamic–pituitary axis that are critical to gonadotropin regulation. Indeed, prolonged treatment of female rodents with antiestrogens that cross the blood–brain barrier (e.g., ZM-189,154, EM-800) and produce an αERKO-like gonadotropin profile lead to a similar ovarian phenotype; whereas treatments with tamoxifen, a receptor antagonist that does not alter gonadotropin secretion generates no such effects in the ovary.[217–219]

A prominent phenotype in αERKO ovaries that may be indicative of an intraovarian role for ERα is their elevated capacity to synthesize androgens.[431] Relative to their wild-type littermates, αERKO females possess plasma levels of androstenedione and T that are increased 3 and 40-fold, respectively.[431] Indeed, LH is the primary stimulus of thecal cell androgen synthesis and plasma LH and thecal cell LH-receptor levels are both significantly increased in αERKO females (as discussed previously). Therefore, it is not totally unexpected that αERKO ovaries exhibit increased expression of the enzymes necessary for androgen synthesis, most notably a remarkable increase of *Cyp17* expression,[431] the enzyme necessary for the final step of androstenedione synthesis (Figure 25.17). However, the elevated plasma androgens found in αERKO females is likely not due solely to LH-mediated hyperstimulation of the theca. E2 from granulosa cells is proposed to mediate an intraovarian short feedback loop upon thecal cells to negatively modulate androgen synthesis during the later stages of folliculogenesis by primarily targeting CYP17 activity.[438] Given that ERα is the dominant ER form expressed in rodent thecal cells, the elevated *Cyp17* expression found in αERKO ovaries suggests ERα mediates this effect of E2 on androgen synthesis. Additional support for a specific ERα-mediated effect on thecal cell steroidogenesis comes from our findings that chronically elevated LH in wild-type and ERβ-null females leads to a much more moderate increase in *Cyp17* expression and androgen synthesis relative to αERKO females.[439] Furthermore, individually cultured ERα-null follicles in vitro secrete substantially more androgens relative to similarly propagated wild-type follicles even though the level of gonadotropin stimulation is held constant.[440] Therefore, it may be concluded that ERα is paramount to maintaining proper androgen synthesis in rodent females via: (1) endocrine actions in the hypothalamic–pituitary axis that negatively modulate LH secretion, and (2) intraovarian actions on thecal cells to negatively modulate *Cyp17* expression.

The granulosa cell–specific enzymes necessary for E2 synthesis, *Cyp19* and *Hsd17b1*, are also expressed at elevated levels in αERKO ovaries.[431] As a result, plasma E2 levels are typically increased almost 10-fold relative to wild-type littermates.[431] It is likely that the plasma androstenedione levels discussed earlier would be even greater in αERKO females if ovarian aromatase activity was not also equally elevated and providing for efficient conversion.[431] Although these findings in αERKO females suggest that ERα may also negatively modulate

E2 synthesis in granulosa cells, there is little precedent for this hypothesis. The chronically elevated plasma LH in αERKO females is also not likely to positively influence granulosa cell E2 synthesis as most evidence indicates that LH stimulation of granulosa cells leads to decreased aromatase activity.[441–443] Furthermore, individually cultured ERα-null follicles in vitro continue to exhibit heightened E2 synthesis relative to similarly propagated wild-type follicles in an environment of controlled FSH and LH stimulation.[440] Instead, increased aromatase activity in ERα-null ovaries is likely due to the positive actions of estrogens on FSH-induced granulosa cell steroidogenesis[442,444–447] which are presumably mediated by ERβ and therefore remain intact in ERα-null ovaries. Androgens have also been shown to augment FSH induction of E2 synthesis[442,448,449] and therefore the elevated androstenedione and T levels characteristic of αERKO females may also contribute to increased E2 synthesis in granulosa cells.

The several attempts to induce ovulation of ERα-null females collectively indicate an age-dependent effect of the loss of ERα. Schomberg et al. report that 4-month-old αERKO females do not successfully ovulate following exogenous treatments with PMSG and hCG, although this conclusion was based solely on the absence of corpora lutea rather than actual oocyte numbers.[373] Two later studies focused on younger αERKO females (3–5 weeks) and produced comparable results of successful ovulation (oocytes in the oviduct) and formation of functional corpora lutea, although the oocyte yield was reduced compared to age-matched wild-type females.[415,430] Oocytes harvested from ERα-null females successfully undergo in vitro fertilization, indicating that ERα may not be important to oocyte function.[415] In contrast, Dupont et al. report that the other line of ERα-null females (ERαKO) fail to ovulate or exhibit corpora lutea following exogenous gonadotropin treatments even at 21–25 days of age.[158] Studies in immature αERKO females of pure C57BL6 background (versus the mixed background of earlier studies) found that these mice can elicit a response to gonadotropin-induced ovulation.[432] This discrepancy in in vivo ovulatory success between the αERKO and ERαKO mice may be due to differences in genetic strain, the considerable disparity in the doses of gonadotropin used, or the small sample size in the Dupont et al. ($n = 3$). In further support of a minor role for ERα in ovulation, Emmen et al. demonstrated that individual ERα-null follicles propagated and induced to ovulate in vitro behave no differently than similarly cultured wild-type follicles.[440]

The discrepancies in the ovulatory response described herein may also be due to the splice variant of ERα expressed in the αERKO that was able to respond to exogenous gonadotropins (albeit reduced number [~15 oocytes] compared to wild-type [~41 oocytes]).[415] These mice were found to have expressed a splice variant in some tissues, and to circumvent this, a new ERα-null mouse model was made by *Cre/loxP*-mediated recombination (Ex3αERKO).[159] The ovarian phenotype and hormonal milieu of these mice is similar to that observed in the αERKO mice,[134,157] however the ability of these animals to respond to exogenous gonadotropins has not been fully studied to date. Preliminary data suggest reduced response to exogenous gonadotropins, as less than 50% ovulate and those that do respond ovulate a significantly lower number of oocytes (preliminary data Korach laboratory); however, further work is necessary to confirm this finding and fully characterize the ovulatory response of the Ex3αERKO mice.

Recently, a woman with homozygous mutation of ERα leading to severe E resistance was reported.[188] It is interesting to note that, similar to the ERα null mouse lines, she had cystic ovaries and elevated E, but unlike the mouse models, her LH and T levels were normal or nominally elevated. This is perplexing in light of the mechanisms discussed previously proposing the key role of LH elevation in causing the hemorrhagic cystic ovarian phenotype, and suggests notable interspecies differences in the mechanisms underlying these processes.

MICE WITH OVARIAN-SPECIFIC DELETION OF ERα

In rodent species, ERα is expressed predominately in theca cells, while ERβ is expressed in granulosa cells. Global loss of ERα in mice leads to a severe ovarian phenotype with large hemorrhagic and cystic phenotype due increased serum LH from loss of negative feedback in the hypothalamic–pituitary axis. While examination of ovarian function in these global ERα-null mice has provided important data on the necessity of ERα for ovarian function, the models make it difficult to delineate the importance of ERα specifically in the theca cells of the ovary. To circumvent this, Bridges et al. generated a mouse model with *Cre-recombinase* under control of the *Cyp17* promoter so that it is expressed specifically in thecal cells in the female mouse, and when crossed with a $ERα^{f/f}$ mouse deletes expression of ERα in thecal cells in the ovary.[361] Further characterization of this mouse and ovarian function in the absence of ERα demonstrated that these mice prematurely lose fertility.[362]

Theca cell–specific ERα knockout (thEsr1KO) mice did not have an altered estrous cycle, fertility as measured by the number of offspring born, or ovarian response to exogenous gonadotropins (superovulation) in animals aged 2 months; however, these measures were significantly reduced in thEsr1KO females at 6 months of age (Table 25.7).[362] In response to exogenous gonadotropins the thEsr1KO animals release similar numbers of oocytes to WT at 2 months of age (27 in WT versus 22 in thEsr1KO); however, by 6 months there are significant reductions in oocytes released (22 in WT versus 6 in

thEsr1KO) and the ovaries show an increase in the number of cystic/hemorrhagic follicles.[362] Additionally, the oocytes collected from the thEsr1KO ampulla appeared to be more degenerated than WT oocytes, however, further studies are required to examine the viability of oocytes collected from thEsr1KO mice. The age-related reduction in fertility coincides with an increase in the length of estrous cycle these animals have at 6 months of age,[362] demonstrating that ERα is important for maintenance of fertility, but its expression in thecal cells is dispensable for normal ovarian response in younger mice.

Global ERα-null mice have increased serum LH, which contributes to the cystic and hemorrhagic phenotype presumably due to loss of negative feedback in the hypothalamic–pituitary axis. Loss of ERα specifically in the theca cell layer leads to a reduction of serum LH in mice at 2 months of age, and a further reduction at 6 months.[362] This reduction in LH in the thEsr1KO animals was not due to reduced gonadotropes in the anterior pituitary, as there was actually an increase in the number of gonadotrope cells. Moreover, no difference is seen in the amount of LH stored in these cells as examined by immunofluorescence.[362] This confirms that the excess LH in the global ERα-null models is due to loss of ER in the hypothalamic–pituitary axis, and suggests that ERα expression in the ovary may be important for positive feedback and/or normal LH secretory patterns, although this needs to be tested experimentally.

Previous work has suggested that androgen synthesis is negatively regulated by estrogen through paracrine signaling actions, which can be examined in the thEsr1KO mouse model. Examination of serum hormone levels at both 2 and 6 months shows that T is increased,[362] confirming that ERα is necessary for maintenance of normal T levels. Examination of the expression of several steroidogenic enzymes found that *Cyp17* expression was increased at both 2 and 6 months following PMSG and hCG stimulation, while *Cyp19* expression was not significantly different between WT and thEsr1KO animals.[362] While expression of *Cyp17* is increased, the protein concentration of CYP17 was also increased in thEsr1KO ovaries in both aged animals and younger mice. CYP17 levels were also examined via immunohistochemistry during diestrus, when CYP17 activity and expression should be minimal, and increased CYP17 was observed in interstitial cells in the thEsr1KO ovary compared to wild-type ovaries.[362] The collective data support the notion that ERα signaling is necessary to support proper androgen synthesis in the ovary.

MICE WITH MUTATED DNA BINDING DOMAINS OF ERα

As discussed earlier in this chapter, the ERs may act as transcription factors on certain genes in the absence of a classical ERE within the regulatory sequences. This alternative mechanism of ER action, often referred to as "tethering", is thought to occur via interaction with transcription factors that are in fact bound directly to DNA (Figure 25.4). To develop an in vivo model for the study of ERα signaling via ERE-independent pathways, Jakacka et al. generated mice that possess a mutant form of the zinc-finger of the ERα DBD.[132] Because this mutation was incorporated into the endogenous *Esr1* gene (i.e., a "knock-in"), transcriptional expression of the mutant ERα is presumably no different than that of an undisrupted *Esr1* gene. Female animals that are heterozygous for the ERα mutation, termed NERKI$^{+/-}$ or ERα$^{AA/+}$ mice, are infertile and exhibit distinct ovarian phenotypes characterized by follicles of all stages of growth but no corpora lutea and lipid-filled cells throughout the stroma (Table 25.7).[132] The latter phenotype is similar to that of female mice lacking functional steroid acute regulatory protein (STAR) and correlates with reduced *StAR* expression in NERKI$^{+/-}$ ovaries.[132] Upon stimulation with exogenous gonadotropins, NERKI$^{+/-}$ ovaries exhibit multiple large hemorrhagic follicles similar to those found in ERα-null ovaries.[132] NERKI$^{+/-}$ females exhibit normal plasma gonadotropin and E2 levels and proper expression of steroidogenic enzymes in the ovary.[132] Still, induced ovulation in NERKI$^{+/-}$ females is only partially successful in causing some oocytes to be released and the formation of corpora lutea, as several preovulatory follicles fail to rupture and become hemorrhagic.[132]

Without further data it is difficult to determine the exact cause of the NERKI$^{+/-}$ ovarian phenotypes. Jakacka et al. postulate that the mutant ERα may act as a dominant-negative form of ER, however, the mutant ERα does not exhibit this property in vitro.[132] Furthermore, prolactin (*Prl*) is a known ERE-regulated gene and exhibits no change in expression in NERKI$^{+/-}$ females.[132] An imbalance between ERE-dependent and -independent pathways or tissue-specific inhibitory effects of the mutant ERα may also explain some of the resulting phenotypes.[132]

To further explore the role of the DNA binding domain in ERα function in the ovary, both the ER$^{AA/-}$ (also called "KIKO") and ER^{EAAE} mouse models (described in the section Mice with Mutated DNA Binding Domains of ERα) were developed,[162,168] both expressing only ERα that is unable to bind directly to ERE DNA motifs. KIKO were found to be anovulatory and infertile (Table 25.7).[162] Further characterization of the ovaries of these mice found follicles at most stages of development, however, there was a lack of corpora lutea in the ovary sections. The lack of corpora lutea and an estrous cycle in these mice suggests that they do not ovulate, however, the ability of these mice to respond to exogenous gonadotropins was not examined. These animals have some of the ovarian phenotypes identified in the NERKI$^{+/-}$,

demonstrating that the DNA binding activity of ERα is important for normal ovarian function. $ER\alpha^{EAAE/EAAE}$ females are infertile and have a hemorrhagic and cystic ovarian pathology (Table 25.7)[164] similar to the ovarian phenotypes observed in the other models that carry a mutation in the DNA binding domain. While further characterization of the ovarian phenotype in these animals is necessary to determine the exact cause of the ovarian phenotypes, the data to date suggest that DNA binding is an essential activity of ERα in normal ovarian function.

The KIKO animals have normal levels of E2 and P (Table 25.7),[162] and hormone levels were not examined in the $ER\alpha^{EAAE/EAAE}$ mouse model although ovarian phenotypes were similar between the two mouse models,[164] suggesting that regulation of serum steroid levels are not dependent on ERα DNA binding activities. Furthermore, the role of the DNA binding domain in ERα in tissues other than the ovary (such as the pituitary) could contribute to some of the observed phenotypes. Tissue-specific knock-in mutations to examine the role of direct DNA binding of ERα in ovarian function experiments could provide more mechanistic properties of the role of ERα, however, these experiments have not been done to date.

MICE WITH MUTATED AF-1 OR AF-2 DOMAINS OF ERα

While the models presented to date discuss the role of the DNA binding domain of ERα, there are other important domains of the nuclear receptor that may also contribute to the role of ERα in the ovary. To examine this, two different mouse models were developed with mutations in the ligand binding domain of ERα. In the first model, a single point mutation was made in the LBD (Figure 25.1) where a leucine was substituted in place of residue 525 where normally a glycine resides. This mutation alters the binding pocket, preventing normal ligand binding due to the increased side chain present in leucine compared to the single methyl group present in glycine. The knock-in mouse model $ER\alpha^{G525L}$, hereafter referred to as ENERKI, is anovulatory with a hemorrhagic and cystic ovarian pathology (Table 25.7).[168] The animals were found to have an increased number of atretic antral follicles, which presumably became the hemorrhagic/cystic follicles observed due to lack of rupture. Furthermore, these animals did not have any corpora lutea present, similar to the ERα suggesting that they do not ovulate,[168] demonstrating the importance of ligand binding ERα for normal ovarian function.

The AF-1 and AF-2 domains of ERα are important for ER function as demonstrated in vitro reporter assays. Mouse models were developed with each AF domain deleted, and uterine function was determined as described earlier in this chapter; however, the ovarian phenotype or hormone levels of these mice were not reported (Table 25.7).[165,167] To examine the importance of the AF-2 domain, a knock-in mutation in the AF-2 domain of ERα was made such that E2 is no longer able to transactivate E-dependent transcription.[169] This two-point mutation in the AF-2 domain was introduced into a knock-in mouse referred to as $AF2ER^{KI/KI}$. The mice are anovulatory and infertile (Table 25.7),[169] similar to the global αERKO knockout mouse models developed to date.[134,157,159] The ovaries of these mice also resemble the ovaries in ERα-null mouse models, in that they are hemorrhagic and cystic and lack presence of corpora lutea.[169] Furthermore, the endocrine milieu of these mice is similar to the global ERα-null animals; the $AF2ER^{KI/KI}$ mice have elevated serum LH and E2 (Table 25.7)[169] presumably due to lack of negative feedback in the hypothalamic–pituitary axis. The ovarian functions of other mice with AF-1 or AF-2 disruptions ($ER\alpha AF\text{-}1^0$ and $ER\alpha AF\text{-}2^0$) were not reported (Table 25.7). These models suggest that the ligand binding domain of ERα is required for normal ovarian function; however, the results differ slightly from the tissue-specific ERα knockout mouse described earlier. Therefore, further work using a tissue-specific knock-in mouse model would be useful in determining the mechanistic role of the different domains of ERα in the ovary.

MICE LACKING ERβ

Several facets of ovarian physiology thought to be dependent on the paracrine actions of E2 are clearly maintained in ERα-null and ERα-mutated ovaries, including granulosa cell proliferation, LH-receptor and aromatase expression, antrum formation, and the attenuation of atresia. The preservation of these estrogen actions in ERα-null ovaries strongly suggests their dependence on ERβ, which continues to be expressed in αERKO granulosa cells.[373,431] Neonatal ovaries from ERβ-null mice exhibit no gross abnormalities,[134,158,171,450] however, there are some differences in the expression of extracellular matrix proteins at this time of development.[451] Adult ERβ-null ovaries possess all stages of folliculogenesis,[158,171,440,450] a slight but perceptible increase in atretic follicles,[171,440,450] and a paucity of corpora lutea[171,440] (Table 25.7; Figure 25.17). Cheng et al. found that among 5-month-old ERβ-null females, less than 30% exhibit corpora lutea compared to 100% incidence in wild-type littermates.[450] Furthermore, when corpora lutea are present in ERβ-null ovaries, there are rarely more than two versus upwards of seven in wild-type females.[450] Continuous mating studies with ERβ-null females indicate a severe impairment in fecundity, but there is discernible variability in this phenotype among age-matched animals.[158,171] Consistent among βERKO pregnancies is reduced litter size of approximately one-third relative to wild-type.[158,171]

However, whereas some βERKO females exhibit the expected number of pregnancies over a 4 month period, others become pregnant only once while still others exhibit total infertility.[171,172] This variability in fecundity among ERβ-null females remains puzzling but is reported in both independently generated lines.[158,171] Given that the ERβ-null lines represent distinct targeting schemes for the *Esr2* gene on different genetic backgrounds, the observed variability in fertility is likely related to the loss of ERβ functions. A similar variability in oocyte yield is found when immature ERβ-null females are induced to ovulate by exogenous gonadotropin treatments.[158,171,172,432] A third ERβ-null mouse model was recently developed in which all mice were found to be infertile and unable to respond to exogenous gonadotropins.[172] These animals also had a significant reduction in the number of corpora lutea present in the ovaries of the ERβ-null mice.[172] While subfertile versus infertile phenotypes are observed in the different ERβ-null mouse lines, it should be noted that although some mice did produce pups and respond to exogenous gonadotropins, the number was significantly lower than that observed in WT mice, demonstrating the importance of ERβ in normal ovarian function. Therefore, the observed sub/infertility in ERβ-null females appears to originate from disrupted ovarian function that is best characterized to date as infrequent and inefficient spontaneous ovulation. A lack of evidence indicating embryo resorption during gestation in mated βERKO females[171] rules out an extraovarian contribution, although such phenotypes have not been categorically studied.

Histological evaluation of ERβ-null ovaries following induced ovulation reveals multiple preovulatory follicles possessing underdeveloped antra and minimal cumulus expansion.[158,171,172,432] Krege et al.[171] report obvious corpora lutea in immature βERKO ovaries 20 h after hCG treatment, but Dupont et al. remark that no luteal cells were observed in similarly treated ERβKO ovaries that did not ovulate.[158] However, immature βERKO females do indeed exhibit functional corpora lutea following induced ovulation, as indicated by histological evaluation of the ovaries and a substantial rise in plasma P levels following induced ovulation.[432] Luteinization and increased P synthesis is also observed when individually propagated ERβ-null follicles are exposed to an hCG bolus in vitro.[440] Furthermore, luteinization occurs in unruptured preovulatory follicles both in vivo and in vitro,[134,171,440] resulting in "trapped" oocytes similar to those observed in other null mouse models.[176,452]

ERβ-null females exhibit a relatively normal reproductive endocrine milieu.[431] Basal gonadotropin levels are within the range of control values,[431] although one report remarks that plasma LH levels are slightly increased in βERKO females.[453] This notwithstanding, ERβ-null females clearly do not exhibit the ovarian phenotypes that are associated with chronically elevated LH.[415,435] Therefore, ERα is clearly the principal receptor involved in mediating the negative feedback effects of E2 on gonadotropin secretion from the hypothalamic–pituitary axis. Sex steroid hormone levels in ERβ-null females are also comparable to wild-type littermates.[431] Additionally, the overtly estrogenized uterine and vaginal tissues observed in ERβ-null females indicate sufficient ovarian estrogen synthesis in the absence of functional ERβ.[134] This preservation of normal E2 levels in βERKO females is surprising given the evidence that estrogens are necessary to maximize FSH stimulation of aromatase activity in granulosa cells.[442,444–447]

Recent studies into the role of ERβ in normal ovulatory function have found that ERβ is necessary in granulosa cells for normal response to both gonadotropins FSH and LH. Granulosa cells isolated from ERβ-null ovaries and grown in culture had reduced levels of cAMP in response to FSH stimulation compared to granulosa cells isolated from WT ovaries.[454] The reduced signaling response to FSH was also observed in granulosa cells from in vitro–stimulated ovaries, and whole follicles grown in vitro.[454,455] The reduced cAMP accumulation after FSH stimulation coincided with a reduction of LH-receptor (officially *Lhcgr*) mRNA accumulation.[455] This suggests that granulosa cells in ERβ-null ovaries are unable to stimulate increased LH-receptor expression, which could contribute to the inability of these cells to respond to LH intracellular signaling components[363,455] and ultimately ovulate. Still, βERKO females clearly do not exhibit a normal ovarian cycle, as evidenced by vaginal smears indicating animals in persistent estrous and the paucity of corpora lutea in the ovary. This suggests the βERKO female mounts a preovulatory rise in E2 that is insufficient to induce the gonadotropin surge from the hypothalamic–pituitary axis, and/or a diminished ability of the βERKO ovary to respond to the LH surge. As mentioned before, androgens also augment FSH-induction of E2 synthesis[442,448,449] and provide for compensatory actions in ERβ-null granulosa cells.

MICE LACKING ER α AND β

Congruent with the loss of ERα, αβERKO females are infertile and do not spontaneously ovulate.[158,174] Adult αβERKO ovaries possess structures appropriate to the normal ovary, including primordial and growing follicles, although the latter possess an underdeveloped antrum, reduced granulosa number and a thin poorly structured theca (Table 25.7).[158,174] Cystic follicles that are somewhat characteristic of those found in ERα-null ovaries are also present but are not as large or as hemorrhagic in αβERKO ovaries and are more apt to exhibit only a thin layer of granulosa cells.[158,174] We have used mice with chronically elevated expression of LH from a transgene (α-LHβCTP mice) to show that the formation

of hemorrhagic and cystic follicles in the mouse ovary are due to ovarian acyclicity compounded by chronically elevated plasma LH in the presence of normal FSH levels.[435,437] The occurrence of hemorrhagic follicles in α-LHβCTP transgenic ERα-null mice and the absence of hemorrhagic follicles in α-LHβCTP transgenic ERβ-null mice[439] demonstrates a role of ERβ in development of the cysts. Therefore, the absence of hemorrhagic and cystic follicles in αβERKO ovaries indicates an important intraovarian role for ERβ in this pathology. A recent compound αβERKO mouse model that does not express the splice variant for ERα[159] has elevated plasma LH and hemorrhagic and cystic ovarian phenotype observed in the ERα-null mice.[456] The discrepancy observed in these mice suggests that the presence of the splice variant, genetic background, or mode of knockout in the mouse models may contribute to the ovarian pathology observed. Further studies are necessary to determine the role of ERα or ERβ in development of hemorrhagic follicles within the ovary.

A most remarkable feature of adult αβERKO ovaries is the presence of seminiferous tubule-like structures that often occupy large portions of the gonad but are conspicuously absent in ERα- or ERβ-null ovaries[158,174] (Figure 25.18). Morphological observations indicate these structures are the "ghosts" of atretic follicles as they possess an intact basal lamina, partial layers of granulosa cells, and an invariably degenerating oocyte[174] (Figure 25.18). Furthermore, these structures are postpubertal as Couse et al. report no such structures in αβERKO ovaries at 10 days of age[174] and Dupont et al. concur in another line of ERαβKO ovary as late as 23 days of age.[158] The defining characteristic of the "ghost" follicles that led to their initial description as testis-like seminiferous tubules is the overt presence of Sertoli-like cells in the lumen (Table 25.7 and Figure 25.18).[158,174] Several morphological features of these cells in both αβERKO and ERαβKO ovaries are strongly indicative of a Sertoli cell phenotype (Figure 25.18), including: (1) alignment with the basal lamina of the follicle wall, (2) a tripartite nucleolus, (3) numerous veil-like cytoplasmic processes extending inward toward the lumen, (4) ectoplasmic specializations that are unique to Sertoli cells in prepubertal testis, and (5) immunoreactivity for Müllerian-inhibiting substance and sulfated glycoprotein-2.

Granulosa and Sertoli cells fulfill analogous gametogenic roles as "nurse" cells in the ovary and testis, respectively, and are postulated to derive from a common embryological precursor cell during early gonadal

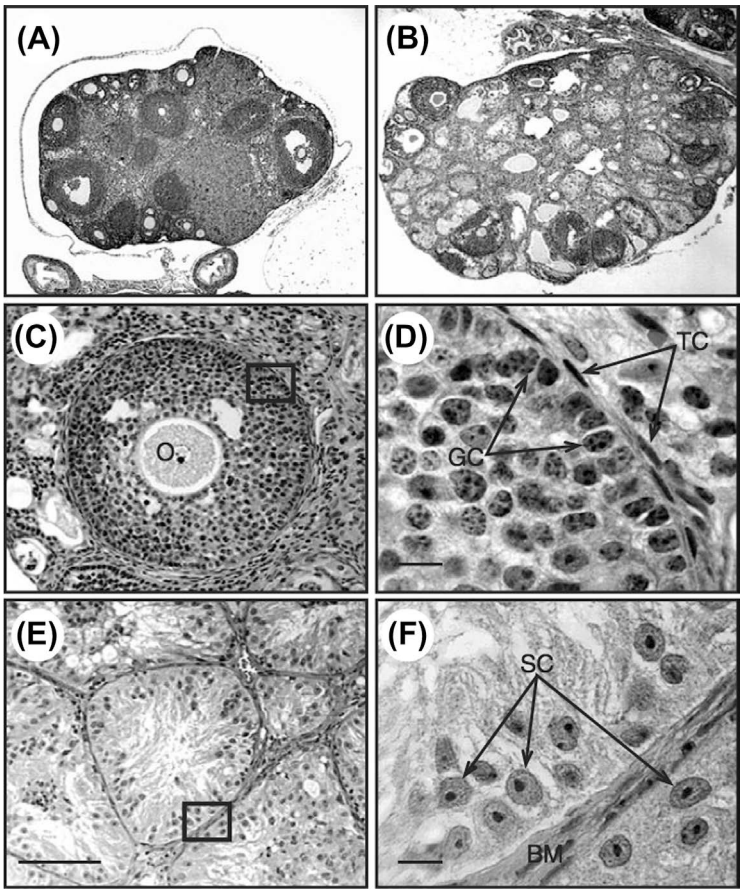


FIGURE 25.18 Ovarian phenotype in adult mice lacking both ERα and ERβ. (A, B) Low-power magnification of representative adult wild-type (A) and ERαβ–null (B) ovaries. The wild-type ovary exhibits all stages of folliculogenesis, including a preovulatory follicle (*right*) and corpus luteum (*right, bottom*). The ERαβ-null ovary exhibits a few relatively healthy maturing follicles but is overwhelmed by multiple "sex-reversed" follicles. (C–F) High-power magnification of representative follicles from adult ERαβ-null ovaries (C, 66×; D, 330×). A healthy follicle shows a single oocyte (O), several layers of granulosa cells (GC), an intact basal lamina, and thecal cells (TC) (E, 66×; F, 330×). A representative "sex-reversed" follicle in which there is no longer evidence of an oocyte and the somatic cells have undergone redifferentiation to a Sertoli-like cell (SC) phenotype. Preservation of the basement membrane (BM) of the follicles provides for the "tubular" appearance that assimilates testicular cords. The Sertoli-like cells (SC) possess the characteristic tripartite nucleolus and veil-like cytoplasmic extensions (F). Scale bars: E ×100 μm; D, F ×10 μm. *Source: Reproduced with permission from Ref. 174.*

differentiation.[457] Thus, the origin of the Sertoli-like cells in αβERKO ovaries remains a perplexing question. Do they originate from a bi-potent precursor population present in the αβERKO ovary at birth, or are they the result of granulosa cell transdifferentiation following oocyte death and follicle atresia? A report of extra-follicular Sertoli cells in the ERαβKO ovary[158,458] supports the existence of an embryological precursor cell population, yet similar interstitial cell populations are not found in ovaries from the other αβERKO.[174] In turn, the following findings that are common to both lines support a pathway of granulosa cell transdifferentiation: (1) the Sertoli-like cells are not apparent prior to 30 days of age, (2) the spherical shape of the "tubules" suggested by two-dimensional histology suggests they originate from a once-healthy follicle in which only the basement membrane remains, and (3) the appearance of Sertoli cells is strongly correlated with oocyte death and follicle atresia.[158,174] Furthermore, *Sox9* is highly expressed in both αβERKO and ERαβKO ovaries[174] and localized to the Sertoli-like cells.[458] SOX9 is an *Sry*-related transcription factor that is critical to normal Sertoli cell differentiation during testis development in rodents and humans.[459,460] Transgenic overexpression of *Sox9* leads to phenotypic male gonadal development in XX mice and therefore acts "downstream" of the Y-linked male-determining gene, *Sry*.[461,462] In strong favor of a pathway of granulosa cell transdifferentiation, *Sox9* expression in ERαβKO ovaries is restricted to the granulosa cells of atretic follicles and precedes the overt appearance of Sertoli-like cells but is not detectable in the granulosa cells of primordial follicles.[458] Furthermore, those cells expressing *Sox9* are initially present with the confines of the follicle "ghosts" and appear in the intrafollicular spaces of the ovary only after the phenotype has progressed to an advanced stage,[458] supporting a follicular (i.e., granulosa) origin.

Similar phenotypes of "sex-reversal" are reported in fetal rodent ovaries following: (1) transplantation of the fetal ovary to an adult host,[463,464] (2) in vitro exposure to purified Müllerian inhibiting substance (MIS),[465] (3) transgenic MIS overexpression in vivo,[453] (4) transgenic *Sox9* overexpression in vivo,[461,462] and (5) following targeted disruption of the *Wnt4* gene in mice.[466] Although the αβERKO ovarian phenotype shares a number of morphological similarities with the previous findings, including aberrant expression of the MIS, SGP-2 and *Sox9* genes, a remarkable distinction in the αβERKO ovary is the postnatal onset of the phenotype. The previous descriptions of ovarian sex reversal are reported to occur or even require *fetal* ovarian tissue.[465,467] Therefore, the observed "sex-reversal" adult αβERKO ovaries is the first to be described in the postnatal mouse gonad, indicating that the potential of female ovarian somatic cells to redifferentiate into Sertoli-like cells may be present throughout life in the mouse. Recently, postnatal reprogramming of ovaries to testis with cells that are positive for SOX9 expression and cells resembling Sertoli cells was reported in adult mice lacking FOXL2.[468] This provided a second postnatal model for this ovarian transdifferentiation, and suggested that FOXL2 is an important transcription factor necessary for granulosa cell differentiation and ovarian function. Interestingly, the Ex3αβERKO mouse model shows loss of *Foxl2*/FOXL2 expression followed by increased *Sox9*/SOX9 expression and appearance of Sertoli-like cells in the ovary of adult mice.[456,469]

A causal link between the loss of all ER function and postnatal morphological ovarian sex reversal is unclear. Dupont et al. report that female mice possessing only one functional ERα allele but lacking functional ERβ also exhibit the αβERKO ovarian phenotype, suggesting a gene-dosage effect for ERα.[158] The majority of documented cases of similar phenotypes in the mammalian ovary are preceded by massive germ cell loss.[470] The remarkable oocyte attrition that occurs in the αβERKO ovaries suggests a similar mechanism occurs in this model; yet it remains unclear if the progressive loss of germ cells in αβERKO ovaries illustrates the loss of critical intraovarian estrogen signaling that is perhaps dependent upon ERα/ERβ cooperation. A role of direct estrogen/ER actions in gonadal differentiation is supported by reports of sex reversal in turtles and whiptail lizards following developmental exposure to aromatase inhibitors.[471,472] The mouse *Sox9* promoter lacks an obvious ERE but does possess two consensus binding sites for GATA-1,[460] a transcription factor expressed in Sertoli cells during differentiation of mouse testis but repressed by germ cells in adult testis.[473] Furthermore, ERα can repress the transcriptional activities of GATA-1,[474] suggesting that aberrant *Sox9* expression leading to Sertoli-cell differentiation in αβERKO ovaries may be due to increased GATA-1 activity following the loss of ERα and other potential oocyte-derived inhibitory factors. As a similar phenotype is observed in FOXL2 knockout ovaries, it has been hypothesized that *Foxl2*/FOXL2 is regulated by ERE-dependent transcription,[468] and loss of this regulation contributes to the Sertoli-cell phenotype in the ovaries of knockout mice. This does not account for the lack of transdifferentiation during ovarian development or in neonatal animals,[158,174,468] which suggests that this "sex-reversal" or transdifferentiation phenotype is a complex trait that requires aberrant regulation/expression of multiple genes. Current studies are underway with the Ex3αβERKO mouse model to look at ovarian gene expression and identify possible candidate genes that may contribute to the granulosa cell transdifferentiation observed.[456,469]

Female αβERKO animals exhibit a gonadotropin profile that is similar to αERKO females, although plasma LH levels are noticeably higher. Therefore, the hormonal

milieu in αβERKO females may impact the ovarian "sex reversal" observed.[174,431] Still, transgenic mice possessing chronically elevated plasma LH also display a progressive loss of oocytes but lack any evidence of ovarian "sex reversal".[435,439,475] Interestingly, αβERKO ovaries do not exhibit αERKO-like increases in ovarian aromatase or E2 synthesis.[431] This finding provides further support that elevated LH and precursor substrates, combined with the positive actions of ERβ, lead to enhanced E2 synthesis in ERα-null ovaries—a scenario that would be disrupted in αβERKO ovaries. The dramatic loss of germ cells in the αβERKO ovary may also impact gonadotropin responsiveness of granulosa cells as oocyte-derived factors are known to positively influence steroidogenesis;[476] further studies are necessary to determine how germ cell loss contributes to the observed phenotype.

MICE LACKING CYP19

In the ovary, CYP19, or aromatase, is expressed exclusively in the granulosa cells (Table 25.6). Initial reports of Cyp19-null ovaries indicated no phenotypic difference from WT, suggesting estrogen-independent signaling mechanisms were involved in eliminating the phenotypes of the ERKO mice.[477] It was not until Cyp19-null mice were fed a soy-free diet that phenotypes appeared. These observations were some of the first to directly demonstrate dietary estrogen contamination in feed. Therefore, to study the phenotypes associated with loss of estrogens, Cyp19 null are kept on soy-free diets. Under these conditions, Cyp19-null mice have ovaries with all stages of folliculogenesis, including multiple large antral follicles but no corpora lutea at 10–14 weeks of age (Table 25.7).[131,156,364] At 21 weeks of age, Cyp19-null ovaries continue to exhibit all stages of folliculogenesis, but larger follicles possess a reduced number of granulosa cells and become enlarged, cystic, and hemorrhagic, similar to ERα-null follicles.[364] By 1 year of age, Cyp19-null ovaries are characterized by a paucity of normal follicles, an increased number of cystic and hemorrhagic follicles, significant collagen deposition, and massive macrophage infiltration into the interstitium (Table 25.7).[364] Several findings indicate infertility among Cyp19-null females due to an inability to spontaneously ovulate, including: (1) absence of corpora lutea at all ages,[131,156,364] (2) lack of pregnancies during continuous mating,[131] and (3) total failure to ovulate following exogenous gonadotropin treatments.[131] Recently, it has been demonstrated that co-treatment of Cyp19-null mice with E2 and PMSG followed by hCG can rescue the anovulatory phenotype,[365] demonstrating the necessity of E2 signaling in addition to the gonadotropins for normal ovulatory function.

Surprisingly, initial descriptions of the ovarian phenotypes in Cyp19-null females did not include remarks of Sertoli-like cells similar to those found in αβERKO females. However, Britt et al. later demonstrated that mutant females maintained on a diet free of phytoestrogens exhibit a clearly exacerbated ovarian phenotype by 6 weeks of age that included hemorrhagic and cystic follicles, seminiferous tubule–like structures possessing Sertoli-like cells, and an almost 40-fold increase in *Sox9* expression.[194] By 16 weeks of age, the tubule-like structures and Sertoli cells occupy up to 80% of the ovary and are strikingly similar to those found in αβERKO ovaries in both morphology and gene expression.[194] Additional supporting evidence of Sertoli-like cells in Cyp19-null ovaries is documentation of Sertoli cell-specific ectoplasmic specializations and immunoreactivity for Espin, an important component of Sertoli cell junctions in testis.[194] Recent microarray analysis has identified 450 genes that are differentially expressed in Cyp19-null ovaries compared to wild-type ovaries, with 291 showing increased expression and 159 showing reduced expression.[478] Several of these genes have been implicated in testis development, however, further studies and confirmation are necessary to determine potential candidates that may contribute to ovarian transdifferentiation. Interestingly, a similar phenotype of "sex reversal" has not been described in the ovaries of a second line of Cyp19-null females.[131]

As expected, the reproductive hormonal milieu in female Cyp19-null mice is severely disrupted. Plasma E2 levels[131] and ovarian aromatase activity[156] are below the level of detection, supporting the existence of a single aromatase-encoding gene in mice. In turn, plasma T levels in Cyp19-null females are 10-fold that found in wild-type littermates.[131,156] Increased plasma androgens in Cyp19-null females are likely the combined effect of an accumulation of androgen precursors that occurs in the absence of aromatization to E2, and increased synthesis due to hyperstimulation of the ovarian theca by five-fold increased plasma LH.[156,364] Thus, increased plasma LH and androgens are characteristic of mice lacking ERα-mediated estrogen actions following the loss of receptor (ERα-null animals) or ligand (Cyp19-null animals). However, one striking difference between Cyp19-null and ERα-null females is the four- to six-fold increase in plasma FSH that is unique to the former.[364] Interestingly, Cyp19-null females exhibit the above abnormal hormone milieu regardless of phytoestrogen content in the diet,[194] suggesting that dietary phytoestrogens may attenuate some but not all of the effects that follow the loss of endogenous E2 synthesis.

Studies have indeed shown that Cyp19-null mice still possess functional ER signaling.[479] Tonic E2 replacement therapy in Cyp19-null females over a period of 21 days leads to restored gonadotropin homeostasis, improved follicular development in the ovary, a reduced presence of Sertoli-like cells and *Sox9* expression, and restoration of ovulation and corpora lutea formation.[479,480] In

a similar study, Toda et al. report that E2 administration to Cyp19-null females every fourth day for 4 weeks also ameliorates the ovarian phenotypes but did not restore ovulation.[131] Ovulation was partially restored when the mice were treated with E2 in addition to exogenous gonadotropins,[365] although oocyte numbers were still significantly lower than those observed in WT mice. Furthermore, treatment of the Cyp19-null mice with a GnRH antagonist and E2 replacement was able to restore normal gonadotropin levels, while treatment with an antiandrogen had no effect. This demonstrates that loss of E2 in these animals contributes to the ovarian phenotype observed and also indicates that an indirect effect from excess androgens does not directly contribute to the excess gonadotropins, hemorrhagic follicles, or Sertoli-like cells present in the Cyp19-null mice.[481]

Intraovarian Roles of Estradiol in Ovarian Function

The importance of the extraovarian or endocrine roles of estrogen signaling in ovarian function has long been recognized and is the precedent for steroid-based oral contraceptives.[482,483] In contrast, the significance of estrogen signaling within the ovary is not well understood due to the inherent difficulties associated with the study of a particular hormone's action within the very tissue from which it is synthesized and secreted. Past in vivo investigations employing ER antagonists[217–219,484] or aromatase inhibitors[484–486] to inhibit hormone receptor actions within the ovary have provided informative but often equivocal findings[217–219] due to the following limitations: (1) the enormous levels of endogenous E2 in the ovary are difficult to overcome by pharmacological administration of a receptor antagonist, (2) the enormous levels of aromatase activity in the ovary are difficult to overcome by pharmacological administration of an enzyme inhibitor, (3) the effects of a pharmacological ER antagonist due to actions directly in the ovary versus actions in the hypothalamus or pituitary are difficult to discern, (4) the well-characterized agonists (e.g., E2 and DES) comparably activate both ERα and ERβ, (5) early antagonists were not selective for the two known ERs, and (6) the two ERs may respond differently to agonist or antagonist ligands. The generation of ER and Cyp19-null mice, and development of improved ER-selective ligands or SERMs[61,487,488] have contributed to better understanding over the years.

The abundance of data on the localization of ERα and ERβ within rodent follicles allows for a simplified model of estrogen actions within the ovary. For example, given that ERα predominates in thecal cells, does it mediate the inhibitory effects of E2 on LH-stimulated androgen synthesis? In turn, as ERβ clearly is the predominant receptor in granulosa cells, is it responsible for mediating the known estrogen augmentation of FSH actions? This section employs the contributions from studies of the pertinent null- or mutated-mouse models as a platform to discuss the current view on intraovarian estrogen/ER signaling.

GRANULOSA CELL PROLIFERATION

A number of studies in immature hypophysectomized rodents have demonstrated that DES or E2 treatments over 2–4 days leads to a marked increase in ovarian weight[489] a significant rise in the number of medium-sized preantral follicles and increased DNA synthesis in both thecal and granulosa cells,[422,490–492] indicating a direct and gonadotropin-independent effect of E2 on the ovary. Similar data have been reported following DES treatment of intact immature rats.[493] However, there is equally convincing data that sequential treatment of immature hypophysectomized rodents with DES followed by FSH results in a synergistic response in terms of increased ovarian weight and DNA synthesis, and elicits several major indices of granulosa cell differentiation, i.e., antrum formation and acquisition of LH-receptor expression, that are not observed with either hormone alone.[359,490,491,494] Furthermore, co-administration of an antiestrogen (*cis*-clomiphene) inhibits FSH-induced increases in ovarian weight and follicular growth in hypophysectomized rats, suggesting that ER-mediated effects are necessary for a full response to gonadotropin.[495] The synergistic actions of E2 on FSH-induced granulosa cell proliferation have been replicated in vitro,[496] although others report that FSH-mediated growth of isolated murine follicles is not affected by anti-E2 antisera or ER antagonist (ICI 182,780).[497] The capacity of E2 to enhance the granulosa cell response to FSH is not believed to rely on estrogen-induced increases in FSH-receptor levels, although some evidence indicates such an effect may play a greater role in mice versus rats.[359,492,494] These differences will be easier to study with the recently described ERα-null rat,[190] which can be used to compare the roles of ERα in rat versus mouse ovarian functions. Still, pretreatment of hypophysectomized rats with antiestrogen (CI628) inhibits FSH-induced increases in granulosa cell FSH receptor levels.[418]

It is generally agreed that the synergistic response of granulosa cells to estrogens and discovery of ERβ and its substantial expression in the granulosa cells of growing follicles and absence in atretic or luteinized follicles strongly suggests that the trophic actions of estrogens are direct and ERβ mediated. Supporting data of a greater importance of ERβ versus ERα in estrogen-induced granulosa cell proliferation comes from a report of Hegele-Hartung et al. in which the ERβ-selective agonist 8-vinylestra-1,3,5-(10)-triene-3,17β-diol induces increased ovarian weight and granulosa cell proliferation to levels comparable to that elicited by

17β-E2 in hypophysectomized rats; whereas the ERα-selective agonist 3,17-dihydroxy-19-nor-17α-pregna-1,3,5 (10)-triene-21,16α-lactone has little effect.[488] However, although these data indicate that E2/ERβ actions may indeed enhance FSH-induced granulosa cell proliferation, several findings question the overall importance of this intraovarian role. First, ERβ-null ovaries exhibit a relatively normal number of growing preantral follicles with no obvious reduction in the granulosa cell population.[158,171,498] Furthermore, immature ERβ-null females treated with PMSG exhibit the expected increase in the number of small and large preantral follicles.[158,440] In addition, granulosa cell tumors, due to the loss of inhibin-α (*Inha*) in mice continue to proliferate regardless of the presence or absence of functional ERβ.[499] It is possible that ERα may provide some compensatory actions as ERβ-null follicles show increased expression of ERα mRNA.[455] Furthermore, Cyp19-null mice are presumably devoid of E2-dependent ER action but exhibit ovaries possessing all stages of follicle growth, each with a normal complement of granulosa cells.[364] Therefore, the data suggest that although estrogen-induced granulosa cell proliferation is likely mediated by ERβ, it is not obligatory to gonadotropin-induced follicle growth in rodent ovaries.

ERβ may facilitate gonadotropin-induced granulosa cell proliferation through the induction of cyclin-D2 (*Ccnd2*), which enhances progression of the cell cycle from the G_0 to S phase. Targeted disruption of the *Ccnd2* in mice indicates this cyclin protein is obligatory to granulosa cell proliferation as Ccnd2-null ovaries lack follicles beyond the small preantral stage.[500] Furthermore, the arrest in folliculogenesis observed in Ccnd2-null mice is not rescued by exogenous FSH treatment in vivo or in vitro, indicating the critical downstream role of cyclin-D2 in gonadotropin-mediated granulosa cell proliferation.[500] In rats, *Ccnd2* expression is localized to the granulosa cells of early growing follicles and is significantly induced by PMSG or FSH treatments.[501] However, E2 also induces *Ccnd2* expression in rat granulosa cells, and although the response is less rapid relative to that elicited by FSH, the overall induction by E2 is higher and better sustained over 24 h.[501] Furthermore, E2 elicits a concomitant decrease in the expression of *Cdkn1b* (p27), a cyclin-dependent kinase inhibitor.[501] E2 induction of *Ccnd2* in undifferentiated rat granulosa cells in vitro is inhibited by the ER antagonist ICI 164,384, indicating this to be an ER-mediated event, presumably ERβ.[501] Therefore, parallel pathways of FSH and E2 converge to induce and maintain *Ccnd2* expression in granulosa cells, thereby forcing cell cycle progression and subsequent proliferation.[501] Interestingly, adult ERβ-null ovaries possess normal granulosa cell expression of *Ccnd2*[450] as well as exhibit the expected induction following PMSG exposure,[432] further supporting the hypothesis that FSH and E2 work in parallel. Therefore, these observations and the absence of any gross deficiency in granulosa cells in ERβ-null ovaries suggests that FSH may play a greater role in inducing *Ccnd2* expression and granulosa cell proliferation. Interestingly, E2 specifically induces *Ccnd2* expression in keratinocytes via the cAMP/PKA signaling pathway that leads to activation of CREB,[502] the same intracellular pathway employed by FSH signaling in granulosa cells. These findings suggest that E2 may also function within the FSH signaling pathway to induce *Ccnd2* in granulosa cells.

A second mechanism by which E2 may amplify FSH-induced granulosa cell proliferation is via interactions with the ovarian IGF1 axis. Like Ccnd2-null mice, Igf1-null mice exhibit a total failure of follicles to progress beyond the small preantral stage.[503,504] This arrest in folliculogenesis in Igf1-null ovaries is primarily due to a >50% reduction in FSH receptor levels in the granulosa cells, severely hampering their response to FSH.[504] However, Kadakia et al. demonstrated that Igf1-null granulosa cells are also refractory to E2-induced proliferation and exhibit a 50% lower rate of DNA synthesis and decreased *Ccnd2* induction following E2 treatment in vivo.[505] The poor response of Igf1-null granulosa cells to E2 may be due to a likewise reduction in ERβ levels as IGF1 is reportedly necessary for the maintenance of ERβ expression in granulosa cells in vitro.[506] There are currently no reports describing the levels of ERβ expression in the ovaries of Igf1–null mice. Interestingly, Richards et al. demonstrated that E2 significantly upregulates the expression of several components of the IGF1 signaling pathway in rat granulosa cells, including the IGF1 receptor-β subunit, the glucose transporter, Glut-1, and the forkhead family member, FKHR (Foxo1).[506] These findings suggest that E2 may enhance IGF1 actions in granulosa cells by regulating targets important for cellular energy flow, glucose metabolism, and cell survival.[506] In summary, IGF1 actions are clearly necessary to provide for sufficient FSH signaling in granulosa cells, which in turn, is necessary for the induction of aromatase activity and E2 synthesis, thereby providing ligand for ERβ to enhance the effects of IGF1, hence forming an autocrine regulatory pathway to promote granulosa cell proliferation.[506] The extent to which these findings may translate to the human ovary remains to be determined since IGFII, rather than IGF1, is the predominant and gonadotropin-regulated IGF form in the human ovary.[507]

The previous discussion provides a compelling argument that the mitogenic actions of E2 on granulosa cells are direct and ERβ mediated. However, thecal cell ER actions may also be involved. For example, ERα in the thecal cells may respond to granulosa cell–derived E2 by inducing the synthesis and secretion of a paracrine-acting growth factor(s) that then becomes the principal stimulus for granulosa cell proliferation. Indeed, Dorrington and

colleagues have proposed that thecal-derived transforming growth factor-β (TGFβ) is estrogen induced and stimulates granulosa cell proliferation.[508,509] Similar examples of growth factor–mediated cooperative actions between theca and granulosa cells are postulated to regulate follicle steroid production.[510,511] Still, ERα-null follicles do not exhibit any gross deficits in granulosa cell number and possess a normal number of preovulatory follicles following gonadotropin stimulation.[415] ERα-null follicles in vitro show normal growth, however, they do not reach the same size as wild-type follicles.[440] It is plausible that a dramatic effect on granulosa cell proliferation may only become apparent following the loss of both putative estrogen pathways, i.e., the direct (ERβ-mediated) and indirect (ERα-mediated) actions—hence explaining the unique somatic cell phenotypes observed in the ovaries of compound ER-null[158,174] and older Cyp19-null mice[364] on a soy-free diet in absence of estrogen ligand.

GRANULOSA CELL DIFFERENTIATION

The process of granulosa cell differentiation that occurs during progression from a preantral to preovulatory follicle has been an area of intense research. Fully differentiated preovulatory follicles in mammalian ovaries are distinctly characterized by: (1) a large fluid-filled antrum, (2) acquisition of LH responsiveness, (3) significant increases in aromatase (CYP19) activity, and (4) increased inhibin synthesis. Although FSH is the primary stimulus for both granulosa cell proliferation and differentiation, these two processes appear to be independent as the undersized follicles in Ccnd2-null ovaries still develop an antrum, possess sufficient aromatase activity, and are responsive to exogenous LH stimulation.[502] Therefore, granulosa cell proliferation and differentiation are separate gonadotropin-induced events during folliculogenesis.

The absolute requirement of FSH signaling in the process of granulosa cell differentiation is illustrated by the lack of antral follicles, aromatase and E2 synthesis, and granulosa cell LH receptor in mice null for FSH action.[420,512,513] Igf1-null follicles exhibit a similar failure to differentiate; this is postulated to be due to a lack of sufficient FSH-receptor expression.[503] However, numerous studies in rat ovaries have demonstrated that FSH induction of antrum formation,[490] aromatase expression and activity,[442,444–447] and LH-receptor expression[484,514,515] require E2 for maximum effect. FSH acts via a classic heterotrimeric G protein–coupled receptor pathway that may activate multiple intracellular second messenger systems, the most well characterized being activation of adenylyl cyclase, which leads to intracellular accumulation of adenosine 3′,5′-monophosphate (cAMP) and activation of protein kinase A (PKA).[416,494] The long-recognized synergism between E2 and FSH on granulosa cell differentiation is believed to be due to the capacity of E2 to both amplify the level of FSH-stimulated intracellular cAMP[359,441,494,515,516] and augment the downstream actions of cAMP itself.[494,515] The mechanisms involved remain poorly understood and are unlikely to involve estrogen-induced increases of FSH receptor levels but appear more likely to rely on E2's capacity to positively modulate the granulosa cell adenylyl cyclase system.[441,494]

ER-mediated estrogen activation of the adenylyl cyclase system and subsequent cAMP-regulated gene expression has been demonstrated in uterine and breast cancer cells.[517] Furthermore, in vitro and in vivo studies indicate that ERα or ERβ can activate ERE-dependent gene transcription via the cAMP/PKA signaling pathway in the absence of E2.[93,103,518] This pathway involves the CREB protein,[519] which is also known to be critical to FSH induction of gene expression.[442,520,521] Therefore, given the existing evidence that E2 is required to maximize the FSH response, combined with the known cooperative activity between the ER and PKA signaling pathways, it may be reasonable to expect that a loss of ER action in the ovary would cause severe deficits in granulosa cell differentiation. Indeed, the ovarian phenotypes in ERβ-null females in terms of antrum formation, aromatase expression, and LH responsiveness collectively represent an attenuated response to FSH-induced granulosa cell differentiation in the absence of ERβ. Additionally, ERβ-null granulosa cells and follicles have a reduced response to FSH marked by reduced cAMP accumulation in vivo and in vitro.[454,455] The reduction in cAMP can be overcome when cells are stimulated with forskolin to activate cAMP accumulation,[455] demonstrating that altered cAMP/PKA signaling contributes to the reduced granulosa cell differentiation observed in ERβ-null ovaries and suggests that maximal activation requires cross talk between FSH and ER-mediated signaling pathways. Possible downstream mediators were recently identified in a microarray study,[363] providing key targets to further study in the future.

ANTRUM FORMATION

Goldenberg et al. first demonstrated that both FSH and estrogen are required for full antrum formation in preovulatory follicles of hypophysectomized rat ovaries.[490] Wang and Greenwald produced similar data in hypophysectomized mice, reporting that FSH plus E2 leads to an average of >70 large antral follicles per ovary versus an average of six or none when treated with FSH or E2 alone, respectively.[492] The requirement of FSH plus E2 for antrum formation holds true when rat preantral follicles are grown in vitro.[522] Others have shown that exposure to aromatase inhibitors during gonadotropin-induced folliculogenesis also reduces the number of healthy antral follicles in rats,[485,486] although contradictory findings are reported when follicles are

similarly exposed in vitro.[523] Independent evaluations of ovaries from three separate lines of ERβ-null mice indicate a paucity of follicles with fully developed antra, even following standard gonadotropin stimulation.[158,172,440] These data strongly suggest that the vital role of E2 in FSH-induced antrum formation is mediated by ERβ. A role for ERα in antrum formation cannot be excluded but is unlikely given the hallmark phenotype in ERα-null ovaries is the invariable presence of severely oversized antral follicles, lending to their description as "cystic".[157–159,373,415] Furthermore, a role of aberrant LH action cannot be discounted, as mice overexpressing a mutant LH receptor have large cystic and hemorrhagic follicles similar to those observed in ERα-null mice.[524] While it is possible to hypothesize that ERβ may contribute to the formation of exaggerated antrum due to hyperstimulation of the receptor, the data supporting this idea are not clear. For example, mice that lack both ER forms (αβERKO),[158,171] or transgenic βERKO females that have elevated LH,[439] do not have large cystic follicles, although a new line of compound αβERKO mice (Ex3αβERKO) do have large cystic follicles[456] as do the Cyp19-null mice.[480] This contradictory data suggests that the mechanism behind antrum formation may not be due solely to overstimulation of ERβ, and multiple mechanisms may exist. Furthermore, mice lacking ERα specifically in the theca cells develop cystic follicles as they age in response to excess gonadotropins, which is not observed in younger mice,[362] suggesting a more complex mechanism contributes to antrum formation.

As mentioned, FSH alone will induce the formation of small antra in growing follicles whereas E2 has no such effect in vivo and in vitro, but both hormones are required for full antrum development.[490,492,522] Compared to the total failure of antrum formation among follicles in the murine models of disrupted FSH signaling,[420,503,512,513,525] similarly sized follicles in ERβ-null ovaries exhibit an initiation of antrum formation but fail to develop to a full preovulatory stage.[158] Quantitative analyses of immature ERβ-null ovaries 48 h after PMSG stimulation indicate an increased number of small antral follicles but fewer large (preovulatory) follicles relative to wild-type females, suggesting an arrest in antrum formation.[440] Likewise, a large percentage of ERβ-null follicles fail to reach maximum size when grown in culture with FSH and E2.[440] These follicles also fail to accumulate maximal cAMP levels after FSH stimulation,[455] suggesting that multiple signaling pathways may contribute to antrum formation. These data support the role of FSH actions in the initiation of antrum formation, but in the absence of ERβ-mediated E2 augmentation, maximum antrum development is prohibited.

Because so little is known about the physiology of antrum formation, the role of ERβ-mediated estrogen actions is speculative. It has long been proposed that antrum development follows gonadotropin-stimulated increases in granulosa cell proteoglycan secretion, which act to increase osmotic pressure within the follicle and cause the influx of water from the ovarian vasculature.[526,527] McConnell et al. report that water movement into mouse follicles in vitro occurs via a transcellular pathway and is likely mediated by aquaporins-7, -8, or -9 on the surface of granulosa cells.[528] Although there is currently no evidence of estrogen regulation of aquaporin expression in the ovary, E2 is known to influence fluid transport in the uterus via regulation of aquaporins -1,[332] -2, and -3,[529] and 5 and 8.[242,530] Furthermore, ERα is principally involved in the regulation of Na^+ and water transport across the efferent ductules in the rodent testis,[531] suggesting ERα may play a similar role in follicles. Antrum formation may not rely totally on hydromechanical mechanisms but is likely to require cell–cell interactions among the granulosa cell layer. Gore-Langton and Daniel demonstrated in vitro that rat preantral follicles lacking an intact basement membrane and therefore unable to provide a sealed environment exhibit a reorganization of granulosa cells that is unmistakably antrum-like in response to FSH plus E2, supporting a role for cell–cell interaction during antrum formation.[522] A lack of antral follicles in the ovaries of mice lacking the gap junction component connexin-37 (*Cx37*) further supports a need for cell–cell interactions among granulosa cells.[532] E2 is known to dramatically increase the number of gap junctions between granulosa cells[533] as well as regulate ovarian expression of certain component proteins.[534–536] Additionally, the extracellular matrix may be important to antrum formation. Mice lacking ERβ exhibit altered expression of several extracellular matrix proteins, including increased expression of Collagen 11a1 and Nidogen 2 at both the mRNA and protein level in both prepubertal and adult ovaries,[451,500] which may alter the follicle composition and contribute to loss of antrum in these mice. Future studies of a potential regulatory role for E2 in the expression of the aquaporins, connexin, and extracellular matrix proteins may reveal a precise role for ERβ in antrum formation.

AROMATASE AND ESTRADIOL SYNTHESIS

Acquisition of aromatase activity and E2 synthesis is a hallmark of healthy preovulatory follicles in mammalian ovaries. In monotocous species, including women, 90% of the circulating E2 is estimated to originate from the dominant follicle in the ovary. Aromatase (*CYP19*) expression in rat, mouse, bovine, marmoset, and human ovaries, via activation of the gonad-specific promoter II,[537] is exclusive to and highest in the granulosa cells of fully differentiated healthy follicles (Table 25.6).[400,538–540] Likewise, E2 levels are consistently highest in the follicular fluid of healthy preovulatory, and substantially

lower in overtly atretic, follicles. The endocrine actions of ovarian-derived E2 are critical to preparing the female reproductive tract for a forthcoming pregnancy and priming the hypothalamic–pituitary axis for generation of the gonadotropin surge. However, there is equally abundant evidence that E2 acts in a paracrine or autocrine manner to augment FSH stimulation of aromatase activity in granulosa cells and thereby positively influences its own rate of synthesis (Figure 25.19). This pattern of *CYP19* expression described herein strongly resembles the profile of ERβ during folliculogenesis, suggesting that the E2-induced enhancement of aromatase activity in granulosa cells is ERβ mediated.[440]

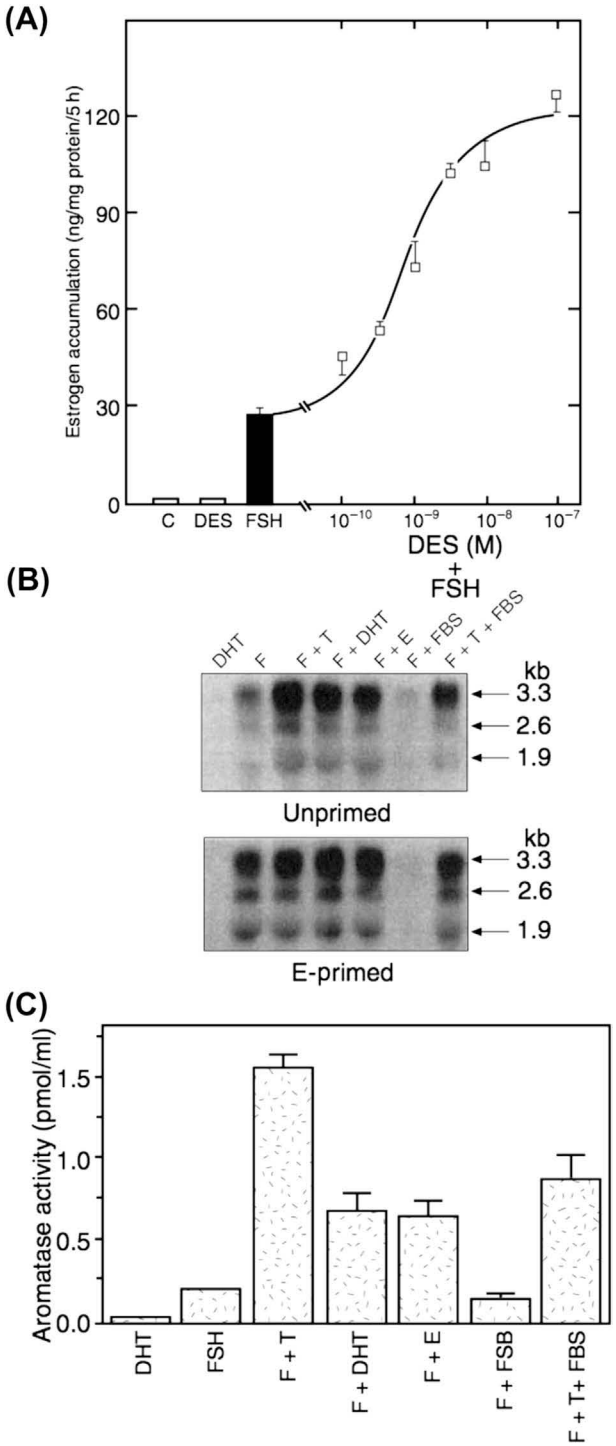


FIGURE 25.19 Synergistic action of sex steroids and FSH in the induction of *Cyp19* expression and aromatase activity in rodent granulosa cells. (A) Shown is the effect of in vitro treatment with the synthetic estrogen DES on FSH-induced aromatase activity in granulosa cells isolated from immature hypophysectomized rats. Granulosa cells were cultured for 3 days in the presence or absence of FSH (10 ng/ml) with or without increasing concentrations of DES. Three days later, the cells were washed with medium and reincubated for a 5-h test interval in medium supplemented with androstenedione. The accumulation of estrogen (as measured by radioimmunoassay) during this test period is taken as a measure of the level of aromatase activity. C, controls. *(Source: Reproduced with permission from Ref. [445].)* (B, C) Shown is the effect of FSH and sex steroids on *Cyp19* expression and aromatase activity in cultured granulosa cells. Granulosa cells were isolated from 26-day-old rats that had been untreated (unprimed) or treated with E2 (1.5 mg/day) for 3 days (E-primed). Cells were incubated in serum-free medium with 5α-dihydroT (DHT) at 20 nM, FSH (50 ng/ml), or FSH in combination with DHT (20 nM), T (20 nM), or E2 (20 nM). Cells were also incubated in medium containing 5% fetal bovine serum (FBS), with FSH alone (50 ng/ml), or in combination with T (20 nM). After 48 h in culture, cells were collected for RNA extraction and evaluation of *Cyp19* expression by Northern Blot analysis (B) or for measurement of aromatase activity (C). *Source: Reproduced with permission from Ref. [442].*

FSH is clearly the principal stimulus for the acquisition of aromatase activity in granulosa cells. Once again, female mice lacking FSH (*Fshb*-null mice)[419,541] or FSH receptor (*Fshr*-null mice)[421,513] exhibit phenotypes that are consistent with a severe reduction in circulating estrogens, including uterine hypoplasia and elevated plasma levels of LH. Furthermore, the ovaries of *Fshb*-null mice exhibit a six-fold reduction in *Cyp19* transcripts,[419] although these data are not corroborated in *Fshr*-null mice.[421] FSH regulation of *Cyp19* expression in granulosa cells is believed to require the actions of multiple transcriptions factors, including steroidogenic factor-1 (SF-1) and β-catenin,[542] CREB protein, GATA-4 and CBP, the former three of which possess cognate response elements within the rodent and human *CYP19* promoter II.[441,520,521,543]

The mechanism by which E2 augments FSH stimulation of aromatase activity or *Cyp19* expression in granulosa cells is unclear but shown to occur in isolated granulosa cell cultures,[442,444–447] ruling out the influence of a thecal cell–derived factor and supporting a direct role for ERβ. Coadministration of FSH and an ER antagonist (tamoxifen) to granulosa cells either in vivo or in vitro inhibits the expected induction of aromatase activity;[544] although others report conflicting data using a different antagonist (ICI 182,780) in whole follicle culture experiments.[497] More recently, Emmen et al.[440] and Rodriguez et al. found that ERβ-null follicles grown in vitro do indeed secrete significantly less E2 relative to wild-type follicles.[455] The absence of a consensus ERE within the rat or human *Cyp19* promoter II makes it unlikely that the effect of E2 is via a direct ERβ mechanism (Figure 25.4). However, ERβ may interact with the cAMP/PKA pathway, SF-1, CREB, GATA-4, CBP, or other unknown transcription factors that act to promote *Cyp19* expression. Indeed, ERβ has been shown to associate with both CREB[519] and CBP[545,546] in heterologous in vitro systems. The ERs are also known to influence GATA-directed gene expression via direct interactions with multiple members of the GATA family of transcription factors.[474,547] Therefore, there is ample evidence to support ER interaction with the multitude of transcription factors known to influence *Cyp19* expression, but the exact nature by which ERβ is involved requires further study.

ACQUISITION OF LUTEINIZING HORMONE RECEPTOR

In contrast to the constitutive expression of LH receptor in thecal cells, granulosa cells express LH receptor only in follicles in the late preovulatory stage. This limited expression of LH receptor to the granulosa cells of healthy preovulatory follicles provides for an intraovarian mechanism that ensures only those follicles that are suitable for ovulation acquire the capacity to respond to the LH surge. As with the other markers of follicle differentiation discussed previously, FSH is the primary stimulus of LH-receptor (*Lhcgr*) expression in preovulatory granulosa cells. However, FSH exposure alone leads to a minimal increase of LH-receptor levels in the ovaries of hypophysectomized rats, and then only after 4 days of treatment; whereas pretreatment of animals with E2 for 4 days prior to FSH exposure leads to an enormous induction of LH-receptor levels that are limited to the granulosa cells of preovulatory follicles (Figure 25.20).[359] This requirement for FSH plus E2 and the temporal pattern of LH-receptor expression has been reproduced in rat granulosa cells in vitro, with maximum levels reached after 48–72 h.[515,548,549] *Lhcgr* mRNA levels mirror the LH-receptor protein levels and exhibit a comparable FSH/E2 regulation in rat granulosa cells.[550] Further confirmation that estrogens are required to augment FSH-induced LH-receptor expression in granulosa cells comes from studies that have collectively shown the following: (1) only estrogens (e.g., E2, diethylstilbestrol) or substrates for aromatization (e.g., androstenedione, T, estrone) are effective,[515,548,549] (2) nonaromatizable androgens (e.g., DHT) and P have little effect (Figure 25.20),[515,549] (3) co-treatment with aromatase inhibitors, ER antagonists, or 17β-E2-specific antisera block FSH induction of LH receptor[484,514,548] and hCG-induced ovulation,[485] and (4) *Lhcgr* levels are decreased five-fold in the ovaries of Cyp19-null mice.[479] The poor ovulatory response of ERβ-null ovaries to an ovulatory dose of the LH analog, hCG,[171,432] strongly suggests that ERβ mediates the synergistic actions of E2 during FSH induction of the *Lhcgr* gene in granulosa cells.[134,171] In contrast, the granulosa cells of large antral follicles in ERα-null ovaries exhibit abnormally high levels of LH receptor, even after becoming enlarged and cystic.[134,373]

The complexity of the *Lhcgr* promoter is illustrated by the differential regulation between thecal and preovulatory granulosa cells. Not surprisingly, FSH induction of LH-receptor expression in granulosa cells occurs via the cAMP/PKA pathway.[551–554] However, the proximal promoter of the *Lhcgr* gene in both humans and rats lacks a consensus cAMP response element (CRE) similar to those that confer FSH regulation of the *Cyp19* promoter II.[555,556] Studies indicate that a GC-rich region possessing a cluster of Sp1 binding sites and an ERE half site contributes to FSH and 8-bromo-cAMP induction of the rat *Lhcgr* promoter in vitro.[557–559] Furthermore, multiple nonconsensus cAMP-response elements within this GC-rich region of the rat *Lhcgr* promoter provide a platform for assembling an undefined complex of nuclear proteins from granulosa cell extracts that confer cAMP induction.[556,560] With such limited data concerning *Lhcgr* regulation, it is difficult to speculate where the actions of ERβ may impact LH responsiveness in granulosa cells. ERβ-null granulosa cells have reduced cAMP accumulation after FSH signaling and also show reduced *Lhcgr* accumulation compared to wild-type granulosa cells.[454]

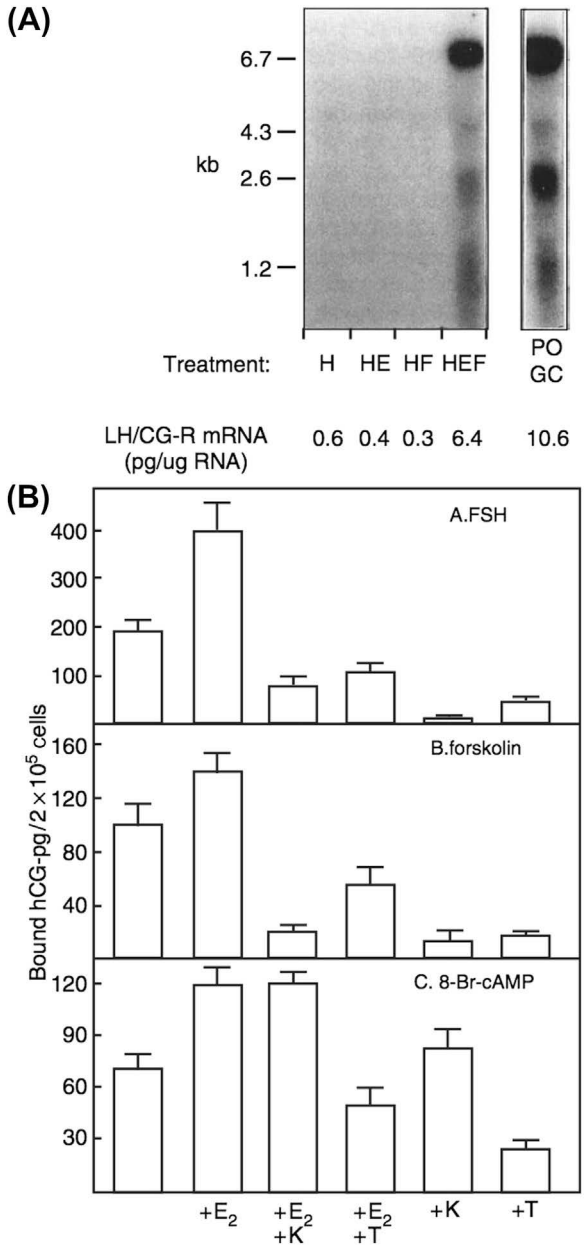


FIGURE 25.20 Synergistic action of E2 and FSH in the induction of LH receptor in rodent granulosa cells. (A) Induction of *Lhcgr* (LH/CG-R) mRNA in granulosa cells of hypophysectomized rats after treatment with FSH and/or E2. Animals were untreated (H) or injected with 1.5 mg E2/day for 3 days (HE), 1 mg FSH twice daily for 2 days (HF), or a sequential combination of E then FSH (HEF). Animals were euthanized after treatment and granulosa cells isolated from the ovaries for total RNA extraction. *Top*: Northern blot of 20 µg RNA/lane probed for *Lhcgr* (LH/CG-R) mRNA. (PO GC: pro-ovulatory granulosa cell RNA) *(Source: Reproduced with permission from Ref.* 550.*)* (B) Effect of antiestrogens on the induction of LH-receptor levels by FSH, forskolin, or 8-bromo-cAMP. Granulosa cells were isolated from immature rats implanted with DES pellets for 4 days. 2×10^5 cells were cultured for 48 h with FSH (100 ng), forskolin (10 µM), or 8-bromo-cAMP (5 nM), with or without E2, 10^{-8} M in the absence or presence of antiestrogens, keoxifene (K, 1 µM), or tamoxifen (T, 1 µM). Media were then removed and cells were analyzed for LH-receptor content by radiolabeled binding assay. The *first bar* on the left side of the figure is FSH (A), forskolin (B), or 8-bromo-cAMP (C) alone. Control cells bound less than 10 pg hCG/2×10^5 cells, and the data are not shown. *Source: Reproduced with permission from Ref.* 514.

The reduced expression suggests it may be downstream of cAMP, and in vitro follicle culture studies found increased *Lhcgr* expression when follicles were treated with forskolin to induce cAMP accumulation.[455]

As discussed before, E2 enhances the level and action of FSH-stimulated increases in intracellular cAMP to maximize *Cyp19* expression, and it is likely that a similar mechanism is employed for induction of LH-receptor levels. However, there are notable differences in the E2/FSH regulation of *Cyp19* versus *Lhcgr* expression. FSH induction of *Lhcgr* expression in preovulatory granulosa cells is augmented by estrogens only,[515,548,549] whereas nonaromatizable androgens or estrogens act through their respective receptor pathways to enhance FSH induction of *Cyp19* expression.[442] This divergence in regulation is critical since androgen augmentation of FSH on *Cyp19* expression allows for FSH/AR-mediated initiation of E2 synthesis in preantral follicles shortly after thecal cells begin synthesizing aromatizable precursors; whereas the specificity for ER/E2 actions in the FSH induction of the *Lhcgr* gene provides that LH receptors are acquired only by those follicles that have sufficient E2 synthesis, and are hence healthy and suitable for ovulation. Secondly, Farookhi and Desjardins[549] demonstrated that FSH induction of *Lhcgr* but not *Cyp19* expression is lost when estrogen-pretreated granulosa cells are dispersed in culture by EGTA-disruption of cell–cell contacts.[549] A requirement for intact cell–cell contacts for E2/FSH-mediated *Lhcgr* expression may ensure that atretic follicles, which often exhibit a fragmented and less compact granulosa cell population, do not acquire the capacity to respond to an LH surge and ovulate an unhealthy oocyte.

ESTRADIOL MEDIATED NEGATIVE FEEDBACK ON THECAL CELL FUNCTION

Like granulosa cells, follicular thecal cells also undergo a process of differentiation during progression from a preantral to preovulatory follicle.[561] Fully differentiated thecal cells are best characterized by their unique capacity to convert cholesterol to C_{19} steroids, primarily androstenedione.[561] A principal element of the *two-cell, two-gonadotropin* paradigm of steroidogenesis in maturing follicles (Figure 25.21) is that thecal cells exclusively possess the 17β-hydroxylase:$C_{17,20}$-lyase activity necessary for synthesizing androgens, forcing the granulosa cell layer to be dependent upon the theca as a source of aromatizable precursors.[562] Thecal cell synthesis of androstenedione is considered the rate-limiting step in E2 production by preovulatory follicles and involves the sequential enzymatic actions of $P450_{scc}$ (CYP11), $P450_{17\alpha/17,20\text{-lyase}}$ (CYP17) and Δ^5-3β-hydroxysteroid dehydrogenase (3β-HSD 1).[562] It is generally accepted that LH regulates thecal cell steroidogenesis via the induction of CYP17 and CYP11 activity,[561] while 3β-HSD 1 is constitutive.[562] The requirement of LH stimulation to induce CYP17 activity in thecal cells

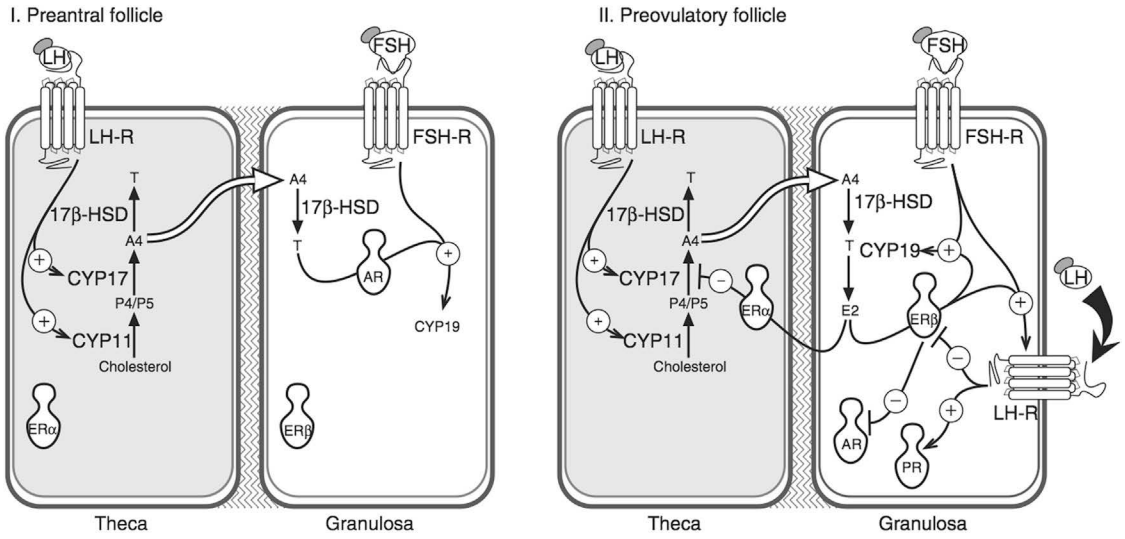


FIGURE 25.21 Model of sex steroid receptor actions in the ovary during follicular differentiation. The process of follicle differentiation from preantral to preovulatory follicle is marked in granulosa cells by the induction of aromatase (CYP19) activity for the synthesis of E2 and LH-receptor (LH-R) expression. This process is principally dependent on stimulation of thecal cells by LH and granulosa cells by FSH. However, there is substantial evidence of a modulatory role for the sex steroid receptors in this process. The above model illustrates the putative expression pattern and actions of the sex steroid receptors during follicle differentiation. Some aspects of the above model are supported by experimental data, whereas others are more speculation at this time (see text for details). (I) Preantral Follicle: In undifferentiated preantral follicles, LH stimulation of thecal cells (which constitutively express LH-R) leads to de novo synthesis of androstenedione (A4) and T. A4 and T diffuse across the basement membrane of the follicle and into the granulosa cells. A4 may be converted to T in granulosa cells. Thecal-cell derived or locally synthesized T activates the androgen receptor (AR), and this action synergizes with FSH to induce aromatase (CYP19) expression in the granulosa cells. The induction of E2 synthesis commences the process of follicle differentiation. (II) Preovulatory Follicle: Increasing E2 synthesis in the granulosa cells leads to activation of ERβ and induces differentiation by synergizing with FSH to further increase CYP19 expression and E2 synthesis and induce LH-R expression in the granulosa cells. Ligand-dependent ERβ actions may also lead to decreased AR expression, allowing the role of A4 and T to shift from that of a ligand for AR-mediated actions to that as substrates for E2 synthesis. Increasing E2 levels eventually activate ERα in thecal cells to reduce further androgen synthesis by decreasing CYP17 activity, forming a negative feedback loop to ensure the proper estrogen-to-androgen ratio in the follicle. LH Surge: Climaxing E2 levels in the circulation facilitate the release of the LH surge from the pituitary, which stimulates ovulation and terminal differentiation of the granulosa cells. Only those follicles that possess properly differentiated granulosa cells (i.e., have acquired sufficient LH-R) are able to respond to the LH surge, which causes a dramatic rise in PR expression that occurs in the preovulatory granulosa cells within hours of the LH surge, whereas ERβ and CYP19 levels are both dramatically decreased.

is effectively illustrated by the 12-fold reduction in *Cyp17* expression in ovaries of LH-receptor (*Lhcgr*)-null mice,[563] as well as the five-fold increase in expression in the ovaries of transgenic mice that possess chronically elevated LH levels.[439] Likewise, chronically elevated plasma LH leads to a comparable increase in *Cyp17* expression and androstenedione synthesis in both ERα-null and ERβ-null ovaries and cultured follicles, indicating that neither ER is obligatory to the positive regulation of thecal cell steroidogenesis.[431,439,564]

Therefore, androgens are necessary to serve as both a stimulus of and substrate for aromatase activity in granulosa cells. However, a proper androgen:estrogen ratio is critical as an intrafollicular milieu of increased androgens is strongly associated with atresia.[565] It is generally believed that an intraovarian mechanism exists to negatively modulate thecal cell androgen synthesis so that levels do not surpass the aromatase capacity of the granulosa cell layer. Indeed, defects in this intraovarian mechanism are postulated to be an underlying cause of the excess thecal cell androgen synthesis that is a hallmark of polycystic ovarian syndrome (PCOS) in women. There is ample evidence that granulosa cell–derived E2 may mediate a feedback loop on thecal cells to decrease further androgen synthesis in thecal cells,[566] however, the mechanism remains unclear. Magoffin posed the question several years ago as to whether E2 inhibition of androgen synthesis occurs at the level of steroidogenic enzyme expression or via direct inhibition of enzymatic activity.[561] Indeed, estrogens have been shown to inhibit CYP17 activity in both thecal and Leydig cell preparations while having no effect on activities of the upstream steroidogenic enzymes.[566–571] Early studies found that E2 can compete with androstenedione for CYP17 enzymatic activity;[568] however, this effect could not be reproduced in ovarian lysates in vitro.[572] Additional studies in dispersed rat ovarian cells found that E2 has no effect on the number of LH receptors or level of hCG-stimulated cAMP synthesis, further supporting a direct E2 inhibition of CYP17 activity as a mechanism for regulating androgen synthesis.[570] However, other evidence supports a role for ER-mediated regulation of *Cyp17* expression at the transcriptional level, including demonstrations that an antiestrogen blocks the expected drop in CYP17

activity that occurs just prior to ovulation in rats,[573] and that E2 significantly reduces *Cyp17* expression in the testes of rats[574] and fish.[575] Our findings that ERα-null ovaries exhibit increased *Cyp17* expression and enzymatic activity despite a milieu of significantly elevated E2 also supports a mechanism of ERα-mediated suppression of *Cyp17* transcription in thecal cells versus direct inhibition of CYP17 activity.[431] Furthermore, Taniguchi demonstrated that in vitro–cultured ERα-null follicles have increased *Cyp17* expression similar to that observed in WT follicles cultured in the presence of an aromatase inhibitor.[564] Treatment of ERα-null follicles with E2 or an ERα-specific agonist as well as an aromatase inhibitor reduced *Cyp17* expression to that observed in wild-type vehicle treated follicles.[564] Therefore, the excess androgen synthesis observed in ERα-null ovaries is due to the loss of ERα-mediated suppression of *Cyp17* expression, indicating the existence of intraovarian feedback actions of E2 that are mediated by ERα and critical to maintaining homeostasis of androgen levels in the female mouse.

OVULATION

E2 clearly facilitates the acquisition of several gonadotropin-dependent effects during folliculogenesis, including: (1) the proper cellular organization of the follicle, e.g., antrum and cumulus oocyte complex; (2) the necessary enzymatic activity, e.g., E2 synthesis; and (3) the essential receptor signaling pathways; e.g., LH receptor. All of these characteristics define a healthy differentiated follicle and are necessary for a proper response to the gonadotropin surge and expulsion of a competent oocyte. However, a need for estrogen signaling in the ovulating follicle or following the postgonadotropin surge remains unclear. Selvaraj et al.[485] found that coadministration of immature rats with PMSG and an aromatase inhibitor (Fadrazole) leads to a reduced number of healthy antral follicles that culminates with a severely reduced ovulatory response to a bolus of hCG. These data support the intrafollicular role of E2 in preparing competent preovulatory follicles for ovulation. However, in an additional experiment, Selvaraj et al. once again treated immature rats with PMSG but delayed administration of the aromatase inhibitor by 40 h such that exposure occurred just 6 h prior to hCG induced ovulation.[485] These animals exhibit an equally poor response in terms of follicular rupture and recoverable oocytes from the oviduct; and this effect is abated by coadministration of E2 just prior to induced ovulation. These data in turn suggest that E2 does facilitate the ovulatory process. However, inhibition of E2 synthesis is known to cause severe intraovarian[485] and intrafollicular[523] accumulations of P and androgens that could also lead to a poor ovulatory response. In contrast to the above in vivo findings, Hu et al. reports that isolated mouse follicles grown exposed to an aromatase inhibitor in vitro exhibit a normal ovulatory response to hCG, including mucification of the cumulus–oocyte complex.[523] Furthermore, detrimental effects of aromatase inhibition such as those reported in the rat ovary are not observed in similarly treated hamsters, rabbits, or monkeys.[486]

Multiple investigations of the ovulatory capacity of ER-null and CYP19-null mice have been conducted. As described before, ERα-null females are anovulatory throughout life and gonadotropin-induced ovulation is unsuccessful in ERα-null females at 4 months of age when the ovarian cystic phenotype is advanced.[373] However, immature (≤28 days of age) ERα-null (αERKO) females do respond to induced ovulation and yield recoverable oocytes in the oviduct at an average yield of 14.5 oocytes/ERα-null female versus 40.6 oocytes/wild-type female.[415] Similar data has been reported in ERα-null females as old as 5 weeks of age.[415,430] Interestingly, Dupont et al. report that immature females from their ERα-null (ERαKO) line exhibit a total failure to ovulate in response to exogenous gonadotropin treatments, although this study involved only three females.[158] Preliminary studies suggest Ex3αERKO mice[159] have a significantly reduced ovulation response, where less than 50% of mice ovulate in response to exogenous gonadotropins, with few oocytes produced from those that do ovulate (Korach laboratory, unpublished data). These observations suggest that the response observed previously[415] was possibly due to the presence of residual ERα splice variant expressed in the αERKO mice,[159,189] thus further studies are underway to confirm these preliminary studies.

The cause of the reduced ovulatory response to exogenous gonadotropins in ERα-null females is unclear and suggests a facilitatory role for ERα in follicular rupture. However, the expected induction of several genes that are critical to follicle rupture, such as P receptor, prostaglandin-synthase-2,[576] occurs in gonadotropin-primed ERα-null ovaries shortly after hCG treatment.[415,432] Furthermore, ERα-null ovaries form functional corpora lutea following induced ovulation, indicating that granulosa cells of ERα-null preovulatory follicles possess the capacity to respond to LH. Therefore, the reduced ovulatory response in ERα-null ovaries is more likely attributable to premature increases in endogenous plasma LH[415] and subsequent ovarian androgen synthesis.[415,430] Although immature ERα-null ovaries do not yet manifest the overt morphological effects of LH-hyperstimulation, e.g., cystic follicles, they do exhibit elevated LH-receptor levels.[415] Cumulative damage in the ovary from chronic LH hyperstimulation likely causes the age-related reduction in gonadotropin-induced ovulatory capacity in ERα-null females discussed previously. Indeed, Armstrong and colleagues demonstrated in rats that treatment with PMSG preparations possessing increased LH activity lead to a reduced ovulatory response to subsequent hCG stimulation[577,578] and that this effect is likely due to inappropriately high stimulation of androgen synthesis during follicle maturation.[578] Studies showing ERα-null follicles allowed to differentiate in vitro under controlled hormonal

stimulation exhibit a rate of hCG-induced rupture that is comparable to similarly cultured wild-type follicles further indicating that any role for ERα in the ovulatory process is minimal.[440] The ERα was found to be necessary for ovulatory response in aging animals, as thEsr1KO mice had normal ovulatory response at 2 months that was severely dampened by the time the mice were 6 months old.[362] The premature loss of fertility in these mice suggests that ERα is important for normal ovulatory function; however, the precise mechanism has yet to be elucidated.

The loss of functional ERβ clearly leads to ovarian defects that are inhibitory to ovulation. Both lines of ERβ-null females exhibit severely reduced fertility due to ovarian defects; however, unlike ERα-null females, ERβ-null animals do spontaneously ovulate.[158,171] Krege et al. found that immature ERβ-null (βERKO) females exhibit a severely reduced oocyte yield of 6 oocytes/female versus 34 oocytes/female among age-matched wild-types following gonadotropin-induced ovulation.[171] Dupont et al. report a similar average yield in immature ERβKO females (17.6 versus 37 oocytes/female), even when excluding those females that do not ovulate at all.[158] A third line of ERβ-null mice was found to be unresponsive to exogenous gonadotropins, and no animals were able to ovulate ($n=19$).[172] Collectively, even though some animals are able to respond with some oocytes ovulated, the models demonstrate the necessity of ERβ for optimal ovulatory response.

Immature ERβ-null ovaries collected 20 h after hCG-induced ovulation reveal multiple preovulatory follicles that retain their oocyte and fail to undergo expansion of the cumulus–oocyte complex.[158,171,432] Furthermore, hCG induction of prostaglandin-synthase 2 and PR, two events that are critical to follicular rupture, are significantly compromised in immature βERKO ovaries.[432] ERβ-null follicles induced to ovulate in vitro exhibit an equally poor rate of rupture and gene induction following hCG exposure.[440] The sum of in vivo and in vitro data from ERβ-null mice strongly indicates an inability to fully respond to an endogenous LH surge or an exogenous bolus of hCG. This is likely due to insufficient acquisition of LH-receptor expression among granulosa cells of ERβ-null preovulatory follicles.[432,440,455] Indeed, Clemens et al. found that the ER-antagonist ICI 164,384 inhibits FSH-induced differentiation of rat granulosa cells in vitro, as illustrated by a severely compromised induction of PR expression following stimulation of the cAMP/PKA signaling pathway.[579] However, if exposure to the ER antagonist is delayed until the time of ovulatory stimulation, little effect is observed,[579] further supporting a role for ERβ in granulosa cell differentiation and LH-receptor expression rather than during ovulation. ERβ-null follicles cultured in vitro also have a reduced ovulatory response and reduced cAMP accumulations after FSH stimulation.[455] Interestingly, treatment of these follicles with forskolin to increase cAMP levels was able to increase the ovulation of these follicles in vitro,[455] suggesting that ERβ is necessary for maximal ovulatory response downstream of gonadotropins, although ERβ is not directly contributing to follicle rupture.

Evidence of any cooperative action between ERα and ERβ in ovulation may be derived from studies of the compound ER-null and CYP19-null mice. The single report of induced ovulation in immature ERα/ERβ-null (ERαβKO) females describes a total failure of ovulation.[158] Not surprisingly, ovaries from these females exhibited underdeveloped preovulatory follicles that failed to exhibit cumulus–oocyte complex expansion or luteinization,[158] indicating an attenuated response to hCG similar to that in ERβ-null ovaries. Investigations in both lines of Cyp19-null females produced comparable results of a total failure to ovulate and luteinize.[131,580] Interestingly, Huynh et al. remarked that cumulus–oocyte complexes lacking oocytes were recovered from the oviducts of 4-week-old Cyp19-null females following induced ovulation, indicating that some elements of follicle rupture may occur after degradation of the oocyte.[580] The more severe ovulatory phenotypes in compound ER-null and Cyp19-null females suggest that the intraovarian functions of both ERα and ERβ are necessary for ovulation but not necessarily for the process of follicular rupture. Recent studies in Cyp19-null mice suggest that treatment with E2 as well as exogenous gonadotropins could stimulate some ovulation,[365] suggesting the importance of the steroid and gonadotropin hormones in regulating ovulation.

ERα MEDIATED REPRESSION OF LEYDIG CELL DEVELOPMENT IN THE OVARY

As discussed in earlier sections, female mice lacking ERα action due either to the loss of receptor (αERKO, thEsr1KO, and αβERKO) or ligand (Cyp19-null) exhibit abnormally high levels of plasma T. For example, the average plasma T level in adult αERKO and αβERKO females is 5.5 ng/ml and 2.1 ng/ml, respectively, versus 0.14 ng/ml in wild-type females.[431] In females of the two existing Cyp19-null lines, Fisher et al. report average plasma T levels in mutant females to be 2.3 ng/ml versus 0.24 ng/ml in wild-type;[156] while Toda et al. report 1.4 ng/ml in the mutant females versus 0.13 ng/ml in wild-type.[131] These levels of plasma T in ERα-null and Cyp19-null females surpass the lower limits characteristic of normal male mice (2–10 ng/ml)[131,156,581] and are obviously very uncharacteristic of normal females. The mouse ovary possesses the enzymatic machinery necessary for T synthesis, although it is not a major product. The *two-cell, two-gonadotropin* paradigm of ovarian steroidogenesis (Figure 25.21) postulates that thecal cell–derived androstenedione serves as a substrate for two sequential reactions in granulosa cells, CYP19-mediated aromatization to estrone followed by a reduction to E2 by 17β-hydroxysteroid dehydrogenase type I

(*HSD17β1*).[561] The murine form of 17β-HSD 1 is reported to efficiently reduce androstenedione to T as well as estrone to E2; whereas the human form may not possess this activity.[582] Therefore, the accumulation of androstenedione that occurs in ERα-null and Cyp19-null ovaries due to LH hyperstimulation of the theca in both, and the absence of aromatization in the latter,[156,431] provides for sufficient substrate for 17β-HSD 1 conversion to T. However, further investigations have discovered that ERα-null[431] and Cyp19-null[479] ovaries uniquely possess substantial levels of 17β-hydroxysteroid dehydrogenase type III (*Hsd17b3*), a Leydig cell–specific enzyme in testes that specifically functions to reduce androstenedione to T.[583–587] Remarkably, the level of *Hsd17b3* expression in αERKO, αβERKO,[431] and Cyp19-null[479] ovaries is comparable to that found in testes of wild-type males. Therefore, the abnormally high capacity of ERα-null and Cyp19-null ovaries to synthesize T may be attributed to the accumulation of androstenedione and its conversion to T by 17β-HSD 3 rather than 17β-HSD 1.

Hsd17b3 expression in Leydig cells is primarily LH regulated.[585] The ectopic expression in ERα-null ovaries is also dependent on LH hyperstimulation as expression is eradicated following prolonged treatment of animals with a GnRH antagonist.[431] In ERα-null females, *Hsd17b3* expression is limited to the ovarian interstitium and possibly the theca.[588] However, it is unclear whether these cells are Leydig-like and suggestive of an "organizational" defect in ERα-null ovaries or are thecal/interstitial type cells within which the loss of ERα leads to loss of *Hsd17b3* repression, i.e., an "activational" defect. Evidence to support the latter hypothesis includes: (1) testes from ERα-null males also exhibit elevated *Hsd17b3* expression and T synthesis,[589] (2) female mice possessing chronically elevated LH but intact ERα signaling do not exhibit ovarian *Hsd17b3* expression,[439] and (3) prolonged E2 therapy in Cyp19-null females eradicates *Hsd17b3* expression.[479] In turn, there exists supporting evidence for the former hypothesis that a defect during ovarian differentiation may occur in the absence of ERα. Thecal and Leydig cells are believed to derive from a common bi-potent embryological precursor cell during gonadal differentiation. In Cyp19-null ovaries, Britt et al. have described the presence of cells resembling the mature Leydig cells of testes, in that they possess a tubulovesicular arrangement of mitochondrial cristae, an annular nucleolus, and an abundance of smooth endoplasmic reticulum in whorl-like formations.[194] These Leydig cell–like ultrastructural features were also observed in ERα-null ovaries.[588] These Leydig-like cells in Cyp19-null ovaries are located in the interstitial regions and often proximal to the basement membrane of atretic follicles that exhibit Sertoli-like cells in the follicle lumen.[194] Indeed, E2 is a potent inhibitor of Leydig cell proliferation in vitro and in vivo,[590] and E2 replacement in Cyp19-null females reduces the presence of Leydig-like cells in the ovaries.[479] Therefore, ERα may be critical to promoting thecal cell differentiation and inhibiting the Leydig cell phenotype during ovarian differentiation. A similar phenotype of Leydig cell differentiation, *Hsd17b3* expression, and "masculinization" of the ovary and reproductive tract is reported in female mice lacking *Wnt4* function.[466]

DISRUPTED ESTROGEN SIGNALING IN HUMANS

There is one case of a human female that is autosomal recessive for an inactivating mutation of the *ESR1* (ERα) but no patients with *ESR2* (ERβ) mutation reported. There are cases of human females that are autosomal recessive for inactivating mutations of the *CYP19* gene and therefore lack the ability to synthesize E2.[591–597] The CYP19 mutated females present with androgen-induced pseudohermaphrodism at birth,[591–593] and amenarche, polycystic ovaries, and an absence of female secondary sex characteristics at puberty.[591,593,594] Three reports describe elevated plasma FSH and LH levels, leading to hypergonadism that manifests as increased plasma androgens and virulization.[591,593,594] The female with Esr1 mutation has polycystic ovaries and elevated E2. The ovarian pathology exhibited is somewhat similar to that observed in ERα-null females and compatible with a diagnosis of PCOS.[593] To date there are no indications of "sex reversal" in terms of male cell types or gene expression in the ovaries of CYP19-null human females. Estrogen and P replacement effectively alleviates all of the above phenotypes.[591,593,594]

Polymorphisms of the human *ESR1* gene have been linked to breast cancer, cardiovascular disease, osteoporosis cognitive function, and multiple reproductive anomalies.[598–603] A polymorphic $(TA)_n$ repeat within the *ESR1* promoter region has been linked to premature ovarian failure[604] but has no effect on plasma levels of E2, sex-hormone binding globulin (SHBG), T, or FSH in premenopausal women during the follicular phase.[605] Two *PvuII* single nucleotide polymorphisms (SNP) in the *ERS1* gene have been described to date. The first of these is a glutamine-to-glycine transformation at amino acid 400[606] and has received little investigative attention. The second is an anonymous SNP located in intron 1, 400 bp upstream of exon 2,[607] and is found in approximately 30% of women.[608–612] Although the intronic *ESR1 PvuII* SNP does not affect ERα receptor levels,[607] it may be associated with: (1) poor performance in women undergoing in vitro fertilization,[608,612] (2) increased plasma E2 and androstenedione levels,[610,612] and (3) late onset of menarche and/or menopause.[609,611,613,614] Two SNP of the *ESR2* gene have been described to date, an *RsaI* SNP leads to a silent nucleotide change in exon 5, and an *AluI* SNP occurs in the 3′-untranslated region of exon 8.[615] Sundarrajan et al. found that Chinese women that are homozygous for either the *RsaI* or *AluI* SNP of the *ESR2* gene are more

prone to exhibit ovulatory defects, menstrual disorders, and elevated LH; while women that are homozygous for both exhibit more severe idiopathic ovulatory defects.[616]

Progesterone Receptor Signaling in Ovarian Function

PR Expression in the Ovary

The first report of saturable, high-affinity P binding sites in the rat ovary is that of Schreiber and colleagues in 1979[617,618] and has since been followed by similar descriptions in human[389–391,619] and cow[619,620] ovaries. Later immunohistochemical studies primarily localized ovarian PR expression to the theca of large preovulatory follicles, the surface epithelia, and stromal/interstitium, and found this expression pattern is well conserved among species, including mice,[621,622] pig,[623] monkeys,[386,624] and humans.[392,625,626] Numerous studies in species ranging from rodents to large domestic animals and primates agree that granulosa cells possess minimal PR expression throughout folliculogenesis except for the period 2–4 h after the preovulatory gonadotropin surge, whereupon an enormous induction of PR expression occurs in preovulatory granulosa cells and peaks just prior to follicle rupture[386,392,415,621–624,627–630] (Figure 25.22). This dramatic increase in PR expression in the granulosa cells of ovulatory follicles is absolutely essential to follicle rupture.[182,631] This induction of PR expression in ovulating follicles is quite transient and lasts less than 12 h in rodent ovaries[415,621,628] but is maintained throughout luteinization and corpus luteum formation in porcine[623] and primate[386,392,624] ovaries.

The regulation of ovarian PR expression prior to the preovulatory surge is poorly characterized. PMSG treatment of immature rats leads to a four-fold increase in cytosolic P binding sites[632] and a modest two-fold increase in *Pgr* mRNA levels[628] after 48 h, indicating that FSH or subsequent E2 signaling may stimulate these basal levels of PR expression. In contrast, the mechanisms underlying the dramatic induction of PR expression in preovulatory granulosa following the gonadotropin surge are relatively well characterized. Over the course of the estrous cycle in rats, *Pgr* mRNA levels are increased >30-fold during a 4 h period (1800–2200 h) on the evening of proestrus and then return to baseline shortly after (2400 h).[628] The increased expression is almost exclusive to the mural granulosa cells of healthy preovulatory follicles in rats,[628,633] indicating that only fully differentiated granulosa cells possess the capacity for such rapid induction of the *Pgr* gene (Figure 25.22). Although PR expression climaxes within hours after plasma E2 and LH levels climax, the following evidence indicates LH is most directly involved: (1) expression peaks just 2 h after plasma LH levels climax versus >8 h after peak plasma E2 levels,[628] (2) hCG treatment 48 h after PMSG exposure leads to a comparable increase in PR expression, whereas PMSG and concomitant increases in E2 have a minimal effect,[415,621,628] (3) treatment of hypophysectomized or immature rats, or differentiated rat granulosa cells with E2 has no marked effect on ovarian PR expression,[422,634] (4) inhibition of the LH surge by pentobarbital abates the increase in PR expression despite having no effect on the rise in plasma E2 levels,[628] (5) LH but not E2 elicits a dramatic PR induction in differentiated granulosa cells from rat[634,635] or porcine[629] ovaries in vitro, (6) an ER antagonist (ICI 164,384) does not inhibit forskolin-induced PR expression in differentiated rat granulosa cells,[579] (7) the putative ERE-like region of the rat *Pgr* promoter does not bind ERα or ERβ in ovarian extracts,[579] and (8) ER (mainly ERβ) levels are rapidly decreasing at the time of rising PR expression in preovulatory granulosa cells.[367,371,380,415]

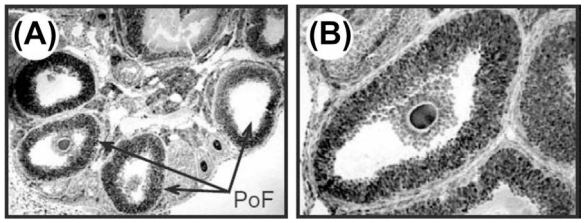


FIGURE 25.22 Regulation of PR expression in mouse ovaries and granulosa cells during gonadotropin-induced ovulation. (A, B) Immunohistochemistry for PR in the ovary of a PMSG-primed mouse 4 h after hCG treatment, illustrating the dramatic induction of PR immunoreactivity in the granulosa cells of preovulatory follicles (POF) but not surrounding preantral follicles. (B, higher magnification of A.)

The above data provide a convincing argument that the rapid and transient induction of PR expression in preovulatory granulosa cells is directly mediated by the LH surge. Clearly only granulosa cells of preovulatory follicles in vivo or fully differentiated granulosa cells in vitro possess the capacity to respond to the LH surge and increase PR expression, presumably due to their unique acquisition of LH receptor.[579,634] Indeed, granulosa cells isolated from estrogen-primed immature rats require a 48-h exposure to physiological concentrations of FSH and T to acquire LH induction of PR expression.[579] LH-mediated induction of the intracellular cAMP/protein kinase-A pathway is likely the principal stimulus for increased PR expression in preovulatory granulosa cells as large doses of FSH, forskolin, or $(Bu)_2$cAMP also elicit a comparable response in rat ovaries in vivo[634] and differentiated rat[634,635] or porcine[629] granulosa cells in vitro. However, GnRH, PMA, or EGF exposure of differentiated rat granulosa cells in vitro elicits a comparable induction and temporal pattern of PR expression, suggesting that stimulation of protein kinase C or tyrosine kinase signaling cascades may also be involved.[635] In fact, Sriraman et al. report that forskolin and PMA synergize to induce increased PR expression in undifferentiated

primary rat granulosa cells.[636] However, studies in fully differentiated rat granulosa cells demonstrate that forskolin-induction of PR expression is fully blocked by co-treatment with a PKA inhibitor (H89) but only minimally affected by a PKC inhibitor (Cal-C),[579] indicating a greater importance for the former signaling pathway.

The mechanisms by which LH activation of the PKA pathway leads to increased PR expression in preovulatory granulosa cells remain unclear. The *Pgr* promoter is complex, consisting of distal and proximal regions and able to give rise to multiple transcripts.[31,33] Initial studies concluded that an estrogen-response element-like region (ERE3) within the proximal rat *Pgr* promoter is important to LH induction,[579] but later studies that better modeled the structure of the rat *Pgr* promoter indicated these sequences are dispensable.[636] Sriraman et al.[636] demonstrated that two Sp1/Sp3 binding sites within the proximal promoter of the mouse *Pgr* gene bind Sp1/Sp3 and are essential to forskolin/PMA-induced expression. Furthermore, LH induction of *Pgr* also involves MAPK activation as mice lacking ERK1/2 in granulosa cells do not induce expression of *Pgr* in response to an hCG ovulatory signal.[637] While these mice show reduced expression, the direct targets of ERK1/2 remain unknown at this time. Recent evidence in porcine granulosa suggests that androgens may regulate *Pgr* expression, where treatment with either T or an AR antagonist alone reduces *Pgr* expression, while co-treatment leads to increased *Pgr* expression.[638] Further characterization into the molecular mechanisms involved in this regulation is necessary. Therefore, unlike the uterus where *Pgr* is estrogen regulated, estrogen does not have the same effect on ovarian *Pgr*.

Differential expression of the two PR isoforms, PRA and PRB, among thecal and granulosa cells further illustrates the complexity of PR expression in the ovary. Western blot analyses following LH induction indicate a PRA:PRB ratio of 3:1 in differentiated granulosa cells from rats[635] and 2:1 ratio in whole ovaries from mice.[621] In rat ovaries, PRA immunoreactivity is restricted to the thecal cells of preantral and antral follicles prior to the gonadotropin surge; whereas PRB immunoreactivity is detected in both thecal and mural granulosa cells throughout the stages of folliculogenesis and shows less variation over the course of the estrous cycle.[622] The enormous induction of PR expression that occurs in preovulatory granulosa cells on the evening of proestrus is primarily representative of PRA, resulting in a 2:1 ratio of PRA:PRB in these cells.[622] In turn, the more moderate LH-induced increases in PR expression in thecal cells is predominantly PRB.[622] During the metestrous stage and rising P synthesis that follow ovulation, PRB is the sole isoform in thecal cells of preantral and antral follicles.[622] The differential expression of PRA and PRB suggests that specific roles exist for each isoform.

To examine this possibility, Sriraman et al. recently examined genes that were induced by PRA or PRB in granulosa cells in vitro. In the absence of P ligand, PRA regulated 41 genes, while PRB only showed differential expression of seven genes compared to control cells.[639] When the granulosa cells were treated with a PR ligand in vitro, PRA-transduced cells showed an increase in 42 genes, while PRB-transduced cells only showed 27 transcripts that were differentially expressed, while only three transcripts were shared between the two PR isoforms.[639] The differential expression of genes in granulosa cells transduced with either PR isoform supports the hypothesis that specific roles exist for each isoform and provide target genes for future study.

Ovarian Phenotypes in Mouse Models of Disrupted Progesterone Signaling

Several mouse lines have been developed that are null for both PR forms and exhibit grossly normal ovaries but are anovulatory and therefore infertile[176–178] (Table 25.8). Furthermore, gonadotropin-induced ovulation of PR-null females is unable to rescue the anovulatory phenotype as no oocytes could be recovered following treatment.[176,178]

TABLE 25.8 Ovarian Phenotypes in PR-Null Mouse Models

Mutated or Null for Sex Steroid Signaling	Ovarian Phenotypes	Hormone Levels	References
$Pgr^{-/-}$ (homozygous null alleles for PRA and PRB: PRKO)	Anovulatory and infertile Failure to respond to superovulation—large follicles present in ovary with trapped oocytes No follicle rupture No CL	Elevated LH Normal FSH	176–178
$PRA^{-/-}$ (homozygous null alleles for PRA: PRAKO)	Anovulatory and infertile Reduced to no response to superovulation Large follicles present with trapped oocytes similar to PRKO No follicle rupture	Not reported	180
$PRB^{-/-}$ (homozygous null alleles for PRB: PRBKO)	Fertile Ovaries appear normal Respond to superovulation similar to wild-type	Not reported	182,227

Instead, PR-null ovaries following induced ovulation exhibit multiple large, preovulatory follicles, each possessing a healthy, competent oocyte but a total failure to rupture[176,178] (Figure 25.23). The follicular cells of the unruptured preovulatory follicles undergo luteinization and form functional corpora lutea within the expected time frame, suggesting that some elements of an LH response are intact[177,178,182,633] (Figure 25.23). Similar experiments in isoform-specific PR-null females indicate a more severe phenotype following the loss of PRA relative to PRB, as females lacking PRA ovulate an average of 9 oocytes/female versus 32 oocytes/female among wild-type littermates; whereas PRB females exhibit no deficits in oocyte yield.[180,182,227] This finding that the PRA isoform is more critical to ovulation[180] is congruent with the above studies of Gava et al. that demonstrate this isoform is much more dramatically induced by the LH surge in preovulatory granulosa cells. This also supports the finding that PRA in the presence of ligand regulates more genes in granulosa cells in vitro compared to cells expressing PRB.[639] Still, the absolute failure of ovulation even after gonadotropin induction in females lacking both PR forms indicates a level of cooperation between PRA and PRB that is vital to follicular rupture.[180] Recently, a mouse model was developed to use for tissue-specific deletion of PR,[177] however to date only global knockout models have been published that show a similar phenotype as previously reported.[176,178]

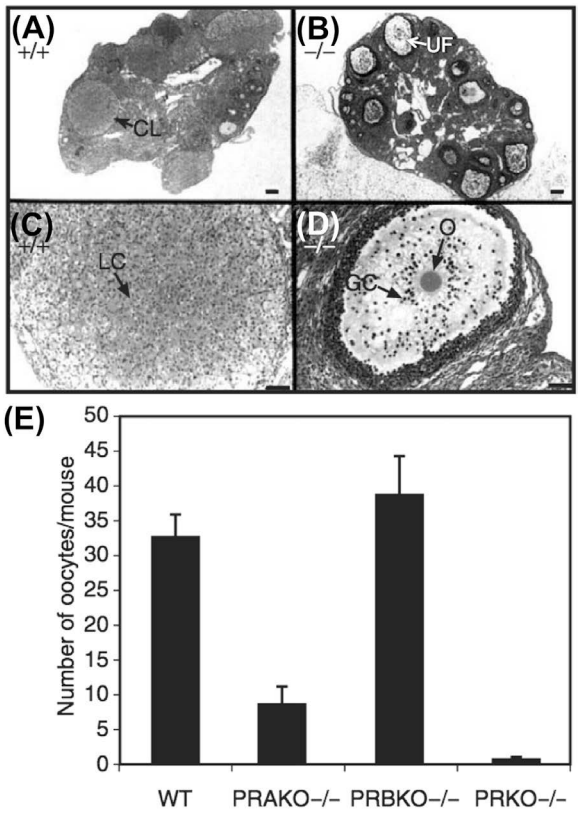


FIGURE 25.23 Ovarian response to gonadotropin-induced ovulation in PR-null mice. (A) Transverse section of a typical ovary isolated from a 6-week-old wild-type mouse (+/+) treated with an intraperitoneal injection of 5 IU of PMSG, followed 48 h later with 5 IU of hCG, and killed 24 h later. Note the presence of numerous corpora lutea (CL). Scale bar = 100 µm. (B) A representative cross-section of an ovary from an age-matched PR-null (−/−) female that was hormonally treated exactly as the wild-type described above. Note the unusual presence of several unruptured follicles (UF) as indicated. Scale bar = 100 µm. (C) High magnification of a corpus luteum in the wild-type ovary, exhibiting the characteristic hypertrophied luteal cells (LC). Scale bar = 50 µm. (D) High magnification of an unruptured follicle present in a PR-null ovary, exhibiting an intact oocyte (O) with a zona pellucida and granulosa cells (GC) that have undergone cumulus expansion. Note the lack of luteinization among the GCs in the PR-null follicle compared to those in the wild-type (C). Scale bar = 50 µm. *(Source: (A–D) reproduced with permission from Ref. 176.)* (E) Average number of oocytes (±SEM) released per mouse after gonadotropin-induced ovulation in wild-type (WT), PRA-null (PRAKO−/−), PRB-null (PRBKO−/−), and PR-null (PRKO−/−) mice; $n = 8$ per test group. *Source: Reproduced with permission from Ref. 182.*

Intraovarian Role of Progesterone in Ovarian Function

FOLLICLE GROWTH AND DIFFERENTIATION

Although immunohistochemical studies indicate that both PR isoforms are expressed at basal levels throughout thecal and granulosa cells,[622] there is little evidence that P action profoundly influences follicle growth and differentiation in the rodent ovary. Administration of P or synthetic progestins to immature or hypophysectomized rats has no obvious effect in the ovary in terms of estrogen receptor levels[358,422] or gonadotropin/estrogen-induced follicle growth and maturation.[358,640] In addition, P has no measurable influence on FSH-induced granulosa cell proliferation[496] or growth and differentiation of whole follicles[497] in vitro. Perhaps most indicative of a minor role for P signaling during folliculogenesis prior to ovulation is the lack of any overt phenotype in follicle growth, maturation, or steroidogenesis in the ovaries of PR-null mice (Table 25.8).[176–178,641] Indeed, Robker et al. demonstrated that the expected FSH- or PMSG-induced increases in *Cyp19* and *Lhcgr* expression in mature follicles occurs in PR-null ovaries.[633]

OVULATION

PR expression in the ovary is relatively low throughout folliculogenesis except during the 4–6 h period immediately after the ovulatory gonadotropin surge, during which an enormous induction of PR expression occurs in granulosa cells of ovulating follicles[628,634,635] and is synchronized with an equally acute increase in ovarian P synthesis. These phenomena alone are indicative of an important role for PR-mediated P signaling during ovulation. Experimental evidence of a critical role for PR in ovulation comes from studies in which ovulation is blocked when peri-ovulatory ovaries or follicles are exposed to: (1) anti-P antisera,[642] (2) inhibitors of P synthesis,[643–645] or (3) PR antagonists.[621,646,647] Finally, the

anovulatory phenotype of PR-null mice discussed before provides definitive evidence that PR actions are required in the mammalian ovary during the period just prior to ovulation.[176–178,180] PR-null females exhibit multiple large, preovulatory but unruptured follicles following induced ovulation.[176] Therefore, follicle growth and differentiation are unaffected by the loss of PR functions as PR-null follicles exhibit fully formed antra and increased *Cyp19* and *Lhcgr* expression following FSH or PMSG treatment.[176,633] Furthermore, although follicle rupture is impaired, PR-null ovaries exhibit several indications that other LH- or hCG-induced responses are intact, including: (1) rapidly decreased *Cyp19* expression,[633] (2) dramatic induction of *Ptgs2* expression,[633] (3) expansion of the cumulus–oocyte complex,[176] and (4) luteinization and formation of functional corpora lutea.[182,633] These findings in PR-null females are congruent with earlier studies using P synthesis inhibitors[645,648] or PR antagonists,[646] which also report that ovulatory levels of prostaglandins and steroids are largely unaffected by inhibition of P signaling.

The previous data clearly indicate that PR actions are highly specific and critical for follicle rupture. Studies have discovered that PR-null ovaries fail to exhibit LH-induced expression of several proteases, including ADAMTS-1, ADAM8, and cathepsin-L, which are postulated to be involved in degradation of the follicle wall and extracellular matrix that is necessary for oocyte extrusion (Figure 25.24). ADAMTS-1 (*Adamts1*) and ADAM8 are members of the A disintegrin and metalloproteinase family of proteases and are dramatically increased in granulosa cells of preovulatory follicles 12 h after hCG treatment, after peak PR expression and coinciding with a period of follicular rupture.[644,649] A similar pattern of peri-ovulatory ADAMTS-1 expression is documented in preovulatory follicles of porcine,[650] equine,[630] and primate[651] ovaries. Cathepsin-L (*Ctsl*) is also significantly increased following hCG exposure in granulosa cells of preovulatory follicles and also peaks 12 h after treatment.[633,652] PR-null females,[633] as well as wild-type female rats exposed to a P synthesis inhibitor near the time of hCG treatment[644,653] fail to exhibit hCG-stimulated increases in *Adamts-1* expression, indicating this effect of LH is primarily dependent on PR-mediated P action. ADAMTS-1-null female mice exhibit a severely compromised ovulation and a phenotype of large, preovulatory but unruptured, follicles following gonadotropin-induced ovulation.[654,655]

Interestingly, PRA-null mice exhibit a severe ovulatory phenotype similar to total PR-null females but possess normal LH-stimulated induction of *Adamts-1* and *Ctsl* during induced ovulation,[182] suggesting possible compensatory actions by PRB but also questioning the importance of ADAMTS-1 and cathepsin-L to follicle rupture. ADAM8 was recently shown to be regulated specifically by PRA in reporter assays, while PRB was unable to regulate the promoter of ADAM8 in vitro.[649] This could explain why PRA-null mice show LH-stimulated induction of *Adamts-1* and *Ctsl* yet are unable to ovulate, although further studies are needed to confirm this.

Several other genes have been identified downstream of PR and suggested to be important in follicle rupture and ultimately ovulation. Since ovulation is discussed more fully in another chapter (Chapter 22) of this volume, we will not provide in-depth description, although

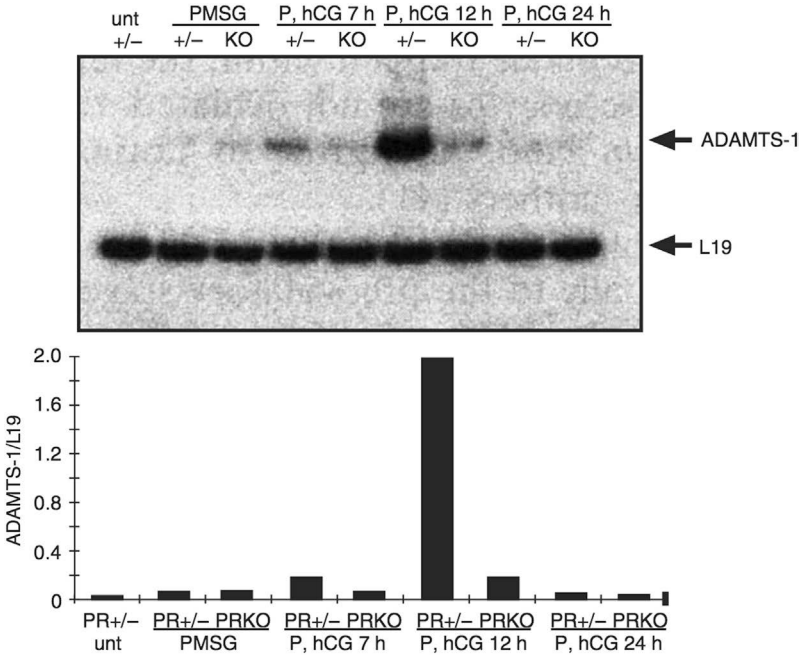


FIGURE 25.24 Ovarian expression of ADAMTS-1 in PR-null ovaries during gonadotropin-induced ovulation. Immature heterozygous (PR+/–) and homozygous PR-null (PRKO) mice were left untreated or injected with PMSG and euthanized 46 h later, or were treated with PMSG for 46 h followed by hCG and euthanized 7, 12, and 24 h later. Reverse transcriptase polymerase chain reaction analysis of total RNA from whole ovaries indicates that ADAMTS-1 expression is dramatically increased by hCG in PMSG-primed mice, but this induction is lacking in PR-null ovaries, indicating dependence on PR function. ADAMTS-1 expression was normalized to expression of ribosomal protein L19. *Source: Reproduced with permission from Ref. 633.*

we will briefly describe what is known to date. These include SNAP25, synaptosomal-associated protein 25, an LH-regulated gene that has decreased expression in PR-null mice but not *Ptgs2* mice.[656] Furthermore, *Snap25* was PR regulated in in vitro reporter assays, and the PR antagonist RU486 was able to inhibit this regulation. SNAP25 acts to alter cytokine and chemokine secretion in granulosa cells, which may act to facilitate follicle rupture.[656] Endothelin 2 (*Edn2*) was identified to be an LH-regulated gene with undetectable levels in PR-null mice that plays a role in follicle rupture.[657,658] Recent studies have shown that *Edn2* is regulated by PRA,[639] and studies in rats have shown that it acts to stimulate smooth muscle contraction and follicle constriction.[659] EDN2 provides the mechanical actions that help to facilitate follicle rupture and successful ovulation. This is supported by its regulation specifically by PRA, which is essential for ovulation, whereas loss of PRB doesn't reduce ovulatory response as described previously.

Numerous transcription factors have also been identified as PR targets, and knockout mouse models have demonstrated that these factors are necessary for ovulation in mice. Hypoxia inducible factor 1 (*Hif1*) is induced by FSH in rat granulosa cells cultured in vitro,[660,661] and have also been found to be regulated by PR.[662] Furthermore, the expression of several HIF family members, including *Hif1a*, *Hif2a*, and *Hif1b*, are reduced in PR-null ovaries[662,663] suggesting an important role in follicle rupture. To further explore this possibility, pharmacological inhibitor of HIF activity was shown to block ovulation and inhibit follicle rupture,[662] demonstrating the importance of HIF activity in regulating ovulation. Another nuclear receptor, PPARG, is also necessary for follicle rupture as demonstrated by targeted deletion in granulosa cells in mice that had no response to exogenous gonadotropins.[664] The action of PPARG was found to be mediated by several PR target genes, including *Il6* and *Edn2*,[664] demonstrating that several targets are regulated downstream of PR and LH signaling to coordinate follicle rupture and ovulation.

Androgen Receptor Signaling in Ovarian Function

AR Expression in the Ovary

AR expression has been documented in the ovaries of multiple species, including mouse,[373,450] rat,[130,665–668] pig,[669,670] sheep,[671] cow,[672] monkey,[274,395,673–675] and human.[395,625,676–679] The ovarian pattern of *AR* expression is well conserved among species, with levels being detectable throughout the stages of folliculogenesis except primordial follicles, with the highest expression in the granulosa cells of small preantral follicles, detectable expression in thecal/interstitial cells, and little to no expression in luteal cells (Table 25.6).

In the ovaries of multiple species, *Ar/AR* expression among growing follicles is inversely correlated with the extent of granulosa cell differentiation (Figure 25.25). A mechanism to decrease AR levels during follicle maturation is consistent with the need to reduce sensitivity to intrafollicular androgens, which rise to levels sufficient for aromatization to E2, as activating AR ligands are detrimental to the health of preovulatory follicles.[680] In the ovaries of gonadotropin-stimulated rats, Testsuka and Hillier found that large antral follicles (>400 μm) exhibit a 2.75-fold higher level of *Cyp19* expression relative to small follicles (<200 μm) but a 51% decrease in *Ar* expression.[665] In rat ovaries, *Ar* expression is first apparent in early postnatal development in follicles of the preantral stage.[681] In adult ovaries, decreasing AR immunoreactivity occurs during follicle differentiation; a decrease is first apparent in mural granulosa cells and then progresses in those cells closest to the antrum.[667] Interestingly, cells composing the cumulus–oocyte complex of preovulatory follicles are believed to be the last to differentiate[682] and maintain *Ar* expression throughout.[667] This gradient of AR expression suggests that AR signaling correlates with the differentiation state of granulosa cells and follicle development.

In primate and human ovaries, *AR* expression is also highest in the granulosa cells of small growing follicles, yet evidence of an inverse correlation with follicle differentiation is conflicting.[625,674,675,678,684] Hillier et al. found AR immunoreactivity in the marmoset ovary is most abundant in granulosa cells of healthy pre- to small antral follicles and low or absent in preovulatory follicles of late follicular stage.[674] In contrast, minimal differences in granulosa cell *AR* expression between preantral and large antral follicles are reported in rhesus monkey ovaries.[675] Furthermore, healthy follicles at late stages of maturation in human ovaries are described to possess significant AR immunoreactivity.[625,678,679] A recent report has suggested that AR expression is highest in small antral follicles and decreases as the follicle matures,[685] suggesting that message levels may not correspond with AR protein levels in human follicles.

The regulatory factors for *AR* expression in the ovary are poorly understood. Small preantral follicles in rats maintain high AR levels after hypophysectomy indicating that gonadotropins are not required to induce *AR* expression.[668] In fact, most evidence indicates that FSH or PMSG-induced differentiation of granulosa cells is the primary cause of reduced *AR* expression; Campo et al. demonstrated that PMSG treatment of rats leads to the replacement of high-affinity, low-capacity androgen binding sites by nonsaturable, low-affinity binding sites in the ovary.[686] Similarly, immature rats treated with recombinant FSH over 48 h exhibit a 65% reduction in ovarian *Ar* mRNA levels and a further decrease when LH is included.[668] However, neither FSH nor (BR)-cAMP

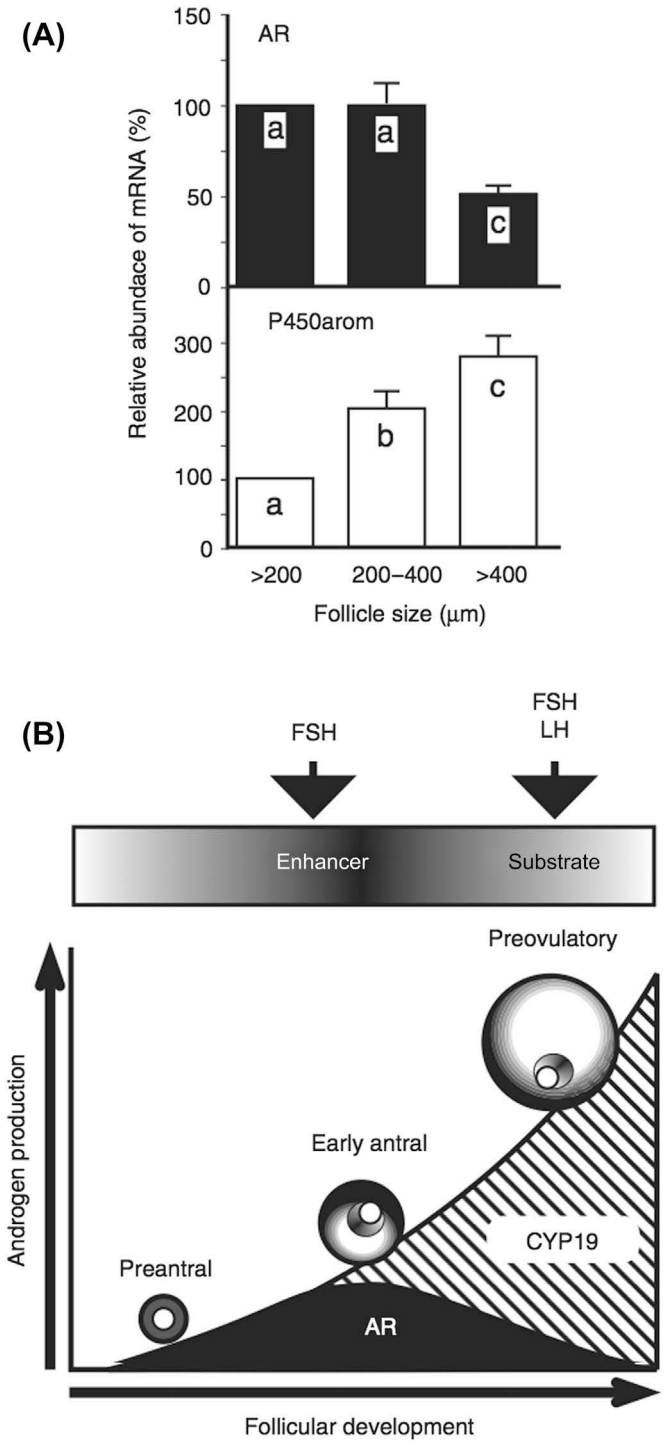


FIGURE 25.25 Relationship between expression of androgen receptor and aromatase (CYP19) during folliculogenesis. (A) Expression of androgen receptor (*Ar*) and aromatase (*Cyp19*) expression in granulosa cells of small (~200 μm), medium (200–400 μm), and large (>400 μm) follicles from immature rats after treatment with PMSG. Expression was quantified by RNase-protection assay and normalized to 18S rRNA (not shown); data are expressed as percentage of control values (SEM of three separate trials, each consisting of 8–10 animals). a, b: $P < 0.05$; a, c: $P < 0.01$ (by ANOVA with the Newman–Keuls test). *(Source: Reproduced with permission from Ref. 491.)* (B) Hypothetical model of androgen utilization during folliculogenesis. As follicular development progresses, thecal androgen production gradually increases. During the early stages of follicular differentiation, androgens act via androgen receptor (AR) to enhance FSH-induced differentiation, including the stimulation of *Cyp19* expression. During the final stages of follicular development, androgens primarily serve as a substrate for CYP19-mediated E2 synthesis under stimulation by FSH and LH. This differential regulation of AR and CYP19 may be important in shifting androgen utilization from action to metabolism, thereby ensuring a healthy transition of a follicle from the early maturation to full maturation stage. *Source: Reproduced with permission from Ref. 683.*

affect *Ar* mRNA levels in cultured rat granulosa cells in vitro.[665] This suggests that a paracrine interaction with theca and/or stromal cells is necessary for reduced *Ar* expression observed in granulosa cells. Interestingly, DHT also elicits a 20% reduction in *Ar* expression in rat granulosa cells in vitro that is prevented by co-treatment with FSH.[665] In contrast, T reportedly has little effect on *AR* expression in rhesus monkey ovaries.[675] There is increasing evidence that E2 may play a role in decreasing granulosa cell AR levels during follicle differentiation. Like DHT, E2 exposure of rat granulosa cells also leads to a 20% reduction in *Ar* transcripts, but this effect is unabated by FSH.[665] Furthermore, granulosa cells retrieved from untreated hypophysectomized rats respond well to DHT plus FSH and exhibit increased *Cyp19* expression accordingly; but the effect of DHT is

lost in granulosa cells isolated from E2-primed hypophysectomized rats, suggesting that estrogen pretreatment decreases *AR* expression.[442] Indeed, mature follicles in ERβ-null ovaries are reported to possess aberrantly high *AR* expression relative to similarly staged follicles in wild-type ovaries,[450] supporting the idea that ER may act to decrease *Ar* expression. While the hormonal regulation of Ar expression is unclear, recent reports suggest that a member of the orphan nuclear receptor family, *Nur77*, is required for maximal *Ar* expression in murine ovaries. *Nur77* regulates several steroidogenic enzymes in the ovary, including those involved in androgen synthesis,[687] and recent reports demonstrate 20% reduction in Ar expression in the granulosa cells from *Nur77*-null mice.[688] Dia and colleagues were able to show that NUR77 bound directly to the promoter regulatory region of the Ar gene in mouse granulosa cells, providing insight into one of the transcriptional factors necessary for maximal AR expression in the ovary.[688]

Ovarian Phenotypes in Mouse Models of Disrupted Androgen Signaling

MICE LACKING AR

Tfm mice were first described in 1970 and are a naturally existing androgen-resistant mutant[283] due to an inactivating mutation of the *Ar* gene.[284,285] With the advent of gene targeting techniques, comparable inactivation mutations of the *AR* gene and resulting phenotypes have been described in rats and humans.[286] *Tfm*/*Tfm* female mice are fertile but exhibit a noticeably shortened reproductive life span that becomes apparent after approximately four litters compared to control (*Tfm*/X or X/X) females; however, individual litter sizes were not different.[287] Histological evaluation of ovaries from the *Tfm/Tfm* breeding females indicated a sparse number of healthy follicles and a hypertrophied interstitium, both illustrative of premature ovarian failure (POF).[287] The difficulties in generating *Tfm/Tfm* female mice limited more extensive studies.

Several global AR-null mouse models have been generated via a *Cre/loxP* targeting scheme that allows for tissue and temporal specific disruption of the *Ar* gene and therefore the generation of fertile male carriers of the targeted *Ar* allele[183,185,288,290] (Table 25.9). In one study, global AR-null females made through deletion of exon 1 exhibit normal fecundity only during the first 12 weeks of continuous breeding, after which they produce a reduced number of litters per female (2.3 versus 3.3 in wild-type) and less offspring per litter (4.5 versus 9.8 in wild-type), while others become totally infertile.[183] The POF observed in AR-null females is remarkably similar to that described before in *Tfm*/*Tfm* females.[287] Adult AR-null females possess ovaries exhibiting a normal number of growing and antral follicles but notably fewer corpora lutea[183] and reduced circulating P (Table 25.9),[689] suggesting that folliculogenesis is preserved but ovulation and luteinization are disrupted. Gonadotropin-induced ovulation of immature AR-null females leads to a severely reduced ovulatory yield and oocytes that often exhibit a less condensed cumulus–oocyte complex.[183] This latter finding is especially interesting in light of the preserved *Ar* expression in the cumulus oophorus cells of preovulatory follicles. Additional evidence that AR-null ovaries fail to fully respond to an ovulatory dose of hCG is an inadequate induction of *Pgr*, hyaluronyl synthetase-2 (*Has2*), and tumor necrosis factor-α-stimulated gene 6 (*Tnfaip6*),[183] all factors that are critical to expansion of the cumulus–oocyte complex and follicle rupture. Furthermore, granulosa cells of ovulatory follicles in AR-null ovaries fail to terminally differentiate and luteinize following hCG-induced ovulation, as indicated by decreased expression of cyclin-dependent kinase inhibitor 1A (*Cdkn1a*) and cytochrome $P450_{scc}$ (*Cyp11*).[183] These animals were generated by targeted deletion of exon 2 and demonstrate that AR signaling is important for normal ovarian function and to prevent POF in rodents.

A second global knockout mouse model was developed by Shiina et al., where AR was deleted through *Cre/loxP* mediated removal of exon 1.[690] This animal model is similar to that described before in that the female mice show an age-dependent decrease in fecundity, however, these mice also have a decrease in number of offspring per litter in animals aged 8 weeks old (4.5 versus 8.3 in wild-type). At 8 weeks of age, these mice also have decreased number of CL and an increased number of unhealthy and atretic follicles.[690] Knockout of AR in exon 1 also causes a POF phenotype where the number of offspring decreases over time concomitant with a decrease in the number of healthy follicles, suggesting defects in folliculogenesis. By 32 weeks of age, 40% of knockout females are infertile, similar to that observed in *Tfm*/*Tfm* females and the exon 2–deleted AR-null mice,[183,283] and at 40 weeks of age all of the mice are infertile.[690] No difference was observed in genes involved in steroidogenesis within the ovary, and serum hormone levels for E2, P, and T are within normal rages similar to WT controls, providing evidence that AR is not necessary for normal steroidogenesis in the ovary (Table 25.9)[690] in contrast to reduced P observed in the exon 2–deleted AR-null female.[689] Microarray analysis was done on ovaries from these animals to find potentially altered transcriptional networks that might contribute to the POF phenotype. Several genes implicated in oocyte-granulosa cell communication were found to be increased,[690] including kit ligand (*Kitl*). Kitl is a downstream target of AR as demonstrated by induction of *Kitl* expression following DHT treatment, while co-treatment with DHT and the antiandrogen flutamide attenuated the increased *Kitl* expression.[690] This report suggests that AR signaling has

TABLE 25.9 Ovarian Phenotypes in AR-Mutant or Null Mouse Models

Mutated or Null for Sex Steroid Signaling	Ovarian Phenotypes	Hormone Levels	References
Tfm/Tfm mice	Fertile but have a reduced number of pups/litter as they age Shorted reproductive life span	Not reported	283
$AR^{-/-}$ (exon 2)	Reduced fertility and a shortened reproductive life span Adults exhibit grossly normal ovaries with reduced number of CL Abnormal estrous cycle Immature females have reduced response to gonadotropin-induced ovulation	Reduced P Other hormones not reported	183,290
$AR^{-/-}$ (exon 1)	Reduced fertility and POF phenotype where animals have a shortened reproductive life span Increased number of atretic follicles and reduced number of CL Altered ovarian gene expression in immature and adult animals	Normal serum E2, P, T, LH, and FSH	690
$AR^{-/-}$ (inframe deletion of exon 3, loss of DNA binding activity)	Delayed production of first litter Decreased pups/litter and reduced number of CL $AR^{+/-}$ animals had age-dependent reduction in pups/litter Increased number of unhealthy antral follicles Normal response to exogenous gonadotropins, but a reduced number of oocytes ovulated during natural mating	Normal serum E2, T, LH and FSH Increased intraovarian T in 10–12 week old mice	185
$AMH^{Cre+}Ar^{f/+};Ar^{f/f}$ (exon 3 floxed, loss of DNA binding activity in large preantral to antral follicles)	Subfertile due to reduced number of litters and age dependent decrease in total number of pups born Decreased fertility over time with a cumulative decrease in pups born per dam 3-month-old animals have increased large preantral and antral follicle numbers 6-month-old animals have increased length of estrous cycle Reduced cumulus expansion and oocyte/embryo viability	Normal serum LH and FSH	691
$Amhr2^{Cre+};Ar^{f/f}$ (exon 2 floxed) GCARcKO	Reduced fertility Altered follicle progression and development 8–9 week mice: normal estrous cycle, reduced pups/litter, reduced number of oocytes from natural mating, however, no differences observed when treated with exogenous gonadotropins 24 weeks: increased length of estrous cycle, reduced number of pups born per dam, reduced number of oocytes from natural mating and treatment with endogenous gonadotropins	Not reported	692
$Gdf9^{Cre+};Ar^{f/f}$ (exon 2 floxed, oocyte specific loss of AR)	Normal female phenotype except androgens were unable to promote oocyte maturation in vitro	Not reported	692
SPARKI (homozygous KI of mutated DBD of AR)	Normal female phenotype	Not reported	186

an important role in the oocyte-granulosa cell regulatory loop,[690] although future work is needed to identify potential mechanisms of action.

These knockout mouse models demonstrated an important role for AR in normal ovarian function and suggest that loss of AR contributes to POF (Table 25.9). Both AR-null models lack all AR protein due to a truncated message and premature stop codon. A third AR-null mouse model was developed by Walters et al. where an in-frame deletion of exon 3 was made that led to production of an AR protein lacking the second zinc finger and is therefore truncated and unable to bind DNA.[185] Mutations in this region of the AR gene have been identified in human patients with androgen insensitivity syndrome (AIS), demonstrating the importance of binding DNA to AR activities in normal physiological state.[693–695] This mouse model allows for analysis of the direct effects of classical genomic AR signaling in the ovary compared to the AR-null models that lack all AR protein. Deletion of the DNA binding domain in female mice led to a subfertile phenotype where the females homozygous for the deletion had less offspring per litter compared to both heterozygous and WT females.[185] Interestingly, the AR(exon 3)-null females had reduced numbers of CL similar to other AR-null mice,[290,690] demonstrating the importance for intact AR signaling for this process.

Furthermore, these mice also had an age-dependent reduction in fecundity similar to the other AR-null models,[183,690] and females heterozygous for the AR(exon3) deletion also showed an age-dependent reduction in offspring born at 6 months of age, suggesting a dosage effect.[185] An increase was observed in the number of unhealthy antral follicles, although AR(exon3)-null mice did not have significant changes in overall follicle development even in animals aged 10 months[185] in contradiction to reports in AR-null ovaries with reduced follicle numbers.[183] While these models have indicated some differences in the role of AR in normal folliculogenesis, all demonstrate an increase in the number of atretic follicles, demonstrating the importance of AR signaling in maintaining ovarian health over time. AR-null mice have reduced response to gonadotropin-induced ovulation,[183] and examination of the AR(exon3)-null animals found that they respond to exogenous gonadotropin stimulation with similar numbers of oocytes ovulated; however, a decrease was observed in oocytes ovulated when examined after natural mating.[185] This suggests that AR is necessary for normal ovulatory response; however, excess gonadotropins are able to override this defect in the presence of a truncated AR protein (lacking the entire DNA binding domain) compared to ovaries that are void of all AR protein. Mutation of the DNA binding domain that alters the ability of AR to bind to selective androgen response elements in the SPARKI mouse model presented by Schauwaers et al. did not lead to a female reproductive defect, suggesting that the ovary does not require selective AR binding to response elements as compared to the male SPARKI mice that have a reproductive phenotype and will not be further discussed herein.[186]

The identification of genes necessary for oocyte-granulosa communication in the ovary as being AR targets[690] provides a possible mechanism that is aberrant in AR-null ovaries, but further studies are necessary to confirm this difference and explore the pathways involved. Loss of the DNA binding domain in AR did not affect circulating hormone levels, as the mice had normal serum levels of E2, T, LH, and FSH; however, an increase in intraovarian T levels was observed in mice aged 10–12 weeks,[185] suggesting alterations in the steroid environment locally may have paracrine effects on ovarian function that was not measured in other AR-null models.[183,690]

MICE WITH OVARIAN-SPECIFIC DELETION OF AR

The global AR knockout mouse models described herein have offered many insights into the important role of AR in female reproduction, specifically in the ovary. However, these models lack AR in all tissues, including all parts of the hypothalamic–pituitary–gonadal axis, which could contribute to the ovarian phenotype observed. To circumvent this, two conditional knockout mouse models have been developed to remove AR specifically in granulosa cells of the ovary.[691,692] These models provide a unique way to explore the role(s) of AR in female fertility and ovarian function.

Sen and Hammes used *Amhr2*-cre to delete exon 2 of AR in the ovary and found that the animals had reduced numbers of offspring born to young dams (2.8 pups in KO versus 7 in WT mice),[692] similar to that observed in the global knockout animals.[183,290] In these granulosa cell AR conditional knockout (GCARcKO) animals, altered follicle progression was observed in animals aged 9 weeks as well as in animals aged 24 weeks (Table 25.9),[692] supporting observations in global knockout animals,[183,290] where numbers of corpora lutea were reduced and atretic follicles were increased. These patterns persisted in older animals, which also showed an increased length of estrous cycle at 24 weeks of age compared to mice aged 9 weeks old that had a normal estrous cycle.

Several other differences were observed in animals at the two ages examined (9 weeks versus 24 weeks), including differences in ovulation. Mice aged 9 weeks had a reduced number of oocytes ovulated during natural mating, but treatment with exogenous gonadotropins was able to circumvent this defect and cause normal numbers of oocytes to be ovulated from these animals. As the animals aged, however, the ability of exogenous gonadotropins to override this defect was lost, and reduced oocyte numbers were observed from natural mating and superovulation paradigms.[692] Failure of the ovary to respond to exogenous gonadotropins concurrent with a reduction in the total number of offspring born over a long-term fertility study (19 in KO versus 119 in WT mice) supports the POF phenotype observed in the global AR-knockout models.[183,185,290]

The GCARcKO presented by Sen and Hammes was made using the *Amhr2*-cre mouse, which has been found to be active in tissues other than granulosa cells including the pituitary,[696] which may complicate the findings. While reduced expression of *Ar* was not reported in the hypothalamus or the pituitary,[692] the heterogeneous population of cells present could mask a reduction in gonadotrope cells that are required for normal female fertility. To eliminate this possibility and explore the classical role of AR in large preantral to antral follicles, Walters et al. used the *Amh*-cre mouse model to delete exon 3 in the ovary.[691] *Amh* is the ligand for *Amhr2* and is expressed specifically in large preantral to antral follicles during folliculogenesis demonstrated by crossing the Amh-cre mouse to R26R (ROSA) reporter mice.[691] This mouse expresses β-galactosidase activity where *Cre recombinase* is expressed, and the X-Gal blue stain can be used to localize cells expressing the β-gal indicator within tissues. The *Amh*-cre mouse was crossed with exon3 floxed AR mouse that creates a truncated protein lacking the DNA binding domain.[185] The use of this

mouse model allowed *Ar*/AR to be deleted in a temporal pattern and to examine whether DNA binding activities are necessary during this stage of folliculogenesis for normal ovarian function.

The *AMH*$^{Cre+}$*Ar(exon3)*$^{f/f}$ females are subfertile, and while the number of offspring born per litter was not significantly altered, an age-dependent decrease in the total number of pups born was observed[185] similar to the POF phenotype observed in other AR-null models (Table 25.9).[183,185,283,690,692] This provides evidence that AR binding to direct AR target genes in large preantral follicles is necessary for normal ovarian function over time. The *AMH*$^{Cre+}$*Ar(exon3)*$^{f/f}$ females also have an increased length of estrous cycle at the age of 6 months that is not observed in younger mice,[691] supporting the POF phenotype observed and the importance of AR in maintaining normal ovarian health. Altered follicle progression was observed in these mice, and at 3 months of age fewer large preantral and small antral follicles were observed; however, this difference was not noted in older animals.[691] While no difference is observed in follicle progression during folliculogenesis in *AMH*$^{Cre+}$*Ar(exon3)*$^{f/f}$ females aged 6 months, a significant increase in unhealthy follicles was observed,[691] supporting the notation that AR signaling is necessary for normal ovarian health and prevention of POF in the ovary.

Communication between granulosa cells and the oocyte are necessary for oocyte health and viability, and disruption of this communication can lead to infertility. Animals lacking functional AR protein have altered expression of several genes implicated in this communication network, including the AR-dependent gene *Kitl*.[690] Pharmacological studies have suggested that T can induce mouse[697,698] and porcine[389,699,700] oocyte maturation presumably through classical AR signaling in vitro. To directly examine the role of AR expression in oocytes, a conditional knockout mouse model was developed using an oocyte-selective *Gdf9*-cre crossed with exon 2–floxed AR.[692] These mice had normal fertility and no overt ovarian phenotype compared to WT controls, demonstrating that AR expression within oocytes is not necessary for female fertility, while granulosa cell specific expression of the AR nuclear receptor is necessary in vivo.[692] Interestingly, when oocytes were removed from immature unprimed females and grown in vitro, DHT was unable to induce oocyte maturation in the absence of AR expression in oocytes.[692] However, P-mediated oocyte maturation was not affected by the loss of AR in vitro, demonstrating that these oocytes could still mature, albeit not through an androgen-mediated mechanism. This suggests that AR expression in oocytes is necessary for in vitro maturation in the absence of granulosa cells, while in vivo expression of AR in the granulosa cells is sufficient for oocyte maturation, possibly due to P-mediated mechanisms.

Loss of AR in granulosa cells contributes to altered ovarian function including a POF phenotype and loss of ability of the ovary to ovulate oocytes through both natural mating or superovulation depending on the age the animals were tested.[183,185,692] While differences were observed depending on the AR-null mouse model examined and the ovulation paradigm presented, the data provide evidence that AR is necessary for normal ovulatory function of the ovary. Interestingly, a decrease was observed in the number of oocytes naturally ovulated by one granulosa cell–specific AR-null model,[692] while no differences were observed in a second model (Table 25.9).[691] These differences may be due to the timing of AR deletion or the different flox animals used (i.e., exon 2 versus exon 3). Further study was done on oocytes ovulated from natural mating in the *AMH*$^{Cre+}$*Ar(exon3)*$^{f/f}$ females to see if these oocytes could be fertilized and progress through early embryo development in vitro.[691] While the number of oocytes ovulated was not different, the number of fertilized oocytes was reduced (38.6% in KO versus 94.5% in WT), as was the number of the embryos able to process to the two-cell stage of embryo development (35.7% in KO versus 89.5% in WT).[691] This supports the necessity of AR expression in granulosa cells to promote oocyte maturation and development, and indicates that DHT-mediated oocyte maturation could be mediated through expression of AR in granulosa cells. It also provides insight into the inability of some oocytes to respond to in vitro fertilization, which has implications in human health in cases where some women fail to respond to assisted reproductive technologies. The variety of AR-null mouse models to date provides evidence of the importance of AR signaling in normal ovarian development, female fertility, and follicle health.

Intraovarian Role of Androgen in Ovarian Function

The intraovarian roles of androgens can be categorized into three distinct functions: (1) as substrates for E2 synthesis, (2) as an enhancer of follicle differentiation, and (3) as a stage-specific inhibitor of follicle growth.[683] The role of thecal-derived androgens, most notably androstenedione, as substrates for E2 synthesis in granulosa cells is obviously critical given the profound importance of the latter hormone to reproductive function. While ovarian steroidogenesis is essential for function of the ovary and female fertility, this topic will not be covered here; instead we focus on the additional intraovarian actions of androgens, which include their role as activating ligands for AR-mediated effects.

GRANULOSA CELL PROLIFERATION

The follicular response to androgens is dependent upon the stage of growth and differentiation. In preantral,

undifferentiated follicles that are unable to synthesize E2, androgens promote gonadotropin-induced granulosa cell proliferation and maturation. In large, differentiated follicles that have acquired aromatase activity, E2 assumes the role of enhancing FSH-induced granulosa cell proliferation and differentiation, and androgens in excess of that required for conversion to E2 are detrimental to follicle health, leading to atresia. This paradox of androgen action during follicle growth and differentiation explains the contradictory results when comparing experimental studies.

Several studies in hypophysectomized rats have shown that coadministration of T inhibits estrogen or gonadotropin-induced granulosa cell proliferation and increased ovarian weight, and promotes degeneration in more mature follicles.[422,489,701–703] In contrast, Armstrong and Papkoff found aromatizable androgens (e.g., T or androstenedione) enhance the promotional effects of FSH in the ovaries of hypophysectomized rats but that the nonaromatizable androgen DHT is inhibitory.[291] Similar treatment with hCG instead of T has a comparable effect on gonadotropin or E2-induced follicle growth in hypophysectomized rats, but is prevented by AR-antagonists, indicating the detrimental effects of hCG are due to stimulation of thecal cell androgen synthesis.[702,704,705] However, Bley et al. found that granulosa cells from preantral follicles of estrogen-primed rats proliferate at comparable rates in response to FSH plus E2 or DHT in vitro[496] and that the promotional effects of the latter steroid are blocked by the antiandrogen hydroxyflutamide.[496] Investigations toward the mechanism by which androgens inhibit granulosa cell proliferation indicate that DHT exposure to estrogen-primed rats prior to granulosa cell isolation causes a blunted response to forskolin-induced cyclin-D2 (*Ccnd2*) expression in vitro, leading to cell-cycle arrest and reduced proliferation.[706] DHT is able to exert its inhibitory effect on *Ccnd2* expression through AMP-activated protein kinase (AMPK) activation, which in turn inhibits phosphorylation of MAPK and ultimately impacts granulosa cell proliferation downstream of FSH.[707,708] AMPK has a role in inhibiting cell proliferation, and pharmacological activation of the kinase can lead to cell cycle arrest, while blocking AMPK activity prior to treatment with DHT was able to overcome the inhibitory effect normally observed,[708] providing insights into the mechanism of action of DHT inhibition on cell cycle progression in estrogen-primed rat granulosa cells. This work also highlights how androgens are able to be stimulatory during early stages of folliculogenesis and then inhibitory during the latter stages when estrogen signaling is the main driver of follicle development.

The stage-specific effects of androgens during folliculogenesis described herein may be more apparent in rat ovaries relative to other species. In hypophysectomized mice, Wang and Greenwald found that T or DHT, in combination with FSH, induces greater levels of DNA synthesis in antral follicles than FSH alone; a similar response is not observed in small- and medium-sized follicles.[492] In preantral mouse follicles grown in vitro, antiandrogen antisera reduce FSH-stimulated growth and DNA synthesis; however, these data must be interpreted with caution since antiandrogen antisera may also remove androgens from the pool of substrates for E2 synthesis. Additional evidence that androgens may promote granulosa cell proliferation in mice comes from the findings of Burns et al. that the AR-antagonist flutamide reduces the growth rate of granulosa cell tumors in animals lacking functional inhibins.[499] In vitro follicle culture studies demonstrated the importance of AR signaling in folliculogenesis, where treating follicles with antiandrogenic compounds reduced follicle growth during the preantral stage of folliculogenesis,[709] demonstrating the importance of androgens in early follicle growth.

In cumulus cells of large antral follicles from porcine ovaries, DHT augments FSH or IGF1-induced proliferation, and this effect is inhibited by flutamide.[710] Rhesus monkey ovaries exhibit a strong correlation ($r = 0.91$) between *AR* expression and cell proliferation[675] and exhibit an increased number of follicles of all stages except large antral following T or DHT treatments.[711] Yang and Fortune reported that T was able to stimulate early follicle maturation in bovine follicles.[712] The collective data support the notion that androgens are important in granulosa cell proliferation during early stages, but at latter stages the effect appears to be species dependent.

GRANULOSA CELL DIFFERENTIATION

Fully differentiated preovulatory follicles in mammalian ovaries are distinctly characterized by a large fluid-filled antrum, significantly increased aromatase (CYP19) activity, and acquisition of LH responsiveness. The absolute requirement of FSH signaling in this process is illustrated by the absence of all three phenotypes in mice null for FSH action.[420,512,513] E2 is also required for full FSH-induced follicle differentiation. However, there is substantial evidence that androgens may be equally efficient as E2 in augmenting certain responses to FSH, most especially the induction of aromatase activity and antrum formation.[683] Both T and DHT enhance FSH induction of aromatase activity in hypophysectomized rat ovaries or isolated granulosa cells in vitro in a dose-dependent fashion.[291,713] Furthermore, pharmacological inhibition of ovarian androgen synthesis in rats simultaneously reduces aromatase activity that can be restored by exogenous T.[714]

Fitzpatrick et al. found that T, and DHT to a lesser extent, significantly enhances FSH induction of *Cyp19* expression and aromatase activity in granulosa cells from

untreated hypophysectomized rats[442] (Figure 25.19). This finding was supported by work done in in vitro cultured granulosa cells isolated from rats where T was able to increase Cyp19 and P450scc expression. This regulation was found to be dependent on liver receptor homolog-1 (*Lrh1*) expression that is induced by T and not DHT through AR binding to *Lrh1* promoter regulatory region[715] and provides evidence that T, and not DHT, affects the expression of genes necessary for estrogen production during folliculogenes. Furthermore, *Lrh1* expression is also induced by FSH in granulosa cells,[715] providing a potential mechanism of action for synergy necessary for differentiation of granulosa cells in the ovary.

Interestingly, this effect is lost in granulosa cells isolated from estrogen-primed hypophysectomized rats,[442] supporting the hypothesis that early growing follicles are more sensitive to androgen action and that estrogens downregulate *AR* expression as part of the process of follicle differentiation. Similar data has been produced in mice. For example, AR antagonists are able to inhibit FSH-induced E2 synthesis in individually cultured murine follicles if included at the beginning of the culture period prior to any indications of differentiation.[716] Similarly, E2 output by large antral murine follicles in vitro is unaffected by DHT.[492] In marmoset ovaries, granulosa cells isolated from small (0.5–1 mm) follicles are highly responsive to the enhancing effects of T or DHT on FSH-induced aromatase activity, but this response is lost in granulosa cells from larger (>2 mm) follicles whereupon DHT exposure becomes inhibitory.[717,718] In contrast, 3 or 10 days of T treatment in rhesus monkeys has no effect on ovarian *CYP19* expression.[684]

The majority of data indicate that healthy preantral follicles are responsive to T and require the steroid for the initiation of *Cyp19* expression by FSH in granulosa cells. Since androgen synthesis in thecal cells precedes the acquisition of aromatase activity in the accompanying granulosa cells, this mechanism of androgen/FSH synergism allows for more efficient induction of *Cyp19* expression than would occur with FSH alone. Once the granulosa cells acquire sufficient aromatase activity, thecal-derived androgens become more important as substrates and ER-mediated E2 actions continue the role of synergizing with FSH to promote follicle differentiation (Figure 25.25). Hence, the hallmark of a healthy preovulatory follicle in rodent ovaries may be the presence of substantial aromatase activity and E2 output, and decreased *AR* expression and androgen sensitivity.

The mechanism by which androgens augment FSH action on granulosa cells is unclear but may differ from that hypothesized for E2. Whereas ER-mediated E2 actions are believed to enhance the amount and effectiveness of FSH-stimulated intracellular cAMP without obvious changes in FSH-receptor levels, evidence suggests that androgens may act at a site prior to adenylyl cyclase. For example, neither T nor DHT synergize with $(Bu)_2$-cAMP to induce aromatase activity in rat granulosa cells[719] whereas E2 does.[442,720] Instead, AR-mediated androgen actions may largely act by increasing the level of FSH receptor in granulosa cells of preantral follicles. T can also restore the losses in FSH receptor and responsiveness that occur in rat granulosa cells cultured without gonadotropins, and this effect is inhibited by AR antagonists.[719] Evidence that androgens increase granulosa FSH-receptor levels comes from reports that ovaries of 10-day-old and prepubertal AR-null females exhibit significantly reduced *Fshr* mRNA levels, even 48 h after PMSG treatment in the latter age group.[183] Similarly, Weil et al. found that 3 or 10 days of T treatment in adult rhesus monkeys increases FSH-receptor expression in follicles of all stages except primary.[684] In human small antral follicles cultured in vitro, a positive correlation was observed in AR expression, androgen concentrations in follicular fluid, and FSHR expression[721] in small follicles demonstrating an association with AR and androgens in immature granulosa cells, and suggests that in human ovaries this association is important for normal follicle development.

AR-mediated androgen actions may also be involved in antrum formation. Murray et al. found DHT potentiates suboptimal doses of FSH to stimulate increased follicle diameter of murine follicles in culture, and this promotional effect is inhibited by AR antagonist.[716] T or DHT are also reported to be almost as effective as E2 in augmenting FSH-induced increases in the number of antral follicles in the ovaries of hypophysectomized mice.[492] However, the ovaries of global AR-null mice do not show differences in the number of antral follicles,[183,185,690] while in the granulosa-specific AR-null mice a reduction in the number of antral follicles was observed at all ages,[692] or only in younger mice (3 months).[691] The conflicting reports of antral formation in AR-null mice demonstrate the complexity of this developmental process in the ovary. Therefore, FSH-dependent antrum formation may be enhanced by either receptor-mediated androgen or estrogen actions, congruent with the process being part of the transition period during which follicles become less sensitive to androgens and more dependent on estrogens.

Not all FSH-dependent processes during follicle differentiation are enhanced by androgens. LH-receptor expression and LH responsiveness by granulosa cells occurs only in preovulatory follicles during the final stages of folliculogenesis, just prior to the LH surge. Unlike the above processes of follicle differentiation, FSH stimulation of LH-receptor expression in preovulatory granulosa cells is facilitated by estrogen or aromatizable androgens only[515,548,549] while DHT has no effect or may even be inhibitory.[515,549] These data

are consistent with the mutually exclusive pattern of AR and LH receptor in preovulatory follicles of rodent ovaries, as *AR* expression is limited to the cumulus cells[667] while LH receptor is predominantly localized to mural and antral granulosa cells.[722–724] In addition, in vivo studies demonstrating that AR antagonists have no effect on LH-receptor expression in the ovaries of FSH-stimulated, DES-primed hypophysectomized rats suggest that AR-mediated actions are not required to promote or maintain LH-receptor expression in preovulatory follicles.[725] The specific requirement of ER-mediated E2 actions to augment FSH-stimulated LH-receptor expression in preovulatory granulosa cells provides a mechanism by which only those follicles that acquire sufficient aromatase activity and are suitable to ovulate acquire the capacity to respond to the LH surge.

THECAL CELL STEROIDOGENESIS

There are few studies on the role of androgen signaling in thecal cell function and steroidogenesis despite these cells being the primary source of androgen synthesis. Several nonsteroidal paracrine factors are known to modulate thecal cell steroidogenesis and have been more thoroughly studied.[476,566] The limited available evidence suggests that AR-mediated androgen actions may negatively regulate thecal cell steroidogenesis, thereby forming an autocrine regulatory feedback loop. Mahesh and colleagues have shown in enriched thecal/interstitial cell cultures from rats that AR activation by nonaromatizable agonists attenuates hCG or hCG/IFG-1–stimulated increases in androstenedione synthesis by 32% and 40%, respectively, via selective inhibition of *Cyp17* expression.[726] Similar experiments demonstrated that AR-antagonists enhance hCG-induced androstenedione synthesis, further indicating a receptor-mediated effect of androgens on thecal cell steroidogenesis.[726] The majority of recent studies published to date focus on the transcriptional regulation of genes involved in androgen biosynthesis in theca cells,[687,688] but not on the autocrine action of the synthesized androgen(s) within the cells. In fact, a recent review entitled "Theca: The forgotten cell of the ovarian follicle" described the various factors that are known to regulate steroidogenesis and androgen production,[727] which also highlights the need for more focused experiments looking at theca cell functions of AR. The use of conditional knockout mouse models to explore the role of AR specifically in theca cells could also shine light on this important question. Therefore, ER-mediated E2 actions (see the section Estrogen Receptor Signaling in Ovarian Function) and AR-mediated androgen actions may both contribute to maintaining homeostasis of androgen synthesis in the thecal cells of developing follicles.

OVULATION

Over the past decade there have been few studies aimed at determining a role for androgen signaling in follicle rupture and ovulation, the majority of these from the AR-null mouse models (Table 25.9). This is especially surprising since Mori et al. demonstrated in 1977 that inhibition of androgen action at the time of ovulation is detrimental to follicle rupture in the rat.[728] These studies employed anti-T and anti-P antisera during induced ovulation in immature or hypophysectomized rats to demonstrate that: (1) acute treatment with anti-T antisera at the time or shortly after hCG-induced ovulation leads to a dose-dependent reduction in the number of ovulated oocytes, and (2) inhibition of ovulation by acute treatment with an anti-P antisera at the time of hCG treatment is rescued by concurrent treatment with T or DHT but not E2.[728] In a similar study, Peluso et al. demonstrated that treatment of immature rats with the AR antagonists, cyproterone acetate or flutamide, 3 h prior to hCG-induced ovulation drastically reduces the number of ovulated oocytes and prevents expansion of the cumulus–oocyte complex.[729] Similar findings are reported in mice.[730] The development of AR-null mouse models has provided some insight into the role of androgen signaling in ovulation. An ovulatory defect in AR-null mice is suggested by the presence of a normal number of preovulatory follicles but few corpora lutea in the ovaries,[183] as well as reduced circulating P levels.[689] Furthermore, gonadotropin-induced ovulation of immature AR-null females indicates a severely reduced response in terms of recoverable oocytes in the oviducts, and may be attributable to their failure to exhibit hCG-induced expression of P receptor, hyaluronyl synthetase-2, and tumor necrosis factor-α-stimulated gene 6.[183] Reduced ovulatory rates were also observed in another AR-null mouse model lacking the DNA binding domain after normal mating,[185] however ovulation rates after treatment with exogenous gonadotropins were normal. A similar finding was observed in granulosa cell–specific AR-null mouse model where oocytes collected after natural mating are reduced but animals respond to exogenous gonadotropins. Interestingly, when these animals were 6 months of age, they didn't ovulate at all.[692] These mouse models suggest that AR plays a role in normal ovulation, but stimulation with excess gonadotropins can overcome these deficiencies only in young mice. Further work to study the role of androgen signaling in other mammalian species including primates and humans could provide insight into possible fertility treatments.

FOLLICLE ATRESIA

Follicle atresia in the mammalian ovary has been thoroughly described in several reviews.[565,680,731] Follicular atresia is a complex hormonally driven process

involving both putative "survival" factors, e.g., insulin-like growth factor-1, epidermal growth factor, basic fibroblast growth factor, E2, and activin; and putative atretogenic factors, e.g., tumor necrosis factor-α, GnRH, and androgens.[565,680,731] The respective action of each of these factors on atresia is dependent on the follicular stage,[731] for example, epidermal growth factor and basic fibroblast growth factor begin to act early on primordial stage follicles, whereas insulin-like growth factor-1 affects early antral follicles.

Circumstantial evidence of the actions of androgen includes the numerous observations that atretic follicles consistently exhibit an increased androgen:estrogen ratio within the intrafollicular fluid.[565] Supporting experimental evidence includes reports that low doses of hCG (to induce endogenous androgen production), T, or DHT following 4 days of E2 treatment in hypophysectomized rats lead to increased numbers of atretic follicles within 24 h.[705,732] The detrimental effects of hCG or exogenous androgens are inhibited by co-treatment with anti-T antisera or AR antagonists, indicating the involvement of androgen receptor in this process.[705,732] Interestingly, large (>150 μm) follicles are more susceptible to the atretogenic effects of androgens than small (<150 μm) follicles, indicating that androgens are largely atretogenic in late-stage follicles.[705,732] In similar experiments on hypophysectomized rats, Billig et al. demonstrated that the follicular atresia that follows the withdrawal of 2 days of continuous estrogen (DES) treatment is enhanced by subsequent T exposure but prevented by E2.[733] Conversely, Flaws et al. show follicular atresia induced by the endocrine-disrupting chemical methoxychlor.[734]

Additionally, AR-null mouse models also have increased atretic follicles,[183,185,690] demonstrating that AR signaling plays a role in follicle atresia; however, further studies are needed to understand the mechanism.

Glucocorticoid Receptor Signaling in Ovarian Function

While the ovary is not considered a classic target tissue of glucocorticoids, altered glucocorticoid production can affect reproduction and fertility throughout the HPG axis. Within the ovary, glucocorticoid signaling through GR can modulate steroidogenesis/gametogenesis and will briefly be discussed here. GR is expressed in the rat ovary, and examination of whole ovary expression shows that levels do not change upon treatment with exogenous gonadotropins.[735] Further examination of GR expression in isolated granulosa cells shows no significant alterations in expression within these cells, although GR expression appears to be elevated in corpora lutea.[735]

Glucocorticoid signaling in the ovary acts to inhibit LH action and steroid biosynthesis during folliculogenesis.[736] Treatment of granulosa cells with cortisol or dexamethasone was able to inhibit FSH-stimulated increases in aromatase activity in vitro, while P production was increased compared to control-treated cells. This finding suggests that GR signaling may affect steroidogenesis of the ovarian steroids in a differential manner.[737] Furthermore, this finding suggests that GR signaling in the ovary is important for P function and ovulation. Dexamethasone was also found to stimulate FSH-mediated increases in P production in porcine granulosa cells,[738] suggesting a key role for GR signaling during late stages of ovulation and for corpora lutea function.

The expression of GR in granulosa cells does not change significantly during early follicle development; however, the treatment of cells with agonist shows differential activity depending on the stage of differentiation of the cells. To circumvent the action of glucocorticoids, expression of two metabolizing enzymes has been found to have stage-specific expression in the ovary.[736] The expression of the type I and type II 11β-hydroxysteroid dehydrogenase (11HSD 1 and 11HSD 2) modulates the action of glucocorticoids by regulating their concentrations within tissues.[739] This is important in the ovary during folliculogenesis since glucocorticoids are not synthesized within the tissue itself. During FSH-stimulated follicle growth, *11Hsd1* is expressed and acts to suppress glucocorticoid action, then as the granulosa cells become luteinized after LH stimulation, *11Hsd2* expression is increased.[735,740] This acts to increase the action of glucocorticoid action on the ovary during luteinization and formation of the corpora lutea, which also shows an increase in GR expression, demonstrating the importance of GR signaling during this process. Interestingly, expression of *11Hsd2* is reduced during luteal regression,[741] demonstrating the importance of GR activity specifically in the corpora lutea. The evidence to date suggests that GR signaling is important in ovarian function, although further work is needed to define the precise activity and necessity of this signaling mechanism specifically in the ovary.

SUMMARY

The salient aspects of the previous discussion are summarized in a model of sex steroid and steroid receptor action during folliculogenesis. Although certain postulated mechanisms and pathways portrayed in this model are supported by substantial evidence, other aspects are much more speculative and remain to be demonstrated. Regardless, the evidence is solid that the receptor-mediated steroid pathways exist in the ovary and their intraovarian functions are critical to female fertility.

CONCLUSION

Within this fairly extensive review we have endeavored to describe as comprehensively as possible what is currently known regarding the important functions of estrogen, progesterone, androgen, and glucocorticoid receptors in ovarian and uterine tissues. Clearly, an abundance of data has been obtained that has greatly advanced our understanding of the roles of these receptors since early descriptions more than five decades ago. Nevertheless, many open questions remain for further elaboration. At the level of the steroid receptors themselves, questions remain regarding how the receptors mediate the spectrum of responses, and what roles tissue and gene specificity of distinct activation domains on the receptors play. Additionally, better understanding of the interactions of receptors with chromatin, which are often quite far from regulated transcripts, and how the interactions lead to changes in transcription, are challenging questions to approach. We can hope to learn more about temporal patterns of changes in the organization of nuclear structures initiated by steroid receptors. At the whole animal level, what remains is to piece together advances gleaned from diverse models and methods into a comprehensive description of the coordinated signaling between receptors for different steroids in cells and tissues that culminate in an optimal environment for fertilization, implantation, gestation, and parturition.

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Color Plates

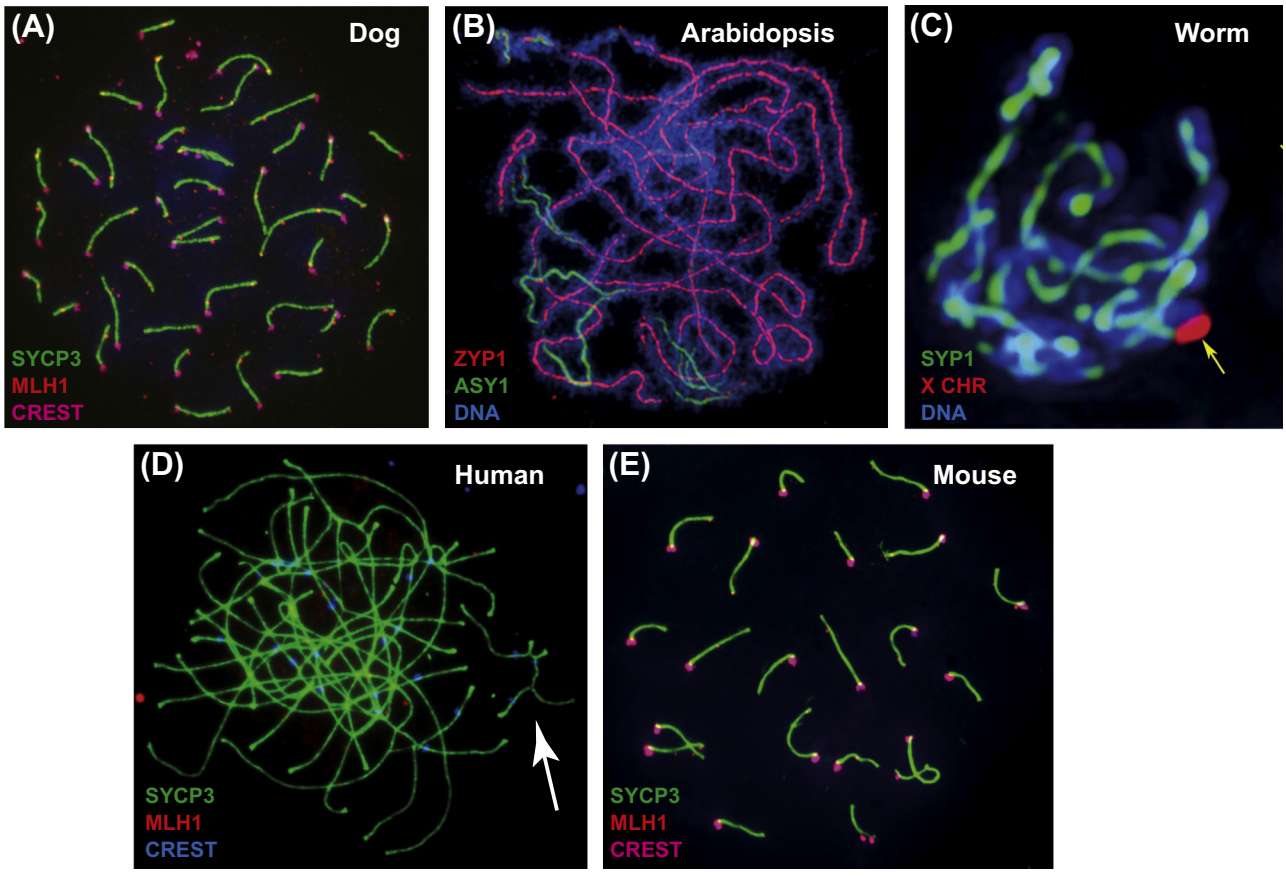

FIGURE 1.2 Comparative meiosis across eukaryotic species. (A) Pachytene cell from an adult dog testis stained with antibodies against SYCP3 (green), MLH1 (red), and CREST (centromeres, pink). (B) Immunolocalization of the *Arabidopsis* synaptonemal complex transverses filament protein ZYP1 (red), polymerizing between the homologous chromosome axes marked by ASY1 (green) and the DNA loops (blue) in a late-zygotene nucleus. The ASY1 signal (green) is reduced in synapsed regions (red), and an interlock is being resolved. (C) A *C. elegans* pachytene nucleus immunostained to show that the synaptonemal complex (green) is normal and that the pairing center ends of the X chromosome are fully paired (red); *(Source: Adapted from Saito et al., PLoS Genet, 2009.)* (D) A trisomic human embryonic oocyte at the pachytene stage stained with antibodies against SYCP3 (green), MLH1 (red), and CREST (centromeres, blue). The three copies of chromosome 21 can be seen attempting to pair (arrow). (E) Mouse pachytene cell stained immunostained with SYCP3 (green), MLH1 (red), and cREST (centromere, pink).

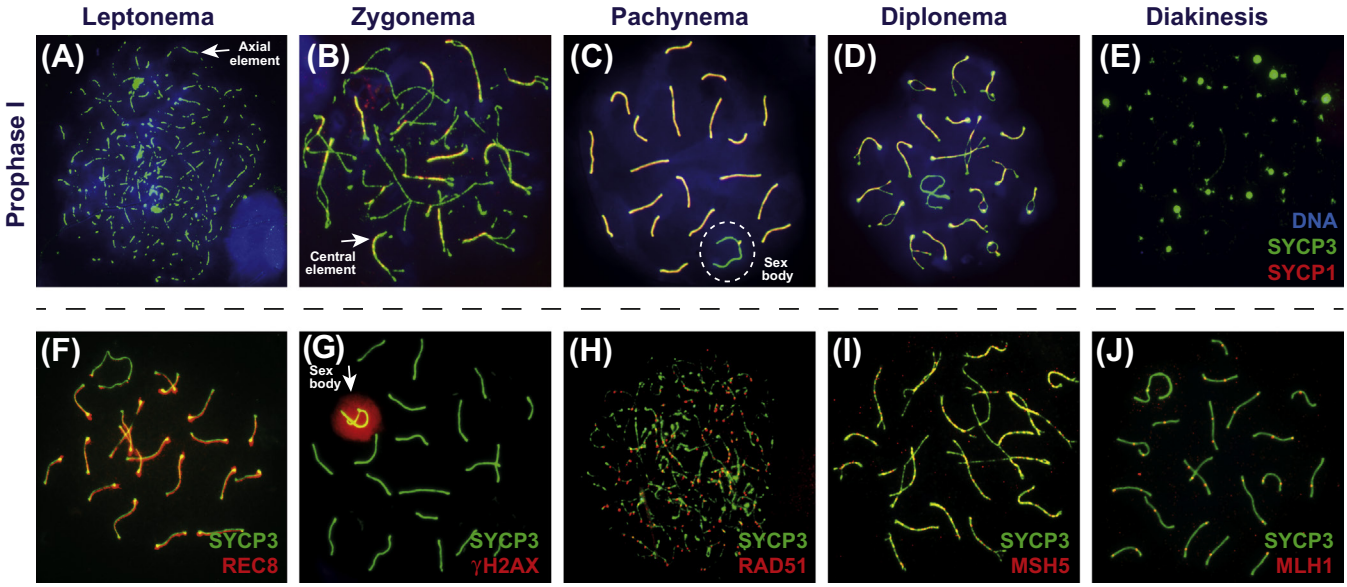

FIGURE 1.7 Immunofluorescent analysis of meiotic prophase I events in mouse spermatocytes. (A–E) Synaptic events in the five substages of prophase I, visualized by staining with antibodies against axial–lateral element protein SYCP3 (green) and central element protein SYCP1 (red), where the overlay between the two appears in yellow. (F–J) Immunolocalization of SYCP3 (green) with key meiotic markers during prophase I; (F) REC8, (G) λH2AX, (H) RAD51, (I) MSH5, and (J) MLH1 all in red, and SYCP3 in green.

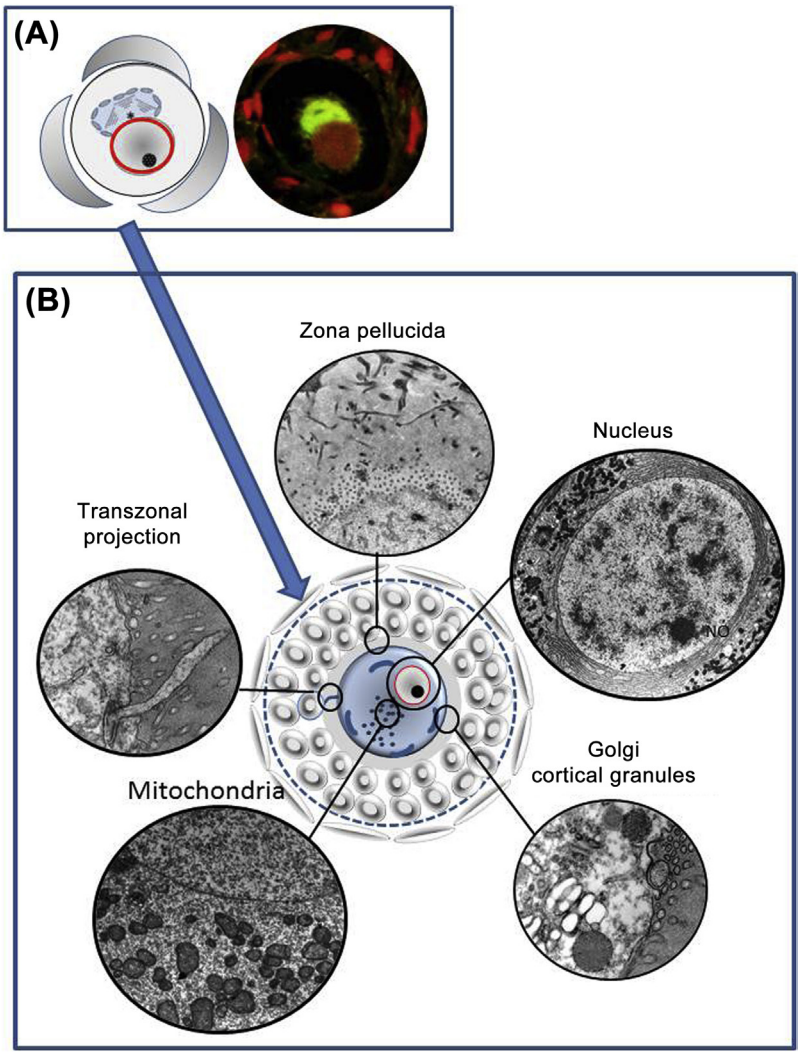

FIGURE 2.1 **Structure of the mammalian oocyte.** (A) depicts the general organization of organelles in a primordial follicle; schematic (left) denotes centrally positioned nucleus and aggregation of organelles known as Balbiani's body (asterisk). Right is an immunofluorescence micrograph illustrating the concentration of the germ cell–specific marker VASA within Balbiani's body (green) located next to the oocyte nucleus (red). *(Image courtesy of Professor Alfredo Vitullo)*. Upon activation, oocytes enter the growth phase of oogenesis (B). (B) summarizes the major ultrastructural features of a growing oocyte within a preantral follicle noting the assembly of the extracellular coat or zona pellucida, the enlarged nucleus or germinal vesicle with prominent nucleoli (NO), subcortical Golgi complexes with associated cortical granules, abundant perinuclear mitochondria, and the elaboration of microvilli on the oocyte plasma membrane interacting with somatic cell projections known as transzonal processes.

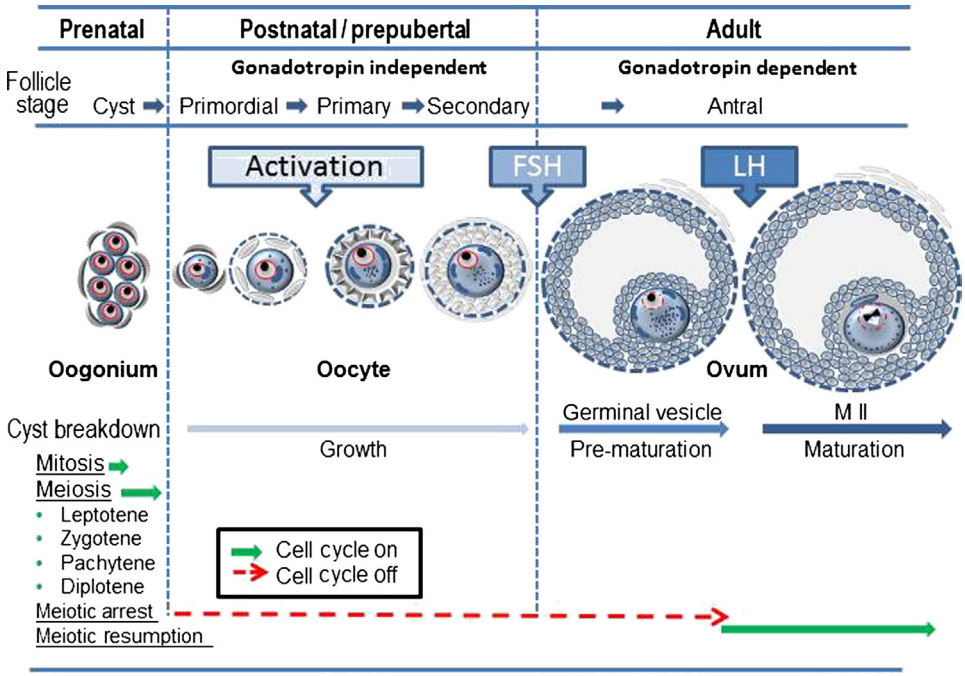

FIGURE 2.2 Stages of oogenesis defined on the basis of the oocyte cell cycle state as it occurs in the mouse. Mitotic proliferation of oogonia occurs in the prenatal gonad and is accompanied by entry into meiotic prophase as follicle formation and cyst breakdown take place. The cell cycle is then turned off for the duration of the growth phase of oogenesis that occurs during the preantral stages of follicle development. While FSH is required to advance follicle development, the oocytes remain in meiotic arrest with a germinal vesicle in the prematuration stage. In response to the LH surge, meiotic arrest is released and the oocyte proceeds through maturation being ovulated at the metaphase two stage of meiosis, which is maintained until the fertilization signal elicits completion of meiosis two. Note that in general the growth phase of oogenesis occurs independent of gonadotropin stimulation, whereas the prematuration and maturation phases require gonadotropin stimulation.

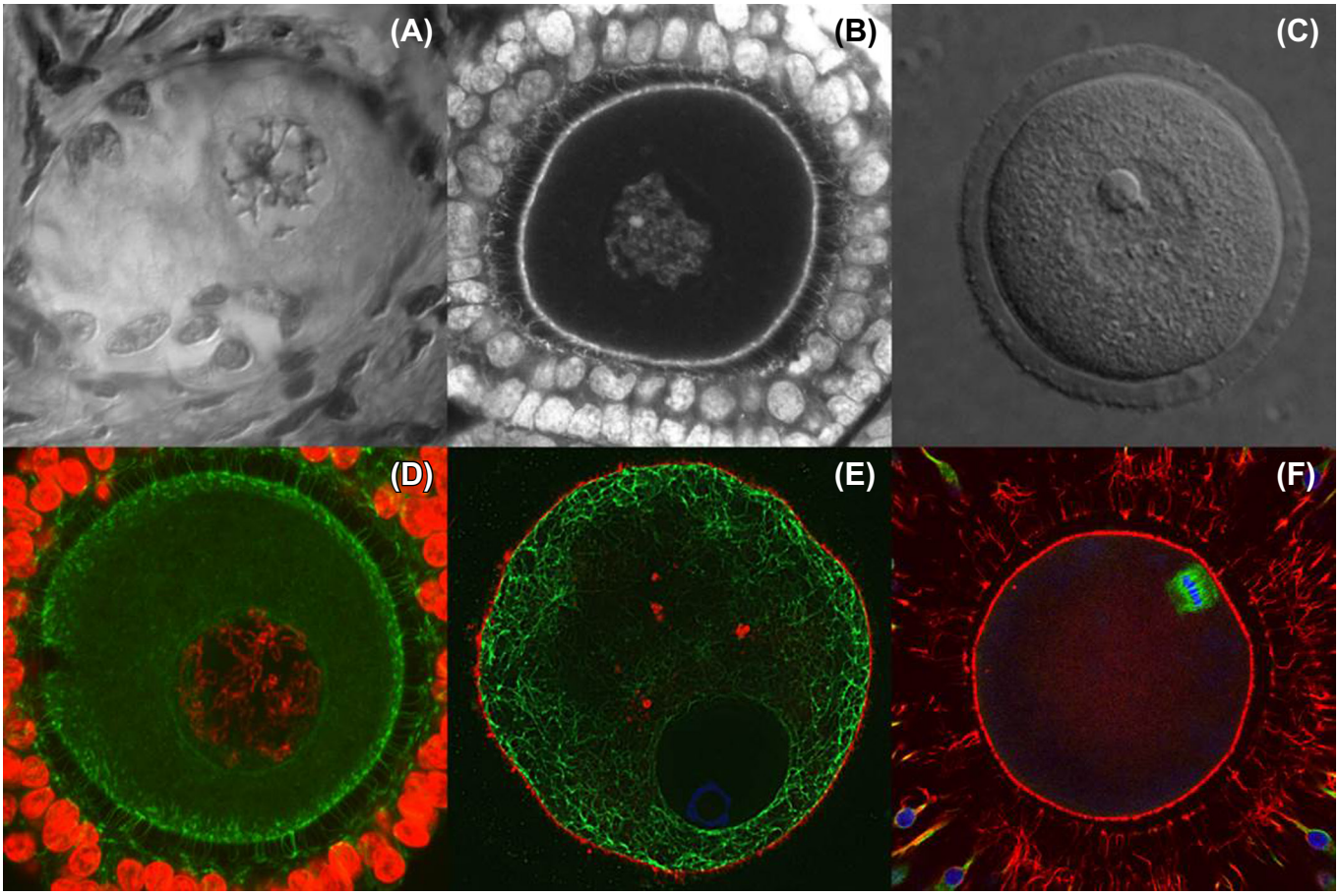

FIGURE 2.3 **Prominent morphological features of the mammalian ovum at various stages of oogenesis in various species.** (A) depicts a resting primordial oocyte within the bovine ovary in which the germinal vesicle chromatin assumes a distinctly fibrillar pattern of organization. (B) represents a primary growing oocyte from the mouse containing a centrally positioned germinal vesicle and illustrating the prominent actin-rich cortex and numerous transzonal projections emanating from surrounding granulosa cells. (C) demonstrates the appearance of a full-grown immature mouse oocyte following isolation from the ovary; note the prominent eccentrically positioned nucleolus within the GV and the cell free zona pellucida. (D) depicts a full-grown immature bovine oocyte isolated from a Graafian follicle; note the clear organization of bivalents in the GV and the presence of numerous foci at the oocyte cell surface, which represent terminal connections with corona TZPs (preparation labels f-actin using rhodamine phalloidin). (E) illustrates an immature GV-stage human oocyte that has been labeled for chromatin and microtubules (fibrillar structures); note that the chromatin is aggregated around the nucleolus of the GV and that a dense network of cytoplasmic microtubules is present in the outer regions of ooplasm. (F) illustrates a mature metaphase two arrested oocyte from a horse following controlled ovarian hyperstimulation; this preparation has been dual-labeled for tubulin (green) that highlights the cortical anchored meiotic spindle and f-actin (red) that labels the prominent corona cell projections or TZPs that appear detached from the oolemma.

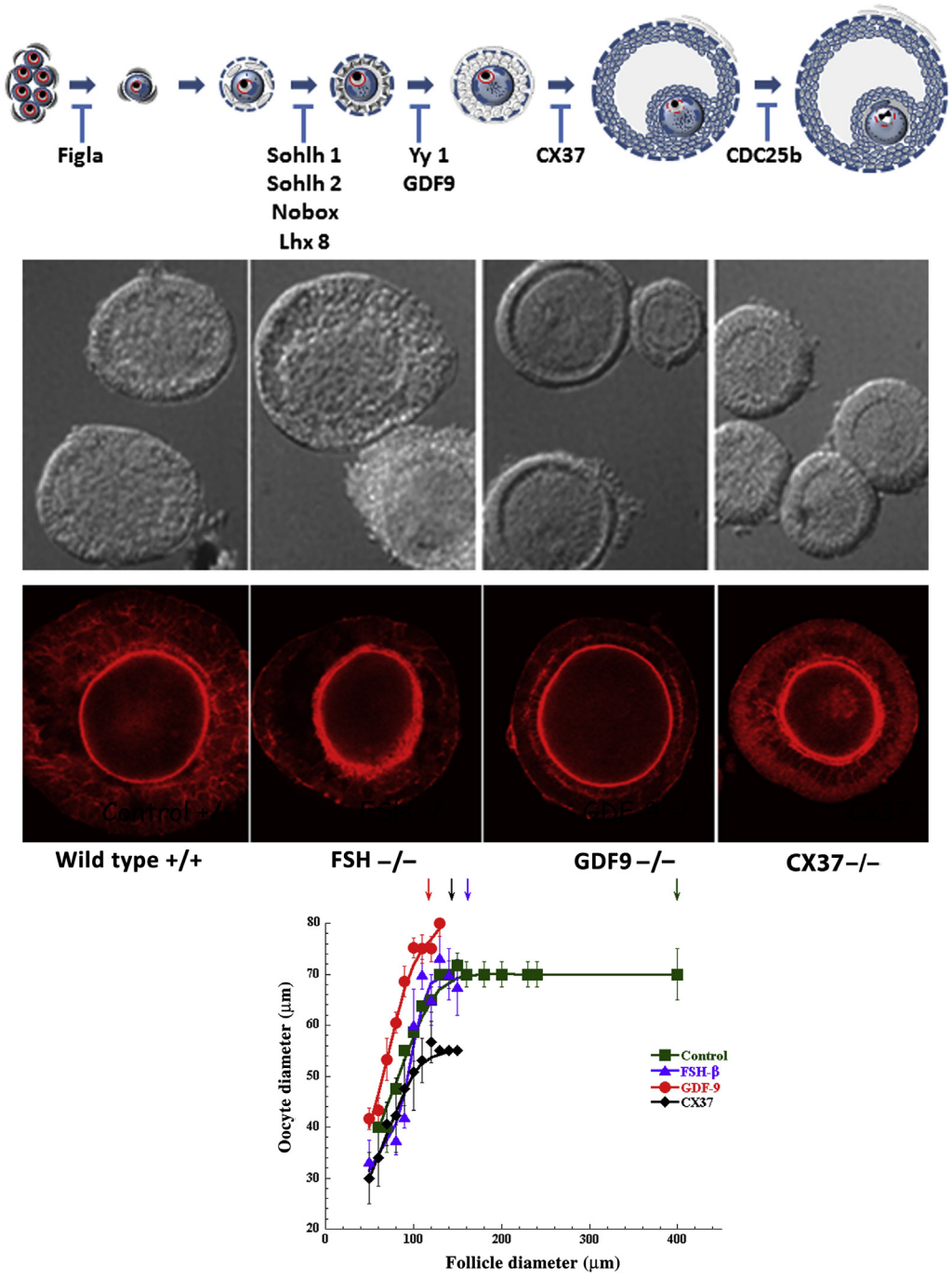

FIGURE 2.10 Mouse knockout phenotypes with distinct defects during the growth phase of oogenesis. Schematic at top shows effects of targeted gene deletion on stage of follicle development. Images show phenotype of wild type (left), GDF9, FSH beta, and CXN 37 in sequence that in the top panel show living follicles, and in the lower panel, the organization of granulosa cell–oocyte interface. Graph (bottom) plots the relationship between follicle and oocyte growth for the same four groups.

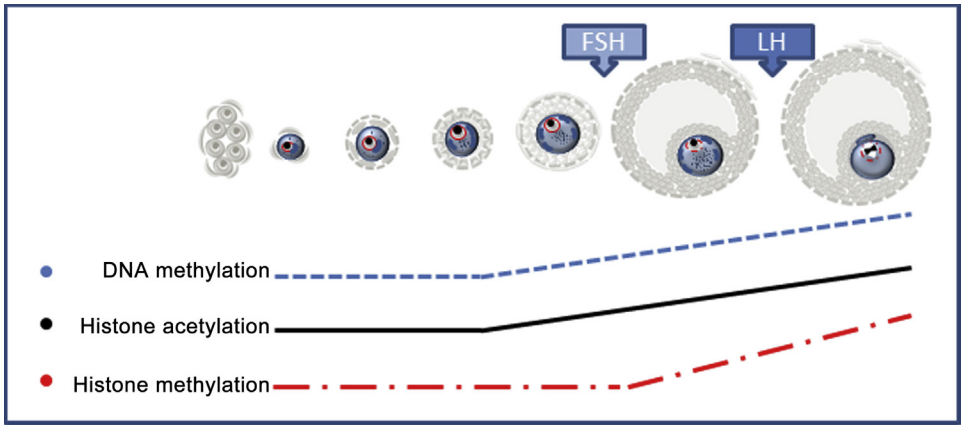

FIGURE 2.11 Epigenetic modifications of chromatin during the course of oogenesis. Genes subject to imprinting show progressive signs of DNA methylation beginning during the growth phase of oogenesis and ending at some point in the preovulatory follicle. Histone acetylation in developing oocytes follows a similar time course as DNA methylation, whereas histone methylation is reported to occur following the growth phase. Data are based primarily on studies in the mouse.

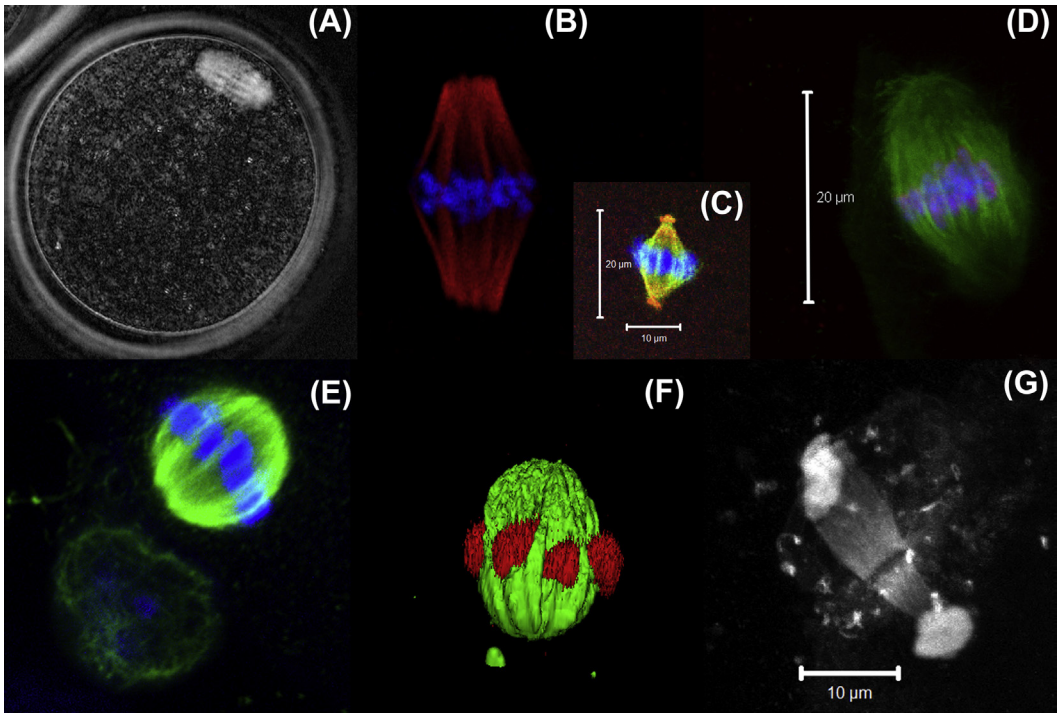

FIGURE 2.15 Meiotic spindles of mammalian oocytes. (A), (B), and (C) depict metaphase two–stage mouse oocytes under polyscope in a living oocyte (A), and after confocal immunofluorescence of tubulin (B, red; C, green); chromosomes aligned on the metaphase plate are rendered blue, and in (C), the poles of the spindle from a naturally ovulated oocyte show gamma-tubulin in red. (D) illustrates kinetochore microtubules as green stripes in a metaphase one horse oocyte that has been stained for chromosomes (blue) and phosphohistone H3 (red). (E), (F), and (G) are human oocytes at metaphase two (E and F, with the latter rendered as a three-dimensional projection); and (G) illustrates a projection of telophase one at the time of polar body extrusion.

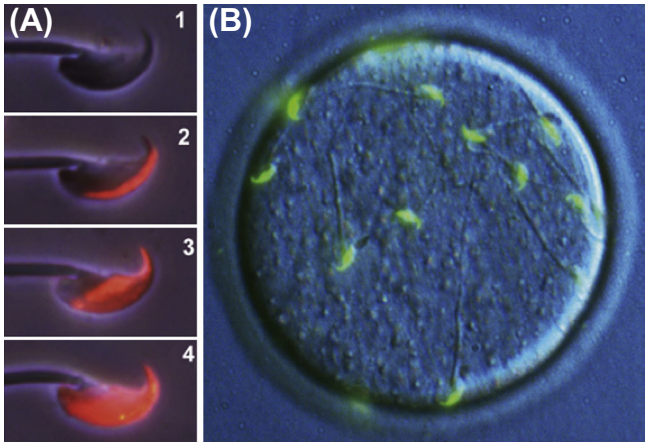

FIGURE 4.7 Gamete fusion-related factor IZUMO1 and localization in sperm. (A) IZUMO1 is an acrosomal membrane protein that is not exposed before the completion of an acrosome reaction (1). Acrosome-reacted sperm can be classified into three major groups by their IZUMO1-staining pattern: acrosomal cap (2), equatorial (3), and whole-head (4). (B) Accumulation of many sperm in the perivitelline space of the eggs recovered from females mated with *Izumo1*$^{-/-}$ males. Sperm in the perivitelline space were labeled with acrosome-reacted sperm-specific monoclonal antibody MN9. *Figure reprinted from Ref. 273 with permission from the* Asian Journal of Andrology. *Courtesy of Dr Masaru Okabe.*

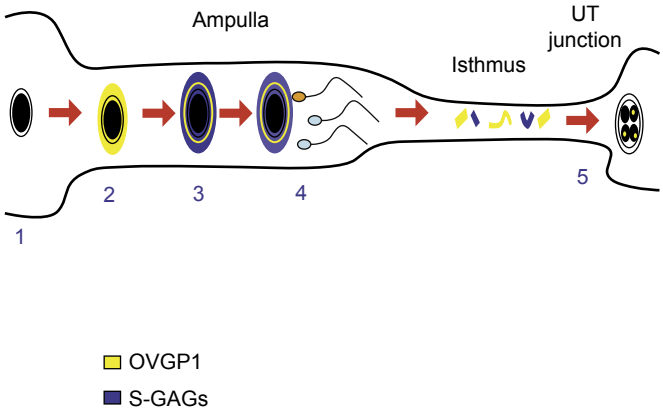

FIGURE 4.8 Depiction of the proposed role of oviductal contents in preventing polyspermy in ungulates. After the egg is shed into the ampulla following ovulation (1), oviduct-specific glycoprotein (OVGP1) surrounds it in a "shell" that renders the zona pellucida (ZP) resistant to proteolysis (2). Other oviduct molecules such as heparin-like glycosaminoglycans (S-GAGs) are thought to aid and strengthen the binding of OVGP1 to the ZP (3), modifying interaction with spermatozoa (4). As the zygote advances to the uterus, the aforementioned factors are shed or absorbed into the egg (5). *Figure reprinted and legend adapted from Ref. 375 with permission from John Wiley & Sons. Courtesy of Dr Pilar Coy.*

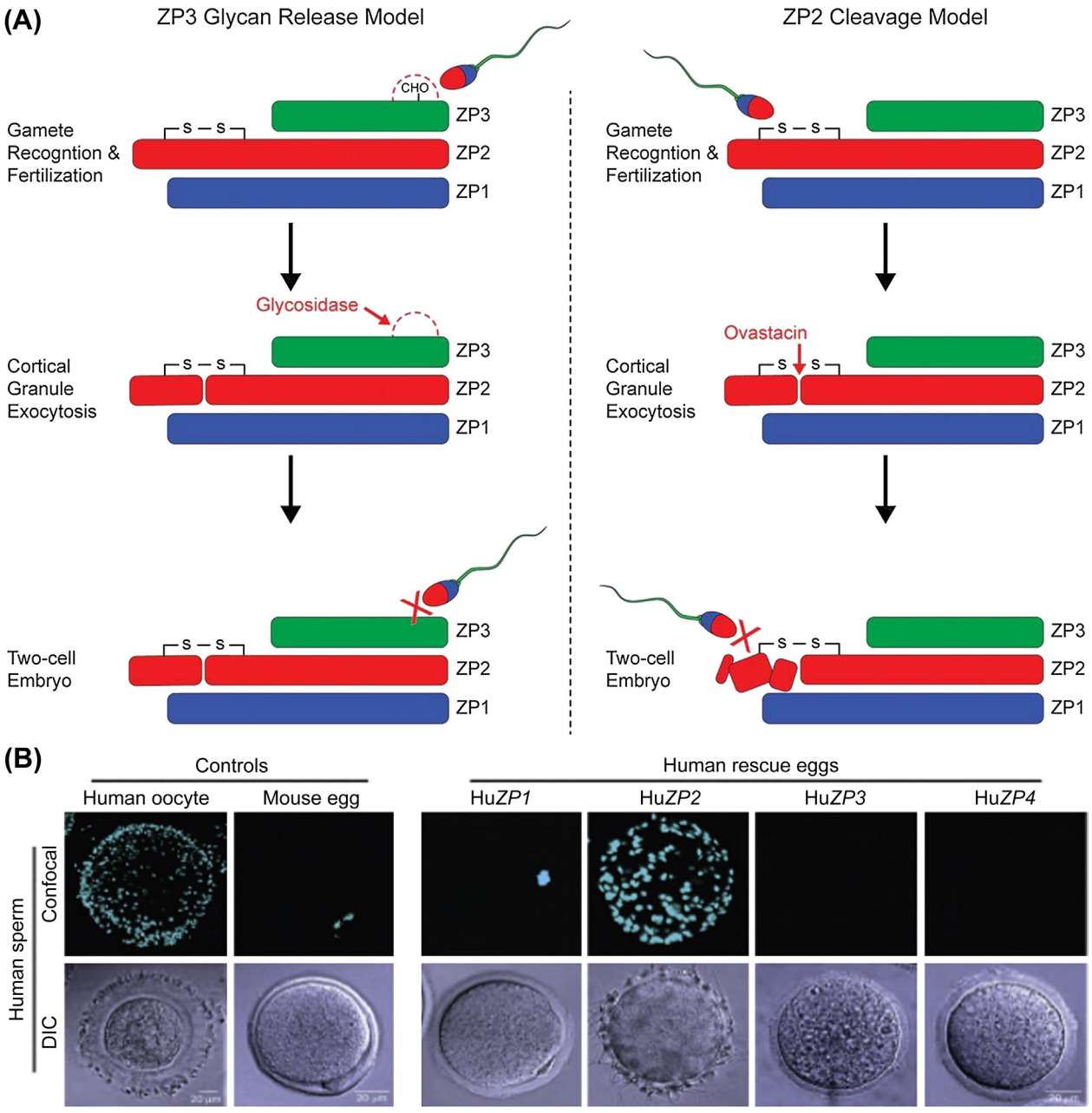

FIGURE 4.9 Models of zona pellucida block to polyspermy. (A) A specific glycan-release model (left panel) proposes that *O*-glycans on ZP3 Ser^{332} and Ser^{334} or possible others act as ligands for a sperm surface receptor. Following fertilization, cortical granules would release a glycosidase that would remove the *O*-glycans and account for the inability of sperm to bind to two-cell embryos. The ZP2 cleavage model (right panel) proposes that sperm bind to an N-terminal domain of ZP2. Following fertilization, ovastacin, a cortical granule metalloendoprotease, is exocytosed and the proteolytic destruction of the ZP2 docking site prevents sperm from binding to two-cell embryos. (B) Human sperm bind to mouse eggs from transgenic strains expressing human ZP2, but not other human ZP proteins, in the ZP. Confocal and differential interference contrast images of capacitated human sperm binding to the ZP of mice expressing human ZP1, human ZP2, human ZP3, or human ZP4, in the absence of the corresponding mouse protein. Human oocytes and mouse eggs served as positive and negative controls, respectively. Specific references and experimental details can be found in the text. *Figure reprinted from Ref. 229 with permission from Oxford Journals. Courtesy of Dr Jurien Dean.*

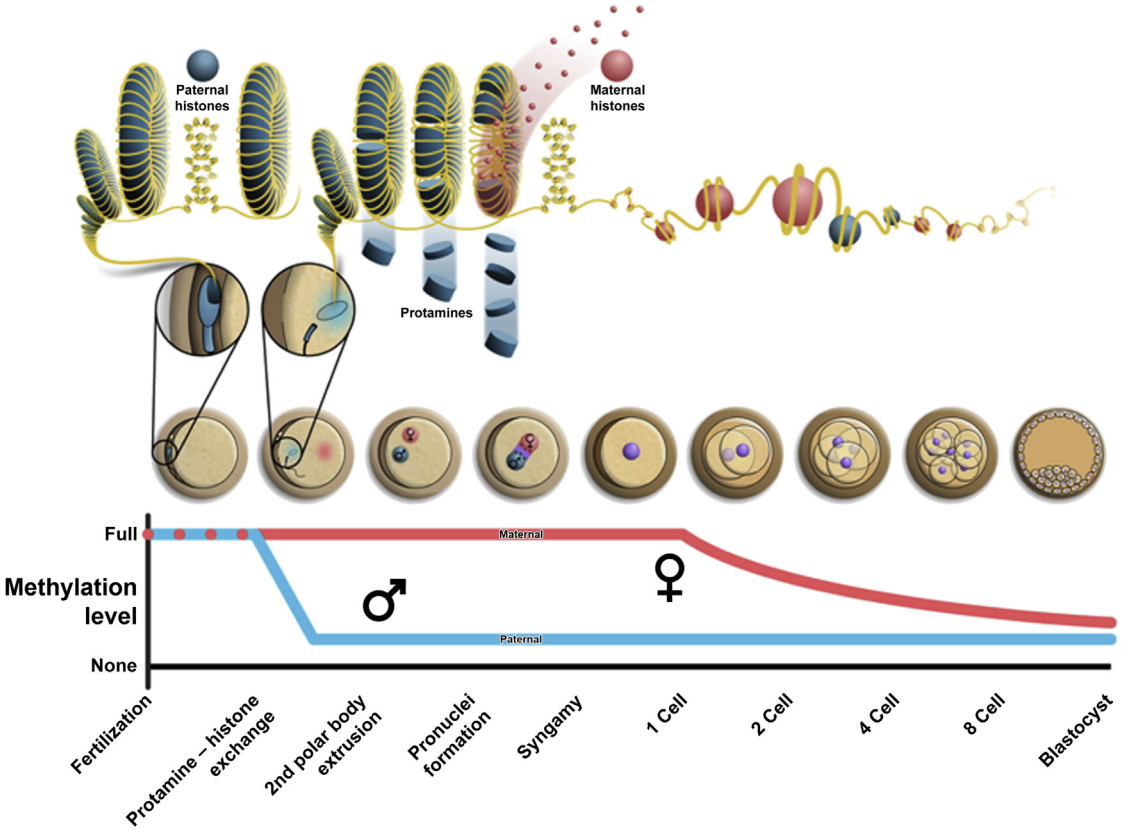

FIGURE 4.13 Reorganization of the male chromatin after fertilization and alterations to the epigenome postfertilization. The top panel illustrates the chromatin structure of the mature sperm immediately following fertilization (highly protaminated with some retention of paternally derived histones). Soon after sperm entry, the protamine to histone transition takes place where maternally derived histones replace protamines resulting in the decondensation of the sperm head. The middle panel illustrates the various stages of early embryonic development. The bottom panel shows the methylation changes that occur over time in the maternal and paternal pronucleus, where the paternal pronucleus undergoes active demethylation and the maternal DNA is demethylated passively in a replication-dependent manner. The approximate chronology of major events in the early embryo is outlined along the bottom of the figure and correlates to the illustrations of embryos above. *Figure reprinted from Ref. 444 with permission from Frontiers. Courtesy of Dr Douglas Carrell.*

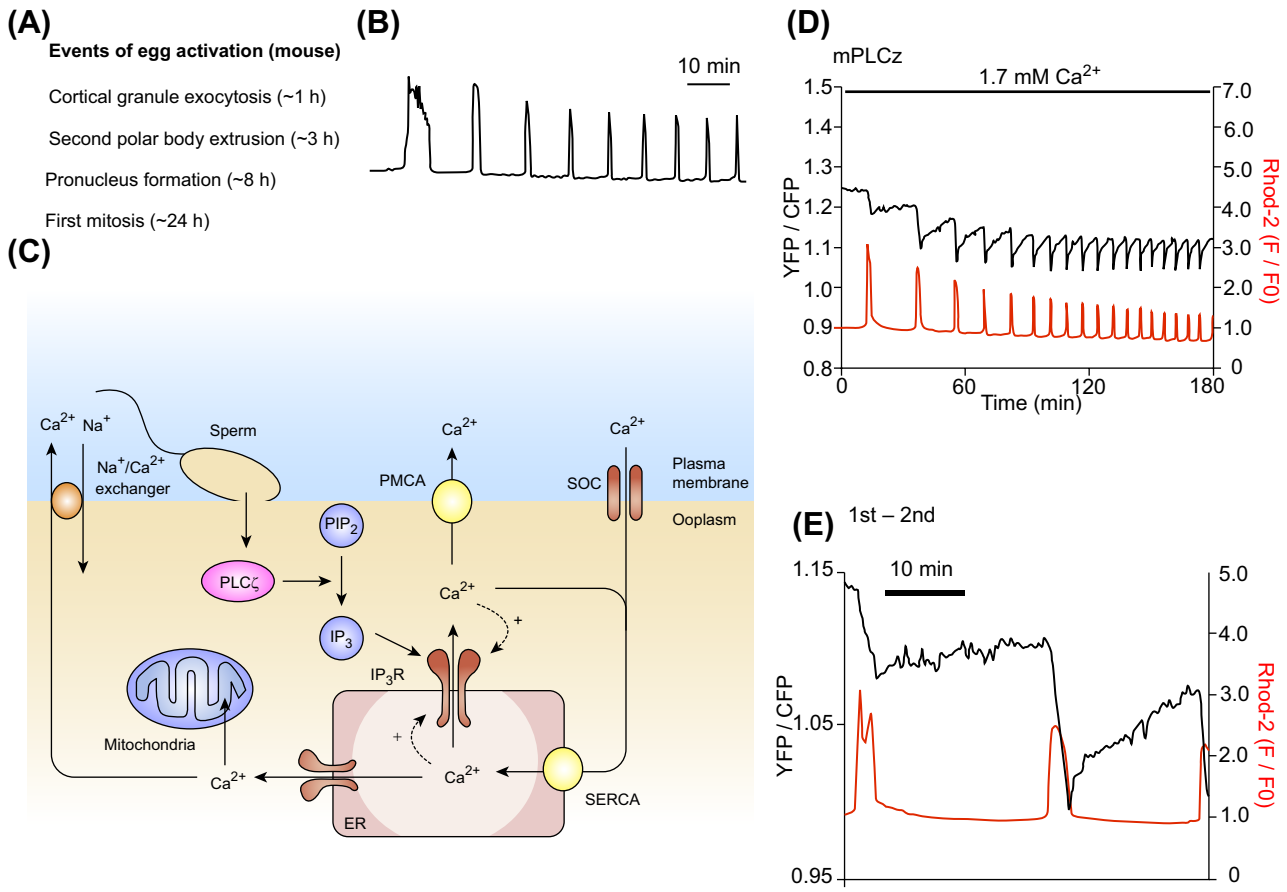

FIGURE 4.14 **Temporal course of activation events in mouse eggs with a characteristic Ca^{2+} response and candidate molecules involved in the initiation oscillations and in Ca^{2+} homeostasis.** (A) Main cellular events of egg activation after sperm entry and approximate time in hours (h) required for their completion. (B) A typical pattern of $Ca^{2+}{}_i$ oscillations associated with fertilization or with injection of PLCζ cRNA. Note that recordings were terminated prematurely. (C) Upon fusion, the sperm is thought to deliver phospholipase C (PLC) ζ, which hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol 1,4,5-trisphospahte (IP_3) and diacylglycerol (DAG). IP_3 binds its receptor, IP_3R1, causing Ca^{2+} release out of the endoplasmic reticulum (ER). Following Ca^{2+} release, basal $Ca^{2+}{}_i$ levels are regulated by the combined action of the sarcoendoplasmic reticulum Ca^{2+} ATPase (SERCA), plasma membrane Ca^{2+} pump (PMCA), Na/Ca^{2+} exchanger, and mitochondria. Store operated Ca^{2+} channels (SOC) or other Ca^{2+} channels are proposed to mediate Ca^{2+} influx, which is required to fill the ER and maintain oscillations. Broken lines suggest feedback action of Ca^{2+} on IP_3R1. *(Adapted from Ref. 507 with permission of CSHLP.)* (D) $Ca^{2+}{}_{ER}$ and $Ca^{2+}{}_i$ undergo simultaneous but opposite changes in concentration during oscillations in mouse MII eggs. $Ca^{2+}{}_i$ responses were induced by injection of 0.05 µg/µl mouse PLCζ cRNA into MII eggs. In vitro transcribed D1ER cRNA was injected into eggs 5 h before the initiation of $Ca^{2+}{}_i$ measurements. The emission ratio of D1ER (YFP/CFP) was used to estimate relative changes in $[Ca^{2+}]_{ER}$ (left axis, black trace). $[Ca^{2+}]_i$ (right axis, red trace) was recorded using Rhod-2. (E) The first and second $Ca^{2+}{}_i$ rises do elicit significant refilling of the ER, but subsequent rises, especially after ~ the sixth rise (D). *Adapted from Ref. 508 with permission from The Company of Biologists.*

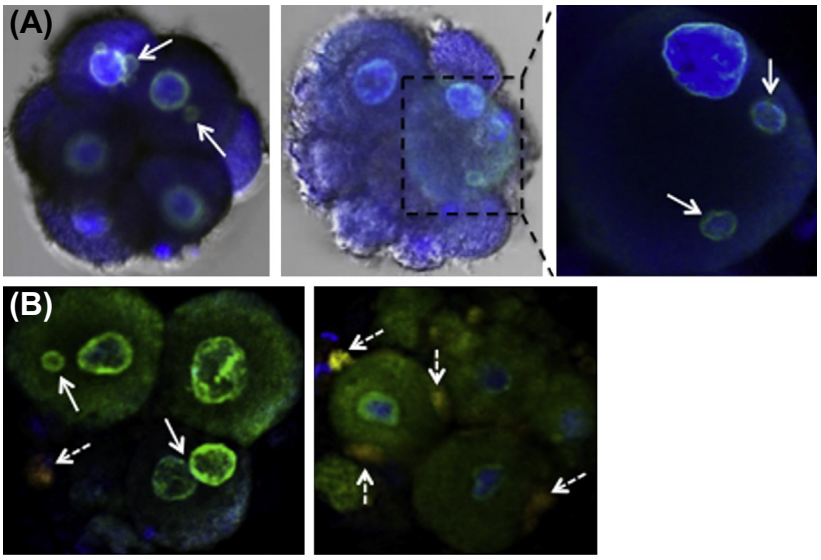

FIGURE 6.4 Detection of embryonic micronuclei in cleavage-stage human embryos. (A) Expression of the nuclear envelope marker, LAMIN-B1 (green), in 4′,6-diamidino-2-phenylindole (DAPI)-stained (blue) cleavage-stage human embryos visualized by immunofluorescent confocal microscopy and differential interference contrast (DIC) imaging at 20× (left and middle) and 40× (right) reveals the presence of several embryonic micronuclei (indicated by white solid arrows) distinct from primary nuclei in human blastomeres. (B) Similarly evaluated additional cleavage-stage human embryos with low (left) and high (right) fragmentation also immunostained for centromere protein E (CENP-E; orange) in the absence of DIC imaging identifies multiple missing chromosomes in cellular fragments (indicated by white dashed arrows) adjacent to blastomeres at this stage of development. *Source: Adapted from Ref. 16.*

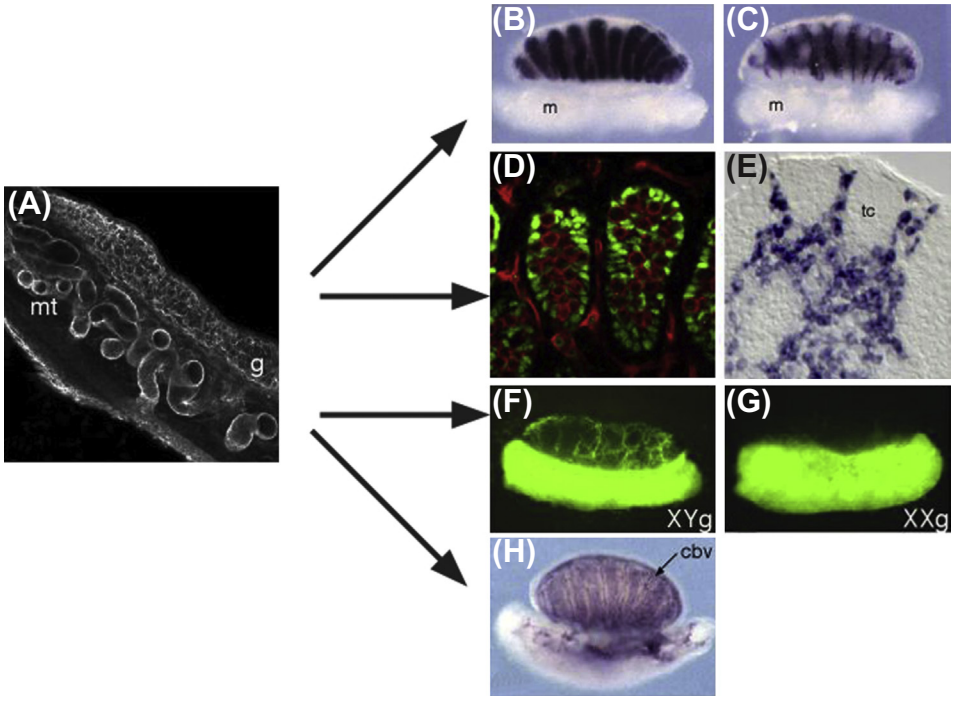

FIGURE 7.6 Differentiation of the embryonic testis from a bipotential precursor. (A) Confocal image of a gonad and mesonephros stained with an antibody to laminin at a stage before morphological changes take place (E11.5). (B–H) Morphology of the embryonic testis. (B, C) The Sertoli and Leydig cells of the E13.5 gonad are marked by whole-mount in situ hybridization for the expression of the Sertoli cell marker AMH (B) and the Leydig cell marker *Cyp11a1* (C). The structure of the testicular cords is seen in the confocal image of an E12.5 gonad that was stained with an antibody against SOX9 in green (D). Germ cells are found within the testicular cords as marked by the antibody stain for PECAM in red (D). In the interstitium, epithelial cells, as marked by the antibody stain for PECAM in red (D), and Leydig cells, as marked by the section of the whole mount in situ hybridization on a gonad from a E14 embryo for the expression of *Cyp11a1* (E), are found. (F, G) Mesonephric cell migration is observed in the XY (F) but not the XX (G) gonad, as shown by organ culture samples where the mesonephros but not the gonad is derived from transgenic mice that express GFP ubiquitously. (H) The formation of male-specific vasculature as shown by a whole-mount antibody stain to PECAM, which marks endothelial cells, of gonad and mesonephros from an E14 embryo. mt, mesonephric tubules; g, gonad; m, mesonephros; tc, testis cord; cbv, coelomic blood vessel. *Source: Adapted from Ref. 252; confocal image (D) courtesy of Blanche Capel.*

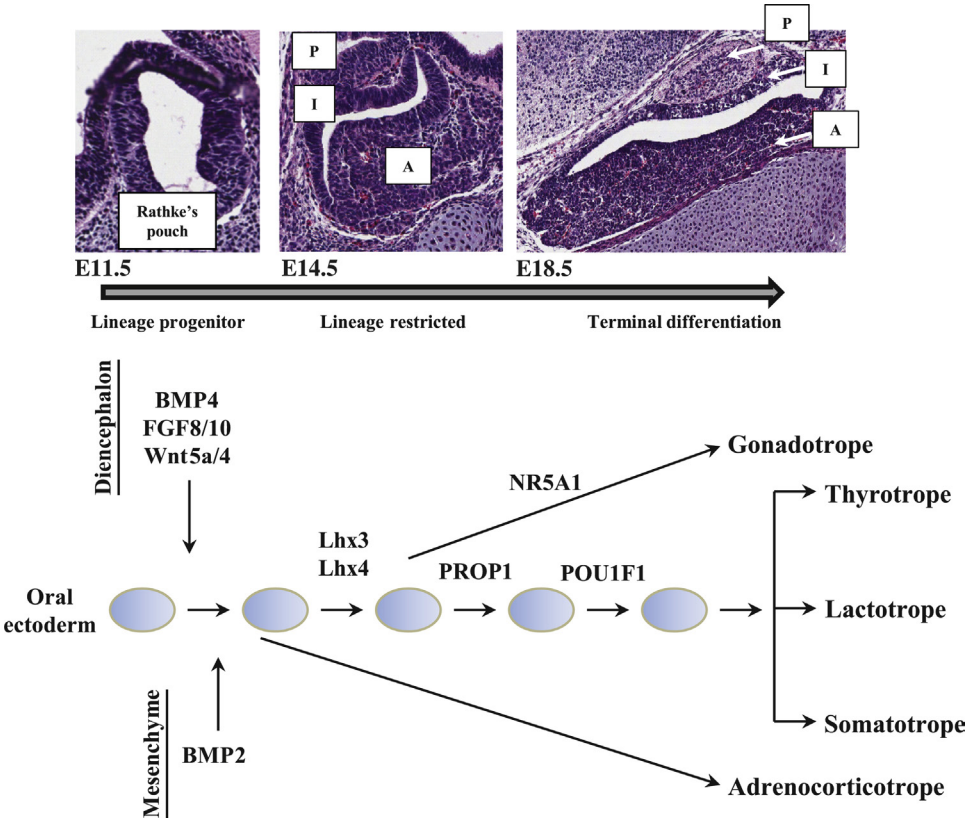

FIGURE 10.1 **Pituitary development.** The pituitary gland emerges from important interactions between the oral and neural ectoderm (diencephalon). Early expansion of the oral ectoderm appears to be functionally related to paracrine signals (such as bone morphogenic proteins (BMPs) and fibroblast growth factors (FGFs)) originating from the neural ectoderm and affecting proliferation and early differentiation of the oral ectoderm. The resulting expansion of tissue forms Rathke's pouch, the anlagen of the presumptive lineage progenitors of the anterior and intermediate lobes of the gland. In addition to signals emerging from the diencephalon, the surrounding mesenchyme provides additional paracrine signaling from BMPs, further restricting lineage identity. Differentiation of the five endocrine cell types within the anterior lobe of the pituitary is controlled by concise expression of a series of transcription factors that serve to restrict cell identity and drive terminal differentiation of the anterior pituitary. For specification of the gonadotrope cell lineage, early expression of Lhx3 (controlled by FGF signaling) and later expression of NR5A1 are central. Interestingly, Lhx3 and NR5A1 play central roles in the regulation of the four gonadotrope signature genes that define gonadotrope function in the adult animal. Sagittal tissue sections shown are from the mouse (unpublished data from M.S. Roberson and A.M. Navratil; e11.5 represents embryonic age in days post conception; P: posterior; I: intermediate; A: anterior).

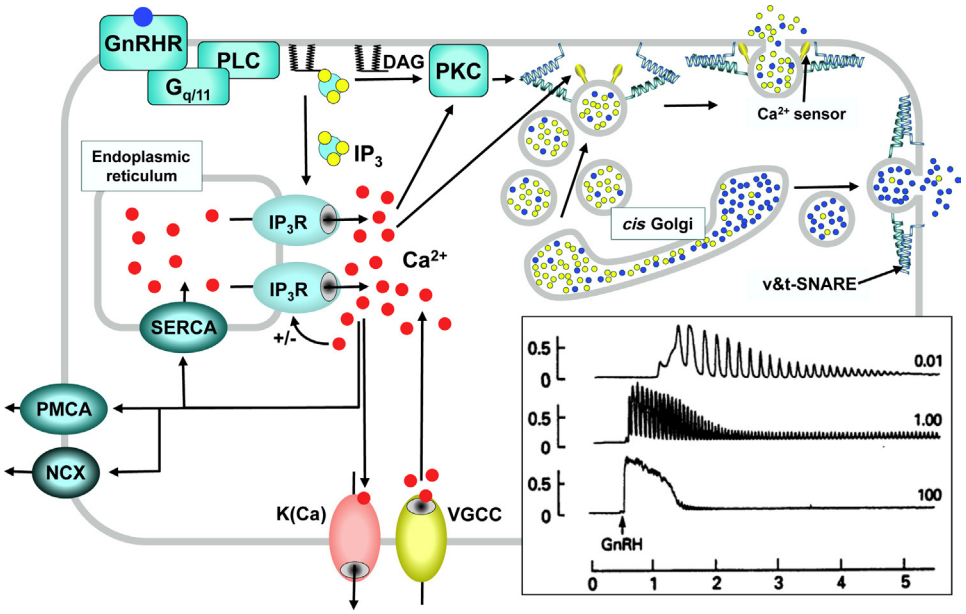

FIGURE 10.2 Acute effects of GnRH on Ca^{2+} and gonadotropin secretion. GnRH acts via Gq/11 coupled GPCRs to activate PLC, with consequent IP_3-mediated Ca^{2+} mobilization and Ca^{2+}–DAG-mediated activation of PKC. The interplay of Ca^{2+} mobilization from intracellular stores and Ca^{2+} entry across the plasma membrane increases $[Ca^{2+}]_i$. This response may be oscillatory or more sustained, often with a spike–plateau-type profile. The response elicited by GnRH varies according to cell type and stimulus intensity but is also variable from cell to cell, even within isogenic cell populations. The GnRHR-mediated increase in $[Ca^{2+}]_i$ drives the secretion of LH and FSH by regulated exocytosis, a process likely involving interaction of a vSNARE (i.e., synaptobrevin) and a t-SNARE (i.e., syntaxin) as well as regulation by a Ca^{2+} sensor (i.e., synaptotagmin). PKC can also stimulate gonadotropin secretion, acting as a positive modulator of Ca^{2+}-driven exocytosis. In addition to regulated exocytosis, gonadotropins may be secreted via the constitutive pathway. The proportion of FSH (blue circles) secreted constitutively is higher than that of LH (yellow circles), and this could reflect sorting into distinct secretory vesicles formed at the Golgi. This principle is illustrated here by the presence of a Ca^{2+} sensor on LH-enriched vesicles but not on FSH-enriched vesicles. The inset shows μM $[Ca^{2+}]_i$ in individual cells stimulated with the indicated concentration of GnRH (plotted against time in min) and is adapted from Ref. 258.

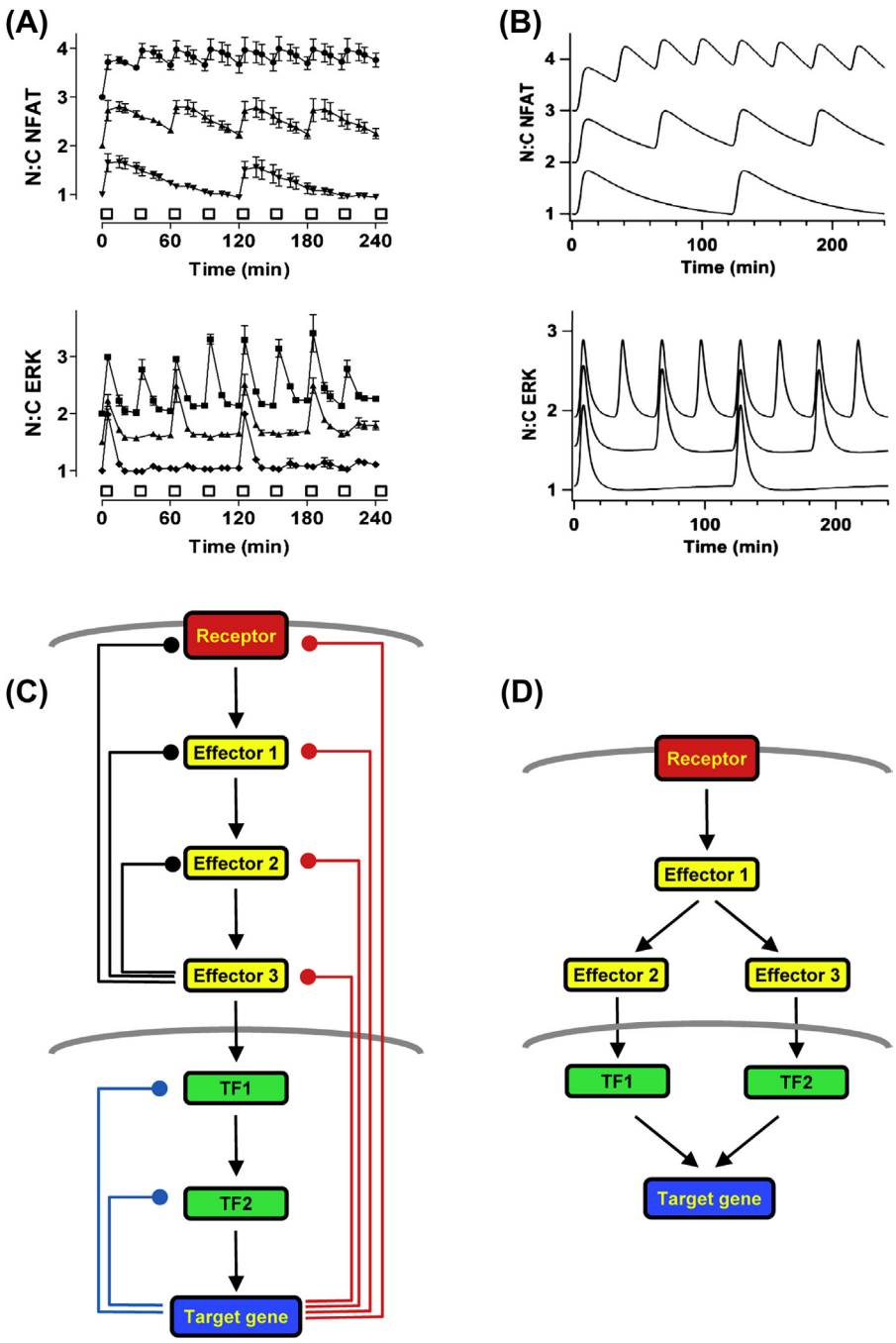

FIGURE 10.7 **GnRH pulse frequency decoding, part I.** Panel (A) shows the effects of pulsatile GnRH (1 nM pulses of 5 min duration and varied intervals) on the nuclear:cytoplasmic ratio (N:C) of NFAT2–EFP (upper chart) or ERK2–GFP (lower chart). In each case, the data are normalized to the prestimulation value and are offset on the vertical axis for clarity. Note that neither of the responses shows desensitization from pulse to pulse and that NFAT–EFP shows a cumulative sawtoothed response at the highest pulse frequency, whereas the ERK2–GFP response is not sawtoothed at any frequency (because it is rapidly reversed on removal of GnRH). Panel (B) shows data from an ordinary differential equation–based mathematical model that mirrors the empirical data for NFAT–EFP and ERK2–GFP translocation. Panel (C) shows a schematic signal transduction cascade in which a receptor in the plasma membrane causes sequential activation of three cytoplasmic proteins (effectors 1–3), and the last of these drives sequential activation of two transcription factors (TF1 and TF2) within the nucleus, which are upstream of a target gene. Also illustrated are multiple negative-feedback loops that could be restricted to the cytoplasm (black) or could involve transcription-dependent feedback to cytoplasmic proteins (red) or nuclear proteins (blue). Panel (D) shows a schematic network in which the receptor activates a bifurcating signal transduction pathway involving three cytoplasmic effectors and two nuclear transcription factors and where these transcription factors converge on the target gene. The empirical and modeled data in panels (A) and (B) suggest that the signal passing from the cytoplasm to the nucleus does not desensitize with pulsatile GnRH, arguing against the upstream negative-feedback pathways shown in black or red in panel (C). *Source: The data in panels (A) and (B) are adapted from Refs 618,620,621.*

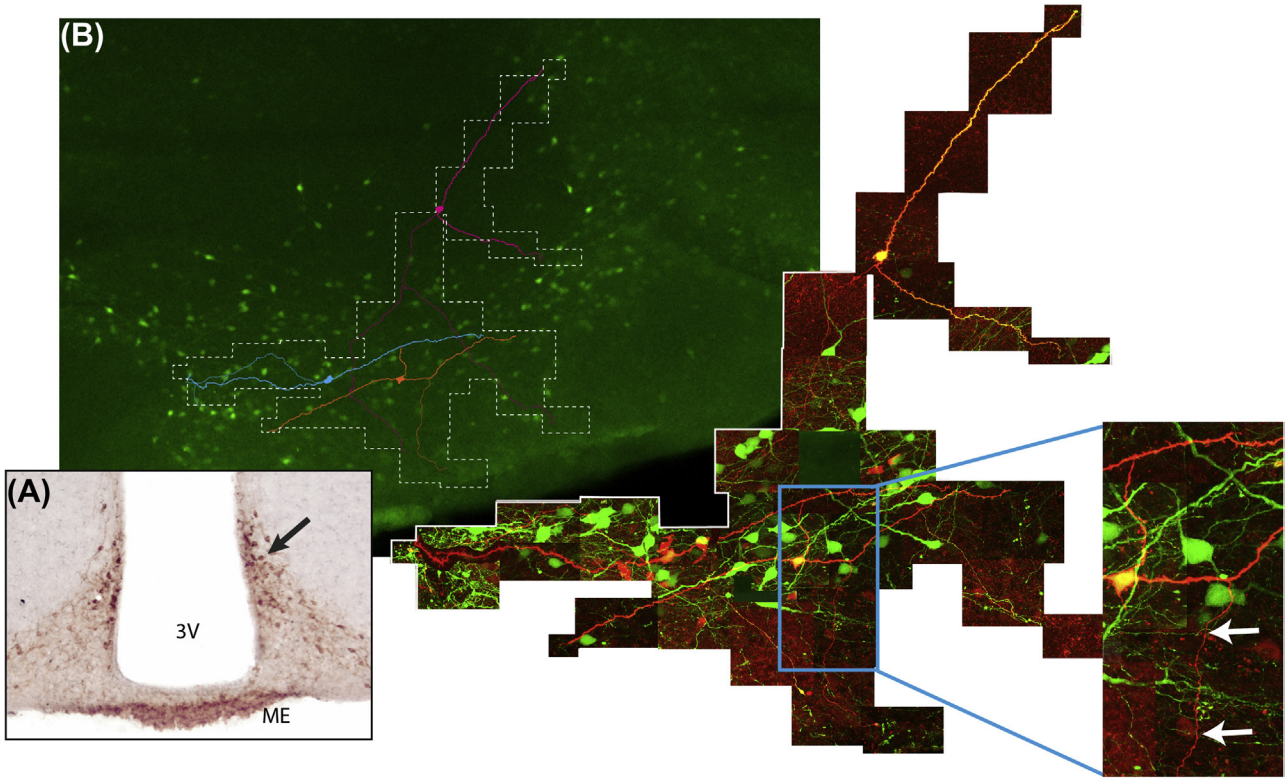

FIGURE 12.3 Neuroanatomy of the TIDA neurons. Panel A shows a coronal section through the mouse hypothalamus, with TIDA neurons identified by tyrosine hydroxylase (TH) immunoreactivity (3V: third ventricle; ME: median eminence). Note the cell bodies in the dorsomedial arcuate nucleus (arrow), as well as strong staining in the nerve terminals in the external zone of the median eminence. Panel B shows a low-power sagittal view of the hypothalamus from a mouse in which green fluorescent protein is expressed under the control of the TH promoter (green). These neurons were visualized in a brain slice, and then three individual TH-positive neurons were filled with neurobiotin (red; but pseudocolored pink, blue and orange in the low power view), allowing visualization of the full extent of the nerve processes. The montage (right) is constructed from multiple high-power views obtained using a laser confocal microscope, illustrating the dentritic tree of three filled cells extending a long distance throughout the nucleus. In the enlargement (right), note the major dendrites coming off the cell body, identified by a wide diameter and multiple spiny processes. The thin, smooth axon (arrow) arises off a primary dendrite and projects down toward the median eminence in the base of the hypothalamus. *Source: Images provided by Drs Michel Herde and Allan Herbison, based on work published in Ref. 86.*

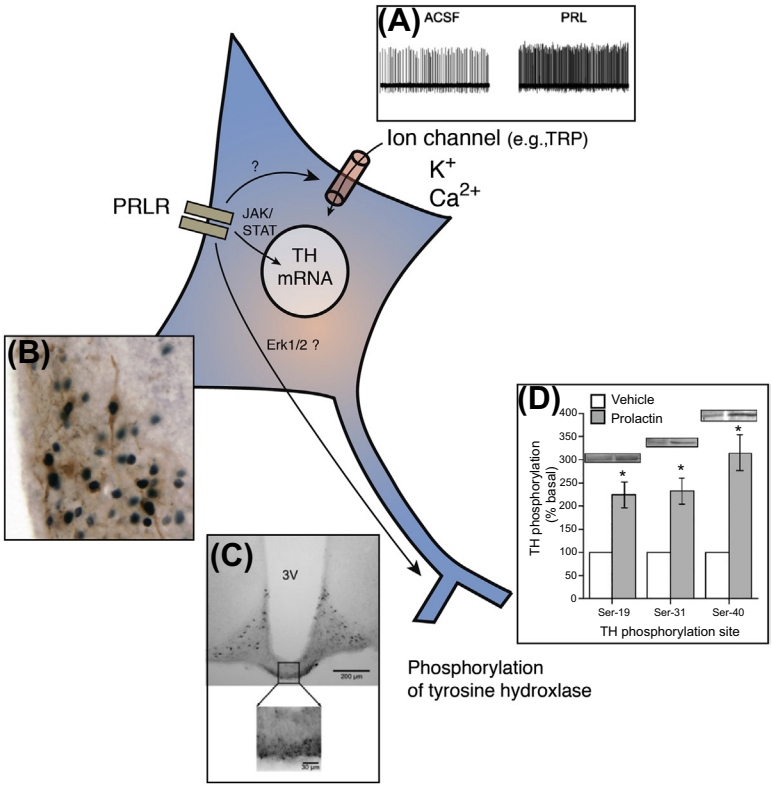

FIGURE 12.4 Diagrammatic representation of the multiple levels of prolactin feedback on NEDA neurons. Prolactin binds to prolactin receptors on NEDA neurons and then acutely (within 5 min) increases the firing rate of those neurons. Inset (A) shows prolactin-induced increase in the firing rate of a TIDA neuron.[92] Prolactin also increases the phosphorylation of STAT5 in TIDA neurons (approximately 15–20 min), inducing a transcriptional response. Inset (B) shows prolactin-induced STAT5 immunoreactivity (black) co-localized with immunoreactivity for tyrosine hydroxylase (TH) (brown), demonstrating direct prolactin action in these cells.[91] Finally, prolactin induces the phosphorylation of TH (approximately 15–30 min) both within the cell body and, more particularly, in the nerve terminals within the median eminence. Inset (C) shows prolactin-induced serine 40 phospho-TH immunoreactivity in the arcuate nucleus and median eminence (unpublished data); inset (D) shows prolactin-induced phosphorylation of TH at serines 19, 31, and 40 in mediobasal hypothalamic cultures.[102] ? indicates an uncertain mechanism; **P* <0.05.

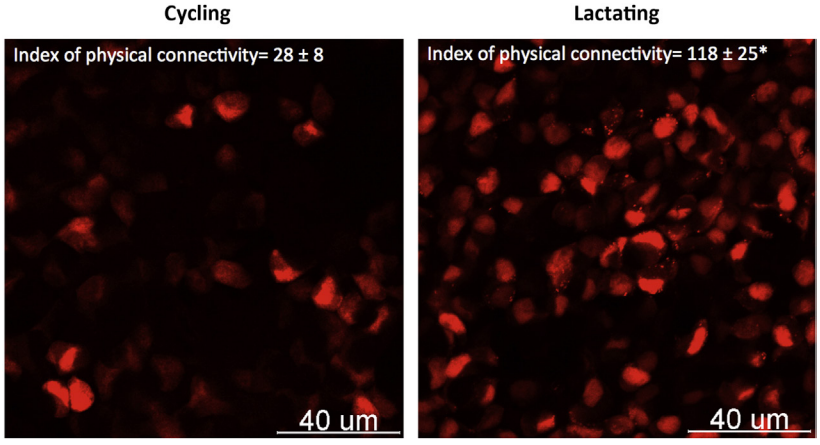

FIGURE 12.5 Biphoton imaging of pituitaries from virgin (left panel) and lactating (right panel) mice with transgenic expression of DsRedExpress fluorescent protein identifying lactotroph cells. In the virgin pituitary, lactotrophs are organized into a network of honeycomb-like structures. In the lactating pituitary, these honeycomb structures become more pronounced, and there is an overall increase in the proportion of lactotrophs with homotypic contacts. The "index of physical connectivity" is generated by three-dimensional reconstructions of the fluorescent label and reflects the degree of structural connections of lactotrophs with other lactotrophs. ? indicates an uncertain mechanism; **P* <0.05. *Source: Image courtesy of Chrystel Lafont, Taoufik El Yandouzi, and Patrice Mollard, Montpellier, France, based on work published in Refs 327,340.*

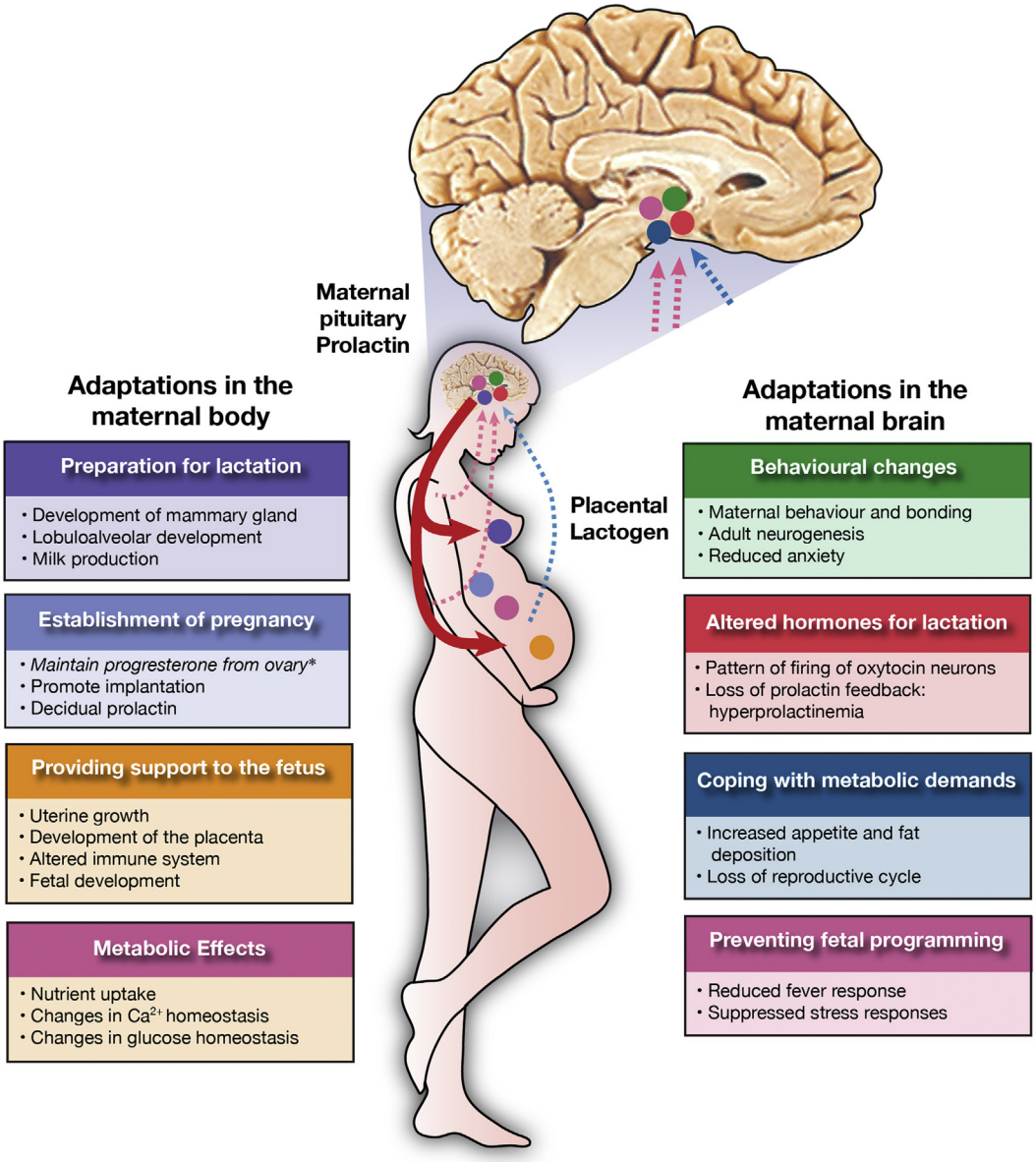

FIGURE 12.8 **Summary of adaptive responses in the pregnant female that may be induced or regulated by prolactin and/or placental lactogen.** Note that while this figure shows a human pregnancy, most of the data are based on experimental data from laboratory animals, and some have not yet been confirmed in women. *Those shown in italics are known to occur only in rodents.

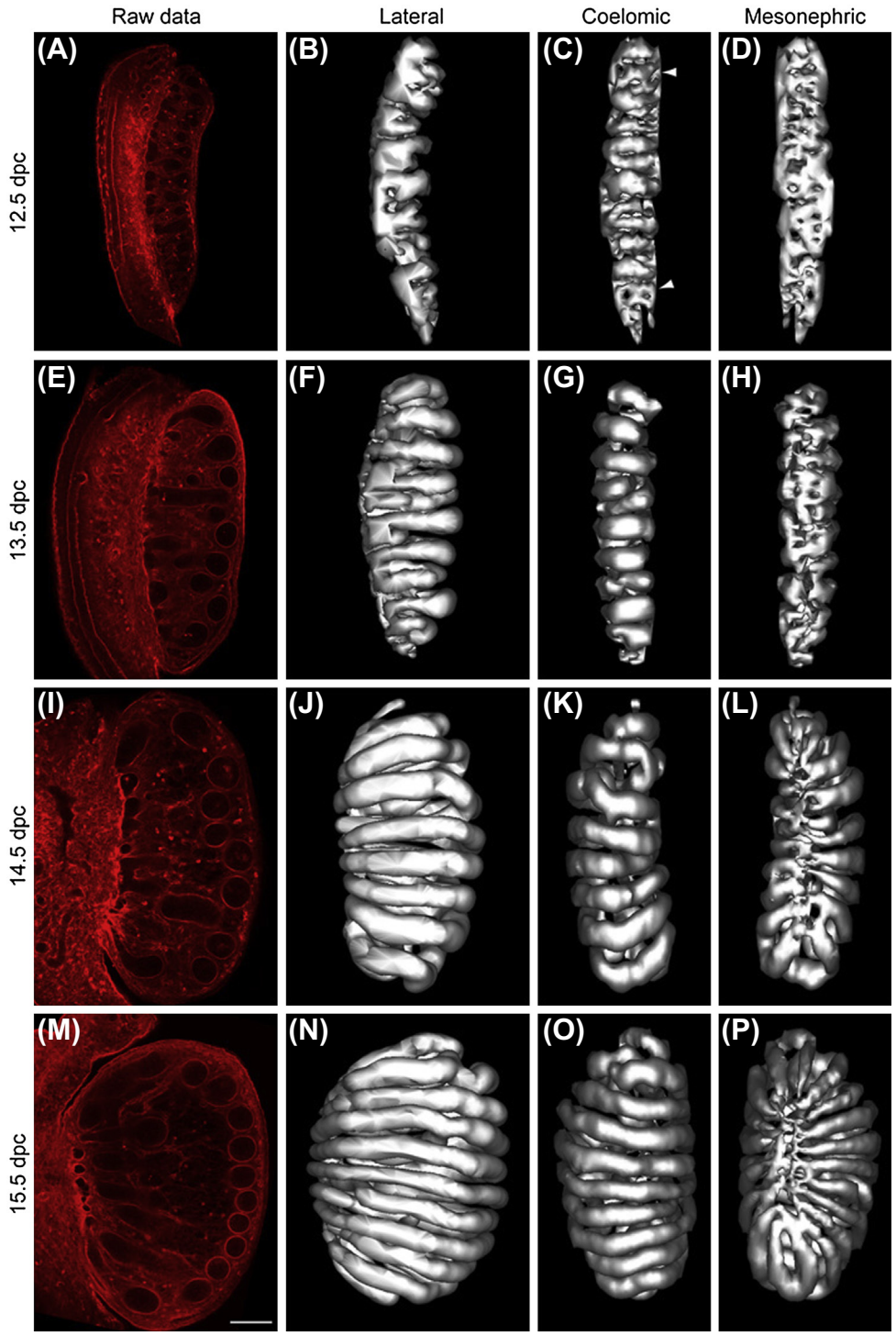

FIGURE 14.2 Modeling of testicular cord development in the mouse. This figure is taken from Ref. 9 and shows lateral (B, F, J, N), coelomic (C, G, K, O), and mesonephric (D, H, L, P) views of models generated from testis cord outlines prepared from whole mount testis. The left panel in each row (A, E, I, M) is a representative image from the data set used to compile the models. The results show that cords initially form as a network of irregular cell clusters that are subsequently remodeled to form regular parallel loops, joined by a flattened plexus at the mesonephric side. Branched, fused, and internalized cords are commonly observed. Parts A–D, E–H, I–L and M–P show models of testicular development at 12.5, 13.5, 14.5 and 15.5 dpc, respectively. Scale bar, 100 μm. *Source: Modified from Ref. 9.*

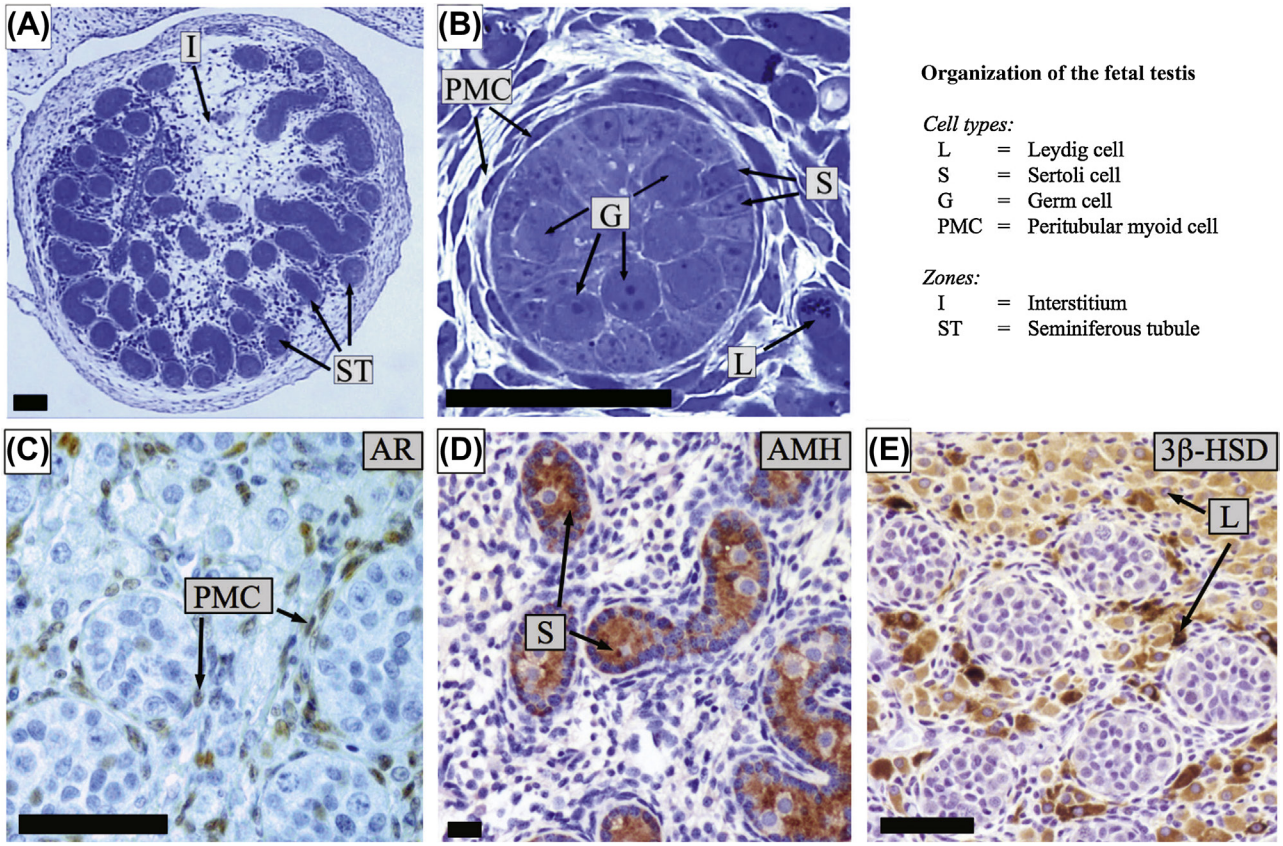

FIGURE 14.3 Histology of the fetal testis. (A) Fetal mouse: low magnification of a semi-thin section showing seminiferous tubules and interstitial tissue (I). (B) Fetal mouse: higher magnification of (A) showing gonocytes (G) in the central part of the sex cord with the Sertoli cells (S) around the periphery. The peritubular myoid cells (PMC) form a concentric layer around the cord, and Leydig cells (L) are present within the interstitium. (C) Fetal human: immunohistochemically labeled for the androgen receptor (AR), which is clearly expressed in PMC and in some interstitial cells. (D) Fetal sheep: immunohistochemically labeled for anti-Mullerian hormone (AMH), which is strongly expressed in fetal Sertoli cells. (E) Fetal human: immunohistochemically labeled for 3β-hydroxysteroid dehydrogenase (HSD3B), which is localized exclusively in the Leydig cells. In all photomicrographs, the bar represents 50 μm. *Source: Reproduced from Ref. 11. © Society for Reproduction and Fertility (2011). Reproduced by permission.*

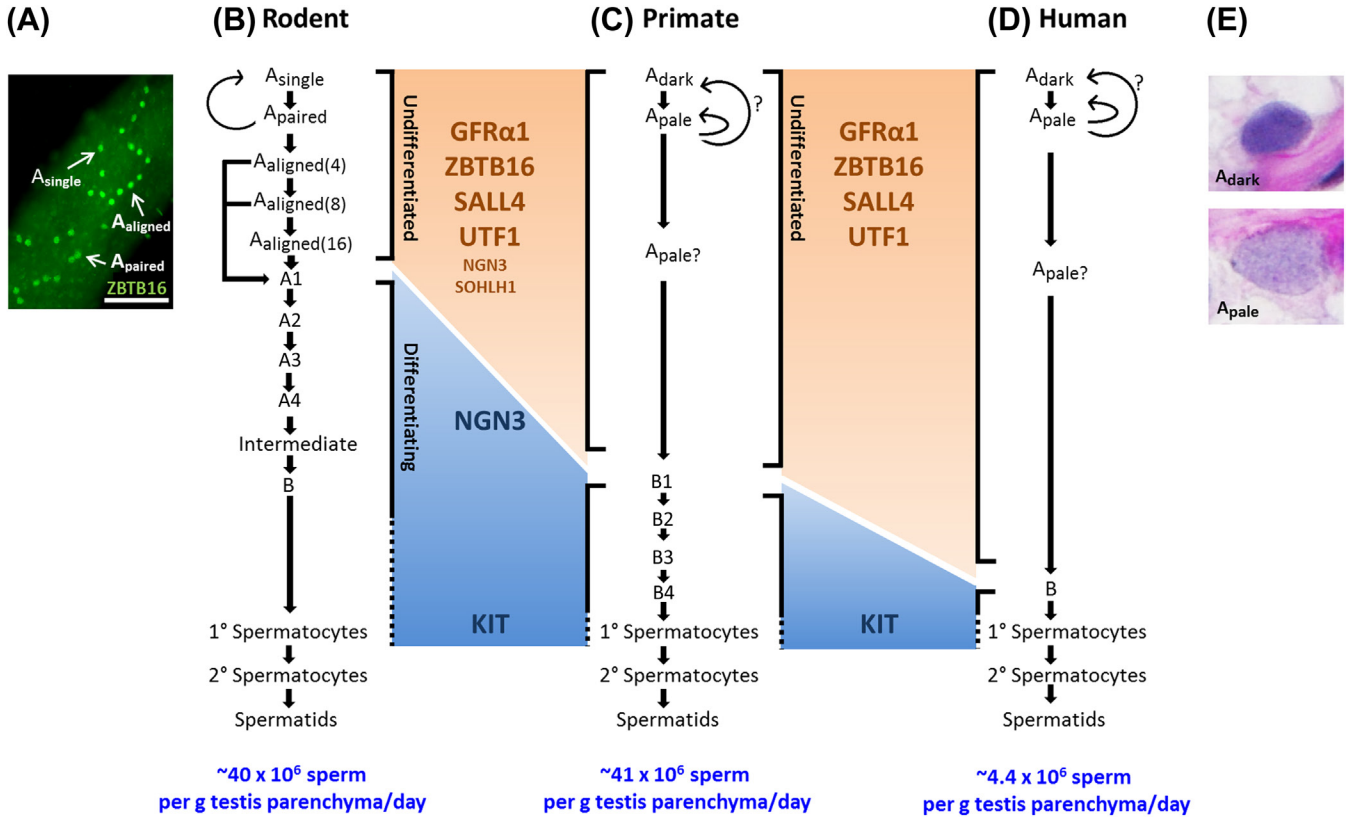

FIGURE 15.2 Current model of rodent, nonhuman primate, and human spermatogenesis. (A) Whole-mount immunohistochemistry staining for ZBTB16 in adult mouse seminiferous tubules. ZBTB16^{+} spermatogonia are identified as A_{single}, A_{paired} or $A_{aligned}$. Scale bar = 100 μm. (B) Rodent undifferentiated spermatogonia, including the SSC pool, is comprised of A_{single} and some A_{paired} spermatogonia and based on whole-mount staining analysis their phenotype is of GFRα1^{+}, ZBTB16^{+}, SALL4^{+}, UTF1^{+}, NGN3$^{+/-}$, and KIT^{-}. Transit amplifying progenitors include some A_{paired} spermatogonia and $A_{aligned}$ spermatogonia (chains of 4–16 cells), with a phenotype of GFRα1^{+}, ZBTB16^{+}, SALL4^{+}, UTF1^{+}, NGN3$^{+/-}$, and KIT$^{+/-}$. The differentiating spermatogonia that are made up of A1–A4, intermediate, and B spermatognia have a phenotype of GFRα1^{-}, ZBTB16^{-}, SALL4^{-}, UTF1^{-}, NGN3$^{+/-}$, and KIT^{+}. (C and D) In nonhuman primate and human testis, the undifferentiated spermatogonia are the type A spermatogonia that are designated A_{dark} and A_{pale} based on nuclear staining intensity with hematoxylin. The B spermatogonia are considered to be the differentiating spermatogonia and in nonhuman primates; they go through four divisions before producing primary spermatocytes, whereas in human there is only one division of B spermatogonia. (E) Sections of human testis stained using periodic acid-Shiff method and counterstained with hematoxylin to show nuclear morphology. ZBTB16, zinc finger and BTB domain containing 16; GFRα1, GDNF family receptor alpha-1; UTF1, undifferentiated embryonic cell transcription factor 1; SALL4, Sal-like 4; NGN3, neurogenin 3; SOHLH1, spermatogenesis and oogenesis specific helix-loop-helix 1. *Adapted from Ref. 75.*

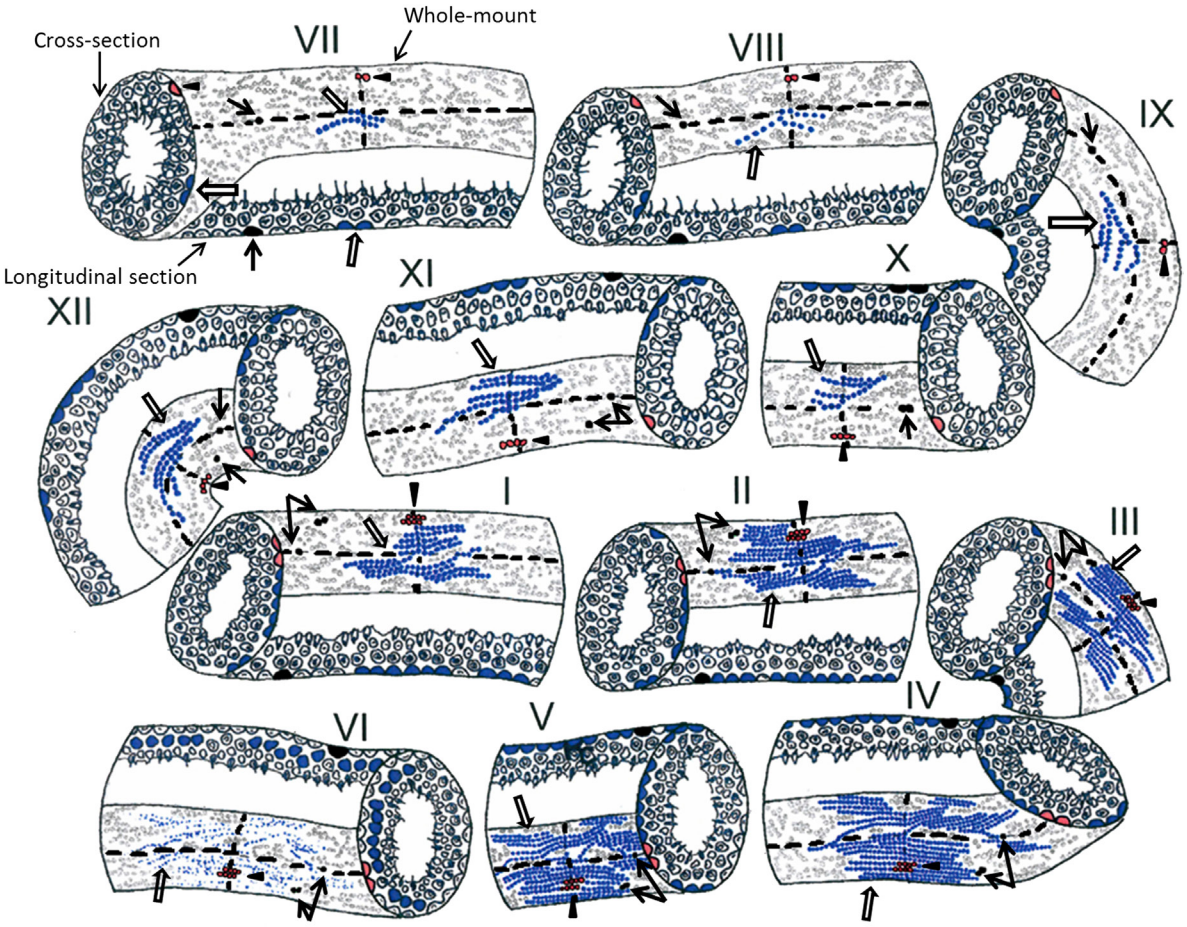

FIGURE 15.3 Schematic of mouse spermatogenic clone development by stage. The seminiferous epithelial cycle of the mouse contains 12 stages (I-XII). Each stage is shown in cross-sectional, longitudinal, and whole-mount perspectives (labeled in stage VII). Three putative spermatogonial clones are highlighted in blue (open arrow), red (arrowhead), and black (black arrow). These clones are only representative examples and do not imply A_{single} spermatogonia divide only in stages X and I. A_{single} and A_{paired} spermatogonia are present in all stages of the seminiferous epithelium and tend to divide more frequently in early and late stages than middle stages. The dotted lines in the whole-mount perspective indicate planes of the cross-section and longitudinal section views. Cells that lie on the focal plane in the whole mount appear in the corresponding cross-section and longitudinal section views; this is indicated by matching color or arrows (arrows shown for stage VII only). For example, in stage VII, the red cell (arrowhead) is in the vertical line and therefore appears in the cross-sectional view. A black cell (black arrow) is in the horizontal line, so it is observed in the longitudinal section view. The development of three putative clones (blue/open arrow, red/arrowhead and black/black arrow) through one cycle of the seminiferous epithelium is shown. Stage VII: $A_{al\text{-}16}$ (blue/open arrow); A_{pair} (red/arrowhead); A_{single} (black/black arrow); stage VIII: A1 (clone of 16) (blue/open arrow); A_{pair} (red/arrowhead); A_{single} (black/black arrow); stage IX: A2 (clone of 32) (blue/open arrow); A_{pair} (red/arrowhead); A_{single} (black/black arrow); stage X: A2 (clone of 32) (blue/open arrow); $A_{al\text{-}4}$ (red/arrowhead); A_{pair} (black/black arrow); stage XI: A3 (clone of 64) (blue/open arrow); $A_{al\text{-}4}$ (red/arrowhead); A_{single} (x2) (black/black arrow); stage XII: A3 (clone of 64) (blue/open arrow); $A_{al\text{-}4}$ (red/arrowhead); A_{single} (x2) (black/black arrow); stage I: A4 (clone of 128) (blue/open arrow); $A_{al\text{-}8}$ (red/arrowhead); A_{single} and A_{pair} (black/black arrow); stage II: intermediate spermatogonia (clone of 256) (blue/open arrow); $A_{al\text{-}8}$ (red/arrowhead); A_{single} and A_{pair} (black/black arrow); stage III: intermediate spermatogonia (clone of 256) (blue/open arrow); $A_{al\text{-}8}$ (red/arrowhead); A_{single} and A_{pair} (black/black arrow); stage IV: Type B spermatogonia (clone of 512) (blue/open arrow); $A_{al\text{-}8}$ (red/arrowhead); A_{single} and A_{pair} (black/black arrow); stage V: Type B spermatogonia (clone of 512) (blue/open arrow); $A_{al\text{-}8}$ (red/arrowhead); A_{single} and A_{pair} (black/black arrow); stage VI: primary spermatocytes (lifting off the basement membrane) (blue/open arrow); $A_{al\text{-}8}$ (red/arrowhead); A_{single} and A_{pair} (black/black arrow). *Reproduced with permission from Ref. 28.*

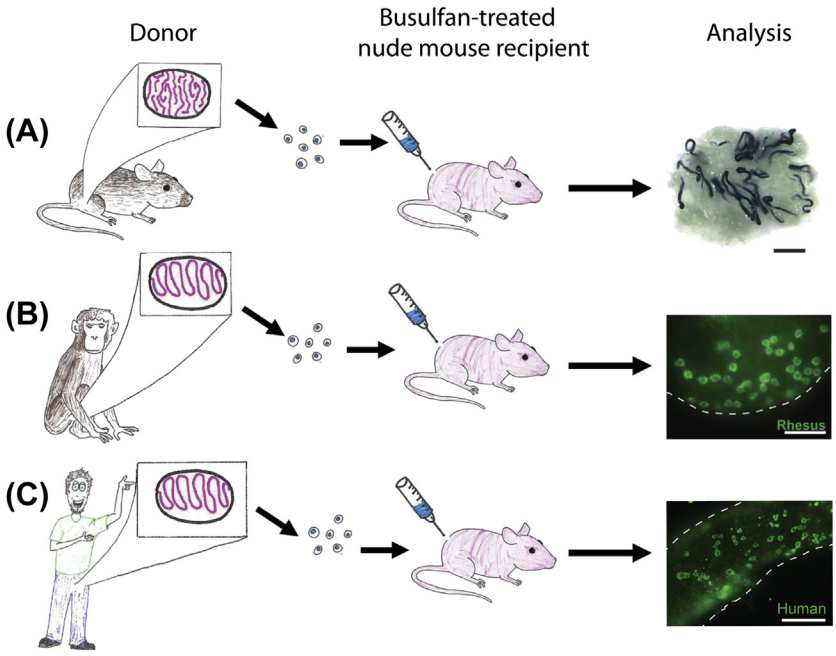

FIGURE 15.6 Spermatogonial stem cell (SSC) transplantation assay. The only way to definitively identify SSCs is to analyze SSC function. (A) In this case, a testicular cell suspension is produced from a *lacZ* donor mouse. The cells are injected into the testis of an infertile recipient mouse and the results are analyzed two to three months after transplantation for *lacZ* expressing donor spermatogenesis (dark colonies). Scale bar = 2 μm. (B, C) Xenotransplantation of nonhuman primate (B) and human (C) testicular cells into busulfan treated nude mouse can be used as an assay to identify SSCs by their ability to initiate the early steps of spermatogenesis in the mouse testis. The colonies of spermatogonia are identified by staining with anti-primate antibody that only recognizes primate cells. The colonies of SSCs are identified by the following criteria—they are located on the basement membrane of the seminiferous tubules, they have four or more cells, and the cells are within 100 μm of each other. Scale bars = 100 μm. In the B and C analysis panel, broken lines indicate the borders of seminiferous tubules.

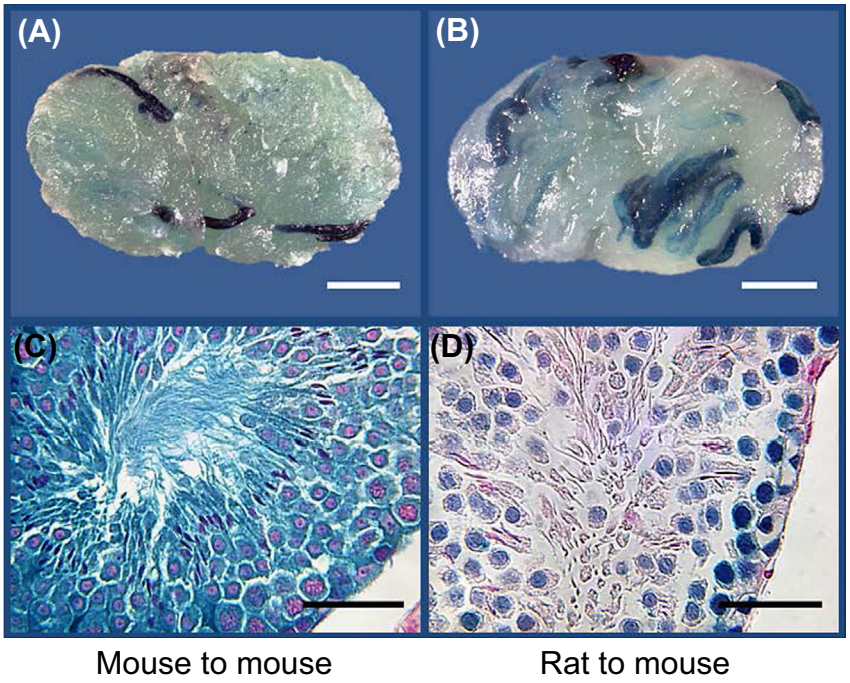

FIGURE 15.7 Macroscopic and histologic appearance of recipient testes transplanted with mouse and rat adult donor testis cells expressing *lacZ*. NCr nude (spontaneous mutant T cell deficient mice) recipient mouse testes were stained with X-gal 3 months after transplantation to visualize mouse (A and C) and rat (B and D) donor-derived spermatogenic colonies. Both mouse and rat donor testis cells generated complete spermatogenesis, characterized by multiple germ cell layers and mature spermatozoa, in recipient testes. Note that the lacZ stain is localized in the cytoplasm of ROSA26 mouse testis cells (C) and the nucleus of MT-lacZ rat testis cells (D). Nuclear fast red counterstain (C and D). Bar = 1 mm (A and B), 40 μm (C and D). *Reproduced with permission from Ref. 134.*

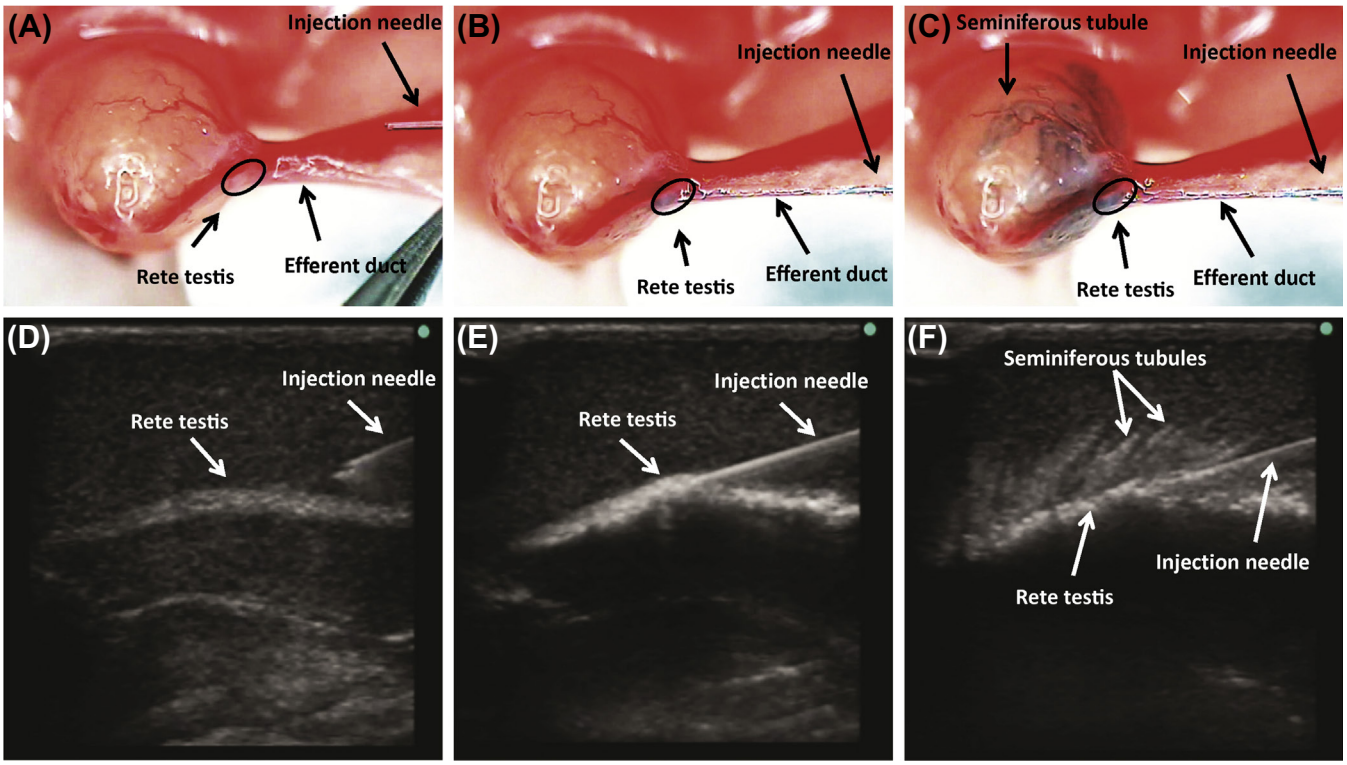

FIGURE 15.8 Testicular cell transplantation. (A–C) In rodents, the testicular cells are injected via the efferent ducts into the rete testis space, which can be visualized on the surface of the testis and is contiguous with all seminiferous tubules. (C) Trypan blue is injected with the testicular cells to visualize the filling of the seminiferous tubules. (D–F) Testis anatomy in large animals is different than rodents, with the rete testis being more centrally localized and therefore more difficult to visualize and access. Therefore, ultrasound is used to guide injections. (D) Rete testis (echo-dense structure) is visible on ultrasound. The injection needle is inserted under ultrasound guidance through the scrotal skin into the rete testis space, which is continuous with the seminiferous tubules. (E) Positive pressure is applied to the needle, so the cells are slowly injected into the rete testis and seminiferous tubules. (F) The filling of the seminiferous tubules is observed using microbubbles.

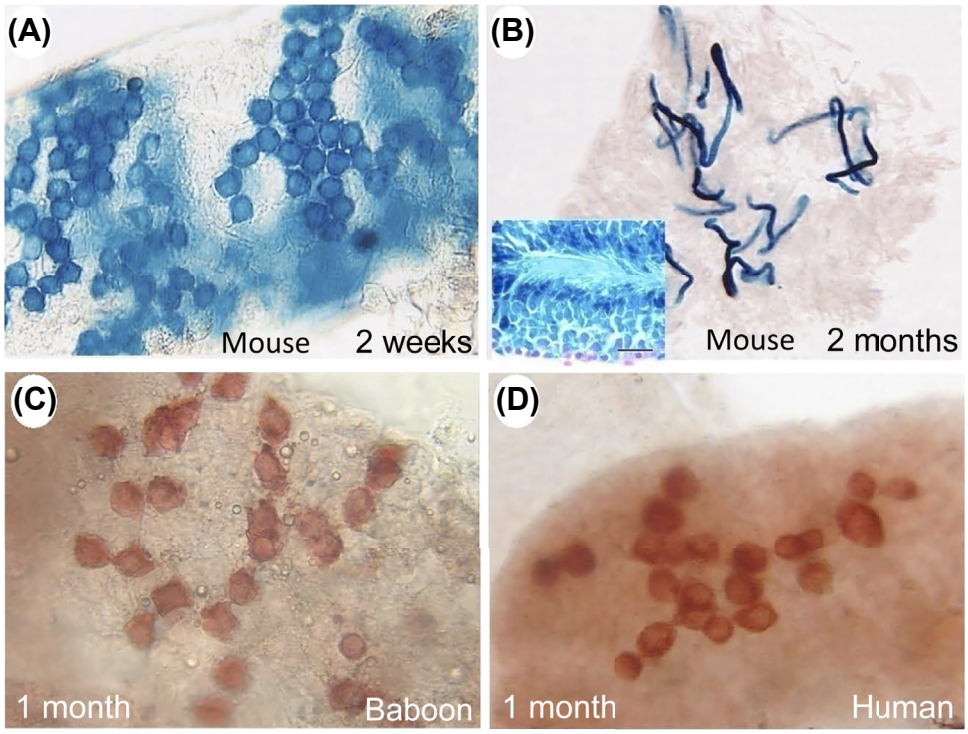

FIGURE 15.9 Colonization of recipient testis. (A) Two weeks after transplantation of transgenic mouse donor cells (stained blue) into a recipient mouse seminiferous tubules, small groups of cells can be seen at low magnification. (B) Two months after the transplantation of mouse donor cells, the blue areas are dark in the center, indicating multiple layers of germ cells and development of spermatogenesis. (B inset) Cross-section of a transplanted mouse seminiferous tubule 2 months after transplant. Scale bar = 30 μm. (C and D) Detection of baboon and human germ cells transplanted into the seminiferous tubules of recipient mouse, using an antibody raised against baboon testis cells. One month after the transplant, clusters of baboon (C) and human (D) germ cells are located on the basement membrane of mouse tubules and exhibit characteristics of undifferentiated spermatogonia. The clusters are similar to the ones seen in mouse 2 weeks after the transplant. *Modified with permission from Refs 104 and 149.*

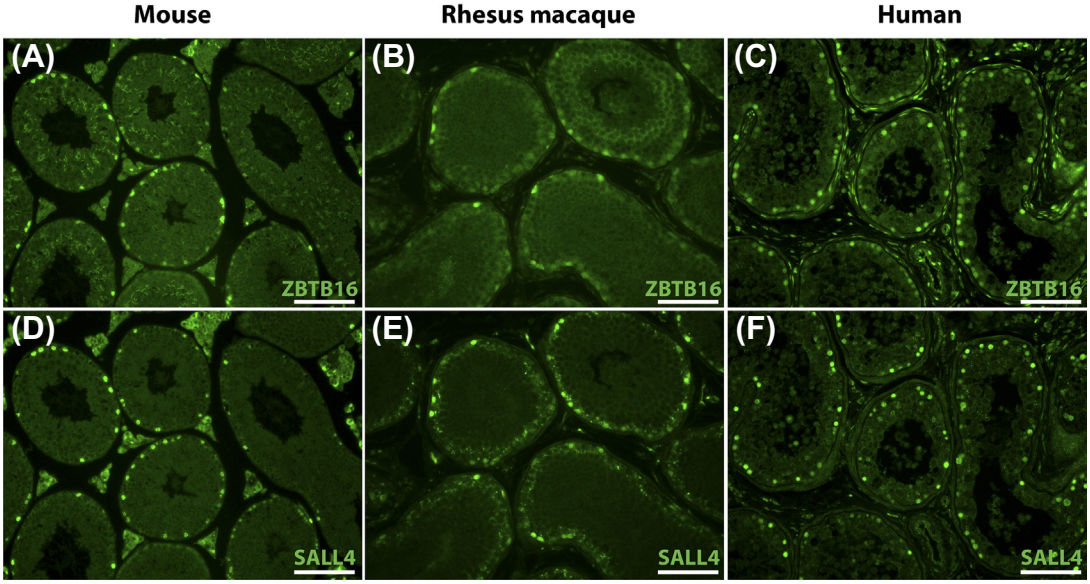

FIGURE 15.10 Conservation of stem and progenitor markers ZBTB16 and SALL4 from mouse to human. ZBTB16 (A–C) and SALL4 (D–F) staining in cross-sections of seminiferous tubules from mouse, rhesus macaque, and human testes shows expression of these transcription factors on the basement membrane of the tubule. Both ZBTB16 and SALL4 are conserved markers from mouse to rhesus macaque to human. Scale bar = 100 μm. ZBTB16, zinc finger, and BTB domain containing 16; SALL4, Sal-like 4.

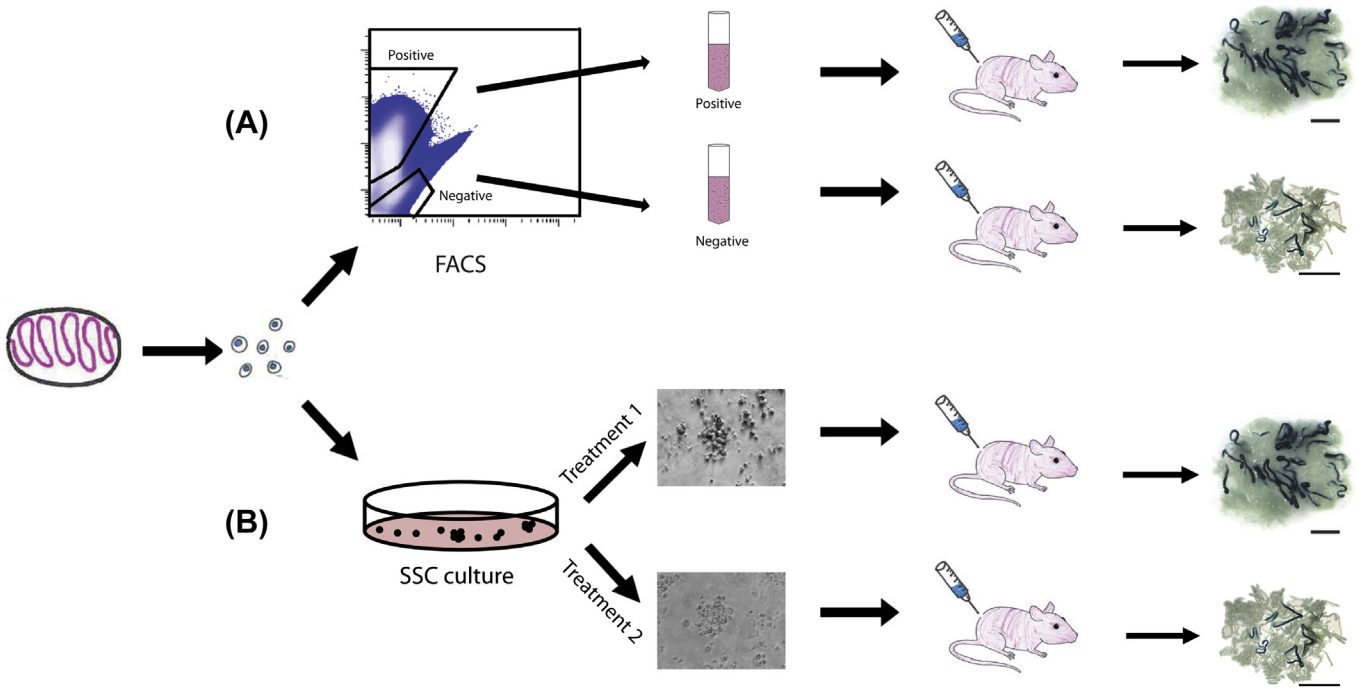

FIGURE 15.11 Experimental designs to study seprmatogonial stem cells. SSC transplantation is used to analyze the effectivness of (A) fluorecsence-activated cell sorting (FACS) for a cell-surface protein or (B) a treatment of SSC culture. (A) FACS is used to fractionate the testicular cell population stained with an antibody against a cell-surface protein, based on marker$^+$ and marker$^-$ status. The sorted fractions can then be transplanted into infertile nude mice to determine which fraction contains the SSCs. (B) SSC cultures are used to identify factors important for SSC self-renewal and maintenance or for SSC differentiation. For example, GDNF can be removed from the culture media to determine whether or not it has an effect on SSC self-renewal and/or maintenance. The cells cultured with or without GDNF are then transplanted into testis of infertile recipient mice and, 2 months later, the testis are analyzed for colony formation. FACS, fluorescence activated cell sorting; SSC, spermatogonial stem cell; GDNF, glial cell-derived neurotrophic factor.

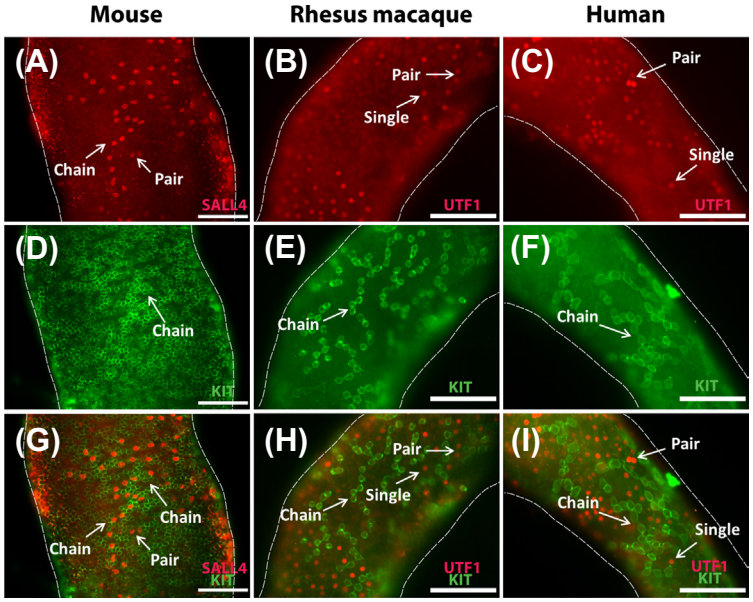

FIGURE 15.12 Whole-mount immunohistochemistry of seminiferous tubules from mouse, rhesus macaque, and human testes. Whole-mount immunofluorescence analysis of the undifferentiated spermatogonia marker, SALL4 (A), differentiating spermatogonia marker, KIT (D) and co-staining of SALL4 and KIT (G) in mouse. Whole-mount immunofluorescence analysis of undifferentiated spermatogonia marker UTF1 (B, C), differentiating spermatogonia marker KIT (E, F), and co-staining of UTF1 and KIT (H, I) in the rhesus macaque (B, E, H) and human (C, F, I). Arrows indicate spermatogonia arranged as single cells, pairs, and larger chains for each species. Scale bar = 100 μm. SALL4, Sal-like 4; UTF1, undifferentiated embryonic cell transcription factor 1.

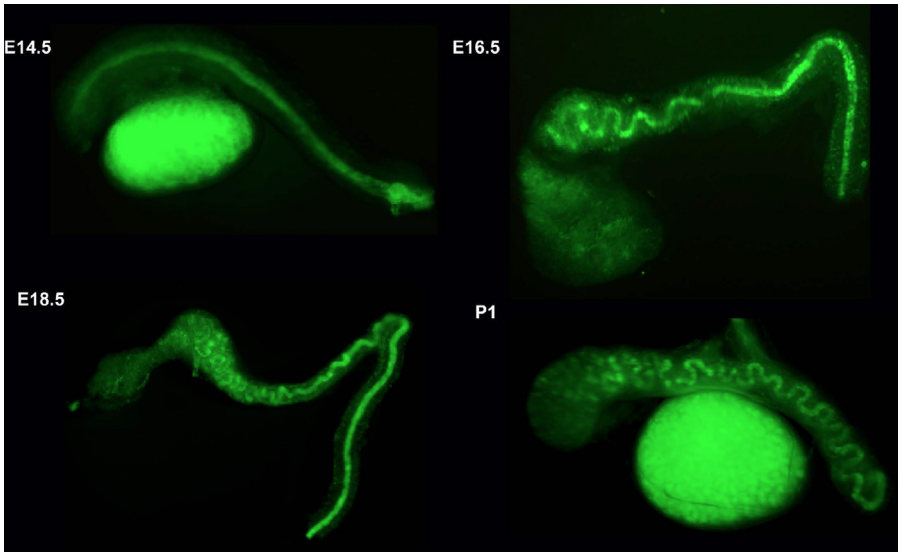

FIGURE 17.2 Wolffian ducts collected from hoxb7-gfp mice at different developmental stages, embryonic (E) days 14.5, 16.5, 18.5 and postnatal (P) day 1. Note how the coiling proceeds from proximal to distal with three-dimensional coiling beginning around 18.5. *Source: Image from Ref. 60.*

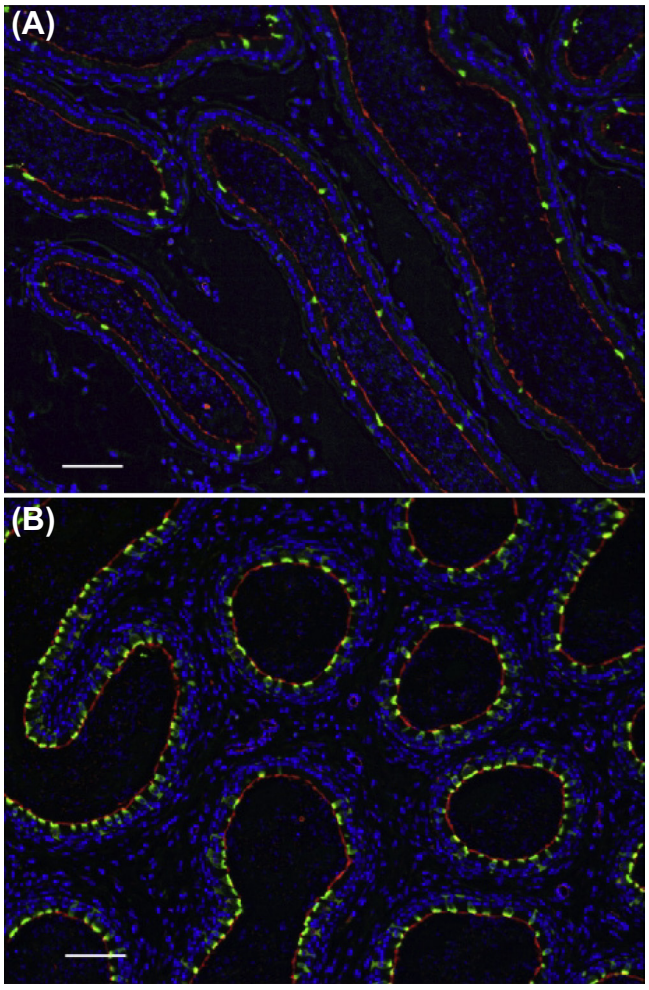

FIGURE 17.8 Relative numbers of clear cells in the rat caput (A) versus cauda (B) epididymidis. Rat epididymis was stained for the V-ATPase B1 subunit (green) to label clear cells, and NHERF1 (red). Nuclei and spermatozoa were stained with DAPI (blue). NHERF1 is located in the apical membrane of both principal cells and clear cells. Clear cells positive for the subunit B-1 or V-ATPase are much more numerous in the cauda (B) than in the caput (A) epididymidis. Scale bars, 50 μm. *Source: Reproduced with permission from Ref. 164.*

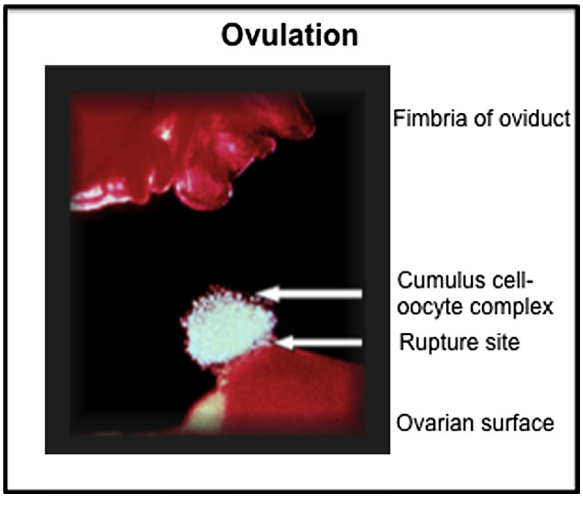

FIGURE 22.1 Moment of rupture of a rabbit follicle. Ovulation of a fertilizable oocyte involves disintegration of the ovarian surface epithelium and extrusion of the expanded cumulus cell–oocyte complex (COC) through the ovulation pore. This is followed by retrieval of the COC by the ciliated cells of the oviduct and its subsequent transport down the oviduct where fertilization occurs. (See text.) *Source: Ref. 3.*

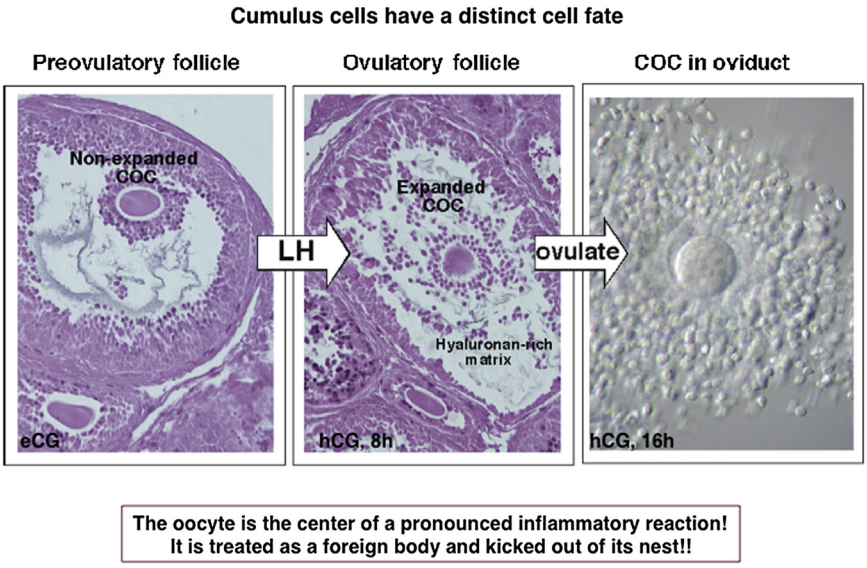

FIGURE 22.3 **The LH surge induces expansion of the cumulus cell-oocyte complex (COC).** Expansion is dependent on the biosynthesis of long polymers of hyaluronan by HAS2. The hyaluronan polymers are stabilized by specific hyaluronan-binding factors including the proteoglycan versican and inflammatory-related factors such as TNFAIP6, IαI, and PTX3. COC expansion is obligatory for release of the enclosed oocyte from the surface of the ovary into the oviduct. (See text.)

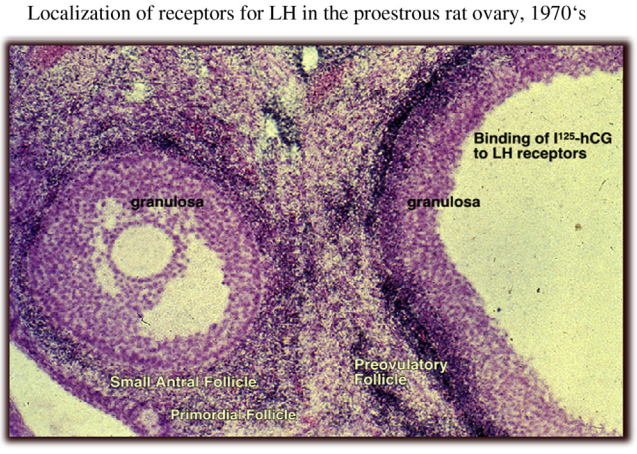

FIGURE 22.5 **Induction of LH receptors (LHR; LHCGR) in granulosa cells is the key distinguishing hallmark of preovulatory follicles.** The presence of LHR in granulosa cells of preovulatory follicles permits them (but not small follicles lacking LHR in granulosa cells) to respond to the LH surge and initiate the molecular programs required for ovulation, COC expansion, resumption of meiosis, and luteinization. This image shows the uptake of radiolabeled hCG (LH) into the theca cells and mural granulosa cells of a preovulatory follicle (right) but only in theca cells of small follicles (left). Primordial follicles do not exhibit detectable uptake of the hormone. Uptake reflects the presence of receptors.

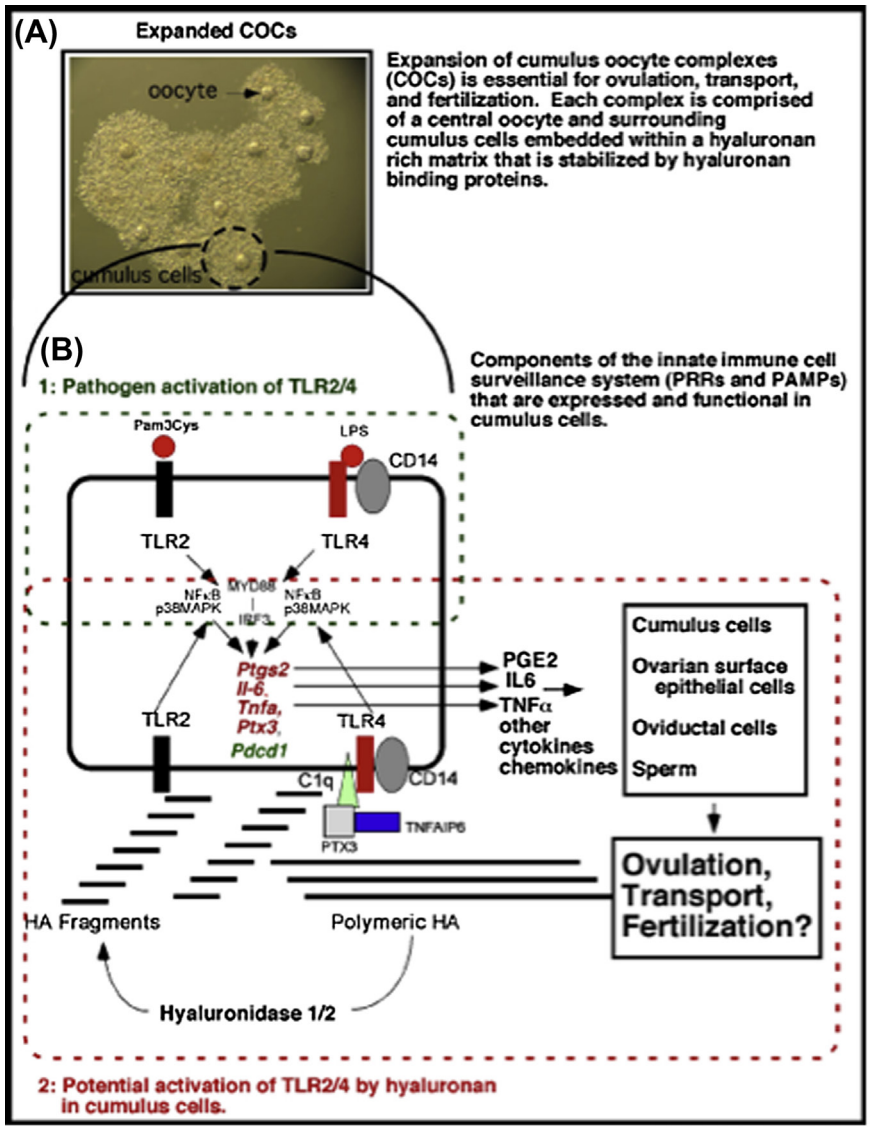

FIGURE 22.12 Schematic of TLR pathway activation and functions in cumulus cells during COC expansion, ovulation, transport and fertilization. (A) In response to the ovulatory surge of LH, cumulus cell–oocyte complexes (COCs) undergo expansion. This process requires the synthesis of a hyaluronan (HA)-rich matrix and factors that bind HA to stabilize the matrix. This process is critical for ovulation. (B) Cumulus cells express members of the Toll-like receptor (TLR) superfamily and can respond to specific ligands (1: pathogen-derived or 2: matrix-derived), leading to the induction of inflammation- and innate immune-related genes. These include: *Ptgs2, Il6, Tnfaip6, Tnfa*, and *Pdcd1*. Prostaglandins (PGE2) synthesized by PTGS2, IL6, and TNFα, as well as other cytokines and chemokines, are released from cumulus cells and can impact the function not only of the cumulus cells but also oviductal cells during transport and sperm during fertilization. The degradation of polymeric HA by hyaluronidases is presumed to lead to the generation of HA fragments that activate TLR2 and TLR4. *Source: Ref.160.*

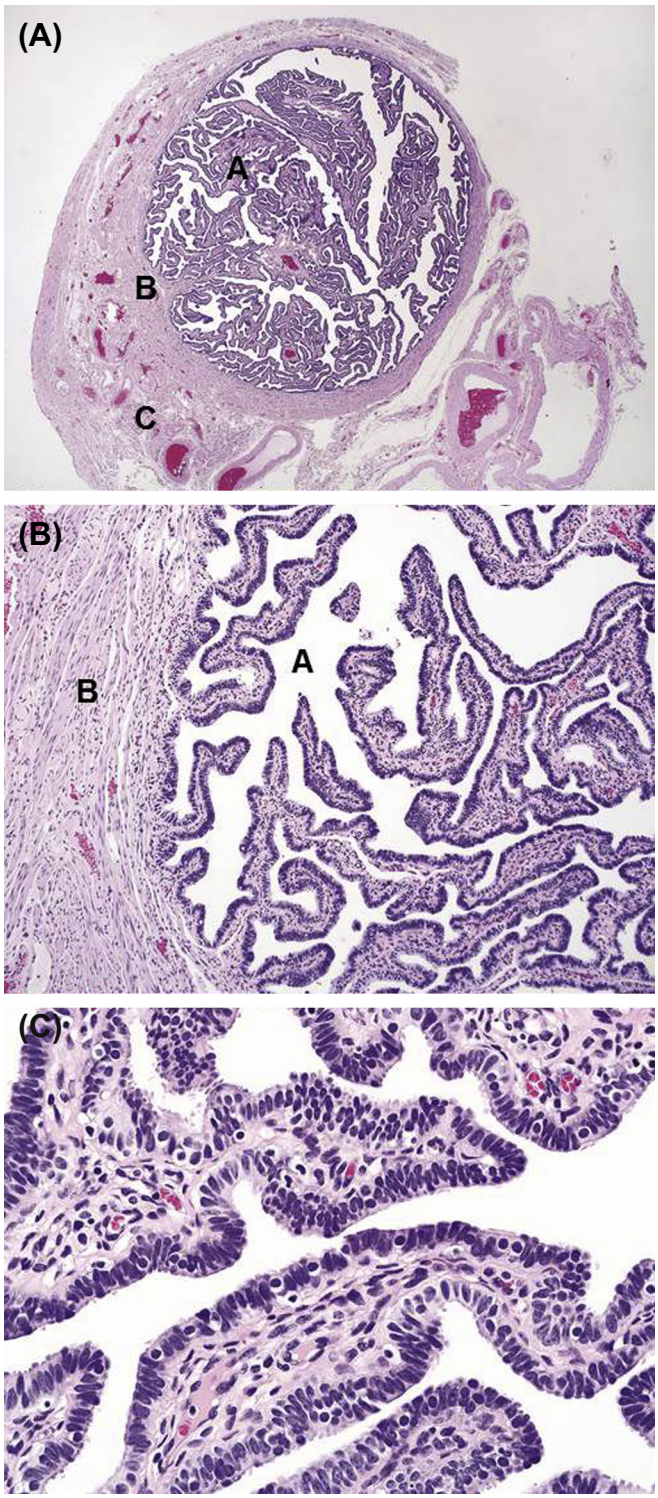

FIGURE 24.2 Cross-section of human fallopian tube, ampulla. Inner mucosa (A) is arranged in longitudinal folds within the middle muscularis (B) and outer serosa (C). The mucosa is lined by a single layer of epithelium (lower panel) composed of ciliated and secretory (nonciliated) cell types. Hematoxylin and eosin (H & E) stain, magnification 20–400×. *Source: Images courtesy of Ramya Massand and Jason Moss.*

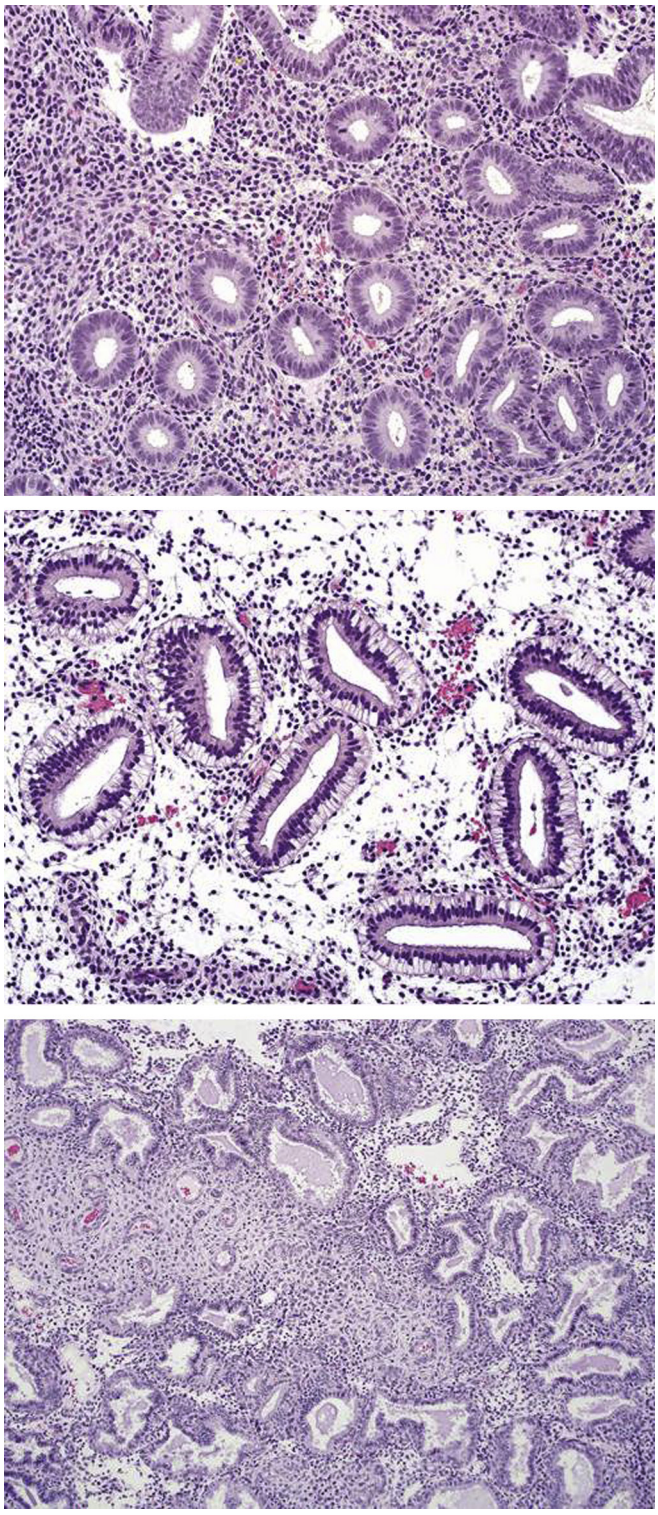

FIGURE 24.4 Human endometrium from proliferative (upper), early-secretory (middle), and late-secretory phases (lower). Note the wide spacing between simple-appearing glands (proliferative), apically displaced nuclei in the glandular epithelium due to glycogen accumulation (middle), and tortuous glands with secretions and a decidualized stroma with abundant pale cytoplasm (lower). H&E stain, magnification 100–200× (Images courtesy of Ramya Massand and Jason Moss).

Color Plates

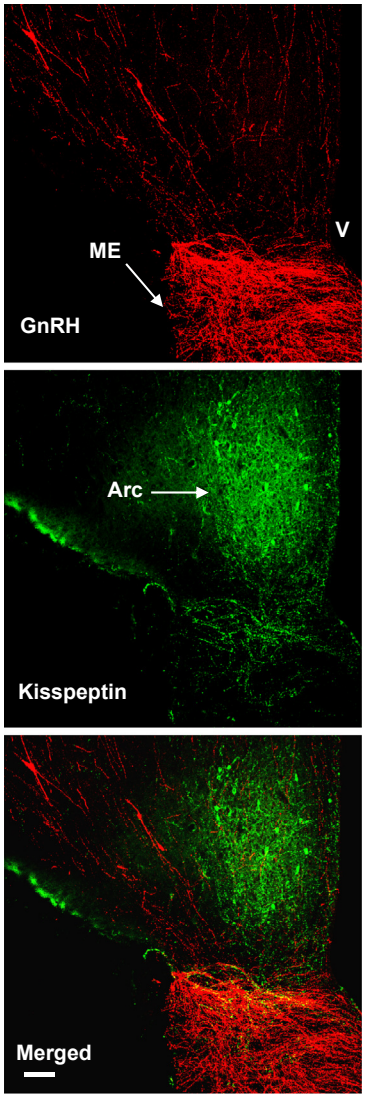

FIGURE 28.3 **A hemi-coronal section through the mediobasal hypothalamus of a castrated rhesus monkey at the level of the arcuate nucleus (Arc), the posited site of the gonadotropin-releasing hormone (GnRH) pulse generator.** The section was doubled labeled by immunofluorescence for GnRH and kisspeptin. Top panel, a confocal image of the distribution of GnRH cell bodies and fibers; middle panel, also a confocal image of the same section showing the corresponding distribution of kisspeptin cell bodies and fibers. The lower panel shows the merged image. Castration results in an upregulation of kisspeptin expression that is associated with robust GnRH pulse generator activity. Note that GnRH cell bodies at this level of the hypothalamus are found lateral to the kisspeptin perikarya in Arc: both sets of neurons send projections to the median eminence (ME) where they closely intermingle. The ependymal lining of the third ventricle (V) may be seen on the right-hand side of the section. Scale bar 100 μm. *Source: Rearranged from Ref. 57, with permission, Copyright 2008, The Endocrine Society.*

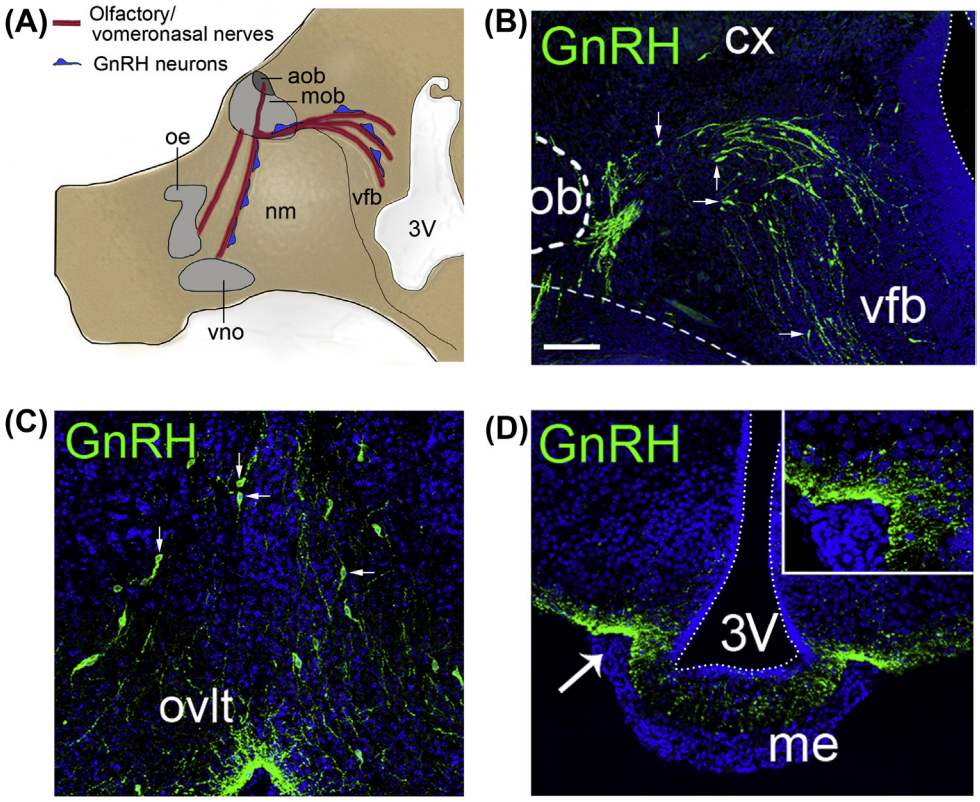

FIGURE 30.1 (A) Schematic representation of the head of a mouse embryo at E14.5, showing the scaffold of vomeronasal/terminal nerve fibers (thick lines) along which GnRH cells (neuron-like shapes) migrate from the nose to the brain. (B) Sagittal section of the rostral and ventral forebrain regions at E14.5, immunolabeled for GnRH. Arrows show GnRH neuronal cell bodies. (C, D) Coronal section of the preoptic region showing GnRH neuroendocrine cells (arrows, C) and their nerve terminals in the median eminence (me, D) in newborn (P0) mice. In D, the inset shows a higher magnification of the area identified by the arrow in the main panel. Abbreviations: oe, olfactory epithelium; vno, vomeronasal organ; nm, frontonasal mesenchyme; mob, main olfactory bulb; aob, accessory olfactory bulb; vfb, ventral forebrain; cx, cerebral cortex; ovlt, organum vasculosum of the lamina terminalis; 3V, third ventricle. Scale bar: 100 μm (50 μm in inset).

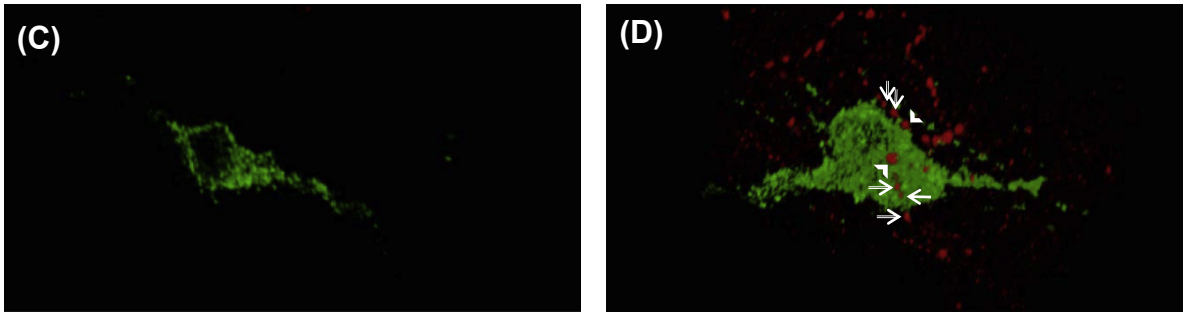

FIGURE 31.8 Changes in LH pulse frequency and kisspeptin during pubertal development. *Panel A*: LH pulse frequency in prepubertal and postpubertal female lambs that were either gonad-intact (Intact) or ovariectomized (OVX). Postpubertal intact lambs were in the early follicular phase. LH pulse frequency was higher in intact postpubertal female lambs than in intact prepubertal female lambs and rose following ovariectomy in the prepubertal, but not postpubertal, lambs. *Panel B*: Kisspeptin-immunopositive cell numbers were greater in postpubertal intact females than prepubertal intact females and increased with ovariectomy in the prepubertal, but not postpubertal, lambs. *Panels C and D*: GnRH neuron from a prepubertal female (panel C) and postpubertal female (panel D). Heavy arrows indicate point of contact between kisspeptin-containing varicosities and the GnRH neuron. Lighter arrows depict kisspeptin-positive nerve fibers. *Panels E and F*: The percentage of GnRH neurons exhibiting at least one kisspeptin close contact (panel E) was greater in intact postpubertal females than intact prepubertal females. This percentage increased with ovariectomy in the prepubertal, but not postpubertal, group. Numbers of contacts per GnRH neuron (panel F) did not differ among groups. *Redrawn from Ref. 72, with permission.*

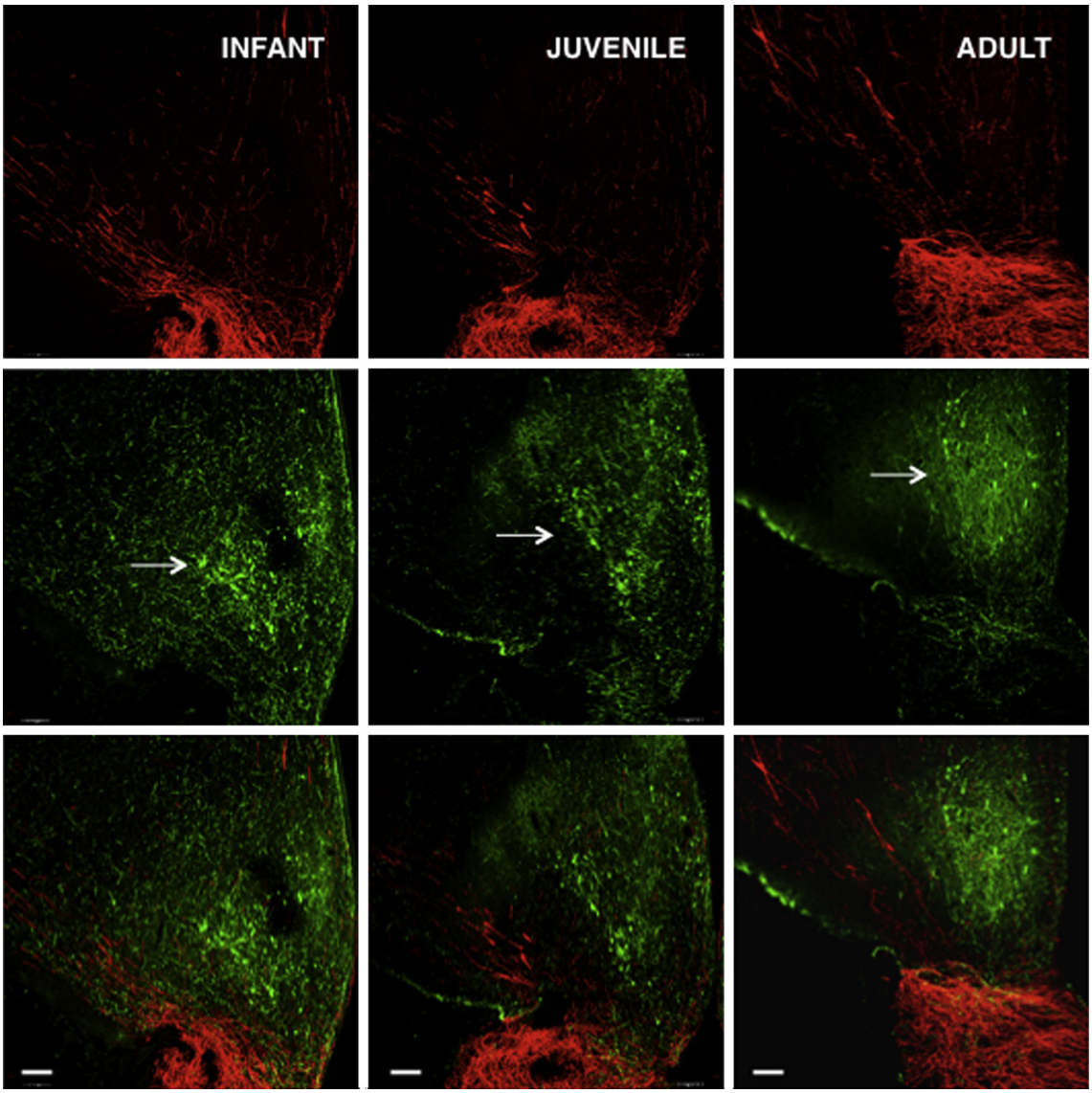

FIGURE 32.6 Confocal images illustrating developmental changes in GnRH (top panels) and kisspeptin (middle panels) neurons (immunopositive perikarya and fibers) in the MBH at the mid-tuberal level during postnatal development in the agonadal male rhesus monkey. The lower panels show the merged images. A hemi-hypothalamic section is shown for an infant (left-hand panels), juvenile (center panels), and adult (right-hand panels) animal; stages of postnatal development corresponding to the on-off-on pattern of GnRH pulse generator activity in primates. Note that kisspeptin immunoactivity is reduced in the juvenile MBH at a time when GnRH pulsatility is arrested but GnRH immunoactivity in the median eminence is maintained during this phase of development. In most cases the ependymal lining of the third ventricle is visible on the right-hand boundary of each hemi-section. *Arrow* indicates kisspeptin neurons in the arcuate nucleus. Scale bar, 100 μm. *Source: Parts of this montage have been reprinted with permission from Refs 170,266.*

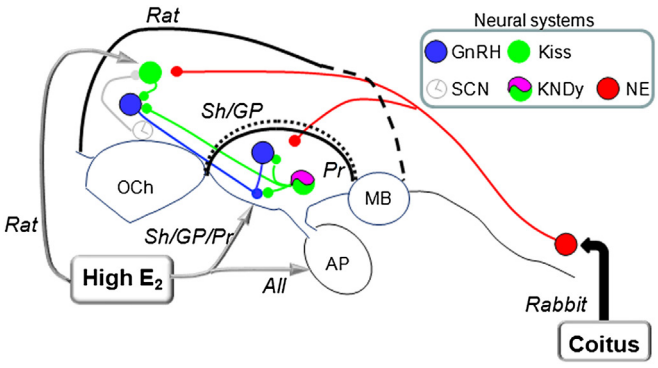

FIGURE 33.8 Schematic of parasagittal section of the hypothalamo–hypophysial unit illustrating the anatomical areas and neural systems participating in the preovulatory GnRH surge and sites of E_2 positive feedback in various species. All structures are required in rabbits; in other species, solid lines depict knife cuts that had no effect on the surge. Dotted lines are cuts that only decreased the amplitude of the LH surge, and the dashed line illustrates that NE input is normally required for the surge in rats but can be compensated for under some circumstances. See the text for more details. AP: anterior pituitary; GP: guinea pig; MB: mammillary body; OCh: optic chiasm; Pr: primate; Sh: sheep.

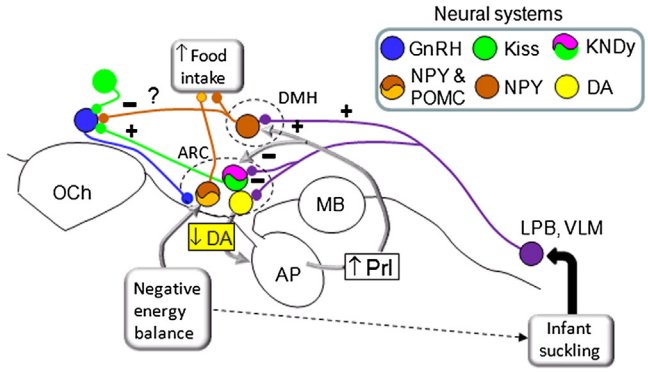

FIGURE 33.12 Neural systems stimulating food intake and inhibiting GnRH secretion during lactation in the rat. The suckling stimulus is transmitted to the hypothalamus via neurons in the lateral parabrachial nucleus (LPB) and ventrolateral medulla (VLM), inhibits DA release from tuberoinfundibular dopaminergic (TIDA) neurons and kisspeptin release from KNDy neurons, and stimulates NPY neural activity in the DMH. The fall in DA allows prolactin secretion to increase, which further inhibits KNDy kisspeptin and stimulates NPY from the DMH. The latter increases food intake, while the former inhibits GnRH secretion. At the same time, the negative energy balance in the mother induced by the demands of the offspring acts via the metabolic homeostatic circuitry in the ARC (increased NPY–AgRP and decreased POMC–CART), which also acts to stimulate food intake and may inhibit GnRH secretion. Note that this circuitry is depicted by a single neural element (NPY and POMC) for simplicity. Similarly, orexogenic neurons in the LHA and anorexogenic CRH neurons in the PVN are not illustrated.

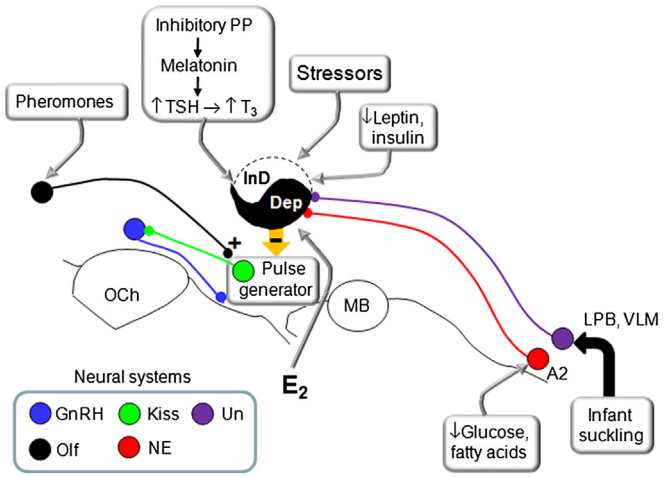

FIGURE 33.13 Schematic of parasagittal section of the hypothalamus illustrating mechanisms by which different external factors alter episodic GnRH secretion. Note that neural inputs are conceptual and do not represent monosynaptic pathways. See the text for more details. Dep: steroid-dependent mechanisms; InD: steroid-independent mechanisms; Kiss: kisspeptin; NE: norepinephrine; Olf: olfactory epithelium; Un: unknown neurotransmitter.

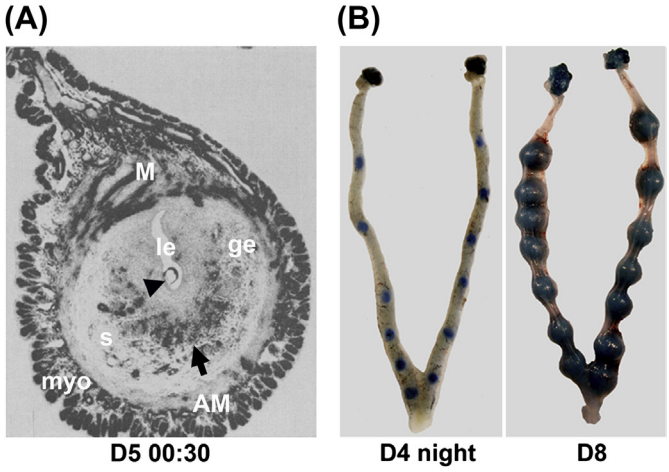

FIGURE 38.1 Increased endometrial vascular permeability upon attachment and implantation in the mouse. (A) Transverse section of uterus on day 5 (D5) early morning (00:30) of pregnancy showing implantation at the antimesometrial pole within a crypt (arrowhead). This section also shows weak alkaline phosphatase activity in the uterine stroma (black, arrow). Note the edema in the outer areas of the mucosa, contrasting with the closely packed cells around the uterine lumen (presumptive primary decidual zone). Le, luminal epithelium; ge, glandular epithelium; s, stroma; myo, myometrium; M, mesometrial pole; AM, antimesometrial pole. *(Source: Reprinted with permission from Ref. 2.)* (B) Increased vascular permeability at the sites of blastocyst attachment with the uterine luminal epithelium was detected after an intravenous injection of a blue dye (Chicago Blue B6) solution in mice at midnight of day 4 (D4) and day 8 (D8) of pregnancy. On day 4, distinct blue bands (dark bands in this picture) indicate that the attachment process has been initiated.

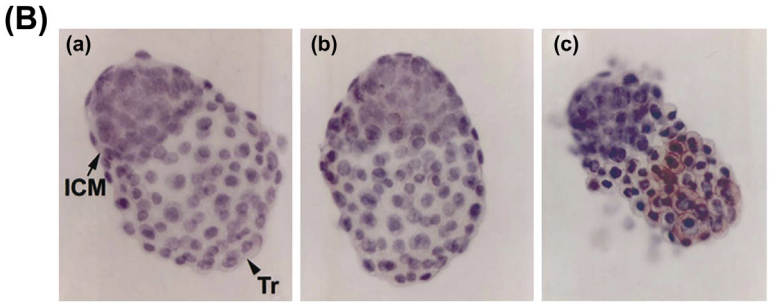

FIGURE 38.4 Molecular markers for blastocyst dormancy and activation. (B) Dormant blastocysts recovered from P_4-primed delayed-implanting mice on day 7 were cultured for 24 h in Whitten's medium, in the presence of (a) vehicle; (b) E_2; or (c) 4-OH-E_2. Blastocysts were processed for immunostaining and counterstained with hematoxylin. Red deposits indicate the sites of immunoreactive COX2. ICM, inner cell mass; Tr, trophectoderm. *Source: Reprinted with permission from Refs 67,137.*

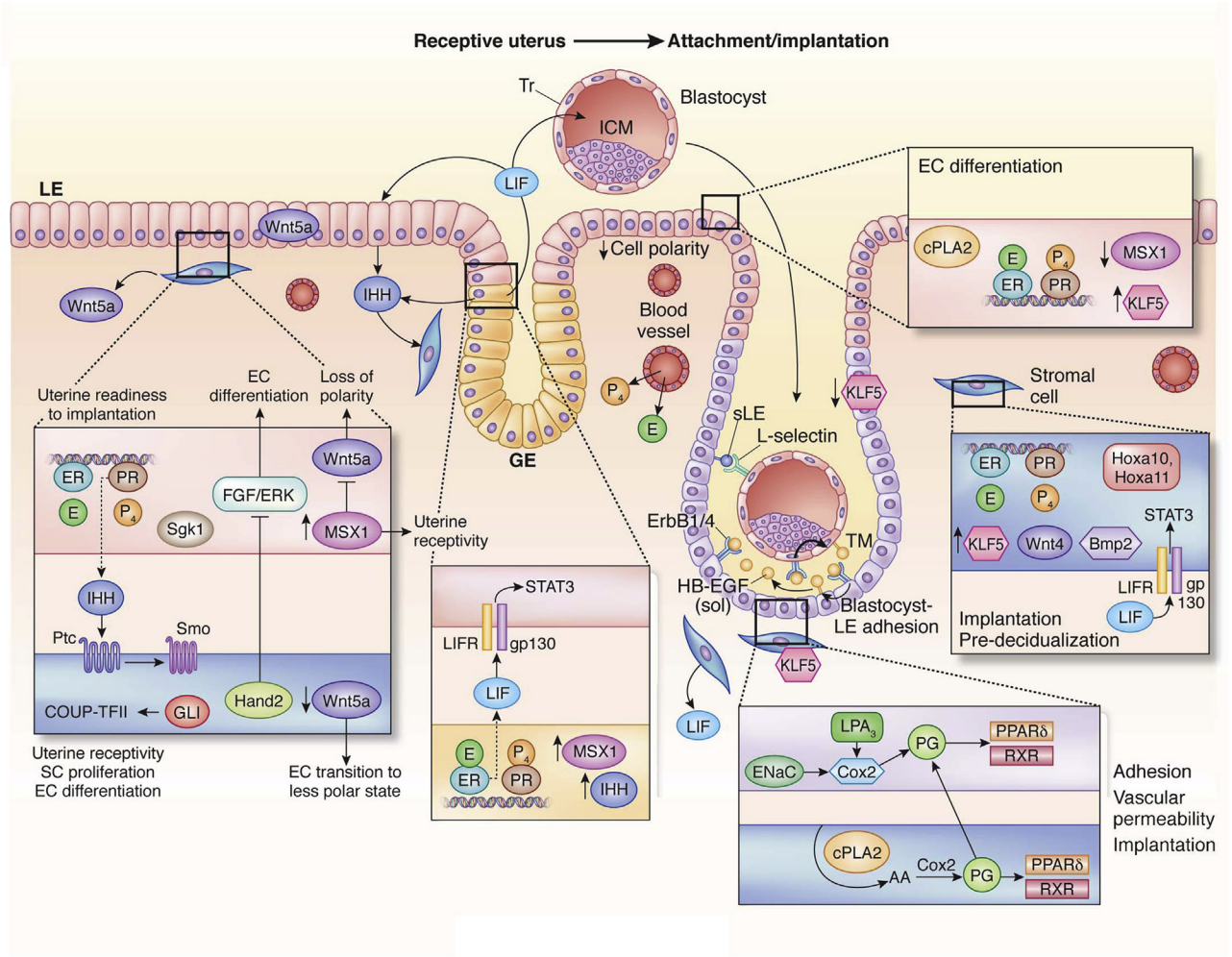

FIGURE 38.5 Signaling network for uterine receptivity and implantation. This is a hybrid cartoon, based on mouse and human studies, portraying compartment- and cell type–specific expression of molecules and their potential functions necessary for uterine receptivity, implantation, and decidualization. Interplay of ovarian P_4- and/or E_2-dependent and P_4- and/or E_2-independent factors in the pregnant uterus in specific compartments contributes to the success of implantation in a juxtacrine–paracrine–autocrine manner. During attachment, interactions between the blastocyst and luminal epithelium (LE) involve ErbB1/4 (blue) and both transmembrane (TM) and soluble (Sol) forms of HB-EGF, as well as L-selectin ligands (sLE) expressed by the luminal epithelium to L-selectin receptors on the blastocyst. The other key signaling pathways for uterine receptivity and implantation are also shown. AA, arachidonic acid; BMP2, bone morphogenetic protein 2; cPLA2α, cytosolic phospholipase A2α; COUP-TFII, chicken ovalbumin upstream promoter transcription factor 2; Cox2, cyclooxygenase 2; E, estrogens; EC, epithelial cell (luminal and glandular epithelia); ENaC, epithelium sodium channel; ER, estrogen receptor; ErbB1/4; epidermal growth factor receptor 1/4; ERK, extracellular signal–regulated kinase; FGF, fibroblast growth factor; GE, glandular epithelium; gp130, glycoprotein 130; Hand2, heart- and neural crest derivatives–expressed protein 2; HB-EGF, heparin-binding epidermal growth factor-like growth factor; Hoxa10/11, homeobox A10/11; ICM, inner cell mass; IHH, Indian hedgehog; KLF5, Kruppel-like factor 5; LIF, leukemia inhibitory factor; LIFR, LIF receptor; LPA3, lysophosphatidic acid receptor 3; MSX1, muscle segment homeobox 1; P_4, progesterone; PG, prostaglandin; PPAR-δ; peroxisome proliferator-activating receptor δ; PR, progesterone receptor; Ptc, Patched; RXR, retinoid X receptor; SC, stromal cell; SGK1, serum- and glucocorticoid-inducible kinase 1; Smo, Smoothened; STAT3, signal transducer and activator of transcription 3; Tr, trophectoderm; Wnt4/5a, Wingless-type MMTV integration site family members 4/5a. Compartment colors: blue, stroma; pink, luminal epithelium; orange, glandular epithelium; purple, epithelium at the attachment site. *Source: Reprinted with permission from Ref. 153.*

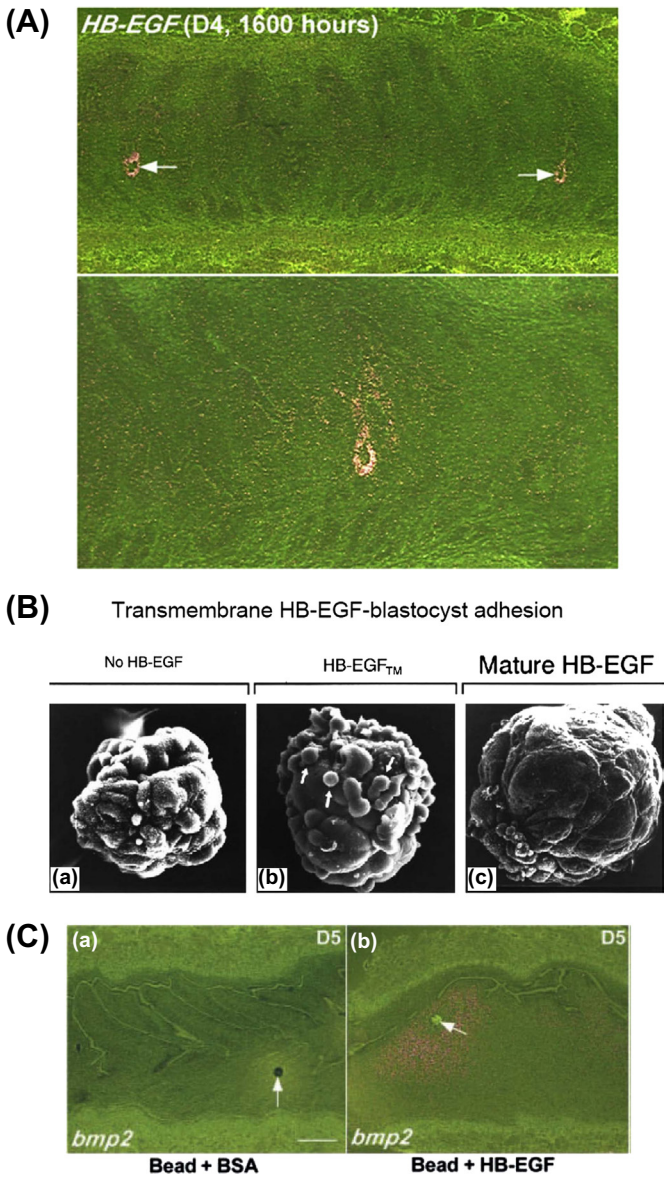

FIGURE 38.6 HB-EGF serves as a reciprocal mediator between the luminal epithelium and activated blastocyst during attachment reaction. (A) In situ hybridization of HB-EGF mRNA (dark-field) in the mouse uterus at 1600 h on day 4 of pregnancy. Note distinct hybridization signals in luminal epithelial cells surrounding two blastocysts in a longitudinal section. Arrows demarcate location of blastocysts. (B) Scanning electron microscopy of blastocysts co-cultured with 32D cells (a murine myeloid cell line). Zona-free day 4 (0900 h) mouse blastocysts were co-cultured with (a) parental 32D cells, (b) 32D cells displaying transmembrane form of HB-EGF^{TM}, or (c) 32D cells synthesizing soluble form of HB-EGF for 36 h. After extensive washing to remove loosely adhering cells, blastocysts were fixed and examined by scanning electron microscopy; magnification 800×. Arrows point to 32D cells. (C) *Bmp2* gene expression in response to beads preloaded with HB-EGF. Beads (7 beads/horn) preabsorbed either in BSA (control) or HB-EGF (100 ng/ml) were transferred into uterine lumens of day 4 pseudopregnant mice. Mice were killed on day 5 to examine *Bmp2* expression at the sites of beads. Arrows indicate the locations of the beads. Note localized stromal expression of *Bmp2* at the sites of beads preabsorbed with HB-EGF. Bar: 75 mm. *Source: Reprinted with permission from Refs 9,135,220.*

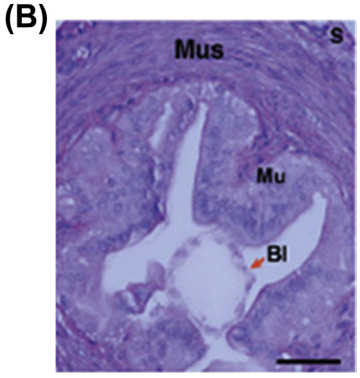

FIGURE 38.7 Impaired oviductal embryo transport causes pregnancy loss in *Cnr1*$^{-/-}$ but not *Cnr2*$^{-/-}$ mice. (B) A representative histological section of a day 7 pregnant *Cnr1*$^{-/-}$ oviduct showing an entrapped blastocyst (Bl, arrow) at the isthmus. Mus, muscularis; S, serosa; Mu, mucosa. Bar, 100 μm. *Source: Reprinted with permission from Ref. 394.*

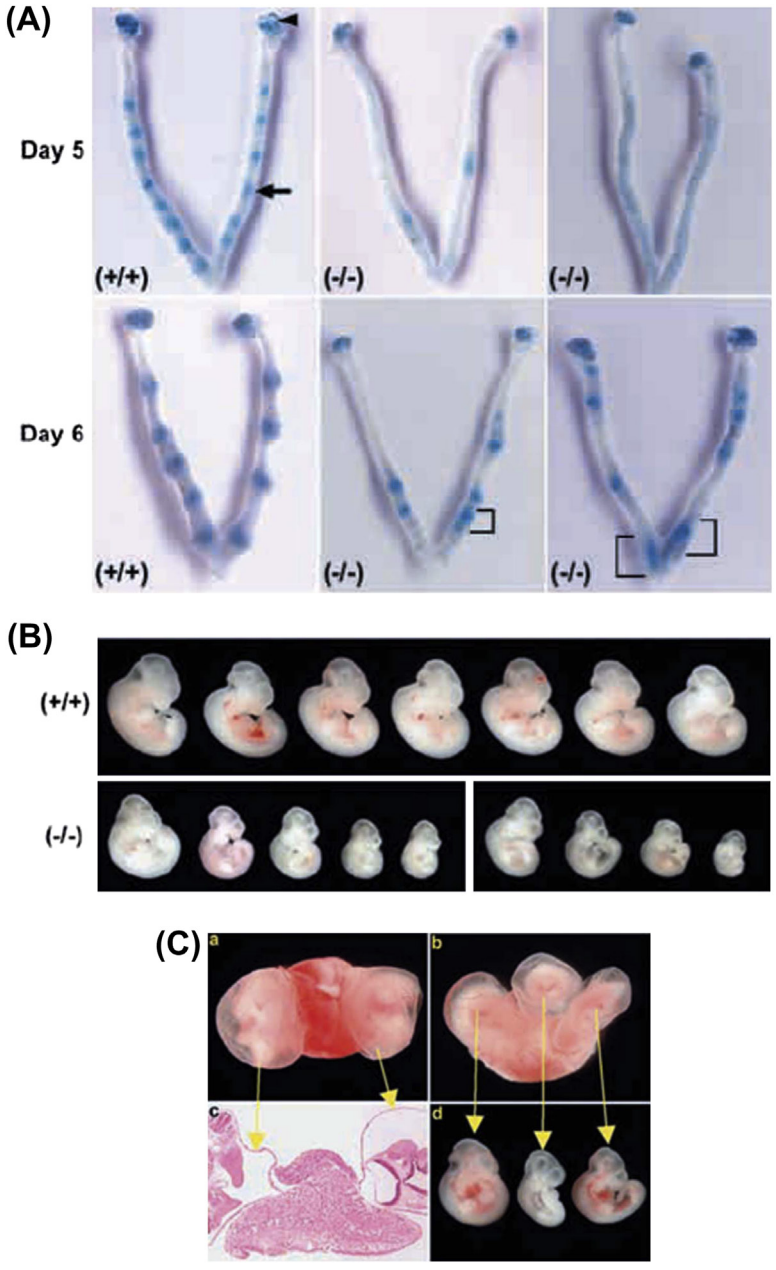

FIGURE 38.8 **Defective postimplantation development in *Pla2g4a*$^{-/-}$ mice.** (A) Representative photographs of uteri with implantation sites (blue bands) on days 5 and 6. Note very few or no implantation sites on day 5, but unevenly spaced implantation sites on day 6 in *Pla2g4a*$^{-/-}$ mice. Arrowhead and arrow indicate ovary and implantation site, respectively. Brackets indicate crowding of implantation sites. (B) Photographs of embryos isolated from implantation sites of one representative wild-type and two *Pla2g4a*$^{-/-}$ mice on day 12. Note retarded and asynchronous development of embryos in *Pla2g4a*$^{-/-}$ mice. (C) Representative photographs of conjoined embryos in a placenta (a, c) and three embryos in the same decidual envelope (b, d) from *Pla2g4a*$^{-/-}$ mice on day 12. (c) A histological section of (a) with two embryos; embryos shown in (d) are from (b). Yellow arrows indicate the source of the embryos from the decidual envelope. *Source: Reprinted with permission from Ref. 47.*

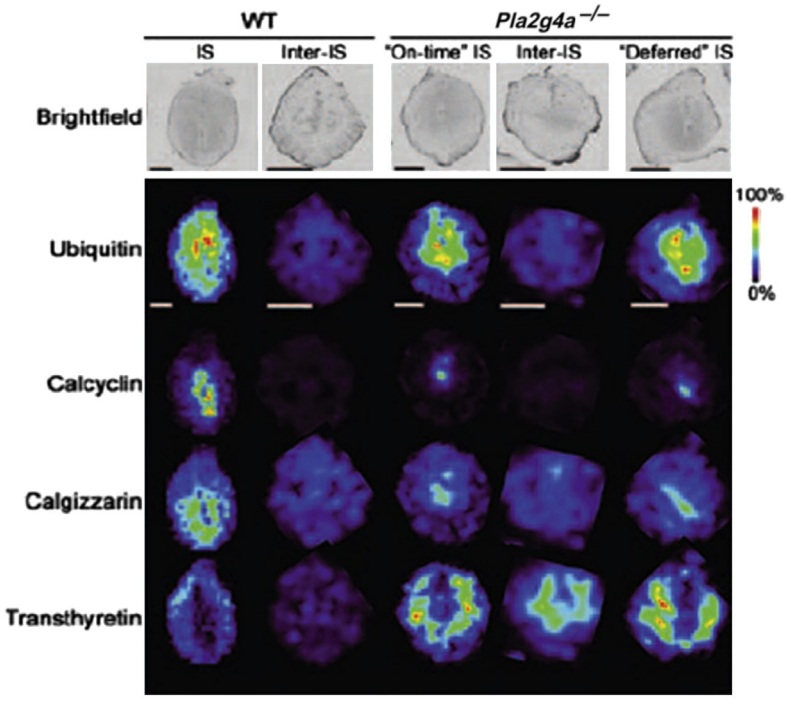

FIGURE 38.9 **Proteome profiles differ between WT and *Pla2g4a*$^{-/-}$ uteri on day 6 of pregnancy, regardless of implantation timing.** Optical images of a WT implantation site (IS) and interimplantation site (inter-IS) and *Pla2g4a*$^{-/-}$ deferred and on-time IS (*upper panel*). Bar, 670 μm. Ion intensity maps are shown below their respective bright-field images. *Source: Reprinted with permission from Ref. 480.*

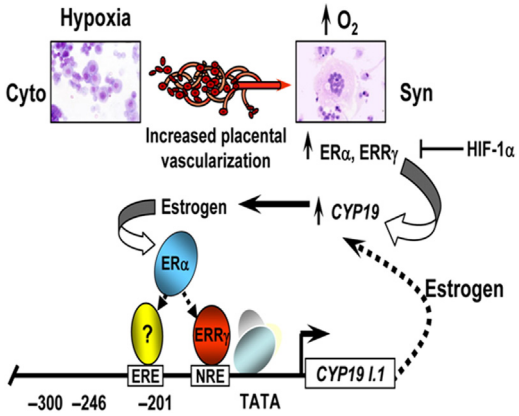

FIGURE 40.12 Model of hCYP19 regulation in human trophoblasts. Early in gestation, the placenta is relatively hypoxic; the elevated levels of HIF-1α inhibit ERRγ transcription via interaction with a response element (HRE) in the ERRγ promoter. This, together with hypoxia-mediated induction of basic helix–loop–helix factors ASCL2 and USF1/2, blocks syncytiotrophoblast differentiation and hCYP19 gene expression. After the ninth week of gestation, increased placental vascularization results in increased oxygen availability to the trophoblast cells. The consequent decrease in HIF-1α levels and the expression of other inhibitory transcription factors cause upregulation of ERRγ and induction of hCYP191.1 transcription. The increased estrogens produced, in turn, activate ERα, which may functionally interact with ERRγ and other activating transcription factors to further upregulate hCYP19 expression. *Source: Reprinted with permission from Ref. 360.*

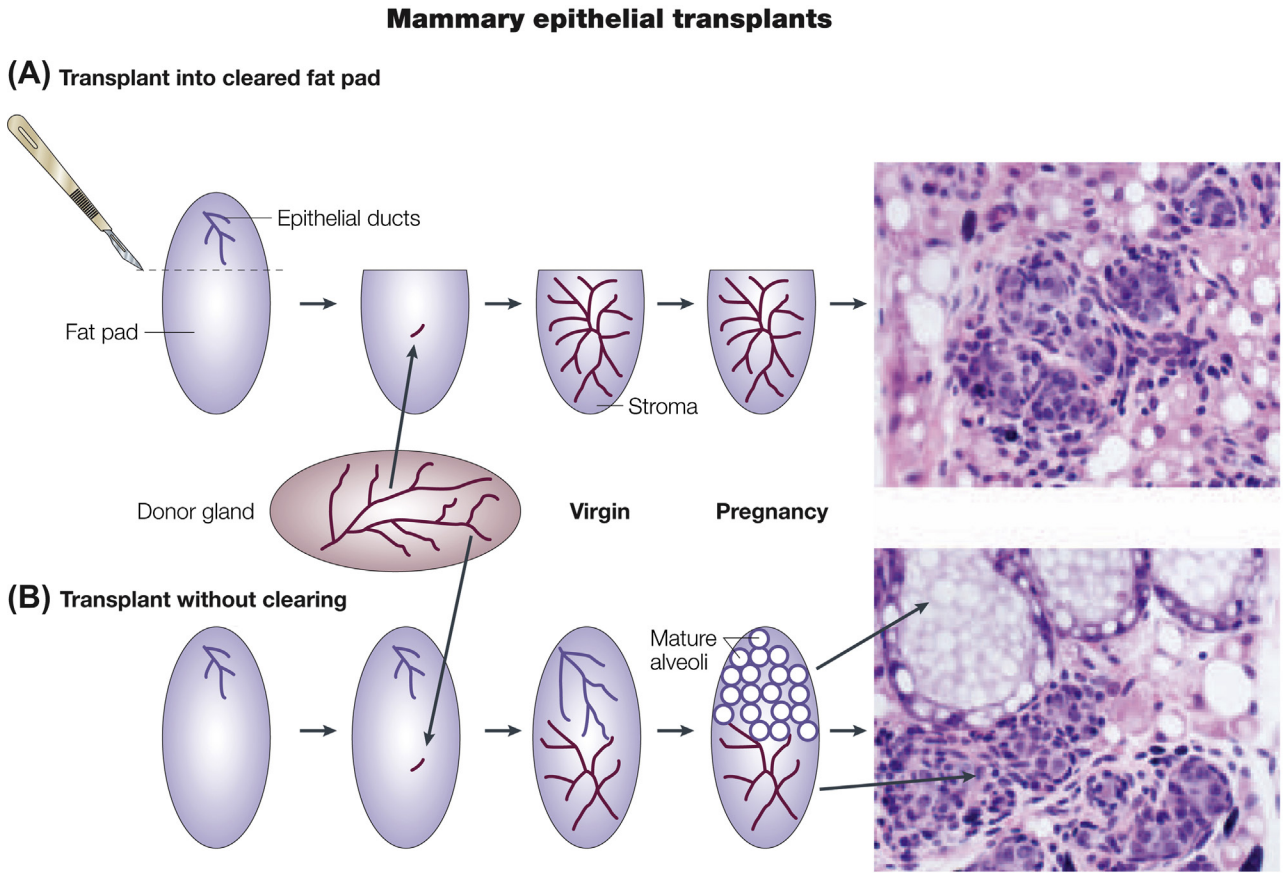

FIGURE 46.2 Use of mammary gland transplantation to assess developmental potential. (A) The area containing the ductal anlage (purple) in a 3-week-old mouse is surgically removed to generate a "cleared fat pad" into which donor tissue (dark pink) is transplanted. The development of the transplanted tissue is monitored at intervals posttransplant by either whole mount or standard histological analysis. (B) An alternative approach involves direct transplantation into a non-cleared mammary gland. Transplanted cells are frequently marked with either immune-fluorescent markers, genetic markers, or infected with viruses expressing detectable markers. *Source: Used by permission from Macmillan Publishing Company; Ref. 14.*

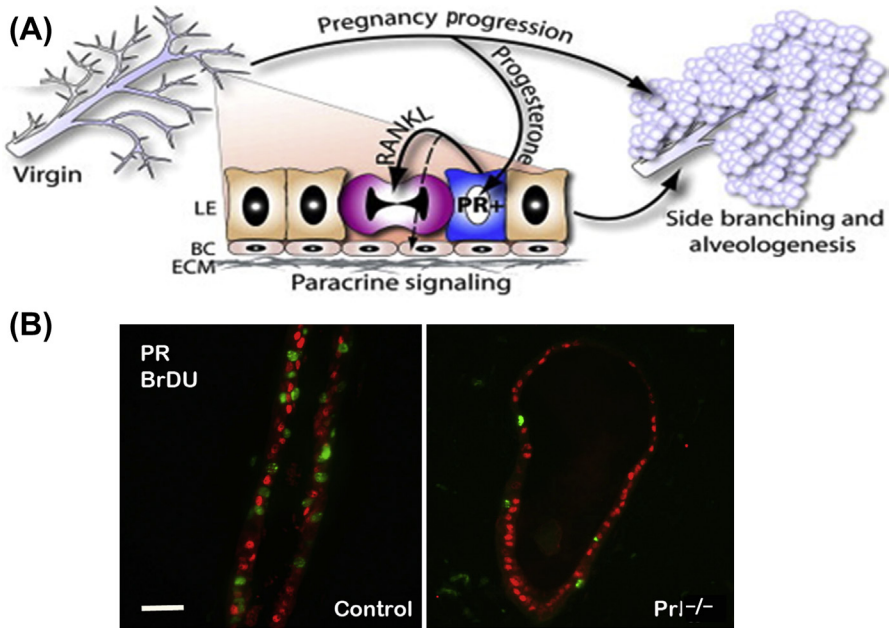

FIGURE 46.4 Role of paracrine signaling in alveologenesis. (A) As pregnancy progresses side branches develop at discreet intervals along the ducts; cells in these side branches proliferate to form alveoli. The progesterone receptor (PR) is expressed in a subset of alveolar cells that are stimulated by increasing P4 from the ovaries to secrete RANKL. This paracrine factor acts on neighboring cells to promote proliferation. (B) Expression of PR and proliferating cells after 2 days of treatment with E2 and P4 in wild-type (control) and PRL-null mice. Proliferating cells (BrDU labeled, green) and PR positive cells (red) are generally not coincident. Note that the number of proliferating cells is reduced in the absence of PRL. Scale bar, 50 μm. *Source: (A) Used by permission from Ref. 93. (B) Original figure from Ref. 64.*

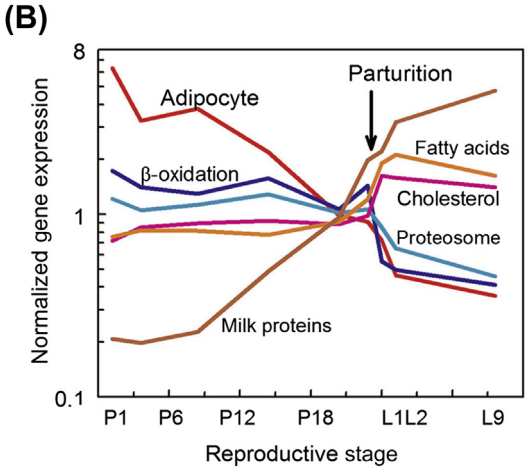

FIGURE 46.5 Changes in the differentiated activity of the mammary gland in pregnancy and lactation. (B) Changes in gene expression of different categories of genes in the mouse mammary gland from microarray studies. Expression of adipocyte-specific genes and collagens (not shown) decreases six- to eightfold during pregnancy and another twofold at parturition, whereas the genes for fatty acid degradation and many components of the proteosome are level during pregnancy and decrease about twofold at parturition. Milk protein genes, on average, increase about fivefold during pregnancy and another threefold around parturition, whereas the genes for fatty acid and cholesterol synthetic enzymes increase about twofold just after parturition. Normalized data for each class were averaged to produce the lines on this graph. *Source: (B) From Ref. 11.*

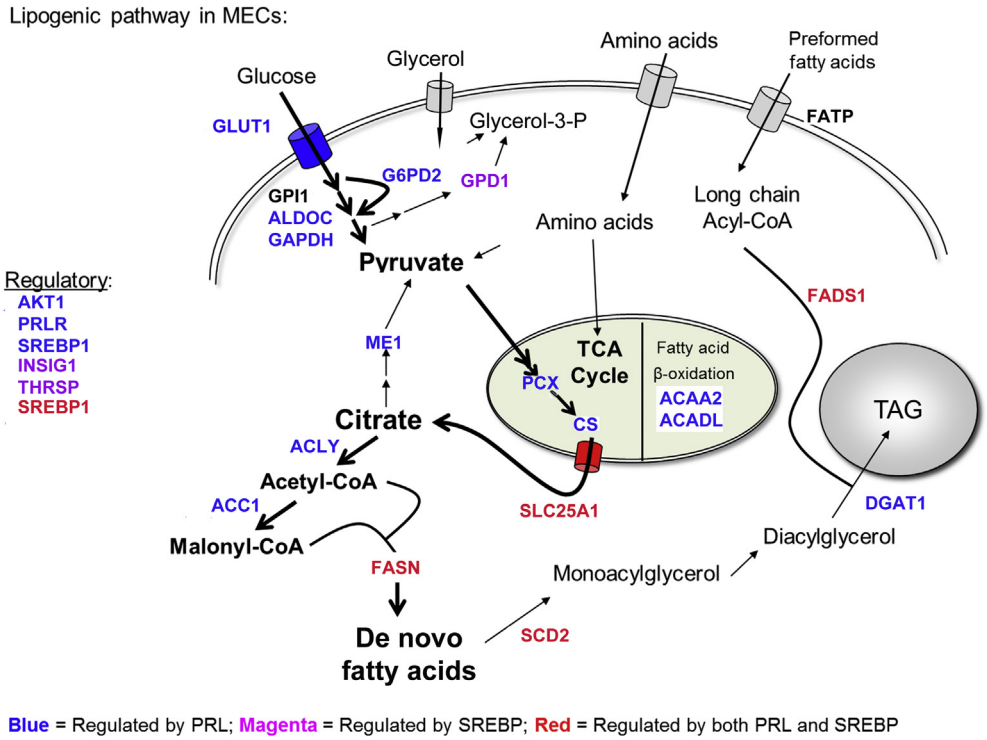

FIGURE 46.7 **Lipid synthesis pathways in mammary epithelial cells during lactation.** Substrates for lipid synthesis enter the cells via the glucose transporter (GLUT1), a glycerol transporter, as amino acids, or as preformed fatty acids via a fatty acid transport protein (FATP). Glycolysis leads to the production of both glycerol-3-phosphate and pyruvate from glucose. The genes for several enzymes in this pathway, fructose bis-phosphate aldolase (ALDOC) and glycerol-3-phosphate dehydrogenase (GAPDH), as well as glycose-6-phosphate dehydrogenase (G6PD2 in the mammary gland) are all upregulated by prolactin along with mitochondrial genes for pyruvate carboxylase (PCX) and citrate synthase (CS). Glycerol-3-phosphate is formed from dihydroxyacetone phosphate, a product of glycolysis, by glycerol-3-phosphate dehydrogenase (GPD1) to be used as a backbone for triacylglyceride (TAG) synthesis. GPD1 is regulated by SREBP. Amino acids are transformed into pyruvate and other substrates that enter the mitochondria to be transformed into citrate, which is exported via the tricarboxylic acid transporter, SLC25A1. Citrate is the major substrate for de novo synthesis of fatty acids in species other than ruminants, which utilize acetate for this purpose. Citrate is transformed into acetyl-CoA by ATP citrate lyase (ACLY), then to malonyl CoA by acetyl-CoA carboxylase (ACC1), and finally to saturated fatty acids with 8–16 carbons (C:8–C:16) by fatty acid synthase (FASN). Cytosolic malic enzyme (ME1) and the enzymes of the pentose phosphate shunt both provide the necessary reducing molecule NADPH that is required for activity of FASN. C:16 fatty acids can be desaturated by stearoyl-CoA desaturase (SCD2) prior to being esterified into monoacylglycerol, then diacylglycerol, and finally into triacylglycerols, with subsequent integration of the fatty acids derived from preformed sources. The final step in the TAG synthesis pathway is catalyzed by diglyceride acyltransferase (DGAT1). The TAG coalesce into lipid droplets. Both prolactin and SREBP have been shown to regulate the genes for FASN, SLC25A1, SCD2, and FADS1. *Source: Diagram derived from data in Refs 19,20.*

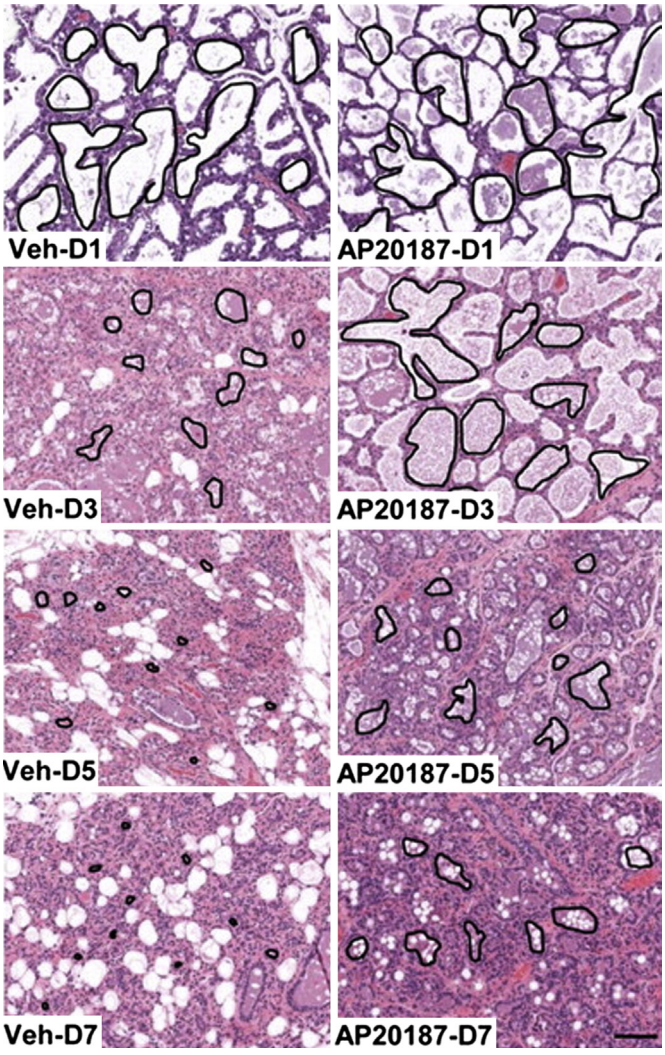

FIGURE 46.9 The role of macrophages in mouse mammary gland involution; histological analysis. Glands from Mafia mice were analyzed on days 1, 3, 5, and 7 after pup removal from a 10-day lactating mouse suckling five to six pups. Three days prior to pup withdrawal experimental dams were given a dose of AP20187, which depletes macrophages in this strain. Left-hand images, vehicle only (macrophages present); right-hand images, AP20187 (macrophages depleted). In both control and experimental mice marked luminal expansion is evident 1 day after removal of pups (some lumens outlined in black for illustration). In vehicle-only mice a marked decrease in lumen size by day three is observed as milk is resorbed. Very small lumens remain on day 7, however significant numbers of lipid filled adipocytes are evident between the alveoli starting on day three and increasing to day 7. These changes occur much more slowly in the glands of AP20197-treated mice, providing evidence that macrophages are required for several aspects of normal involution. *Source: Reproduced by permission from Ref. 183; link at Development:* dev.biologists.org.

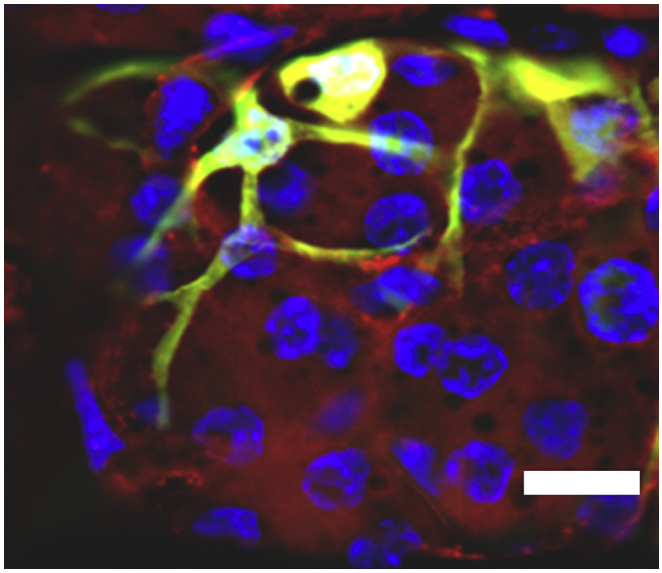

FIGURE 46.19 Myoepithelial cell in the mammary gland of a lactating mouse. The cell has been transduced with adenoviral GFP (yellow) showing processes embracing the luminal epithelial cells (red). Nuclei stained with DAPI (blue). Scale bar 10 μm. *Source: From Russell T, Fischer A, Beeman N, Freed E, Neville MC, Schaack J. Transduction of the mouse mammary epithelium with adenoviral vectors in vivo.* J Virol *2003;77(10):5801–09.* http://dx.doi.org/10.1128/JVI.77.10.5801–5809.2003, *© 2003, American Society for Microbiology.*

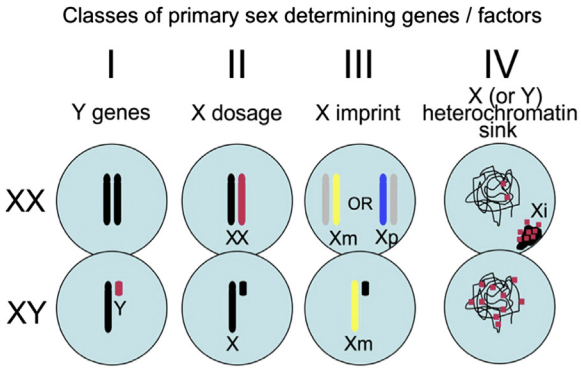

FIGURE 47.7 Four ways that sex chromosomes could affect gene expression. Four possible classes of primary sex-determining factors are recognized. Class I comprises Y genes that have a male-specific effect on one or more tissues. Class II is X genes that are expressed at a higher level in females than males by virtue of the 2:1 ratio in the number of X chromosomes. Class III is X genes that receive a parental imprint. The X chromosome receiving a maternal imprint (Xm, yellow) is active in half of XX cells and all of XY cells, whereas the X chromosome receiving a paternal imprint (Xp, blue) is active in half of XX cells and no XY cells. Class IV comprises proposed regions of the sex chromosome heterochromatin (the heterochromatic inactive X (Xi) is illustrated here) that act as sex-specific sinks for factors (red dots) that regulate the amount of euchromatin–heterochromatin at interphase and therefore affect gene expression throughout the genome. To date, specific members of only Class I have been identified (*Sry* and spermatogenesis genes). Although evidence indicates that the number of X chromosomes leads to some sex differences in phenotype, the specific genes or chromosome regions that explain these X effects have not been identified. Class IV is particularly speculative at present because it is based on a limited number of studies. Future studies are likely to expand the importance of Classes II–IV. *Source: Adapted with permission of Elsevier from Ref. 58.*

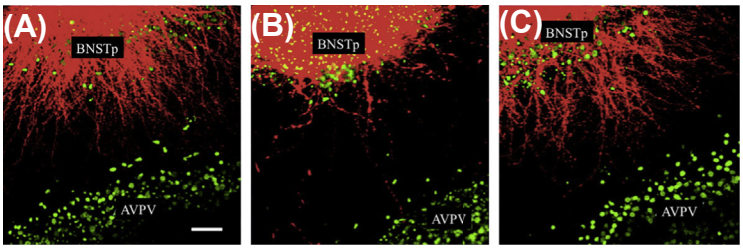

FIGURE 47.10 Target-dependent induction of neurite outgrowth in co-cultures of the principal nucleus of the bed nucleus of the stria terminalis (BNSTp) and anteroventral periventricular nucleus (AVPV). Confocal images of BNSTp-AVPV co-cultures show DiI labeling of neuritis (pseudo-colored red) that extend from the BSTp toward the AVPV. The BSTp explant was derived from a male rat on postnatal day 5 (PN5) and co-cultured with an AVPV explant (pseudo-colored green) derived from a PN9 (A) male, (B) female, or (C) androgenized female. A marked difference in the density of neurites between the co-cultures explants was observed, with many more neuritis seen when the AVPV explant was derived from a male (A) versus a female (B) rat. Treatment of neonatal female rats with testosterone during the first 9 days of life in vivo masculinized (i.e., increased) neurite outgrowth from BSTp explants (C), suggesting that the target-dependent formation of neuritis extending from the BSTp to AVPV is determined by exposure to sex steroid hormones during the neonatal period. Scale bar: 15 μm. *Source: Adapted with the permission from Ref. 217.*

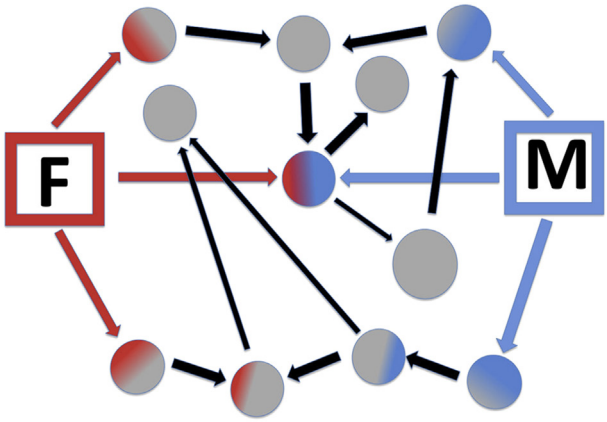

FIGURE 47.21 Schematic view of the sexome. Male- and female-biasing factors (chromosomes, hormonal profiles, etc.) are represented by the M and F boxes, respectively. These factors act on specific nodes in the gene network causing male and female bias in gene expression represented by differently colored shading. Sex bias can be propagated to other nodes, thereby increasing or decreasing gene expression and in some cases canceling out sex-biasing effects. Arnold and Lusis define the sexome as the total of all sex-biasing actions within the network.[404] Although they focus on sex bias in gene products,[404] the circles and arrows in this figure may just as well represent interactions between different organ systems. *Source: Adapted from Ref. 404.*

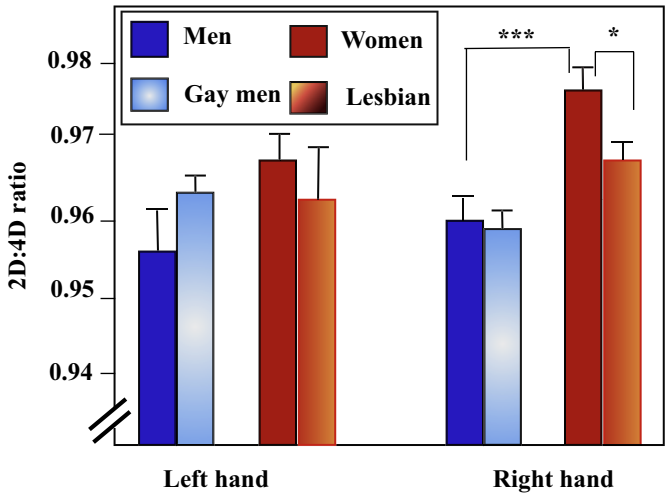

FIGURE 48.7 **Ratio of lengths of the index (2D) and ring finger (4D) as a function of sex and sexual orientation.** The 2D:4D ratio is greater in women than in men, probably due to in utero exposure to testosterone of male embryos. It is close to the male level in homosexual women, suggesting that they have been exposed to abnormally high levels of testosterone during a part of their embryonic life. For unknown reasons, these differences are more pronounced in the right hand. * = $p < 0.05$, *** = $p < 0.001$. *Source: Redrawn from Ref. 117.*

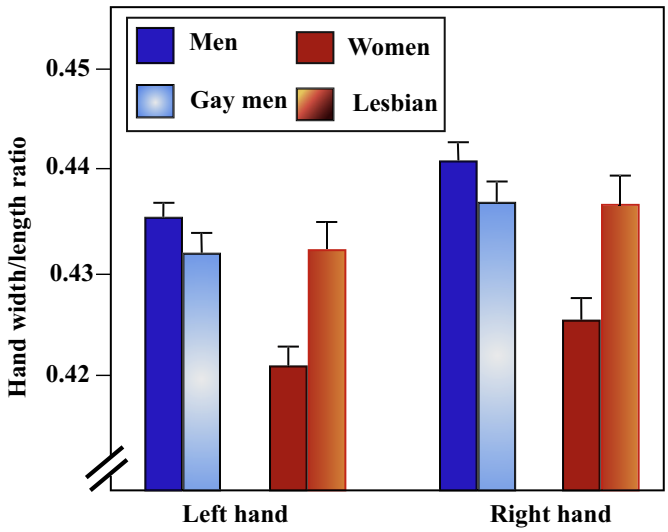

FIGURE 48.8 **Relationship between sex and sexual orientation and the ratio of width to length of the hand.** *Source: Redrawn from data in Ref. 123.*

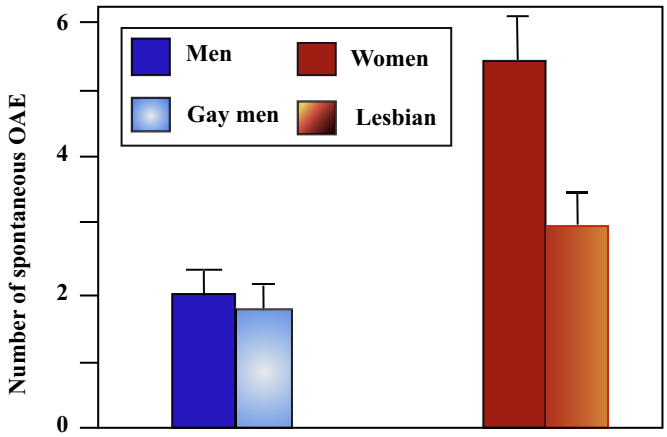

FIGURE 48.9 **Frequency of oto-acoustic emissions (OAE) evoked by click sounds measured in the right ear in men and women as a function of sexual orientation.** OAE are sexually differentiated and significantly masculinized in homosexual females. *Source: Redrawn from data in Refs 124,127.*

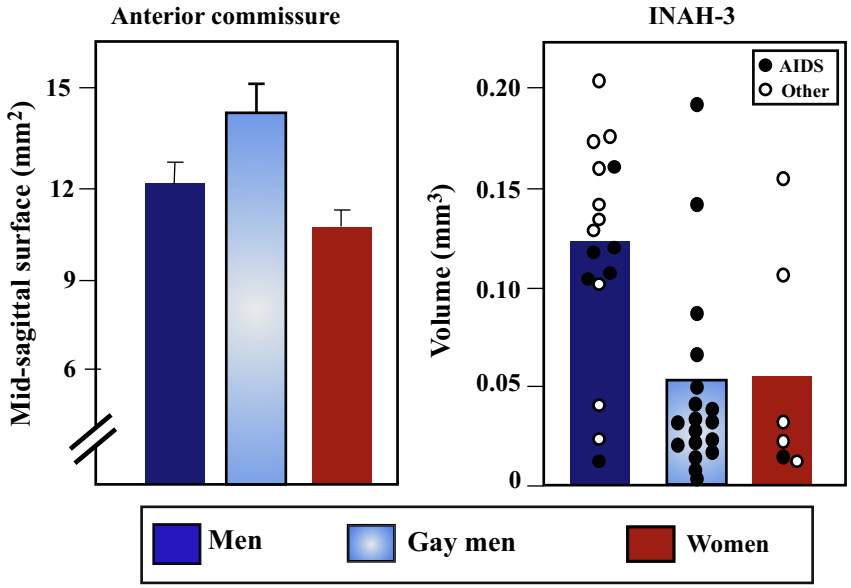

FIGURE 48.11 **Average size of the anterior commissure as estimated by its surface in the mid-sagittal plane (left) and volume of the 3rd interstitial nucleus of the anterior hypothalamus (INA-3) (right) in men, gay men, and women.** Data for the anterior commissure are presented as means ± SEM, whereas for INAH-3 the average value in each group, as well as all individual data points, are shown to clearly illustrate the partial overlap between groups despite the significant differences between men and women and between men and gay men. Individual data points are filled in subjects who died from AIDS and open in subjects who died from other causes. The smaller average size of INAH-3 in gay men cannot be accounted for by the fact that all subjects included in the study had died from AIDS since in the control group, the INAH-3 of men who died from AIDS is not smaller than in men who died from other causes. *Source: Redrawn from data in Refs 140,141.*

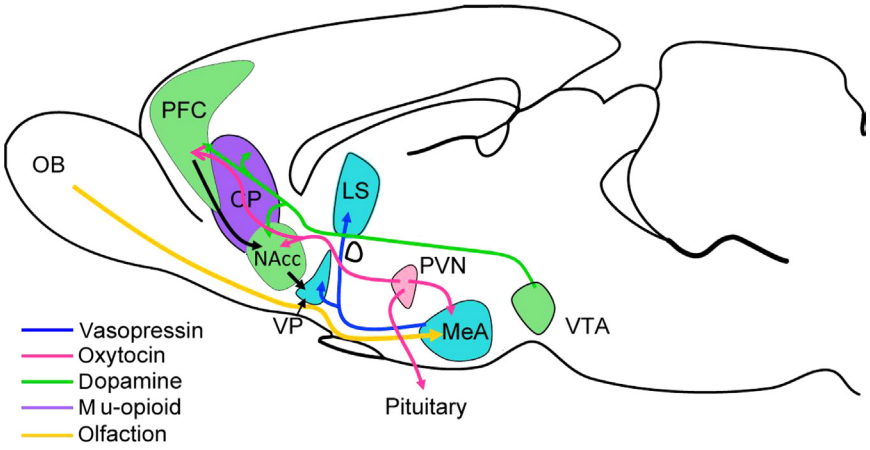

FIGURE 48.21 A schematic of the proposed neural circuitry of pair bond formation in prairie voles. Mating stimulates the release of oxytocin from neurons in the paraventricular nucleus of the hypothalamus (PVN) into the nucleus accumbens (NAcc) and prefrontal cortex (PFC) in females, and the release of vasopressin from neurons in the medial amygdala (MeA) into the ventral pallidum (VP) and lateral septum (LS) in males. Mating also stimulates the release of dopamine from neurons in the ventral tegmental area (VTA) into the NAcc and PFC. The MeA receives olfactory input from the partner and conveys the neural encoding of the olfactory signature of the partner to the striatum and LS. Mu-opioid receptors in the CP stimulate the rewarding aspects of mating. OT and AVP facilitate the processing of the olfactory signatures of the mate, and dopamine facilitates an association between the neural encoding of the social cues of the partner and the opioid-based reward and reinforcement. The actions of these neurochemicals in these circuits lead to a conditioned partner preference. *Source: Adapted from Refs 271,334.*

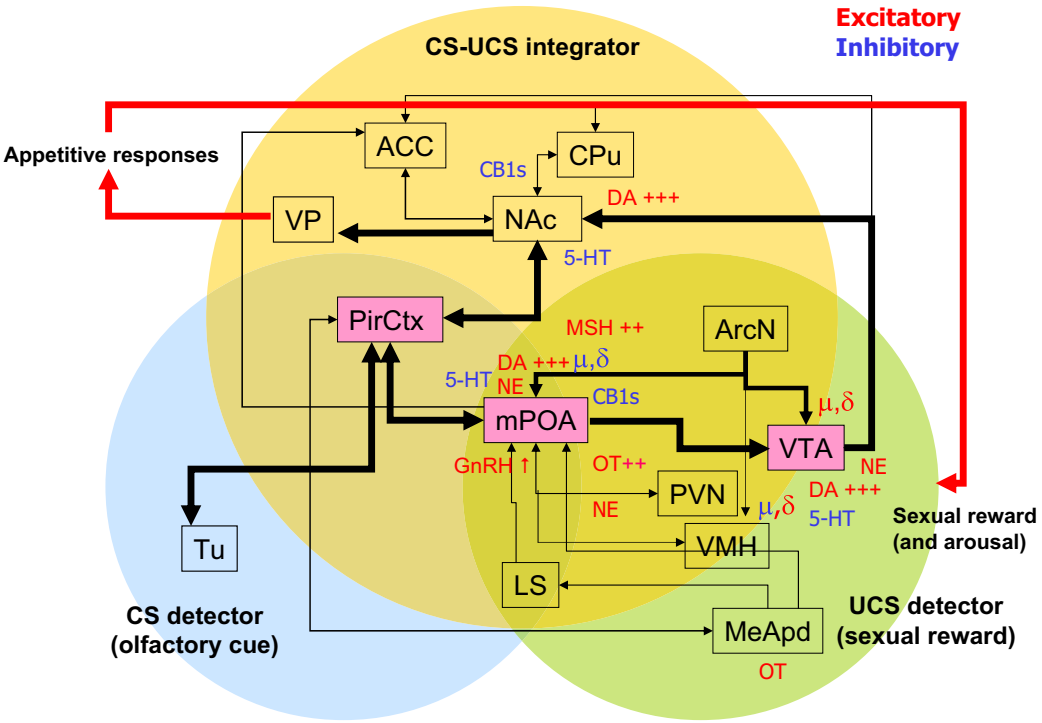

FIGURE 50.28 **Neural systems critical for the display of sexual behavior in the rat.** Appetitive behaviors made toward unconditioned or conditioned sexual incentive stimuli lead to sexual reward that is processed by three interactive systems. Two systems process olfactory stimuli and sexual reward relatively independently, whereas a third, mesolimbic DA system, acts to integrate both the conditioned olfactory cue and its rewarding sexual outcome. Three common regions, the piriform cortex, mPOA, and VTA, are activated in male and female rats by conditioned olfactory stimuli. Opioid actions in the VTA potentiate mesolimbic DA activation, whereas opioid actions in the mPOA inhibit sexual arousal and desire. Neurotransmitter systems or their receptors in red are excitatory for sexual motivation whereas those in blue are inhibitory. Black pathways signify major inputs and outputs of the system. Note that opioids can be excitatory in the VTA, inhibitory in the mPOA, or either in the VMH (depending on the receptor type). A similar system is activated in humans.[63] Abbreviations: ACC, anterior cingulate cortex; ArcN, arcuate nucleus of the hypothalamus; CB1, cannabinoid Type 1 receptor; CPu, caudate-putamen (striatum); DA, dopamine; δ, delta opioid receptors; GnRH, gonadotropin releasing hormone; LS, lateral septum; MeApd, posterior-dorsal nucleus of the medial amygdala; mPOA, medial preoptic area; MSH, melanocyte stimulating hormone; μ, mu opioid receptors; NAc, nucleus accumbens; NE, norepinephrine OT, oxytocin; PirCtx, piriform cortex; PVN, paraventricular nucleus of the hypothalamus; Tu, olfactory tubercle; VMH, ventromedial nucleus of the hypothalamus; VP, ventral pallidum; VTA, ventral tegmental area; 5-HT, serotonin. *Source: Adapted from Pfaus et al.*[683]

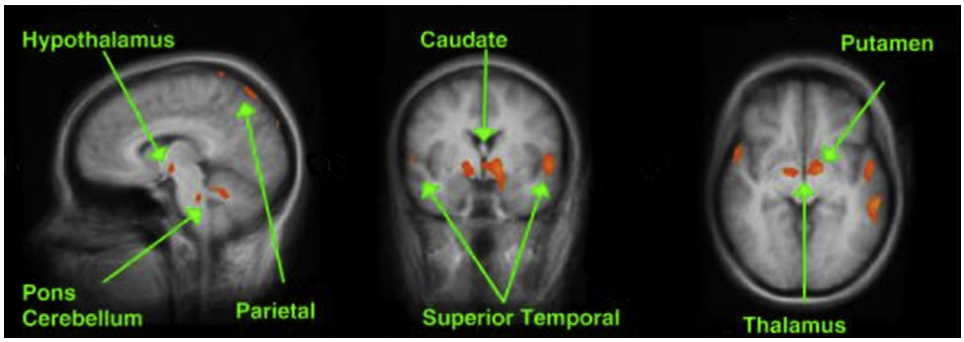

FIGURE 51.20 Sites of the maternal brain where fMRI activity was higher (indicated by red) in mothers who delivered vaginally compared to via Caesarean section while listening to their own infant crying. *Source: Modified from Swain et al., 2008.*[98]

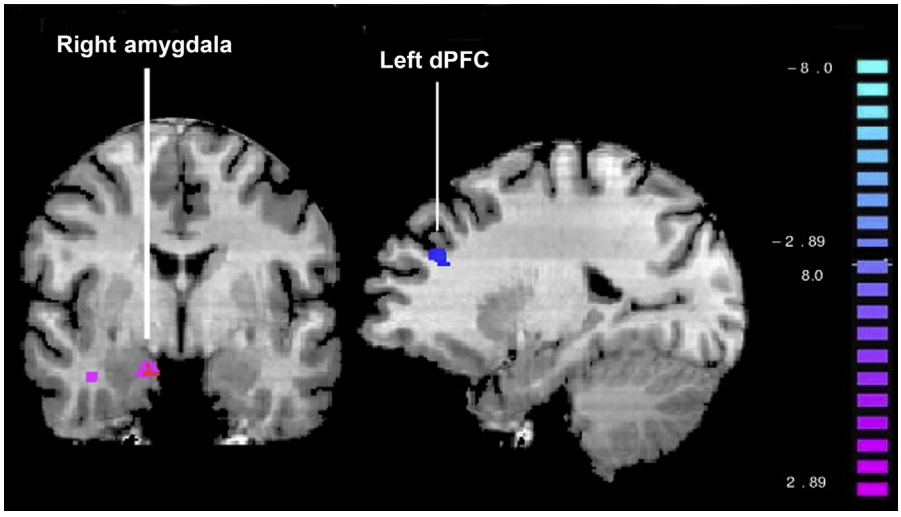

FIGURE 51.21 Sites in the parental brain where mothers (left) or fathers (right) showed greater fMRI activity in response to their own infant video compared to viewing an unfamiliar infant. Mothers showed greater activity in the right amygdala and temporal, occipital, and parietal cortices compared to fathers, while fathers had greater activity in the dorsal prefrontal cortex (dPFC). *Source: Modified from Atzil et al., 2012.*[711]

Index

Note: Page numbers followed by "b", "f" and "t" indicate boxes, figures and tables respectively.

A

A disintegrin and metalloprotease 3 (ADAM3), **I:**208
A-kinase anchoring protein (AKAP), **I:**41, **I:**121, **I:**323, **I:**896, **II:**1789
A/B domain, **I:**1103–1105
AANAT. *See* Arylalkylamine-*N*-acetyltransferase
ABC transporters. *See* ATP-binding cassette transporters
ABCA1. *See* ATP-binding cassette transporter 1
ABCG2. *See* ATP-binding cassette transporter G2; Breast cancer resistance protein (BCRP)
AbdominalB (AbdB), **II:**1717
Abnormal flagella, **I:**131
  gene knockouts, **I:**132
  heritable effects
    human sperm flagellar structure, **I:**131–132
    mouse sperm flagellar structure, **I:**132
ABP. *See* Androgen binding protein; Androgen-binding protein
Abs. *See* Antibodies
*AC*3 gene. *See* Adenylyl cyclase type 3 gene
ACAT1. *See* A–cholesterol acyl transferase
ACC. *See* Anterior cingulate; Anterior cingulate cortex
Accessory glands, **I:**809–810
  development regulation
    androgens, **I:**782–783
    estrogens, **I:**783
    neural regulation, **I:**784
    retinoids, **I:**784
  immune cells in, **I:**825–826
  inflammation on male reproduction, **I:**845–847
Accessory olfactory bulb (AOB), **II:**2232
Accessory sex glands in male
  adult prostate regulation, **I:**792–794
  anatomy and histology
    bulbourethral glands, **I:**779
    prostate anatomy, **I:**775–778, **I:**776f
    prostate histology, **I:**778–779
    seminal vesicle, **I:**779
  anterior and sagittal views, **I:**774f
  embryology and development
    accessory gland development regulation, **I:**782–784
    developmental genes, **I:**784–789
    prostate gland development, **I:**779–781
    prostate stem cells, **I:**781–782, **I:**781f
    seminal vesicle development, **I:**782
  posterior view, **I:**774f
  prostate gland diseases, **I:**794–797
  sex accessory tissues function, **I:**789–792
ACE3. *See* Angiotensin-converting enzyme-3
Acetylcholine (ACh), **II:**2227, **II:**2289
  direct application of drugs effects, **II:**2246–2247
A–cholesterol acyl transferase (ACAT1), **I:**1035
Acid phosphatase, **I:**791
Acinus structure, **I:**776
Acrosin (ACR), **I:**114
Acrosin-binding protein (ACRBP), **I:**114
Acrosomal cap, **I:**101
Acrosome, **I:**113
  components, **I:**115t–116t
  contents, **I:**114–117
  structure, **I:**113–114
ACTH. *See* Adrenal corticotropic hormone; Adrenocorticotropic hormone
Actin-related protein T2 (ACTRT2), **I:**105–106
Actins, **I:**105–106
  cytoskeleton, **I:**896
Activating protein 1 (AP1), **II:**1887
Activating transcription factor 1 (ATF1), **I:**645
Activation function-1 (AF-1), **I:**1103
Activation of the anaphase-promoting complex/cyclosome (APC/C), **I:**82–83
Active labor, **II:**1875–1876
Active transport, **II:**1754
Activin receptor-like kinase (ALK), **I:**833–834, **I:**966
Activin-regulated gene expression, **I:**668–669
Activins, **I:**375–376, **I:**787–788, **I:**964, **I:**966–967, **II:**1317, **II:**1543–1544, **II:**1795. *See also* Inhibins
Activity regulated cytoskeletal-associated protein (ARC), **I:**105–106
ACTRT2. *See* Actin-related protein T2
Acyl ghrelin (AG), **II:**1814
AD. *See* Androstenedione
ADAM3. *See* A disintegrin and metalloprotease 3
*Adamts*1 protein, **I:**1011
ADAMTS10 protein, **I:**105
ADCY. *See* Adenylyl cyclase
Adenine phosphosulfate (APS), **I:**304
Adenosine monophosphate (AMP), **II:**1625
Adenosine triphosphate (ATP), **I:**304, **I:**654, **II:**1879
Adenosine triphosphate–sensitive potassium channel (K-(ATP) channel), **II:**1625–1626
Adenosinetriphosphatase (ATPase), **I:**20–21, **I:**316
Adenylyl cyclase (ADCY), **I:**134
Adenylyl cyclase type 3 gene (*AC*3 gene), **II:**2398
ADH. *See* Antidiuretic hormone
ADHD. *See* Attention deficit-hyperactivity disorder
Adipocyte triglyceride lipase (ATGL), **II:**1767–1768
Adipokines, **II:**1811, **II:**1967
  adiponectin, **II:**1813
  expression in pregnancy complications
    GDM, **II:**1815
    preeclampsia, **II:**1814–1815
  ghrelin, **II:**1814
  leptin, **II:**1811–1813
  resistin, **II:**1814
Adiponectin, **II:**1813
Adipose tissues, **II:**2085–2086
ADM. *See* Adrenomedullin (AM)
ADP-ribosylation factor (ARF), **I:**921–922
Adrenal cortex, **I:**295
Adrenal corticotropic hormone (ACTH), **I:**337
Adrenal function during puberty, **II:**1491
Adrenal steroids
  humans, **II:**2386–2387
  laboratory rodents, **II:**2381
  sheep, **II:**2385
Adrenocorticotropic hormone (ACTH), **I:**671, **I:**671, **II:**1638, **II:**1893, **II:**1960, **II:**2168, **II:**2301
Adrenogenital syndrome. *See* Congenital adrenal hyperplasia (CAH)
Adrenogonadal primordium (AGP), **I:**272
Adrenomedullin (AM), **II:**1723, **II:**1807, **II:**1963
  placental peptide hormone, **II:**1810–1811
Adult granulosa cell tumor (AGCT), **I:**961
Adult Leydig cells, **I:**580–581
  humans, **I:**643
  LH in, **I:**642–643
  LH signaling, **I:**643–644, **I:**644f
  mouse models, **I:**642–643
Adult neurogenesis, **I:**500
Adult prostate regulation, **I:**792
  EDC, **I:**794
  protein hormones, **I:**793–794
  retinoids, **I:**793
  steroid hormones, **I:**792–793
  vitamin D, **I:**793

Adult testis function, **I:**650
AE-PCOS. *See* Androgen Excess and PCOS Society
AEA. *See* Arachidonoylethanolamine
AF-1. *See* Activation function-1
AFC. *See* Antral follicle count
Affection scale, **II:**2199
Afferent pathway mediating milk ejection, **I:**533
  DMH, **I:**540
  forebrain regions modulating, **I:**539
  hypothalamic circuitry, **I:**536–538, **I:**536f
  PVN and SON interconnections, **I:**538–539
  spinal cord and brainstem, **I:**533–536
Afferents expressing vasopressin (AVP), **II:**1624
AFM. *See* Atomic force microscopy
After-hyperpolarization (AHP), **II:**1993
AG. *See* Acyl ghrelin
AGCT. *See* Adult granulosa cell tumor
AGD. *See* Anogenital distance
Aggression, **II:**2134–2136
Aging. *See also* Female reproductive aging; Hypothalamic-pituitary aging; Male reproductive aging; Ovarian aging
  changes in spermatozoa, **I:**738–739
  of epididymal epithelium, **I:**736–737
  mechanism, **I:**738
  molecular and functional, **I:**737–738
Ago. *See* Argonaute
AGO-like proteins, **I:**31–32
Agouti gene, **II:**2042
Agouti-related protein (AGRP), **I:**538, **II:**1557, **II:**2446
  neurons, **II:**1620–1622
AGP. *See* Adrenogonadal primordium
AGRP. *See* Agouti-related protein
Ah. *See* Aryl hydrocarbon
AHA. *See* Anterior hypothalamic area
AHP. *See* After-hyperpolarization
AhR. *See* Aryl hydrocarbon receptor
AIRE. *See* Autoimmune regulator
Airn gene, **II:**2036
AIS. *See* Androgen insensitivity syndrome; Androgen-insensitivity syndrome
AKAP. *See* A-kinase anchoring protein
AKR. *See* Aldo-keto reductase
AKT. *See* Protein kinase B
Albumin excretion, **II:**1943–1944
Aldh1a2. *See* Retinaldehyde dehydrogenase 2 (*Raldh2*)
Aldo-keto reductase (AKR), **I:**297–298, **I:**1046
Aldolase a (ALDOA), **I:**130
ALK. *See* Activin receptor-like kinase
Alkaline phosphatase (AP), **I:**247
Allopregnanolone, **II:**1991
  cognitive function
    in animals, **II:**1997
    in women, **II:**1999
  hormone secretion profiles, **II:**1958f
Allostasis, **II:**1637
Alpha-activator element (α-ACT), **II:**1785
α-amino-3-hydro-5-methyl-4-isoxazole-propionic acid (AMPA), **II:**1223
α/β-hydrolase domain containing-5 (ABHD5). *See* Comparative gene identification-58 (CGI58)
α-chlorohydrin, **I:**740–741
α-glycoprotein hormone subunit (αGSU), **I:**335–336
αGSU. *See* α-glycoprotein hormone subunit
α-melanocyte-stimulating hormone (α-MSH), **I:**820, **II:**1414, **II:**1458, **II:**1620, **II:**1965, **II:**2230, **II:**2329
5α-reductase activity, **I:**721
5α-reductase deficiency, **II:**2170
20α-hydroxyprogesterone (20α-OH-P), **II:**1205
5α-hydroxyT (DHT), **I:**1106
AM. *See* Adrenomedullin
AMH. *See* Anti-Müllerian hormone
Amino acid
  anionic, **II:**1766
  cationic, **II:**1766
  neurotransmitters, **II:**1505–1507
  neutral, **II:**1765–1766
  transporter regulation, **II:**1766
Aminoaciduria, **II:**1940
AMP. *See* Adenosine monophosphate
AMPA. *See* α-amino-3-hydro-5-methyl-4-isoxazole-propionic acid
AMP-activated protein kinase (AMPK), **I:**1171, **II:**1625–1626
Amphiregulin (*Areg*), **I:**976, **I:**998, **I:**1000–1001, **II:**1328–1329
Amphitelic attachment, **I:**36
AMPK. *See* AMP-activated protein kinase
Amygdala, **II:**2234, **II:**2234f
  amygdaloid efferents, **II:**2238
  c-fos activation, **II:**2236–2237
  effects of lesions, **II:**2235–2236
  hormone implants effects, **II:**2237–2238
  microinjections of drugs, **II:**2238
Anandamide. *See* Arachidonoylethanolamine (AEA)
Anaphase-promoting complex/centrosome (APC/C), **I:**36–37
Anchoring villi, **II:**1747
Androgen binding protein (ABP), **I:**714, **I:**720–721
Androgen Excess and PCOS Society (AE-PCOS), **II:**1375–1376
Androgen insensitivity syndrome (AIS), **I:**1168–1169
Androgen receptor (AR). *See also* Estrogen receptor (ER), **I:**572–573, **I:**645, **I:**719–720, **I:**977, **I:**1100, **I:**1102–1103, **II:**1316, **II:**2112–2113, **II:**2380, **II:**2454
  cell-specific ablation, **I:**647–648
  expression in testis, **I:**646
  inducible AR ablation, **I:**650
  in Leydig cells, **I:**649
  in peritubular myoid cells, **I:**648–649
  in Sertoli cells, **I:**648
  signaling in ovarian function
    AR expression in ovary, **I:**1165–1167
    disrupted androgen signaling mouse models, **I:**1167–1170
    intraovarian role, **I:**1170–1174
    ovarian phenotypes, **I:**1168t
  signaling in uterine function
    AR expression in uterus, **I:**1127
    mice lacking AR, **I:**1127–1128
  in testicular vasculature, **I:**650
Androgen response element (ARE), **I:**320, **I:**646, **I:**785
Androgen-binding protein (ABP), **I:**646
Androgen-insensitivity syndrome (AIS), **II:**2134
Androgen(s), **I:**300–301, **I:**313, **I:**577, **I:**856–857, **II:**2112
  accessory gland development regulation, **I:**782–783
  epididymis development, **I:**696–697
  signaling, **I:**977
  steroid hormones, **I:**792
1,4,6-androstatriene-3,17-dione (ATD), **II:**2391–2392
Androstenedione (AD), **I:**302
Androtin, **II:**1396
Anemia of pregnancy, **II:**1936–1937
Anestrous season, **II:**1459–1460
Anestrus, **II:**1294–1295
Aneuploidy, **I:**36
  environmental effects on, **I:**44
Angelman syndrome (AS), **II:**2037, **II:**2040
Angiogenesis, **I:**1027, **I:**1029–1031, **I:**1323, **II:**1721–1722
Angiogenic factors, **I:**1031–1032
Angiolytic factors, **I:**1031–1032
Angiopoietin (ANGPT), **I:**1031
Angiotensin
  angiotensin II, **II:**2231
  AT2 receptor, **II:**1947
  1–7 receptor, **II:**1947, **II:**1947f
Angiotensin-converting enzyme-3 (ACE3), **I:**162
ANGPT. *See* Angiopoietin
Anionic amino acids, **II:**1766
Annulus, **I:**126
  DNAJB13, **I:**127
  SEPT, **I:**126–127
  SLC26A8, **I:**127
  during spermatogenesis, **I:**127
Anogenital distance (AGD), **I:**578
ANP. *See* Atrial natriuretic peptide
Anterior cingulate (ACC), **II:**2414
Anterior cingulate cortex (ACC), **II:**2308
Anterior hypothalamic area (AHA), **I:**400, **II:**1211, **II:**1266
Anterior pituitary gland, **I:**481
Anterior prostate, **I:**777–778
Anteroflexions, **II:**2214
Anteroventral periventricular nucleus (AVPV nucleus), **I:**285, **II:**1550, **II:**1610, **II:**1647
  cell populations in, **II:**2476
  ejaculation-activated neurons, **II:**2264–2265
  enzyme neuronal nitric oxide in, **II:**2114–2116
  GnRH neurons and, **II:**1409
  in rodent hypothalamus, **II:**1673
Anti-Müllerian hormone (AMH), **I:**274–275, **I:**567, **I:**663–664, **I:**953, **I:**959–960
  measurement of serum concentrations, **II:**1365
  production from male testes, **II:**1662
  secretion by Sertoli cells, **II:**2112
  sex differences in brain, **II:**2113f

Antibodies (Abs), **I:**103, **I:**1057
Antidiuretic hormone (ADH), **II:**1960
Antimicrobial peptides, **I:**732–733
Antiprogestins, **I:**1110
antisense morfolino oligonucleotide (as-MON), **II:**1624–1625
Antral follicle count (AFC), **II:**1668
Antral follicle development, **II:**1314–1315
  abnormal
    granulosa, **II:**1380, **II:**1382f
    theca, **II:**1379–1380
  connexins, **I:**977–979
  gap junction membrane channel proteins, **I:**977–979
  HPG, **I:**973–979
  steroid-signaling pathways, **I:**976–977
Antrum formation, **I:**1152–1153
AOB. *See* Accessory olfactory bulb
AP. *See* Alkaline phosphatase
AP1. *See* Activating protein 1
APC/C. *See* Activation of the anaphase-promoting complex/cyclosome; Anaphase-promoting complex/centrosome
Apical cells, **I:**698, **I:**700f
Apical membrane transport, **II:**2082
apo. *See* Apolipoprotein
Apolipoprotein (apo), **I:**727
Apolipoprotein B, **II:**1767
Apoptosis, **I:**483–484
Appetite regulation, **I:**494–496
Apposition, **II:**1699
APS. *See* Adenine phosphosulfate; Autoimmune polyglandular syndrome
APS-I. *See* Autoimmune polyendocrine syndrome type I
Aquaporins (AQP), **I:**730, **II:**1760–1761
  AQP5, **II:**2081
AR. *See* Androgen receptor
Arachidonoylethanolamine (AEA), **II:**1719
2-arachidonoylglycerol (2AG), **II:**1719
ARC. *See* Activity regulated cytoskeletal-associated protein
ArcN. *See* Arcuate nucleus (ARN)
Arcuate kisspeptin neurons, **I:**421–422
Arcuate nucleus (ARN), **I:**419–420, **II:**2312–2313
  infusion of kisspeptin receptor antagonist, **II:**1222
  and pregnancy, **II:**1975
  in rodent hypothalamus, **II:**1673
Arcuate POMC, **II:**1972
ARE. *See* Androgen response element
Areg. *See* Amphiregulin
ARF. *See* ADP-ribosylation factor
Argali (*Ovis ammon*), **II:**1441
Arginine vasopressin (AVP), **I:**730–731, **II:**1638, **II:**2184
  in male prairie voles, **II:**2186–2188
  neuroendocrine activation effect, **II:**1638
Argonaute (Ago), **II:**2031
Argonaute 4 (*Ago*4), **I:**15
ArKO. *See* Aromatase knockout
ARN. *See* Arcuate nucleus
ARNT. *See* Aryl hydrocarbon receptor nuclear translocator
Aromatase, **I:**917–918, **I:**976–977
  Aromatase synthesis, **I:**1153–1155
  expression, **I:**1153–1154, **I:**1154f
Aromatase knockout (ArKO), **I:**976–977
Arterial blood pressure, **II:**1934–1935
Aryl hydrocarbon (Ah), **I:**327–328
Aryl hydrocarbon receptor (AhR), **I:**327–328
Aryl hydrocarbon receptor nuclear translocator (ARNT), **II:**1751
Arylalkylamine-*N*-acetyltransferase (AANAT), **II:**1581
AS. *See* Angelman syndrome
as-MON. *See* antisense morfolino oligonucleotide
ATF1. *See* Activating transcription factor 1
ATGL. *See* Adipocyte triglyceride lipase
Atomic force microscopy (AFM), **I:**103
ATP. *See* Adenosine triphosphate
ATP-binding cassette transporter 1 (ABCA1), **I:**1035–1036
ATP-binding cassette transporter G2 (ABCG2), **I:**107
ATP-binding cassette transporters (ABC transporters), **I:**107, **I:**732, **II:**1767
ATPase. *See* Adenosinetriphosphatase
Atresia, **II:**1202
Atrial distension, **II:**1932–1933
Atrial natriuretic peptide (ANP), **II:**1931, **II:**1960
Attention deficit-hyperactivity disorder (ADHD), **II:**2136
Atypical inputs, **II:**1414–1419
Auditory system, **II:**2234
Aurora kinase (AURK), **I:**21
Autoimmune orchitis
  etiology, **I:**849–850
  experimental, **I:**850–851
Autoimmune polyendocrine syndrome type I (APS-I), **II:**1841
Autoimmune polyglandular syndrome (APS), **II:**1371
Autoimmune regulator (AIRE), **I:**816–817
Autoimmunity, **I:**817
  in epididymis, vas deferens, and prostate, **I:**851–852
  orchitis, **I:**849–851
  sperm antibodies, **I:**848–849
Autophagy, **I:**1039–1040
Autosomal recessive brachyolmia. *See* Pakistani type dysplasia
Autotransfusion, **II:**1929–1930
AVP. *See* Afferents expressing vasopressin; Arginine vasopressin
AVPV nucleus. *See* Anteroventral periventricular nucleus
Axon outgrowth, **II:**2121–2122
Axoneme
  dyneins, **I:**121
  microtubules, **I:**120–121
  tektins, **I:**121–122
5-AZA compounds, **II:**2483–2484

B

B lymphocyte stimulator (BlyS), **II:**1853
B-cell chronic lymphocytic leukemia–lymphoma 2 (BCL2), **I:**1039
B-cell leukemia/lymphoma 2 (Bcl2), **I:**955
B-cell regulation and functions, **I:**815–816
B-upstream segment (BUS), **I:**316
"Baby X" experiments, **II:**2113
Balbiani's body, **I:**64
Basal cells, **I:**700–701
Basal membrane (BM), **II:**1743–1745, **II:**1805. *See also* Microvillus membrane (MVM)
Basal metabolic rate (BMR), **II:**1928
  during pregnancy, **II:**1929f
Basal plasma membrane. *See* Basal membrane (BM)
Basal plate, **II:**1747
  blood-derived fibrinoid, **II:**1747
BASC. *See* BRCA1-associated genome surveillance complex
Base excision repair (BER), **II:**2450–2451
basic helix–loop–helix (bHLH), **I:**317
basic helix–loop–helix–leucine zipper (bHLH-Zip), **I:**327
basic leucine zipper domain (bZIP), **I:**320
Basic leucine zipper transcription factor 3 (BATF3), **I:**321
Basolateral amygdala (BLA), **II:**2407
Basolateral early endosome (BEE), **II:**2079
Basolateral membrane transporters, **II:**2081–2082
BAT. *See* Brown adipose tissue
BATF3. *See* Basic leucine zipper transcription factor 3
BCL-2 homology (BH), **I:**912
BCL2. *See* B-cell chronic lymphocytic leukemia–lymphoma 2
Bcl2. *See* B-cell leukemia/lymphoma 2
BCRP. *See* Breast cancer resistance protein
BDNF. *See* Brain-derived neurotrophic factor
Beckwith–Wiedemann syndrome (BWS), **II:**2037, **II:**2039
  incidence with ART, **II:**2039–2040
Beclin 1 (BECN1), **I:**1039–1040
Bed nucleus of stria terminalis (BNST), **I:**539, **II:**1638–1640, **II:**1975, **II:**2238, **II:**2299
  c-fos activation, **II:**2238–2239
  effects of lesions, **II:**2238
  hormonal effects, **II:**2239
BEE. *See* Basolateral early endosome
Benign prostate hyperplasia (BPH), **I:**349, **I:**773, **I:**796
BER. *See* Base excision repair
Beta-adrenergic receptor agonists, **II:**1907
Betacellulin (BTC), **I:**976, **I:**1000–1001, **I:**1003
1-beta-D-ribofuranosyl-2(1H)-pyrimidinone. *See* Zebularine
17β-estradiol, **II:**1609–1610
3β-hydroxysteroid dehydrogenase (3β-HSD), **I:**281, **I:**896
11β-hydroxysteroid dehydrogenase (11β-HSD), **II:**1796f
  11β-HSD-1 and 2 expression, **II:**1804–1805
  clinical correlations, **II:**1806–1807
  cortisol–cortisone metabolism
    developmental pattern, **II:**1804
    impact of E2-regulated placental, **II:**1805–1806
  estrogen on, **II:**1806f
  physiological relevance, **II:**1806–1807

17 beta-hydroxysteroid dehydrogenase (17βHSD), **I:**490–491, **II:**1799–1800
β-microseminoprotein. *See* Prostate-specific protein 94 (PSP94)
BH. *See* BCL-2 homology
bHLH. *See* basic helix–loop–helix
bHLH-Zip. *See* basic helix–loop–helix–leucine zipper
Bi-orientation. *See* Amphitelic attachment
Bicarbonate, **II:**1760
Binder of Sperm 1 (BSP1), **I:**211–212
Binding protein (Bip), **II:**1709–1710
Bioactive lipids, **I:**857–858
Biomechanical influences, **II:**1899
Bip. *See* Binding protein
2,2-bis(4- chlorophenyl)-1,1,1-trichloroethane (DDT), **II:**2472
Bisphenol A (BPA), **I:**44, **I:**794, **II:**2473
Bisulfite
  conversion, **II:**2487–2488, **II:**2488f
  sequencing, **II:**2470, **II:**2489
BLA. *See* Basolateral amygdala
Blastocyst, **II:**1697, **II:**1750–1751
  implantation-competent, **II:**1713–1714
  state of activity, **II:**1702
BLM helicase. *See* Bloom's syndrome mutated helicase
Blood urea nitrogen, **II:**1940
Blood volume, **II:**1929–1930
  altitude, **II:**1931
  atrial distension, **II:**1932–1933
  average red cell mass, **II:**1931f
  circulating factors, **II:**1931–1932
  hemodilution of pregnancy, **II:**1930
  plasma ANP, **II:**1932f
Blood–brain barrier, prolactin transport, **I:**478
Blood–epididymis barrier, **I:**702
  functions, **I:**703–704
  junctional proteins, **I:**703
  structure, **I:**702–703
Blood–testis barrier (BTB), **I:**615, **I:**852
Bloom's syndrome mutated helicase (BLM helicase), **I:**28–29
BlyS. *See* B lymphocyte stimulator
BM. *See* Basal membrane
BMI. *See* Body mass index
BMP. *See* Bone morphogenic protein
*Bmp*2 expression, **II:**1720
BMR. *See* Basal metabolic rate
BNST. *See* Bed nucleus of stria terminalis
BNSTp. *See* Principal nucleus of bed nucleus of the stria terminalis
Body fatness, **II:**1605
Body mass index (BMI), **II:**1376, **II:**1682, **II:**1901, **II:**1928
  weight gain during pregnancy, **II:**1929t
Body weight, **I:**494
Bohr effect, **II:**1756
Bone, **I:**497
Bone morphogenic protein (BMP), **I:**666, **I:**786–788, **I:**961–962, **I:**1037, **I:**1092
  BMP15, **I:**925, **I:**955, **I:**970–971, **I:**1003–1004
  Bmp2, **II:**1711
  BMP4, **I:**338, **I:**693, **I:**957–958
Bovine serum albumin (BSA), **I:**135
BPA. *See* Bisphenol A
BPH. *See* Benign prostate hyperplasia
Brain
  actions in of prolactin, **I:**498–502, **I:**498f–499f
  cells, **I:**412–413
  prolactin, **I:**501–502
  sexual differentiation, **II:**2479–2480
  structures, **II:**2174–2175
Brain-derived neurotrophic factor (BDNF), **I:**960, **II:**2452
Branching, **II:**2122–2123
BRCA1-associated genome surveillance complex (BASC), **I:**320–321
BrdU. *See* Bromodeoxyuridine
Breast cancer, **I:**504. *See also* Prostate cancer
Breast cancer resistance protein (BCRP), **II:**1769
Breast milk production, **II:**2058–2059
Breeding season, **II:**1459–1460
Bromodeoxyuridine (BrdU), **I:**605, **II:**2063, **II:**2412
Brothers effect, **II:**2178
Brown adipose tissue (BAT), **II:**1967, **II:**2084
Bruce effect, **II:**1997
BSA. *See* Bovine serum albumin
BSP1. *See* Binder of Sperm 1
BST. *See* Bed nucleus of stria terminalis (BNST)
BTB. *See* Blood–testis barrier
BTC. *See* Betacellulin
Bulbourethral glands, **I:**779
Bursting cells, **I:**404–405
BUS. *See* B-upstream segment
Butch, **II:**2171–2172
BWS. *See* Beckwith–Wiedemann syndrome
bZIP. *See* basic leucine zipper domain

C

C domain, **I:**1105
c-Jun N-terminal kinases 1–3 (JNK1–3), **I:**350–351
C-ROS receptor, **I:**726–727
c-Src hormone, **II:**1892
C-type natriuretic peptide (CNP), **I:**925
C/EBPα. *See* CCAAT enhancer-binding proteins alpha
C/EBPβ. *See* CCAAT enhancer-binding proteins beta
C14MC. *See* Chromosome 14 miRNA cluster
Ca^{2+} metabolism, **I:**346–349, **I:**347f
Ca^{2+}–calmodulin (CaM), **I:**643–644
Caenorhabditis elegans (*C. elegans*), **I:**18–19
CAH. *See* Congenital adrenal hyperplasia
Calcitonin gene-related peptide (CGRP), **II:**1807, **II:**2219
  placental peptide hormone, **II:**1811
Calcium, **II:**1761–1762
  channel blockers, **II:**1906
  homeostasis, **I:**497
Calcium–activated 3 (Clca3), **I:**1120
Calicin (CCIN), **I:**105–106
California Mice, **II:**2389–2390
CalM. *See* Calmodulin
Calmodulin (CalM), **II:**1709–1710
  CALM1, **I:**322–323
CalP. *See* Calpactin I
Calpactin I (CalP), **II:**1709–1710
Calpain-1 (CALPN1), **I:**105–106
Calpains, **I:**1007
Calreticulin (CALR), **I:**104
CaM. *See* Ca^{2+}–calmodulin
cAMP. *See* cyclic adenosine monophosphate
cAMP response element (CRE), **I:**1155–1156
cAMP response elementbinding protein (CREB), **I:**1092
cAMP responsive element-binding protein 1 (Creb1), **II:**1625
cAMP-dependent signaling, **I:**899–915
cAMP-response element binding (CREB), **I:**1102–1103
Candidate gene approach, **II:**2176
Cannabinoid receptor 1 (CB1), **II:**1965
Cannabinoids, **I:**544
  implantation in mice, **II:**1720
  psychoactive, **II:**1719
CAP. *See* Contraction-associated protein
Capacitation, **I:**149–150
Carbon dioxide, **II:**1757
Carbonic anhydrase IV, **I:**729–730
Carboxy terminal extension (CTE), **I:**1105
5-carboxycytosine (5caC), **II:**2463–2464
Carboxyl terminus, **II:**1624
CARD. *See* Caspase activation and recruitment domain
Cardiovascular adaptations. *See also* Metabolic adaptations
  blood volume, **II:**1929–1933
  circulatory adaptations, **II:**1933–1938
CART. *See* Cocaine-and amphetamine-regulated transcript
Caruncular foldings, **II:**1700
Casein kinase 1δ/ε (CK1), **I:**40
Caseins, **II:**2076
Caspase activation and recruitment domain (CARD), **I:**811–814
Caspases, **I:**326
Castration-resistant prostate cancer (CRPC), **I:**794–795
Catechol-*O*-methyl transferase (COMT), **II:**1977
Catecholestrogen, **II:**1705
Cathepsin Q (*Ctsq*), **II:**1750–1751
Cation channels, sperm associated family (CATSPER family), **I:**103–104
Cationic amino acids, **II:**1766
CATSPER family. *See* Cation channels, sperm associated family
Caudal-type homeobox 2 (CDX2), **II:**1750
CAV. *See* Congenital absence of vas
Caveolin1 (CAV1), **I:**107, **I:**326
CB1. *See* Cannabinoid receptor 1
CBG. *See* Corticosterone binding globulin
CBP. *See* CREB-binding protein
CBV. *See* Coelmic blood vessel
CCAAT enhancer-binding proteins alpha (C/EBPα), **I:**1009
CCAAT enhancer-binding proteins beta (C/EBPβ), **I:**728, **I:**1009, **I:**1088

CCIN. *See* Calicin
CCK. *See* Cholecystokinin
Ccnd2. *See* Cyclin-D2
CCV. *See* Clathrin-coated vesicle
CD. *See* Cluster designation; Common docking
CDK. *See* Cyclin-dependent kinase
Cdk inhibitor (CKI), **II:**1716
CDKI. *See* Cyclin-dependent kinase inhibitor
CDKN1A. *See* Cyclin-dependent kinase inhibitor 1A
CDX2. *See* Caudal-type homeobox 2
Cell cycle proteins, **I:**324
Cell death, **II:**2120–2121, **II:**2121f
Cell kinase cascades, **I:**322–324
Cell migration, **II:**2119–2120
Cell–cell cross talk, **I:**701–702, **I:**702f
Cell–oocyte complex (COC), **I:**998–1000
  cGMP regulatory pathway, **I:**1007–1008
  EGF receptor loop in, **I:**1005–1006
  expansion, **I:**1000–1001
  MAPK14-PTGS2 connection in, **I:**1006–1007
  nongenomic processes, **I:**1007
Cellular retinoic acid–binding protein (CRABP), **I:**10b
Cellular retinol-binding protein (CRBP), **I:**10b
Central integrative areas
  LH, **II:**2257
  mesocorticolimbic DA tract, **II:**2251
    c-fos expression, **II:**2252
    castration effects, **II:**2251–2252
    chemical changes, **II:**2252–2253
    direct application of drugs effects, **II:**2252
    electrolytic or cell body lesions effects, **II:**2251
  MPOA, **II:**2240
    drugs effects, direct application of, **II:**2243–2247
    effects of lesions, **II:**2240–2241
    electrical or chemical stimulation effects, **II:**2242
    of steroids effects, direct applications, **II:**2242–2243
  nigrostriatal DA tract, **II:**2253
  PVN, **II:**2253–2256
  VMH, **II:**2257–2258
Central nervous system (CNS), **I:**313, **I:**528, **II:**1226, **II:**1308–1309, **II:**1489, **II:**1765, **II:**1967
  GnRH peptide distribution, **I:**345
  prolactin actions, **I:**492
Central tegmental field (CTF), **II:**2239
  c-fos activation, **II:**2239
  connections, **II:**2240
  effects of lesions, **II:**2239
  presence of steroid receptors, **II:**2240
Centrin (CETN1), **I:**119
Centrobin (CNTROB), **I:**120
Centrosome, **I:**119–120
CER. *See* Common endosome recycling
Cerebrospinal fluid (CSF), **I:**478, **II:**1277, **II:**1967–1968
Cervical
  dilation, **II:**1884
  mucus, **I:**202–203
  repair, **II:**1884
  ripening, **II:**1883
  softening, **II:**1883
  stimulation, **II:**2304–2305
Cervix, **II:**1882–1884
CETN1. *See* Centrin
CFTR. *See* Cystic fibrosis transmembrane conductance regulator
CG. *See* Chorionic gonadotropin
CGI58. *See* Comparative gene identification-58
cGMP. *See* cyclic guanosine monophosphate
CGRP. *See* Calcitonin gene-related peptide
Chaperones, **I:**104
Chaperones calmegin (CLGN), **I:**104
Chemokines, **I:**1052–1053, **II:**1857
  parturition hormonal regulation, **II:**1897
Chemosensory circuits, **II:**2263–2264
Chemotaxis, **I:**217–218
Chiasmata, **I:**38–39
Chicken ovalbumin upstream promoter transcription factor II (COUP-TFII), **I:**578, **I:**1089–1090, **II:**1710–1711
  PR-mediated decidualization, **I:**1122
  uterine ER function, **I:**1119
Childhood, **II:**1488
Chinese hamster ovarian (CHO), **I:**1119
ChIP. *See* Chromatin immunoprecipitation
ChIP sequencing (Chip-seq), **I:**1129–1131, **I:**1132f, **II:**2486–2487
ChIP-on-Chip, **II:**2485–2486
Chloride, **II:**1758–1760
CHO. *See* Chinese hamster ovarian
Cholecystokinin (CCK), **I:**535, **II:**1508, **II:**1967, **II:**2231, **II:**2335
Cholesterol, **I:**297, **I:**791
  esterase, **I:**1035
Chorionic gonadotropin (CG), **I:**1031, **II:**1344f, **II:**1701
  mechanism of action, **II:**1344–1346
  role in corpus luteum, **II:**1343–1344
Chorionic somatomammotropin (CS), **II:**1789
Chorionic somatomammotropin. *See* human placental lactogen (hPL)
Chromatin, **II:**2028
  organization, **II:**2028–2029, **II:**2029f
Chromatin immunoprecipitation (ChIP), **I:**318–319, **I:**901
Chromatin remodeling, **II:**2441, **II:**2479–2480
  factors, **I:**276
  histone-modifying enzymes, **II:**2442–2448
  histones, **II:**2441
    "histone code", **II:**2442
    modifications, **II:**2441–2442
  nucleosomes, **II:**2441
Chromogranin A, **II:**1810
Chromosomal sex, **I:**267. *See also* Gonadal sex
  dosage compensation, **I:**269
  heteromorphic sex determination evolution, **I:**267–269
  sex chromosome-related disease, **I:**269–271
Chromosome
  aneuploidy, **I:**42–43
  instability, **I:**238–239
  movements, **I:**17–18
Chromosome 14 miRNA cluster (C14MC), **II:**1751–1752
Chronic hypoxia, **II:**1746–1747
Chronic lymphocytic leukemia (CLL), **I:**1039
Chronic pain, **I:**863
Chronic stress exposure, **II:**1639–1640
Chylomicron, **II:**2085
CIAS. *See* Complete androgen-insensitivity syndrome
Circadian, **I:**428, **I:**432
  clock, **II:**1209–1210
  rhythms, **I:**478–479
Circannual rhythms, **II:**1577–1578
Circulatory adaptations, **II:**1933
  arterial blood pressure, **II:**1934–1935
  cardiac output, **II:**1933–1934, **II:**1934f
  cardiovascular responses, **II:**1937–1938
  coagulation proteins, **II:**1938t
  hematological factors, **II:**1936–1937
  hemodynamic changes, **II:**1933t
  hemostasis, **II:**1937
  pulsatile characteristics, **II:**1936
  twin pregnancies, **II:**1936
  vascular changes, **II:**1935
CITED2 transcriptional cofactor, **I:**274
Citric acid, **I:**790
CK1. *See* Casein kinase 1δ/ε
CKI. *See* Cdk inhibitor
CKIp21. *See* Cyclin-dependent kinase inhibitor 1A (CDKN1A)
CL. *See* Corpus luteum
Clathrin-coated vesicle (CCV), **I:**363–364
Claudins, **II:**2072–2073
Clca3. *See* Calcium–activated 3
CLD. *See* Cytoplasmic lipid droplets
Clear cells, **I:**699, **I:**701f
CLGN. *See* Chaperones calmegin
Clitoral stimulation (CLS), **II:**2298–2299, **II:**2304
CLL. *See* Chronic lymphocytic leukemia
Cloacal exstrophy, **II:**2169–2170
CLS. *See* Clitoral stimulation
Cluster designation (CD), **I:**810–811, **I:**812t
CNP. *See* C-type natriuretic peptide
CNS. *See* Central nervous system
CNS1. *See* Conserved noncoding sequence-1
CNTROB. *See* Centrobin
Co-regulatory complexes, **I:**1106
Co-stimulation, **II:**1860–1861
Coagulation, **I:**789–790
COC. *See* Cell–oocyte complex
Cocaine-and amphetamine-regulated transcript (CART), **II:**1557, **II:**1620, **II:**1965
  neurons, **II:**1620
Coelmic blood vessel (CBV), **I:**280

Cognition during pregnancy, **II:**1995
in animals, **II:**1995
behavioral tests, **II:**1996
neurogenesis, **II:**1998
neuroplasticity, **II:**1997–1998
pregnancy-related hormones effects, **II:**1996–1997
investigations, **II:**2000
rodent behavioral tests, **II:**1995
in women, **II:**1998
hippocampal volume, **II:**1999
hormonal modulators, **II:**1999
Cognitive function, **II:**2133–2134
Cognitive systems, **II:**2412. *See also* Motivation systems
CA1 pyramidal cells, **II:**2413
cerebral cortex, **II:**2413
DG, **II:**2412
female rats' spatial memory, **II:**2412
mPOA, **II:**2414
parental interactions, **II:**2412
PFC, **II:**2413–2414
rodent mPFC, **II:**2414
SVZ, **II:**2412–2413
COH. *See* Controlled ovarian hyperstimulation
Cohesins, **I:**21
during cell division, **I:**21
mouse meiocytes, **I:**21–22, **I:**22f
phylogenetic analyses, **I:**23
RAD21, **I:**22–23
RAD21L, **I:**22
STAG3, **I:**23
Cohesion function, **I:**39
Colliculus tubarius, **I:**207
Colony stimulating factor-1 (CSF1), **I:**823, **I:**958, **II:**1840
Combined pituitary hormone deficiency (CPHD), **I:**341
Common docking (CD), **I:**351
Common endosome recycling (CER), **II:**2079
Comparative gene identification-58 (CGI58), **II:**1767–1768
Complete androgen-insensitivity syndrome (CIAS), **II:**2380
Computer-based modeling, **II:**1994
COMT. *See* Catechol-*O*-methyl transferase
Conditioned place preference (CPP), **II:**2339
Conditioned stimulus (CS), **II:**2213
Congenital absence of vas (CAV), **I:**825
Congenital adrenal hyperplasia (CAH), **I:**295–296, **II:**1514–1515, **II:**1899, **II:**2133–2134, **II:**2168–2169
Congenital virilizing hyperplasia. *See* Congenital adrenal hyperplasia (CAH)
Connecting stalk, **II:**1749–1750
Connective tissue growth factor (*Ctgf*), **I:**966
Connexins (Cx), **I:**977–979, **II:**1721
Cx37, **I:**1153
Cx43, **I:**574, **II:**1282
Conserved noncoding sequence-1 (CNS1), **II:**1857
Constitutive heterochromatin, **II:**2441
Context-dependent epigenetic changes, **II:**2440
Contraction-associated protein (CAP), **II:**1880–1881
Controlled ovarian hyperstimulation (COH), **I:**86–87
Controlled ovarian stimulation, **II:**1384
Convection, **II:**1755
Copper transporter 1 (CTR1), **II:**2080
Copulatory plugs, **I:**200–201
Corpora lutea, **I:**1025, **I:**1026f
long-lived, **I:**1025–1026
short-lived, **I:**1026–1027
ultrashort-lived, **I:**1027
Corpus luteum (CL), **I:**1023, **II:**1332, **II:**1537, **II:**1889
development, **I:**1028f
angiogenesis, **II:**1333–1334
luteal function preovulatory determinants, **II:**1332–1333
luteinization, **II:**1333
endocrine secretions, **II:**1334
gonadotropin secretion neuroendocrine control, **II:**1341–1343
historical perspective, **I:**1023–1025
long-lived, **I:**1025–1026
luteal cell types, **I:**1059f
function and regulation, **I:**1057–1059
small luteal cells *vs*. large luteal cells, **I:**1057
luteal function control
autocrine and paracrine regulation, **II:**1336
cellular regulation, **II:**1336–1338
luteotropic hormones, **II:**1334–1336
luteal structure regulation, **I:**1040
function in early pregnancy, **I:**1053–1057
luteolytic factors, **I:**1045–1053
luteotropic factors, **I:**1040–1045
luteinization, **I:**1027–1037
luteolysis, **I:**1037–1040
pregnancy maternal recognition, **II:**1343
CG mechanism of action, **II:**1344–1346
CG role, **II:**1343–1344
luteal–placental shift, **II:**1346–1347
primate corpus luteum regression
estradiol as luteolysin, **II:**1339
LH secretion pattern, **II:**1338–1339, **II:**1339f
luteal regression cellular correlates, **II:**1340–1341
prostaglandins, **II:**1340
PRL
mating-induced PRL control, **II:**1235–1238
secretion pattern, **II:**1235
rat estrous cycle
activation, **II:**1204
formation, **II:**1204
rescue of, **II:**1234–1235
of sheep ovaries drawing, **I:**1024f
short luteal phase, **II:**1238
short-lived, **I:**1026–1027
types, **I:**1025, **I:**1026f
ultrashort-lived, **I:**1027
Corticosteroid control, **I:**818–819
Corticosterone binding globulin (CBG), **II:**1982
Corticotropin-releasing factor (CRF), **II:**1638, **II:**2326
Corticotropin-releasing hormone (CRH), **I:**842, **II:**1491, **II:**1560, **II:**1608, **II:**1763–1764, **II:**1886, **II:**1960, **II:**2231, **II:**2390, **II:**2446
Cortisol, **II:**1889
Cortisol–cortisone metabolism, **II:**1804–1807
"Cost of producing males", **II:**2109
COUP-TFII. *See* Chicken ovalbumin upstream promoter transcription factor II
COX-1. *See* Cyclooxygenase 1
CPEB. *See* Cytoplasmic polyadenylation binding element
CPHD. *See* Combined pituitary hormone deficiency
cPLA2α. *See* Cytosolic phospholipase A2α
CPP. *See* Conditioned place preference
CRABP. *See* Cellular retinoic acid–binding protein
CRBP. *See* Cellular retinol-binding protein
CRE. *See* cAMP response element
Creatinine, **II:**1940
CREB. *See* cAMP response elementbinding protein; cAMP-response element binding
CREB-binding protein (CBP), **II:**2443
Creb1-regulated transcription coactivator-1 (Crtc1), **II:**1625–1626
Creb1. *See* cAMP responsive element-binding protein 1
Cre–LoxP strategy, **II:**1615–1616
CRES. *See* Cystatin-related epididymal spermatogenic
CRF. *See* Corticotropin-releasing factor
CRF-R1. *See* Type 1 CRF receptor
CRH. *See* Corticotropin-releasing hormone
CRISP. *See* Cysteine-rich secretory proteins
Critical fat mass hypothesis, **II:**1605
Critical weight hypothesis, **II:**1510
CRPC. *See* Castration-resistant prostate cancer
Crtc1. *See* Creb1-regulated transcription coactivator-1
Cryopreservation
embryo, **II:**1384
human oocytes, **I:**87
oocyte, **II:**1384–1385
ovarian tissue, **II:**1385
CS. *See* Chorionic somatomammotropin; Conditioned stimulus
CSF. *See* Cerebrospinal fluid; Cytostatic factor
CSF1. *See* Colony stimulating factor-1
CTE. *See* Carboxy terminal extension
CTF. *See* Central tegmental field
Ctgf. *See* Connective tissue growth factor
CTLA-4. *See* Cytotoxic T-lymphocyte associated protein 4
CTR1. *See* Copper transporter 1
Ctsq. *See* Cathepsin Q
Cuddling, **II:**2298–2299
Cullin 2 (CUL2), **I:**323
Cumulus cells, **I:**1011
expression and functions of cytokines, **I:**1011–1012
immune-related factors role, **I:**1012–1013

Cumulus expansion, **I:**919
Cumulus GCs, **I:**77–78
Cumulus granulosa cells, **I:**919, **I:**952. *See also* Mural granulosa cells
  expansion, **I:**923f, **I:**924–926
Cumulus oophorus, sperm interaction with, **I:**156–157
Cx. *See* Connexins
Cx26 expression, **II:**1721
6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), **II:**2330
Cycle of seminiferous epithelium, **I:**808
cyclic adenosine monophosphate (cAMP), **I:**41, **I:**342–343, **I:**482–483, **I:**899–915, **I:**1034–1035, **I:**1083, **II:**1263, **II:**1703, **II:**1743–1745, **II:**1880
cyclic guanosine monophosphate (cGMP), **I:**41, **I:**1050, **II:**1788, **II:**1880
Cyclin D3, **II:**1716
Cyclin-D2 (Ccnd2), **I:**1151
Cyclin-dependent kinase (CDK), **I:**21, **I:**909, **I:**965, **II:**1716
  CDK5, **I:**123–125
Cyclin-dependent kinase inhibitor (CDKI), **I:**663, **I:**965, **I:**1033
Cyclin-dependent kinase inhibitor 1A (CDKN1A), **I:**1033
Cyclooxygenase 1 (COX-1), **I:**700–701, **I:**730–731, **I:**731f, **I:**998, **II:**1718
Cyclooxygenase-2 (COX-2), **I:**497, **II:**1703, **II:**1718, **II:**2123
Cyclophosphamide, **I:**741
CYP. *See* Cytochrome P450
*CYP*19 expression. *See* Aromatase expression
Cyp19-null mice, **I:**1115
Cyp19-null ovaries, **I:**1149–1150
CYP19A1. *See* Cytochrome P450 aromatase
Cyst, **I:**40–41
Cystatin-related epididymal spermatogenic (CRES), **I:**736
*C*ysteine-*ri*ch *s*ecretory *p*roteins (CRISP), **I:**151
Cysteine-rich serine protein 1 (CRISP1), **I:**104
Cystic fibrosis transmembrane conductance regulator (CFTR), **I:**694–695, **I:**702
Cytochrome P450 (CYP), **II:**1705
  enzymes, **I:**296
Cytochrome P450 aromatase (CYP19A1), **I:**723
Cytochrome P450-oxidoreductase deficiency (POR deficiency), **I:**297
Cytokine-induced inflammation models, **I:**841
Cytokines, **I:**816f, **I:**1051–1053, **II:**1281
  expression and functions, **I:**1011–1012
  of IL6 type, **I:**832
  of TGFβ family, **I:**832
  interleukin-18 and interleukin-1 family, **I:**828–829
  parturition hormonal regulation, **II:**1897
  regulation of leukocytes, **II:**1840–1841
  response to pregnancy, **II:**1850
Cytoplasmic composition
  classes of filaments, **I:**70
  electron microscopy protocols, **I:**69
  Golgi lysosome system, **I:**67–69
  hamster oocytes, **I:**70
  of mammalian oocytes, **I:**66–67
  maternal inheritance, **I:**69–70
  oocyte mitochondria, **I:**67
  PADI6, **I:**70
  ultrastructural feature, **I:**69, **I:**69f
Cytoplasmic lipid droplets (CLD), **II:**2056
Cytoplasmic polyadenylation binding element (CPEB), **I:**75–76
Cytoskeleton, **I:**325–326
Cytosolic phospholipase A2α (cPLA2α), **II:**1717, **II:**1723
Cytostatic factor (CSF), **I:**39
Cytotoxic T lymphocytes (Tc cells), **II:**1835–1837
Cytotoxic T-lymphocyte associated protein 4 (CTLA-4), **II:**1854–1855
Cytotrophoblasts, **II:**1743–1745

D

D domain, **I:**1105–1106
D-box. *See* Distal box
D-cycloserine (DCS), **II:**2196
DA. *See* Dopamine
DAG. *See* Des-acyl-ghrelin; Diacylglycerol
Damage-associated molecular pattern (DAMP), **II:**1845–1846
DAMP. *See* Damage-associated molecular pattern
Danger theory, **II:**1845–1846
DAP. *See* Depolarizing afterpotential
DAT. *See* Dopamine transporter
days post-coitum (dpc), **I:**567
DAZL. *See* Deleted-in-azoospermia-like
DBB. *See* Diagonal band of Broca
DBD. *See* DNA-binding domain
Dbf4-dependent Cdc7 kinase (DDK), **I:**40
DBH. *See* Dopamine β; hydroxylase
DCS. *See* D-cycloserine
DCV. *See* Dense core vesicle
DD. *See* Death domain
DDK. *See* Dbf4-dependent Cdc7 kinase
DDR. *See* DNA damage and repair response
De novo lipogenesis, **II:**2085
Death domain (DD), **I:**830
Death effector domain-containing protein (DEDD), **II:**1717
Death receptor (DR), **I:**830
Decidua basalis, **II:**1846
Decidua membrane, **II:**1884–1888
Decidual
  cells, **II:**1716
  dendritic cells, **II:**1852
  hemorrhage, **II:**1770, **II:**1904
  luteotropin, **I:**1040
  macrophages, **II:**1851–1852
Decidualization, **I:**1090, **II:**1716–1717, **II:**1843–1844
  adverse effects, **II:**1724–1725
  extracellular matrix, **I:**1092–1093
  immune system, **I:**1092–1093
  mediators and products, **I:**1091–1092
  stromal cell, **I:**1091
  vascular remodeling, **I:**1092–1093
DEDD. *See* Death effector domain-containing protein
DEFB126. *See* Defensin beta 126
DEFB22. *See* Defensin beta 22
Defensin beta 126 (DEFB126), **I:**105
Defensin beta 22 (DEFB22), **I:**105
Defensins, **I:**862
Dehydroepiandrosterone (DHEA), **II:**1799, **II:**1999, **II:**2390
Delayed implantation. *See* Embryonic diapause
Delayed puberty, **II:**1461f
  disorders, **II:**1521–1522
  spring-born *vs*. autumn-born lamb, **II:**1461–1463
Deleted-in-azoospermia-like (DAZL), **I:**247, **I:**282
Delta opioids, **II:**2335–2336
Delta-like ligand 4 (DLL4), **I:**1031–1032
Delta-like ligand 1 (Dll1), **I:**786
Dendrite, **I:**412–413
Dendritic cells, **I:**701
Dendritic growth, **II:**2122–2123
Dendron, **I:**407–408, **I:**412–413
DENN. *See* Differentially expressed in normal and neoplastic cells
Dense core vesicle (DCV), **I:**543
21-deoxycortisol (21dF), **I:**305
Dentate gyrus (DG), **II:**2412
Depolarizing afterpotential (DAP), **II:**1993
DES. *See* Diethylstilbestrol
Des-acyl-ghrelin (DAG), **II:**1814
Desert hedgehog (Dhh), **I:**277, **I:**571, **I:**961
Desmosomes, **II:**2074
Detergents, **I:**296–297
Developmental Origins of Health and Disease (DOHaD), **II:**2040, **II:**2040f
  maternal constraints, **II:**2041t
    dietary exposure, **II:**2043–2044
    exposure to famine, **II:**2040–2041
    maternal obesity, **II:**2043–2044
    maternal protein restriction, **II:**2042–2043
    metastable epialleles, **II:**2042
    MTSE, **II:**2044–2045
  paternal constraints, **II:**2045–2046
Developmental pluripotency-associated 3 (DPPa3), **II:**2460
DFS. *See* Dysplasia of fibrous sheath
DG. *See* Dentate gyrus
DGK. *See* Diacylglycerol kinase
DHEA. *See* Dehydroepiandrosterone
Dhh. *See* Desert hedgehog
dHJ. *See* Double Holliday junction
DHT. *See* Dihydrotestosterone
Diabetes, **II:**1772
Diacylglycerol (DAG), **I:**177–178, **I:**346, **I:**654, **I:**1043
Diacylglycerol kinase (DGK), **I:**349–350
Diagonal band of Broca (DBB), **II:**1211, **II:**1266
Diarylpropionitrate (DPN), **I:**1106
Dickkopf (Dkk), **I:**788
Dictyate arrest. *See* Dictyotene
Dictyotene, **I:**40
Diestrus-1, **II:**1201

Diestrus-2, **II:**1201
Diet-induced obesity (DIO), **II:**1967
  evidence for impaired lactation in humans, **II:**2088
  high fat feeding and maternal, **II:**2087
  mammary gland development and function, **II:**2087–2088
Dietary exposure, **II:**2043–2044
Diethylstilbestrol (DES), **I:**953, **I:**1106, **II:**2169, **II:**2472
  treatment of pregnant mothers with, **II:**2169
Differentially expressed in normal and neoplastic cells (DENN), **I:**965–966, **II:**1379
Differentially methylated region (DMR), **II:**2036, **II:**2475–2476
Differentiating spermatogonia, **I:**595, **I:**618, **I:**624
Diffusing capacity, **II:**1939–1940
Diffusing capacity for carbon monoxide (DLCO), **II:**1939–1940
"Diffusion-limited" transport, **II:**1753
1,1′-dihexadecyl-3,3′-tetramethyl-indocarbocyanine perchlorate (C16diI), **I:**103
Dihydrotestosterone (DHT), **I:**284, **I:**714, **I:**783, **II:**1680, **II:**2300
3,4-dihydroxyphenylacetic acid (DOPAC), **II:**2326–2327
Dilation, **II:**1884
6,7-dinitroquinoxaline-2,3-dione (DNQX), **II:**2330
DIO. *See* Diet-induced obesity
Diphenylamine-2-carboxylic acid (DPC), **II:**1758–1760
Direct benefit, **II:**2180
Disorders of sexual development (DSDs), **I:**269, **II:**2137–2139
Disrupted estrogen signaling, **I:**1112–1119
  in humans, **I:**1160–1161
  vaginal and cervical phenotypes, **I:**1121
Disrupted progesterone signaling mouse models
  ovarian phenotypes in, **I:**1162–1163
  uterine phenotypes in, **I:**1112–1121
Distal box (D-box), **I:**1105
Divalent metal transporter protein (DMT1), **II:**1768–1769
Dkk. *See* Dickkopf
DLCO. *See* Diffusing capacity for carbon monoxide
Dll1. *See* Delta-like ligand1
DLL4. *See* Delta-like ligand 4
DLN. *See* Dorsolateral nucleus
DLT. *See* Dorsolateral tegmentum
DMH. *See* Dorsomedial hypothalamus
DMN. *See* Dorsomedial nucleus
DMR. *See* Differentially methylated region
DMRT1. *See* Doublesex and mab-3-related transcription factor 1
DMT1. *See* Divalent metal transporter protein
DNA binding, **I:**1104f–1105f, **I:**1105
DNA damage and repair response (DDR), **I:**84–85
DNA demethylation, **II:**2450, **II:**2451f
  in brain, **II:**2452
  GADD45, **II:**2451
  histone modifications interaction, **II:**2452–2453
  5hmC, **II:**2450
  TET proteins, **II:**2451–2452
DNA methylation, **I:**109, **II:**2030–2031
  enzymes, **II:**2448, **II:**2449f
  MBD, **II:**2448–2449
DNA methyltransferase (DNMT), **I:**236–237, **II:**2127, **II:**2448–2450
  inhibitors, **II:**2483, **II:**2484f
    5-AZA, **II:**2483–2484
    RG108, **II:**2484–2485
    Zebularine, **II:**2484
DNA methyltransferase 1 (*Dnmt*1), **I:**955
DNA methyltransferase 3-like (*Dnmt3L*), **I:**14
DNA-binding domain (DBD), **I:**315–316, **I:**645–646
DNMT. *See* DNA methyltransferase
DOHaD. *See* Developmental Origins of Health and Disease
Domestic animals, **I:**1111–1112
  embryos in, **I:**1700
  luteal function in early pregnancy, **I:**1053–1054
  luteal oxytocin role in, **I:**1036
  ovarian function
    ERα in, **I:**1135–1136
    ERβ in, **I:**1139–1140
  semen characteristics, **I:**201t
Dopamine (DA), **I:**546–547, **II:**1266–1267, **II:**2222–2223, **II:**2334, **II:**2407
  contradictory effects, **II:**2224
  daily injections of amphetamine, **II:**2223
  direct application of drugs effects, **II:**2243–2247
  ejaculation, **II:**2223–2224
  Esr1 gene, **II:**2223
  haloperidol, **II:**2223
  receptors, **II:**2223
  sexual motivation, **II:**2224–2225
Dopamine transporter (DAT), **I:**474–475
Dopamine β hydroxylase (DBH), **II:**1224, **II:**2318
Dormant blastocysts
  activation, **II:**1705–1706
    of autophagy, **II:**1706
  molecular markers, **II:**1707f
  survival in ovariectomy model, **II:**1706
Dorsolateral nucleus (DLN), **II:**2261
Dorsolateral tegmentum (DLT), **II:**2239
Dorsomedial hypothalamus (DMH), **II:**1266, **II:**1965
  neurons, **II:**1557–1558
  nucleus, **I:**536–537
Dorsomedial nucleus (DMN), **II:**1226
Dosage compensation, **I:**269, **II:**2464
Double Holliday junction (dHJ), **I:**25
Doublesex and mab-3-related transcription factor 1 (DMRT1), **I:**574–575
Down syndrome, **I:**44
DP. *See* Prostaglandin D
dpc. *See* days post-coitum
DPC. *See* Diphenylamine-2-carboxylic acid
DPL19l2. *See* Dpy-19-like-2
DPN. *See* Diarylpropionitrate
DPPa3. *See* Developmental pluripotency-associated 3
Dpy-19-like-2 (DPL19l2), **I:**110
DR. *See* Death receptor
Drug-induced erections, **II:**2214
DSDs. *See* Disorders of sexual development
Dual-specific phosphatase 6 (DUSP6), **I:**696
Dual-specificity phosphatase (DUSP), **I:**371–372
Dwarf hamsters, **II:**2392–2393
Dyneins, **I:**121
Dynorphin (Dyn), **II:**1617–1618
Dysplasia of fibrous sheath (DFS), **I:**131

E

E domain, **I:**1106
E-cadherin, **II:**1712
E-RABP. *See* Epididymal retinoic acid binding protein
E74-like factor 5 (ELF5), **II:**1750
EAA. *See* Essential amino acids
EAP1. *See* Enhanced at puberty 1
Early growth response factor 1 (Egr1), **I:**343, **I:**896, **I:**1053
Early life stress (ELS), **II:**2469–2470
EBs. *See* Embryoid bodies
eCB. *See* Endocannabinoid
ECE1. *See* Endothelin-converting enzyme
ECFV. *See* Extracellular fluid volume
ECM. *See* Extracellular matrix
Ectoplasmic specialization (ES), **I:**654
EDC. *See* Endocrine disrupting compound
EDL. *See* Efferent duct ligation
EDN. *See* Endothelin (ET)
Edn2. *See* Endothelin 2
EDNRA. *See* Endothelin receptor type A
EDS. *See* Ethane dimethane sulfonate
EEF2. *See* Eukaryotic translation elongation factor 2
Effective renal plasma flow (ERPF), **II:**1940, **II:**1940f
Efferent duct ligation (EDL), **I:**717
EG. *See* Endocrine gland
EGC. *See* Embryonic germ cell
EGF. *See* Endothelial growth factor; Epidermal growth factor
EGFR. *See* Epidermal growth factor receptor
Egg activation
  Ca^{2+}, **I:**175–176
  egg phospholipase C, **I:**177–179
  IP_3, IP_3R, **I:**176–177
  sperm phospholipase C, **I:**179–180
  SRC family kinases, **I:**177–179
Egg phospholipase C, **I:**177–179
Egr1. *See* Early growth response factor 1
eIF-IA. *See* Eukaryotic initiation factor-IA
eIF4E. *See* eukaryotic translation initiation factor 4E
Ejaculation, **I:**789, **II:**2217–2218
Electrophoresis mobility shift assays (EMSA), **I:**1133–1135
Electrophysiology
  burst firing, GnRH neuron, **I:**405–406

dendrite electrophysiology, GnRH neuron, **I:**407–408
electrical properties, **I:**407f
firing patterns, GnRH neuron, **I:**404–405
ion channels expression, **I:**406–407, **I:**406t
ELF5. *See* E74-like factor 5
ELS. *See* Early life stress
EMAS. *See* European Male Aging Study
Embryo implantation, **II:**1697–1698
comparative aspects, **II:**1700
embryo–uterine interactions, **II:**1698
connexins, **II:**1721
decidualization, **II:**1716–1717
endocannabinoid signaling, **II:**1719–1720
matrix remodeling and angiogenesis, **II:**1721–1723
molecular aspects, **II:**1708
molecular signature for attachment reaction, **II:**1712–1716
morphogens, **II:**1720–1721
prostaglandins, **II:**1718–1719
signaling network, **II:**1709f
signaling spanning different phases, **II:**1717–1723
uterine responses to factors independent of estrogen and P_4, **II:**1711–1712
uterine responsiveness to hormone-dependent factors, **II:**1708–1711
endometrial glands and secretions, **II:**1706–1707
endometrial vascular permeability, **II:**1698f
evolving technologies and discoveries, **II:**1725–1726
hormonal requirements, **II:**1700–1702
immunology
decidual response, **II:**1843–1844
leukocyte response, **II:**1844–1845
maternal–fetal interface, **II:**1844f
ripple effects from early-pregnancy events, **II:**1723
consequences of defects in attachment, **II:**1723–1724
effects from dysregulated decidualization, **II:**1724–1725
stages, **II:**1698–1700
apposition, **II:**1699
attachment, **II:**1699–1700
penetration, **II:**1700
timing, **II:**1699t
uterine receptivity, **II:**1698
window of implantation, **II:**1702–1703
Embryo self-correction, **I:**239
Embryogenesis, **I:**61
advances in embryology, **I:**243–244
AMH during, **I:**959
cavitation, **I:**241–242
chromosomal instability, **I:**238–239
compaction, **I:**241–242
deletion of *Dicer*, **I:**32
early mitotic divisions, **I:**240–241
embryonic genome activation, **I:**239–240
epigenetic reprogramming, **I:**236–238
mammalian preimplantation, **I:**233–234
ovarian germ cells initiate meiosis, **I:**13f
TE lineage specification, **I:**242–243
Embryoid bodies (EBs), **I:**252
Embryonic CB1, **II:**1719
Embryonic diapause, **II:**1704–1705
embryo in diapause, **II:**1705–1706
environmental, neural, and pituitary regulation, **II:**1705
molecular markers, **II:**1707f
obligate diapauses, **II:**1704–1705
ovarian regulation in rodents, **II:**1705–1706
strategies for marsupials and carnivores, **II:**1704f
uterine factors, **II:**1707–1708
Embryonic genome activation, **I:**239–240
Embryonic germ cell (EGC), **I:**245
Embryonic stem cell (ESC), **I:**88, **I:**244–245, **II:**2028, **II:**2451–2452
biology of, **I:**244–245
ethical and technical concerns, **I:**246–247
induced pluripotency, **I:**246
nuclear reprogramming, **I:**246
Emotion regulation systems, **II:**2409–2410
intact mPOA, **II:**2410
lower anxiety behavior, **II:**2411
maternal behavior, **II:**2411
MeA lesions, **II:**2410
suppression of anxiety, **II:**2411–2412
EMSA. *See* Electrophoresis mobility shift assays
Endocannabinoid (eCB), **II:**1964–1965
signaling, **II:**1719–1720
Endocrine disrupting compound (EDC), **I:**794, **II:**2472, **II:**2475t
BPA, **II:**2473–2474
DEHP, **II:**2473
DES, **II:**2472
DNA mutations, **II:**2473
effects on reproduction, **II:**2472
in F3 offspring, **II:**2473
HPLC, **II:**2473–2474
manmade, **II:**2472–2473
method of delivery, **II:**2474
Endocrine disruptors, **II:**1472–1473
Endocrine gland (EG), **I:**1031–1032, **II:**1784
EG-VEGF, **II:**1798–1799
Endocrine profiles of pregnancy
humans, **II:**2385–2388
laboratory rodents, **II:**2379–2384
sheep, **II:**2384–2385
Endocytosis–transcytosis, **II:**1754
Endogenous nutrients, **II:**2085
adipose tissues, **II:**2085–2086
liver, **II:**2086–2087
Endogenous opioid actions, **II:**1980
Endogenous opioid peptide (EOP), **II:**1226, **II:**1268, **II:**1546, **II:**1959, **II:**1978
Endoplasmic reticulum (ER), **I:**346, **I:**697–698
Endoreduplication, **I:**239
Endothelial growth factor (EGF), **II:**1743–1745
EGF-like factors, **I:**998–999
EGF-like growth factors, **II:**1712–1713
receptor loop in COCs, **I:**1005–1006
Endothelial-tanycyte-GnRH nerve terminal interactions, **I:**426
Endothelin (ET), **I:**1049–1050, **II:**1810
Endothelin 1 (ET1), **II:**1285, **II:**1944–1945
Endothelin 2 (Edn2), **I:**1010
Endothelin receptor type A (EDNRA), **II:**1285
Endothelin-converting enzyme (ECE1), **I:**1049
Energy partitioning, **I:**493–494
ENERKI. *See* Estrogen-nonresponsive ERα; Knock-in
Enhanced at puberty 1 (EAP1), **II:**1517, **II:**2477
Enkephalins, **II:**1975–1976
Environmental Protection Agency (EPA), **I:**794
Eomesodermin (EOMES), **II:**1750
EOP. *See* Endogenous opioid peptide
EPA. *See* Environmental Protection Agency
EPAC. *See* Exchange proteins activated by cAMP
Epiblast-like cell stage (EpiLCs), **I:**252–255
Epidermal growth factor (EGF), **I:**617–618, **I:**896, **I:**976, **II:**1228, **II:**1784, **II:**1795–1796
Epidermal growth factor receptor (EGFR), **I:**323, **I:**725–726, **II:**1706, **II:**2059–2060
Epididymal cell types, **I:**697
apical cells, **I:**698, **I:**700f
basal cells, **I:**700–701
cell–cell cross talk, **I:**701–702
clear cells, **I:**699, **I:**701f
dendritic cells, **I:**701
halo cells, **I:**701
narrow cells, **I:**698–699
principal cells, **I:**697–698, **I:**699f
Epididymal protease inhibitor (EPPIN), **I:**201
Epididymal retinoic acid binding protein (E-RABP), **I:**733–734
Epididymis, **I:**691, **I:**809–810
aging
changes in spermatozoa, **I:**738–739
of epididymal epithelium, **I:**736–737
mechanism, **I:**738
molecular and functional, **I:**737–738
autoimmunity in, **I:**851–852
cell specific expression, **I:**719f
cells in epithelium, **I:**698f
development
androgens, **I:**696–697
coiling during embryonic period, **I:**694–695
elongation during embryonic period, **I:**694–695
mesonephric formation, **I:**692–694
nephricduct formation, **I:**692–694
postnatal development, **I:**695–696
testis and male reproductive tract, **I:**694f
tubules formation, **I:**692–694
wolffian ducts, **I:**695f
wolffian formation, **I:**692–694
epididymal secretions, **I:**692

Epididymis (*Continued*)
functions in luminal compartment, **I:**704–714
immune cells in, **I:**824–825
immunology, **I:**859–860
inflammation on male reproduction, **I:**845–847, **I:**846f
protein family
antimicrobial peptides, **I:**732–733
general principles, **I:**717–718
ligands, **I:**718–727
lipocalins, **I:**733–736
transcription factors, **I:**727–729
transporters and channels, **I:**729–732
regulation
hormones, **I:**714–716
of SRD5A and androgen action, **I:**716f
testicular factors, **I:**716–717
structural organization, **I:**697f
anatomy, **I:**697
blood–epididymis barrier, **I:**702–704
epididymal cell types, **I:**697–702
as target for xenobiotics, **I:**739–742
Epididymitis, **I:**851
Epidural anesthesia, **II:**2377
Epigenesis, **I:**109
Epigenetics, **I:**109, **II:**2028, **II:**2028f, **II:**2177–2178. *See also* Reproduction
alterations to DNA, **II:**2028
ART, **II:**2466
chromatin and specific genes, **II:**2485, **II:**2486f
ChIP sequencing, **II:**2486–2487
ChIP-on-Chip, **II:**2485–2486
DNA methylation and histones, **II:**2487
chromatin organization, **II:**2028–2029, **II:**2029f
chromatin remodeling, **II:**2441–2448
CpG methylation measurement, **II:**2487
DNMT enzyme activity, **II:**2487
global methylation approaches, **II:**2487
sequence-specific methylation approaches, **II:**2487–2489
DNA methylation, **II:**2030–2031
of fertilization, **II:**2031–2032, **II:**2032f
oocyte epigenetics, **II:**2033–2034
postfertilization epigenetics, **II:**2034–2035
sperm epigenetics, **II:**2032–2033
germ cell and early embryo development, **II:**2464–2466
global demethylation phases, **II:**2465f
histone measurement, **II:**2485
histone modifications, **II:**2029–2030, **II:**2030t, **II:**2485
historical overview, **II:**2439–2440, **II:**2441f
mechanisms, **II:**2453–2464
modifications to DNA, **II:**2448
CpG islands, **II:**2448
DNA methylation enzymes, **II:**2448–2452
methylated cytosine, **II:**2448f
ncRNAs, **II:**2031
noncoding RNAs, **II:**2031
pharmacology, **II:**2483
chromatin modification, **II:**2483
DNMT inhibitors, **II:**2483–2485
publishing trends for, **II:**2440f
reprogramming, **I:**236–238
and sexual differentiation, **II:**2477–2482
social play, **II:**2482f
EpiLCs. *See* Epiblast-like cell stage
Epimutations, **II:**2465
Epiregulin (*Ereg*), **I:**976, **I:**1000–1001
EPOR. *See* Excitement-Plateau-Orgasm-Resolution
EPPIN. *See* Epididymal protease inhibitor
EPSP. *See* Excitatory postsynaptic potential
"Equational" meiosis division, **I:**5
Equatorin (EQTN), **I:**114
equine CG (eCG). *See* Pregnant mare serum gonadotropin (PMSG)
ER. *See* Endoplasmic reticulum; Estradiol receptor; Estrogen receptor
ERE. *See* Estrogen response element
Ereg. *See* Epiregulin
ERK. *See* Extracellular signal-regulated kinase
ERM. *See* Ezrin/Radixin/Moesin
Eroticization, **II:**2372
ERP. *See* Event related cortical potential
ERPF. *See* Effective renal plasma flow
*Errfi*1 gene, **I:**1120
ERV. *See* Expiratory reserve volume
ERα. *See* Estrogen receptor α
ERα immunoreactivity, **I:**1112
ERα-null mice, **I:**1114–1115
ERαAF-1^0 domain, **I:**1118
ERβ. *See* Estrogen receptor β
ERβ-null mice, **I:**1114–1115
ES. *See* Ectoplasmic specialization
ESC. *See* Embryonic stem cell
*Esco*2. *See* Establishment of cohesion 1 homolog 2
ESHRE–ASRM. *See* European Society for Human Reproduction and Embryology–American Society for Reproductive Medicine
ESR. *See* Estrogen receptor (ER)
*ESR*1 gene, **I:**1101
Essential amino acids (EAA), **II:**1764f
EST. *See* Expressed sequence tag
Establishment of cohesion 1 homolog 2 (*Esco*2), **I:**12–13
Estradiol, **II:**1663
ER signaling in ovarian function, **I:**1150
antrum formation, **I:**1152–1153
aromatase synthesis, **I:**1153–1155
disrupted estrogen signaling, **I:**1160–1161
ERα mediated repression, **I:**1159–1160
granulosa cell differentiation, **I:**1152
granulosa cell proliferation, **I:**1150–1152
luteinizing hormone receptor acquisition, **I:**1155–1156
ovulation, **I:**1158–1159
negative feedback, **I:**1156–1158
cellular mechanism, **II:**1325–1326
in human menstrual cycle, **II:**1326–1327
molecular mechanism, **II:**1325–1326
pituitary sites, **II:**1324–1325
oviducts, **I:**1081
synthesis, **I:**1153–1155
uterine response
changes in physiology, **I:**1123–1124
E2 treatment, **I:**1125f
estrogen-induced uterine proliferation, **I:**1124, **I:**1126f
estrogen–growth factor, **I:**1124–1125, **I:**1130f
Estradiol receptor (ER), **II:**1315–1316
Estrin, **II:**1395–1396
Estrogen imprinting, **I:**783
Estrogen receptor (ER), **I:**650, **I:**722–723, **I:**1081, **I:**1099–1102, **I:**1877–1878, **I:**2112–2113
endometrial compartmental cross-talk, **I:**1088
human and mouse, **I:**1102f
signaling in endometrial proliferation, **I:**1086–1088, **I:**1087f
signaling in ovarian function
disrupted estrogen signaling mouse models, **I:**1141–1150
ER-null mice, **I:**1141f
ERα, **I:**1133–1136
ERβ, **I:**1136–1141, **I:**1138f
estradiol intraovarian roles, **I:**1150–1161
expression patterns, **I:**1133, **I:**1133t
signaling in uterine function, **I:**1111
domestic animals, **I:**1111–1112
primates, **I:**1112
rodents, **I:**1111
Estrogen receptor activity (REA), **I:**1119
PR-mediated function, **I:**1122–1123
uterine ER function, **I:**1119
Estrogen receptor α (ERα), **I:**479, **I:**1146–1149, **I:**1147f, **II:**1610, **II:**1666
AF-1 or AF-2 domains, **I:**1145
domestic animals, **I:**1135–1136
estrogen action for uterine receptivity and implantation, **II:**1708
mediated repression, **I:**1159–1160
mutated DNA binding domains, **I:**1144–1145
ovarian-specific deletion, **I:**1143–1144
primates, **I:**1136
rodents, **I:**1133–1135
Estrogen receptor β (ERβ), **I:**953, **I:**1146–1149, **I:**1147f, **II:**1409
domestic animals, **I:**1139–1140
ERβ-null mice, **I:**1145–1146
primates, **I:**1140–1141
rodents, **I:**1136–1139
Estrogen reprogramming, **I:**783
Estrogen response element (ERE), **I:**642, **I:**1086, **I:**1105, **II:**1722
Estrogen-nonresponsive ERα Knock-in (ENERKI), **I:**1118, **I:**1145
Estrogen(s), **I:**300–301
accessory gland development regulation, **I:**783
action, **I:**665–666
aromatase, **I:**664
control, **I:**665
for blastocyst activation and implantation, **II:**1703

cognitive function
in animals, **II:**1996
in women, **II:**1999
effects in uterus, **II:**1708
estrogen-induced uterine proliferation, **I:**1124, **I:**1126f
estrogen–growth factor, **I:**1124–1125, **I:**1130f
executing uterine functions, **II:**1710
humans, **II:**2386
laboratory rodents, **II:**2379
nongenomic effects, **I:**666
parturition hormonal regulation, **II:**1892–1894
placenta steroid hormone function, **II:**1800–1802
hCYP19 regulation, **II:**1801f
regulation, **II:**1800, **II:**1800f
synthesis, **II:**1799–1800, **II:**1799f
sheep, **II:**2384
signaling, **I:**976–977
steroid hormones, **I:**792–793
surge, **II:**1705
target tissues, **I:**1099–1100
uterine responses to factors independent, **II:**1711–1712
Estrous behavior control
hormonal control, **II:**1276–1277
neural control, **II:**1277
Estrous cycle plasticity, **I:**431–432
circadian inputs, **I:**432
GnRH neurons, **I:**432
direct estradiol actions, **I:**433
indirect non-neuronal sites, **I:**434
indirect transsynaptic pathways, **I:**433–434
integrated model, **I:**441–443, **I:**442f
negative feedback, **I:**437–440
positive feedback, **I:**434–437
progesterone, **I:**440–441
Estrous cycles, **II:**1537–1538
Estrus, **II:**1200
Estrus termination, **II:**2337
role of PRs, **II:**2337–2338
VMH glutamate disinhibition, **II:**2338, **II:**2339f
ET. *See* Endothelin
Ethane dimethane sulfonate (EDS), **I:**822–823
Eukaryotic initiation factor-IA (*eIF-IA*), **I:**240
Eukaryotic translation elongation factor 2 (EEF2), **I:**1044
eukaryotic translation initiation factor 4E (eIF4E), **I:**958
European Male Aging Study (EMAS), **II:**1681–1682
European Society for Human Reproduction and Embryology–American Society for Reproductive Medicine (ESHRE–ASRM), **II:**1375–1376
Event related cortical potential (ERP), **II:**2308
Events of egg activation, **I:**164–165
EVT. *See* Extravillous trophoblast
Exchange proteins activated by cAMP (EPAC), **I:**899
Excitatory postsynaptic potential (EPSP), **I:**539
Excitatory system activation, **II:**2331
ACH and histamine, **II:**2334–2335
cholecystokinin, **II:**2335
delta opioids, **II:**2335–2336
dopamine, **II:**2334
GABA and glutamate, **II:**2331–2334
GNRH, **II:**2335
norepinephrine, **II:**2334
oxytocin, **II:**2335
prolactin, **II:**2335
substance P, **II:**2335–2336
Excitement-Plateau-Orgasm-Resolution (EPOR), **II:**2292–2293, **II:**2293f
ExE. *See* Extraembryonic ectoderm
Exocytotosis, **II:**2077
calcium, **II:**2078
citrate, **II:**2078
lactose, **II:**2077–2078
oligosaccharides, **II:**2077–2078
phosphate, **II:**2078
proteins, **II:**2077
Exogenous nutrients, **II:**2083
adaptations in expended energy, **II:**2084
dietary fat *vs*. de novo derived lipid, **II:**2085
increasing energy intake, **II:**2083–2084, **II:**2084f
Exonization, **II:**2458–2459
Expiratory reserve volume (ERV), **II:**1938–1939
Expressed sequence tag (EST), **I:**954–955
External determinants timing puberty, **II:**1459
photoperiod and, **II:**1460–1467
seasonal reproduction, **II:**1459–1460
sexual maturity *vs*. puberty *vs*. breeding season onset, **II:**1468–1469
Extracellular fluid volume (ECFV), **II:**1931–1932
Extracellular matrix (ECM), **I:**1029, **I:**1092–1093, **II:**1714–1715, **II:**1843–1844, **II:**1875–1876
Extracellular nucleotides, **I:**377
Extracellular signal-regulated kinase (ERK), **I:**323, **I:**472, **I:**898, **I:**1047–1048, **II:**1892, **II:**2405
Extraembryonic ectoderm (ExE), **I:**247
Extrapituitary prolactin, **I:**488–489
Extraplacental membranes, **II:**1846–1847
Extravillous trophoblast (EVT), **II:**1747, **II:**1785
Ezrin/Radixin/Moesin (ERM), **I:**83

F

F domain, **I:**1106
Faah. *See* Fatty acid amide hydrolase
FABP. *See* Fatty acid binding protein
Facilitated diffusion. *See* Facilitated transport
Facilitated transport, **II:**1754
FACS. *See* Fluorescence-activated cell sorting
FAD. *See* Flavin adenine dinucleotide
FAK. *See* Focal adhesion kinase
Familial hyperaldosteronism type 1 (FHA1), **I:**300
FAS. *See* Free alpha subunit
FAS ligand (FASL), **I:**1038–1039
FASN. *See* Fatty acid synthase
FAT/CD36. *See* Fatty acid translocase and cluster of differentiation 36
FATP. *See* Fatty acid transport protein
Fatty acid amide hydrolase (Faah), **II:**1720
Fatty acid binding protein (FABP), **II:**1767
Fatty acid synthase (FASN), **II:**2066–2067
Fatty acid translocase and cluster of differentiation 36 (FAT/CD36), **II:**1767
Fatty acid transport protein (FATP), **II:**1767
FBGRKO. *See* Forebrain glucocorticoid receptor knockout
FBN3. *See* Fibrillin 3
FCG model. See Four Core Genotypes model
FcRn. *See* Neonatal Fcg receptor
FDG. *See* Flurodeoxyglucose
FDXR. *See* Flavoprotein ferredoxin reductase
Female reproductive aging. *See also* Male reproductive aging
hormonal, follicular, and endometrial dynamics, **II:**1663f
hypothalamic-pituitary axis aging
in animal models, **II:**1671–1678
and changes in steroid feedback, **II:**1667–1668
reproductive axis integration, **II:**1668–1671
response to loss of ovarian feedback, **II:**1664–1665
standardization of perimenopause/menopause terminology, **II:**1671
ovarian aging in women, **II:**1664–1665
reproductive function, **II:**1663–1664
Female reproductive cycle, **II:**1649. *See also* Reproductive system
FHA, **II:**1650–1651
menstrual and estrous cycles, **II:**1649–1650
Female sexual behavior, **II:**2287. *See* Male sexual behavior
analysis, **II:**2289
animal models, **II:**2295
gerbils, **II:**2298
guinea pigs, **II:**2298
hamsters, **II:**2298
humans, **II:**2300–2301
macaques and nonhuman primates, **II:**2299–2300
mice, **II:**2297–2298
musk shrews, **II:**2299
rabbits, **II:**2299
rat copulation in several chambers, **II:**2297f
rats, **II:**2296–2297
voles, **II:**2298–2299
circular model, **II:**2294f
sexual brain, **II:**2290–2292
cytological changes in vaginal epithelium, **II:**2288–2289
drugs of abuse, **II:**2289
hormonal priming and control, **II:**2308–2309
ARs and aromatase, **II:**2323–2324
binding of ERs, **II:**2313–2314

Female sexual behavior (*Continued*)
classic ERs and distribution in brain, **II:**2309–2311, **II:**2310t, **II:**2314–2316
ER co-regulators, **II:**2312–2313
genitosensory stimulation activation, **II:**2323
hormone replacement therapy in women, **II:**2324–2325
membrane PRs, **II:**2317
necessity of ERs, **II:**2311, **II:**2316
neurotransmitters, **II:**2317–2320
nonclassic mechanisms of P action, **II:**2317
pattern of estradiol exposure, **II:**2311–2312
regulation of ERs, **II:**2311
role of membrane ERs, **II:**2314
steroid hormone receptors, **II:**2309, **II:**2317–2320
ultrastructural changes, **II:**2320–2323
upregulation of PRs, **II:**2316–2317
hysteria, **II:**2288
medicalization of women's bodies, **II:**2290
sexual stimulation consequences, **II:**2339–2349
sophistication growth, **II:**2289–2290
structure, **II:**2292–2294
Female-orientated rams (FOR), **II:**2167
Feminization, **II:**2130
Ferguson reflex pathway, **II:**1988–1989
Fertilization, **I:**149
egg activation, **I:**175–180
epigenetics, **II:**2031–2032, **II:**2032f
oocyte epigenetics, **II:**2033–2034
postfertilization epigenetics, **II:**2034–2035
sperm epigenetics, **II:**2032–2033
sperm capacitation, **I:**149–155
sperm–egg
interaction, **I:**155–158
membrane fusion, **I:**164–175
plasma membrane interactions, **I:**158–164
Fetal
blood vessels, **II:**1749–1750
demand, **II:**1773f
development, **I:**491–492, **II:**1495–1496
gonad, **I:**957
interface, **II:**1846–1847
Leydig cells, **I:**577–579
membrane, **II:**1884–1888
programming, **II:**1518
semi-allograft, **II:**1845–1846
Fetal antigens, maternal awareness of, **II:**1849
Fetal contribution
immunomodulatory molecules regulated expression
B7 family co-stimulatory molecules, **II:**1860–1861
HLA molecules, **II:**1858–1860
IDO, **II:**1861
TNF and receptor superfamilies, **II:**1861–1862
with maternal cells, **II:**1857–1858
Fetal epigenetic origins of disease, **II:**2027
DOHaD, **II:**2040–2045, **II:**2040f
emerging interest areas
microbiome, **II:**2046–2047
micronutrient supplementation, **II:**2047
epigenetics, **II:**2028
chromatin organization, **II:**2028–2029
DNA methylation, **II:**2030–2031
histone modifications, **II:**2029–2030
noncoding RNAs, **II:**2031
fertilization epigenetics, **II:**2031–2032
genomic changes, **II:**2027
imprinting, **II:**2035–2036
PWS, **II:**2027
Fetal growth restriction (FGR), **II:**1784
Fetal hemoglobin (HbF), **II:**1756
Feto-placental unit, **II:**1893
FGF. *See* Fibroblast growth factor
FGFR. *See* Fibroblast growth factor receptor
FGR. *See* Fetal growth restriction
FHA. *See* Functional hypothalamic amenorrhea
FHA1. *See* Familial hyperaldosteronism type 1
Fibrillin 3 (FBN3), **II:**1378–1379
Fibrin-type fibrinoid. See Basal plate blood-derived fibrinoid
Fibroblast growth factor (FGF), **I:**243, **I:**339, **I:**693, **I:**786, **I:**957–958
FGF2, **I:**1030
FGF4, **II:**1706
FGF7, **II:**1711
FGF9, **I:**14, **I:**247
Fgf-10, **I:**787
FGF21, **II:**1649
Fibroblast growth factor receptor (FGFR), **I:**725, **I:**1120–1121
FGFR2, **II:**1711
Fibrous sheath. *See also* Mitochondrial sheath
composition, **I:**127–130
fibrous sheath-associated proteins, **I:**128t
formation, **I:**127
function, **I:**130–131
general features, structure, and formation, **I:**127
structure, **I:**127
15q11q13 region, **II:**2037–2038
"Fight or flight" response, **II:**1637
Filipin, **I:**103
FK506 binding proteins, **I:**1123
Flagellar
dyskinesia, **I:**131–132
motion
flagellar wave, **I:**132–133
sperm motility regulation, **I:**133–136
wave, **I:**132–133
Flagellum, **I:**117–118
abnormal flagella, **I:**131–132
annulus, **I:**126–127
axoneme, **I:**120–122
centrosome, **I:**119–120
connecting and proximal middle piece regions, **I:**118f
connecting piece, **I:**119
cytoskeletal components, **I:**118f
fibrous sheath, **I:**127–131
flagellar motion, **I:**132–136
mitochondrial sheath, **I:**122–123
outer dense fibers, **I:**123–126
Flavin adenine dinucleotide (FAD), **I:**297–298
Flavin mononucleotide (FMN), **I:**297–298
Flavoprotein ferredoxin reductase (FDXR), **I:**297–298
Flotillin2 (FLOT2), **I:**107
"Flow-limited" transport, **II:**1753
Fluid-phase endocytosis, **II:**1754
Fluorescence recovery after photobleaching (FRAP), **I:**106–107, **I:**346
Fluorescence resonance energy transfer (FRET), **I:**176, **I:**346
Fluorescence-activated cell sorting (FACS), **I:**252, **I:**612
Flurodeoxyglucose (FDG), **II:**1666–1667
Flushing, **II:**1279–1280
FMN. *See* Flavin mononucleotide
fMRI. *See* functional magnetic resonance imaging
Focal adhesion kinase (FAK), **I:**164, **I:**364, **I:**654, **II:**1794, **II:**2122
FOG. *See* Friend of GATA
Folate, **II:**1768
Folate receptor alpha (FRα), **II:**1768
Follicle. *See* Preovulatory follicle selection
atresia, **I:**1013, **I:**1173–1174
cell differentiation, **I:**278–279
control of follicular development, **I:**1277–1278
development in human ovary, **II:**1365
gonadotropin-dependent follicle development, **II:**1367
preantral follicle development, **II:**1365–1367
primordial follicles, **II:**1366f
steroidogenesis in, **II:**1367
follicular dynamics, **II:**1261–1263
follicular rupture, **II:**1263
follicular waves and hormonal patterns, **II:**1264f
folliculogenesis, **II:**1201–1203
antral follicular development, **II:**1314–1315
follicular estradiol biosynthesis control, **II:**1314–1315
gonadotropic control, **II:**1317–1318
preantral follicular development, **II:**1313–1314
growth, **I:**1163
inhibin production by, **II:**1264–1265
ovarian, **II:**1307, **II:**1310
Follicle-stimulating hormone (FSH), **I:**61, **I:**572, **I:**637, **I:**655, **I:**809, **I:**895, **I:**973–974, **I:**1043, **II:**1199, **II:**1308, **II:**1363, **II:**1443, **II:**1539, **II:**1605, **II:**1641–1642, **II:**1701–1702. *See also* Thyroid-stimulating hormone (TSH)
AKT pathway, **I:**657
cAMP–PKA pathway, **I:**655–656
circulating concentrations, **II:**1309
desensitization, **I:**658
differentiated Sertoli cells, **I:**658–659

estradiol negative feedback action
cellular mechanism, **II:**1325–1326
in human menstrual cycle, **II:**1326–1327
hypothalamic and pituitary sites, **II:**1324–1325
molecular mechanism, **II:**1325–1326
feedback inhibition, **II:**1320
FSH-regulated signaling pathways, **I:**655, **I:**656f, **I:**916
aromatase, **I:**917–918
inhibin-α, **I:**917–918
LH receptor, **I:**916–917
FSH-responsive gene targets, **I:**916f
FSHR, **I:**659–660, **I:**662f
GDNF regulation, **I:**661
gene regulation, **I:**660–661
hypothalamo–hypophysial unit, **II:**1261
integration, **I:**661–662
intracellular Ca^{2+} pathway, **I:**658
knockout of FSHβ, **I:**659–660
MAPK pathway, **I:**656–657
mTOR pathway, **I:**657
neuroendocrine control, **II:**1323–1324
gonadotropin secretion *vs*. ovarian hormones, **II:**1324
p70S6K pathway, **I:**657
PI3K pathway, **I:**657
PLA_2 pathway, **I:**658
profiles of concentrations, **II:**1309f
rat estrous cycle, **II:**1207
receptor expression in testis, **I:**655
reproductive aging in men, **II:**1680
secretion
arcuate nucleus, **II:**1309
suppression, **II:**1319–1320
sequential secretion, **II:**1539–1540
sertoli cell proliferation regulation, **I:**659–660
signals, **I:**896–899
follicle maturation, **I:**896
granulosa cell differentiation and proliferation, **I:**896–899
receptor, **I:**896–899
testosterone-signaling pathway, **I:**661–662
threshold model, **II:**1327f
Follicle-stimulating hormone receptor (FSHR), **I:**974–975
Follicular
development, **II:**1649
dynamics, **II:**1261–1263
phase, **I:**1083
rupture, **II:**1263
Follicular estradiol biosynthesis control
autocrine and paracrine factors, **II:**1315
activin, **II:**1317
IGF1, **II:**1316–1317
IGF2, **II:**1316–1317
inhibin, **II:**1317
steroid hormones, **II:**1315–1316
gonadotropic hormones, **II:**1315
Folliculogenesis, **I:**947–948, **I:**956f
antral follicle development, **I:**973–979, **II:**1314–1315
coordination of oogenesis with, **I:**77–80
follicular estradiol biosynthesis control, **II:**1315–1317
gonadotropic control
FSH, **II:**1317–1318
LH, **II:**1318
metabolic influences, **I:**979
GH, **I:**980
IGF-signaling system, **I:**980
leptin, **I:**979
morphology
female reproductive phenotypes, **I:**949t–950t
human, **I:**952
mouse follicles, **I:**951f
murine, **I:**948–952
in mouse, **I:**951f
ovarian events, **II:**1201–1203
preantral follicular development, **II:**1313–1314
primordial follicle formation, **I:**953–957
primordial to primary follicle transition, **I:**957–961
regulation by TGFβ family signaling, **I:**961–962, **I:**962f
activins, **I:**966–967
BMP receptor type IB receptor, **I:**971
BMP signaling SMADs, **I:**967–968
follistatin, **I:**967
inhibins, **I:**962–966
oocyte-specific, **I:**968–971
transcription factors in preantral-stage
Foxo transcription factors, **I:**972
GATA transcription factors, **I:**972–973
Taf4b RNA polymerase II, **I:**971–972
TBP, **I:**971–972
Tbp2, **I:**972
Follistatin (Fst), **I:**280, **I:**375–376, **I:**967, **I:**1120, **II:**1543–1544
FOR. *See* Female-orientated rams
Forebrain glucocorticoid receptor knockout (FBGRKO), **II:**1639
Forkhead (Foxo), **I:**972
Forkhead box genes (*Fox*), **I:**785
Forkhead box L2 (Foxl2), **I:**960–961
Forkhead box O1 (FOXO1), **I:**1091, **II:**1969–1970
Forkhead box O3 (Foxo3), **I:**958
Formins, **I:**83–84
5-formlycytosine (5fC), **II:**2463–2464
Fos induction, **II:**2306
Four core genotype model (FCG model), **I:**270–271, **I:**271f, **II:**2116, **II:**2117f
4311. *See* Trophoblast-specific protein alpha (TPBPA)
Fox. *See* Forkhead box genes
FOXI1 factor, **I:**729
Foxl2. *See* Forkhead box L2
Foxo. *See* Forkhead
FOXO1. *See* Forkhead box O1
FOXO3. *See* Forkhead box O3
FP. *See* Prostaglandin F
FRAP. *See* Fluorescence recovery after photobleaching
FRC. *See* Functional residual capacity
Free alpha subunit (FAS), **II:**1666
Freeze-fracture, **I:**103
FRET. *See* Fluorescence resonance energy transfer
Friend of GATA (FOG), **I:**275
Frisch hypothesis, **II:**1606
Fructose, **I:**790
FRα. *See* Folate receptor alpha
FSH. *See* Follicle-stimulating hormone
FSHR. *See* Follicle-stimulating hormone receptor
Fst. *See* Follistatin
Functional hypothalamic amenorrhea (FHA), **II:**1374, **II:**1650–1651
functional magnetic resonance imaging (fMRI), **II:**1999, **II:**2401
Functional residual capacity (FRC), **II:**1938–1939
Fungicides, **I:**741

G

G-protein coupled receptor (GPCR), **I:**321–322, **I:**335–336, **I:**642, **I:**896–897, **II:**1584, **II:**1807
cloning, **II:**1584
GPR56, **I:**567–568
seasonal neuroendocrine synchronization, **II:**1584
"G-spot", **II:**2304
GABA. *See* Gamma-aminobutyric acid
GAD. *See* Glutamic acid decarboxylase
GAD67. *See* Glutamic acid decarboxylase 67
GADD45. *See* Growth arrest and DNA damage inducible 45
Galactopoiesis. *See* Lactation
Galanin, **I:**412, **I:**483, **II:**1227
Galanin-like peptide (GALP), **II:**2231
neurons, **II:**1623
Gamete
interaction models, **I:**155–156
transport facilitation, **I:**1082–1083
Gametogenesis, **I:**31–32
Gametogenetin 1 (GGN1), **I:**125
Gamma 1 tubulin (TUBG1), **I:**119
Gamma-aminobutyric acid (GABA), **I:**133, **I:**538, **II:**1222–1223, **II:**1266–1267, **II:**1412, **II:**1505, **II:**1546, **II:**1639, **II:**1674, **II:**2227, **II:**2404
direct application of drugs effects, **II:**2245
neuromodulators mediating milk ejection, **I:**546
synaptic plasticity, **I:**551–552
Gap junctions, **I:**1001
membrane channel proteins, **I:**977–979
GAPDHS subunit. *See* Glyceraldehyde 3-phosphated dehydrogenase, spermatogenic subunit
GAS5. *See* Growth arrest specific 5
Gastrointestinal peptides
CCK, **II:**1973
resistance in pregnancy, **II:**1974
leptin-CCK interactions, **II:**1974
prolactin-releasing peptide, **II:**1974
GC. *See* Granulosa cells
GCC. See Germ cell cluster; Germ cell cyst
GCM1. *See* Glial cells missing homolog 1
GCNA-1. *See* Germ cell nuclear antigen-1
GDF. *See* Growth and differentiation factor

GDF9. *See* Growth differentiation factor 9
GDM. *See* Gestational Diabetes Mellitus
GDNF. *See* Glial cell line–derived neurotrophic factor
GDP. *See* Guanosine diphosphate
Gene expression
  AR-dependent regulation, **I:**651–652
  GnRH pituitary actions
    αGSU, **I:**356–357
    FSHβ subunit gene promoter, **I:**359–361
    gene promoter, **I:**354–356, **I:**354f
    GnRH-induced gene network, **I:**353
    gonadotrope signature genes, **I:**353–354
    LHβ subunit gene promoter, **I:**357–359
  molecular basis for differential regulation, **I:**929–930
  transcriptional activators to regulating, **I:**916–918
General transcription factors (GTFs), **I:**319–320
Genetic influences. *See also* Sexual partner preference
  candidate gene approach, **II:**2176
  direct *vs*. indirect genetic effects, **II:**2176–2177
  epigenetics, **II:**2177–2178
  genome-wide screening, **II:**2177
  sexual orientation heritability, **II:**2176
Genetic integrity regulation, **I:**84
  DDR, **I:**85f
  DNA double-strand breaks, **I:**85–86
  metazoans, **I:**84–85
  TAp63, **I:**85
Genetic mutations, **I:**965–966
Genital ridge, **I:**248
Genitosensory stimulation, **II:**2304
  cervical stimulation, **II:**2304–2305
  clitoral and cervical overlap, **II:**2306
  clitoral stimulation, **II:**2304
Genome-wide association study (GWAS), **I:**965–966, **II:**1379
Genome-wide location analysis (GWLA), **I:**321–322
Genome-wide screening, **II:**2177
Genome–epigenome interaction, **II:**1726
Genomic imprinting, **II:**1751, **II:**2459, **II:**2459f
  ART, **II:**2460
  functions, **II:**2459–2460
  ncRNAs, **II:**2460
  "parental conflict" hypothesis, **II:**2460
  *Peg*3 gene, **II:**2460–2461
Gerbils, **II:**2298
Germ cell cluster (GCC), **I:**953
Germ cell cyst (GCC), **I:**953
Germ cell nuclear antigen-1 (GCNA-1), **I:**248–249
Germ cell-less homolog 1 (*Gmcl*1), **I:**110
Germ cell-less homolog 1 (*Drosophila*)-like (GMCL1), **I:**126
Germ cells, **I:**281–283, **I:**571–572
Germ line development, **I:**234–236, **I:**236f
  germ cell colonization of gonads, **I:**248–249
  PGC
    developmental remodeling, **I:**249
    specification, **I:**247–248
Germ line stem cell (GSC), **I:**249–250
Germinal vesicle (GV), **I:**64
  chromatin remodeling and transcriptional silencing, **I:**65, **I:**66f
  *Gdf*9, **I:**65
  immature mammalian oocyte nucleus, **I:**65f
  mammalian ova, **I:**64
  meiotic competence, **I:**64
  SN, **I:**64–65
Germinal vesicle breakdown (GVBD), **I:**41, **I:**76, **I:**971–972
Germline alpha (*Figla*), **I:**954
Germline-dependent epigenetic changes, **II:**2440
Gestational Diabetes Mellitus (GDM), **II:**1815
GFAP. *See* Glial fibrillary acidic protein
GFP. *See* Green fluorescent protein
GFR. *See* Glomerular filtration rate
GGN1. *See* Gametogenetin 1
GH. *See* Growth hormone
GH-secreting cells, **II:**1627–1628
GH-V. *See* Growth hormone variant
GHF1. *See* Growth hormone factor 1
GHR. *See* Growth hormone receptor
Ghrelin, **II:**1514, **II:**1609, **II:**1628
GHRH. *See* Growth hormone-releasing hormone
GHS-R type 1a, **II:**1628
GIOT-1. *See* Gonadotropin inducible ovarian transcription factor-1
Glial cell line–derived neurotrophic factor (GDNF), **I:**666
Glial cells missing homolog 1 (GCM1), **II:**1751
Glial fibrillary acidic protein (GFAP), **II:**2403–2404
Glial-derived growth factors, **II:**1508–1509
Global methylation approaches, **II:**2487
Global transcriptomic platforms, **I:**328
Glomerular filtration rate (GFR), **II:**1940, **II:**1940f
GLP1. *See* Zinc finger GATA-like protein 1 (ZGLP1)
Glucocorticoid modulatory element–binding protein 1 (GMEB1), **I:**961
Glucocorticoid receptor (GR), **I:**727, **I:**1100, **I:**1103
  expression, **II:**1639–1640
  *GAS*5 and, **II:**2454
  signaling, **I:**1174
    expression in uterus, **I:**1128
    uterine functions, **I:**1128–1129
Glucocorticoid response element (GRE), **I:**671
Glucocorticoids, **II:**1763–1764
  cognitive function in women, **II:**1999
  effect on LH pulsatility, **II:**1643–1644
  metabolism, **II:**1886–1887
  receptor immunoreactivity identification, **II:**1647–1648
  role in HPA axis, **II:**1640
  signaling, **I:**671–672
Glucose, **II:**1762–1764, **II:**2087
  deprivation with 2-deoxyglucose, **I:**303
  homeostasis, **I:**496–497
  uptake, **I:**2198
Glucose transporter (GLUT), **II:**1763
GLUT1 transporters, **II:**1763
GLUT3 transporters, **II:**1763
GLUT4 transporters, **II:**1763
GLUT9 transporters, **II:**1763
GLUT12 transporters, **II:**1763
Glutamate, **II:**1223–1224, **II:**1673–1674, **II:**2331–2334
  direct application of drugs effects, **II:**2246
  GnRH neuron, **I:**417–418
  neuromodulators mediating milk ejection, **I:**540–541
  neurotransmitters co-expression, **I:**412
  synaptic plasticity, **I:**550–551
  transporters, **I:**732
Glutamate receptor interacting protein 1 (GRIP1), **I:**342–343
Glutamic acid decarboxylase (GAD), **II:**1412, **II:**2236–2237
Glutamic acid decarboxylase 67 (GAD67), **II:**1674
Glutamine, **II:**1764–1765
Glutathione S-transferase (GST), **I:**698–699, **II:**1769
Glyceraldehyde 3-phosphated dehydrogenase, spermatogenic subunit (GAPDHS subunit), **I:**129
Glycogen synthase kinase (GSK), **I:**899
Glycogen synthase kinase 3 beta (GSK3B), **I:**135
Glycolysis, **I:**131
Glycosuria, **II:**1940
Glycosyl phosphatidylinositol (GPI), **I:**162–163
GM-CSF. *See* Granulocyte macrophage colony-stimulating factor
*Gmcl*1. *See* Germ cell-less homolog 1
GMCL1. *See* Germ cell-less homolog 1 (*Drosophila*)-like
GMEB1. *See* Glucocorticoid modulatory element–binding protein 1
GN11 cells. *See* GnRH-secreting neuronal 11 cells
GnIH. *See* Gonadotropin-inhibitory hormone
GnRH. *See* Gonadotropin-releasing hormone
GnRH neurons, **I:**413, **I:**418, **I:**432, **II:**1400, **II:**1672–1673, **II:**1676–1677
  burst firing, **I:**405–406
  dendrite electrophysiology, **I:**407–408
  direct estradiol actions, **I:**433
  embryonic development, **II:**1400–1402, **II:**1400f
  female HPG axis, **II:**1405f
  firing patterns, **I:**404–405
  GABA, **I:**415–417
  genes encoding for proteins, **II:**1401t
  glutamate, **I:**417–418
  indirect non-neuronal sites, **I:**434
  indirect transsynaptic pathways, **I:**433–434

kisspeptin, **I:**418–422, **I:**419f
male HPG axis, **II:**1408f
metabolic targeting, **II:**1610
ablation of insulin receptors, **II:**1611
Cre–LoxP approaches, **II:**1610–1611
direct effects of key metabolic hormones, **II:**1611
ghrelin role, **II:**1611
modes of GABA action, **I:**415f
morphology, **I:**400–402, **I:**402f
neuroanatomy, **I:**400
cell bodies distribution, **I:**400, **I:**401f
morphology, **I:**400–402, **I:**402f
projections, **I:**402–404
neurokinin B, **I:**422–423
neuropeptide actions, **I:**414t–415t
non-neuropeptidergic neurotransmitter actions, **I:**413t
NPY, **I:**423–424
postnatal maturation, **II:**1402–1409
RFRP-3, **I:**425–426
structural maturation, **II:**1409
transcriptional activity, **II:**1403t
GnRH receptor (GnRHR), **I:**335
GnRH secretion
energy metabolism pathways, **II:**1457–1459
neuroendocrine integrative mechanism, **II:**1221f
neuropeptide modulators control, **II:**1220–1227
neurotransmitters control, **II:**1220–1227
prepubertal hiatus in, **II:**1509–1516
transition into adulthood, **II:**1443
estradiol feedback inhibition, **II:**1447–1451
hypothalamus competency, **II:**1446–1447
LH in OVX, **II:**1446f
LH pulse frequency, **II:**1446f, **II:**1450f
ovary competency, **II:**1444–1445
pituitary competency, **II:**1445–1446
preovulatory competency, **II:**1444
GnRH-green fluorescent protein (GnRH-GFP), **I:**401
GnRH-secreting neuronal 11 cells (GN11 cells), **II:**1611
GnRH1 neurons. *See* Gonadotropin-releasing hormone (GnRH)
GnRHR. *See* GnRH receptor
Golgi lysosome system, **I:**67–69, **I:**68f
Gonadal function during puberty
ovary, **II:**1490–1491
testis, **II:**1489–1490
Gonadal hormones, **II:**2112–2113
effects, **II:**2118
cell death, **II:**2120–2121
cell migration, **II:**2119–2120
morphology and number of microglia, **II:**2119f
neurogenesis, **II:**2118–2119
epigenetic factors role, **II:**2125
DNA methylation, **II:**2127–2128
histone modifications, **II:**2126–2127
neural morphology and neurochemistry differentiation, **II:**2121
axon outgrowth, **II:**2121–2122
branching, **II:**2122–2123
dendritic growth, **II:**2122–2123
neurochemical phenotype, **II:**2124–2125
Gonadal independence of postnatal switches, **II:**1500–1501
Gonadal sex, **I:**272. *See also* Phenotypic sex
plasticity, **I:**283–284
sex determination, **I:**272
genes implication in, **I:**272
testis and ovary development, **I:**276–277
cell migration and vasculature formation, **I:**279–280
germ cells, **I:**281–283
interstitial cell differentiation, **I:**280–281
supporting cell differentiation, **I:**277
Gonadal stem cells, **I:**249–250
exploration, **I:**250–251
hESC, **I:**250
pluripotent stem cells, **I:**250
SSC, **I:**250
Gonadal steroid action, **I:**313–314, **I:**314f
coregulators and gonadal steroid physiology, **I:**327–328
historical perspectives, **I:**314
informatic approaches and transcriptional regulation, **I:**328–329
nontranscriptional effectors
caspases, **I:**326
cell cycle proteins, **I:**324
cell kinase cascades, **I:**322–324
cytoskeleton, **I:**325–326
ubiquitin pathway, **I:**324–325
steroid signaling via membrane receptors, **I:**326–327
transcriptional actions, **I:**314–315
gonadal steroid receptors, **I:**315f, **I:**316
steroid receptor transcriptional coregulators, **I:**316–320
steroid transcriptional targets, **I:**321–322
transcription factor cross talk, **I:**320–321
Gonadal steroid hormones, **I:**479
Gonadotrope-specific element (GSE), **II:**1785
Gonadotropes, **II:**1661–1662
autocrine and paracrine regulation, **I:**353–354
activins, **I:**375–376
extracellular nucleotides, **I:**377
follistatin, **I:**375–376
guanylyl cyclases, **I:**377–378
inhibin, **I:**375–376
natruiretic peptides, **I:**377–378
NO, **I:**377–378
PACAP, **I:**376–377
changes in biosynthesis, **I:**344
lineage, and fertility, **I:**337
NR5A1, **I:**342–344
paracrine factors, **I:**338–341
pituitary development, **I:**338–341, **I:**339f
lineage specification, **I:**341–342
transcription factor network regulation, **I:**341–342
pituitary organogenesis, **I:**337
inductive model, **I:**338
signature genes, **I:**353–354
Gonadotropin inducible ovarian transcription factor-1 (GIOT-1), **I:**896
Gonadotropin secretion
developmental pattern
fetal, **II:**1495–1496
infant, **II:**1496–1497
juvenile, **II:**1497–1498
moment-to-moment changes, **II:**1497f
neonatal, **II:**1496–1497
pubertal, **II:**1498–1499
disruptions of reproductive function, **II:**1552–1553
lactation, **II:**1555–1558
puberty, **II:**1553–1555
feedback control, **II:**1538
early models, **II:**1538–1539
secretion of FSH and LH model, **II:**1539–1540
tonic and surge secretion model, **II:**1540–1541
follicular estradiol patterns, **II:**1538f
gonadal function control, **II:**1537
gonadotropin surge hypothalamic control, **II:**1549–1552
endogenous preovulatory LH and GnRH surges, **II:**1549f
hypothalamic areas, **II:**1550
hypothalamo–hypophysial unit, **II:**1550f
neural circuits for GnRH surge control, **II:**1550–1552
neuroendocrine control, **II:**1493–1495
neuroendocrine systems mediating steroid feedback, **II:**1544
hypothalamic systems, **II:**1545–1546
ovarian steroids effects on pituitary, **II:**1544–1545
ovarian cycles, **II:**1537–1538
species differences in gonadal feedback, **II:**1541
LH surge control, **II:**1542
steroid inhibition of episodic LH secretion, **II:**1542–1543
tonic FSH secretion control, **II:**1543–1544
tonic LH secretion control, **II:**1542
suppression of reproduction, **II:**1558
nutritional, **II:**1560–1561
parasagittal section of hypothalamus, **II:**1562f
psychosocial and pheromonal control, **II:**1561–1562
seasonal breeding, **II:**1558–1559
stressors, **II:**1559–1560
tonic gonadotropin secretion hypothalamic control
anatomical sites of action, **II:**1546–1548
negative feedback actions of E_2, **II:**1547f
neural circuits mediating negative feedback actions, **II:**1548–1549

Gonadotropin signaling in ovary, **I:**895.
See also Ovary
FSH signals
follicle maturation, **I:**896
FSH and FSH receptor, **I:**896–899
FSH-regulated signaling pathways, **I:**916–918
granulosa cell differentiation and proliferation, **I:**899–915
LH signals, **I:**919
LH surge
LH receptor, **I:**920–922
luteinization, **I:**919–920
oocyte maturation, **I:**919–920
ovulation, **I:**919–920
LH-stimulated signaling pathways, **I:**923f
cumulus granulosa cell expansion, **I:**924–926
luteinization, **I:**927–929
oocyte maturation, **I:**924–926, **I:**924f
ovulation, **I:**926–927
in preovulatory cells, **I:**922–924
molecular basis for differential regulation, **I:**929–930
Gonadotropin surge hypothalamic control, **II:**1549–1552
endogenous preovulatory LH and GnRH surges, **II:**1549f
hypothalamic areas, **II:**1550
hypothalamo–hypophysial unit, **II:**1550f
neural circuits for GnRH surge control, **II:**1550–1552
Gonadotropin-dependent follicle development, **II:**1367
Gonadotropin-inhibitory hormone (GnIH), **I:**425, **I:**809, **II:**1266–1267, **II:**1592
RF-amide orthologue, **II:**1214
Gonadotropin-releasing hormone (GnRH), **I:**335, **I:**399, **I:**480, **I:**642, **II:**1199, **II:**1259, **II:**1308–1309, **II:**1364, **II:**1441, **II:**1489, **II:**1544, **II:**1575–1576, **II:**1605, **II:**1672–1673, **II:**2229–2230
attenuation with aging, **II:**1670–1671
biosynthesis, **I:**409f
GnRH-1 gene, **I:**408–409
GnRH-1 mRNA, **I:**409–410
GnRH-1 peptide, **I:**410–411
GnRH-associated peptide, **I:**411
regulation, **I:**411
estrous cycle plasticity, **I:**431–443
expression in MBH, **II:**1666
gene expression, **II:**1649
GnRH1, **I:**964
heterogeneity, **I:**431
hypothalamic GnRH pulse generator, **II:**1310–1313
hypothalamic neural and glial networks, **II:**1673
juvenile hiatus neurobiological bases, **II:**1501
amino acid neurotransmitters, **II:**1505–1507
confocal images, **II:**1502f
glial-derived growth factors, **II:**1508–1509
hypogonadotropic state, **II:**1503f
kisspeptin, **II:**1502–1505, **II:**1504f
neuronal factors, **II:**1508
NPY, **II:**1507–1508
opioid peptides, **II:**1508
structural remodeling, **II:**1509
LHRH, **I:**399
metabolic targeting, **II:**1610
ablation of insulin receptors, **II:**1611
Cre–LoxP approaches, **II:**1610–1611
direct effects of key metabolic hormones, **II:**1611
ghrelin role, **II:**1611
negative feedback actions of E_2, **II:**1547f
neural network, **II:**1409
components, **II:**1412–1414
embryonic development, **II:**1409–1411
postnatal development, **II:**1410f, **II:**1411–1412
transcriptional repression mechanism, **II:**1419
neurobiology, **I:**399–400
neuron
electrophysiology, **I:**404–408
neuroanatomy, **I:**400–404
neurotransmitters co-expression, **I:**411–412
neuronal network, **I:**412–413
glial elements, **I:**426
modes of GABA action, **I:**415f
neuronal elements, **I:**413–426
neuropeptide actions, **I:**414t–415t
non-neuropeptidergic neurotransmitter actions, **I:**414t
neuropeptide, **II:**1661–1662
non-GnRH neurons, **II:**1547
pattern, **II:**1261
pituitary actions
acute regulation, **I:**346–349
Ca^{2+} metabolism, **I:**346–349, **I:**347f
context-dependent GnRHR signaling, **I:**372–375
decoding GnRH pulse frequency, **I:**368–372, **I:**368f, **I:**370f
desensitization, **I:**361–363
extrapituitary GnRHRs, **I:**372–375
heterotrimeric G-protein-independent signaling, **I:**363–364
heterotrimeric G-proteins, **I:**346–349
intracellular GnRHRs, **I:**364–365, **I:**366f
ligand bias, **I:**372–375
lipid rafts, **I:**365–368
MAPK, **I:**350–353
multiple lipid-derived messengers, **I:**349–350
receptors, **I:**345–346, **I:**361–363
regulation of gene expression, **I:**353–361
placental peptide hormone, **II:**1808–1809
pulse generator, **II:**1641–1642
fasting effects, **II:**1644–1645
follicular development, **II:**1649
neural components identification, **II:**1642
stress-induced suppression, **II:**1638, **II:**1645–1646, **II:**1648
stressors and, **II:**1648
signaling from plasma membrane, **I:**351f
stress impact, **II:**1638
surges, **II:**1549f
generation, **II:**1651
preovulatory, **II:**1649
system, **II:**1471–1472
synchronization and pulsatility, **I:**428f
extrinsic pulse generation, **I:**429
intrinsic pulse generation, **I:**428–429
pulse generation, **I:**426–428
surge generation, **I:**429–431
treatment with E_2 on pituitary response, **II:**1545f
Gonadotropins
acute actions, **II:**1337
pituitary competency, **II:**1445–1446
deficiency, **II:**1385–1386
and disordered regulation, **II:**1372–1374
functional disorders, **II:**1374
long-term actions, **II:**1337–1338
pattern during menstrual cycle, **II:**1309–1310
Gonads, **I:**295
Gonocytes, **I:**10–12
GPCR. *See* G-protein coupled receptor
GPI. *See* Glycosyl phosphatidylinositol
GPR54. *See* KISS1-derived peptide receptor (KISS1R)
GR. *See* Glucocorticoid receptor
Graafian follicle, **II:**1201–1202, **II:**1363
Granulocyte macrophage colony-stimulating factor (GM-CSF), **II:**1714
Granulosa cells (GC), **I:**77–78
apoptosis, **I:**1013
AR signaling
differentiation, **I:**1171–1173
proliferation, **I:**1170–1171
cAMP/PKA activates ERK, **I:**903f
differentiation and proliferation
FSH increases intracellular Ca^{2+}, **I:**904–905
FSH signaling pathways, **I:**899, **I:**899f
functions in preovulatory follicles, **I:**1002–1003
linking LH receptor
ERK1/2central role, **I:**1005
LH–EGF receptor connection, **I:**1003–1004
LH–EGF receptor pathway, **I:**1004–1005
RAS, **I:**1005
mitochondrial-dependent apoptotic pathway, **I:**913f
PKA
AKAPs with, **I:**900–901, **I:**900f
holoenzymes, **I:**899
signals to activate ERKs, **I:**902–904
signals to activate PI-3K pathway, **I:**905–915
in undifferentiated granulosa cells, **I:**901–902
PR signaling
differentiation, **I:**1152
proliferation, **I:**1150–1152
signal connections, **I:**1005
testosterone synergizes, **I:**915
transcriptional activators, **I:**916
aromatase, **I:**917–918
inhibin-α, **I:**917–918
LH receptor, **I:**916–917
Gravid uterus, **II:**1876, **II:**1878
cervix, **II:**1882–1884
changes in PTGS2, **II:**1885f
decidua membrane, **II:**1884–1888

fetal membrane, **II:**1884–1888
myometrium, **II:**1879–1882, **II:**1881f
parturition process, **II:**1878–1879
synthesis of biologically active PGs, **II:**1886f
*Grb*10. *See* Growth factor receptor-bound 10
GRE. *See* Glucocorticoid response element
Green fluorescent protein (GFP), **I:**103, **I:**252, **I:**474, **I:**718, **II:**1409
GRIP1. *See* Glutamate receptor interacting protein 1
Growth and differentiation factor (GDF), **I:**666
Growth arrest and DNA damage inducible 45 (GADD45), **II:**2451
Growth arrest specific 5 (*GAS*5), **II:**2454
Growth differentiation factor 9 (GDF9), **I:**65, **I:**249, **I:**968–970, **II:**1278
Growth factor receptor-bound 10 (*Grb*10), **II:**1751
Growth factor receptors, **I:**725–726
Growth hormone (GH), **I:**337, **I:**793–794, **I:**980, **I:**1044–1045, **II:**1281, **II:**1457, **II:**1511–1512, **II:**1789, **II:**2059
Growth hormone factor 1 (GHF1), **I:**337
Growth hormone receptor (GHR), **I:**793–794, **I:**980, **II:**2063
Growth hormone variant (GH-V), **II:**1791–1792
Growth hormone-releasing hormone (GHRH), **II:**1457
GSCGerm line stem cell
GSE. *See* Gonadotrope-specific element
GSK. *See* Glycogen synthase kinase
GSK3B. *See* Glycogen synthase kinase 3 beta
GST. *See* Glutathione S-transferase
GTFs. *See* General transcription factors
GTP. *See* Guanosine triphosphate
Guanosine diphosphate (GDP), **I:**346, **I:**655
Guanosine triphosphate (GTP), **I:**346, **I:**655
Guinea pigs, **II:**2298
GV. *See* Germinal vesicle
GVBD. *See* Germinal vesicle breakdown
GWAS. *See* Genome-wide association study
GWLA. *See* Genome-wide location analysis

H

H19 long intergenic noncoding RNA (H19 lincRNA), **II:**1751
HA. *See* Hyaluronic acid; Hypothalamic amenorrhea
*Hairy/Enhancer-of-split*1 (*Hes*1), **I:**281
Halo cells, **I:**701
Hamsters, **II:**2298
HAND1. *See* Heart and neural crest derivatives expressed protein 1
Hand2. *See* Heart-and neural crest derivatives–expressed protein 2
*Has*2. *See* Hyaluronan synthase 2; Hyaluronyl synthetase-2
HAT. *See* Histone acetyltransferase
HB-EGF. *See* Heparin-binding-epidermal growth factor
HbF. *See* Fetal hemoglobin
HbA. *See* Maternal hemoglobin
hCG. *See* human chorionic gonadotropin
HCG-H. *See* Hyperglycosylated HCG
HDAC. *See* Histone deacetylase; Histone deacetyltransferase
HDC. *See* Histidine decarboxylase
HDL. *See* High-density-lipoprotein
HDLR. *See* High-density lipoprotein receptor
"Head-to-head" position, **I:**1105
"Head-to-tail" fashion, **I:**1105
Heart and neural crest derivatives expressed protein 1 (HAND1), **II:**1750–1751
Heart-and neural crest derivatives–expressed protein 2 (Hand2), **I:**1086, **I:**1120–1121, **II:**1711
Heat shock 70 kDa protein 1 (*Hsp*70.1), **I:**240
Heat shock protein (HSP), **I:**315–316
Heat-killed *Escherichia coli* (HKE), **II:**1903–1904
HEK cells. See Human embryonic kidneycells
helper T lymphocytes (Th cells), **II:**1835–1837
Hematological factors, **II:**1936–1937
Heme-oxygenase 1 (HO1), **II:**1797–1798
Hemochorial placentation, **II:**1838
Hemodilution of pregnancy, **II:**1930
Hemostasis, **II:**1937
Heparin-binding-epidermal growth factor (HB-EGF), **II:**1706, **II:**1712
autoinduction loop, **II:**1713–1714
as reciprocal mediator, **II:**1713f
Hepatocyte growth factor (HGF), **I:**726
Hepatocyte growth factor-like protein (HGFL), **I:**726
*Hes*1. *See* Hairy/Enhancer-of-split1
hESC. *See* Human embryonic stem cell
HESC. *See* Human endometrial stromal cell
Heteromorphic sex determination evolution, **I:**267–269
Heteromorphism, **I:**267–268
Heterotrimeric G-protein-independent signaling, **I:**363–364
Heterotrimeric G-proteins, **I:**346–349
Hexokinase type 1, spermatogenic cell variant (HK1S), **I:**129
HFD. *See* High fat diet
HGF. *See* Hepatocyte growth factor
HGFL. *See* Hepatocyte growth factor-like protein
HIF. *See* Hypoxia-inducible factor
High fat diet (HFD), **II:**2043
High mobility group (HMG), **II:**2457
High performance liquid chromatography (HPLC), **I:**724
High-density lipoprotein receptor (HDLR), **I:**327
High-density-lipoprotein (HDL), **II:**1767
High-mobility group (HMG), **I:**319
Highly stress resilient (HSR), **II:**1650–1651
Hinge region, **I:**1105–1106
HIOMT. *See* Hydroxyindole-*O*-methyltransferase
Hippocampus, **II:**1959
Histamine, **I:**547, **II:**1702–1703, **II:**2334–2335
Histidine decarboxylase (HDC), **II:**1702
Histone acetyltransferase (HAT), **I:**316, **I:**318, **II:**2442–2443
Histone deacetylase (HDAC), **I:**316–317, **II:**2126
Histone deacetyltransferase (HDAC), **II:**2442–2443
Histone methyltransferase (HMT), **II:**2446, **II:**2447t
Histone-modifying enzymes, **II:**2442–2443, **II:**2444t
HATs, **II:**2443
HDAC, **II:**2443–2446
HMT, **II:**2446
Histones, **II:**2441, **II:**2442f
demethyltransferase, **II:**2446–2448
isolation, **II:**2485
modifications, **I:**109, **II:**2029–2030, **II:**2030t, **II:**2441–2442
visualization, **II:**2485
"Histones Code", **II:**2441–2442, **II:**2443f
HK1S. *See* Hexokinase type 1, spermatogenic cell variant
HKE. *See* Heat-killed *Escherichia coli*
HLA. *See* Human leukocyte antigen
HMG. *See* High mobility group; High-mobility group
hMG. *See* human menopausal gonadotropin
HMT. *See* Histone methyltransferase
HO1. *See* Heme-oxygenase 1
Hofbauer cells, **II:**1745
HOMA-IR. *See* Homeostasis model assessment of insulin resistance
Homeobox (Hox), **I:**784, **II:**1717
Homeodomain-containing transcription factors, **I:**728–729
Homeostasis, **II:**1637
Homeostasis model assessment of insulin resistance (HOMA-IR), **II:**1967–1968
Hormonal influence, **I:**795–796
Hormonal regulation, **I:**739–740
Hormone action
changes in, **II:**2175–2176
principles, **II:**2219
genomic effects, **II:**2219
nongenomic effects, **II:**2219–2220
Hormone replacement therapy (HRT), **II:**1372
Hormone response element (HRE), **I:**1103–1105
Hormone signaling in testis
cell associations, **I:**640f
endocrine disruptors, **I:**637
estrogen, **I:**664–666
FSH, **I:**655–662
germ cell progression, **I:**641f
glucocorticoid signaling, **I:**671–672
hormone action, **I:**640–642, **I:**641f
IGF signaling, **I:**669–670
inhibin–activin signaling system, **I:**666–669
insulin–IGF–Insl3 signaling, **I:**669
leptin signaling, **I:**672
LH, **I:**642–645
mineralocorticoids, **I:**672
osteocalcin, **I:**669
prolactin, **I:**672–673
spermatogenesis, **I:**638–640
testis anatomy, **I:**638, **I:**639f
testosterone, **I:**645–655
thyroid hormone, **I:**662–664

Hormone-sensitive lipase (HSL), **II:**1767–1768
Hormone(s), **II:**1839–1840
epididymis regulation, **I:**714–716
lactogenic, **II:**1374
LH, **II:**1334–1335
FSH, **II:**1335
PRL, **II:**1335–1336
luteotropic, **I:**1024–1025
metabolic, **II:**1513–1514
neuropeptide, **II:**1985
for onset of maternal behavior, **II:**2376–2377
endocrine profiles of pregnancy, **II:**2379–2385
epidural anesthesia, **II:**2377
mothering without pregnancy, **II:**2378–2379
nonhormonal maintenance, **II:**2377–2378
ovarian steroid, **I:**1077
peripheral, **II:**1609–1610
placental, **II:**1751
placental peptide, **II:**1809
primary hypothalamic, **II:**1638
AVP, **II:**1638
CRF, **II:**1638
UCNs, **II:**1638–1639
PRL-like luteotropic, **I:**1040–1042
Horsetail motion, **I:**17–18
Hox. *See* Homeobox
Hox genes, **I:**784–785
Hoxa genes, **I:**1092
HPA axis. *See* Hypothalamic–pituitary–adrenal axis
HPA axis aging
in animal models, **II:**1671–1672
GABA, **II:**1674
glial-GnRH regulation, **II:**1675–1676
glutamate, **II:**1673–1674
GnRH neurons, **II:**1672–1673, **II:**1676–1677
hypothalamic neural and glial networks, **II:**1673
kisspeptin, **II:**1673, **II:**1677
NKB, **II:**1677
nonhuman primates, **II:**1675–1676
ovarian aging, **II:**1678
PDYN, **II:**1677
pituitary aging in females, **II:**1677–1678
of reproductive senescence in females, **II:**1678–1679
rodents, **II:**1672
steroid hormone actions and receptors, **II:**1677–1678
steroid hormone receptors and feedback, **II:**1674–1675
and changes in steroid feedback, **II:**1667
estrogen positive feedback with aging, **II:**1668
hypothalamus, **II:**1667–1668
pituitary, **II:**1668
steroid negative feedback with aging, **II:**1668
reproductive axis integration, **II:**1668
early ovarian aging, **II:**1668–1669
menopause transition, **II:**1669–1671, **II:**1670f
uterine aging, **II:**1671
response to loss of ovarian feedback, **II:**1665–1666
hypothalamus, **II:**1666–1667
pituitary, **II:**1667
steroid negative feedback, **II:**1666–1667
standardization of perimenopause/menopause terminology, **II:**1671, **II:**1672f
HPD. *See* Hypothalamo-pituitary disconnection
HPG axis. *See* Hypothalamic–pituitary–gonadal axis
HPG mouse. *See* Hypogonadal mouse
hPL. *See* human placental lactogen
HPLC. *See* High performance liquid chromatography
HPO axis. *See* Hypothalamic–pituitary–ovarian axis
HPV. *See* Human papilloma virus
hpy. *See* Hydrocephalic-polydactyl
HRE. *See* Hormone response element
HRE-independent steroid receptor activation. *See* Indirect/tethered actions
HRT. *See* Hormone replacement therapy
Hsd17b3 expression, **I:**1160
HSDs. *See* Hydroxysteroid dehydrogenases
HSL. *See* Hormone-sensitive lipase
hSMVT. *See* Human Na^+–multivitamin transporter
HSP. *See* Heat shock protein
Hsp70. 1. *See* Heat shock 70 kDa protein 1
HSR. *See* Highly stress resilient
5-HT transporter-linked promoter region (5-HTTLPR), **II:**1640–1641, **II:**1651
Human assisted reproductive technologies (Human ART), **I:**60–61, **I:**165, **II:**2460
COH, **I:**87
estradiol and, **I:**1086
free βhCG, **I:**1055
human oocyte cryopreservation, **I:**87
imprinting disorders and, **II:**2039
BWS, **II:**2039
BWS with ART, **II:**2039–2040
implications, **II:**2040
AS with ART, **II:**2040
IVM human oocytes, **I:**87
lifestyle factors and oocyte quality, **I:**86–87
oocyte biology
context of fertility preservation, **I:**88, **I:**88f
and regenerative medicine, **I:**88–89
oocyte in, **I:**86
human chorionic gonadotropin (hCG), **I:**243, **I:**642, **I:**896–897, **I:**974, **I:**1029, **II:**1206, **II:**1315–1316, **II:**1683, **II:**1701–1702, **II:**1784, **II:**1843
function, **II:**1787–1789
hypothesis, **II:**1788f
secretion, **II:**1785–1787
serum concentrations, **II:**1786f
stimulation, **II:**1798f
structure, **II:**1784, **II:**1785f
α-hCG, **II:**1784–1785
β-hCG, **II:**1785
synthesis, **II:**1785–1787
variants of, **II:**1790t
Human embryonic kidney cells (HEK cells), **I:**897
Human embryonic stem cell (hESC), **I:**233–234
Human endometrial stromal cell (HESC), **I:**1128
Human endometrium, **I:**1078, **I:**1083, **I:**1084f
compartmental cross-talk mediators
ESR, **I:**1088
PGR, **I:**1089–1090
decidualization, **I:**1090
extracellular matrix, **I:**1092–1093
immune system, **I:**1092–1093
mediators and products, **I:**1091–1092
stromal cell, **I:**1091
vascular remodeling, **I:**1092–1093
genomic profiling, **I:**1085–1086
histology, **I:**1083–1085
steroid hormone signaling, **I:**1086
ESR signaling, **I:**1086–1088, **I:**1087f
PGR signaling, **I:**1088–1089
stromal cells in vitro, **I:**1085f
structure, **I:**1083–1085
Human evolution, **II:**1876–1878
Human female sexual behavior, **II:**2300–2301
Human folliculogenesis, **I:**952. *See also* Murine folliculogenesis
Human lactation studying, **II:**2058–2059
Human leukocyte antigen (HLA), **I:**817, **II:**1837, **II:**1858
class I genes, **II:**1858, **II:**1858f
class IB HLA, **II:**1859–1860
and immune cell interactions, **II:**1859f
trophoblast HLA-C, **II:**1859
trophoblast repression, **II:**1858–1859
Human maternal structural features, **II:**1846
extraplacental membranes, **II:**1846–1847
placenta development, **II:**1846–1847
syncytial shedding, **II:**1847
human menopausal gonadotropin (hMG), **II:**1386
Human menstrual cycle, **II:**1367–1368, **II:**1368f
Human Na^+–multivitamin transporter (hSMVT), **II:**1760
Human oocyte cryopreservation, **I:**87
Human oviducts
anatomy, **I:**1077–1079
embryology, **I:**1077–1079
estradiol, **I:**1081
fluid, **I:**1082–1083
gamete transport facilitation, **I:**1082–1083
histology, **I:**1077–1081
human fallopian tube
anatomic regions, **I:**1078f
cross-section of, **I:**1079f
human uterus, **I:**1080f
ovarian steroid hormones, **I:**1077
progesterone, **I:**1081
in reproduction, **I:**1079–1080

Human papilloma virus (HPV), **I:**1121
Human parturition pathophysiology
  decidual hemorrhage, **II:**1904
  postterm birth, **II:**1904–1905
  preterm birth, **II:**1900–1902
    animal models, **II:**1902–1904
    progestin therapy to prevention, **II:**1902
  uterine atony, **II:**1905
Human placenta, **II:**1741, **II:**1742f. *See also* Placental transport function
  amino acid transport systems, **II:**1759t
  glucose transporter isoform protein expression, **II:**1758t
  perfusion, **II:**1749f
  villus, **II:**1741–1742
Human placental development
  developmental milestones, **II:**1747–1748
    lacunar stage, **II:**1748–1749
    nonvillous placental components, **II:**1749–1750
    prelacunar stage, **II:**1748
    villous stage, **II:**1749
  molecular control, **II:**1750
    blastocyst, **II:**1750–1751
    C19MC, **II:**1752
    GCM1, **II:**1751
    genomic imprinting, **II:**1751
    noncoding RNAs, **II:**1751–1752
    origin of trophoblastic cell lineage, **II:**1750–1751
    placental hormones, **II:**1751
    polar trophectoderm, **II:**1750–1751
    TPBPA, **II:**1751
    trophectoderm, **II:**1750
  stages, **II:**1748f
human placental lactogen (hPL), **I:**487–488, **II:**1789–1790
Human placental morphology. *See also* Placental transport function
  basal plate, **II:**1747
  extravillous trophoblast, **II:**1747
  junctional zone, **II:**1747
  nonvillous support, **II:**1747
  placental villous tree, **II:**1745–1747
  placental villus, **II:**1743–1745
  terminal villus, **II:**1744f
Human pregnancy. *See also* Pregnancy
  glomerular capillary permselectivity, **II:**1943
  intraglomerular hemodynamics, **II:**1942
  postpartum glomerular hemodynamics, **II:**1942–1943
Human steroid biosynthesis, **I:**295
  cholesterol conversion, **I:**297–300, **I:**299f
  classes, **I:**298t
  comparison with species, **I:**308–309
  composite steroidogenic pathway, **I:**299f
  cytochrome P450 enzymes, **I:**296, **I:**300f
  detergents, **I:**296–297
  gene mutations, **I:**297
  HSD, **I:**297, **I:**302–303
  microsomal P450 enzymes, **I:**300–302
  pathways, **I:**305–308
    adrenal, **I:**306f
    alternate or backdoor pathway, **I:**308f
    gonads and placenta, **I:**307f
  steroid
    degradation, **I:**304–305
    excretion, **I:**304–305
    hydroxylation, **I:**300–302
    sulfonation, **I:**303–304
  steroidogenesis, **I:**296f
HYAL5. *See* Hyaluronoglucosaminidase 5
Hyaluronan synthase 2 (*Has*2), **I:**1000–1001
Hyaluronic acid (HA), **I:**221, **I:**969
Hyaluronoglucosaminidase 5 (HYAL5), **I:**114
Hyaluronyl synthetase-2 (*Has*2), **I:**1167
Hydrocephalic-polydactyl (*hpy*), **I:**132
6-hydroxydopamine (6-OHDA), **II:**2217
4-hydroxyestradiol (4-OH-E_2), **II:**1705
Hydroxyindole-*O*-methyltransferase (HIOMT), **II:**1581
21-hydroxylase deficiency (21OHD), **I:**295–296
5-hydroxymethyl-cytosine (5hmC), **I:**249, **II:**2450, **II:**2463–2464
3-hydroxy-3 methylglutaryl CoA (HMG–CoA), **I:**1034
17-hydroxyprogesterone (17OHP), **I:**301
Hydroxysteroid dehydrogenases (HSDs), **I:**297, **I:**302–303
5-hydroxytryptamine (5-HT). *See* Serotonin
Hyperglycosylated HCG (HCG-H), **II:**1789
Hypermethylation, **II:**1641
Hyperprolactinemia, **I:**503–504, **II:**1374, **II:**1386
Hyperventilation, **II:**1938
Hypocretins, **II:**1227–1228
Hypogonadal mouse (HPG mouse), **I:**335, **I:**576
Hypogonadism, **II:**1681–1682
Hypogonadotropic hypogonadism, **II:**1373–1374
Hypoperfusion lesions, **II:**1769–1770
Hypophysial clamp, **II:**1544–1545
Hypophysiotropic clamps, **II:**1324–1325
Hypothalamic
  deiodinase mediation, **II:**1587–1589
  neural and glial networks, **II:**1673
  pulse generator, **I:**426–427
Hypothalamic amenorrhea (HA), **II:**1373
Hypothalamic–pituitary–adrenal axis (HPA axis), **II:**1560, **II:**1959, **II:**1980–1981
  activation, **II:**1637
  adaptations at the anterior pituitary, **II:**1982
  adrenomedullary, **II:**1986
  allopregnanolone-opioid mechanism, **II:**1981f
  basal activity, **II:**1981–1982
  central mechanism regulation, **II:**1982–1983
  inhibitory endogenous opioid mechanism, **II:**1983–1984
  neural systems mediating stress responses, **II:**1639–1640
  Penka gene expression, **II:**1986
  pregnancy hormones role, **II:**1984
    glucocorticoid negative feedback, **II:**1985–1986
    neuropeptide hormones, **II:**1985
    sex steroids, **II:**1984–1985
  primary hypothalamic hormones, **II:**1638
    AVP, **II:**1638
    CRF, **II:**1638
    UCNs, **II:**1638–1639
  responses to stress in pregnancy, **II:**1982
  stress response modulation, **II:**1640–1641
  sympathetic responses, **II:**1986
Hypothalamic–pituitary–gonadal axis (HPG axis), **I:**952, **I:**973, **II:**1396–1397, **II:**1560, **II:**1605, **II:**1641–1642. *See also* Reproduction; Reproductive system
  AMH production, **II:**1662
  changes with aging in male, **II:**1681f
  FSH, **I:**973–974
  FSHR, **I:**974–975
  human mutations, **I:**974t
  LH, **I:**975
    receptor, **I:**975–976
  molecular mediators for central control, **II:**1625
    AMPK, **II:**1626
    Crtc1, **II:**1626
    K-(ATP) channel, **II:**1627
    leptin-induced STAT3 signaling, **II:**1627
    mTOR, **II:**1625–1626, **II:**1626f
    PI3K role, **II:**1627
  pubertal process, **II:**1662
  reproduction control regulation levels, **II:**1661–1662
  reproductive aging process, **II:**1661
Hypothalamic–pituitary–ovarian axis (HPO axis), **II:**1364
Hypothalamo-pituitary disconnection (HPD), **II:**1585
Hypothalamo–hypophysial function control
  FSH secretion control, **II:**1275–1276
  GnRH and LH secretion control, **II:**1268–1269
    estradiol actions, **II:**1270
    estradiol negative feedback, **II:**1271–1272, **II:**1272f
    KNDy neurons, **II:**1269f
    progesterone actions, **II:**1270
    progesterone negative feedback, **II:**1270–1271
    pulse generator, **II:**1269–1270
  GnRH and LH surges control
    estradiol action, **II:**1272–1275
    estrogen-induced LH, **II:**1275f
    hormonal control, **II:**1272
    progesterone action, **II:**1275
    time course of changes, **II:**1273f
    transmitters implicated in induction, **II:**1274t
  neuroanatomy, **II:**1266–1268, **II:**1267t
Hypothalamo–hypophysial unit
  FSH Secretion, **II:**1261
  LH and GnRH Patterns, **II:**1261
  parasagittal section, **II:**1550f

Hypothalamus, **II:**1552, **II:**1965
aging effects, **II:**1683
NEDA neurons in, **I:**473f
ovarian feedback, **II:**1667–1668
parasagittal section of, **II:**1562f
prolactin secretion inhibition, **I:**472–474
regulator of thyrotrophs, **I:**483
reproductive aging in men, **II:**1682
steroid feedback, **II:**1666–1667
Hypoxia, **I:**1010
Hypoxia-inducible factor (HIF), **II:**1722–1723
HIF1, **I:**693, **I:**897–898
HIF1A, **I:**318–319

I

IAM. *See* Inner acrosomal membrane
IAP. *See* Intracisternal A particle element
IAPs. *See* Inhibitors of apoptosis
IC. *See* Imprinting center
ICAM1. *See* Intercellular adhesion molecule-1
ICE. *See* Imprinting control element
ICM. *See* Inner cell mass
ICR. *See* Imprinting control region
ICS. *See* Immotile-cilia syndrome
ICSI. *See* Intracytoplasmic sperm injection
ICV injection. *See* Intracerebroventricular injection
ID domain. *See* Intrinsically disordered domain
IDO, 3-dioxygenase. *See* Indoleamine 2
IFITM proteins. *See* Interferon-induced transmembrane proteins
IFN regulatory factor 2 (IRF2), **II:**1703–1704
IFNγ. *See* Interferon-γ
IFN&epsiInterferon-ε
IFNτ. *See* Interferon tau
IGF. *See* Insulin-like growth factor
IGF binding protein 1 (IGFBP1), **II:**1717
IHC studies. *See* Immunohistochemical studies
IHH. *See* Indian hedgehog
IL region. *See* Infralimbic region
IL-1α. *See* Interleukin-1 alpha
IL-1β. *See* Interleukin-1beta
IL1. *See* Interleukin 1
IL6. *See* Interleukin-6
ILCs. *See* Immature Leydig cell population
IMM. *See* Inner mitochondrial membrane
Immature intermediate villi, **II:**1745–1746
Immature Leydig cell population (ILCs), **I:**281
Immediate-early gene expression, **II:**2404–2405
Immotile-cilia syndrome (ICS), **I:**131
Immune cells, **I:**820
in accessory glands, **I:**825–826
dendritic cells, **I:**820–823
eosinophils, **I:**824
in epididymis, **I:**824–825
lymphocytes, **I:**823–824
macrophages, **I:**820–823
mast cells, **I:**824
quantification, **I:**820t
in urethra, **I:**825–826
in vas deferens, **I:**825–826
Immune function sex-specific regulation, **I:**819–820
Immune privilege in male reproduction, **I:**852
blood–testis barrier, **I:**852
epididymis, **I:**859–860
immune tolerance, **I:**853–855
immunoregulation, **I:**853–855
spermatogenic cell, **I:**858
testicular immune privilege, **I:**859
testicular immunoregulatory mechanism, **I:**855–858
testis transplantation, **I:**858
transplantation studies, **I:**852–853
vas deferens, **I:**859–860
Immune response, adaptive, **I:**814, **I:**815f, **II:**1852
autoimmunity, **I:**817
B-cell regulation and functions, **I:**815–816
immunological tolerance, **I:**816–817
initiation and control, **I:**817–818
NK cell regulation and functions, **I:**816
NK T-cell regulation and functions, **I:**816
placental contribution to tolerance, **II:**1854f
regulatory lymphocyte subsets, **I:**816–817
rejection responses in immunity, **I:**817
T cells, **II:**1853–1854
regulation and functions, **I:**815–816
regulatory, **II:**1854–1857
Treg cells, **II:**1853f, **II:**1855f–1856f
Immune system, **I:**1092–1093
adaptive, **II:**1835–1837
immune response, **I:**814–818
general principles, **I:**810–811
innate immune response, **I:**811
inflammation, **I:**814
innate immunity activation, **I:**811–814
pattern recognition receptors, **I:**811–814
mucosal immunity, **I:**818
regulation of immunity, **I:**818–820
Immune tolerance, **I:**853
FAS ligand, **I:**854
immunological responses, **I:**855
MHC, **I:**854–855
Immune-related factors, **I:**1012–1013
Immunity regulation
corticosteroid control, **I:**818–819
immune function sex-specific regulation, **I:**819–820
Immunocontraception, **I:**863
Immunoglobulins, **I:**203, **I:**791, **I:**862, **II:**1762, **II:**2079
Immunohistochemical studies (IHC studies), **II:**1612–1613
Immunohistochemistry, **I:**612–614
Immunological tolerance, **I:**816–817
Immunology of pregnancy
embryo implantation, **II:**1843–1845
features, **II:**1839
female reproductive organs, **II:**1838–1839
fetal contribution, **II:**1857–1862
fetal–maternal interface features, **II:**1837–1838
genetic disparity
fetal antigens maternal awareness, **II:**1849
fetal semi-allograft, **II:**1845–1846
human maternal structural features, **II:**1846–1847
maternal–fetal cell coexistence and exchange, **II:**1847–1849, **II:**1848f
immune system, **II:**1835
innate immune system, **II:**1835–1837
leukocyte cell lineages with key roles, **II:**1836b
maternal contribution, **II:**1849–1857
maternal tolerance of fetus, **II:**1838f
nonpregnant uterus features, **II:**1839
female immune response, **II:**1841–1842
hormones, **II:**1839–1840
leukocytes, **II:**1839–1840
leukocytes cytokine regulation, **II:**1840–1841
oocyte development, **II:**1841
preimplantation embryo development, **II:**1842–1843
origins of pregnancy disorders, **II:**1862
deficiency in Treg cells, **II:**1863–1864
disturbances to HLA-G, **II:**1864
dysfunction in uNK cells, **II:**1864
placental responses to infection, **II:**1863
preventing fetal immune rejection, **II:**1837
"Th1/Th2" hypothesis, **II:**1837
Immunomodulators, **I:**1051–1053
Immunoregulation, **I:**853
FAS ligand, **I:**854
immunological responses, **I:**855
MHC, **I:**854–855
by seminal plasma, **I:**860–861
Immunotherapy, **I:**863
Implantation fossa, **I:**117–118
Implantation-competent blastocysts, **II:**1713–1714
Imprinting, **II:**2035
disorders, **II:**2036–2037
AS, **II:**2037
and ART, **II:**2039–2040
15q11q13 region, **II:**2037–2038
implications, **II:**2038–2039
PWS, **II:**2037
SRS, **II:**2038
TNDM1, **II:**2038
genes and disease, **II:**2035t
insulator model, **II:**2036
mechanism, **II:**2035–2036
noncoding RNA model, **II:**2036
Imprinting center (IC), **II:**2036
Imprinting control element (ICE), **II:**2036
Imprinting control region (ICR), **II:**2036, **II:**2460
In situ hybridization (ISH), **II:**1211
in vitro 2-cell block, **I:**240
In vitro fertilization (IVF), **I:**165, **I:**234, **I:**706, **I:**1136, **II:**1668, **II:**1701–1702, **II:**2039, **II:**2460
In vitro maturation (IVM), **I:**1014
In vitro oocyte maturation (IVM), **I:**60
INAH3. *See* Interstitial nucleus of anterior hypothalamus
Indian hedgehog (IHH), **I:**281, **I:**1086
Indirect transsynaptic actions, **I:**438–440
Indirect/tethered actions, **I:**1109

Indoleamine 2, 3-dioxygenase (IDO), **I:**856, **II:**1843, **II:**1861
Indomethacin, **II:**1906
induced pluripotent stem cells (iPSCs), **I:**88, **I:**233–234, **I:**624, **II:**1726
Infancy, **II:**1488
Infant development, **II:**1496–1497
Infantile period, females, **II:**1404
Infertility, **I:**257, **I:**607–608, **I:**980, **I:**1014
  anovulatory, **I:**1014
  socially induced, **II:**1646
Inflammation, **I:**814, **I:**818, **I:**847–848
  corticosteroid control, **I:**818–819
  cytokine-induced, **I:**841
  effects
    on Leydig cell, **I:**840–843
    on spermatogenesis, **I:**840–845
  enzymes involved in, **I:**813t–814t
  in epididymis, vas deferens, and accessory glands, **I:**845–847
  of immune response, **I:**826
  in myometrium, cervix, and decidua, **II:**1876
  sterile, **I:**863
  of testis, **I:**806–807
  vascular, **I:**843
Inflammatory mediators, **I:**845–847
Inflammatory signaling pathways, **I:**838
  for male fertility, **I:**839–840
  seminiferous epithelium cycle, **I:**838–839
  spermatogenesis, **I:**838–839
  steroidogenesis, **I:**838
Infralimbic region (IL region), **II:**2414
Infundibular nucleus, **II:**1494
Inha. *See* Inhibin-α
Inhibin B, **II:**1668–1669
Inhibin beta B (*Inhbb*), **I:**280
Inhibin-α (Inha), **I:**917–918, **I:**1150–1151
  gene expression, **I:**918
Inhibin–activin signaling system
  components, **I:**666–667
  functional studies, **I:**667
  regulation, **I:**668
  transgenic mouse models, **I:**667–668
Inhibins, **I:**375–376, **I:**962, **II:**1264–1265, **II:**1317, **II:**1396, **II:**1543–1544, **II:**1795
  female double mutants, **I:**963t
  human disease, **I:**965–966
  mouse models, **I:**963–965
  mutations, **I:**965–966
  serum inhibin, **I:**962
Inhibitors of apoptosis (IAPs), **I:**914
Inhibitory systems, **II:**2336–2337
Initiation of lactation. *See* Secretory activation
Innate immune response, **I:**811
  inflammatory signaling pathways, **I:**813f
  innate immune system, **II:**1835–1837
  pattern recognition receptors, **I:**811–814
Inner acrosomal membrane (IAM), **I:**159–160
Inner cell mass (ICM), **I:**234–236, **II:**1697, **II:**1699, **II:**2035
Inner mitochondrial membrane (IMM), **I:**298
Insemination
  artificial, **I:**201
  copulatory plugs and gels, **I:**200–201
  timing, **I:**199
  uterine, **I:**201
  vaginal, **I:**199–200
INSL3. *See* Insulin-like factor 3
Insulin, **I:**669–670, **I:**1608–1609, **II:**1628
  receptor family, **I:**275
  resistance, **II:**1382–1383
  signaling to brain, **II:**1967
Insulin response substrate 1 (IRS1), **I:**657, **I:**1047–1048
Insulin-like factor 3 (INSL3), **I:**671, **II:**2112
Insulin-like growth factor (IGF), **I:**321–322, **I:**896, **II:**1228, **II:**1278, **II:**1717, **II:**2180–2181
  IGF1, **I:**481, **I:**794, **I:**976, **I:**1030, **I:**1088, **I:**1118–1119, **II:**1457, **II:**1511–1512, **II:**1609–1610, **II:**1784, **II:**1792–1794, **II:**2059
  IGF2, **I:**1030, **II:**1751, **II:**1784, **II:**1792–1794, **II:**2459–2460
Integrin beta 3 binding protein (ITGB3BP), **I:**325
Integrins, **II:**1714–1715
Interacting protein 3 (IP3), **I:**326–327
Intercellular adhesion molecule-1 (ICAM1), **I:**814
Interferon tau (IFNτ), **II:**1703–1704
Interferon-induced transmembrane proteins (IFITM proteins), **I:**247
Interferon-γ (IFNγ), **II:**1840
Interferon-ε (IFNε), **II:**1840
Interferons, **I:**862
Interleukin 1 (IL1), **I:**668, **I:**826
  and IL18, **I:**828–829
  intratesticular actions, **I:**827–828
  intratesticular production, **I:**826–827
Interleukin-1 alpha (IL-1α), **II:**1643
Interleukin-1beta (IL-1β), **II:**1643
Interleukin-6 (IL6), **I:**832
Internal determinants, **II:**1453. *See also* External determinants timing puberty
  energy metabolism pathways, **II:**1457–1459
  leptin, **II:**1454–1456
  metabolic signals, **II:**1456–1457
  partitioning of metabolic fuel, **II:**1454f
  sexual maturation energetics, **II:**1453–1454
Interstitial cell differentiation, **I:**280–281
Interstitial nucleus of anterior hypothalamus (INAH3), **II:**2132, **II:**2174
Intracellular Ca^{2+} pathway, **I:**658
Intracerebroventricular injection (ICV injection), **I:**479–480, **I:**538–539, **II:**1269
Intracerebroventricularly administered drug effects, **II:**2222. *See also* Male sexual behavior
  α-MSH, **II:**2230
  ACh, **II:**2227
  dopamine, **II:**2222–2223
    contradictory effects, **II:**2224
    daily injections of amphetamine, **II:**2223
    ejaculation, **II:**2223–2224
    Esr1 gene, **II:**2223
    haloperidol, **II:**2223
    receptors, **II:**2223
    sexual motivation, **II:**2224–2225
  GABA, **II:**2227
  GnRH, **II:**2229–2230
  nitric oxide, **II:**2228–2229
  norepinephrine, **II:**2225
  opioids, **II:**2227–2228
  oxytocin, **II:**2228
  peptides, **II:**2230–2231
  serotonin, **II:**2225–2227
Intracisternal A particle element (IAP), **I:**172–173
Intracytoplasmic sperm injection (ICSI), **I:**161–162, **I:**252, **I:**708, **I:**1014, **II:**2039
Intratesticular dendritic cells, **I:**822
Intrauterine fetal growth restriction (IUGR), **II:**1724–1725, **II:**2038
Intravenous (IV), **II:**1268
Intrinsically disordered domain (ID domain), **I:**1103, **I:**1105–1106
Invasive mouse trophoblasts, **II:**1715
Involution, **II:**2068–2069
  apoptotic process, **II:**2069–2070
  lysosome-mediated cell death, **II:**2070
  M2 macrophages, **II:**2070
  milk stasis, **II:**2070
  phases, **II:**2069, **II:**2071
  TGFβ, **II:**2070–2071
Iodide, **II:**1760
Ion transport, **I:**729–731
Ionic microenvironment, **I:**712
IP. *See* Prostaglandin I
IP3. *See* Interacting protein 3
iPSCs. *See* induced pluripotent stem cells
IREG1. *See* Iron-regulated gene
IRF2. *See* IFN regulatory factor 2
Iron, **II:**1768–1769
Iron-regulated gene (IREG1), **II:**1768–1769
IRS1. *See* Insulin response substrate 1
ISH. *See* In situ hybridization
ITGB3BP. *See* Integrin beta 3 binding protein
IUGR. *See* Intrauterine fetal growth restriction
IV. *See* Intravenous
IVF. *See* In vitro fertilization
IVM. *See* In vitro maturation; In vitro oocyte maturation
Izumo (IZUMO1), **I:**114

J

Jansen's ring. *See* Annulus
Janus kinase (JAK), **I:**325, **II:**1791
Janus kinase 2 (JAK2), **I:**1041
JM. *See* Joint molecule
JNK1–3. *See* c-Jun N-terminal kinases 1–3
Joint molecule (JM), **I:**27
Junctional proteins, **I:**703
Junctional regulatory element (JRE), **II:**1785
Junctional zone, **II:**1747
Juvenile development, **II:**1497–1498
  development hypogonadotropic state, **II:**1500–1501
  hiatus neurobiological bases, **II:**1501
    amino acid neurotransmitters, **II:**1505–1507
    confocal images, **II:**1502f
    glial-derived growth factors, **II:**1508–1509

Juvenile development (*Continued*)
hypogonadotropic state, **II:**1503f
kisspeptin, **II:**1502–1505, **II:**1504f
neuronal factors, **II:**1508
NPY, **II:**1507–1508
opioid peptides, **II:**1508
structural remodeling, **II:**1509
Juvenile period, females, **II:**1406
Juxtacrine signaling, **I:**786

K

Kallmann syndrome, **II:**1372–1374, **II:**1521
Kappa-opioid receptors (KOR), **II:**2194
Karyorrhexis, **II:**1203
KASH. *See* Klarsicht, ANC1, syne homology
K-(ATP) channel. *See* Adenosine triphosphate–sensitive potassium channel
Keratinocyte growth factor. See Fibroblast growth factor 7 (FGF7)
KI. *See* Knocked-in
KIKO domain, **I:**1144–1145
Killer inhibitory receptor (KIR), **II:**1859
Kinase light chain 3 (KLC3), **I:**123–125
Kinetochore orientation, **I:**39
KIR. *See* Killer inhibitory receptor
Kiss1 neurons, **I:**1575–1576, **II:**418–422, **II:**1221–1222, **II:**1413, **II:**1502–1505, **II:**1504f, **II:**1555, **II:**16115, **II:**1673, **II:**2476
in basal forebrain, **II:**1612
central pathways and transmitters, **II:**1618–1619
changes in, **II:**1503
estrogen-induced suppression, **II:**1326
expression studies, **II:**1612–1614
functional analyses, **II:**1614–1615
GALP neurons, **II:**1623
leptin signaling and, **II:**1615–1617
MCH and orexin neurons, **II:**1623
impact of metabolic stress conditions, **II:**1613f
molecular forms, **II:**1611
nesfatin 1 and SF1 neurons, **II:**1624–1625
neuronal efferents from SCN, **II:**1624
neuropeptides, **II:**1619–1620, **II:**1621t
NPY and AgRP neurons, **II:**1620–1622
placental peptide hormone, **II:**1809
PMV neurons, **II:**1622–1623
POMC and CART neurons, **II:**1620
RF-amide related peptides, **II:**1624
KISS1-derived peptide receptor. *See* Kisspeptin receptor (KISS1R)
Kisspeptin receptor (KISS1R), **II:**1269, **II:**1308–1309, **II:**1373, **II:**1489, **II:**1606, **II:**1647
antagonist, **II:**1271, **II:**1273–1274, **II:**1292
Kisspeptin-neurokininB-dynorphin neurons (KNDy neurons), **II:**1221–1222, **II:**1313, **II:**1326, **II:**1342, **II:**1442, **II:**1550–1551, **II:**1591
in ARC, **II:**1551
cells, **II:**1548–1549
Kisspeptins (*KISS1*), **I:**418–419, **I:**436–437, **II:**1221–1222, **II:**1268, **II:**1372, **II:**1413, **II:**1502–1505, **II:**1575–1576, **II:**1673, **II:**1809, **II:**2476
ablation of, **II:**1409–1411
anatomy, **I:**419–420
ARC, **II:**1548–1549
ARC KNDy neurons, **II:**1292
arcuate kisspeptin neurons, **I:**421–422
in arcuate nucleus, **II:**1591–1592
changes in LH pulse frequency and, **II:**1450f
continuous infusions of, **II:**1311
direct apposition, **II:**1414
effects of suckling on, **II:**1557–1558
estrogen-responsive, **II:**1326
expression in ARC KNDy neurons, **II:**1292
hypothalamic, **II:**1647–1648
neurons, **I:**1273–1274
physiology, **I:**420–421
putative roles, **II:**1611
role of, **II:**1269, **II:**1273
RP3V kisspeptin neurons, **I:**421
KIT ligand (KITL), **I:**247, **I:**617, **I:**957–959, **I:**1167–1168
KITLG. *See* KIT ligand (KITL)
Klarsicht, ANC1, syne homology (KASH), **I:**17
KLC3. *See* Kinase light chain 3
KLF4. *See* Krupple-like transcription factor 4
KNDy neurons. *See* Kisspeptin-neurokininB-dynorphin neurons
Knocked-in (KI), **I:**1117, **II:**1217
Knockout (KO), **II:**1217, **II:**1610, **II:**2289
KOR. *See* Kappa-opioid receptors
Kruppel-like factor 15 (KLF15), **I:**1086
Kruppel-like factor 5 (Klf5), **II:**1712
Kruppel-like factor 9 (KLF9), **I:**321
Krupple-like transcription factor 4 (KLF4), **I:**1125–1126

L

L-carnitine transporter, **I:**731–732
L-dihydroxyphenylalanine (L-DOPA), **II:**1977
L-PGDS. *See* Lipocalin prostaglandin D synthase
L-pgds gene, **I:**735
Labor induction, **II:**1907
Lactate, **II:**1757–1758
Lactate dehydrogenase (LDH), **I:**657
Lactate dehydrogenase A (LDHA), **I:**130
Lactate dehydrogenase-C (LDHC), **I:**130
Lactation, **I:**490, **II:**1555–1558, **II:**1976–1980, **II:**2055, **II:**2067–2068. *See also* Prolactin system
changes in pituitary function, **I:**483–484
competency assessment, **II:**2057–2058
energy requirements, **II:**2083–2088
mammary epithelium, **II:**2071–2075
milk composition, **II:**2075–2083
milk ejection, **II:**2088–2091
milk secretion, **II:**2056–2071
OT neurons
electrophysiological properties, **I:**547–550
plasticity, **I:**547–550
phases in placental mammals, **II:**2067
synaptic plasticity, **I:**550
GABA, **I:**551–552
glutamate, **I:**550–551
IPSC, **I:**552f
neurohypophysial plasticity, **I:**553
noradrenaline, **I:**552–553
during osmotic activation, **I:**553
Lactational delay, **II:**1705
Lactoferrin (LF), **II:**1709–1710
Lactogenesis I. *See* Secretory differentiation
Lactogenesis II. *See* Secretory activation
Lactogenic hormone, **II:**1374
Lactose, **II:**2067, **II:**2076
Lactotransferrin (Ltf), **I:**1119
Lactotroph function, **I:**481
changes in pituitary function, **I:**483–484
dopamine inhibition, **I:**482
organization, **I:**484
prolactin gene expression, **I:**481
prolactin secretion in pituitary, **I:**482–483
proliferation
via D2 receptor, **I:**482
stimulation, **I:**481
Lamin B receptor (LBR), **I:**110
Lamina terminalis complex, **II:**1961
Lamina-associated polypeptide 2 (LAP2), **I:**110
Large for gestational age (LGA), **II:**2043
Large neutral amino acid transporter 1 (LAT1), **II:**1765–1766
Laser capture microdissection (LCM), **I:**1133–1135
Lateral hypothalamic area (LHA), **II:**1557–1558, **II:**1619–1620
MCH and orexin neurons, **II:**1623
Lateral hypothalamus (LH), **II:**2257. *See also* LH secretion
change detection, **II:**2257
direct applications of drugs effects, **II:**2257
orexin/hypocretin neurons, **II:**2257
LBD. *See* Ligand-binding domain
LBR. *See* Lamin B receptor
LCM. *See* Laser capture microdissection
LDH. *See* Lactate dehydrogenase
LDHA. *See* Lactate dehydrogenase A
LDHC. *See* Lactate dehydrogenase-C
LDL. *See* Lipoprotein; Low-density lipoprotein
Lectins, **I:**103
LEF. *See* Lymphoid enhancer factor
Left ventricular outflow tract (LVOT), **II:**1936f
Leptin, **I:**979, **II:**1420, **II:**1607–1608, **II:**1628, **II:**1768, **II:**1967
adipokines, **II:**1811–1813
estradiol mechanism, **II:**1812f
LEP gene, **II:**1510–1511
receptors, **II:**1967
as reproduction, **II:**1454–1456
resistance, **II:**1967, **II:**1971
intracellular mechanism, **II:**1970–1971
pregnancy in women, **II:**1967–1968
in pregnant rodents, **II:**1968
as signal linking metabolism, **II:**1454–1456
signaling, **I:**672, **II:**1615–1617

adiponectin, **II:**1971
insulin signaling, **II:**1970
intracellular mechanism, **II:**1969–1970, **II:**1970f
Leptonema, **I:**15
Letdown reflex, **II:**2089
Leucine-rich repeats (LRRs), **I:**642
Leukemia inhibitory factor (LIF), **I:**245, **I:**693, **I:**832, **I:**957–958, **I:**1086, **II:**1707, **II:**1840, **II:**2070
Leukocytes, **II:**1201
in cycling uterus, **II:**1839–1840
cytokine regulation, **II:**1840–1841
Leydig cell(s), **I:**281, **I:**571, **I:**856–857. *See also* Peritubular cells; Sertoli cells; Spermatogenic cells
adult, **I:**580–581
AR in, **I:**649
depletion, **I:**822–823
development
inflammation effects, **I:**840–843
macrophages role, **I:**840
fetal, **I:**577–579
IL1β effect, **I:**828
inhibition, **I:**842–843
from interstitium, **I:**280
macrophage ratio in rat and mouse, **I:**821
murine, **I:**828–829
role, **I:**856–857
steroidogenic pathways, **I:**579–580, **I:**579f
T3 regulation, **I:**664
testosterone
biosynthesis regulation, **I:**809f
secretion, **II:**2219
LF. *See* Lactoferrin
Lfng. *See* Lunatic fringe
LG. *See* Licking and grooming
LGA. *See* Large for gestational age
LH. *See* Lateral hypothalamus; Luteinizing hormone
LH receptor gene (*Lhr*), **I:**77–78, **I:**916–917
LH secretion
arcuate nucleus, **II:**1309
estradiol negative feedback action
cellular mechanism, **II:**1325–1326
in human menstrual cycle, **II:**1326–1327
hypothalamic and pituitary sites, **II:**1324–1325
molecular mechanism, **II:**1325–1326
during estrous cycle
neuropeptide modulators control, **II:**1220–1227
neurotransmitters control, **II:**1220–1227
gonadotropin secretion *vs.* ovarian hormones, **II:**1324
neuroendocrine control, **II:**1323–1324
suppression, **II:**1319–1320
LH surge, **I:**952
events, **II:**1451–1452
LH receptor, **I:**920–922
luteinization physiology, **I:**919–920
oocyte maturation physiology, **I:**919–920
ovulation physiology, **I:**919–920
postpubertal shift in steroid control, **II:**1452
postpubertal sterility, **II:**1452–1453
sexual receptivity, **II:**1452
LH-receptor (Lhcgr), **I:**1155
LHA. *See* Lateral hypothalamic area
LH–CG. *See* Luteinizing hormone–chorionic gonadotropin
Lhcgr. *See* LH-receptor
LHCGR. *See* LH–choriogonadotropin receptor; Luteinizing hormone–chorionic gonadotropin receptor
LH–choriogonadotropin receptor (LHCGR), **I:**965–966
Lhr. *See* LH receptor gene
LHRH. *See* Luteinizing hormone-releasing hormone
Lhx8. *See* LIM Homeodomain Protein 8
Licensing process, **I:**282
Licking and grooming (LG), **II:**2466–2467
LIF. *See* Leukemia inhibitory factor
Lif expression, **II:**1717
Ligand bias, **I:**372–375
Ligand-binding domain (LBD), **I:**315–316, **I:**645–646, **I:**1100. *See also* N-terminal domain (NTD)
steroid receptor structure, **I:**1106
Ligand-dependent actions, **I:**1107–1109
Ligand-independent actions, **I:**1108f, **I:**1110
Ligands, **I:**718–727
LIM Homeodomain Protein 8 (Lhx8), **I:**956–957
LINE. *See* Long interspersed nucleotide element
LINE1. *See* Long interspersed element 1
Linker DNA, **II:**2028–2029
Lipid microdomains. *See* Lipid rafts
Lipid rafts, **I:**107, **I:**365–368
Lipids, **I:**791, **II:**1766–1768, **II:**2076
bioactive, **I:**857–858
composition, **I:**106
diffusion of, **I:**106–107
with maturation and capacitation, **I:**107
secretion, **II:**2078–2079
sperm plasma membrane
changes with maturation and capacitation, **I:**107
diffusion, **I:**106–107
lipid composition, **I:**106
Lipocalin prostaglandin D synthase (L-PGDS), **I:**277
Lipocalins, **I:**733–736
function, **I:**735–736
LCN 2, **I:**734–735
Lipopolysaccharide (LPS), **I:**811, **II:**1612–1613, **II:**1643, **II:**1845–1846, **II:**1903–1904
Lipopolysaccharide-induced inflammation models, **I:**841
Lipoprotein (LDL), **I:**1043
Lipoprotein lipase (LPL), **II:**1771–1772, **II:**2085
Liquefaction. *See* Coagulation
Liquid-ordered domain, **I:**365–366
Liver receptor homolog (LRH), **I:**896
LRH1, **I:**1010, **I:**1171–1172
Liver x receptor (LXR), **I:**1035–1036
Lobuloaveolar development, **I:**489–490
Localized sperm membrane components, **I:**103–104
ADAMTS10, **I:**105
chaperones, **I:**104
CRISP1, **I:**105
GLUT8, **I:**104
PCSK4, **I:**105
PMCA4, **I:**104
sperm proteins, **I:**104
T21 antigen, **I:**105
Locus ceruleus, **II:**1640
Long day breeders, **II:**1575–1576
Long interspersed element 1 (LINE1), **I:**269
Long interspersed nucleotide element (LINE), **II:**2455
effects of SRY, **II:**2457
HMG, **II:**2457
neural progenitor cells, **II:**2456–2457
PGCs, **II:**2456
retrotransposition, **II:**2456, **II:**2456f
Long terminal repeats (LTR), **II:**2455
Long-lived corpora lutea, **I:**1025–1026. *See* Short-lived corpora lutea; Ultrashort-lived corpora lutea
Long-term depression (LTD), **II:**1996–1997
Long-term potentiation (LTP), **II:**1959, **I:**1996–1997
Lordosis onset
excitatory system activation, **II:**2331
ACH and histamine, **II:**2334–2335
delta opioids and substance P, **II:**2335–2336
dopamine, **II:**2334
GABA and glutamate, **II:**2331–2334
GNRH, prolactin, and cholecystokinin, **II:**2335
norepinephrine, **II:**2334
oxytocin, **II:**2335
modular control, **II:**2332f
Lordosis quotient (LQ), **II:**1234
Low-density lipoprotein (LDL), **I:**327, **II:**1333, **II:**1767, **II:**1787–1788
*Lpar*3 receptor, **II:**1723
LPL. *See* Lipoprotein lipase
LPS. *See* Lipopolysaccharide
LQ. *See* Lordosis quotient
LRH. *See* Liver receptor homolog
LRRs. *See* Leucine-rich repeats
LTD. *See* Long-term depression
Ltf. *See* Lactotransferrin
LTP. *See* Long-term potentiation
LTR. *See* Long terminal repeats
Luminal compartment functions, **I:**704
microenvironment, **I:**711
ionic, **I:**712
organic solute, **I:**712–714
protein, **I:**714
water movement, **I:**712
sperm protection, **I:**710–711
spermatozoa
maturation, **I:**706–709
storage, **I:**709–710
transport, **I:**704–706
storage and protection, **I:**711–714

Lunatic fringe (*Lfng*), **I:**953–954
Luteal cell types, **I:**1059f
  function and regulation, **I:**1057–1059
  small luteal cells *vs*. large luteal cells, **I:**1057
Luteal neovascularization
  angiogenic factors, **I:**1031–1032
  angiolytic factors, **I:**1031–1032
  regulation and role, **I:**1029–1030
  VEGF, **I:**1030–1031
Luteal oxytocin, **I:**1036–1037
Luteal progesterone secretion control, **II:**1281
  affecting factors, **II:**1286
  development, **II:**1281
  regression, **II:**1282–1286
  support, **II:**1281–1282
Luteal structure regulation, **I:**1040
  function in early pregnancy, **I:**1053
    domestic animals, **I:**1053–1054
    primates, **I:**1054–1057
  luteolytic factors, **I:**1045–1046
    immunomodulators, **I:**1051–1053
    $PGF_{2\alpha}$, **I:**1046–1049
    vasoactive factors, **I:**1049–1050
  luteotropic factors, **I:**1040
    estradiol, **I:**1044
    GH, **I:**1044–1045
    growing evidence, **I:**1043–1044
    LH-like luteotropins, **I:**1042–1043
    PGR, **I:**1045f
    PRL-like luteotropic hormones, **I:**1040–1042
Luteal–placental shift, **II:**1346–1347, **II:**1889
Luteinization, **I:**1002, **I:**1027, **II:**1333
  LH surge promoting, **I:**920
  LH-stimulated signaling pathways, **I:**927–929
  luteal neovascularization regulation and role, **I:**1029–1032
  morphologic events, **I:**1027–1029
  steroid-secreting cells differentiation, **I:**1032–1037
Luteinizing hormone (LH), **I:**61, **I:**298, **I:**399, **I:**485, **I:**637, **I:**642, **I:**809, **I:**895, **I:**952, **I:**975, **I:**1024–1025, **I:**1082, **II:**1199, **II:**1363, **II:**1395, **II:**1441, **II:**1605, **II:**1641–1642, **II:**2173
  adult leydig cell development
    humans, **I:**643
    LH signaling, **I:**643–644, **I:**644f
    mouse models, **I:**642–643
  LHCGR
    genes regulation, **I:**644–645
    localization, **I:**642
    structure and function, **I:**642
  LH-like luteotropins, **I:**1042–1043
  LH-stimulated signaling pathways, **I:**923f
    cumulus granulosa cell expansion, **I:**924–926
    luteinization, **I:**927–929
    oocyte maturation, **I:**924–926, **I:**924f
    ovulation, **I:**926–927
    in preovulatory cells, **I:**922–924
  positive feedback response, **II:**2173–2174
  rat estrous cycle, **II:**1206–1207
    Clock/Clock mutants fail, **II:**1210f
    LH-release mechanism, **II:**1209f
  receptor acquisition, **I:**1155–1156
  signals, **I:**919
Luteinizing hormone-releasing hormone (LHRH), **I:**399, **II:**1200
  immunoassayable, **II:**1212f
Luteinizing hormone–chorionic gonadotropin (LH–CG), **II:**1784
Luteinizing hormone–chorionic gonadotropin receptor (LHCGR), **I:**642
  genes regulation, **I:**644–645
  localization, **I:**642
  structure and function, **I:**642
Luteolysis, **I:**1037–1040, **II:**1260, **II:**1282
Luteolytic factors, **I:**1045–1046
  immunomodulators, **I:**1051–1053
  $PGF_{2\alpha}$, **I:**1046–1049
  vasoactive factors, **I:**1049–1050
Luteolytic hormone, **I:**1024–1025
Luteotropic factors, **I:**1040
  estradiol, **I:**1044
  GH, **I:**1044–1045
  growing evidence, **I:**1043–1044
  LH-like luteotropins, **I:**1042–1043
  PGR, **I:**1045f
  PRL-like luteotropic hormones, **I:**1040–1042
Luteotropic hormones, **I:**1024–1025
  FSH, **II:**1335
  LH, **II:**1334–1335
  PRL, **II:**1335–1336
LVOT. *See* Left ventricular outflow tract
LXR. *See* Liver x receptor
Lybrido®, **II:**2338–2339
Lymphoid enhancer factor (LEF), **I:**911
Lyso-glycerophosphatidylcholine (lyso-GPC), **I:**857
lyso-GPC. *See* Lyso-glycerophosphatidylcholine

M

MACS. *See* Magnetic activated cell sorting
MADD. *See* MAPK activating death domain
Magnesium sulfate, **II:**1906
Magnetic activated cell sorting (MACS), **I:**612
Magnocellular oxytocin neuron system, **II:**1986–1987
  computer-based model, **II:**1995
  consequences of sustained inhibition, **II:**1994
  early OT release prevention
    allopregnanolone, **II:**1991–1992
    auto-inhibition, **II:**1989
    changes in OT neuron responses, **II:**1991–1992
    endogenous opioid actions, **II:**1992
    progesterone, **II:**1990–1991
    retrograde signaling, **II:**1989–1990
  Ferguson reflex pathway, **II:**1988–1989
  microenvironment of magnocellular oxytocin neuron, **II:**1990f
  morphological plasticity, **II:**1993
  OT, **II:**1987–1988
    neuron electrical properties, **II:**1988
    neuron electrophysiological properties, **II:**1993–1994
    neuron projections and afferents, **II:**1987f
    provision, **II:**1989
  parturition, **II:**1987–1988, **II:**1988f
  readiness to burst-fire, **II:**1992–1993
  restraints removal, **II:**1992–1993
maGSC. *See* multipotent adult germ line stem cells
Main olfactory bulb (MOB), **II:**2232
Major histocompatibility complex (MHC), **I:**815, **I:**1051, **II:**1835, **II:**2183
  in testis, **I:**854–855
Major vault protein (MVP), **I:**325
Male reproduction immunophysiology
  autoimmunity, **I:**848–852
  developmental timeline of spermatogenesis, **I:**806f
  historical aspects, **I:**806–807
  human testis, **I:**805
  immune cells, **I:**820–826
  immune privilege, **I:**852–861
  immune system endocrine control
    adaptive immune response, **I:**814–818
    effects, **I:**840–848
    general principles, **I:**810–811
    innate immune response, **I:**811–814
    mucosal immunity, **I:**818
    regulation of immunity, **I:**818–820
  immunological mediators in testis, **I:**826–840
  immunoregulatory environment, **I:**805–806
  implications and applications, **I:**862–863
  inflammation
    in accessory glands, **I:**845–847
    effects, **I:**840–843
    in epididymis, **I:**845–847
    macrophages role, **I:**840
    semen, **I:**847–848
    seminal leukocytes, **I:**847–848
    spermatogenesis, **I:**843–845
    in vas deferens, **I:**845–847
  inflammatory mediators in testis, **I:**826–840
  innate immunity, **I:**861–862
  key enzymes involved in inflammation, **I:**813t–814t
  structure and function, **I:**807
    accessory glands, **I:**809–810
    epididymis, **I:**809–810
    testis, **I:**807–809
    vas deferens, **I:**809–810
Male reproductive aging. *See also* Female reproductive aging
  aging effects on HPG axis
    hypothalamus and pituitary, **II:**1683
    testis, **II:**1683–1684
  in animals
    spermatogenesis, **II:**1683
    steroidogenesis, **II:**1683
  gender differences, **II:**1679
  in men
    FSH and sertoli cell regulation, **II:**1680

spermatogenesis, **II:**1679–1680
steroidogenesis, **II:**1680–1682
methodological challenges, **II:**1679
testosterone decline mechanism with age
hypothalamus and pituitary, **II:**1682
testis, **II:**1682–1683
Male sexual behavior, **II:**2211. *See also* Intracerebroventricularly administered drug effects
brain areas implication in control
amygdala, **II:**2234–2238
BST, **II:**2238–2239
CTF, **II:**2239–2240
sensory systems, **II:**2231–2234
SPFp, **II:**2239–2240
central integrative areas, **II:**2240–2258
circuitry and anatomical interconnections, **II:**2262
chemosensory circuits, **II:**2263–2264
efferents from MPOA, **II:**2266
ejaculation-related circuit, **II:**2264–2265
sexual behavior, **II:**2266
somatosensory input from genitals, **II:**2264
gonadal hormones
dependence of copulation, **II:**2220–2221
hormone deprivation effects, **II:**2222
replacement on *ex-copula* penile responses, **II:**2222
role of T metabolites, **II:**2221–2222
hormone action principles, **II:**2219–2220
motor outputs, **II:**2258–2262
patterns of male mammals
behavioral elements description, **II:**2212–2213
postejaculatory behavior, **II:**2213
rats copulation, **II:**2213f
satiety, **II:**2213
sexual experience effects, **II:**2213
penile function measurement, **II:**2213–2215
role of penis
anatomy and mechanics of erection, **II:**2215–2216, **II:**2215f
cellular mediators of erection, **II:**2218–2219
ejaculation, **II:**2217–2218
neural innervation, **II:**2216–2217
Male specific lethal (*Msl*), **II:**2464
Male-oriented rams (MOR), **II:**2167
Male-specific region (MSY), **I:**268–269
MALT. *See* Mucosaassociated lymphoepithelial tissue
Mammalian homeobox genes, **I:**1119
Mammalian meiosis, **I:**5
comparison with mitosis, **I:**5–7
conservation, **I:**7
disorders, **I:**42–43
environmental effects on aneuploidy, **I:**44
human diseases, **I:**44
maternal age effect, **I:**43–44
eukaryotic species, **I:**8f
history and discovery of, **I:**7
induction, **I:**11f, **I:**12–14
initiation, **I:**9
RA, **I:**9–12
small RNAs, **I:**15
sry induction of male germ cell program, **I:**14–15
Stra8, **I:**12–14
metaphase-to-anaphase transition, **I:**37f
MI and meiotic progression, **I:**36–42
prophase I, **I:**15–36
sexual dimorphism and timing, **I:**8–9
stages, **I:**6f
Mammalian oocyte, **I:**59
cell biology, **I:**63–64
Balbiani's body, **I:**64
cytoplasmic composition, **I:**66–70
extracellular coats, **I:**70–73, **I:**71f
GV, **I:**64–65
membrane specializations, **I:**70–73, **I:**71f
clinical parameters, **I:**84
genetic integrity regulation, **I:**84–86
human ARTs, **I:**86–89
reproductive aging and human oocyte, **I:**86
competencies, **I:**61t
electron microscopic investigations, **I:**59–60
maternal effect genes, **I:**61
oogenesis, **I:**61
structure, **I:**60f
ultrastructural investigations, **I:**60
of vertebrates, **I:**59, **I:**62–63
Mammalian preimplantation development, **I:**234, **I:**235f
mammalian target of rapamycin (mTOR), **I:**905–906, **II:**1625, **II:**1626f
signaling pathway, **II:**1766
Mammalian target of rapamycin complex 1 (mTORC1), **I:**958, **II:**1724–1725
Mammary epithelial cell (MEC), **II:**2059
Mammary epithelium
adherens junctions, **II:**2074
cell–cell interactions, **II:**2071
desmosomes, **II:**2074
gap junctions, **II:**2074–2075, **II:**2075f
mammary tight junctions
claudins, **II:**2072–2073
epithelial permeability, **II:**2073–2074
hormone dependence, **II:**2073
occludin, **II:**2072–2073
and regulation, **II:**2071, **II:**2074
tricellulin, **II:**2072–2073
Mammary gland, **I:**489–490
Mammary tight junctions
claudins, **II:**2072–2073
epithelial permeability, **II:**2073–2074
hormone dependence, **II:**2073
occludin, **II:**2072–2073
and regulation, **II:**2071, **II:**2074
tricellulin, **II:**2072–2073
Mammosomatotrophs, **I:**483
MAO. *See* Monoamine oxidase
MAP. *See* Mitogen-activated protein
MAP2D. *See* Microtubule-associated protein 2D
MAPK. *See* Mitogen-activated protein kinase
MAPK activating death domain (MADD), **I:**965–966
Mapk14, **I:**1006–1007
Masculinization, **II:**2113–2114
Masculinization programming window (MPW), **I:**578
Massachusetts Male Aging Study (MMAS), **II:**1680
Mate choice
different facets of, **II:**2160
evolutionary significance of, **II:**2158–2159
nature *vs*. nurture, **II:**2160–2161
pair bond, **II:**2159
percentages of mating choices, **II:**2161f
sensory modalities, **II:**2161
audition, **II:**2162
olfaction, **II:**2162
vision, **II:**2161–2162
species specificity in, **II:**2160
specific individual features, **II:**2179–2180
choosing healthy mate, **II:**2182
indirect markers of fertility in females, **II:**2181–2182
markers of quality in males, **II:**2180–2181
optimal outbreeding achievement, **II:**2182–2183
Maternal adaptations to pregnancy
cardiovascular adaptations, **II:**1929–1938
metabolic adaptations, **II:**1928–1929
pharmacokinetics of pregnancy, **II:**1947–1948
pregnancy literature, **II:**1928
renal adaptations, **II:**1940–1947
reproductive strategies, **II:**1927
respiratory system adaptations, **II:**1938–1940
Maternal age effect, **I:**43–44
Maternal and fetal blood flow, **II:**1743f
Maternal behavior, **II:**2000. *See also* Parenting behavior
brain control
in humans, **II:**2414–2415
in nonhuman animals, **II:**2401
for lactation, **II:**2004
mammalian, **II:**2000
neural circuitry, **II:**2001f
mesocorticolimbic dopamine system, **II:**2002
mPOA, **II:**2001–2002
neurogenesis, **II:**2002–2003
olfactory bulbs, **II:**2000–2001
PRL, **II:**2002–2003
VTA, **II:**2002
opioids and, **II:**2003
OT actions postpartum, **II:**2003
PAG, **II:**2003–2004
postpartum disturbances, **II:**2004
postpartum emotionality, **II:**2004
significance of hormones, **II:**2376–2377
endocrine profiles of pregnancy, **II:**2379–2385
epidural anesthesia, **II:**2377
mothering-like behavior, **II:**2378–2379
nonhormonal maintenance, **II:**2377–2378

Maternal brain adaptations in pregnancy
  actions on brain hormones, **II:**1957
  cognition, **II:**1995–2000
  food intake and metabolism, **II:**1964
    adipose signaling, **II:**1967
    allopregnanolone, **II:**1969
    appetite, **II:**1964, **II:**1971–1973, **II:**1976
    estradiol, **II:**1968
    gastrointestinal peptides, **II:**1973–1974
    hypothalamic circuitry, **II:**1976
    insulin signaling, **II:**1967
    leptin resistance, **II:**1967–1968
    leptin signaling, **II:**1969–1971
    mediators, **II:**1965–1967
    metabolic regulation, **II:**1964
    mid-to-late pregnancy, **II:**1969
    modulators, **II:**1965–1967
    network components, **II:**1965–1967
    oxytocin, **II:**1971–1973
    pregnancy in rats, **II:**1968
    progesterone, **II:**1968–1969
    reward circuitry, **II:**1975–1976
  hormone secretion profiles, **II:**1958f
  HPA axis, **II:**1980–1986
  magnocellular oxytocin neuron system, **II:**1986–1995
  maternal behavior, **II:**2000–2004
  osmoregulation
    decreased blood osmolarity central control, **II:**1960–1963
    increased volume in pregnancy, **II:**1960–1963
    relaxin action, **II:**1963
    RXFP1 expression, **II:**1963
  prolactin, **II:**1959, **II:**1976–1980
Maternal care, **I:**492
  hormonal regulation, **II:**2375–2376
  sensory control, **II:**2395
    olfaction, **II:**2397–2399
    somatosensation, **II:**2399–2401
    taste, **II:**2399
    vision and hearing, **II:**2396
Maternal contribution, **II:**1849
  adaptive immune response, **II:**1852–1857
  chemokines, **II:**1857
  cytokine response to pregnancy, **II:**1850
  innate immune response, **II:**1850–1852
  selective decidual leukocyte recruitment, **II:**1857
Maternal effect genes, **I:**63–64
Maternal hemoglobin (HbA), **II:**1756
Maternal low protein (MLP), **II:**2042
Maternal obesity, **II:**2043–2044
Maternal photoperiodic programming, **II:**1578–1579, **II:**1579f
Maternal protein restriction, **II:**2042–2043
Maternal recognition of pregnancy, **I:**1053–1054
Maternal separation, **II:**2469–2470
Maternal tobacco smoke exposure (MTSE), **II:**2044–2045
Maternal–fetal
  barrier interface, **II:**1742–1743
  exchange
    cell coexistence and, **II:**1847–1849, **II:**1848f
    factors influencing placental transfer, **II:**1755–1756
    placental barrier properties, **II:**1755f
    transcellular transport, **II:**1753–1755
    transtrophoblastic channels, **II:**1752–1753
  glucose transfer, **II:**1754
  interdigitation, **II:**1742–1743
Mating strategies, **II:**2159–2160
Matrix metalloproteinase (MMP), **I:**351–352, **I:**1029, **II:**1263, **II:**1329, **II:**1721, **II:**2071
  MMP2, **I:**114
  MMP8, **II:**1877–1878
Matrix remodeling, **II:**1721
Matrix-type fibrinoid, **II:**1747
Maturation promoting factor (MPF), **I:**41, **I:**168–169, **I:**924
Mature intermediate villi, **II:**1745–1746
MBD proteins. *See* Methyl binding domain proteins
MBH. *See* Medial basal hypothalamus; Mediobasal hypothalamus
MC. *See* Melanocortin
MC2R. *See* Melanocortin type 2 receptor
MC4. *See* Melanocortin-4 receptors
MCH. *See* Melanin-concentrating hormone
MCM. *See* Minichromosome maintenance proteins
MCP1. *See* Monocyte chemoattractant protein 1
MCR. *See* Melanocortin receptor
MCT. *See* Monocarboxylate transporter
MD. *See* Methyl donor
MD2. *See* Myeloid differentiation 2 protein
MDR1. *See* Multidrug resistance protein 1
MeA. *See* Medial amygdala
MEApv. *See* Posteroventral region of medial amygdala
MEC. *See* Mammary epithelial cell
MeCP2. *See* Methyl CpG binding protein 2
MED. *See* Mediator
Medial amygdala (MeA), **II:**1997, **II:**2397
Medial basal hypothalamus (MBH), **II:**1211–1213, **II:**1310, **II:**1312f, **II:**1494–1495, **II:**1666
Medial prefrontal cortex (mPFC), **II:**2290–2292, **II:**2407
Medial preoptic area (MPOA), **II:**1211, **II:**1959–1960, **II:**2240, **II:**2389–2390, **II:**2402–2403
  active oral components, **II:**2403
  with BSTv, **II:**2405–2406
  direct application
    of drugs effects, **II:**2243–2247
    of steroids effects, **II:**2242–2243
  effects of lesions, **II:**2240–2241
  electrical or chemical stimulation effects, **II:**2242
  elicitation of maternal behaviors
    cognitive systems, **II:**2412–2414
    emotion regulation systems, **II:**2409–2414
    motivation systems, **II:**2407–2409
  in mothering, **II:**2403–2404
  neurotransmitters, **II:**2404
  role of, **II:**2403
  site for hormone-sensory integration, **II:**2406–2407
  transient inactivation, **II:**2403
Medial preoptic nucleus (MPN), **II:**1211, **II:**2240
Medial septum (MS), **I:**400
Mediated transport, **II:**1754
Mediator (MED), **I:**318–319
Mediobasal hypothalamus (MBH), **I:**400, **II:**1266
MeDip. *See* Methylation-dependent immunoprecipitation
Medium stress resilient (MSR), **II:**1650–1651
Meiosis, **I:**1001. *See also* Mammalian meiosis
Meiotic recombination
  CO, **I:**30
  DNA mismatch repair pathway, **I:**29–30
  double-strand break repair model, **I:**24f–25f
  initial DSB processing, **I:**27–29
  initiation of DSBs, **I:**26–27
  multistage regulation of CO, **I:**30–31
  overview of, **I:**23–25
  physical events in, **I:**25–26
  ZMM proteins, **I:**29
Meiotic sex chromosome inactivation (MSCI), **I:**33–34
Meiotic silencing, **I:**33
  MSCI, **I:**33–34
  purpose, **I:**33
  small RNA participation, **I:**34–35
Meiotic silencing of unpaired chromatin (MSUC), **I:**33–34
Meiotic spindle, **I:**81–83, **I:**82f
Melanin-concentrating hormone (MCH), **II:**1619–1620, **II:**1623, **II:**1965
Melanocortin (MC), **II:**2326
Melanocortin receptor (MCR), **II:**1458–1459
Melanocortin type 2 receptor (MC2R), **I:**305
Melanocortin-4 receptors (MC4), **II:**1965
Melanocyte-stimulating hormone (MSH), **I:**547
Melanotan-II (MT-II), **II:**2329
Melatonin, **II:**1464–1466, **II:**1465f, **II:**1512–1513
  action
    hypothalamic lesions and implants, **II:**1587
    pars tuberalis as site of, **II:**1585–1586
    photoperiodic variation, **II:**1586f
    prolactin secretion, **II:**1586f
  synthesis pathway, **II:**1581–1582, **II:**1581f
Melatonin receptors, **II:**1583
  binding studies, **II:**1583–1584
  and energy homeostasis, **II:**1585
  GPCR
    cloning, **II:**1584
    seasonal neuroendocrine synchronization, **II:**1584–1585
  melatonin receptor autoradiography, **II:**1583–1584
  PT, **II:**1584f
Membrane transport, **II:**2080–2081
  apical, **II:**2082
  basolateral membrane transporters, **II:**2081–2082

ductal epithelium role, **II:**2081
phosphate transport in mammary gland, **II:**2083
purinergic receptors, **II:**2083
water permeability of mammary tissues, **II:**2081
Membrane-only estrogen receptor (MOER), **I:**666
Membrane-spanning transporters, **II:**1769
Menarche, **II:**1555
Menopause, **II:**1666, **II:**1671
Menopause transition, **II:**1668–1671, **II:**1670f
decline in ovarian function, **II:**1667
failure of positive feedback, **II:**1668
STRAW, **II:**1662
Menstrual cycle, **I:**1077–1078, **I:**1081. *See also* Human oviducts; Human endometrium
CNS, **II:**1308–1309
corpus luteum, **II:**1332–1343
endocrine functions, **II:**1307
folliculogenesis, **II:**1313–1318
gonadotropins patterns, **II:**1309–1310
hypothalamic GnRH pulse generator, **II:**1310–1313
inhibin levels, **II:**1309–1310
ovarian events, **II:**1307
ovarian steroids, **II:**1309–1310
ovulation, **II:**1328–1332
Pelvic clock concept, **II:**1310
preovulatory follicle selection, **II:**1318–1328
Mental Rotations Test, **II:**2133–2134
mEpiSCs. *See* mouse epiblast stem cells
mESCs. *See* mouse embryonic stem cells
Mesenchymal villi, **II:**1745–1746
Mesocorticolimbic DA tract, **II:**2251
c-fos expression, **II:**2252
castration effects, **II:**2251–2252
chemical changes, **II:**2252–2253
direct application of drugs effects, **II:**2252
electrolytic or cell body lesions effects, **II:**2251
Mesonephric formation, **I:**692–694
Metabolic adaptations
diet, **II:**1929
energy costs of pregnancy, **II:**1928
energy expenditure, **II:**1930f
fetal programming, **II:**1929
maternal body composition, **II:**1929
placental phenotypes, **II:**1931f
Metabolic control improvement, **II:**2087
Metabolic effects, **I:**493–497
Metabolic hormone reproductive effects, **II:**1627
direct pituitary effects, **II:**1627–1628
Ghrelin, **II:**1628
GHS-R type 1a, **II:**1628
leptin and insulin, **II:**1628
Metabolic hormones, **II:**1513–1514
Metabolic perturbations, **II:**1645
Metabolic stressors, **II:**1637, **II:**1644–1645
Metabolic syndrome (MetS), **I:**582–583
Metabolic targeting of GnRH neurons, **II:**1610
ablation of insulin receptors, **II:**1611
Cre–LoxP approaches, **II:**1610–1611
direct effects of key metabolic hormones, **II:**1611
ghrelin role, **II:**1611
Metal transport protein (MTP1), **II:**1768–1769
Metaphase-to-anaphase transition, **I:**37f, **I:**39–40
Metastable epialleles, **II:**2042
Metazoans, **I:**84–85
Metestrus, **II:**1200
Methyl binding domain proteins (MBD proteins), **II:**2448–2449
sex differences in, **II:**2481–2482
Methyl chloride, **I:**741
Methyl CpG binding protein 2 (MeCP2), **II:**2125, **II:**2447–2448
Methyl donor (MD), **II:**2047
Methyl-seq, **II:**2489
Methylation-dependent immunoprecipitation (MeDip), **II:**2475–2476, **II:**2489
Methylation-specific PCR, **II:**2488–2489
Methylation-specific restriction enzyme digest, **II:**2488
MethylCap-Seq, **II:**2489
MetS. *See* Metabolic syndrome
MFG. *See* Milk fat globule
MHC. *See* Major histocompatibility complex
Mice, **II:**1903, **II:**2297–2298
micro-RNA (miRNA), **I:**31, **I:**654, **I:**1129, **II:**2031
Microbiome, **II:**2046–2047
Microbiota characterization, **II:**1726
Micronutrient supplementation, **II:**2047
Microsomal P450 enzymes, **I:**300–302
Microtubule organizing center (MTOC), **I:**69–70
Microtubule-associated protein 2D (MAP2D), **I:**896
Microtubules, **I:**120–121
Microtus cabrerae (*M. cabrerae*), **II:**2457
Microvillous plasma membrane (MVM), **II:**1743–1745, **II:**1805
of human trophoblasts, **II:**1767
L-type calcium channels, **II:**1761
normal and preeclamptic syncytial, **II:**1754f
passive properties, **II:**1755–1756
Midbrain periaqueductal gray, **II:**2258
electrolytic or cell body lesions effects, **II:**2258
steroid receptors and connections, **II:**2258
Mig-6. *See* Mitogen-inducible gene 6
Milieu intérieur, **II:**1637
Milk composition, **II:**2075
milk components, **II:**2075
lactose, **II:**2076
lipids, **II:**2076
oligosaccharides, **II:**2076
proteins, **II:**2076
milk secretion pathways, **II:**2076–2083, **II:**2076f
Milk ejection, **II:**2088–2089. *See also* Non-milk ejection
afferent pathway mediation, **I:**533
brainstem, **I:**533–536
DMH, **I:**540
forebrain regions modulating, **I:**539
hypothalamic circuitry, **I:**536–538, **I:**536f
PVN and SON interconnections, **I:**538–539
spinal cord, **I:**533–536
lesions in intercollicular zone, **I:**534f
letdown reflex, **II:**2089
model for, **I:**553–554
myoepithelial cell contraction, **II:**2090
neuroendocrinology of OT synthesis and release, **II:**2089–2090
neurotransmitters/neuromodulator mediating, **I:**540
antidromic activation of PVN, **I:**545f
dopamine, **I:**546–547
GABA, **I:**546
glutamate, **I:**540–541
histamine, **I:**547
MSH, **I:**547
noradrenaline, **I:**541–542
oxytocin, **I:**542–546
peptide neurotensin, **I:**547
serotonin, **I:**546–547
OT neurons behavior
burst establishment, **I:**531
bursts synchronizing, **I:**532–533
and maternal behavior, **II:**2090–2091
receptor, **II:**2090
SON, **I:**531–532
in response to suckling, **I:**529–531
SON and PVN neurons, **I:**532f
Milk fat globule (MFG), **II:**2058
Milk secretion, **II:**2076–2077, **II:**2076f
exocytotosis, **II:**2077
calcium, **II:**2078
citrate, **II:**2078
lactose, **II:**2077–2078
oligosaccharides, **II:**2077–2078
phosphate, **II:**2078
proteins, **II:**2077
functional differentiation
paracrine signaling in alveologenesis, **II:**2062f
proliferative phase, **II:**2061–2063, **II:**2062f
secretory activation, **II:**2065–2067
secretory differentiation, **II:**2063
hormonal control of anatomical development
endocrine and paracrine regulation, **II:**2059–2060
mammary development in pregnancy, **II:**2060–2061
lipid secretion, **II:**2078–2079
mammary gland development and function, **II:**2057f
histological methods, **II:**2056
human lactation studying, **II:**2058–2059
lactation competency assessment, **II:**2057–2058
transplantation methods, **II:**2056–2057
membrane transport, **II:**2080–2081
apical, **II:**2082

Milk secretion (*Continued*)
basolateral membrane transporters, **II:**2081–2082
ductal epithelium role, **II:**2081
phosphate transport in mammary gland, **II:**2083
purinergic receptors, **II:**2083
water permeability of mammary tissues, **II:**2081
transcytosis, **II:**2079
immunoglobulins, **II:**2079
serum albumin, **II:**2079–2080
trace elements, **II:**2080
Mineralocorticoid receptor, **I:**727, **II:**1639–1640. *See also* Glucocorticoid receptor (GR)
Mineralocorticoids, **I:**672
Minichromosome maintenance proteins (MCM), **I:**1124
MCM2, **I:**1089
miR-371–3 cluster, **II:**1751–1752
miRNA. *See* micro-RNA
Mitochondrial sheath, **I:**122–123
Mitogen-activated protein (MAP), **I:**643–644, **II:**2314
Mitogen-activated protein kinase (MAPK), **I:**41, **I:**316, **I:**350–353
FGFR2 activating, **II:**1711
prolactin activating, **I:**472
signaling, **I:**243, **I:**275–276
Mitogen-inducible gene 6 (Mig-6), **I:**1120
Mitosis, **I:**38f, **I:**45
chromosome segregation, **I:**36
cohesion in, **I:**21
meiosis comparison with, **I:**5–7
SAC, **I:**36–37
MLCK. *See* Myosin light-chain kinase
MLL. *See* Myeloid/lymphoid
MLP. *See* Maternal low protein
MMAS. *See* Massachusetts Male Aging Study
MMP. *See* Matrix metalloproteinase
MMTV. *See* Mouse mammary tumor virus
MOB. *See* Main olfactory bulb
MOER. *See* Membrane-only estrogen receptor
MOF. *See* Multiple oocyte follicle
Mongolian gerbils, **II:**2391
Monoamine oxidase (MAO), **II:**1977
Monoamines, **II:**1224–1226, **II:**1810
Monocarboxylate transporter (MCT), **II:**1757–1758
Monocyte chemoattractant protein 1 (MCP1), **I:**497, **I:**1052–1053, **II:**1840
Monogamous mating systems, **II:**2159
Mononucleated cytotrophoblasts, **II:**1741–1742
MOR. *See* Male-oriented rams; Mu-opioid receptor
Morphogens, **II:**1720–1721
Morphologic events, **I:**1027–1029
Mosaic sperm surface, **I:**101
freeze-fracture image, **I:**102f
postacrosomal region, **I:**101
posterior ring, **I:**101–103
regions of plasma membrane, **I:**101
secretions from epididymis, **I:**103
Motility
activation of, **I:**133
calcium and, **I:**133–134
cAMP and, **I:**134
phosphorylation and, **I:**135–136
PKA and, **I:**134–135
Motivation systems, **II:**2407
DA neurotransmission, **II:**2408–2409, **II:**2408f
finer temporal analysis, **II:**2408
hormone and pup sensory information, **II:**2409
maternal behavior, **II:**2407–2408
NA, **II:**2407
VTA, **II:**2409
Motor outputs
brain areas, **II:**2259
midbrain periaqueductal gray, **II:**2258
electrolytic or cell body lesions effects, **II:**2258
steroid receptors and connections, **II:**2258
nucleus paragigantocellularis of medulla, **II:**2258
electrical stimulation effects, **II:**2259
electrolytic or cell body lesions effects, **II:**2259
immunocytochemistry, **II:**2259
spinal cord, **II:**2259
chemical changes, **II:**2261
direct applications of drugs effects, **II:**2260–2261
immunocytochemistry, **II:**2262
lesions or transections effects, **II:**2259–2260
presence of steroid receptors, **II:**2261–2262
ventral premammillary nucleus, **II:**2258
Mouflon (*Ovis musimon*), **II:**1441
mouse embryonic stem cells (mESCs), **I:**244–245
mouse epiblast stem cells (mEpiSCs), **I:**245
Mouse genetic models, **I:**611
Mouse mammary tumor virus (MMTV), **I:**318, **II:**2063
MPF. *See* Maturation promoting factor
mPFC. *See* Medial prefrontal cortex
MPN. *See* Medial preoptic nucleus
MPOA. *See* Medial preoptic area
MPW. *See* Masculinization programming window
MRP proteins. *See* Multidrug resistance–associated proteins
MS. *See* Medial septum
MSCI. *See* Meiotic sex chromosome inactivation
MSH. *See* Melanocyte-stimulating hormone
Msl. *See* Male specific lethal
MSR. *See* Medium stress resilient
MSUC. *See* Meiotic silencing of unpaired chromatin
Msx genes, **II:**1711–1712
MSY. *See* Male-specific region
MT-II. *See* Melanotan-II
MTOC. *See* Microtubule organizing center
mTOR. *See* mammalian target of rapamycin
mTORC1. *See* Mammalian target of rapamycin complex 1
MTP1. *See* Metal transport protein
MTSE. *See* Maternal tobacco smoke exposure
Mu-opioid receptor (MOR), **II:**1975–1976
MUA. *See* Multiunit activity; Multiunit electrical activity
Muc1. *See* Mucin 1
Mucification, **I:**998
Mucin 1 (Muc1), **I:**1119, **II:**1714
Mucosaassociated lymphoepithelial tissue (MALT), **I:**818
Mucosal immunity, **I:**818
Mucosal tolerance, **I:**818
Müllerian-inhibiting hormone. *See* Anti-Müllerian hormone (AMH)
Mullerian-inhibiting substance (MIS). *See* Anti-Müllerian hormone (AMH)
Multidrug resistance protein 1 (MDR1), **II:**1758–1760
Multidrug resistance–associated proteins (MRP proteins), **II:**1769
Multiple lipid-derived messengers, **I:**349–350
Multiple oocyte follicle (MOF), **I:**953
multipotent adult germ line stem cells (maGSC), **I:**250
Multiunit activity (MUA), **I:**336, **II:**1269, **II:**1310, **II:**1311f, **II:**1546–1547
volley, **II:**1642
Multiunit electrical activity (MUA). *See* Multiunit activity (MUA)
Mural granulosa cells, **I:**77–78, **I:**952
Murine folliculogenesis, **I:**948–952
Musk shrews, **II:**2299
MVM. *See* Microvillous plasma membrane
MVP. *See* Major vault protein
Myeloid differentiation 2 protein (MD2), **I:**811
Myeloid/lymphoid (MLL), **II:**2453–2454
Myoepithelial cell contraction, **II:**2090
Myometrial cells, **II:**1879
Myometrium, **II:**1879–1882, **II:**1881f
Myosin, **I:**105–106
Myosin light-chain kinase (MLCK), **II:**1879, **II:**2090
Myosin phosphatase targeting subunit 3 (MYPT3), **I:**120

N

N-ethylmaleimide sensitive fusion protein (NSF), **I:**111–113, **II:**2079
N-ethylmaleimide sensitive fusion protein attachment protein alpha (NAPA), **I:**111–113
N-methyl-D-aspartate receptor (NMDA), **II:**1223, **II:**2196, **II:**2246, **II:**2331
N-methyl-D-aspartate receptors (NMDAR), **II:**1673–1674
N-terminal domain (NTD), **I:**645–646, **I:**1100, **I:**1103
steroid receptor structure, **I:**1103–1105
NA. *See* Nucleus accumbens (NAc)
Na^+–Ca^{2+} exchanger, **II:**1761–1762
NAc. *See* Nucleus accumbens

NAcc. *See* Nucleus accumbens (NAc)
NAD. *See* Nictotinamide adenine dinucleotide
NAFLD. *See* Nonalcoholic fatty liver disease
Nanos homolog 2 (*Nanos*2), **I:**14
*Nanos*2. *See* Nanos homolog 2
NAPA. *See N*-ethylmaleimide sensitive fusion protein attachment protein alpha
Narrow cells, **I:**698–699
Natriuretic peptide precursor C (NPPC), **I:**925, **I:**1007–1008
Natruiretic peptides, **I:**377–378
Natural killer cells (NK cells), **I:**808, **I:**1051, **II:**1754–1755, **II:**1835–1837
  regulation and functions, **I:**816
NCAM. *See* Neural cell adhesion molecule
NCE. *See* Noncontact erections
NCO. *See* Non-crossover
NCOA. *See* Nuclear coactivator
NCOA-6. *See* Nuclear receptor co-activator-6
Ncoa2. See Steroid receptor co-activator 2 (SRC2)
NCOR. *See* Nuclear receptor co-repressor
ncRNA. *See* Noncoding RNA
NE. *See* Norepinephrine
NEAA. *See* Nonessential amino acids
NEDA. *See* Neuroendocrine dopamine
Negative energy balance, **II:**1614–1615
Neonatal development, **II:**1496–1497
Neonatal Fcg receptor (FcRn), **II:**1762
Neonatal period
  females, **II:**1404
  males, **II:**1407
Nephricduct formation, **I:**692–694
NER. *See* Nucleotide excision repair
NERKI. *See* Nongenomic ER knockin
Nerve growth factor (NGF), **I:**957–958, **I:**960, **II:**1228
  NGF1-A, **II:**2467–2468
Nesfatin 1 neurons, **II:**1624–1625, **II:**1973
NET. *See* Neutrophil extracellular trap; Norepinephrine transporter
Neural cell adhesion molecule (NCAM), **I:**550, **II:**1373
Neural events during estrous cycle
  circadian control mechanism, **II:**1209–1210
  control by GnRH, **II:**1210–1211
  GnRH neurosecretion, **II:**1211–1214
  LH and FSH secretion, **II:**1214–1215
Neural organization, **II:**2325–2326. *See also* Female sexual behavior
  disinhibition, **II:**2328
  dual control model, **II:**2327f
  estrus termination, **II:**2337–2338
  excitation, **II:**2326–2327
  inhibition, **II:**2327–2328
    inhibitory systems, **II:**2336–2337
  lordosis, **II:**2330–2336
  pacing behavior, **II:**2330
  sexual approach behaviors, **II:**2328–2329
  sexual desire disorders, **II:**2338–2339
  solicitations, **II:**2329–2330
Neural regulation, **I:**784
Neuregulin 1 (NRG1), **I:**617–618, **I:**1003
Neurite, **II:**2122–2123
Neuroanatomy, **I:**400
  cell bodies distribution, **I:**400, **I:**401f
  morphology, **I:**400–402, **I:**402f
  projections, **I:**402–404
Neuroendocrine control, **II:**1493–1495
  developmental pattern
    fetal, **II:**1495–1496
    infant, **II:**1496–1497
    juvenile, **II:**1497–1498
    moment-to-moment changes, **II:**1497f
    neonatal, **II:**1496–1497
    pubertal, **II:**1498–1499
  FSH secretion, **II:**1323–1327
  gonadotropin secretion, **II:**1341–1343, **II:**1493–1495
  LH secretion, **II:**1323–1327
  neuroendocrine systems mediating steroid feedback, **II:**1544
    hypothalamic systems, **II:**1545–1546
    ovarian steroids effects on pituitary, **II:**1544–1545
  prolactin secretion
    inhibition by dopamine, **I:**472–474
    NEDA neurons, **I:**473f, **I:**474–475, **I:**478–480
    prolactin-releasing factors, **I:**480
    short-loop negative feedback, **I:**475–477
    TIDA neuronal system development, **I:**477–478
    transport across blood–brain barrier, **I:**478
Neuroendocrine dopamine (NEDA), **I:**473f, **I:**474
  prolactin feedback on, **I:**477f
  regulation, **I:**478–480
Neuroendocrine model, **II:**1469–1470
Neurogenesis, **II:**2118–2119
Neurogenin 3 (*Neurog*3), **I:**250, **I:**606
Neurohypophysial plasticity, **I:**553
Neurokinin B (NKB), **I:**413, **I:**422–423, **II:**1312–1313, **II:**1548–1549, **II:**1617–1618, **II:**1677, **II:**1807, **II:**1810
  characterization of putative roles of, **II:**1619f
  intracerebroventricular injection effects of agonists, **II:**1618f
Neuromedin-S (NMS), **II:**1624
Neuromedin-U (NMU), **II:**1624
Neuronal elements, **I:**413
  GABA, **I:**415–417
  glutamate, **I:**417–418
  GnRH, **I:**418
  kisspeptin, **I:**418–422, **I:**419f
  modes of GABA action, **I:**415f
  neurokinin B, **I:**422–423
  neuropeptide actions, **I:**414t–415t
  non-neuropeptidergic neurotransmitter actions, **I:**413t
  NPY, **I:**423–424
  RFRP-3, **I:**425–426
Neuronal nitric oxide synthase (nNOS), **II:**1989–1990
Neuronal regulation, **I:**706, **I:**740
Neuronal-glial interactions, **I:**426
Neuropeptide mediators, **II:**1965
Neuropeptide Y (NPY), **I:**413, **I:**423–424, **I:**536–538, **I:**1214, **II:**1227, **II:**1458, **II:**1507–1508, **II:**1546, **II:**2231
  neurons, **II:**1620–1622
  placental peptide hormone, **II:**1809
Neuropilin 1 (NRP1), **II:**1722
Neurotransmitter systems, **II:**1645
Neurotransmitters/neuromodulator mediating milk ejection reflex, **I:**540. *See also* Milk ejection
  antidromic activation of PVN, **I:**545f
  dopamine, **I:**546–547
  GABA, **I:**546
  glutamate, **I:**540–541
  histamine, **I:**547
  MSH, **I:**547
  noradrenaline, **I:**541–542
  oxytocin, **I:**542–546
  peptide neurotensin, **I:**547
  serotonin, **I:**546–547
Neutral amino acids, **II:**1765–1766
Neutrophil extracellular trap (NET), **I:**206–207
NFY. *See* Nuclear factor Y
NGF. *See* Nerve growth factor
NGN3. *See* Neurogenin 3 (*Neurog*3)
NHANES III. *See* Third National Health and Nutrition Examination Survey
NICD. *See* Notch intracellular domain
Nictotinamide adenine dinucleotide (NAD), **II:**2443
Nidation, **II:**1697
Nigrostriatal DA tract, **II:**2253
NIS. *See* Sodium iodide symporter
Nitabuch's fibrinoid. *See* Basal plate blood-derived fibrinoid
Nitric oxide (NO), **I:**377–378, **I:**1010, **I:**1048–1050, **I:**1228, **II:**1414–1415, **II:**2228–2229
  direct application of drugs effects, **II:**2246
Nitric oxide synthase (NOS), **I:**378, **I:**836–838, **I:**1049–1050, **II:**1228, **II:**1267–1268, **II:**1415, **II:**2218–2219
  NOS-3, **I:**1008
NK cells. *See* Natural killer cells
NK T-cell regulation and functions, **I:**816
NKB. *See* Neurokinin B
Nkx3. 1 gene, **I:**785
NLS. *See* Nuclear localization signal
NMDA. *See* N-methyl-D-aspartate receptor
NMDAR. *See* N-methyl-D-aspartate receptors
NMS. *See* Neuromedin-S
NMU. *See* Neuromedin-U
nNOS. *See* Neuronal nitric oxide synthase
NO. *See* Nitric oxide
Nobox gene, **I:**73, **I:**954–955
NOD. *See* Nucleotide binding and oligomerization domain
Non-crossover (NCO), **I:**25
Non-milk ejection, **I:**531
  phasic-bursting, **I:**532
Non-pathogen-mediated inflammation, **I:**839
Nonalcoholic fatty liver disease (NAFLD), **II:**2043
Nonclassical testosterone signaling, **I:**653–654
Noncoding RNA (ncRNA), **II:**2031, **II:**2453
  long ncRNA, **II:**2453–2454
  microRNAs, **II:**2454–2455

Noncontact erections (NCE), **II:**2211–2212, **II:**2214
Nondisjunction, **I:**36
Nonessential amino acids (NEAA), **II:**1764f
"Nonfunctional" corpora lutea, **II:**1204
Nongenomic actions, **I:**1109–1110
Nongenomic ER knockin (NERKI), **I:**1117
"Nongenomic" pathways, **I:**1109
Nonhuman primate model, **II:**1650–1651
Nonhuman primates
  animal models of preterm birth, **II:**1904
  female sexual behavior, **II:**2299–2300
Nonmediated transport, **II:**1753
Nonspecific flagellar anomalies (NSFAs), **I:**131
Nonvillous support, **II:**1747
Noradrenaline, **I:**541–542, **I:**552–553
Norepinephrine (NE), **I:**413, **II:**1266–1267, **II:**1546, **II:**2225, **II:**2334
  direct application of drugs effects, **II:**2245
  in regulation of estrous cyclicity, **II:**1224
Norepinephrine transporter (NET), **II:**1769
NOS. *See* Nitric oxide synthase
Notch intracellular domain (NICD), **I:**953–954
Notch signaling, **I:**953–954
nPGi. *See* nucleus paragigantocellularis
NPM. *See* Nucleoplasmin
NPPC. *See* Natriuretic peptide precursor C
NPY. *See* Neuropeptide Y
NR. *See* Nuclear receptor
Nr2f2. See Chicken ovalbumin upstream promoter–transcription factor (COUP-TFII)
NR5A1. *See* Nuclear receptor subfamily 5, group A, member 1
NRG1. *See* Neuregulin 1
*Nrip*1 expression, **I:**1005
NRP1. *See* Neuropilin 1
NSF. *See* N-ethylmaleimide sensitive fusion protein
NSFAs. *See* Nonspecific flagellar anomalies
NTD. *See* N-terminal domain
NTS. *See* Nucleus of solitary tract; Nucleus tractus solitarii
Nuclear coactivator (NCOA), **I:**316
Nuclear envelope, **I:**109
Nuclear factor Y (NFY), **I:**343
Nuclear lacunae, **I:**110
Nuclear lamina, **I:**110
Nuclear localization signal (NLS), **I:**645–646
Nuclear mitotic apparatus protein 1 (NUMA1), **I:**119
Nuclear pocket, **I:**110
Nuclear proteins, **I:**108–109
Nuclear receptor (NR), **I:**313, **I:**1099–1100
  response elements, **I:**1104f–1105f
Nuclear receptor co-activator-6 (NCOA-6), **I:**1120
Nuclear receptor co-repressor (NCOR), **II:**2127
  NCOR1, **I:**317–318
Nuclear Receptor Signaling Atlas (NURSA), **I:**328
Nuclear receptor subfamily 5, group A, member 1 (NR5A1), **I:**342–344
Nuclear vacuoles. *See* Nuclear lacunae
Nucleobindin 2. *See* Nesfatin-1
Nucleoplasmin (NPM), **I:**172
  *Npm*2, **I:**955
Nucleosomes, **II:**2441, **II:**2442f
Nucleotide binding and oligomerization domain (NOD), **I:**811
Nucleotide excision repair (NER), **II:**2450–2451
Nucleus accumbens (NAc), **II:**2184, **II:**2213, **II:**2292, **II:**2407
Nucleus of solitary tract (NTS), **I:**535
nucleus paragigantocellularis (nPGi), **II:**2256
Nucleus paragigantocellularis of medulla, **II:**2258
  electrical stimulation effects, **II:**2259
  electrolytic or cell body lesion effects, **II:**2259
  immunocytochemistry, **II:**2259
Nucleus tractus solitarii (NTS), **II:**1965, **II:**1973
NUMA1. *See* Nuclear mitotic apparatus protein 1
NURSA. *See* Nuclear Receptor Signaling Atlas
Nutrient uptake, **I:**493
Nutritional suppression of reproduction, **II:**1560–1561

O

OAE. *See* Oto-acoustic emissions
OAZ. *See* Ornithine decarboxylase antizyme
Obesity, **II:**1771–1772
Occludin, **II:**2072–2073
Octamer-binding transcription factor 4 (*Oct*4). *See* POU-domain class 5 transcription factor 1 (*Pou5f*1)
ODC. *See* Ornithine decarboxylase
ODF. *See* Outer dense fibers
Oestrin, **II:**1395–1396
OFC. *See* Orbitofrontal cortex
OHSS. *See* Ovarian hyperstimulation syndrome
Olfaction, **II:**2397
  amniotic fluid, **II:**2398
  bulbectomy, **II:**2397–2398
  odor profile, **II:**2399
  steroid hormone–primed nulliparous rats, **II:**2398
  virgin female rats, **II:**2397
  vomeronasal organ removal, **II:**2398
Olfactory bulbs, **II:**2231
  auditory system, **II:**2234
  C-FOS activation, **II:**2233–2234
  effects of lesions, **II:**2232–2233
  somatosensory systems, **II:**2234
Olfactory receptor 1D2 (OR1D2), **I:**133
Oligosaccharides, **II:**2076
OMM. *See* Outer mitochondrial membrane
Oocyte arrest, **I:**924
Oocyte cortex, **I:**83
  ERM, **I:**83
  FYN kinase, **I:**83
  microtubule organizing centers, **I:**83–84
  SCNT, **I:**84
Oocyte development immunology, **II:**1841
Oocyte epigenetics, **II:**2033–2034. *See also* Postfertilization epigenetics; Sperm epigenetics
Oocyte maturation, **I:**924–926
  LH surge promoting, **I:**919
  LH-stimulated signaling pathways, **I:**924–926, **I:**924f
Oocyte pickup
  adhesive interactions, **I:**220–222
  hamster oocyte cumulus complexes, **I:**221f
  interaction of infundibular cilia, **I:**221f
  muscle and ciliary action in, **I:**220
Oocyte transport
  muscle and ciliary action, **I:**222–223
  ovulatory products fit oviductal lumen, **I:**222
Oocyte-specific TGFβ members, **I:**968
  Bmp15, **I:**970–971
  GDF9, **I:**968–970
  sheep mutations, **I:**970t
Oogenesis, **I:**61
  of coordination with folliculogenesis, **I:**77–80
    metabolic substrate sharing, **I:**78t
    modifications in cell communication, **I:**80f
    TZP, **I:**79f
  growth phase
    epigenetic modifications of chromatin, **I:**74f
    epigenetic modulation of genome, **I:**76–77
    maternal effects genes, **I:**75t
    patterns of gene expression, **I:**73–75
    protein synthesis regulation, **I:**75–76
    PTM, **I:**76
  maturation phase
    meiotic spindle, **I:**81–83
    oocyte cortex, **I:**83–84
    spatial patterning, **I:**80–81
  morphological features, **I:**63f
  ovarian events, **II:**1201
  stages, **I:**62f
Ooplasm, **I:**66–67
Open reading frame (ORF), **II:**2448–2449
Open-loop setting, **II:**1667
OPG. *See* Osteoprotegerin
Opioids, **II:**2227–2228
  differential effect of receptors, **II:**2330
  direct application of drugs effects, **II:**2245
  endogenous, **II:**1226
  inhibiting TIDA neurons, **II:**2330
  peptides, **I:**479–480, **II:**1508
OPN. *See* Osteopontin
Optimal outbreeding achievement, **II:**2182–2183
OR1D2. *See* Olfactory receptor 1D2
Orbitofrontal cortex (OFC), **II:**2308
Orexin receptor (OX1R), **II:**1623
Orexins, **II:**1623
  A peptide, **II:**1227–1228
  direct application of drugs effects, **II:**2247
ORF. *See* Open reading frame
Organic solute
  microenvironment, **I:**712–714
  transporters, **I:**731–732

Organum vasculosum of lamina terminalis (OVLT), **I:**400–401, **II:**1211, **II:**1409, **II:**1961
Ornithine decarboxylase (ODC), **I:**791
Ornithine decarboxylase antizyme (OAZ), **I:**120
OSC. *See* Ovarian stem cell
oSDN. *See* Ovine sexually dimorphic nucleus
OSE. *See* Ovarian surface epithelium
Osmoregulation. *See also* Pregnanacy
  blood osmolarity central control, **II:**1960
    adrenomedullin, **II:**1963
    lamina terminalis complex, **II:**1961
    oxytocin, **II:**1960–1963
    relaxin, **II:**1961–1963
    salt appetite, **II:**1963
    sex steroids, **II:**1963
    vasopressin, **II:**1960–1961
  decreased blood osmolarity central control, **II:**1960–1963
  increased volume in pregnancy, **II:**1960–1963
  relaxin action, **II:**1963
  RXFP1 expression, **II:**1963
Osteocalcin, **II:**1609–1610
Osteopontin (OPN), **II:**1715
Osteoprotegerin (OPG), **I:**497
OT. *See* Oxytocin
Oto-acoustic emissions (OAE), **II:**2172–2173
OTR. *See* Oxytocin receptor
Otxr. *See* Oxytocin receptor (OTR)
Outer dense fibers (ODF), **I:**123–125
  composition, **I:**123–126
  function, **I:**126
  general features, structure, and formation, **I:**123
  ODF-associated proteins, **I:**124t
  satellite fibers, **I:**126
Outer mitochondrial membrane (OMM), **I:**298
Ovarian aging
  in animal models, **II:**1678
  early, **II:**1668–1669
  in women, **II:**1664–1665
Ovarian cyclicity seasonal control
  pheromones and social interactions, **II:**1292–1294
  seasonal breeding, **II:**1289–1292, **II:**1289f–1290f
Ovarian dysfunction, **II:**1374
Ovarian events, **II:**1202f
  rat estrous cycle, **II:**1201–1206
Ovarian function, SR in, **I:**1131
  AR signaling, **I:**1165–1174
  ER signaling, **I:**1133–1161
  during follicular differentiation, **I:**1157f
  glucocorticoid receptor signaling, **I:**1174
  PR signaling, **I:**1133–1161
Ovarian function control, **II:**1277
  follicular
    development control, **II:**1277–1278
    hormone secretion control, **II:**1280–1281
  luteal progesterone secretion control, **II:**1281–1286
  ovulation rate control, **II:**1278–1280
Ovarian function pathophysiology
  female infertility, **II:**1363
  GnRH structure, **II:**1364
  Graafian follicle, **II:**1363
  human ovarian function physiology, **II:**1364–1365
    follicle development, **II:**1365–1367
    human menstrual cycle, **II:**1367–1368, **II:**1368f
  ovulation disorders, **II:**1368–1387
  reproductive hormones, **II:**1363–1364
Ovarian hyperstimulation syndrome (OHSS), **I:**1014, **II:**1387
Ovarian stem cell (OSC), **I:**250–251
Ovarian steroids
  during estrous cycle, **II:**1218–1220
  negative feedback control of LH secretion, **II:**1215–1216
  steroid receptors mediation, **II:**1217–1218
  stimulation of preovulatory, **II:**1216–1217
Ovarian surface epithelium (OSE), **I:**999–1000
Ovary, **I:**276–284, **I:**490–491, **II:**1490–1491. *See also* Gonadotropin signaling in ovary; Polycystic ovary syndrome (PCOS)
  age-related decrease, **II:**1668
  AR expression, **I:**1165–1167
  development, **I:**276–284
    in XY embryos, **I:**275
  estrogen receptor expression, **I:**1133
  follicular, **II:**1264f
    development, **II:**1365–1367
    dynamics, **II:**1261–1263
    rupture, **II:**1263, **II:**1265f
  folliculogenesis regulation by TGFβ family signaling, **I:**961–971
  Gata4 expression in postnatal ovary, **I:**972–973
  hormone secretion, **II:**1263–1265
  newborn ovary homeobox gene, **I:**954–956
  Ngf in rat ovary, **I:**960
  PR expression, **I:**1161–1162
  steroid receptor expression localization, **I:**1133t
  steroidogenesis, **II:**1367
OVGP1. *See* Oviduct-specific glycoprotein 1
Oviduct, **I:**220
  embryo transport, **I:**223
    cigarette smoke interferes, **I:**223–224
  oocyte pickup
    adhesive interactions, **I:**220–222
    hamster oocyte cumulus complexes, **I:**221f
    interaction of infundibular cilia, **I:**221f
    muscle and ciliary action in, **I:**220
  oocyte transport
    muscle and ciliary action, **I:**222–223
    ovulatory products fit oviductal lumen, **I:**222
Oviduct-specific glycoprotein 1 (OVGP1), **I:**165–166
Oviductal fluid, **I:**1082–1083
Oviductal sperm reservoir
  bull sperm on glass slides, **I:**214f
  carbohydrates on oviductal epithelium, **I:**211
  entrapment, **I:**215–216
  fate of nonfertilizing sperm, **I:**217
  marsupials and insectivores, **I:**216
  release of sperm, **I:**213–216
  sperm fertility during storage preservation, **I:**213
  sperm ligands and, **I:**211–213
  sperm storage
    and polyspermy prevention, **I:**210
    in women, **I:**216–217
Ovine estrous cycle, **II:**1260
  analysis, **II:**1265
    estrous behavior control, **II:**1276–1277
    hypothalamo–hypophysial function control, **II:**1266–1276
    ovarian function control, **II:**1277–1286
  estrous behavior, **II:**1260–1261
  hypothalamo–hypophysial unit, **II:**1261
  ovary, **II:**1261–1265
  resynthesis
    model for cycle, **II:**1286–1288, **II:**1287f
    retrospective historical analysis, **II:**1288–1289
  uterine prostaglandins, **II:**1265
Ovine sexually dimorphic nucleus (oSDN), **II:**2167
OVLT. *See* Organum vasculosum of lamina terminalis
Ovulation, **I:**1001–1002, **II:**1649–1650
  AR signaling, **I:**1173
  cell types and organization, **I:**999f
  COC during, **I:**1005
    cGMP regulatory pathway, **I:**1007–1008, **I:**1008f
    EGF receptor loop, **I:**1005–1006
    MAPK14-PTGS2 connection in, **I:**1006–1007
    nongenomic processes, **I:**1007
  EGF-like factors, **I:**998–999
  ER signaling, **I:**1158–1159
  granulosa cell functions, **I:**1002–1003
  LH receptors, **I:**997
  LH surge promoting, **I:**919
  LH-stimulated signaling pathways, **I:**926–927
  menstrual cycle control, **II:**1328
    EGF-like ligands, **II:**1328–1329
    mid-cycle gonadotropin surge neuroendocrine control, **II:**1329–1332, **II:**1331f
    progesterone, **II:**1329
    prostaglandins, **II:**1329
    proteolytic enzymes, **II:**1329
    ERK1/2central role, **I:**1005
    LH–EGF receptor connection, **I:**1003–1004
    LH–EGF receptor pathway, **I:**1004–1005
    RAS, **I:**1005
    cGMP regulatory pathway, **I:**1007–1008
    EGF receptor loop in COCs, **I:**1005–1006
    EGF-PTGS2 regulatory loop, **I:**1006, **I:**1006f
    MAPK14-PTGS2 connection in COC, **I:**1006–1007
    nongenomic processes, **I:**1007
    progesterone, **I:**1008

Ovulation (*Continued*)
C/EBPα, **I:**1009
C/EBPβ, **I:**1009
hypoxia-related events, **I:**1010
nuclear receptor Nr5a2, **I:**1010
Pparg, **I:**1010
RUNX1, **I:**1009–1010
RUNX2, **I:**1009–1010
WNT/FZD/CTNNB1 pathway, **I:**1010–1011
oocyte, **I:**999
COC expansion, **I:**1000–1001
gap junctions, **I:**1001
luteinization, **I:**1002
meiosis, **I:**1001
PGs, **I:**997–998
PR signaling, **I:**1163–1165
progesterone, **I:**998
cytokines expression and functions, **I:**1011–1012
immune-related factors, **I:**1012–1013
stimulus, **II:**1540
translational clinical considerations, **I:**1013
anovulatory infertility, **I:**1014
ICSI, **I:**1014
LH receptor mutants, **I:**1014
PCOS, **I:**1014
unique biological process, **I:**997
Ovulation disorders, **II:**1368–1369
causes of anovulation, **II:**1370t
clinical management
investigation, **II:**1383–1384
treatment, **II:**1384–1385
gonadotropins
deficiency, **II:**1372–1374, **II:**1385–1386
disordered regulation, **II:**1372–1374
functional disorders, **II:**1374
PCOS, **II:**1375–1383, **II:**1386–1387
POI, **II:**1369–1372
Ovulatory follicle "rupture", **I:**997, **I:**998f
Ovum donation, **II:**1384
OX1R. *See* Orexin receptor
Oxidative enzymes, **I:**836–838
Oxidative stress, **II:**1770
Oxygen, **II:**1756–1757
Oxytocin (OT), **I:**483, **I:**527, **II:**1214, **II:**1227, **II:**1876, **II:**1960, **II:**2057–2058, **II:**2228, **II:**2247, **II:**2335
c-fos activation, **II:**2250
chemical changes, **II:**2247–2250
electrophysiological properties, **I:**547–550
AHPs, **I:**548f, **I:**550f
rebound depolarization, **I:**548f
electrophysiological recordings, **II:**2247
intracerebral grafts effects, **II:**2250
laboratory rodents, **II:**2383
magnocellular, **II:**1960
milk ejection, **I:**529–531, **I:**541f
burst establishment, **I:**531
bursts synchronizing, **I:**532–533
neuromodulators mediation, **I:**542–546
reflex, **I:**543f
SON, **I:**531–532
MPOA functional roles, **II:**2251
neuron burst firing, **II:**1994
neurons, **II:**1972–1973
neurotransmitter receptors activation, **II:**2251
partner preference formation, **II:**2184–2185
parturition hormonal regulation, **II:**1894–1895
placental peptide hormone, **II:**1809–1810
receptors, **II:**1907
release in SON, **I:**544f
reward system in pair bonding, **II:**2185–2186
sheep, **II:**2385
social information processing, **II:**2185
and sodium balance, **II:**1962
and vasopressin, **II:**2184
Oxytocin receptor (OTR), **I:**725, **II:**1971–1973, **II:**2468
Oxytocinase, **II:**1894, **II:**1994

P

P box. *See* Proximal box
p-chlorophenylalanine (pCPA), **II:**2226
P-elementinduced wimpy testis-interacting RNAs (PIWI), **I:**20
P-interacting RNA (piRNA), **I:**20
p38MAPKα. *See* Mapk14
P_4. *See* Progesterone
PA. *See* Phosphatidic acid
PA. *See* Plasminogen activator
PACAP. *See* Pituitary adenylyl cyclase-activating polypeptide
Pachynema, **I:**15–16
Pachytene checkpoint 2 (PCH2), **I:**35
Pacing behavior, **II:**2330
PAF. *See* Platelet activating factor
PAG. *See* Periaqueductal gray
PAH. *See* Para-aminohippurate
Pair bonding, **II:**2159, **II:**2183
and addiction, **II:**2195
in birds, **II:**2197
early-life experiences, **II:**2196–2197
genetic mechanisms contribution, **II:**2188–2189
in humans, **II:**2198–2200
model of social cognition, **II:**2195–2196
neural mechanisms, **II:**2193
CRF, **II:**2194–2195
dopamine, **II:**2194
KOR, **II:**2194
selective aggression, **II:**2194
separation-induced negative effect, **II:**2194–2195
neurochemistry and neural circuitry, **II:**2184–2193
in nonhuman primates, **II:**2197–2198
Prairie voles, **II:**2183–2184
sex differences, **II:**2190
stress and, **II:**2192–2193
Pakistani type dysplasia, **I:**304
PAMP. *See* Pathogen-associated molecular pattern
Pancreatic duodenal homeobox 1 (PDX1), **II:**1791
PAP. *See* Prostatic acid phosphatase
PAPS. *See* Phosphoadenine phosphosulfate
PAPSS. *See* Phosphoadenine phosphosulfate synthetase
PAR. *See* Pseudoautosomal region
Para-aminohippurate (PAH), **II:**1940
Paracellular transport, **II:**1752–1753
Parathyroid hormone (PTH), **II:**1762
Parathyroid hormone–related peptide (PTHrP), **II:**1762, **II:**1807
placental peptide hormone, **II:**1811
Paraventricular nucleus (PVN), **I:**485, **I:**527, **II:**1606, **II:**1638, **II:**1959, **II:**2253–2254, **II:**2292, **II:**2469–2470. *See also* Supraoptic nucleus (SON)
c-fos expression, **II:**2256
chemical changes, **II:**2255
direct application of drugs effects, **II:**2254–2255
efferent projections, **II:**2256
electrolytic or cell body lesions effects, **II:**2254
mRNA expression, **II:**2256
presence of steroid receptors, **II:**2255–2256
Parental care components, **II:**2373–2375
Parental influences on offspring, **II:**2466
female offspring, **II:**2468
humans, **II:**2471
impaired maternal care, **II:**2468
importance of fathers, **II:**2470–2471
LG, **II:**2466–2468
maternal behavior, **II:**2468
maternal separation, **II:**2469–2470
prenatal stress, **II:**2468–2469
Parenting behavior, **II:**2371
brain control
nonhuman animals, **II:**2416–2417
in primates, **II:**2417–2418
historical perspective, **II:**2372–2373
parental care components, **II:**2373–2375
potential caregivers, **II:**2371
significance of hormones, **II:**2388
California mice, **II:**2389–2390
dwarf hamsters, **II:**2392–2393
laboratory mice, **II:**2390–2391
Mongolian gerbils, **II:**2391
Prairie voles, **II:**2391–2392
primates, **II:**2393–2395
Wild House mice, **II:**2390
Particulate guanylyl cyclases, **I:**377–378
Partner preference
classical conditioning, **II:**2192
dopamine and, **II:**2190–2191
in male prairie voles, **II:**2186–2187
opioids and, **II:**2191–2192
Parturition, **II:**1875, **II:**1878–1879
birth timing, **II:**1876–1878
cephalo-pelvic ratio, **II:**1877f
clinical control, **II:**1905–1906
beta-adrenergic receptor agonists, **II:**1907
calcium channel blockers, **II:**1906
labor induction, **II:**1907
magnesium sulfate, **II:**1906
OT receptors, **II:**1907
PG synthesis inhibitors, **II:**1906–1907
gravid uterus, **II:**1876, **II:**1878–1888
hormonal regulation, **II:**1888
biomechanical influences, **II:**1899
chemokines, **II:**1897

cytokines, **II:**1897
estrogens, **II:**1892–1894
OT, **II:**1894–1895
PG, **II:**1895–1897
placental peptide hormones, **II:**1897–1899
plasma CRH levels, **II:**1898f
progesterone, **II:**1889–1892
signals from fetus, **II:**1899–1900
human evolution, **II:**1876–1878
human parturition pathophysiology, **II:**1900–1905
positive-feedback hormonal loops, **II:**1891f
progesterone block hypothesis, **II:**1875
across viviparous species, **II:**1875–1876
Patched homolog 1 (PTCH1), **I:**1089–1090
Paternal stress, **II:**2474
Pathogen-associated molecular pattern (PAMP), **I:**811, **II:**1845–1846
Pattern recognition receptor (PRR), **II:**1845–1846
PBE. *See* Polar body extrusion
PBP. *See* Prostate-binding protein
PCB. *See* Polychlorinated biphenyls
PCD. *See* Primary ciliary dyskinesia
PCFT. *See* Proton-coupled folate transporter
PcG. *See* Polycomb group
PCH2. *See* Pachytene checkpoint 2
PCNA. *See* Proliferating cell nuclear antigen
PCNT. *See* Pericentrin
PCOS. *See* Polycystic ovarian syndrome
pCPA. *See* p-chlorophenylalanine
PCR. *See* Polymerase chain reaction
PCSK4. *See* Proprotein convertase subtilisin/kexin type 4
PDE. *See* Phosphodiesterase
PDGF. *See* Platelet-derived growth factor
PDGFR. *See* Platelet derived growth factor receptors
PDHB. *See* Pyruvate dehydrogenase E1 component β
PDK-1. *See* Phosphoinositide-dependent kinase 1
PdPN. *See* Posterodorsal preoptic nucleus
PDX1. *See* Pancreatic duodenal homeobox 1
PDYN. *See* Prodynorphin
PDZ. *See* Primary decidual zone
PE. *See* Persistent estrus
PEA3. *See* Polyomavirus enhancer activator 3
*Peg*3 gene, **II:**2460–2461
PEI. *See* Postejaculatory interval
Pelvic clock concept, **II:**1310
Pendrin (SLC26A4), **II:**1760
Penetration of blastocyst trophectoderm, **II:**1700
Penile function measurement
ex-copula measures
anteroflexions, **II:**2214
drug-induced erections, **II:**2214
NCE, **II:**2214
reflexive erections, **II:**2214f
seminal emissions, **II:**2214
spontaneous erections, **II:**2214
touch-based erections, **II:**2214
UG reflex, **II:**2214–2215
observations during copulation, **II:**2213–2214
Pentraxin 3 (PTX3), **I:**969, **I:**1000–1001
PEP. *See* Propyl endopeptidase
PEPCK. *See* Phosphoenolpyruvate carboxykinase
Peptide hormones, **II:**1807
AM, **II:**1810–1811
CGRP, **II:**1811
chromogranin A, **II:**1810
CRH, **II:**1807–1808
ET, **II:**1810
GnRH, **II:**1808–1809
kisspeptin, **II:**1809
monoamines, **II:**1810
NKB, **II:**1810
NPY, **II:**1809
OT, **II:**1809–1810
PTHrP, **II:**1811
Ucn, **II:**1807–1808
Peptide ligand receptor (PLR), **I:**323
Peptide YY (PYY), **II:**1974
Perforatorium cytoskeleton, **I:**110
Periaqueductal gray (PAG), **II:**2003–2004, **II:**2404
Pericentrin (PCNT), **I:**119
Perinatal lethality, **I:**966
Perinuclear theca, **I:**110
Perinuclear zone (PNZ), **I:**537
Peripheral benzodiazepine receptor, **I:**1033–1034
Peripheral hormones, **II:**1609–1610
Peripubertal period, **II:**1406
Peritubular cells, **I:**720, **I:**830, **I:**838, **I:**841–842
Peritubular myoid cells (PTM cells), **I:**571, **I:**581, **I:**638, **I:**648–649
Periventricular hypophysial dopaminergic neurons (PHDA neurons), **I:**474, **II:**1233
Peroxiredoxin 6 (Prdx6), **II:**1710
Peroxisome proliferator-activated receptor (PPAR), **II:**1718
PPARγ, **I:**1010, **II:**1787
PPARδ, **II:**1719
Persistent estrus (PE), **II:**1674
PET. *See* Positron emission tomography
PFC. *See* Prefrontal cortex
pFF. *See* porcine follicular fluid
PG. *See* Prostaglandin
PGA. *See* Polyglandular autoimmune
PGBE. *See* Pituitary glycoprotein hormone basal element
PGC. *See* Primordial germ cell
PGD2. *See* Prostaglandin D2
PGDS. *See* Prostaglandin D Ssynthase
PGE_1. *See* Prostaglandin E_1
PGE_2. *See* Prostaglandin E_2
PGES. *See* Prostaglandin E synthase
PGI synthase (PGIS), **II:**1719
PGIS. *See* PGI synthase
PGR. *See* Progesterone receptor (PR)
PGRMC1. *See* Progesterone receptor membrane component 1
PGS. *See* Prostaglandin synthase
PGT. *See* Prostaglandin transporter
PH. *See* Pleckstrin homology
PHDA neurons. *See* Periventricular hypophysial dopaminergic neurons
Phenotypic sex
internal and external genitalia, **I:**285f
sexual differentiation
nervous system, **I:**285–286
of reproductive organs, **I:**284–285
Phenylethanolamine-*N*-methyl-transferase (PNMT), **II:**1225
Pheromones, **II:**1420–1421
PHGPX. *See* Phospholipid hydroperoxide glutathione peroxidase
Phosphatidic acid (PA), **I:**349–350
Phosphatidyl inositol (PI), **I:**1058
Phosphatidylinositol 3-kinase–protein kinase B (PI3K–AKT), **I:**958
Phosphatidylinositol-3-kinase (PI3K), **I:**472, **I:**899, **I:**1041, **II:**1625–1627, **II:**1970
Phosphatidylinositol-specific phospholipase C (PI-PLC), **I:**163
Phosphoadenine phosphosulfate (PAPS), **I:**304
Phosphoadenine phosphosulfate synthetase (PAPSS), **I:**304
Phosphodiesterase (PDE), **I:**896
PDE3A, **I:**41
PDE5, **II:**1788, **II:**2338–2339
Phosphoenolpyruvate carboxykinase (PEPCK), **I:**980
Phosphoinositide 3 kinase (PI3K), **I:**325, **I:**657
Phosphoinositide-dependent kinase 1 (PDK-1), **I:**899
Phospholipase A_2 (PLA_2), **I:**658, **I:**730–731
Phospholipase C (PLC), **I:**111, **I:**654, **I:**897
Phospholipase C zeta (PLCz), **I:**87
PLCZ1, **I:**104
Phospholipases D (PLD), **I:**349–350
Phospholipid hydroperoxide glutathione peroxidase (PHGPX), **I:**122–123
Phosphorylated signal transducer and activator of transcription 5b (pSTAT5b), **II:**1978
Phosphorylated STAT3 (pSTAT3), **II:**1615
Phosphorylcholine, **I:**790–791
Photoperiod
ontogeny, **II:**1466
sequence for puberty, **II:**1463–1464, **II:**1464f
sex differences in response, **II:**1473–1474
Photoperiodic history dependence, **II:**1578–1579
Photorefractoriness, **II:**1578
Physiological sexual arousal, **II:**2302
PI. *See* Phosphatidyl inositol
PI-PLC. *See* Phosphatidylinositol-specific phospholipase C
PI3K. *See* Phosphatidylinositol-3-kinase; Phosphoinositide 3 kinase
PI3K–AKT. *See* Phosphatidylinositol 3-kinase–protein kinase B
PIAS. *See* Protein inhibitor of activated STAT
Pineal gland, **II:**1464–1466, **II:**1466f

Pineal hormone melatonin, **II:**1579
  melatonin synthesis pathway, **II:**1581–1582, **II:**1581f
  photoneuroendocrine system, **II:**1580f
  pineal gland activity control, **II:**1579–1581
  role of, **II:**1579
  SCN, **II:**1580f
  timed infusion experiments, **II:**1582–1583, **II:**1583f
Pinopodes, **II:**1715
piRNA. *See* P-interacting RNA
piRNA. *See* Piwi-interacting RNA
*Pit*1 factor. *See* Pou1*f*1 factor
Pit1. *See* Pituitary-specific transcription factor 1
Pituitary
  aging effects, **II:**1683
  gonadotrope function, **II:**1492–1493
  gonadotropin subunit, **II:**1208
  ovarian feedback, **II:**1667
  reproductive aging in men, **II:**1682
  steroid feedback, **II:**1668
  tumors, **II:**1374
Pituitary adenylyl cyclase-activating polypeptide (PACAP), **I:**376–377, **II:**1579–1581
Pituitary glycoprotein hormone basal element (PGBE), **I:**356–357
Pituitary hormone secretion control, **II:**1215
  FSH, **II:**1229, **II:**1229f
    nonsteroidal hormones secretion, **II:**1229–1231
    ovarian steroid hormones secretion, **II:**1229–1231
    steroid *vs*. nonsteroidal hormones, **II:**1231
  LH secretion
    anorexigenic factors, **II:**1227–1228
    gas NO, **II:**1228
    growth factors, **II:**1228–1229
    neuropeptide modulators, **II:**1220–1227
    neurotransmitters, **II:**1220–1227
    orexigenic factors, **II:**1227–1228
    ovarian steroids, **II:**1215–1220
  PRL, **II:**1231–1232
    candidate prolactin-releasing hormones, **II:**1233–1234
    dopamine, **II:**1233–1234
    luteolytic role, **II:**1234
    ovarian steroids in control, **II:**1232–1233
Pituitary-specific transcription factor 1 (Pit1), **I:**337
Pituitary–gonadal axis, **II:**2476
  *Eap*1, **II:**2477
  GnRH, **II:**2476, **II:**2476f
  *Kiss*1 neurons, **II:**2476–2477
  PcG genes, **II:**2477
PIWI. *See* P-elementinduced wimpy testis-interacting RNAs
Piwi-interacting RNA (piRNA), **II:**2031
PK. *See* Pyruvate kinase
PK2. *See* Prokineticin-2
PKA. *See* Protein kinase A
PKA-dependent signaling, **I:**899–915
PKC. *See* Protein kinase C
PKG. *See* Protein kinase G
PL. *See* Placental lactogen
PLA_2. *See* Phospholipase A2
*Pla*2*g*4*a* expression, **II:**1723
Placenta, **II:**1741. *See also* Placental transport function
  comparative placentation, **II:**1742–1743
  interdigitation between maternal and fetal tissues, **II:**1743f
Placental dysfunction disorders, **II:**1769–1771
Placental endocrine function
  endocrine roles, **II:**1783
  peptide hormones, **II:**1807
    AM, **II:**1810–1811
    CGRP, **II:**1811
    chromogranin A, **II:**1810
    CRH, **II:**1807–1808
    ET, **II:**1810
    GnRH, **II:**1808–1809
    kisspeptin, **II:**1809
    monoamines, **II:**1810
    NKB, **II:**1810
    NPY, **II:**1809
    OT, **II:**1809–1810
    PTHrP, **II:**1811
    Ucn, **II:**1807–1808
  protein hormones, **II:**1784
    GH-V, **II:**1791–1792
    hCG, **II:**1784–1789
    placental growth factors, **II:**1792–1799
    placental lactogen, **II:**1789–1791
  steroid hormones, **II:**1799
    11β-HSD, **II:**1804–1807
    estrogens, **II:**1799–1802
    progesterone, **II:**1802–1804
  structure and development, **II:**1783–1784
Placental growth factor (PlGF), **II:**1746
  EG-VEGF, **II:**1798–1799
  EGF, **II:**1795–1796
  endostatin-mediated suppression, **II:**1794f
  IGF-I, **II:**1792–1794
  IGF-II, **II:**1792–1794
  placental–fetal growth, **II:**1794f
  TGFβ, **II:**1794–1795
  VEGF, **II:**1796–1798
  VEGF–VEGFR system, **II:**1796f
Placental hypothalamic-like neuropeptide, **II:**1807–1811
Placental ion transporters, **II:**1771
Placental lactogen (PL), **II:**1784, **II:**1978, **II:**2063
  expression, **II:**1789–1790
  function, **II:**1791
  production, **II:**1790
  structure, **II:**1789–1790
  synthesis regulation, **II:**1790–1791
Placental micro-and nanovesicles, **II:**1754–1755
Placental peptide hormones, **II:**1897–1899
Placental shape, **II:**1742–1743
Placental transport function, **II:**1752
  alterations
    in human fetal growth restriction, **II:**1771t
    in maternal diabetes, **II:**1772t
  amino acids, **II:**1764–1766
    anionic amino acids, **II:**1766
    cationic amino acids, **II:**1766
    neutral amino acids, **II:**1765–1766
    transporter regulation, **II:**1766
  bicarbonate, **II:**1760
  calcium, **II:**1761–1762
  carbon dioxide, **II:**1757
  chloride, **II:**1758–1760
  complications of pregnancy
    diabetes, **II:**1772
    disorders associated with placental dysfunction, **II:**1769–1771
    obesity, **II:**1771–1772
  drugs, **II:**1769
  folate, **II:**1768
  glucose, **II:**1762–1764
  immunoglobulins, **II:**1762
  iodide, **II:**1760
  iron, **II:**1768–1769
  lactate, **II:**1757–1758
  lipids, **II:**1766–1768
  maternal–fetal exchange
    factors influencing placental transfer, **II:**1755–1756
    placental barrier properties, **II:**1755f
    transcellular transport, **II:**1753–1755
    transtrophoblastic channels, **II:**1752–1753
  oxygen, **II:**1756–1757
  potassium, **II:**1758
  protons, **II:**1757
  sodium, **II:**1758
  vitamin B12, **II:**1768
  water, **II:**1760–1761
  xenobiotics, **II:**1769
Placental vascularity, **II:**1742–1743
Placental villous tree, **II:**1745–1747
Placental villus, **II:**1743–1745
Plasma membrane Ca^{2+} ATPase (PMCA), **I:**177, **II:**1761–1762
Plasminogen (PLG), **I:**106, **I:**1029
Plasminogen activator (PA), **II:**1329
Platelet activating factor (PAF), **I:**223
Platelet derived growth factor receptors (PDGFR), **I:**725
Platelet-derived growth factor (PDGF), **I:**580
  PDGF-A, **I:**660
PLC. *See* Phospholipase C
PLC. *See* Progenitor Leydig cells
PLCz. *See* Phospholipase C zeta
PLD. *See* Phospholipases D
Pleckstrin homology (PH), **I:**323
Plf. *See* Proliferin
PLG. *See* Plasminogen
PlGF. *See* Placental growth factor
Plin2 protein, **II:**1767–1768
PLK1. *See* Polo-like kinase 1
PLR. *See* Peptide ligand receptor
Pluripotent stem cells
  gametes generation, **I:**251–252, **I:**253t–254t
  germ cells derivation, **I:**252–256
  germ line differentiation, **I:**256
  in vitro-derived gametes, **I:**256–257
PMCA. *See* Plasma membrane Ca^{2+} ATPase

PMSG. *See* Pregnant mare serum gonadotropin
PMV. *See* Premammillary nucleus
PN-1. *See* Protease nexin-1
PNMT. *See* Phenylethanolamine-*N*-methyl-transferase
PNZ. *See* Perinuclear zone
POA. *See* Preoptic area
POF. *See* Premature ovarian failure
POI. *See* Primary ovarian insufficiency
Polar body extrusion (PBE), **I:**38–39
Polar trophectoderm, **II:**1750–1751
Polo-like kinase 1 (PLK1), **I:**21
Polychlorinated biphenyls (PCB), **I:**704, **I:**794, **II:**1472–1473, **II:**2473
Polycomb group (PcG), **II:**2446–2447
Polycomb-repressive complex 1 (PRC1), **I:**74–75
Polycystic ovarian syndrome (PCOS), **I:**957, **I:**1086, **I:**1157–1158, **II:**1363–1364, **II:**1375, **II:**1627–1628
  antiestrogens, **II:**1387
  diagnosis, **II:**1375–1376, **II:**1376t
  diet, **II:**1386–1387
  etiology, **II:**1377–1383, **II:**1378f
  gonadotropin therapy, **II:**1387
  laparoscopic ovarian diathermy, **II:**1387
  lifestyle modification, **II:**1386–1387
  metabolic abnormalities, **II:**1376–1377
  pathogenesis, **II:**1377–1383
  presentation, **II:**1376
  prevalence, **II:**1376
Polyglandular autoimmune (PGA), **I:**817
Polymerase chain reaction (PCR), **I:**344, **I:**1085, **II:**1264–1265, **II:**2475–2476
Polyomavirus enhancer activator 3 (PEA3), **I:**727–728
POMC. *See* Proopiomelanocortin
POR deficiency. *See* Cytochrome P450-oxidoreductase deficiency
porcine follicular fluid (pFF), **II:**1229
Porosomes, **II:**2077
Positron emission tomography (PET), **II:**1666–1667, **II:**2179, **II:**2289
Postejaculatory interval (PEI), **II:**2229
Posterodorsal preoptic nucleus (PdPN), **II:**2240
Posteroventral region of medial amygdala (MEApv), **II:**2306
Postfertilization epigenetics, **II:**2034–2035. *See also* Oocyte epigenetics; Sperm epigenetics
Postnatal development, **I:**695–696
Postpartum emotionality, **II:**2004
Postprandial thermogenesis (PPT), **II:**1383
Postpubertal sterility, **II:**1452–1453
Postsynaptic current (PSC), **I:**415
Postterm birth, **II:**1904–1905
Posttranslational modification (PTM), **II:**2442
Potassium, **II:**1758
POU-domain class 5 transcription factor 1 (*Pou5f*1), **I:**12
*Pou1f*1 factor, **I:**481
*Pou5f*1. *See* POU-domain class 5 transcription factor 1
PPAR. *See* Peroxisome proliferator-activated receptor
PPROM. *See* Preterm premature rupture of membranes
PPT. *See* Postprandial thermogenesis
PPT. *See* Propylpyrazole
PR. *See* Progesterone receptor
PR-A. *See* Progestin receptor A
Prader–Willi syndrome (PWS), **II:**2027, **II:**2037. *See also* Beckwith–Wiedemann syndrome (BWS)
Prairie voles, **II:**2183–2184, **II:**2184f, **II:**2391–2392
  mate guarding in male, **II:**2187–2188
PRAP. *See* Prolactin receptor–associated protein
PRC1. *See* Polycomb-repressive complex 1
Prdx6. *See* Peroxiredoxin 6
PRE. *See* Progesterone response element
Preantral follicle development, **II:**1313–1314, **II:**1365–1367
  abnormal, **II:**1381–1382
Precocious puberty, **II:**1520–1521
Predecidualization, **II:**1701, **II:**1717
Preeclampsia, **II:**1770, **II:**1814–1815
Preformation, **I:**281–282
Prefrontal cortex (PFC), **II:**2407
Pregnancy, **I:**494–496, **II:**1723. *See also* Immunology of pregnancy; Maternal brain adaptations in pregnancy
  luteal function in, **I:**1054–1057
  placental transport in complications
    diabetes, **II:**1772
    disorders associated with placental dysfunction, **II:**1769–1771
    obesity, **II:**1771–1772
  prolactin secretion patterns during, **I:**485–487, **I:**486f
  rat
    glomerular hemodynamics in, **II:**1942
    relaxin paradigm in, **II:**1944–1946
"Pregnancy block". *See* Bruce effect
Pregnant mare serum gonadotropin (PMSG), **I:**913, **I:**1042–1043, **II:**1406
Pregranulosa cells, **I:**948–950
Preimplantation embryo development, **II:**1842–1843
Prelimbic region (PrL region), **II:**2414
Premammillary nucleus (PMV), **II:**1409, **II:**1616
Premature ovarian failure (POF), **I:**958, **I:**1167
Prenatal androgen correlation, **II:**2139–2143
Prenatal steroids
  GnRH controls by, **II:**1471
    GnRH surge system, **II:**1471–1472
    progesterone feedback system, **II:**1472
  GnRH rise by, **II:**1470–1471
Preoptic area (POA), **II:**1266, **II:**1330–1331, **II:**1540–1541, **II:**1612, **II:**2456–2457
Preovulatory follicle selection, **II:**1318
  cessation during luteal phase, **II:**1318–1319
  clinical implications, **II:**1327–1328
  initiation, **II:**1319
  maturing follicle, **II:**1320
    dependency on FSH, **II:**1322–1323
    estradiol, **II:**1320
    FSH secretion neuroendocrine control, **II:**1323–1327
    FSH secretion suppression, **II:**1321–1322
    inhibin, **II:**1320–1321
    LH secretion neuroendocrine control, **II:**1323–1327
  ovulatory induction, **II:**1324f
Preovulatory granulosa cells
  FSH in preantral and LH, **I:**929–930
  LH-stimulated signaling pathways in, **I:**922–924
Preterm birth, **II:**1900–1902
  animal models, **II:**1902–1904
  progestin therapy to prevention, **II:**1902
Preterm premature rupture of membranes (PPROM), **II:**1887
PRF. *See* Prolactin releasing factor
Primary ciliary dyskinesia (PCD), **I:**131
Primary decidual zone (PDZ), **II:**1716
Primary ovarian insufficiency (POI), **I:**965, **II:**1364–1365, **II:**1369, **II:**1664
  autoimmune causes, **II:**1371–1372
  diagnosis, **II:**1370
  genetic causes, **II:**1370–1371
  genetic syndromes, **II:**1371t
  management, **II:**1372
  miscellaneous causes, **II:**1372
  pathogenic mechanism, **II:**1370t
Primate corpus luteum regression, **II:**1346f
  cellular correlates of luteal regression, **II:**1340–1341
  estradiol as luteolysin, **II:**1339
  LH secretion pattern, **II:**1338–1339, **II:**1339f
  prostaglandins, **II:**1340
Primates, **I:**1112, **II:**2393
  endocrinology of human fathering, **II:**2394–2395
  ERα in ovarian function, **I:**1136
  ERβ expression, **I:**1112
  ERβ in ovarian function, **I:**1140–1141
  estradiol, **II:**2395
  glucocorticoid levels, **II:**2395
  Hylobatidae, **II:**2394
  luteal function in early pregnancy, **I:**1054–1057
  mate's pregnancy, **II:**2393
  nonhuman, **I:**1112
  testosterone and PRL, **II:**2393, **II:**2395
  uterine phenotypes in mouse models, **I:**1112–1119
Primordial follicles, **II:**1201
  factor in germline, **I:**954
  formation, **I:**953
  Lhx8, **I:**956–957
  notch signaling, **I:**953–954
  ovary homeobox gene, **I:**954–956
  prenatal exposures, **I:**957
  *Sohlh*1, **I:**956
  *Sohlh*2, **I:**956
  steroid hormone signaling, **I:**953
  ZGLP1, **I:**957
Primordial germ cell (PGC), **I:**9, **I:**234, **I:**269, **I:**622, **II:**2033

Primordial to primary follicle transition, **I:**957
AMH, **I:**959–960
Foxl2, **I:**960–961
Kitl, **I:**958–959
NGF, **I:**960
PI3K–AKT pathway, **I:**958
Principal cells, **I:**697–698, **I:**699f
Principal nucleus of bed nucleus of the stria terminalis (BNSTp), **II:**2119–2120
PRKO. *See* Progesterone receptor knockout
PRL. *See* Prolactin
PrL region. *See* Prelimbic region
PRL-like luteotropic hormones, **I:**1040–1042
PRL-R. *See* Prolactin receptor
PRMT. *See* Protein arginine N-methyltransferase
Procreation in prokaryotes and eukaryotes, **II:**1697
Prodynorphin (PDYN), **II:**1677
Progenitor cells, **I:**484
Progenitor Leydig cells (PLC), **I:**281
Progesterone (P_4), **I:**440, **I:**998, **I:**1008, **I:**1081
changes in ovarian secretion, **II:**1663
cognitive function
in animals, **II:**1997
in women, **II:**1999
effects in uterus, **II:**1708
estrous cycle plasticity
modes of action, **I:**440
negative feedback, **I:**440–441, **I:**441f
positive feedback, **I:**441
executing uterine functions, **II:**1710
feedback system, **II:**1472
as hormone of pregnancy, **II:**1700
humans, **II:**2386
inducing *Ihh* in uterus, **II:**1710–1711
inhibitory actions of, **II:**1542
laboratory rodents, **II:**2380–2381
parturition hormonal regulation, **II:**1889–1892
comparison of tissue synthesis, **II:**1890f
human PR-B and PR-A, **II:**1890f
placenta steroid hormone
clinical correlations, **II:**1803–1804
function, **II:**1803
physiological relevance, **II:**1803–1804
regulation, **II:**1803
synthesis, **II:**1802–1803
production, **I:**927, **I:**1033–1036
sheep, **II:**2384
stimulatory actions of, **II:**1542
treatment and reactivation, **II:**1698
uterine response
in absence of ER, **I:**1127
antiproliferative functions factor, **I:**1125–1126
in rodent uterus, **I:**1126–1127
uterine responses to factors independent, **II:**1711–1712
Progesterone receptor (PR), **I:**953, **I:**998, **I:**1081, **I:**1100, **I:**1102, **II:**1266–1267, **II:**1674, **II:**1877–1878
endometrial compartmental cross-talk, **I:**1089–1090
expression in uterus, **I:**1121
signaling, **I:**1087f, **I:**1088–1089
intraovarian role of progesterone, **I:**1163–1165
ovarian phenotypes in mouse models, **I:**1162–1163
PR expression in ovary, **I:**1161–1162, **I:**1161f
uterine phenotypes in mouse models with disrupted progesterone signaling, **I:**1121–1122
with mutations in transcriptional proteins, **I:**1122–1123
Progesterone receptor knockout (PRKO), **I:**998, **II:**1218, **II:**2381
Progesterone receptor membrane component 1 (PGRMC1), **I:**1109, **II:**2317
Progesterone response element (PRE), **I:**1086
Progestin receptor A (PR-A), **II:**2309
Progressive motility, **I:**132–133
Prokineticin 1 (PROK1), **I:**1031–1032
Prokineticin-2 (PK2), **II:**1624
Prolactin (PRL), **I:**469, **I:**672–673, **I:**793, **I:**1024–1025, **I:**1084, **II:**1624, **II:**1971, **II:**1976–1977, **II:**2057, **II:**2231, **II:**2335
breadth, **I:**469–470
control of prolactin surges, **II:**1978–1979
gene expression, **I:**481
humans, **II:**2387
hypothalamo-pituitary prolactin system, **II:**1977f
laboratory rodents, **II:**2382
lactotroph function, **I:**481–484
mRNAs, **II:**1208
multiple signal transduction pathway, **I:**471f
negative feedback, **II:**1979
pituitary hormone secretion control
candidate prolactin-releasing hormones, **II:**1233–1234
dopamine, **II:**1233–1234
luteolytic role, **II:**1234
ovarian steroids in control, **II:**1232–1233
prolactin-induced adaptive responses, **I:**493
bone, **I:**497
brain actions, **I:**498–502, **I:**498f–499f
calcium homeostasis, **I:**497
metabolic effects, **I:**493–497
prolactin-releasing factor, **II:**1980
in rat, **II:**1977
rat estrous cycle, **II:**1207–1208
receptor, **I:**470–472
releasing factors, **II:**1978
reproductive actions in male, **I:**502
fertility, **I:**502
paternal behavior, **I:**503
prostate gland, **I:**502–503
and reproductive disorders, **I:**503–505
reproductive functions, **I:**489
behaviors, **I:**492–493
fetal development, **I:**491–492
mammary gland, **I:**489–490
ovary, **I:**490–491
reproductive tract, **I:**491
sheep, **II:**2385
signal transduction, **I:**470–472
TIDA neurons regulation, **II:**1977–1978, **II:**1980
Prolactin receptor (PRL-R), **II:**1978
Prolactin receptor–associated protein (PRAP), **I:**1041–1042
Prolactin releasing factor (PRF), **II:**1959
Prolactin releasing peptide (PrRP), **II:**1978
Prolactin secretion, **II:**1977
from mid-pregnancy, **II:**1979–1980
neuroendocrine control
inhibition by dopamine, **I:**472–474
NEDA neurons, **I:**473f, **I:**474–475, **I:**478–480
prolactin-releasing factors, **I:**480
short-loop negative feedback, **I:**475–477
TIDA neuronal system development, **I:**477–478
transport across blood–brain barrier, **I:**478
patterns
extrapituitary, **I:**488–489
in lactating women, **I:**487–488
during pregnancy in rodents, **I:**485–487, **I:**486f
in pregnant, **I:**487–488
in pituitary, **I:**482–483
Prolactin-releasing factors, **I:**480, **II:**1559
Prolactinomas, **I:**503–504
Proliferating cell nuclear antigen (PCNA), **I:**605
Proliferative phase, **I:**1083, **II:**1879
Proliferin (*Plf*), **II:**1750–1751
Proopiomelanocortin (POMC), **I:**494–496, **II:**1226, **II:**1342, **II:**1414, **II:**1458–1459, **II:**1557, **II:**1610, **II:**1620, **II:**1897, **II:**1965, **II:**2230, **II:**2446
Prophase I, **I:**15–16
checkpoint control of synapsis and recombination, **I:**35–36
chromosome dynamics and pairing
chromosome movements, **I:**17–18
pairing centers and noncanonical mechanisms, **I:**18–19
SUN–KASH proteins, **I:**18
telomere bouquet formation, **I:**17–18
telomere clustering, **I:**17–18
cohesins, **I:**21–23
immunofluorescent analysis, **I:**17f
meiotic recombination, **I:**23–31
meiotic silencing, **I:**33–35
recombination and synapsis in, **I:**16f
small RNA pathways, **I:**31
gametogenesis, **I:**31–32
PIWI-interacting RNAs, **I:**32–33
synaptonemal complex, **I:**19–21
Proprotein convertase subtilisin/kexin type 4 (PCSK4), **I:**104
Propyl endopeptidase (PEP), **I:**410–411
Propylpyrazole (PPT), **I:**1106
Prostaglandin (PG), **I:**791, **I:**997–998, **I:**1045, **I:**1718–1719, **II:**1722, **II:**1876
metabolism, **II:**1885
parturition hormonal regulation, **II:**1895–1897
$PGF_{2\alpha}$, **I:**1046–1049
synthesis inhibitors, **II:**1906–1907

Prostaglandin D (DP), **II:**1718–1719
Prostaglandin D Ssynthase (PGDS), **I:**734
Prostaglandin D2 (PGD2), **I:**277
Prostaglandin E synthase (PGES), **II:**1896
Prostaglandin E_1 (PGE_1), **II:**2219
Prostaglandin E_2 (PGE_2), **I:**223, **I:**702, **I:**969, **I:**1092, **II:**1415, **II:**1850, **II:**2230
  direct application of drugs effects, **II:**2247
Prostaglandin F (FP), **II:**1718–1719
Prostaglandin I (IP), **II:**1718–1719
Prostaglandin synthase (PGS), **I:**998, **I:**1046
Prostaglandin synthase1 (PTGS1). *See* Cyclooxygenase 1 (COX-1)
Prostaglandin transporter (PGT), **II:**1282
Prostate
  anatomy, **I:**775–778, **I:**776f
  autoimmunity in, **I:**851–852
  histology, **I:**778–779
Prostate cancer, **I:**504–505, **I:**773, **I:**794–795
  stem cells, **I:**795
Prostate gland, **I:**502–503
  development, **I:**779
    human, **I:**779–780
    rodent, **I:**780–781
    stem cells, **I:**781–782, **I:**781f
  diseases, **I:**794–797
    BPH, **I:**796
    hormonal influence, **I:**795–796
    prostate cancer, **I:**794–795
    prostatitis, **I:**796–797
Prostate-binding protein (PBP), **I:**778
Prostate-specific antigen (PSA), **I:**200, **I:**318, **I:**778, **I:**791–792
Prostate-specific protein 94 (PSP94), **I:**778
Prostatic acid phosphatase (PAP), **I:**778
Prostatic urethra, **I:**775–776
Prostatitis, **I:**773, **I:**796–797
Protease nexin-1 (PN-1), **I:**200
Protein arginine N-methyltransferase (PRMT), **I:**316, **I:**318, **II:**2446
Protein hormones, **II:**1784
  corpus luteum endocrine secretions, **II:**1334
  GH, **I:**793–794
  GH-V, **II:**1791–1792
  hCG, **II:**1784–1789
  Igf, **I:**793–794
  placental growth factors, **II:**1792–1799
  placental lactogen, **II:**1789–1791
  PRL, **I:**793
Protein inhibitor of activated STAT (PIAS), **I:**325
Protein kinase A (PKA), **I:**41, **I:**896, **I:**1103, **I:**1152, **II:**1314–1315, **II:**1416–1417, **II:**1880, **II:**2219, **II:**2314
  AKAPs with, **I:**900–901, **I:**900f
  holoenzymes, **I:**899
  signals
    to activate ERKs, **I:**902–904
    to activate PI-3K Pathway, **I:**905–915
  in undifferentiated granulosa cells, **I:**901–902
Protein kinase B (AKT), **I:**899–900, **I:**905–906
  substrate, **I:**907, **I:**907f
Protein kinase C (PKC), **I:**408, **I:**900, **I:**1058, **I:**1109–1110, **II:**1283
Protein kinase G (PKG), **II:**2219
Protein posttranslational modification (PTM), **I:**76
Protein tyrosine kinase (PTK), **I:**327
Protein tyrosine phosphatase (PTP), **I:**352, **I:**903
Protein(s), **II:**2076
  excretion, **II:**1940
  microenvironment, **I:**714
  synthesis regulation, **I:**75–76
Proteomics, **II:**1725–1726
Proton-coupled folate transporter (PCFT), **II:**1768
Protons, **II:**1757
Proximal box (P box), **I:**1105. *See also* Distal box (D-box)
PRR. *See* Pattern recognition receptor
PrRP. *See* Prolactin releasing peptide
PRSS21. *See* Serine protease 21
PSA. *See* Prostate-specific antigen
PSC. *See* Postsynaptic current
Pseudoautosomal region (PAR), **II:**2461
Pseudopregnancy, **II:**1204
PSP94. *See* Prostate-specific protein 94
pSTAT3. *See* Phosphorylated STAT3
pSTAT5b. *See* Phosphorylated signal transducer and activator of transcription 5b
Psychological stressors, **II:**1637, **II:**1645–1647
PT-specific cells, **II:**1586
PTCH1. *See* Patched homolog 1
PTGS. *See* Prostaglandin synthase (PGS)
PTH. *See* Parathyroid hormone
PTHrP. *See* Parathyroid hormone–related peptide
PTK. *See* Protein tyrosine kinase
PTM. *See* Posttranslational modification
PTM. *See* Protein posttranslational modification
PTM cells. *See* Peritubular myoid cells
PTP. *See* Protein tyrosine phosphatase
PTX3. *See* Pentraxin 3
Pubarche, **II:**1487
Puberty, **II:**1553–1555
  adrenal function, **II:**1491
  comparative considerations, **II:**1442–1443
  determinants, **II:**1453
    external determinants, **II:**1459–1469
    internal determinants, **II:**1453–1459
  disorders, **II:**1520
    delayed puberty, **II:**1521–1522
    precocious puberty, **II:**1520–1521
  epigenetics, **II:**1516–1518
  exteroceptive cues
    food availability, **II:**1519–1520
    season, **II:**1518–1519
    social factors, **II:**1519
  female sheep, **II:**1443
    estradiol on LH secretion, **II:**1444f
  fetal programming, **II:**1518
  genes, **II:**1516–1518
  genetics, **II:**1516–1518
  GnRH
    neural network, **II:**1409–1419
    neurons, **II:**1400–1409
  gonad-dependent, **II:**1398–1399
  gonadal function
    ovary, **II:**1490–1491
    testis, **II:**1489–1490
  gonadal independence of postnatal switches, **II:**1500–1501
  gonadotropin secretion
    developmental pattern, **II:**1495–1499
    neuroendocrine control, **II:**1493–1495
  historical overview, **II:**1441–1442
  HPG, **II:**1396–1397
  juvenile development hypogonadotropic state, **II:**1500–1501
  juvenile hiatus neurobiological bases, **II:**1501
    amino acid neurotransmitters, **II:**1505–1507
    confocal images, **II:**1502f
    glial-derived growth factors, **II:**1508–1509
    hypogonadotropic state, **II:**1503f
    kisspeptin, **II:**1502–1505, **II:**1504f
    neuronal factors, **II:**1508
    NPY, **II:**1507–1508
    opioid peptides, **II:**1508
    structural remodeling, **II:**1509
  male sheep, **II:**1443
  in man, **II:**1488
  model for timing control, **II:**1506f
  modulatory influences, **II:**1419–1420
    leptin, **II:**1420
    pheromones, **II:**1420–1421
  neuroendocrine axis, **II:**1397–1398
  neuroendocrine sequence
    events after first LH surge, **II:**1451–1453
    onset of high-frequency GnRH secretion, **II:**1443–1451
  onset of, **II:**1518
  ovulation for, **II:**1442f
  pars distalis, **II:**1396
  photoperiod and timing, **II:**1459f
  pituitary gonadotrope function, **II:**1492–1493
  prepubertal hiatus in GnRH secretion, **II:**1509
    pubertal clock, **II:**1515–1516
    somatometer, **II:**1510–1515
  in primates, **II:**1488
  pubertal clock, **II:**1515–1516
  pubertal development, **II:**1498–1499
  rats and mice, **II:**1399
    females, **II:**1399
    males, **II:**1399–1400
  secular trends, **II:**1492
  sex differences, **II:**1469
    endocrine disruptors, **II:**1472–1473
    GnRH controls by prenatal steroids, **II:**1471–1472
    GnRH rise by prenatal steroids, **II:**1470–1471
    GnRH rise in pulse frequency, **II:**1469–1470
    response to growth cues, **II:**1474–1475
    response to photoperiod, **II:**1473–1474
  sexual reproduction, **II:**1395
  staging, **II:**1491–1492

Pulsatility, GnRH, **I:**428f
extrinsic pulse generation, **I:**429
intrinsic pulse generation, **I:**428–429
pulse generation, **I:**426–428
surge generation, **I:**429–431
Pulse generation, **I:**426–428
extrinsic, **I:**429
intrinsic, **I:**428–429
Purring, **II:**2298
PVN. *See* Paraventricular nucleus
PWS. *See* Prader–Willi syndrome
Pyknosis, **II:**1203
Pyruvate dehydrogenase E1 component β (PDHB), **I:**135
Pyruvate kinase (PK), **I:**130
PYY. *See* Peptide YY

Q

Quantitative polymerase chain reaction (qPCR), **I:**652
Quantitative reverse-transcriptase (qRT), **II:**1264–1265

R

R-spondin1 (RSPO1), **I:**14–15, **I:**278–279
RA. *See* Retinoic acid
RA receptor (RAR), **I:**9–10
RAB3A. *See* ras oncogene family member 3a
Rabbits, female sexual behavior, **II:**2299
Radioimmunoassay (RIA), **II:**1493, **II:**1540–1541
RALDH. *See* Retinaldehyde dehydrogenase
RANKL. *See* Receptor activator of nuclear factor kappa-B ligand
RAR. *See* RA receptor
RARE. *See* Retinoic acid response elements
Ras homolog protein (RHO), **I:**129
ras oncogene family member 3a (RAB3A), **I:**111–113
Rat estrous cycle, **II:**1199
corpus luteum, **II:**1234–1238
laboratory rat, **II:**1200
molecules chemical nature, **II:**1200
mRNA levels, **II:**1206–1208
neural events, **II:**1209–1215
neuroendocrine regulation, **II:**1199–1200
ovarian events, **II:**1202f
corpus luteum, **II:**1204
folliculogenesis, **II:**1201–1203
oogenesis, **II:**1201
ovulation, **II:**1203–1204
steroids secretion, **II:**1204–1206
pituitary hormone secretion
control, **II:**1215–1234
patterns, **II:**1206–1208
Rats
female sexual behavior, **II:**2296–2297
and mice as models, **II:**1399
species differences, **II:**1399–1400
pregnancy
glomerular hemodynamics in, **II:**1942
relaxin paradigm in, **II:**1944–1946
RB. *See* Retinoblastoma
Rbx1. *See* Ring-box 1
RCT. *See* Reverse cholesterol transport
Rdh10. *See* Retinol dehydrogenase 10
RDLN. *See* Retrodorsolateral nucleus
REA. *See* Estrogen receptor activity
Reactive oxygen species (ROS), **I:**710–711, **I:**814, **I:**836–838
Rearranged during transfection (RET), **I:**693
Receptor activator of nuclear factor kappa-B ligand (RANKL), **I:**322, **II:**2061
Receptor tyrosine kinase (RTK), **I:**275
Recognition helix, **I:**1105
Reduced folate carrier (RFC), **II:**1768
Reduced uterine perfusion pressure (RUPP), **II:**1810
Reduction in intrarenal interstitial hydrostatic pressure (RIHP), **II:**1945
"Reductional" meiosis division, **I:**5
Regulatory lymphocyte subsets, **I:**816–817
Regulatory T cells (Treg cells), **II:**1835–1837, **II:**1854–1857
deficiency in, **II:**1863–1864
and reproductive dysfunctions, **II:**1863f
Reichert membrane, **II:**1752
Relative length
index, **II:**2171–2172
long bones, **II:**2172
Relaxin (RLN), **I:**1037, **II:**1961
and oxytocin neurons, **II:**1962–1963
receptors in brain, **II:**1962
relaxin-3, **II:**1973
Relaxin family peptide (RXFP), **I:**1037
Relaxin-like factor (RLF), **I:**1037
Renal adaptations, **II:**1940. *See also* Cardiovascular adaptations
albumin excretion, **II:**1943–1944
aminoaciduria, **II:**1940
blood urea nitrogen, **II:**1940
creatinine, **II:**1940
ERPF, **II:**1940, **II:**1940f
genitourinary tract, **II:**1944
GFR, **II:**1940, **II:**1940f
glycosuria, **II:**1940
human pregnancy
glomerular capillary permselectivity, **II:**1943
intraglomerular hemodynamics in, **II:**1942
postpartum glomerular hemodynamics in, **II:**1942–1943
increasing GFR mechanism, **II:**1941–1942
integrated view, **II:**1946–1947
kidney, **II:**1944
protein excretion, **II:**1940
rat pregnancy
glomerular hemodynamics in, **II:**1942
relaxin paradigm in, **II:**1944–1946
renal hemodynamic changes, **II:**1941
renin release, **II:**1940
sodium handling, **II:**1940
structural changes, **II:**1941
urinary protein, **II:**1943–1944
water retention, **II:**1940
Renal hemodynamic adaptations mediators, **II:**1946–1947
Renal tubular function, **II:**1940
Renal vascular resistance (RVR), **II:**1941
Renin release, **II:**1940
Replication protein A (RPA), **I:**27
Repressive element silencing transcription (REST), **II:**2443–2446
Reproduction, **II:**1605, **II:**1638
Kiss1 neurons, **II:**1611
in basal forebrain, **II:**1612
central pathways and transmitters, **II:**1618–1619
expression studies, **II:**1612–1614
functional analyses, **II:**1614–1615
GALP neurons, **II:**1623
leptin signaling and, **II:**1615–1617
MCH and orexin neurons, **II:**1623
impact of metabolic stress conditions, **II:**1613f
nesfatin 1 and SF1 neurons, **II:**1624–1625
neuronal efferents from SCN, **II:**1624
neuropeptides, **II:**1619–1620, **II:**1621t
NPY and AgRP neurons, **II:**1620–1622
PMV neurons, **II:**1622–1623
POMC and CART neurons, **II:**1620
RF-amide related peptides, **II:**1624
metabolic hormone reproductive effects, **II:**1627
direct pituitary effects, **II:**1627–1628
Ghrelin, **II:**1628
GHS-R type 1a, **II:**1628
leptin and insulin, **II:**1628
metabolic hormones regulating, **II:**1606–1607
ghrelin, **II:**1609
insulin, **II:**1608–1609
leptin, **II:**1607–1608
peripheral hormones, **II:**1609–1610
regulatory roles of key hormonal signals, **II:**1607f
metabolic targeting of GnRH neurons, **II:**1610–1611
stress-induced suppression, **II:**1647
in viviparous mammals, **II:**1697
Reproductive aging, **II:**1661. *See also* Female reproductive aging; Male reproductive aging
historical perspective, **II:**1662–1663
HPG axis, **II:**1661–1662
Reproductive disorders, **I:**503–505
Reproductive system, **II:**1641–1642
GnRH pulse generator components, **II:**1642
inhibitory effects of stressors, **II:**1642–1643
HPA and HPG axes relationship, **II:**1643
immunological/inflammatory stressors, **II:**1643–1644
influencing reproductive function, **II:**1643
metabolic stressors, **II:**1644–1645
psychological stressors, **II:**1645–1647
suppression of pulsatile GnRH/gonadotropin secretion, **II:**1644f
stress impact on hypothalamic Kp and KNDy system, **II:**1647
adverse early life environments, **II:**1648

exogenous corticosterone, **II:**1647
glucocorticoid receptor immunoreactivity identification, **II:**1647–1648
GnRH pulse generator frequency, **II:**1648
inhibitory neurotransmitter GABA, **II:**1648
stress-induced suppression, **II:**1647
stress-related neuropeptide systems, **II:**1648
Residual volume (RV), **II:**1938–1939
Respiratory system adaptations, **II:**1938. *See also* Cardiovascular adaptations
diffusing capacity, **II:**1939–1940
hyperventilation, **II:**1938
lung volumes and function, **II:**1938–1939, **II:**1939f
REST. *See* Repressive element silencing transcription
Restraint, **II:**1645–1646
Resynthesis
model for cycle, **II:**1286–1288, **II:**1287f
retrospective historical analysis, **II:**1288–1289
RET. *See* Rearranged during transfection
Retinaldehyde dehydrogenase (RALDH), **I:**10b
*Raldh*2, **I:**9–10
Retinoblastoma (RB), **I:**619
RB1, **I:**318
Retinoic acid (RA), **I:**9–10, **I:**252, **I:**617, **I:**793
germ cells, **I:**12
in male mice, **I:**10–12
receptors, **I:**723–725
synthesis and action, **I:**10b
Retinoic acid response elements (RARE), **I:**10b, **I:**723–724
Retinoid X receptor (RXR), **I:**9–10, **II:**1719
Retinoids, **I:**784
Retinol dehydrogenase 10 (Rdh10), **I:**9–10, **I:**10b
Retinol-binding protein (RPB), **I:**10b
Retrodorsolateral nucleus (RDLN), **II:**2261
Retrograde signaling, **II:**1989
eCBs, **II:**1990
nNOS, **II:**1989–1990
Reverse cholesterol transport (RCT), **I:**1035–1036
Reward circuitry, **II:**1975–1976
26RFamide (26RFa), **II:**1624
43RFamide (43RFa), **II:**1624
Rf-amide signaling pathways, **II:**1590–1591
Kisspeptin, **II:**1591–1592, **II:**1593f
RFRP neurons, **II:**1592–1594
thyrotropin-dependent control, **II:**1594
RF-amide-related peptide (RFRP), **I:**425, **II:**1592, **II:**1623
RFRP-3, **I:**413, **II:**1226, **II:**1266–1267, **II:**1624
RFRP1, **II:**1624
RFC. *See* Reduced folate carrier
RFRP. *See* RF-amide-related peptide
RG108 compound, **II:**2484–2485
Rhesus macaques, **II:**2299–2300
RHO. *See* Ras homolog protein
Rhophilin (RHPN1), **I:**129
RIA. *See* Radioimmunoassay
Ribosomal protein S6 kinase 1 (RPS6K1), **I:**1047–1048
RIHP. *See* Reduction in intrarenal interstitial hydrostatic pressure
Ring fingers, **II:**2171–2172
Ring-box 1 (Rbx1), **I:**323
RISC. *See* RNA-induced silencing complex
Ritodrine, **II:**1907
RLF. *See* Relaxin-like factor
RLN. *See* Relaxin
RNA helicases, **I:**319
RNA interference (RNAi), **I:**20
RNA-induced silencing complex (RISC), **I:**31, **II:**2031
RNAi. *See* RNA interference
Rodents, **I:**1111
A0/A1 model, **I:**600–605, **I:**604f
ovarian function
ERα in, **I:**1133–1135
ERβ in, **I:**1136–1139
germ cell markers in, **I:**601t–603t
hypothalamic-pituitary aging in, **II:**1672
laboratory
adrenal steroids, **II:**2381
estrogen, **II:**2379
oxytocin, **II:**2383
progesterone, **II:**2380–2381
prolactin, **II:**2382
testosterone, **II:**2380
A_s model, **I:**598–600, **I:**604f
prolactin secretion during pregnancy, **I:**485–487, **I:**486f
spermatogenic clone development, **I:**600f
Rohr's fibrinoid. See Basal plate blood-derived fibrinoid
Ropporin 1 (ROPN1), **I:**109
ROS. *See* Reactive oxygen species
ROS1. *See* C-ROS receptor
Rostral periventricular area of the third ventricle (RP3V), **II:**1550
rostral preoptic area (rPOA), **I:**400
RP3V. *See* Rostral periventricular area of the third ventricle
RP3V kisspeptin neurons, **I:**421
RPA. *See* Replication protein A
RPB. *See* Retinol-binding protein
rPOA. *See* rostral preoptic area
RPS6K1. *See* Ribosomal protein S6 kinase 1
RSPO1. *See* R-spondin1
RTK. *See* Receptor tyrosine kinase
RUNX1, **I:**1009–1010
RUNX2, **I:**1009–1010
RUPP. *See* Reduced uterine perfusion pressure
Russell–Silver syndrome. *See* Silver–Russell syndrome (SRS)
RV. *See* Residual volume
RVR. *See* Renal vascular resistance
RXFP. *See* Relaxin family peptide
RXR. *See* Retinoid X receptor
Ryanodine receptor (RyR), **I:**176

S

S-adenosyl methionine (SAM), **II:**2446
S-GAG. *See* Sulfated-glycosaminoglycan
sAC. *See* Sensitive adenylate cyclase
SAC. *See* Spindle assembly checkpoint
Sal-like 4 (*Sall*4), **I:**955
*Sall*4. *See* Sal-like 4
Salt appetite, **II:**1963
SAM. *See* S-adenosyl methionine
Sarcoplasmic/endoplasmic reticulum calcium ATPase (SERCA), **I:**177, **I:**347
Satellite fibers, **I:**126
SCAP. *See* SREBP cleavage-activating protein
SCARKO. *See* Sertoli cell–specific AR knockout
Scavenger receptor class B type I (SRBI), **I:**327
Scavenger receptors A (SRA), **II:**1767
SCC. *See* Side chain cleavage
SCD2. *See* Stearoyl-CoA-desaturase
SCF. *See* Stem cell factor
SCMC. *See* Subcortical maternal complex
SCN. See Superchiasmatic nucleus
SCN. *See* Suprachiasmatic nucleus
SCNT. *See* Somatic cell nuclear transfer
SCP2. *See* Sterol carrier protein 2
SDA. *See* Sexually dimorphic area
SDN-POA. *See* Sexually dimorphic nucleus of the preoptic area
SDR. *See* Short-chain dehydrogenase/reductase
SDSA pathway. *See* Synthesis-dependent strand annealing pathway
SDZ. *See* Secondary decidual zone
Seasonal breeding, **II:**1558–1559
Seasonal regulation of reproduction in mammals, **II:**1575. *See also* Reproduction
ecological context, **II:**1576
innate long-term timing, **II:**1577–1578
maternal photoperiodic programming, **II:**1578–1579
nutrition x photoperiod interactions, **II:**1577f
photoperiodic cues, **II:**1577–1578
photoperiodic history dependence, **II:**1578–1579
geophysical basis of seasonality, **II:**1576f
melatonin
action, **II:**1585–1587
receptors, **II:**1583–1585
pineal hormone melatonin, **II:**1579
melatonin synthesis pathway, **II:**1581–1582, **II:**1581f
photoneuroendocrine system, **II:**1580f
pineal gland activity control, **II:**1579–1581
SCN, **II:**1580f
timed infusion experiments, **II:**1582–1583, **II:**1583f
PT and thyroid hormone-dependent control, **II:**1587–1590
Rfamide signaling pathways, **II:**1590–1594
tanycytes, **II:**1594–1596
timing of breeding season, **II:**1576f
Secondary decidual zone (SDZ), **II:**1716

Secreted factors, **I:**862
Secreted frizzled related protein (sFRP), **I:**788
  SFRP2, **II:**1709–1710
Secreted protein, acidic, cysteine-rich (SPARC), **I:**1029
Secreted signaling
  molecules, **I:**786
  regulators, **I:**788–789
Secretory activation, **II:**2065–2067
Secretory differentiation hormonal regulation, **II:**2063
  progesterone, **II:**2065
  prolactin, **II:**2064–2065
Selective estrogen receptor degrader (SERD), **I:**1110–1111
Selective estrogen receptor modulator (SERM), **I:**1109
Selective serotonin reuptake inhibitors (SSRI), **II:**1651, **II:**2225
Self-priming, **I:**361–362
Self–nonself, **II:**1845–1846
Semaphorins, **II:**1418
Semen, **I:**847–848
Semenogelin I (SEMG1), **I:**200
semenogelin II (SEMG2), **I:**200
Seminal colliculus, **I:**775–776
Seminal emissions, **II:**2214
Seminal leukocytes, **I:**847–848
Seminal plasma
  components, **I:**790
    acid phosphatase, **I:**791
    cholesterol, **I:**791
    citric acid, **I:**790
    fructose, **I:**790
    immunoglobulins, **I:**791
    lipids, **I:**791
    phosphorylcholine, **I:**790–791
    prostaglandins, **I:**791
    PSA, **I:**791–792
    spermine, **I:**791
    zinc, **I:**790
  human components, **I:**789t
  immunoregulation by, **I:**860–861
Seminal vesicle
  anatomy, **I:**779
  development, **I:**782
Seminal vesicle-secreted proteins (SVS), **I:**200
Seminiferous epithelium, **I:**638, **I:**838–839
Sensitive adenylate cyclase (sAC), **I:**699
Sensory modalities, **II:**2161, **II:**2179
  audition, **II:**2162
  olfaction, **II:**2162
  vision, **II:**2161–2162
Sensory systems, olfactory bulbs, **II:**2231
  auditory system, **II:**2234
  c-fos activation, **II:**2233–2234
  effects of lesions, **II:**2232–2233
  somatosensory systems, **II:**2234
Septin (SEPT), **I:**126–127
Sequence-specific methylation approaches
  bisulfate
    conversion, **II:**2487–2488, **II:**2488f
    sequencing, **II:**2489
  methyl-seq, **II:**2489
  methylation-dependent immunoprecipitation, **II:**2489
  methylation-specific PCR, **II:**2488–2489
  methylation-specific restriction enzyme digest, **II:**2488
  MethylCap-Seq, **II:**2489
SERCA. *See* Sarcoplasmic/endoplasmic reticulum calcium ATPase
SERD. *See* Selective estrogen receptor degrader
Serine protease 21 (PRSS21), **I:**107
SERM. *See* Selective estrogen receptor modulator
Serotonin, **I:**546–547, **II:**1225–1226, **II:**1581, **II:**1640, **II:**1965–1967, **II:**2225–2227, **II:**2336
  copulation, **II:**2226
  direct application of drugs effects, **II:**2245
  5-HT_{2C} receptors, **II:**2227
  8-OH-DPAT's effects, **II:**2226
  opposing effects, **II:**2227
  SSRI fluoxetine to rats, **II:**2225–2226
Serotonin transporter (SERT), **II:**1769
Sertoli cell(s), **I:**568–571, **I:**648. *See also* Spermatogenic cells
  activin-regulated gene expression in, **I:**668–669
  AR-dependent regulation, **I:**651–652
  cell proliferation hormonal control, **I:**572–574
  development, **I:**278
  during development, **I:**574–576, **I:**575f
  GDNF regulation, **I:**661
  nongenomic actions, **I:**664
  proliferation regulation, **I:**659–660
  regulation of peripubertal, **I:**576–577
  reproductive aging in men, **II:**1680
  T3 downstream target, **I:**663–664
Sertoli cell–specific AR knockout (SCARKO), **I:**648
Serum albumin, **II:**2079–2080
Serum glucocorticoid kinase (SGK), **I:**896, **I:**973
Serum inhibin, **I:**962
Sex
  accessory tissue function, **I:**789
    coagulation, **I:**789–790
    human seminal plasma components, **I:**789t
    seminal plasma components, **I:**790–792
  body, **I:**33
  chromosomes, **II:**2112, **II:**2115t
    complement, **II:**2114
    FCG model, **II:**2116
    gonad-independent effects, **II:**2114, **II:**2116
    role of, **II:**2113–2114
    SDN-POA, **II:**2114–2116
    SF1 knockouts, **II:**2116, **II:**2116f
    X and Y gene dosage, **II:**2118
    X-escapees, **II:**2117–2118
  reversal, **I:**269, **I:**1148
  steroids, **II:**1963
Sex chromosome-related disease, **I:**269
  SRY, **I:**269–271
  X-dosage
    XO, **I:**271
    XXY, **I:**271
  Y-specific genes, **I:**271
Sex determination, heteromorphic, **I:**267–269
Sex hormone–binding globulin (SHBG), **I:**646, **I:**720–721, **I:**792, **I:**841, **II:**1376, **II:**1680
Sex-determining region of the Y chromosome gene (Sry gene), **I:**14–15, **I:**269–271, **II:**1752, **II:**2112
Sexome, **II:**2144, **II:**2144f
Sexual attitudes, **II:**2136–2137
Sexual brain, **II:**2290–2292
Sexual differentiation
  age of onset, **II:**2143
  agents, **II:**2111–2112
    environmental factors, **II:**2113
    gonadal hormones, **II:**2112–2113
    sex chromosomes, **II:**2112
  for behavioral and neurological disorders, **II:**2143
  in brain, **II:**2143
    function of, **II:**2144
    key genes, **II:**2144
    whole-body approach, **II:**2144–2145
  of brain and behavior, **II:**2109–2110, **II:**2478f
  course of disease, **II:**2143
  critical periods, **II:**2128–2129
  in different contexts, **II:**2129–2131
  environment play in, **II:**2143
  gonadal hormone levels, **II:**2110
  hormones role and biological factors, **II:**2133
    aggression, **II:**2134–2136
    cognitive function, **II:**2133–2134
    DSDs, **II:**2137–2139
    prenatal androgen correlation, **II:**2139–2143
    sex ratios in criminal offenses, **II:**2135t
    sexual attitudes, **II:**2136–2137
    sexual orientation, **II:**2137
  "organizational" and "activational" hypothesis, **II:**2110, **II:**2111f
  periadolescent testosterone exposure effects, **II:**2129f
  structure and function relationship, **II:**2131–2132
  vulnerability, **II:**2143
Sexual maturation, **II:**1395–1396, **II:**1399–1400, **II:**1453–1454
Sexual orientation, **II:**2137, **II:**2163
  endocrine control, **II:**2175
  heritability of, **II:**2176
Sexual partner preference
  biological and social factor interactions, **II:**2178–2179
  changes in hormone action, **II:**2175–2176
  endocrine control in animals, **II:**2163
    activational effects of adult hormones, **II:**2163–2164
    brain mechanisms, **II:**2166
    homosexuality, **II:**2166–2167
    organizational effects of steroids, **II:**2164–2166
  endocrine control in humans, **II:**2167

clinical studies, **II:**2168–2171
sexually differentiated characteristics, **II:**2171–2175
older brothers effect, **II:**2178
sensory modalities, **II:**2179
Sexual receptivity, **II:**1452
Sexual sensory systems. *See also* Female sexual behavior
anatomy and physiology of, **II:**2301, **II:**2302f
autonomic control, **II:**2301–2302
clitoral complex, **II:**2303f
erogenous zones, **II:**2307–2308
genitosensory stimulation, **II:**2304
cervical stimulation, **II:**2304–2305
clitoral and cervical overlap, **II:**2306
clitoral stimulation, **II:**2304
primary visual and olfactory senses, **II:**2308
spinal pathways, **II:**2306
Fos induction, **II:**2306
tract tracing, **II:**2306
ultrastructural changes in brain, **II:**2306–2307
Sexual stimulation consequences, **II:**2339
conditioned sexual
arousal in women, **II:**2347–2348
partner preferences, **II:**2343–2347, **II:**2344f
responding, **II:**2348–2349
Coolidge effects, **II:**2339–2340
CPP, **II:**2340, **II:**2340f
lordosis and sexual behaviors, **II:**2340–2341
selective activation of Fos, **II:**2346f
sexual CPPs in females, **II:**2342–2343
sexual experience in females, **II:**2341
spine densities, **II:**2341f
Sexually dimorphic area (SDA), **II:**2238
Sexually dimorphic nucleus of the preoptic area (SDN-POA), **I:**285
SF. *See* Steroidogenic factor
SFO. *See* Subfornical organ
sFRP. *See* Secreted frizzled related protein
SGA. *See* Small for gestational age
sGC. *See* Soluble guanylate cyclase
SGK. *See* Serum glucocorticoid kinase
SH2. *See* Src homology 2
SHBG. *See* Sex hormone–binding globulin; Steroid hormone binding globulin
Sheep ovarian cycle
analysis methods, **II:**1260
estrogen and progesterone, **II:**2384
estrous cycle of ewe, **II:**1260
ovarian cyclicity seasonal control, **II:**1289–1294
ovine estrous cycle, **II:**1260
analysis, **II:**1265–1286
estrous behavior, **II:**1260–1261
hypothalamo–hypophysial unit, **II:**1261
model for, **II:**1286–1289
ovary, **II:**1261–1265
uterine prostaglandins, **II:**1265
ovine reproduction, **II:**1259–1260
oxytocin, **II:**2385
preterm birth animal models, **II:**1902–1903
prolactin, **II:**2385
Shh. *See* Sonic hedgehog
"Short day breeders", **II:**1575–1576
short hairpin RNA (shRNA), **II:**2406
Short interspersed nucleotide element (SINE), **II:**2455
and Alu elements, **II:**2457–2458
primate-specific, **II:**2458–2459
Short-chain dehydrogenase/reductase (SDR), **I:**297–298
Short-lived corpora lutea, **I:**1026–1027. *See also* Long-lived corpora lutea; Ultrashort-lived corpora lutea
Short-loop negative feedback, **I:**475–477
shRNA. *See* short hairpin RNA
SHTAP. *See* Sperm head and tail associated protein
Shugoshin gene, **I:**40
Side chain cleavage (SCC), **I:**896
Signal transducers and activators of transcription (STAT), **II:**1791, **II:**1887
STAT3, **II:**1610, **II:**1766
STAT5, **II:**1233
Sik-similar protein (Sik-SP), **II:**1709–1710
"Silencer" proteins, **II:**1517
Silencing mediator of retinoid and thyroid receptors (SMRT), **I:**317–318
Silver–Russell syndrome (SRS), **II:**2037–2038
Sim1 gene. *See* Single-minded 1 gene
SINE. *See* Short interspersed nucleotide element
Single nucleotide polymorphism (SNP), **II:**1516–1517, **II:**2003, **II:**2199
Single-minded 1 gene (Sim1 gene), **II:**1971–1972
single-stranded DNA (ssDNA), **I:**27
siRNA. *See* small interfering RNA
Sirtuins (SIRT1–7), **II:**2446
SLC. *See* Stem Leydig cell
SLC11A2. *See* Divalent metal transporter protein (DMT1)
SLC19A1. *See* Reduced folate carrier (RFC)
SLC2. *See* Glucose transporter (GLUT)
SLC26A4. *See* Pendrin
SLC2A1. *See* GLUT1 transporters
SLC2A3. *See* GLUT3 transporters
SLC2A4. *See* GLUT4 transporters
SLC2A9. *See* GLUT9 transporters
SLC46A1. *See* Proton-coupled folate transporter (PCFT)
SLC5A5. *See* Sodium iodide symporter (NIS)
SLC5A6. *See* Human Na^+–multivitamin transporter (hSMVT)
SLC7A5. *See* Large neutral amino acid transporter 1 (LAT1)
SLC7A8. See Large neutral amino acid transporter 2 (LAT2)
SLE. *See* Systemic lupus erythematosus
Sma-and Mad-related proteins (SMAD), **I:**961
Small for gestational age (SGA), **II:**1518, **II:**2044–2045
small interfering RNA (siRNA), **I:**31, **I:**1034–1035
Small nuclear RING finger (SNURF), **I:**343
Small ubiquitin modifier (SUMO), **I:**1091
SUMO1, **I:**324–325
SMC1β. *See* Structural maintenance of chromosomes 1 beta
SMCP. *See* Sperm mitochondria-associated cysteine-rich protein
SMRT. *See* Silencing mediator of retinoid and thyroid receptors
SN. *See* Surrounded nucleolus
SNARE. *See* Soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor
SNAT1. *See* Sodium-dependent neutral amino acid transporter 1
SNB. *See* Spinal nucleus of bulbocavernosus
SNP. *See* Single nucleotide polymorphism; Sodium nitroprusside
SNS. *See* Sympathetic nervous system
SNURF. *See* Small nuclear RING finger
SOC. *See* Store-operated channels
SOCE. *See* Store-operated Ca^{2+} entry
Sociosexuality, **II:**2136
SOCS1. *See* Suppressor of cytokine synthesis-1
SOD2. *See* Superoxide dismutase 2
Sodium, **II:**1758
balance, **II:**1962
handling, **II:**1940
Sodium chloride (NaCl), **II:**1957
Sodium iodide symporter (NIS), **II:**1760
Sodium nitroprusside (SNP), **II:**2246
Sodium-dependent neutral amino acid transporter 1 (SNAT1), **II:**1765
*Sohlh*1. *See* Spermatogenesis and oogenesis-specific helix–loop–helix 1
Solicitations, **II:**2329
glutamate in ventrolateral VMH, **II:**2330
incertohypothalamic DA, **II:**2329
MC, **II:**2329
opioid receptors, **II:**2330
Soluble guanylate cyclase (sGC), **I:**702
Soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor (SNARE), **I:**348–349
Solute carrier family 9, **I:**133–134
Somatic cell nuclear transfer (SCNT), **I:**83–84
Somatometer, **II:**1510
GH, **II:**1511–1512
GnRH pulse generator, **II:**1514
IGF-1, **II:**1511–1512
LEP gene, **II:**1510–1511
melatonin, **II:**1512–1513
metabolic hormones, **II:**1513–1514
serum LH concentrations, **II:**1512f
substrates, **II:**1513–1514
Somatosensation, **II:**2399
importance of suckling, **II:**2401
nursing behavior, **II:**2399–2400
perioral
anesthesia or nerve cuts, **II:**2399
tactile inputs, **II:**2399
sensory events regulation, **II:**2400f
tactile senses, **II:**2401
termination of nursing bout, **II:**2400–2401
Somatosensory systems, **II:**2234
SON. *See* Supraoptic nucleus
Sonic hedgehog (Shh), **I:**338, **I:**786–787

Sorbitol dehydrogenase (SORD), **I:**130
*Sox*9 gene, **I:**277–278, **I:**785–786
SP1. *See* Specificity protein 1
SPA. *See* Surfactant protein A
SPACA3. *See* Sperm acrosome associated 3
SPAG5. *See* Sperm associated antigen 5
SPAM1. *See* Sperm adhesion molecule 1
SPARC. *See* Secreted protein, acidic, cysteine-rich
SPARKI domain, **I:**1128
Specificity protein 1 (SP1), **I:**318–319, **I:**1091, **II:**1790
Sperm acrosome associated 3 (SPACA3), **I:**114–117
Sperm adhesion molecule 1 (SPAM1), **I:**114
Sperm antibodies, **I:**848
  binding sites and effects, **I:**848f
  clinical significance, **I:**849
  measurement, **I:**849
  origins, **I:**848–849
Sperm associated antigen 5 (SPAG5), **I:**568–570
Sperm capacitation
  and comments, **I:**149–151
  consequences, **I:**151
    hyperactivated motility, **I:**151–153, **I:**152f
    sperm chemoattraction, **I:**153–154
    sperm–egg interaction regulation, **I:**154
  considerations, **I:**154–155
  general features, **I:**150
Sperm chemotaxis in oviduct, **I:**217–218
  BSP proteins, **I:**219
  chambers, **I:**219
  chemoattractants, **I:**219
  marine invertebrates, **I:**219
  odorant receptors, **I:**218
  physical factors, **I:**219
  reports of, **I:**219
  turning, **I:**218
Sperm chromatin, **II:**2034
Sperm domains, **I:**103
Sperm epigenetics, **II:**2032–2033. *See also* Oocyte epigenetics; Postfertilization epigenetics
Sperm head and tail associated protein (SHTAP), **I:**125
Sperm head cytoskeleton, **I:**107–108, **I:**110
  abnormal, **I:**117
  acrosome, **I:**113–117
  composition, **I:**111
  cytoplasm, **I:**108
  cytoskeleton, **I:**110–113
  domains and cytoskeletal proteins, **I:**112t
  function, **I:**111–113
  postacrosomal region, **I:**111f
  sperm nucleus, **I:**108–110
  structural aspect, **I:**110–111
Sperm hyaluronidase, **I:**114
Sperm maturation regulation, **I:**708–709
Sperm mitochondria-associated cysteine-rich protein (SMCP), **I:**122–123
Sperm nucleus, **I:**108
  epigenetics, **I:**109
  freeze-fracture image of membranes, **I:**109f
  nuclear
    envelope, **I:**109
    lacunae, **I:**110
    lamina, **I:**110
    pocket, **I:**110
    proteins, **I:**108–109
  RNA, **I:**109
Sperm phospholipase C, **I:**179–180
Sperm phosphoproteins, **I:**155
Sperm protection, **I:**710–711
Sperm transport
  through cervix
    bovine cervix, **I:**204f
    cervical mucus, **I:**202–203
    flagellar beating patterns, **I:**204f
    immune responses, **I:**203
    preferential pathways, **I:**204
  deer mouse sperm, **I:**198f
  insemination
    artificial, **I:**201
    copulatory plugs and gels, **I:**200–201
    timing of, **I:**199
    uterine, **I:**201
    vaginal, **I:**199–200
  oviductal sperm reservoir
    bull sperm on glass slides, **I:**214f
    carbohydrates on oviductal epithelium, **I:**211
    entrapment, **I:**215–216
    fate of nonfertilizing sperm, **I:**217
    marsupials and insectivores, **I:**216
    release of sperm, **I:**213–216
    sperm fertility during storage preservation, **I:**213
    sperm ligands and, **I:**211–213
    sperm storage and polyspermy prevention, **I:**210
    sperm storage in women, **I:**216–217
  sperm chemotaxis in oviduct, **I:**217–218
    BSP proteins, **I:**219
    chambers, **I:**219
    chemoattractants, **I:**219
    marine invertebrates, **I:**219
    odorant receptors, **I:**218
    physical factors, **I:**219
    reports of, **I:**219
    turning, **I:**218
  sperm competition and cooperation, **I:**198–199
  through uterotubal junction, **I:**207f
    entrance to oviduct, **I:**207
    as filter, **I:**207–208
    proteins requirement, **I:**208–210
    rapid transport of sperm, **I:**210
  through uterus
    immunological responses, **I:**206–207
    rate of transport, **I:**205
    uterine peristalsis, **I:**205–206
  vaginal defenses
    leukocytic response, **I:**202
    vaginal pH, **I:**202
Spermatogenesis, **I:**595, **I:**638–640, **I:**673, **I:**838–839, **I:**956, **II:**1679–1680, **II:**1683
  cessation of, **I:**597
  generations, **I:**597
  inflammation effects on, **I:**843
    lipopolysaccharide-induced, **I:**843–844
    vascular inflammation models, **I:**844–845
  initiation, **I:**582
  mammalian, **I:**596f
  model, **I:**598f
  stem cell renewal theory, **I:**595
  testicular tissues producing, **I:**615
Spermatogenesis and oogenesis-specific helix–loop–helix 1 (*Sohlh*1), **I:**13–14, **I:**956
Spermatogenic cells, **I:**805, **I:**858
  apoptosis, **I:**831, **I:**844
  destruction, **I:**861
  FAS expression, **I:**832
Spermatogenic lineage development
  macroscopic and histologic appearance, **I:**608f
  molecular markers, **I:**606f
  nonhuman primates and humans A_{dark}/A_{pale} model, **I:**605–607
  rodents
    A0/A1 model, **I:**600–605, **I:**604f
    germ cell markers in, **I:**601t–603t
    A_s model, **I:**598–600, **I:**604f
    spermatogenic clone development, **I:**600f
Spermatogonial stem cell (SSC), **I:**247–248, **I:**567, **I:**595, **I:**638
  aging, **I:**621
  culture, **I:**614–615
  developmental fate
    cell cycle regulation, **I:**618–619
    posttranscriptional regulation, **I:**620–621
    signal transduction, **I:**618
    transcriptional regulation, **I:**619–620, **I:**620f
  experimental designs, **I:**613f
  immunohistochemistry, **I:**612–613
  macroscopic and histologic appearance, **I:**608f
  molecular characteristics
    from mouse genetic models, **I:**611
    from sorting, **I:**612
    from transplantation, **I:**612
  molecular markers, **I:**606f
  niche factors, **I:**615, **I:**616f
    differentiation, **I:**617–618
    maintenance and renewal, **I:**617
    transplant paradigm, **I:**616
  nonhuman primates and humans A_{dark}/A_{pale} model, **I:**605–607
  recipient testis colonization, **I:**610f
  regenerative potential, **I:**622–624
  rodents
    A0/A1 model, **I:**600–605, **I:**604f
    germ cell markers in, **I:**601t–603t
    A_s model, **I:**598–600, **I:**604f
    spermatogenic clone development, **I:**600f
  testicular cell transplantation, **I:**609f
  transgenesis, **I:**621–622
  transplantation, **I:**607–609, **I:**608f
  whole-mount immunohistochemistry, **I:**613–614, **I:**614f
  xenografting, **I:**615
  xenotransplantation, **I:**609–611

Spermatozoa, **I:**99–101, **I:**717, **I:**1835
with abnormal flagella, **I:**131
in cauda epididymis, **II:**1407–1409
changes during aging, **I:**738–739
of mammalian species, **I:**107–108
maturation
ability fertilization, **I:**706–708, **I:**706f–707f
changes, **I:**708
microenvironment, **I:**711–714
motility, **I:**708
sperm maturation regulation, **I:**708–709
storage, **I:**709–710
transport, **I:**704–706
Spermatozoon, **I:**99–100
characteristics, **I:**101
components, **I:**100
flagellum, **I:**117–136
general features, **I:**100f
sperm head, **I:**107–117
sperm plasma membrane
formation and maintenance of domains, **I:**105–106
identification of domains, **I:**103
lipids, **I:**106–107
localized sperm membrane components, **I:**103–105
mosaic sperm surface, **I:**101–103
Sperm–egg interaction
cumulus oophorus, **I:**156–157
gamete interaction models, **I:**155–156
obligatory sequence, **I:**158
plasma membrane interactions, **I:**159f
candidate egg proteins, **I:**162–164
candidate sperm proteins, **I:**160–162
domains and factors, **I:**158–160
IZUMO1 and localization in sperm, **I:**161f
sperm incorporation fertilization, **I:**164
regulation, **I:**154
with ZP, **I:**157–158
Sperm–egg membrane fusion, **I:**164–165
blocks to polyspermic fertilization, **I:**165–168
cell cycle progression, **I:**168–170
completion of meiosis, **I:**168–170
cytoplasmic events, **I:**173–175
egg activation signaling diagram, **I:**168f
pronuclear stage, **I:**170–173
Spermine, **I:**791
SPFp. *See* Subparafascicular nucleus
Spinal cord, **II:**2259
chemical changes, **II:**2261
direct applications of drugs effects, **II:**2260–2261
immunocytochemistry, **II:**2262
lesions or transections effects, **II:**2259–2260
presence of steroid receptors, **II:**2261–2262
Spinal nucleus of bulbocavernosus (SNB), **I:**285, **II:**2116–2117, **II:**2261
Spinal pathways, **II:**2306
Fos induction, **II:**2306
tract tracing, **II:**2306
ultrastructural changes in brain, **II:**2306–2307
Spindle assembly checkpoint (SAC), **I:**36
APC/C, **I:**36–37
BUB1, **I:**37–38
classic laser ablation studies, **I:**37
MAD2, **I:**39
during meiosis, **I:**38–39
sister chromatid interactions, **I:**38f
Spindlin gene, **I:**74
Spondyloepimetaphyseal dysplasia, **I:**304
Spontaneous erections, **II:**2214
SR. *See* Steroid receptor
SRA. *See* Scavenger receptors A
SRA. *See* Steroid receptor RNA activator
SRBI. *See* Scavenger receptor class B type I
SRC. *See* Steroid receptor co-activator
Src homology 2 (SH2), **I:**470–471
SREBP. *See* Sterol-response element binding protein
SREBP cleavage-activating protein (SCAP), **II:**2057
SRS. *See* Silver–Russell syndrome
Sry gene. *See* Sex-determining region of the Y chromosome gene
SS. *See* Stress sensitive
SSC. *See* Spermatogonial stem cell
ssDNA. *See* single-stranded DNA
SSRI. *See* Selective serotonin reuptake inhibitors
STAG3. *See* Stromalin antigen 3
Stages of Reproductive Aging Workshop (STRAW), **II:**1662
STAR protein. *See* Steroidogenic acute regulatory protein
STAT. *See* Signal transducers and activators of transcription
Stearoyl-CoA-desaturase (SCD2), **II:**2066–2067
Stem cell factor (SCF), **I:**824
Stem cell renewal theory, **I:**595
Stem Leydig cell (SLC), **I:**281
Stem villi, **II:**1745–1746
Sterile inflammation, **I:**863
Sterile-mediated inflammation, **I:**839
Steroid 5α-reductases, **I:**721–722
Steroid degradation, **I:**304–305
Steroid excretion, **I:**304–305
Steroid hormone binding globulin (SHBG), **II:**2300
Steroid hormones, **I:**313, **II:**1315–1316, **II:**1334, **II:**1336, **II:**1661–1662. *See also* Protein hormones
androgens, **I:**792
corpus luteum endocrine secretions, **II:**1334
estrogens, **I:**792–793
gonadal, **I:**479
impacting LHβ subunit promoter activity, **I:**359
lipophilic, **I:**645
placenta, **II:**1799
11β-HSD, **II:**1804–1807
estrogens, **II:**1799–1802
progesterone, **II:**1802–1804
receptors, **II:**2309
cross-talk between neurotransmitters and, **II:**2317–2320
and feedback, **II:**1674–1675
genitosensory stimulation activation, **II:**2323
signaling, **I:**953, **I:**1086
ESR signaling, **I:**1086–1088, **I:**1087f
PGR signaling, **I:**1088–1089
Steroid hydroxylation, **I:**300–302
Steroid receptor (SR), **I:**1106
co-regulator complexes, **I:**1103t
and endogenous ligands, **I:**1101f
gene promoters methylation, **II:**2480–2481
genes, mRNA, and regulation
AR, **I:**1102–1103
ER, **I:**1101–1102
GR, **I:**1103
PR, **I:**1102
mechanism of action, **I:**1106–1107
agonists *vs*. antagonists, **I:**1107t
indirect/tethered actions, **I:**1109
ligand-dependent actions, **I:**1107–1109, **I:**1108f
ligand-independent actions, **I:**1108f, **I:**1110
nongenomic actions, **I:**1109–1110
receptor antagonists, **I:**1110–1111
NRs family, **I:**1100
nuclear receptor response elements, **I:**1104f–1105f
ovarian function, **I:**1131
AR signaling, **I:**1165–1174
ER signaling, **I:**1133–1161
during follicular differentiation, **I:**1157f
glucocorticoid receptor signaling, **I:**1174
PR signaling, **I:**1133–1161
ovarian-derived sex, **I:**1099–1100
receptor-mediated actions, **I:**1100
structure, **I:**1103
A/B domain, **I:**1103–1105
C domain, **I:**1105
co-regulatory complexes, **I:**1106
D domain, **I:**1105–1106
DNA binding, **I:**1104f–1105f, **I:**1105
E domain, **I:**1106
F domain, **I:**1106
hinge region, **I:**1105–1106
LBD, **I:**1106
NTD domain, **I:**1103–1105
transcriptional coregulators, **I:**316
DNA and chromatin modification, **I:**319
GTF, **I:**319–320
histone acetylases and deacetylases, **I:**316–318
RNA helicases, **I:**319
transcriptional cointegrators, **I:**318–319
in uterine function
AR signaling, **I:**1127–1128
changes in uterine gene expression, **I:**1129–1131
chip-seq, **I:**1129–1131, **I:**1132f
ER signaling, **I:**1111–1121
GR signaling, **I:**1128–1129
microarray studies, **I:**1130t
miRNA in uterine reproductive function, **I:**1129
PR expression, **I:**1121–1123
response to estradiol, **I:**1123–1127
response to progesterone, **I:**1123–1127

Steroid receptor antagonists, **I:**1110–1111
Steroid receptor co-activator (SRC), **I:**1106, **II:**2443
SRC-1, **I:**901–902, **II:**2312
SRC2, **II:**1710
Steroid receptor RNA activator (*SRA*), **II:**2454
Steroid sulfonation, **I:**303–304
Steroid transcriptional targets, **I:**321–322
Steroid-secreting cell differentiation
progesterone production, **I:**1033–1036, **I:**1036f
steroidogenic cell proliferation suppression, **I:**1032–1033
Steroid-signaling pathways
androgen signaling, **I:**977
estrogen signaling, **I:**976–977
Steroidogenesis, **II:**1680–1683
conceptual model for, **I:**296f
inflammation effects, **I:**840–843
Leydig cell, **I:**838
macrophages role, **I:**840
Steroidogenic acute regulatory protein (STAR protein), **I:**297, **I:**808, **I:**961, **I:**1033–1034, **I:**1144, **II:**1282, **II:**1802–1803
Steroidogenic cell proliferation suppression, **I:**1032–1033
Steroidogenic factor (SF), **I:**897–898, **II:**1624–1625
Steroidogenic factor 1 (SF1), **I:**14, **I:**272, **I:**274–275, **II:**1610, **II:**2114
Steroidogenic pathways, **I:**579–580
Sterol carrier protein 2 (SCP2), **I:**1041–1042
Sterol-response element binding protein (SREBP), **II:**2066–2067
STIM1. *See* Stromal interaction molecule 1
Stimulated by retinoic acid 13 (STRA13), **II:**1750–1751
Stimulated by retinoic acid gene 8 (*Stra*8), **I:**9–10, **I:**12, **I:**282
DMRT1, **I:**13–14
induction, **I:**12–13
meiotic initiation in mice, **I:**13f
Store-operated Ca^{2+} entry (SOCE), **I:**177
Store-operated channels (SOC), **II:**1761
*Stra*8. *See* Stimulated by retinoic acid gene 8
STRAW. *See* Stages of Reproductive Aging Workshop
STRAW+10 staging system, **II:**1671
Stress response, **II:**2301
Stress sensitive (SS), **II:**1650–1651
Stress-induced amenorrhea, **II:**1637
Stressors, **II:**1559–1560, **II:**1637–1638
immunological/inflammatory, **II:**1643–1644
influencing reproductive function, **II:**1643
inhibitory effects on pulsatile GnRH/LH release, **II:**1642–1643
metabolic, **II:**1644–1645
psychological, **II:**1645–1647
Stromal cell decidualization, **I:**1091
Stromal interaction molecule 1 (STIM1), **I:**177
Stromalin antigen 3 (STAG3), **I:**23
Structural maintenance of chromosomes 1 beta (SMC1β), **I:**18
Subcortical maternal complex (SCMC), **I:**241
Subfornical organ (SFO), **II:**1961
Subparafascicular nucleus (SPFp), **II:**2239
c-fos activation, **II:**2239
connections, **II:**2240
effects of lesions, **II:**2239
presence of steroid receptors, **II:**2240
Substance P, **II:**2335–2336
Subventricular zone (SVZ), **I:**492–493, **II:**2412–2413
"Suckling reflex", **I:**528–529
Sulfated-glycosaminoglycan (S-GAG), **I:**165–166
Sulfonates, **I:**741–742
SUMO. *See* Small ubiquitin modifier
SUN–KASH proteins, **I:**18, **I:**19f
Superoxide dismutase 2 (SOD2), **I:**1091
Suppressor activity, **II:**1785
Suppressor of cytokine synthesis-1 (SOCS1), **II:**1851
Suprachiasmatic nucleus (SCN), **I:**415, **II:**1409, **II:**1550
GABA inputs, **I:**415
neuronal efferents from, **II:**1624
Supraoptic nucleus (SON), **I:**527, **II:**1959, **II:**2292
Surface charge, **I:**103
Surfactant protein A (SPA), **II:**1904
Surgical deafferentation, **II:**1509
Surrounded nucleolus (SN), **I:**64
SVS. *See* Seminal vesicle-secreted proteins
SVZ. *See* Subventricular zone
SYCP3. *See* Synaptonemal complex protein 3
Symmetric self-renewal, **I:**781
Sympathetic nervous system (SNS), **II:**1940
Sympathetic orchiopathia, **I:**850–851
Synapse formation, **II:**1411–1412
Synaptic plasticity during lactation, **I:**550. *See also* Oxytocin (OT)
GABA, **I:**551–552
glutamate, **I:**550–551
IPSC, **I:**552f
neurohypophysial plasticity, **I:**553
noradrenaline, **I:**552–553
during osmotic activation, **I:**553
Synaptonemal complex, **I:**19–21
Synaptonemal complex protein 3 (SYCP3), **I:**18
Synaptotagmins (SYT), **I:**111–113
Syncytial knots, **II:**1769–1770
Syncytial shedding, **II:**1847
Syncytin, **II:**1743–1745
Synthesis-dependent strand annealing pathway (SDSA pathway), **I:**25
Synthetic phase, **II:**1879
System A activity, **II:**1771
System L amino acid transporter, **II:**1765–1766
Systemic lupus erythematosus (SLE), **II:**1371
SYT. *See* Synaptotagmins

T

T cell factor (TCF), **I:**901–902
T-cell, **II:**1853–1854
regulation and functions, **I:**815–816
subsets, **I:**816
T-cell receptor (TCR), **I:**815
T21 antigen, **I:**105
T2DM. *See* Type 2 diabetes mellitus
Taf4b. *See* TATA Box Binding Protein-Associated Factor
TAG. *See* Triglycerides
TALE. *See* Three-amino-acid loop extension
Tanycytes, **II:**1575–1576, **II:**1594
energy sensing, **II:**1595
hypothalamic neurogenesis, **II:**1595–1596
hypothalamic neuronal excitability, **II:**1594
PT and deiodinase expression, **II:**1589–1590
Target-primed reverse transcription, **II:**2457–2458
TATA Box Binding Protein-Associated Factor (Taf4b), **I:**971–972
TATA-binding protein (TBP), **I:**319–320, **I:**971–972
TATA-binding protein 2 (Tbp2), **I:**972
Taurine transporters, **I:**732
TBI. *See* Traumatic brain injury
TBP. *See* TATA-binding protein
Tc cells. *See* Cytotoxic T lymphocytes
TCF. *See* T cell factor
TCII. *See* Transcobalamin II
TCR. *See* T-cell receptor
tda1. *See* Testis determining autosomal 1
TDG. *See* Thymine–DNA glycosylase
TDLU. *See* Terminal duct lobular unit
TE. *See* Trophectoderm
TEAD4. *See* Transcriptional enhancer factor domain family member 4
TEB. *See* Terminal end buds
TEF. *See* Transcription enhancer factors
TEKT1. *See* Tektin 1
Tektin 1 (TEKT1), **I:**121–122, **I:**249
Telomere bouquet formation, **I:**17–18
Telomere clustering, **I:**17–18
Ten-eleven translocation (TET), **II:**2450–2452
Ten-eleven translocation 3 (TET-3), **I:**172–173
Teratozoospermia, **I:**117
Terminal duct lobular unit (TDLU), **II:**2059
Terminal end buds (TEB), **II:**2056
Terminal villi, **II:**1745–1746
TES. *See* Testis-specific enhancer element
Testicular development
and adult health, **I:**582–583
AMH, **I:**567
anatomical, **I:**567–572
eutherian mammals, **I:**567
germ cells, **I:**582
histological, **I:**567–572
morphometric development, **I:**567–568
developmental landmarks, **I:**568f
germ cells, **I:**571–572
Leydig cells, **I:**571
modeling of testicular cord development, **I:**569f
peritubular myoid cells, **I:**571
Sertoli cells, **I:**568–571
testicular macrophages, **I:**571
testis development control, **I:**572–582
Testicular factors, **I:**716–717
Testicular feminization mutation (*tfm*), **I:**647–648, **II:**2246

Testicular immunoregulatory mechanism, **I:**855
androgens, **I:**856–857
bioactive lipids, **I:**857–858
immune privilege, **I:**859
Leydig cell, **I:**856–857
Sertoli cell, **I:**855–856
soluble immunosuppressive activities, **I:**857–858
Testicular macrophages, **I:**571, **I:**582
distribution and properties, **I:**820–821
heterogeneity, **I:**821–822
recruitment and regulation, **I:**822–823
Testis, **I:**808f, **II:**1489–1490
aging effects, **II:**1683–1684
anatomy, **I:**609, **I:**638, **I:**639f
AR-regulated gene expression, **I:**652–653, **I:**653t
development, **I:**276–284
endocrine regulation, **I:**809
eosinophils, **I:**824
FSH receptor expression, **I:**655
hormone signaling in
cell associations, **I:**640f
endocrine disruptors, **I:**637
estrogen, **I:**664–666
FSH, **I:**655–662
germ cell progression, **I:**641f
glucocorticoid signaling, **I:**671–672
hormone action, **I:**640–642, **I:**641f
IGF signaling, **I:**669–670
inhibin–activin signaling system, **I:**666–669
insulin–IGF–Insl3 signaling, **I:**669
leptin signaling, **I:**672
LH, **I:**642–645
mineralocorticoids, **I:**672
osteocalcin, **I:**669
prolactin, **I:**672–673
spermatogenesis, **I:**638–640
testosterone, **I:**645–655
thyroid hormone, **I:**662–664
IGF signaling in, **I:**669–670
immunological mediators in, **I:**826–840
immunological responses, **I:**855
inflammatory mediators in, **I:**826
eicosanoids, **I:**835–836
IL1 family, **I:**826–830
IL6, **I:**832
inflammatory signaling pathways, **I:**838–840
interferons, **I:**835
NO synthases, **I:**836–838
oxidative enzymes, **I:**836–838
ROS, **I:**836–838
testicular function, **I:**838–840
TGFβ family, **I:**832–835
TLR, **I:**829–830, **I:**829t
TNF family, **I:**830–832
intratesticular dendritic cells, **I:**822
LH and FSH act on, **II:**1661–1662
lymphocytes in, **I:**823–824
mast cells, **I:**824
MHC in, **I:**854–855
reproductive aging in men, **II:**1682–1683
structural organization, **I:**807–809
testicular macrophage population distribution and properties, **I:**820–821
heterogeneity, **I:**821–822
recruitment and regulation, **I:**822–823
testosterone-regulated gene expression, **I:**650–651
thyroid hormone receptor expression, **I:**663
transplantation, **I:**858
Testis determining autosomal 1 (tda1), **I:**273
Testis development control, **I:**572
during fetal and perinatal development, **I:**573f
Leydig cell
adult, **I:**580–581
fetal, **I:**577–579
steroidogenic pathways, **I:**579–580, **I:**579f
peritubular myoid cells, **I:**581
sertoli cell
cell proliferation hormonal control, **I:**572–574
during development, **I:**574–576, **I:**575f
regulation of peripubertal, **I:**576–577
testicular macrophages, **I:**582
Testis-specific enhancer element (TES), **I:**272
Testis-specific serine kinase (TSSK), **I:**119–120
Testis-specific serine kinase substrate (TSKS), **I:**119–120
Testosterone (T), **I:**313, **I:**645, **II:**1407, **II:**1469–1470
adult testis function, **I:**650
AR
AR-dependent regulation, **I:**651–652
AR-regulated gene expression, **I:**652–653, **I:**653t
cell-specific ablation, **I:**647–648
expression in testis, **I:**646
inducible AR ablation, **I:**650
in Leydig cells, **I:**649
in peritubular myoid cells, **I:**648–649
in Sertoli cells, **I:**648
in testicular vasculature, **I:**650
genes regulation, **I:**652
humans, **II:**2386
laboratory rodents, **II:**2380
levels and functions, **I:**646
miRNAs androgen regulation, **I:**654
nonclassical testosterone signaling, **I:**653–654
prenatal exposure, **II:**1470f
testosterone-regulated
gene expression, **I:**650–651
meiotic proteins, **I:**654–655
testosterone-signaling pathways, **I:**646–647, **I:**647f
Testosterone-regulated meiotic proteins, **I:**654–655
Testosterone-signaling pathway, **I:**661–662
TET. *See* Ten-eleven translocation
TFIID. *See* Transcription factor IID
tfm. *See* Testicular feminization mutation
TFN. *See* Transferrin
TFNR. *See* Transferrin receptor
TGF-α. *See* Transforming growth factor alpha
TGF-β. *See* Transforming growth factor beta
TH. *See* Thyroid hormone
THADA. *See* Thyroid adenoma-associated gene
Th cells. *See* helper T lymphocytes
THDA. *See* Tuberohypophysial dopaminergic
Theca cell, **I:**950–952, **I:**968–969
gland, **II:**1201–1202
LH signals, **I:**919
steroidogenesis, **I:**1173
Thiamine pyrophosphate (TPP), **I:**130
Thioredoxins, **I:**130
Third National Health and Nutrition Examination Survey (NHANES III), **II:**1490
Three-amino-acid loop extension (TALE), **I:**318
Thrombospondin 2 (TSP2), **II:**1883
Thromboxane A (TP), **II:**1718–1719, **II:**1896
Thymine–DNA glycosylase (TDG), **I:**319
Thyroid adenoma-associated gene (THADA), **I:**965–966
Thyroid hormone (TH), **I:**574, **I:**662
nongenomic actions, **I:**664
sertoli cell number regulation, **I:**663
T3
downstream targets, **I:**663–664
regulation, **I:**664
testis development regulation, **I:**663
TRs expression in testis, **I:**663
Thyroid hormone receptor (TR), **I:**662
Thyroid hormone receptor interactor 13 (TRIP13), **I:**20–21
Thyroid-stimulating hormone (TSH), **I:**337, **I:**477–478, **I:**642, **I:**1043, **II:**1206, **II:**1788
Thyrotrophin-releasing hormone (TRH), **I:**480, **II:**1215, **II:**1978
Thyroxine (T4), **I:**662
TIDA neurons. *See* Tuberoinfundibular neurons
Tidal volume (TV), **II:**1938
Timing mechanisms, **II:**2336–2337
TIMP. *See* Tissue inhibitors of metalloprotease
TIP39. *See* Tuberoinfundibular peptide of 39 residues
Tissue inhibitor of metalloproteinase 2 (TIMP2), **II:**1883–1884
Tissue inhibitors of metalloprotease (TIMP), **II:**1721, **II:**2071
tissue plasminogen activator (tPA), **I:**660
TLC. *See* Total lung capacity
TLR. *See* Toll-like receptor
TM. *See* Transmembrane
TMD. *See* Transmembrane domain
TNDM1. *See* Transient neonatal diabetes mellitus 1
TNF. *See* Tumor necrosis factor
TNF-α. *See* Tumor necrosis factor alpha
TNFRSF. *See* Tumor necrosis factor receptor superfamily
TNFαIP6. *See* Tumor necrosis factor alpha induced protein 6
Tocolytics, **II:**1905–1907

Toll-like receptor (TLR), **I:**668, **I:**811, **I:**829–830, **I:**829t, **II:**1845–1846
Tonic gonadotropin secretion hypothalamic control
  anatomical sites of action, **II:**1546–1548
  negative feedback actions
    of E_2, **II:**1547f
    neural circuits mediating, **II:**1548–1549
TOPGAL mice, **II:**1720
TORC1. *See* Creb1-regulated transcription coactivator-1 (Crtc1)
Total lung capacity (TLC), **II:**1938
Touch-based erections, **II:**2214
TP. *See* Thromboxane A
TP1. *See* Trophoblast protein 1
tPA. *See* tissue plasminogen activator
TPBPA. *See* Trophoblast-specific protein alpha
TPH. *See* Tryptophan hydroxylase
TPI. *See* Triose phosphate isomerase
TPP. *See* Thiamine pyrophosphate
TR. *See* Thyroid hormone receptor
Trace elements, **II:**2080
Transcellular transport, **II:**1752–1753
  endocytosis–transcytosis, **II:**1754
  mediated transport, **II:**1754
  nonmediated transport, **II:**1753
  normal and preeclamptic syncytial MVM, **II:**1754f
  placental micro-and nanovesicles, **II:**1754–1755
Transcobalamin II (TCII), **II:**1768
Transcription enhancer factors (TEF), **II:**1790
Transcription factor IID (TFIID), **I:**319–320
Transcription factors, **I:**727–729, **I:**784, **I:**784f
  cross talk, **I:**320–321
  ovulation impact, **I:**1008–1009
  in preantral-stage folliculogenesis
    Foxo transcription factors, **I:**972
    GATA transcription factors, **I:**972–973
    Taf4b RNA polymerase II, **I:**971–972
    TBP, **I:**971–972
    Tbp2, **I:**972
Transcription-requiring complex (TRC), **I:**240
Transcriptional enhancer factor domain family member 4 (TEAD4), **II:**1750
Transcriptional proteins
  uterine phenotypes in mouse models
    COUP-TFII, **I:**1119
    Hand2, **I:**1120–1121
    mammalian homeobox genes, **I:**1119
    Mig-6, **I:**1120
    NCOA-6, **I:**1120
    REA, **I:**1119
  uterine phenotypes in mouse models
    COUP-TFII, **I:**1122
    FK506 binding proteins, **I:**1123
    REA, **I:**1122–1123
    SRCs mediate P-dependent action, **I:**1122
Transcriptional start sites (TSS), **I:**1131
Transcytosis, **II:**2079
  immunoglobulins, **II:**2079
  serum albumin, **II:**2079–2080
  trace elements, **II:**2080
Transdifferentiation, **I:**483
Transducer of regulated Creb1. *See* Creb1-regulated transcription coactivator-1 (Crtc1)
Transferrin (TFN), **II:**2080
Transferrin receptor (TFNR), **II:**2080
Transforming growth factor alpha (TGF-α), **I:**426, **II:**1416, **II:**1712
Transforming growth factor beta (TGF-β), **I:**787–788, **I:**833f, **I:**897–898, **I:**1037, **II:**1794–1795
  activin, **I:**833–835
  autocrine and paracrine actions, **II:**1664
  folliculogenesis regulation, **I:**961–962
    activins, **I:**966–967
    BMP receptor type IB receptor, **I:**971
    BMP signaling SMADs, **I:**967–968
    follistatin, **I:**967
    inhibins, **I:**962–966
    oocyte-specific, **I:**968–971
  forms, **I:**832
  GDNF, **I:**611
  growth inhibitory effects, **II:**2059
  immune-deviating cytokine, **II:**1842
  oocyte and granulosa cells, **II:**1201
  plausible candidates, **II:**1365–1367
  signaling molecules secretion, **I:**786
  thecal-derived, **I:**1151–1152
Transgenerational epigenetic inheritance, **II:**2471, **II:**2472f
  EDC, **II:**2472–2474
  mechanisms, **II:**2474–2476
  multigenerational, **II:**2471
  paternal stress, **II:**2474
  reprogramming, **II:**2471–2472
Transgenesis, **I:**621–622
Transgenic mouse models, **II:**1708
Transient neonatal diabetes mellitus 1 (TNDM1), **II:**2037–2038
Transient receptor potential (TRP), **I:**177, **I:**476, **I:**700–701
Transient receptor potential 2 (TRP2), **II:**2232–2233
Transient receptor-1 homolog (TRPC1), **I:**107
Transit-amplifying cells, **I:**781–782
Transmembrane (TM), **I:**896–897
Transmembrane domain (TMD), **I:**345–346
Transplantation medicine, **I:**863
Transplantation studies, **I:**852–853
Transsynaptic inputs, **II:**1412–1414, **II:**1417f
Transtrophoblastic channels, **II:**1752–1753
Transzonal projections (TZPs), **I:**70–71, **I:**80, **I:**978–979
Traumatic brain injury (TBI), **I:**503
TRC. *See* Transcription-requiring complex
Treg cells. *See* Regulatory T cells
TRH. *See* Thyrotrophin-releasing hormone
Tricellulin, **II:**2072–2073
Trichostatin A (TSA), **II:**2467–2468
3,5,3-triiodo-L-thyonine (T3), **I:**662
  downstream targets, **I:**663–664
  regulation, **I:**664
Triglycerides (TAG), **II:**1767, **II:**2085
Triose phosphate isomerase (TPI), **I:**130
TRIP13. *See* Thyroid hormone receptor interactor 13
Trithorax group (TrxG), **II:**2446–2447
Trophectoderm (TE), **I:**233–234, **II:**1750, **II:**2035
Trophinin, **II:**1715–1716
Trophoblast protein 1 (TP1), **I:**1054
Trophoblast specific element (TSE), **I:**357
Trophoblast-specific protein alpha (TPBPA), **II:**1751
Trophoblasts, **II:**1767–1768
TRP. *See* Transient receptor potential
*Trp*53–/–. *See* Tumor suppressor protein p53
TRPC1. *See* Transient receptor-1 homolog
TrxG. *See* Trithorax group
Tryptophan hydroxylase (TPH), **II:**2067–2068
TSA. *See* Trichostatin A
TSC. *See* Tuberous sclerosis
TSE. *See* Trophoblast specific element
TSH. *See* Thyroid-stimulating hormone
TSKS. *See* Testis-specific serine kinase substrate
TSP2. *See* Thrombospondin 2
TSS. *See* Transcriptional start sites
TSSK. *See* Testis-specific serine kinase
Tuberalin. *See* Prolactin-releasing factor
Tuberohypophysial dopaminergic (THDA), **I:**474, **II:**1233
Tuberoinfundibular neurons (TIDA neurons), **I:**474, **II:**1233
  neuroanatomy, **I:**475f
  prolactin effect, **I:**477–478
Tuberoinfundibular peptide of 39 residues (TIP39), **I:**487
Tuberous sclerosis (TSC), **I:**907
TUBG1. *See* Gamma 1 tubulin
Tuberous sclerosis complex 1 (TSC1), **I:**958
Tubules formation, **I:**692–694
Tumor necrosis factor (TNF), **I:**811–814
  estrogen-regulated cytokines, **II:**1840
  FAS, **I:**831–832
  intratesticular production and actions, **I:**830–831
  luteal expression, **I:**1038–1039
Tumor necrosis factor alpha (TNF-α), **I:**322, **I:**668, **II:**1263, **II:**1643
Tumor necrosis factor alpha induced protein 6 (TNFαIP6), **I:**969
Tumor necrosis factor receptor superfamily (TNFRSF), **I:**1038–1039
Tumor suppressor protein p53 (*Trp*53–/–), **II:**1710
Tumor-initiating cell, **I:**795
Turner syndrome, **I:**44, **II:**1370–1371
TV. *See* Tidal volume
Twin pregnancies, **II:**1936
"Two-cell, two-gonadotropin" model, **II:**1204–1205, **II:**1315
Type 1 CRF receptor (CRF-R1), **II:**1638
Type 2 CRF receptor (CRF-R2), **II:**1638–1639
Type 2 diabetes mellitus (T2DM), **II:**1375
TZPs. *See* Transzonal projections

U

Ubiquitin pathway, **I:**324–325
UCN. *See* Urocortin
UCS. *See* Unconditioned stimulus

UG reflex. *See* Urethrogenital reflex
UGKO. *See* Uterine gland knockout
UGM. *See* Urogenital mesenchyme
UGS. *See* Urogenital sinus
UGT. *See* Uridine diphosphate glucuronyltransferase
Ultrashort-lived corpora lutea, **I:**1027. *See also* Long-lived corpora lutea; Short-lived corpora lutea
Ultrasound Doppler, **II:**1746
Unconditioned stimulus (UCS), **II:**2340
Undifferentiated granulosa cells
  FSH increases intracellular Ca^{2+}, **I:**904–905
  PKA
    signals to ERK activation, **I:**902–904
    targets in, **I:**901–902
Undifferentiated spermatogonia, **I:**619–620
uNK cells. *See* Uterine natural killer cells
"Up-suck" reflex, **II:**2304
uPA. *See* urokinase plasminogen activator
Upstream regulatory element (URE), **II:**1785
Urethra, immune cells in, **I:**825–826
Urethrogenital reflex (UG reflex), **II:**2214–2215
Urial sheep (*Ovis orientalis*), **II:**1441
Uridine diphosphate glucuronyltransferase (UGT), **I:**304
Urinary protein, **II:**1943–1944
Urocortin (UCN), **II:**1638–1639, **II:**1807–1808
Urogenital mesenchyme (UGM), **I:**787
Urogenital sinus (UGS), **I:**779
urokinase plasminogen activator (uPA), **I:**727–728, **II:**1263
Uterine
  aging, **II:**1671
  atony, **II:**1905
  blue reaction, **II:**1699
  gene expression, **I:**1129–1131
  insemination, **I:**201
  luminal epithelial competency, **II:**1716–1717
  peristalsis, **I:**205–206
  phenotypes in mouse models
    COUP-TFII, **I:**1122
    FK506 binding proteins, **I:**1123
    REA, **I:**1122–1123
    SRCs mediate P-dependent action, **I:**1122
  prostaglandins, **II:**1265
  receptivity, estrogen action, **II:**1698, **II:**1708, **II:**1709f
  reproductive function, **I:**1129
  veins, **II:**1746
Uterine function
  AR signaling, **I:**1127–1128
  changes in uterine gene expression, **I:**1129–1131
  chip-seq, **I:**1129–1131
  ER signaling, **I:**1111
    domestic animals, **I:**1111–1112
    primates, **I:**1112
    rodents, **I:**1111
  GR signaling, **I:**1128–1129
  microarray studies, **I:**1130t
  miRNA in uterine reproductive function, **I:**1129
  PR expression, **I:**1121–1123
  response to
    estradiol, **I:**1123–1127
    progesterone, **I:**1123–1127
Uterine gland knockout (UGKO), **II:**1703
Uterine natural killer cells (uNK cells), **I:**1093, **I:**1128–1129
  dysfunction in, **II:**1864
  innate immune response, **II:**1850–1851
Uterine phenotypes in mouse models, ER signaling
  of disrupted estrogen signaling, **I:**1112
    cervical phenotypes, **I:**1121
    Cyp19-null mice, **I:**1115
    ERα AF-1 or AF-2 domains, **I:**1118–1119
    ERα mutated DNA binding domains, **I:**1117
    ERα uterine-specific deletion, **I:**1116–1117
    ERα-null mice, **I:**1114–1115
    ERβ-null mice, **I:**1114–1115
    in global, **I:**1115–1116
    gross uterine morphology, **I:**1116f, **I:**1118f
    for sex SR and signaling, **I:**1113t–1114t
    vaginal phenotypes, **I:**1121
  with mutations in transcriptional proteins
    COUP-TFII, **I:**1119
    Hand2, **I:**1120–1121
    mammalian homeobox genes, **I:**1119
    Mig-6, **I:**1120
    NCOA-6, **I:**1120
    REA, **I:**1119
  paracrine and autocrine mechanism, **I:**1120f
Uterus, **II:**1701
  AR expression in, **I:**1127
  estrogen effects in, **II:**1708
  gravid, **II:**1876, **II:**1878
    cervix, **II:**1882–1884
    changes in PTGS2, **II:**1885f
    decidua membrane, **II:**1884–1888
    fetal membrane, **II:**1884–1888
    myometrium, **II:**1879–1882, **II:**1881f
    parturition process, **II:**1878–1879
    synthesis of biologically active PGs, **II:**1886f
  human, **I:**1080f
  P_4 signaling induces *Ihh*, **II:**1710–1711
  sperm transport
    immunological responses, **I:**206–207
    rate, **I:**205
    uterine peristalsis, **I:**205–206

V

V1a. *See* Vasopressin 1a
Vacuolar type H^+ATPase (V-ATPase), **I:**730
VAD. *See* Vitamin A deficiency
Vaginal mucosa, **I:**1121
Vaginal pulse amplitude (VPA), **II:**2347–2348
Vaginocervical stimulation (VCS), **II:**2298
Valosin-containing protein (VCP), **I:**135
Valproic acid (VPA), **II:**2477
Varicocele, **I:**844–845
Vas deferens, **I:**697, **I:**809–810
  autoimmunity in, **I:**851–852
  immune cells in, **I:**825–826
  immunology, **I:**859–860
  inflammation on male reproduction, **I:**845–847
Vascular adhesion molecule-1 (VCAM1), **I:**814
Vascular beds, **II:**1756–1757
Vascular changes, **II:**1935
Vascular endothelial cells (VE cells), **I:**650
Vascular endothelial growth factor (VEGF), **I:**896, **I:**1030–1031, **II:**1721–1722, **II:**1784, **II:**1796–1799
  inhibition in gonad culture, **I:**280
  in testis and epididymis, **I:**725
Vascular endothelial growth factor receptor (VEGFR), **I:**725
Vascular inflammation
  effects on spermatogenesis, **I:**844–845
  Leydig cell development, **I:**843
Vascular permeability factor (VPF), **I:**1030
Vascular remodeling, **I:**1092–1093
Vascular smooth muscle (VSM), **I:**650
VASH. *See* Vasohibin
Vasoactive factors, **I:**1049–1050
Vasoactive intestinal peptide (VIP), **I:**483, **II:**1215, **II:**1978, **II:**2219, **II:**2230–2231
Vasohibin (VASH), **I:**1031–1032
Vasomotion, **I:**650
Vasopressin (VP), **I:**527, **II:**1960
  magnocellular, **II:**1960
  neurons, **II:**1961
Vasopressin 1a (V1a), **II:**2298
VC. *See* Vital capacity
VCAM1. *See* Vascular adhesion molecule-1
Vcan. *See* Versican
VCP. *See* Valosin-containing protein
VCS. *See* Vaginocervical stimulation
VDCCs. *See* Voltage-dependent calcium channels
VDR. *See* Vitamin D receptor
VE cells. *See* Vascular endothelial cells
VEGF. *See* Vascular endothelial growth factor
VEGFR. *See* Vascular endothelial growth factor receptor
Ventral premammillary nucleus, **II:**2258
Ventral tegmental area (VTA), **II:**1975, **II:**2002, **II:**2292, **II:**2406
Ventrolateral medulla (VLM), **II:**1983
Ventromedial hypothalamus (VMH), **I:**537–538, **II:**1610, **II:**1965, **II:**2257
  brain lesion studies, **II:**2289
  c-fos expression, **II:**2258
  electrolytic or cell body lesions effects, **II:**2257
  GnRH surge, **II:**1550
  using Golgi technique, **II:**2123
  hormonal manipulations effects, **II:**2257–2258
  MPOA, **II:**2250
Ventromedial nucleus (VMN). *See* Ventromedial hypothalamus
Venus figurines (*V. figurines*), **II:**1605
Versican (*Vcan*), **I:**1000–1001
Very low density lipoproteins (VLDL), **II:**2086

VGCCs. *See* Voltage-gated Ca^{2+} channels
VHL. *See* Von Hippel–Lindau
Villus, **II:**1741–1742
4-vinylcyclohexene diepoxide (VCD), **II:**1678
VIP. *See* Vasoactive intestinal peptide
Viral oophoritis, **II:**1372
Vital capacity (VC), **II:**1938
Vitamin A deficiency (VAD), **I:**723
Vitamin B12, **II:**1768
Vitamin D, **I:**793
Vitamin D receptor (VDR), **I:**793
VLDL. *See* Very low density lipoproteins
VLM. *See* Ventrolateral medulla
VMH. *See* Ventromedial hypothalamus
VNO. *See* Vomeronasal organ
Voles, female sexual behavior, **II:**2298–2299
Voltage-dependent calcium channels (VDCCs), **II:**1761
Voltage-gated Ca^{2+} channels (VGCCs), **I:**482, **II:**1989
Vomeronasal organ (VNO), **II:**2232
Von Hippel–Lindau (VHL), **I:**323
VP. *See* Vasopressin
VPA. *See* Vaginal pulse amplitude
VPA. *See* Valproic acid
VPF. *See* Vascular permeability factor
VSM. *See* Vascular smooth muscle
VTA. *See* Ventral tegmental area

W

WAT. *See* White adipose tissues
Water, **II:**1760–1761
  retention, **II:**1940
  transport, **I:**729–731
WBP2 N-termina like protein (WBP2NL), **I:**111
"Weigh-suckle-weigh" method, **II:**2057
Weismann barrier, **I:**7
Weismann's germ plasm theory, **I:**7
White adipose tissues (WAT), **II:**2085–2086
WHO. *See* World Health Organization
WIF. *See* Wnt inhibitor factors
Wild House Mice, **II:**2390
Wild-type receptor (WT receptor), **I:**1101–1102
Wilm's tumor 1 (*Wt*1), **I:**273–274
Wnt genes, **I:**788–789, **II:**1721
Wnt inhibitor factors (WIF), **I:**788
Wolffian formation, **I:**692–694
Women
  with chronic hyperprolactinemia, **I:**503
  with FSH mutations, **I:**973–974
  hyperandrogenism in, **I:**977
  hypothalamic-pituitary axis aging
    and changes in steroid feedback, **II:**1667–1668
    reproductive axis integration, **II:**1668–1671
    response to loss of ovarian feedback, **II:**1664–1665
    standardization of perimenopause/menopause terminology, **II:**1671
  with mutations in GHR, **I:**980
  ovarian aging in, **II:**1664–1665
  PCOS effect, **I:**1014
  prolactin secretion patterns, **I:**487–488
  reproductive function, **II:**1663–1664
  sperm storage in, **I:**216–217
World Health Organization (WHO), **I:**848
WT receptor. *See* Wild-type receptor
*Wt*1. *See* Wilm's tumor 1

X

X-box binding protein 1 (XBP1), **I:**320
X-chromosome inactivation, **II:**2461, **II:**2462f–2463f
  dosage compensation, **II:**2464
  early preimplantation, **II:**2463–2464
  genes escape, **II:**2462–2463
  mechanisms, **II:**2462
  Sry gene
    in mammals, **II:**2461
    and Y chromosome, **II:**2461f
Xanthine oxidoreductase (XOR), **II:**2078
XBP1. *See* X-box binding protein 1
Xenobiotic response element (XRE), **II:**2045
Xenobiotics, **II:**1769
  epididymis as, **I:**739
    chemicals, **I:**739–742
Xenografting, **I:**615
XOR. *See* Xanthine oxidoreductase
XRE. *See* Xenobiotic response element

Y

Y-specific genes, **I:**271
Yes-associated protein 1 (Yap1), **I:**242–243
Yin Yang1 (Yy1), **I:**956–957, **I:**1047–1048
Yohimbine, **II:**2225

Z

ZAN. *See* Zonadhesin
*Zar*1. *See* Zygotic arrest 1
Zebularine, **II:**2484
ZF. *See* Zona fasciculata
ZFN. *See* Zinc finger nuclease
ZG. *See* Zona glomerulosa
ZGA. *See* Zygote gene activation
ZI. *See* Zona incerta
Zinc, **I:**790
  as bacteriostatic factors, **I:**789
  sparks, **I:**175–176
Zinc finger GATA-like protein 1 (ZGLP1), **I:**957
Zinc finger nuclease (ZFN), **I:**1114
Zinc import proteins (ZIP), **II:**2080
Zinc transporters (ZnT), **II:**2080
ZIP. *See* Zinc import proteins
Zona fasciculata (ZF), **I:**300
Zona glomerulosa (ZG), **I:**298
Zona incerta (ZI), **II:**2329
Zona pellucida (ZP), **I:**70–71
  promoter elements, **I:**954
  sperm interaction with, **I:**157–158
Zona reticularis (ZR), **I:**303–304
Zonadhesin (ZAN), **I:**113–114
ZP. *See* Zona pellucida
ZR. *See* Zona reticularis
Zygote gene activation (ZGA), **I:**239–240
Zygotic arrest 1 (*Zar*1), **I:**955

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Section Editors David F. Albertini, Robert L. Goodman, Allan E. Herbison, Margaret M. McCarthy, Louis J. Muglia and JoAnne S. Richards

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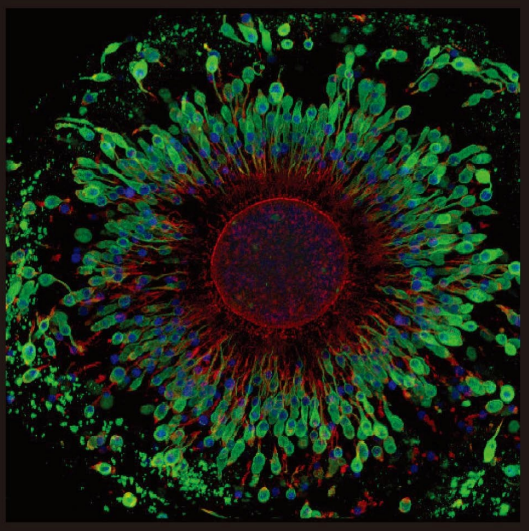

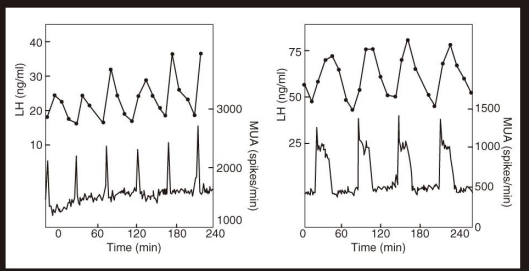

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