The Laboratory Mouse
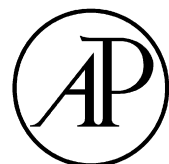
Edited by

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Foreword

In 2004 when the first edition of *The Laboratory Mouse* was published the genome of the mouse had been sequenced. Now at the time of its second edition, only a few years later, the mouse has become the most common species used in biomedical investigation because the systematic manipulation of its genome gives us the capacity to create most animal models for human or animal diseases.

Following genome sequencing came the creation of integral databases, the description of harmonized systems of phenotyping and the development of organizations for the conservation and distribution of mutant strains. In 2007, the International Knock out Mouse Consortium (IKMC) was set up as an amalgam of four different initiatives from Europe and North America to make publicly available tools to inactivate any single mouse gene.

No other experimental animal has managed to engender such international collaboration to create tools available for use by the scientific community. These efforts confirm the key importance of the mouse in biomedical research and the fact that no other animal species offers the same resources or versatility.

However, while mice are a valuable tool, it is imperative to recognize that unless their physiological complexity, behavioural needs, health status and genetic background are properly taken into account, there is the danger that experimental results will be unreliable.

The great value of *The Laboratory Mouse* is that it provides, in just one volume, the essential knowledge to enable both scientists and technicians to be better able to assess and monitor the genetic and microbiological quality of the mice they use in research.

The book, which has been extensively revised since its last publication, contains contributions from internationally recognized experts and covers the key areas of genetics, infectious diseases and basic experimental procedures. A significant part of the book is dedicated to biology with special attention to the anatomy and function of the mouse body systems, knowledge that is vital for pharmacological studies. The book also deals comprehensively with husbandry and maintenance – two areas where knowledge is critical in order to guarantee genetic and health consistency of the experimental model.

Fortunately, scientists are becoming increasingly aware of the need to know more about the experimental animal models they use in research, and training courses for animal users are also becoming increasingly available in more countries. These trends underlie the need for *The Laboratory Mouse*, both as a very useful handbook for scientists, trainers and trainees and as an indispensable reference.

Professor Dr. Patri Vergara D.V.M., Ph.D., DipECLAM
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Preface

With the immense amount of published knowledge available on the laboratory mouse as an experimental tool, one may well ask, "Why publish a second edition of *The Laboratory Mouse*?" When Elsevier approached me with this proposal, I was actually first somewhat reluctant, but recognizing that the first edition was out of print prompted me to accept the offer, despite several other books related to the mouse having been published recently. This second edition, however, is not just another book, or a reprint of the first edition. Immense care has been taken to select topics and authors to define and illustrate the most important features of this species.

The volume has been divided up in such a way that the new and/or established researcher can easily track down the most up-to-date information in any one area. While headline-grabbing topics such as mouse genomics and the generation of mouse mutants sit comfortably with the analysis of the total mouse genome, equal importance has been given to the basis of mouse development, pathological anatomy and pathophysiology. Further consideration has been given to husbandry, methodological aspects, alleviation of pain, as well as legal aspects.

All chapters retained from the first edition, except "Gross Anatomy" and "The Gastrointestinal System and Metabolism", have been thoroughly revised by their previous authors. Quite a number of chapters are either completely rewritten (1.4, 1.5, 2.10, 2.11, 2.12, 5.6, 6.1), or cover aspects that were lacking in the first edition (2.5, 3.3, 3.4, 4.2, 4.5). On practical grounds and due to time constraints, the coverage of certain aspects/topics unfortunately had to be skipped: translational aspects of mouse experimentation as well as basics in experimental mouse surgery. Nevertheless, I believe the new edition has not only updated but has also provided new information on *Mus laboratorius*.

I am extremely grateful to all my friends and colleagues who have helped me to put together this volume. The authors, being associated with both universities and applied research organizations, came from a wide range of countries, thus providing a global, well-balanced approach.

In particular I am very much indebted to my staff member Marie-Luise Enss, who served as my liaison-officer to the authors and Elsevier. Without her constant patience and excellent contacts with all who participated in this piece of work, I would not have been able to manage the task of a second edition of *The Laboratory Mouse*.

Finally, I would like to thank Janice Audit, Mary Preap and Julia Haynes from Elsevier for their patience and support during the development of this second edition.

Professor Hans J Hedrich

Origins and Phylogenetic Relationships of the Laboratory Mouse

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Introduction

On the basis of zooarcheological data it has been established that humans and mice were already in close contact at the end of the last glacial period, at the time of the Neolithic revolution, i.e. about 12 000 years ago [1–3]. Archaeological records have confirmed that by the Bronze Age (3000 years BC) house mice were quasi-obligatory commensals of established farming communities, and as a consequence they were transported almost everywhere the maritime trade could carry them at that time, i.e. essentially around the Mediterranean [1, 3–5]. A further step was taken with the grand circumnavigations of the last five centuries, which transported mice almost everywhere around the world. Finally, historical records indicate that mice were bred as pet animals as early as three millennia ago in several parts of the world, particularly Japan and China [1, 3–5]. It was thus totally logical that this small mammal, like the rat and some small birds, should be used by early scientists for performing their experiments. Even if this choice was opportunistic rather

than being based on purely scientific considerations, it nonetheless appears nowadays to be an excellent one in the context of modern biomedical research where the house mouse has become a preferred model.

Mice are easy to breed. Because they are rodents, they eat rather large quantities of food for their size but do not have very specific or costly nutritional requirements. Under favourable conditions they breed all year round, with a short generation time. They give rise to relatively large progenies and tolerate inbreeding rather well compared to other mammalian species.

Over the years hundreds of mutations—most of them resulting in alleles with deleterious effects—have been collected and most, not to say all, of these mutations have contributed and still contribute to a better understanding of gene function(s). Because these mutant mice often represent animal models of human genetic diseases, several programs of intensive mutagenesis making use of the powerful mutagen ethylnitrosourea (ENU) have been developed worldwide to increase further this invaluable resource [6, 7].

Finally, two other very important advantages must be credited to the mouse as a model organism. (i) In this species, it is possible to grow *in vitro*, for several generations, totipotent embryonic stem (ES) cells that can be genetically modified in a number of ways but retain their capacity to participate in the formation of a germ line once inserted into the blastocystic cavity of a developing embryo [8, 9]. (ii) Importantly, the complete sequence of the mouse genome is now available on line, with a high-quality annotation, allowing comparisons with other mammalian genomes, including rat and human, to be performed with great accuracy [10]. In short, the mouse is one of the very few mammalian species to date whose genomic sequence has been entirely determined and for which technical procedures are available for the generation of a virtually unlimited number of genetic alterations, some of which are conditional, i.e. expressed only when the experimenter wishes.

In this chapter we describe the origins of laboratory mice, starting with their phylogenetic relationships with other mammalian species. We also discuss the advantage of strains established from recently trapped wild specimens as a source of polymorphisms for scientific research.

Phylogenetic relationships of the house mouse

The position of rodents among mammalian species

Mice are rodents. They belong to the most abundant (around 40%) and diversified order of living placental mammals, with some 2277 species grouped in 33 families [11]. Because of a relative homogeneity in their external appearance, the phylogenetic relationships between the different species of this order have sometimes been a matter of controversy, especially when morphological characters (tail, body shape, coat colour, etc.) were the only criteria available for the establishment of these relationships. Nowadays, with the use of various molecular markers (mostly DNA) and possible references to the complete genomic sequence of numerous orthologous genes, the situation has been much clarified. Figure 1.1.1, which is based on comparisons at the level of nuclear DNA sequences, represents the most likely phylogenetic tree for a sample of 28 different vertebrate species including the murid rodents (*Mus* and *Rattus* genera). The divergence between humans and mice of the *Mus* genus occurred somewhere between 70 and 75 Myr ago [10].

The position of mice among rodents

The rodent family *Muridae* encompasses at least 1326 species grouped in 281 genera [11]. The establishment of the evolutionary systematics in this group has also been controversial because of similarities in size and shape of the different species. Here again, studies making use of DNA sequences of various types have greatly contributed to clarify the situation [11–15]. Figure 1.1.2 represents the evolutionary relationships among a sample of 32 species of rodents including the
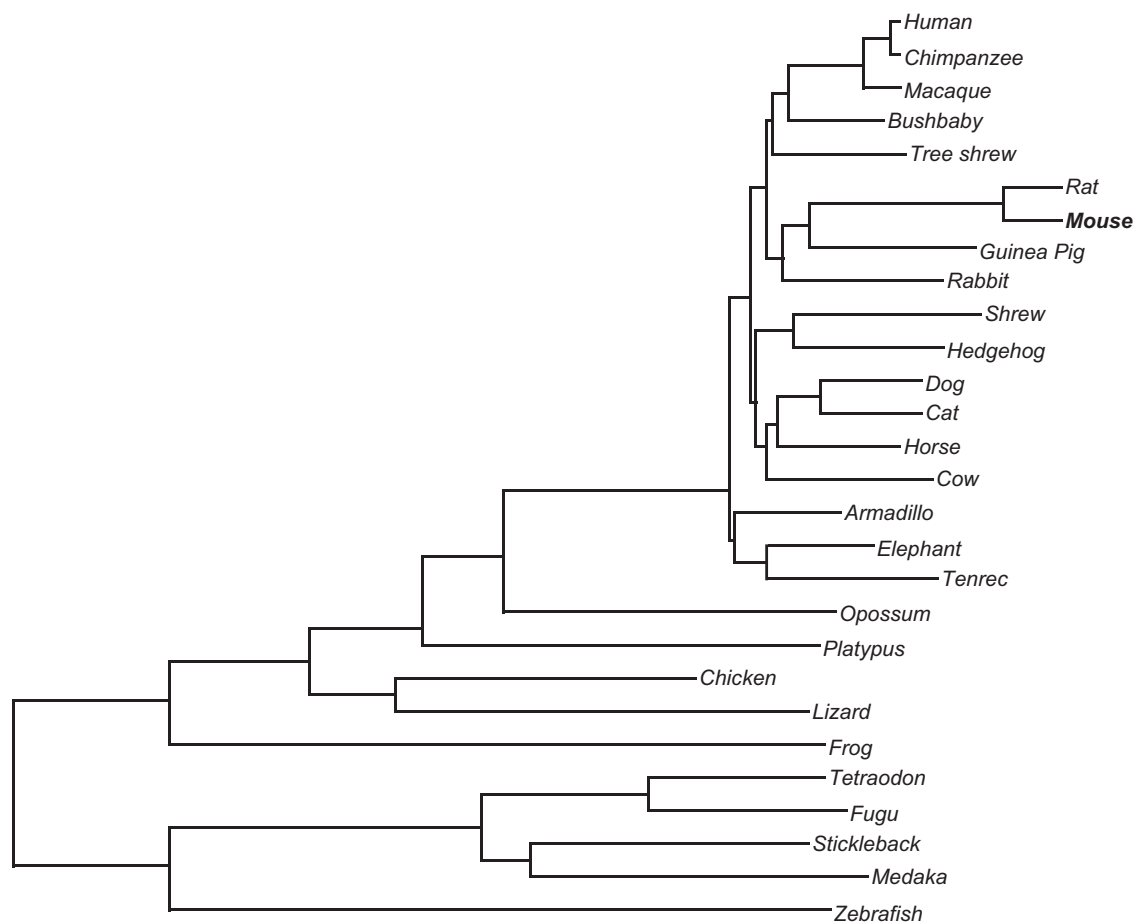
Figure 1.1.1 Tree indicating evolutionary relationships among 28 vertebrate species. Branch lengths are proportional to the number of base pair substitutions at a certain number of specific sites. The estimated time of divergence between human, and rodents was set approximately 75 Myr ago. *Redrawn from Miller W.* et al., Genome Res. **17**, 1797–1808, 2007.

mouse (*Mus musculus*) and rat (*Rattus norvegicus*). The divergence between the *Mus* and *Rattus* genera probably occurred around 10-12 Myr ago [14, 15], while the divergence of these two genera from *Peromyscus maniculatus*, the deer mouse (subfamily *Sigmodontinae*), occurred around 25 Myr ago. This should be kept in mind because deer mice, which are abundantly used as laboratory models in the USA, are often as considered close relatives of the laboratory mice of the genus *Mus* while, in fact, they are no more related to them than hamsters are.

Systematics in the genus *Mus*

The genus *Mus* contains four subgenera: *Mus*, *Coelomys*, *Pyromys* and *Nannomys*, totalling 41 species at present [16]. The individualization of the subgenus *Mus sensu stricto* occurred around

6.5 Myr ago with the split from three other different subgenera [17].

Members of this genus can be distinguished from other genera belonging to the same murine subfamily by a series of morphological characters [18, 19]. An accurate and up-to-date description of the genus with its different species and their geographical distribution is provided in Auffray and Britton-Davidian [16]. Briefly, the original geographic distribution of the genus *Mus* encompasses most of Eurasia and Africa, while its presence elsewhere results from human-mediated introductions during recent millennia. The highest taxonomic diversity occurs in Asia (with 3 subgenera and 19 species) where this genus likely originated [18-21].

Considering the high number of taxa that have been described recently and thanks to the
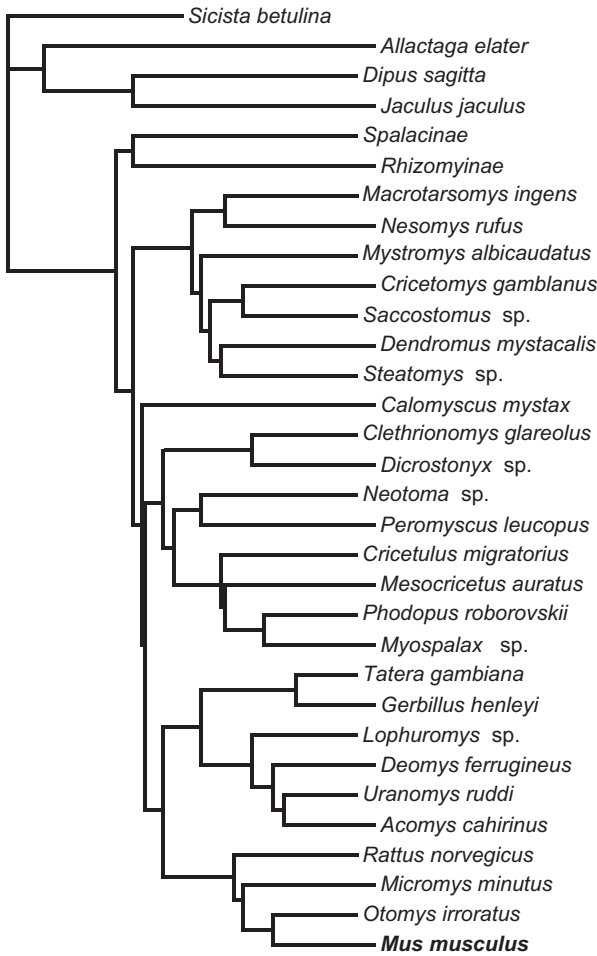
**Figure 1.1.2 Phylogenetic relationships among 32 species of rodents representing 14 subfamilies of Muridae.** *Redrawn from Michaux* et al., Mol. Biol. Evol. *18, 2017–2031, 2001.*

use of molecular markers, it is reasonable to think that the number of species in the subgenus *Mus* may still increase further. South East Asia, which has provided four of the five new species recently characterized, appears to be the key geographic area where new species may be found. In this context the case of *Mus cypriacus*, described in Cyprus, is noteworthy [22]. New species are even more likely to be discovered if we consider that the habitat of some of these new species is extremely limited and sometimes embedded in the wider habitat of another species [22, 23]. The subgenus *Nannomys*, which thrives in Africa, is also very likely to give rise to many new taxa as its systematics is studied more carefully [24].

Figure 1.1.3 summarizes the phylogenetic relationships within the genus *Mus* (subfamily *Murinae*). The subgenus *Mus* contains several species that are extremely similar in size and shape but seldom hybridize in the wild. Among the Asian species are *Mus cervicolor, Mus cookii* and *Mus caroli*, which form a group. The Indian pigmy mice related to *Mus terricolor* (formerly *Mus dunni*) together with *Mus famulus* from India as well as the more recently discovered species *Mus fragilicauda* [23] from Thailand and *Mus nitidulus* from Laos [25] should also be cited as forming a second group. The third group is that of *Mus musculus* and the other Palearctic species. Within this group, *Mus spicilegus* and *Mus macedonicus* are short-tailed mice that are found from the Caucasus to central Europe and the eastern Mediterranean, respectively, while mice belonging to the species *Mus spretus* are common in the western Mediterranean regions (south-eastern France, Spain, Portugal and North Africa). Finally, the recently rediscovered species *Mus lepidoides* from Burma [26] forms a fourth group of its own. For a more accurate description of the phylogeny within the subgenus see Suzuki and colleagues [20, 21].

Within the subgenus *Mus* there is a set of closely related subspecies of special interest to us because they constitute the house mouse (*M. musculus*) complex. These subspecies have their evolutionary origins in the Indian subcontinent and surrounding regions [27, 28]. The best-known representatives are those from the periphery of the original range that have been transported to the five continents since they became human commensals during the Neolithic: *Mus m. domesticus*, common in western Europe, Africa and the Near East and transported by humans to the Americas and Australia (Figure 1.1.4); *Mus m. musculus*, whose habitat ranges from eastern Europe to Japan, across Russia, and northern China; and *Mus m. castaneus*, which is found from Sri Lanka to South East Asia including the Indo-Malayan archipelago. Various molecular criteria discriminate easily between these different species at the periphery of the species range [5, 29]. It is, however, more difficult to understand what exactly happened in the centre of the range, where a mixture of primary differentiation and secondary intergradation seems to be the rule, especially in the highly mountainous and fragmented areas between Iran and northern India [30]. This is why a certain
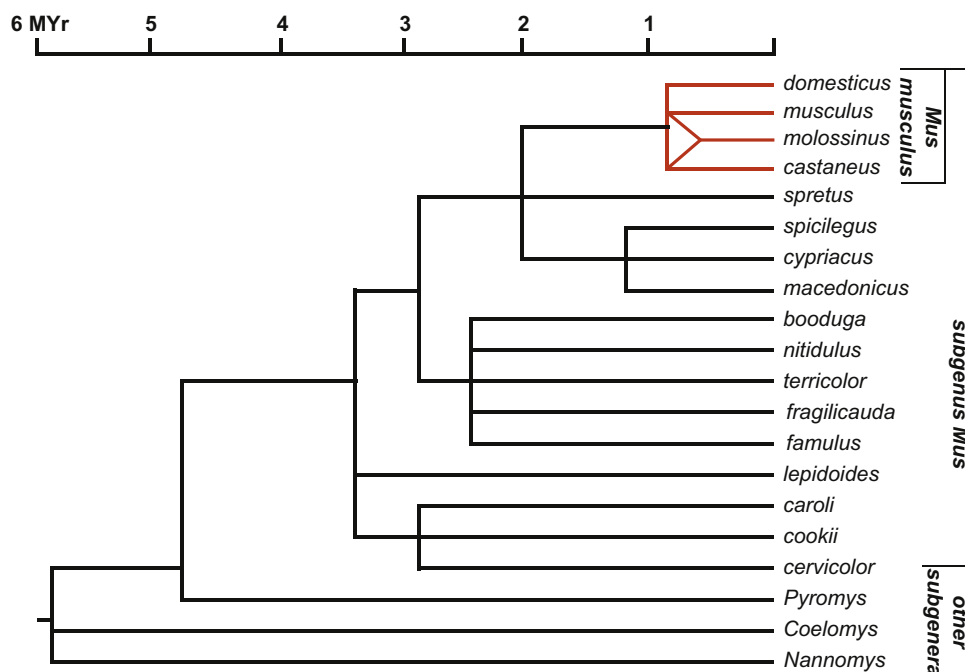
Figure 1.1.3 Consensus phylogenetic tree of the genus *Mus* issued from a compilation of all extant studies (see text). The approximate time scale (in Myr) stems from the recent calibration of Lecompte et al. [17].
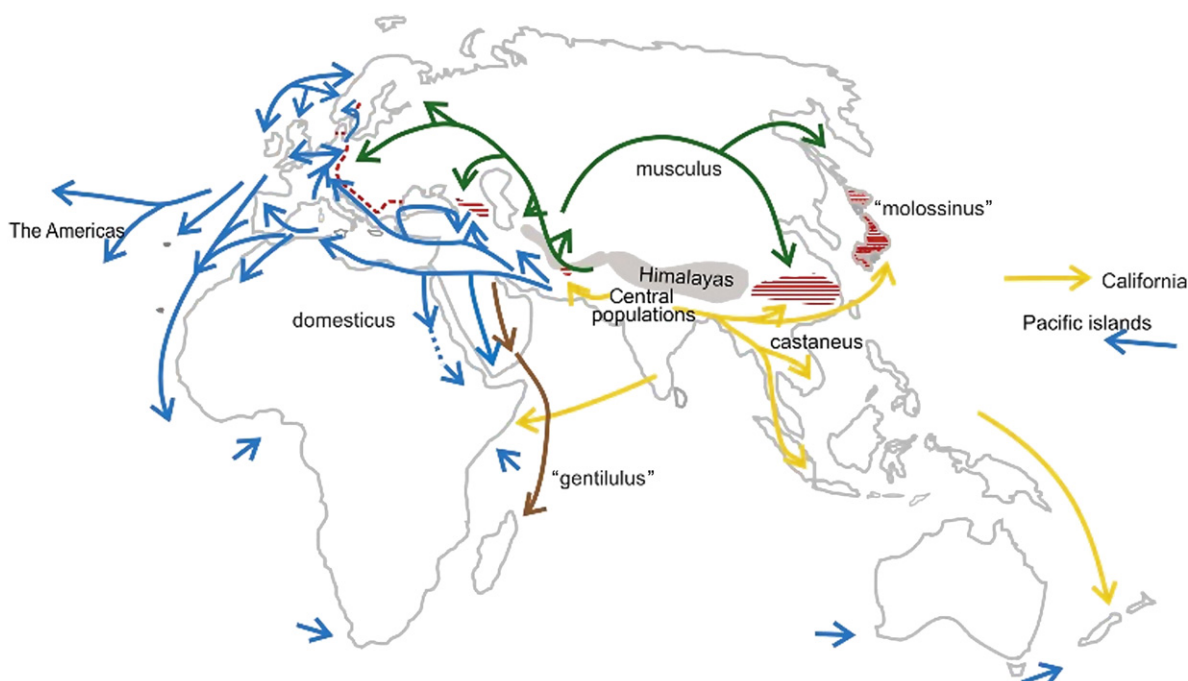
Figure 1.1.4 Summary of the inferred colonization history of the house mouse (*M. musculus*). The history starts with an origin, and differentiation in the vicinity of the Indian subcontinent, and with arrows indicating the colonizing movements of the *M. m. castaneus* (yellow), *M. m. domesticus* (blue) and *M. m. musculus* (green) subspecies and the *'gentilulus'* lineage (brown). These inferences are based primarily on mitochondrial DNA studies. Red dashes and shading indicate regions of hybridization between the subspecies including the generation of the *'molossinus'* form in Japan. For maps showing more detail of the inferred colonization history in Europe see Gabriel et al. [3].
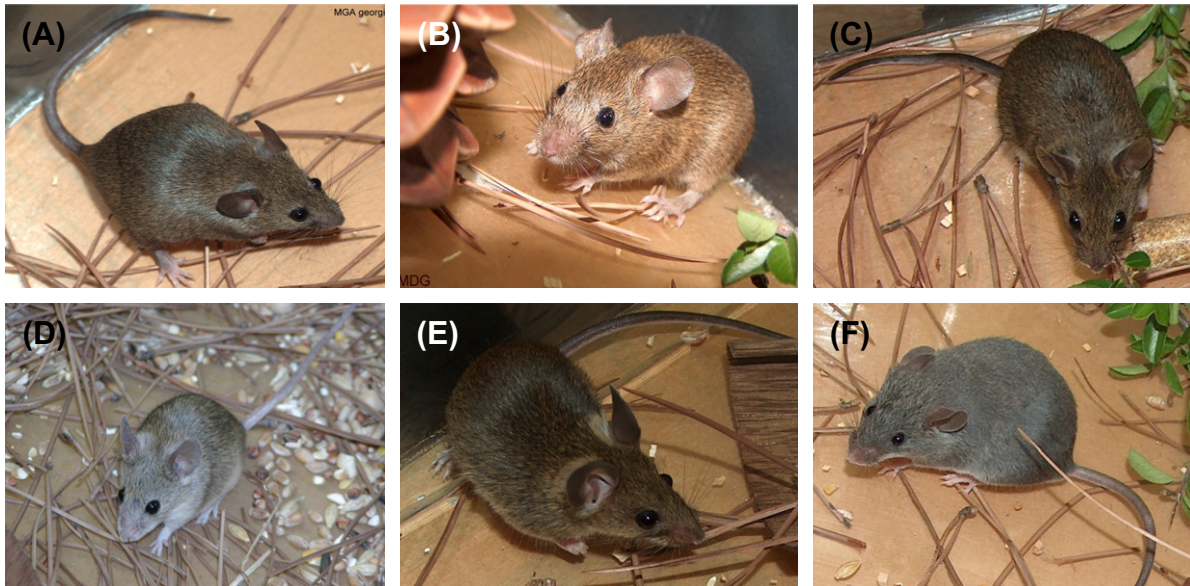
Figure 1.1.5 Six physically similar but only distantly related species of mice. In spite of close similarities in size, and body shape, the mice represented here are distantly related species. Only *M. m. castaneus* (B) and *M. spretus* (C) can produce viable and fertile hybrids with *M. m. domesticus* (A) or laboratory strains. Hybrids with the *M. spretus* species are fertile only in the female (Haldane's effect) and have been extensively used for the development of the mouse genetic map. Hybrids generated from the laboratory females and sperms from *M. caroli* (E) completed fetal development and a very low percentage of them even survived to maturity but none reproduced. *M. caroli* embryonic cells can survive in the uterus of a laboratory mouse when associated in a chimera with cells of the laboratory species [31, 32]. Hybrids between *Coelomys pahari* (F) and laboratory strains have never been produced and probably would not be viable. The possibility of obtaining hybrids between *M. cypriacus* (D) and laboratory strains has not yet been assayed. Several of these species, and subspecies, have been established as laboratory colonies. One of the most diverse collections of these wild-derived strains can be found at http://www.isem.cnrs.fr/spip.php?rubrique272.

number of relatively infrequently used Latin trinomens (e.g. *M. m. bactrianus*, *M. m. homourus*, *M. m. urbanus*) are described from this region, although it is currently impossible to disentangle real differentiation from artificial synonymy (Figure 1.1.5).

Mouse interspecific hybridization

Hybrids between mice of the subgenus *Mus* and the other subgenera *Nannomys*, *Coelomys* or *Pyromys* have never been reported and can probably no longer occur. Hybrids between *M. cervicolor*, *M. caroli*, *M. terricolor* and mice of the *M. musculus* complex have never been found in the wild, but hybrids between the former three wild species and laboratory mice have been produced by artificial insemination [31]. In these experiments hybrids generated by insemination of female

laboratory mice with *M. cervicolor* sperm failed to complete more than a few cleavage divisions. Hybrids generated from *M. dunni* (now *M. terricolor*) sperm and laboratory female oocytes implanted, but died *in utero* at a very early developmental stage. Hybrids generated from the same laboratory females and sperm from *M. caroli* completed fetal development and a very low percentage of them even survived to maturity, but none reproduced. Embryonic cells of *M. caroli* can survive in the uterus of a laboratory mouse when associated in a chimera with cells of the laboratory species; however, *M. caroli* embryos die around day 11–16 of pregnancy in the same conditions [32].

Although they are sympatric (i.e. share the same territory) with some *M. musculus* subspecies, the short-tailed species *M. spretus*, *M. spicilegus* and *M. macedonicus* exceptionally produce hybrids in nature. However, evidence from studies on mitochondrial DNA [28] and LINE transposable

elements [33] indicates that exchanges could occur sporadically that would allow alleles with a selective advantage to circulate outside the species in which they originated [34]. The three species mentioned above produce viable offspring with laboratory mice but male offspring of these crosses are sterile in compliance with Haldane's rule [35]. Male hybrids born from a *M. musculus* × *M. spretus* cross, for example, are invariably sterile regardless of the direction of the cross. This sterility is controlled by a relatively small number of genes since fertile males are frequently observed in the backcross progeny of F1 females with a male of one or the other parental species [35-39].

Mice of the *M. musculus* complex are not genetically isolated and, in those locations where they meet, there is evidence of gene exchanges ranging from limited introgression to more or less complete blending [28]. The best-documented cases of such gene exchanges are those occurring between *M. m. musculus* and *M. m. domesticus* in Europe, along a narrow hybrid zone, and between *M. m. musculus* and *M. m. castaneus* in Japan. The two subspecies have hybridized extensively in Japan, giving rise to a unique population often referred to as *Mus musculus molossinus* [40]. Other possibilities for gene exchanges probably also exist in many other places (e.g. southern Caucasus, Iran, China) and wherever mice of several subspecific origins have been transported by humans, as on many Pacific islands. These gene exchanges, which indicate that the speciation process is in progress but not yet completed, also explain the use of Latin trinomens for the designation of the different subspecies in the *M. musculus* complex.

The house mouse as a laboratory model: a historical perspective

Mice, rats and other small vertebrates have been used in biomedical research since the beginning of the 16th century when biology gradually shifted from a descriptive to an experimental science. Morse [41] reported that William Harvey (1578-1657) used mice for his fundamental studies on reproduction and blood circulation and, according to Berry [42], the earliest record of the use of mice in scientific research seems to have been in England, in 1664, when Robert Hooke (1635-1703) used mice to study the biological consequences of an increase in air pressure. Much later, Joseph Priestley (1733-1804) and his intellectual successor, Antoine Lavoisier (1743-1794), both used mice repeatedly in their experiments on respiration.

During the 19th century several fanciers in Europe, in Japan, and the United States were breeding and exchanging pet mice, segregating for a variety of coat colour or behavioural mutations (e.g. the famous 'dancing mice'—homozygous for the *Cdh23^v* or *waltzer* mutant allele). According to Hans Grüneberg [43], one of these fanciers, Louis-Théodore Colladon, (1792-1862), a Geneva pharmacist, reported results from his breeding experiments that were in perfect agreement with the Mendelian ratios—but this was 36 years before the publication of Mendel's own results on peas. As mentioned by Kenneth Paigen [44, 45] in his notes on the history of mouse genetics, it seems that Mendel's first experiments on the transmission of heritable characters were made using mice, segregating for coat colour markers (e.g. agouti, albino, brown) but Mendel was asked by his ecclesiastical hierarchy to stop breeding smelly creatures that copulated in his monastic cell. Mendel changed his experimental material to peas and in 1866 published his observations in a botanical journal where they had a much lower impact than might have been possible and remained virtually ignored until the beginning of the 20th century. Once rediscovered by H. de Vries, C. E. Correns and E. von Tschermak-Seysenegg, the three of them working independently with plants, it was really tempting to check whether the so-called Mendel's laws were also valid for animals. In 1902 L. Cuénot [46], a professor of biology at the University of Nancy, published experiments indicating that this was indeed the case. Cuénot's observations were shortly confirmed and extended to other species as well as for other genetic traits by G. Bateson, E. R. Saunders, A. Garrod, W. E. Castle and C. C. Little [44].

Mice have been instrumental for research in immunology, oncology and genetics because the

breeding systems that are used to produce them allow the establishment of highly standardized strains whose characteristics are precisely known and monitored generation after generation. (Among the genetically standardized strains, inbred strains are the most widely used. They result from the systematic and uninterrupted mating of brothers to their sisters for at least 20 generations, which leads to complete homozygosity for the same allele in all members of the strain.) Most laboratory strains originate from a few pet dealers who progressively became suppliers of 'laboratory' mice. For many years, and even today, many of the non-inbred albino strains used in laboratories are collectively designated 'Swiss' mice to recall their Helvetian origin.

Strain DBA/2 (formerly dba, then DBA) is the most ancient of all inbred strains since it was established by C. C. Little in 1909 [47], by intercrossing mice homozygous for the coat colour markers non-agouti (*a*), brown (formerly *b*, now *Tyrp1*) and dilute (formerly *d*, now *Myo5a*). About 10 years later strain C57BL/6 was established by Miss Abbie Lathrop of Granby, Massachusetts (USA) intercrossing the 'black' offspring of her female 57, while strains C3H, CBA and A were created by L. C. Strong, a cancer geneticist established at Cold Spring Harbor Laboratory [48]. At this point it is interesting to note that, among the strains established by L. C. Strong, strains CBA and C3H stemmed from the offspring of an out-cross with a few wild specimens trapped in a pigeon coop in Cold Spring Harbor. This probably explains how the wild allele at the agouti locus (*A*) was reintroduced into laboratory strains.

With a few exceptions historical records concerning the genealogy of most laboratory inbred strains are well documented and several interesting reviews on this subject are available [49, 50]. A chart describing the genealogy of these strains, including the recently established ones, has been published [51] and regularly updated information is available from The Jackson Laboratory website (see details in the References section). In addition to the chart published by Beck and co-workers [51], which was based mostly on historical records, a 'mouse family tree' was recently published by Petkov and co-workers [52], which is exclusively based on a set of 1638 informative single nucleotide polymorphism (SNP) markers, located 1.5 Mb apart, tested in 102 mouse strains (Figure 1.1.6). This family tree is an invaluable document for researchers who are willing to make interstrain comparisons because it makes it easy to select inbred strains that are more (or less) distantly related and compare specific phenotypic traits. It also provides an effective way of performing genome scans and quantitative trait loci analyses. Additionally, the SNP markers revealed several subtle differences (indels—base pair substitutions, etc.) between closely related mouse strains, including the groups of several 129, BALB, C3H, C57 and DBA strains.

The mouse has been closely associated with many important discoveries in biology during the 20th century. To cite just a few, we could say that our understanding of the genetic determinism underlying the success or failure of tissue transplantations is a consequence of the many experiments performed with inbred mouse strains by P. A. Gorer [53], then by G. D. Snell and co-workers, who developed a series of congenic resistant strains that were all genetically identical to the C57BL/10Sn background strain, with the exception of single short chromosomal regions determining graft rejection [54]. The discovery and genetic interpretation of the phenomenon of X-inactivation in female mammals, by M. F. Lyon [55], was facilitated by the existence and use of several X-linked mutations in the mouse and the observation of variegation in the coat colour of these mutations. The first chimeric organisms, produced by A. K. Tarkowski in Warsaw [56] and B. Mintz in Philadelphia [57], were mice. The observation of a particularly high frequency of testicular teratocarcinomas in strain 129 [58] and the *in vitro* culture of cell lines derived from these tumours [59], which for almost a decade represented the material of choice for investigating the processes at work in tissue differentiation [60], undoubtedly opened the way to the establishment of the so-called ES cells by M. J. Evans and M. H. Kaufman [6] and, almost simultaneously, by G. R. Martin [7]. The discovery of parental imprinting of some chromosomal regions was a consequence of experiments performed by J. McGrath and D. Solter [61] and M. A. Surani and co-workers [62], who demonstrated that a normal mouse embryo can only result from the fusion of a male and a female pronucleus,
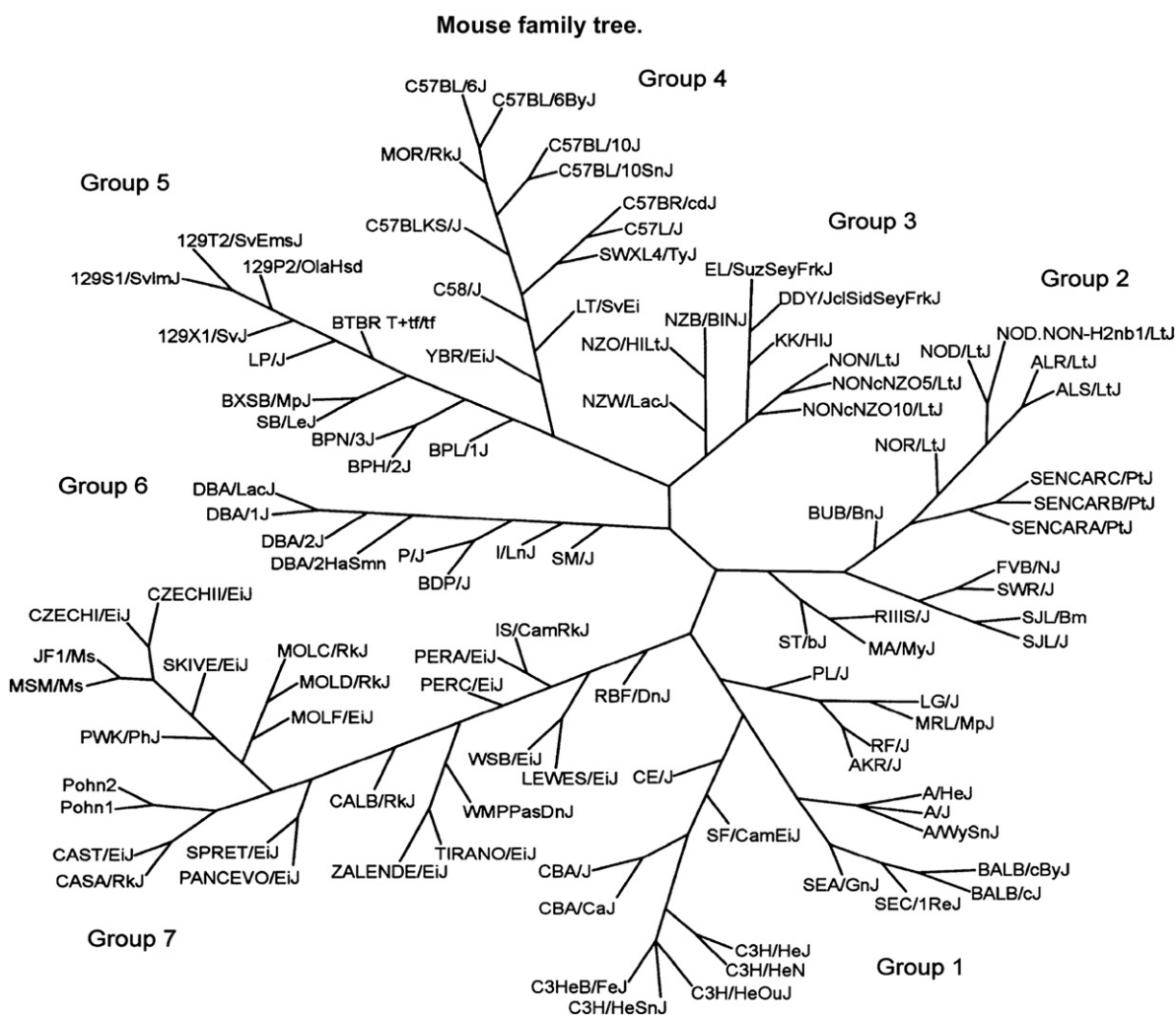
Mouse family tree.

Figure 1.1.6 Mouse family tree concerning 102 laboratory inbred strains, as established by Petkov et al. [52]. All these mice are organized into seven groups. A detailed description of each group is given in Petkov et al. [52]. The length and angle of the branches do not reflect the actual evolutionary distances between strains.

while B. M. Cattanach and M. Kirk [63] demonstrated that the parental origin of the two elements of a given chromosome pair was sometimes not genetically equivalent. The first transgenic mammal created by pronuclear injection of cloned DNA was a mouse [64-70], as was the first *in vitro* genetically engineered mammalian organism [71]. Although the first cloned mammal was not a mouse, this type of uniparental procreation has also been achieved in the mouse [72].

Information about many aspects of the biology of the mouse considered as a laboratory model, in particular about its genetics, has been published in the 95 issues of the *Mouse News Letter*. First issued in 1949 and published regularly every semester till 1997, this informal publication, edited by the scientists from the Medical Research

Council unit at Harwell and distributed throughout the world, was for several decades the major medium for the dissemination of information among the community of mouse geneticists. (The name *Mouse News Letter* was changed to *Mouse Genome* in 1990, when this publication became a peer reviewed journal. In 1998 *Mouse Genome* merged with *Mammalian Genome*.) The *Mouse News Letter* will forever remain the best place to find information about the history of mouse genetics, and in particular, about the history and location of most inbred strains, the progressive development and refinement of the linkage map and the discovery of hundreds of spontaneous mutations.

The Jackson Laboratory, which was founded in 1929 by C. C. Little in Bar Harbor (Maine,

USA), has played a pivotal role in the promotion of the mouse as a laboratory model and still is a unique centre for mouse genetics. The Jackson Laboratory, a non-profit organization entirely dedicated to basic research on the genetics of mammals, is nowadays almost exclusively dedicated to the mouse. It is, at the same time, a top-ranked research institution, a meeting place where courses and conferences are organized on various aspects of mouse genetics, and the world's largest genetic repository for mouse material where a great variety of genotypes and biological samples of all kinds are stored, in the form of frozen embryos or sperm cells, for distribution to the scientific community. Several other institutions, like the Oak Ridge National Laboratory in Tennessee (USA) and the MRC centre at Harwell in England, have also played a very important role in the development of the mouse as a laboratory model for research in genetics, oncology and immunology. More recently the European Union has decided to support the establishment of a network of genetic repositories (the so-called European Mouse Mutant Archive or EMMA), with major nodes in Italy (EMMA headquarters is in Monteretondo, near Rome), England (Harwell), France (Orléans-la-Source) and Germany (Munich). Finally, and even more recently, Japanese scientists have established a bioresource centre at the RIKEN Institute, in Tsukuba, with teaching and research activities focused on mouse genetics. More information about all these centres is available at the websites provided at the end of this chapter.

Since the completion of the mouse genome sequencing project several other collaborative projects have been developed worldwide that will undoubtedly result in a wealth of invaluable tools for research in mouse (mammalian) genetics in the future: here we review some of the most important.

To complement and expand the large-scale ENU mutagenesis projects described above [6, 7], several other projects have been launched with the aim of generating a comprehensive and publicly available resource of mouse ES cells containing a null mutation, generated by gene trapping or gene targeting, in every gene in the genome. The Knockout Mouse Project (KOMP) for example is a trans-NIH initiative

of this kind. Several similar projects exist in other institutions such as European Conditional Mouse Mutagenesis (EUCOMM) involving, in particular, the Wellcome Trust Sanger Institute in Hinxton (UK) and the Helmholtz Zentrum in Munich (Germany). So far approximately 10 000 conditional targeted alleles have been generated by the EUCOMM consortium in the highly germline-competent C57BL/6N ES cell line. The EUCOMM project is still in progress, in association with other partners constituting the International Knockout Mouse Consortium (IKMC) [73].

Alongside these large-scale projects for producing a collection of catalogued and ready-to-use genetic alterations, several phenotyping projects, often called 'mouse clinics', have been developed in dedicated facilities where experienced scientists use the most sophisticated equipment to detect all kinds of phenotypic differences. The International Mouse Phenotyping Consortium (IMPC) for example consists of a group of major mouse genetics research institutions and plans to address the challenge of developing an 'encyclopedia' of mammalian gene function. Each mutant line generated by the IKMC will undergo a broad suite of high-throughput tests to identify developmental, anatomical, physiological, behavioural and pathological phenotypes. The Japan Mouse Clinic, established at the RIKEN Institute in Tsukuba, has developed a similar project. There is no doubt that these programmes will result in a better understanding of the genetic basis of disease.

Finally, it is important to mention here the development of a completely new and original genetic resource that will be of great value for the analysis of complex traits: the so-called Collaborative Cross. This project was first proposed in 2001, has been implemented at the Oak Ridge National Laboratory (USA) since May 2005 and has now spread to several other laboratories. The Collaborative Cross is a randomized cross of eight inbred mouse strains that features a randomized assortment of eight remotely related genomes: A/J, C57BL/6J, 129S1/SvImJ, NOD/LtJ, NZO, CAST/Ei, PWK/Ph, WSB/Ei. The lines are first crossed pairwise to make all 56 possible G1 parents. A set of possible four-way crosses is performed, keeping Y chromosome and mitochondrial balance.

Finally, all eight genomes are brought together in G2:F1, and the offspring of this cross are inbred. Currently there are 650 lines in production, and close to 200 lines are now beyond their seventh generation of inbreeding. The project is to breed around 1000 inbred strains with a unique assortment of the eight parental genomes. While the breeding is in progress, samples of the lines that have reached or passed the seventh generation of inbreeding progressively enter a high-throughput phenotyping protocol and DNA samples are banked for sequencing, analyses of recombination history, allele drift and loss, and population structure. The inbred strains of the Collaborative Cross will be able to detect biologically relevant correlations among thousands of measured traits, and the 1000 strains, considered together, will represent 135 000 recombination events, which is an enormous and unprecedented power of resolution. Such a panel would indeed represent a valuable community resource in which information will progressively accumulate over time [74].

The house mouse and its wild relatives

As discussed above, the classical laboratory inbred strains of mouse have many advantages relating to their great genetic homogeneity. For example, a population of F1 hybrids, born from an intercross between two highly inbred strains, is genetically equivalent to a population of cloned mice since this population displays no genotypic variation from one animal to the next. Unfortunately, the coin has another side: because they are derived from a relatively small pool of ancestors, these inbred strains do not exhibit a great variety of genetic polymorphisms of natural origin. This relative genetic homogeneity is well reflected in the fact that most of the classical strains possess the same maternally inherited molecule of mitochondrial DNA [75-77] and relatively reduced polymorphisms for the Y chromosome [78, 79]. Aside from this relative genetic homogeneity and paucity in terms of variability, a careful analysis of the genetic polymorphism segregating among the different strains revealed that these strains have a mosaic genome derived from more than one species [80, 81]. Today's classical laboratory strains should be regarded as interspecific recombinant strains derived from three parental components: *M. m. domesticus*, *M. m. musculus* and *M. m. castaneus*. For this reason, and to point to their relatively unnatural genetic constitution, it would probably be more appropriate to designate them collectively as *M. m. 'laboratorius'*!

The existence of this genetic 'mosaicism' has been recently confirmed and assessed with great precision [82, 83]. It was established that, on average, 92% of the genome of *M. 'laboratorius'* is *M. m. domesticus* in origin, while the remainder is mostly of Japanese origin (*M. m. molossinus*). Another important observation was that the distribution of diversity is markedly non-random among the chromosomes, with large regions of extremely low diversity and hot spots of diversity [83].

To compensate for this relative lack of variability, a collection of strains derived from small breeding nuclei of wild specimens, trapped in well-defined geographical areas and belonging to characterized species, have been developed in various laboratories over the last 20 years. A list of the strains that are completely inbred, i.e. that have been propagated by strictly unrelaxed brother × sister mating for more than 20 generations (the so-called wild-derived inbred strains or WDIS), is given in Bonhomme and Guénet [84], but other such WDIS are being derived, their number being now close to 50. Other useful stocks of wild mice are also maintained in various laboratories and a description of these stocks has been published by Potter [85]. These 'new' inbred strains have played an important role in recent years because they represent a virtually unlimited reservoir of genetic polymorphisms.

Wild mice have been useful in providing geneticists with polymorphisms such as electrophoretic variants, restriction fragment length polymorphisms (RFLPs), or more generally SNPs that are much less numerous in standard inbred strains. With the introduction of strains derived from wild progenitors, in particular from *M. spretus*, the genetic map of the mouse has dramatically increased its resolution [86]. Comparisons of non-coding orthologous regions at the sequence level indicate that any inbred

strain derived from *M. spretus* exhibits, on average, one SNP at every 80-100 bp when compared with any of the classical laboratory strains [87]. This wealth of polymorphisms represents a considerable advantage in experiments where the aim is positional cloning of a gene identified only by phenotype because it allows an accurate delineation of the targeted locus [88]. The high density of polymorphisms turns out to be an even greater advantage when quantitative traits are mapped, because every animal with a relevant phenotype can be genotyped for a very large number of markers. In this respect, the mouse is unique since the frequency of SNPs between humans is roughly one order of magnitude lower than that of *M. spretus* compared to laboratory strains [89, 90]. The high frequency of SNPs in coding regions means that the genome of *M. spretus* or *M. m. musculus* is full of ready-made quantitative trait loci (QTL) point mutations waiting for functional genomic studies!

Wild mice have also been invaluable in providing cytogeneticists with a large collection of robertsonian translocations (or centric fusions) recovered from the many populations of *M. m. domesticus* where they occur in homozygous conditions [91]. These translocations are characterized by the fusion of two acrocentric chromosomes by their centromeres and they often interfere with the normal process of meiosis resulting in the production of gametes with an aneuploid (unbalanced) complement. Using carefully designed crosses involving these centric fusions, it has been possible to produce and study trisomies and monosomies for all mouse chromosomes [92].

In addition to their homogeneity in terms of chromosome morphology, laboratory strains have only long telomeres while, for instance, strains of *M. spretus* origin have both long and short telomeres [93, 94]. This peculiarity might be helpful for investigating the significance of the still-mysterious variations in telomere size found in mammalian cells.

When infectious agents of various kinds are injected into mice it is common to observe that some strains are more susceptible than others and that wild-derived strains are in general (but not always) more resistant than classical laboratory strains. A commonly accepted, although not demonstrated, explanation is that some alleles of laboratory strains that are essential for determining innate or acquired mechanisms of defence have been by chance replaced by a defective mutant allele without any consequences for the mice because these animals are kept in protected environments. Even if in most cases the level of susceptibility or resistance is controlled by several genes interacting together (QTLs) or having an additive effect [95, 96], the situation is sometimes under the control of a single gene, making its analysis relatively simple. This is the case, for example, when mice of most laboratory strains die after an injection with an appropriate dose of orthomyxoviruses while most wild strains are resistant [97]. This phenotype is controlled by a single gene (*Mx1* on chromosome 16) with two alleles: $Mx1^+$ (resistant, dominant) and $Mx1^-$ (susceptible, recessive) and the discrepancy between wild mice and laboratory mice in terms of susceptibility indicates that the mutated allele of *Mx1* is over-represented in laboratory strains, probably due to a sampling effect. A similar example exists with experimental flavivirus infections where all laboratory inbred strains, except strain PL/J, are susceptible while most wild-derived inbred strains are resistant [98]. Here again the allele responsible for susceptibility is a null at the *Oas1b* locus (chromosome 5), which has been fortuitously selected in laboratory strains while it is rare or absent in wild mice. Similar phenotypes of resistance/susceptibility have also been reported for a variety of pathogens [99, 100], and, even if in most instances genetic differences have been observed among classical laboratory strains, these differences also exist between laboratory and wild-derived strains, making the genetic analysis much easier. Wild mice have also proved particularly useful for investigating the biology of murine leukaemia viruses and both new *Fv* loci and new alleles at the *Fv1* and *Fv2* loci have been discovered in wild strains [101, 102].

The comments concerning the susceptibility of mice to infectious diseases also apply to carcinogenesis, and comparisons between classical laboratory strains allowed the complex influences of genetic background on tumour susceptibility to be unravelled; several genes modifying tumour susceptibility have been identified. However, while the phenotype of F1 hybrids between any two classical laboratory strains is

generally intermediate between the two parental strains, it is often identical to the phenotype of the wild parent when crosses are performed with WDIS, indicating dominance of the wild-derived allele [103].

Besides their use in mapping, interspecific crosses also offer an opportunity for analysing the effects of bringing together the products of genes separated by divergent evolution in the cells of an offspring. This can help identify the genetic functions that are subject to rapid divergence and to pinpoint the functions that eventually promote speciation. Those functions that are mostly unaffected during the evolution of the taxa are most likely to be basic functions that are under more constraint. This last point will be important in the comparison of orthologous regions between human and mouse genomes, and even more so between the genomes of various species of the genome *Mus* that have now been resequenced.

Questions concerning epistatic interactions can also be addressed by investigating offspring of interspecific crosses at the genomic level. So far we have no clear answers to this question but data exist indicating that some combinations of alleles are strongly counter-selected in the offspring of some interspecific crosses [104].

A less dramatic but still very interesting situation is frequently observed when wild mice are used for the mapping of mutations with deleterious phenotypic effects. In this case the interspecific offspring, homozygous for the mutant allele, often exhibit a wide range of variations in the degree of severity of their phenotypes, with severe forms and weaker ones. In these cases, genes or loci with a modifying effect can be identified, mapped and eventually cloned [105, 106]. Genes of this kind, which are potentially of great value, cannot be recognized in an animal with a normal genotype.

Because many different inbred strains belonging to several more or less related taxa of the genus *Mus* are now easily available, it is possible to address questions aimed at a better understanding of genome structure and functions. For example: are all the genes present in one strain also present in the others, or are there differences and/or variations in the copy number? If the answer is that a particular gene exists in one strain and not in a closely related

one, then what use is the gene in question? Examples of this kind have already been reported [107], and have allowed fundamental questions to be answered in a very elegant way.

It would also be interesting to study certain categories of orthologous genes in closely related species to see how their pattern of spatiotemporal expression evolves and in what sort of sequence variation this evolution is involved. This can be particularly interesting when adaptive traits are concerned.

Investigations at the genomic level using inbred strains derived from wild mice are bound to become very popular in the near future because they can be achieved with a high level of refinement and can be correlated in a very reliable way to the phenotype of the living animal. At this point it is no exaggeration to say that this new type of mouse strain is bound to be of expanding interest and it is predictable that, in the future, the house mouse and its related species will be even more useful for scientific research than it has been over the last centuries, especially since more than one complete genome is now available for comparative purposes in the genus *Mus*.

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Morse 3rd HC. Origins of Inbred Mice. http://
www.informatics.jax.org/morsebook/
Silver LM. Mouse Genetics. www.informatics.jax.
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Theiler K. The House Mouse, Atlas of Embryonic Development. http://www.emouseatlas.org/
Atlas/Theiler_book_download.html

Websites

Ensembl Genome Browser. http://useast.
ensembl.org/index.html

International Mammalian Genome Society.
(IMGS) http://www.imgs.org
International Knockout Mouse Consortium.
(IKMC) http://www.knockoutmouse.org
International Mouse Strain Resources (IMSR).
http://www.findmice.org
Montpellier wild mice genetic respository. http://
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MRC Harwell (UK). http://www.har.mrc.ac.uk
Nomenclature of Mouse and Rat Strains. http://
www.informatics.jax.org/mgihome/nomen/
strains.shtml
RIKEN Mouse Mutagenesis Program. http://
www.yokohama.riken.jp/english/index.html
The Jackson Laboratory. http://www.jax.org

Historical Foundations

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Introduction

The laboratory mouse (derived from the common house mouse) has played a key role in mammalian genetic and biomedical research. The mouse is a powerful model organism for research on human disease because it is a mammal and because of the high degree of conservation between the mouse and human genomes. After the first report of a conserved mouse and human autosomal linkage in 1976 [1], intense comparative mapping of the mouse and human genomes [2-4] culminated in the sequencing of both genomes [5-7], demonstrating high DNA coding sequence conservation.

Research using the mouse spanned the 20th century, from the birth of mammalian genetics to sequencing the mouse genome at the beginning of the 21st century. The future of the laboratory mouse promises to continue to be invaluable for biomedical research in the coming years as new technologies enable rapid genome sequencing and large-scale generation of new mouse models. Figure 1.2.1 highlights a sampling of discoveries, resources and milestones in mouse genetics, shaping the past, present and future of biomedical research.

The ability to selectively modify the mouse genome increased the power of the mouse as a research tool for understanding the genetic basis of human health and disease [8-10]. Mutant and inbred mice frequently have syndromes similar to human inherited diseases because of their close metabolic and internal anatomical similarities to human beings. Hence, the mouse provides models for research not only on mammalian biology but also on a wide variety of human diseases including cancer, diabetes,
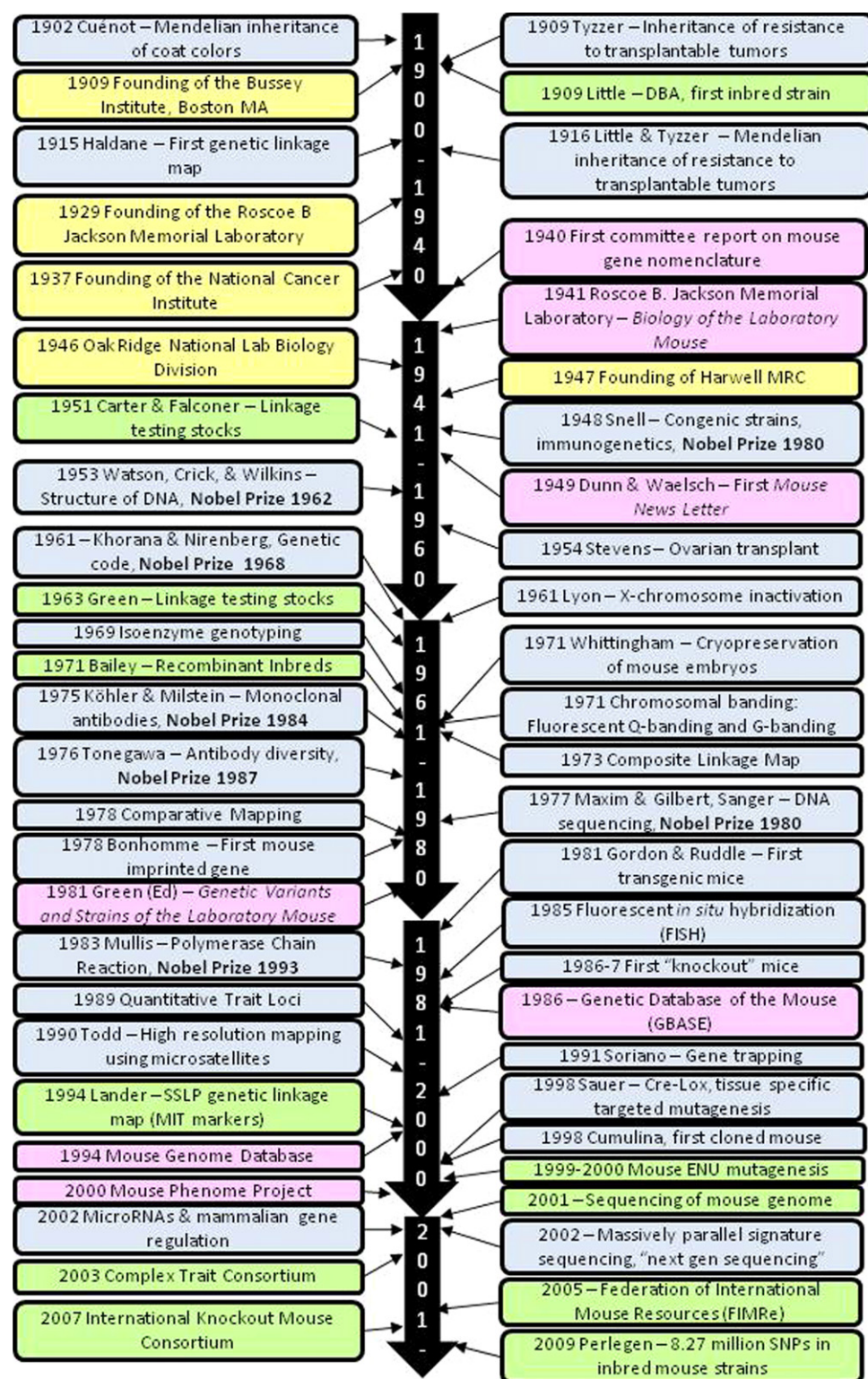
Figure 1.2.1 History of mouse genetics. The timeline highlights more than a century of seminal experiments and discoveries (blue), creation and characterization of new mouse resources (green), founding of mouse-related research institutions (yellow) and mouse information resources (pink). The history of mouse genetics can be roughly categorized into five periods: 1902–1940, birth of mouse genetics; 1941–1960, discovery and expansion of mouse resources; 1961–1980, mapping the mouse genome; 1981–2000, genetic mapping advances and manipulation of the mouse genome; and 2001–2011, the mouse genome sequence and beyond.

aging, atherosclerosis, endocrine diseases, immunological diseases, autoimmunity, neurological dysfunction and numerous others. The ultimate recognition of the value of the mouse was its selection as the first model organism to have its genome sequenced in the Human Genome Initiative [7, 11]. In 2011 we have the complete genome sequence of at least 15 inbred strains, thousands of genetically engineered mutant mice have been generated and high-throughput sequencing is leading to rapid identification of new spontaneous mutation models.

# 1902–1940: the birth of mouse genetics

Origins of the laboratory mouse

The laboratory mouse originates from ancestors in the Middle East in the area that is now Pakistan. A commensal organism, the mouse has emigrated to most corners of the world as human beings' travelling companion. For a detailed history of the origins of the house mouse see Silver [12] and Chapter 1.1.

On the tiny Turkish island of Tenedos at the mouth of the Dardanelles stands a temple to Apollo, God of Mice, that predates the Trojan War. Albino mice were used in auguries for Egyptian rulers [13]. The earliest drawings of mice may be seen in Chinese prints as early as 300 AD and

mutant mice, such as albino and waltzer, appear in 18th and 19th century Asian prints.

Mouse fanciers of the late 19th and early 20th centuries were the origin of most laboratory mice of today. The mouse fancy hobby originated in Asia and later spread to Europe and from there to America. Because of their origins in the mouse fancy trade, laboratory mouse strains are a genetic mix of four different subspecies: *Mus musculus musculus* (eastern Europe), *Mus musculus domesticus* (western Europe), *Mus musculus castaneus* (south-east Asia), and *Mus musculus molossinus* (Japan). The latter is thought to be a hybrid between *M. m. castaneus* and *M. m. musculus*. Genome analysis has confirmed that the laboratory mouse is a blend of these four different species or subspecies of the genus *Mus* [14, 15]. Analysis of whole genome sequence and high-density single nucleotide polymorphism (SNP) maps in several inbred traditional and wild-derived laboratory strains shows that the laboratory mouse genome is mostly from *M. m. domesticus*, ranging from estimates of 68% [16] to 92% [17] on average. As might be expected based on the origins of laboratory strains, mouse genomes are a mosaic of segments of different subspecific origins. Phylogenetically, the house mouse (*Mus*) belongs to the family Muridae, along with several other species of mice and the common rat (Figure 1.2.2).

Many inbred laboratory strains derive from those of Miss Abbie Lathrop, a mouse fancier who bred and sold mice in Granby, Massachusetts (USA) from around 1900 to her death in 1918. She obtained her mice from dealers and European
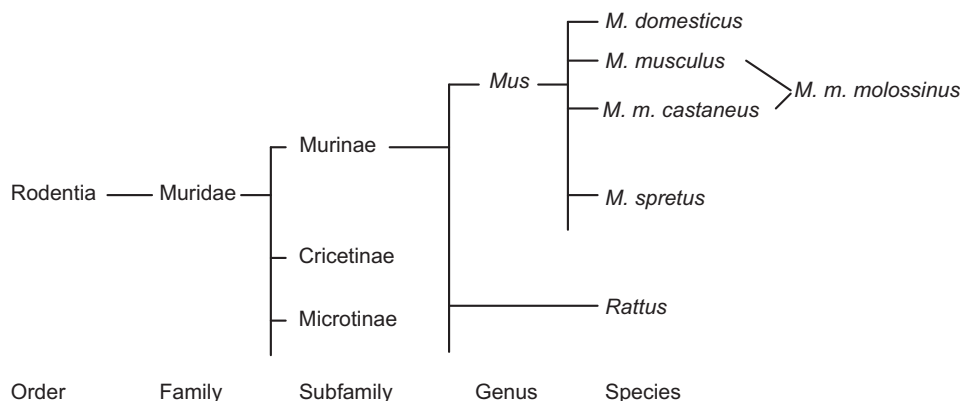
Figure 1.2.2 Abbreviated diagram of the phylogenetic origin of laboratory mouse strains. Note the laboratory rat also is in the subfamily Murinae, which is why 'murine' is an inappropriate adjective for the laboratory mouse. *Derived from Moriwaki and colleagues [114].*

fanciers, and animals captured in the wild. Although Miss Lathrop is often mentally pictured as a little old lady who collected fancy mice, she was an experimentalist and observer. She carried out cancer research experiments, collaborating with Dr Leo Loeb at the University of Pennsylvania [18-20]. This collaboration grew out of her observation of tumour growths in her mice and her curiosity to learn more [21]. She also carried out breeding experiments in collaboration with William Castle, and later Clarence Cook ('C. C.') Little, who were at the Bussey Institute at Harvard. Miss Lathrop's breeding records and notebooks, including such observations, are preserved in the library at The Jackson Laboratory.

The history of mouse genetics might have begun in the 1860s if an Augustinian bishop had not forbidden the breeding of mice within the monastery where Gregor Mendel did his classic genetic studies in plants [22]. Thus, the first proof that mice, like sweet peas, had genes and showed genetic transmission of traits was when a French geneticist, Lucien Cuénot, demonstrated that mammals show Mendelian inheritance, using the inheritance of coat colours in mice [23]. He went on to demonstrate that a gene can have multiple alleles [24] and that some alleles, like the yellow allele of the agouti gene (A^y), can be lethal [25]. In 1903 William Castle at Harvard also published a paper on coat colour genetics in mice [26]. He and his student C. C. Little are often credited with the first cogent report and explanation of a lethal allele, also A^y [27].

Mouse genetic research was initiated at the Bussey Institute for Research in Applied Biology at Harvard in 1909. William Ernest Castle directed the mammalian research programme [28, 29]. Sewall Wright and C. C. Little were two of Castle's first students. Most of the well-known names in the history of mouse genetics, at least in the United States, can be traced to the Bussey Institute. Examples include L. C. Dunn (developmental biology); L. C. Strong, C. C. Little, and Lloyd Law (cancer genetics); Clyde Keeler (behavioural genetics); Paul Sawin (quantitative biology); and George Snell (immunogenetics). Other geneticists from Europe and Asia regularly visited the Bussey. A 'genealogical' tree of mouse geneticists drawn by Elizabeth ('Tibby') Russell and modified by Sandy Morse shows the extent to which

students of the Bussey determined the future of mouse genetics [21]. Genetic research using mice in the early 20th century centred on coat colour genetics, cancer and tumour transplantability.

The development of inbred strains of laboratory mice was central to mouse genetics because inbred strains enabled the genetic analysis of individual mutations and traits by eliminating the 'noise' of heterogeneous segregating genetic backgrounds. C. C. Little is credited with conceiving of and creating the first inbred strain, DBA (dilute, brown, non-agouti), which was maintained and protected from loss by his mentor E. E. Tyzzer at Harvard while Little was in the army during World War I [30]. Breeders from C. C. Little's Line C (derived from Miss Lathrop's mice) founded the C57/C58 family of strains; females 57 and 58 were mated to male 52 to give rise to the C57BL, C57BR, and C58 inbred strains. Others, including Miss Lathrop and Leonell Strong, also began inbreeding mice at about the same time.

Biomedical research

The earliest biomedical research using the mouse involved the genetics of tumour transplantability and cancer susceptibility. Initial studies suggested that the genetic component in these traits was weak or non-existent because of the use of outbred mice and the complexity of the trait [31, 32]. The development of defined genetic backgrounds by inbreeding encouraged the continuation and growth of research on cancer.

C. C. Little and others continued to develop inbred strains to analyse the genetics of susceptibility and resistance to cancer and tumour transplantation [33]. In 1933 J. B. S. Haldane suggested that cancer had a genetic component [34]. Jacob Furth, at the University of Pennsylvania, developed the high leukaemia strain AKR [35]. Leonell C. Strong studied the genetics of susceptibility and resistance to tumour transplantation. Howard Andervont studied the genetics and viral aetiology of cancer and went on to head the National Cancer Institute until 1961 [36]. Walter Heston began his early work on lung cancer [37].

In 1933 the staff of The Jackson Laboratory published a paper describing maternal inheritance of mammary tumours in mice [38]. This phenomenon was later shown to be non-Mendelian

transmission by a factor in the dam's milk [39], subsequently identified as a virus [40, 41]. Ultimately, it was shown that the virus could be integrated into the mouse genome and transmitted as a gene by both females and males.

1941–1960: discovery and expansion of mouse resources

The growing number of inbred strains and mutant mice developed and characterized in early 20th century were in high demand. Early pioneers distributed their mice widely to other investigators, who in turn established breeding colonies creating substrains, or bred mutations on to different genetic backgrounds. Standardization of mouse nomenclature became important. L. C. Dunn, Hans Grüneberg and George Snell served as the first mouse nomenclature committee and published their first report in 1940 [42]. There also was a need for resources to facilitate transfer of information concerning animal care, husbandry and the genetics of the strain or mutation. The first edition of the *Biology of the Laboratory Mouse* was published in 1941 and the first *Mouse News Letter* 'advertised' the latest mutants in 1949.

From 1915 to 1970 the mouse genetic map was composed of linkage groups in which two or more genes or 'visible markers' were linked together. In the mid 20th century most mouse genetic and biological research was carried out at a triumvirate of mouse research centres: the Harwell Medical Research Council (MRC) Genetics Unit (UK), the Biology Unit at the Atomic Energy Commission's facility in Oak Ridge, Tennessee (USA), and The Jackson Laboratory in Bar Harbor, Maine (USA). During the 1940s the two major focuses for genetic mapping were identification of histocompatibility genes and discovery and characterization of visible, morphological markers resulting from spontaneous mutations. Study of the latter also provided the first mouse models of human inherited diseases.

Biomedical research

George Snell, inspired by Little and his early work on tumour transplantation, began his life-long study of the genetics of transplantation that became the basis for all histocompatibility and tissue transplantation research. He joined the staff at The Jackson Laboratory in 1935. During the 1940s Snell created congenic strains (strains differing at a locus of interest and a linked chromosomal segment carried over during backcrossing) to isolate, identify and map genes involved in tissue rejection or acceptance [43]. He rediscovered the mouse major histocompatibility complex (MHC), described by Peter Gorer in 1938 as a red blood cell antigen affecting transplantation. Snell and Gorer's joint research on histocompatibility genes [44] became the foundation for the medical field of tissue and organ transplantation in humans. Because Gorer died prematurely, it was Snell who was awarded the Nobel prize in 1980 for this research.

During the 1940s and 1950s spontaneous and induced mutations, created in radiation risk assessment studies following World War II, were used to map genes in the mouse. As the effects of these mutations were studied, the potential of mutant mice to provide research tools for studying human inherited disease became evident and the types of biomedical research broadened. Areas of research expanded from cancer genetics and histocompatibility to haematopoietic stem cell research, haematological disorders, skeletal abnormalities, neurological and neuromuscular diseases, kidney disease and many more.

1961–1980: mapping the mouse genome

Most principles of genetics in the mid 20th century were established in non-mammalian species. Studies in *Drosophila* and microorganisms led to the understanding of chromosomal theory, the nature of mutation, the discovery of the genetic code and gene structure and function. During this period, however, the study of mouse genetics and gene mapping grew steadily, creating a solid foundation for the future of mouse genetics. In

the 1960s and 1970s mouse genetics became prominent again with the recognition of the need for a mammalian model for biomedical research, the development of efficient genetic mapping tools in mice and the realization of the high degree of genomic conservation between the mouse and human genomes.

Genetic map of the mouse

Genetic maps, the road maps of genetics, are of two types: linkage and physical. The 'signposts' on the maps are loci, any location or marker in the genome that can be detected by genetic or DNA analysis. The term 'gene' is more restrictive than locus and refers to DNA segments that encode proteins or can be linked to phenotypes. Linkage maps are recombinational or probability maps; they are constructed by carrying out linkage crosses that measure the recombination frequency (plus or minus a standard error) between genes or loci on the same chromosome. Physical maps were based first on chromosomal bands in metaphase chromosomes and now on genomic DNA sequence. The first genetic linkage in the mouse (the first autosomal linkage in mammals) was described in 1915 in the classic paper on the linkage of pink-eyed dilution and albino [45]. This proof of linkage between these two genes was the beginning of the genetic linkage mapping effort that continued through the rest of the 20th century and into the 21st. The discovery and genetic analysis by mapping crosses of spontaneous mutations that caused visible phenotypes was the basis for virtually all genetic mapping in the mouse from 1940 to 1970.

Genetic mapping with spontaneous mutations that created visible phenotypes, such as changes in coat colour/texture (e.g. albino, Tyr^c; piebald, $Ednrb^s$; satin, sa ; fuzzy, fz) or behaviour (e.g. waltzer, v; reeler, $Reln^{rl}$; shiverer, Mbp^{shi}) was laborious and sometimes took years. This was because crosses between mice carrying recessive mutations yielded so few informative progeny, and genes on only one or two chromosomes could be scored in each cross. Determining linkage demanded sophisticated statistical analysis and large numbers of progeny were required to obtain statistical significance [46]. It was not uncommon to generate thousands of intercross (F2) progeny—but the results did not lead to the

high-resolution maps that such crosses with today's genetic markers provide. During the 1950s and 1960s, linkage-testing stocks (e.g. V/Le, SB/Le) combining multiple visible markers were created to speed the mapping process.

The first real breakthrough in linkage mapping, enabling the scoring of many test markers and chromosomes in the same cross, was the discovery and use of codominant biochemical (isoenzyme) genes (e.g. glucose phosphate isomerase 1, $Gpi1$) [47–49]. This transition from visible markers to polymorphisms revolutionized gene mapping in the mouse. Also, in the 1970s Donald W. Bailey conceived of and began developing recombinant inbred (RI) strains, panels of inbred strains derived from a mating of mice from two inbred strains [50]. Because they are inbred and derived from common ancestors, mice from these strains can be characterized for any trait or genetic marker that differs between the parental strains, and mapping data are cumulative [51].

The development of chromosomal banding techniques in the late 1960s enabled the assignment of gene linkage groups to physical chromosomes of the mouse in the early 1970s. Visualization of banding patterns was critical to this advance because all laboratory mouse chromosomes are telocentric (the centromere is located at one end) making the identification of most individual chromosomes virtually impossible. Fluorescence quinacrine (Q)-banding [52] and Giemsa (G)-banding [53] were used to produce karyotypes in which all 21 chromosomes were identified by their unique banding patterns. Linkage groups were assigned to physical chromosomes by cytological identification of the chromosomes involved in reciprocal translocations already associated with specific linkage groups [54]. This approach is credited to John Hutton but drew on the accumulated data of many laboratories. Prior to chromosomal banding, however, Eva Eicher assigned the first linkage group to a chromosome, based on the size and unusual unbanded cytological appearance of chromosome 19 and linkage analysis of linkage group XII [55].

Because genetic crosses are possible in mice, somatic cell hybrid panels, enabling the assignment of genes to chromosomes, were never used in mouse gene mapping to the extent they were in human mapping. Somatic cells of two

species are fused and one species' chromosomes are segregated out during cell line propagation, creating a panel of cell lines, each with one or a few chromosomes of the species of interest. Radiation hybrid panels, in which the chromosomes of the species of interest have been fragmented by irradiation prior to fusion, have been used effectively in the mouse as well as human to physically locate and order genes in chromosomal segments. The T31 mouse radiation hybrid (RH) mapping panel was made available through The Jackson Laboratory mapping resource [56].

During the 20th century, composite linkage maps were compiled at The Jackson Laboratory (Margaret Dickie, Margaret Green, James Womack, Thomas Roderick and Muriel Davisson) and at the MRC Genetics Unit at Harwell (C. Beechey, J. Butler, S. Hawkes and R. Meredith) by statistically combining data from all scientists' linkage crosses. The first composite linkage map showing both chromosome numbers and linkage groups was published by Margaret Green [57]. Davisson and Roderick [58] published the first linkage map in which linkage groups were adjusted to chromosome size based on physical measurements of the chromosomes [59].

The first report of conserved mouse and human autosomal linkage was published in 1976 [1] and the mouse was represented in the first report of a Comparative Committee at the Third International Workshop on Human Gene Mapping [60]. A flurry of intense comparative mapping of the mouse and human genomes followed (summarized in references 2-4). This effort culminated in the sequencing of both genomes [5-7] demonstrating high DNA coding sequence conservation.

1981–2000: genetic mapping advances and manipulation of the mouse genome

In the 1980s and 1990s mouse genomics again burst into the limelight, centre stage, with the development of powerful methods to map and manipulate the mouse genome.

Genetic mapping advances

The final advance in the cytological physical map was the development of fluorescent *in situ* hybridization (FISH) [61]. FISH allows mapping of single genes to cytological bands on the physical chromosome and identification of chromosomal rearrangements using paints [62, 63]. For many years Mary Lyon and her colleagues at Harwell maintained and published in *Mouse News Letter* (which later became *Mouse Genome*) the composite 'Chromosome Atlas' map, which combined linkage data and physical mapping by FISH or cytological location of chromosomal rearrangement breakpoints. As more and more genes were identified and mapped, the number of linkage groups and genes mapped within them grew increasingly rapidly until it was impossible to depict the whole mouse linkage map graphically in print publications. In 1990 Davisson and colleagues published the last print copy of the linkage map [64] and in 1997 Mary Lyon and colleagues published the last print copy of the Chromosome Atlas [65].

During this period as well, several scientists developed inbred strains from wild populations to increase genetic variability in mapping crosses [66-68]. Verne Chapman (Roswell Park, Buffalo, NY, USA), Michael Potter (National Institutes of Health, Bethesda, MD, USA), Jean-Louis Guénet (Institute Pasteur France), and Eva Eicher and Tom Roderick (The Jackson Laboratory) all developed wild-derived inbred strains. *M. m. castaneus* (e.g. CAST/Ei), *M. spretus* (e.g. SPRET/Ei) and *M. m. molossinus* (e.g. MOLD/Rk) were the most widely used. Johnson and colleagues improved mapping mutations with RFLP (restriction fragment polymorphisms) loci by combining intersubspecific intercrosses (F2) using inbred CAST/Ei with RFLPs for gene families, allowing multiple genes to be detected on a single Southern blot [69]. The efficiency of mapping mutations with polymorphic markers was further improved by pooling F2 progeny DNAs for the initial genome screen with PCR (polymerase chain reaction) [70]. Panels of backcross DNAs were developed for efficient mapping of polymorphic markers that

could be typed in DNA. The C57BL/6Ros × *Mus spretus* panel of Neal Copeland and Nancy Jenkins [71] and the C57BL/6J × SPRET/Ei backcross panel at The Jackson Laboratory were mapping resources available to investigators [72].

In the 1980s and 1990s DNA markers revolutionized genetic mapping. Their use was greatly facilitated by the development of the concept of the PCR in 1983. DNA polymorphic markers, RFLPs [73] and later simple sequence length polymorphisms (SSLPs), such as the MIT markers [74], are widespread throughout the genome. One of the biggest advantages of DNA markers for mapping is that newly discovered markers can be typed in indefinitely stored DNAs from linkage crosses, such as the C57BL/6 × *M.spretus* crosses above, or mapping panels, such as RI [50, 51] and chromosome substitution strains [75]. Subsequently, SNPs, of which millions are present in the mouse genome, increased the density of available DNA markers [16, 76, 77].

One of the most rapidly growing areas of mouse genomic research in the latter 20th century was the genetic analysis of complex traits and diseases. Ironically, this field combined the latest advances in molecular mapping, such as SNPs, with strain panels developed in previous decades. Larger panels of RI strains and their variant recombinant congenic strains and panels of consomic (chromosome substitution) strains were developed [75, 78]. In the latter one can search for complex trait genes on individual chromosomes and then narrow the analysis with segmental congenic strains. The Complex Trait Consortium was established in 2002 [79] and a large RI strain panel, called the Collaborative Cross, was initiated with a goal of generating 1000 strains derived from an eight-way cross [80].

Gene identification

Identification (or cloning) of mutated genes in the last decades of the 20th century was made possible by the development of libraries of artificial chromosomes containing inserts of mammalian DNA: plasmid clones (PACs), bacterial artificial chromosomes (BACs) and yeast artificial chromosomes (YACs), listed in order of size from small to large. High-resolution

genetic crosses with sometimes thousands of progeny were used to narrow the chromosomal interval harbouring the gene of interest. Once a mutant gene was mapped to a segment less than a fraction of a centimorgan, contigs of overlapping clones were constructed across the region by hybridizing PACs, BACs and/or YACs to each other. Although laborious, this approach to identifying mutated genes became increasingly successful as DNA sequencing technologies and the quality of libraries improved. With the electronic publication and annotation of the entire mouse genome sequence [7, 81, 82], candidate gene cloning became more common and yielded more rapid gene identification than positional cloning did. It became a simple matter to electronically examine the chromosomal interval identified by the high-resolution genetic cross for candidate genes whose mutation might have led to the phenotype observed. With both methods, candidate genes were tested by assessing RNA expression levels or examining the gene itself by Southern blotting for large DNA alterations and, ultimately, by sequencing exons. The 21st century has seen the development of high-throughput and massively parallel signature sequencing (i.e. next-gen sequencing) technologies, greatly reducing the time needed to identify the gene of interest [83]. However, the cost of next-gen sequencing remains prohibitive for most laboratories and generates massive amounts of sequence data requiring software analysis tools that are currently insufficiently robust for finding many single gene mutations. The array-based sequence capture approach based on a coarse genetic map position makes gene identification using high-throughput sequencing more efficient [83]. Independent of the technology used, once a mutation is identified in the candidate gene one must validate that the mutation causes the phenotype. This can be accomplished by (i) sequencing the candidate gene to look for mutations in other alleles identified by complementation testing, (ii) demonstrating in a cross or mutation-segregating colony that the mutation and the phenotype co-segregate, (iii) rescuing the phenotype by overexpressing the wild-type allele in a transgenic mouse or (iv) creating a targeted mutation of the candidate gene.

Bioinformatics

The development of large, comprehensive databases must be recognized as a milestone because without their development the rapidly increasing accumulation of genetic and biological data in the last three decades of the 20th century would be overwhelming and impossible to manage. In the late 1970s the linkage data Margaret Green had accumulated on 4×6 inch cards was entered into a computer program known as GBASE (the Genetic Database of the Mouse) developed by Thomas Roderick, Muriel Davisson and Carolyn Blake at The Jackson Laboratory. The data were proofread by Mary Lyon while she was on an extended visit to the laboratory. GBASE became the first online database of mouse genetic information, released in 1986. Subsequently, Margaret Green's catalogue describing mouse genes [84] was added as the *Mouse Locus Catalog*, which was maintained for many years by Donald Doolittle. At about this time Thomas Roderick coined the term 'genomics' for the new journal of that name, providing a name for the expanded science that encompasses genetic mapping, sequencing and genome analysis. In 1994 GBASE was combined with a linkage analysis database developed by Janan Eppig and a homology database developed by Joseph Nadeau to become the predecessor of the Mouse Genome Database (MGD). Today's Mouse Genome Informatics program at The Jackson Laboratory encompasses the MGD database of genomic and phenotype information [85], the Gene Expression Database (GXD) [86], the Mouse Tumor Biology database (MTB) [87] and MouseCyc, a compilation of records for biochemical pathways that are specific to mice or mammals [88]. MGD is part of the Gene Ontology consortium [89] and links to a variety of inbred strain information and sequence analysis tools [90]. The advent of DNA sequencing, culminating in the sequencing of entire genomes, has generated sophisticated bioinformatics systems beyond the scope of this chapter to describe. The many sequence databases and analysis software packages that are available are valuable tools for the mouse geneticist. Examples include the Ensembl (www.ensembl.org), the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov), and the University of California Santa Cruz Genome Browser (genome.ucsc.edu) projects. A critical aspect of all genetic mapping and the bioinformatics programs that support it is the use of controlled genetic nomenclature. Conventions of genetic nomenclature are described in Chapter 1.3 (Strains, Stocks and Mutant Mice) and the complete guidelines are found on the MGI website at http://www.informatics.jax.org/mgihome/nomen/index.shtml.

Genetic manipulation of the mouse genome

Genetic engineering has catapulted the mouse into the leading position as a mammalian model organism for biomedical research. With transgenesis and gene targeting it is possible to selectively modulate the amount or composition of a gene product (see Chapter 1.5, Generation of Mouse Mutants by Genotype-Driven Mutagenesis, for a detailed explanation). The first technology introduced was the insertion of foreign genes into mouse chromosomes to produce gain-of-function mutants. The first transgenic mouse was created and described by Jon Gordon in Frank Ruddle's laboratory in 1980 [8]. Creation of loss-of-function mutations followed the pioneering demonstration by Leroy Stevens that embryonic teratocarcinoma cell lines could give rise to differentiated tissues, which led to the discovery that pluripotent embryonic stem (ES) cells could be grown in culture [91]. In the late 1980s two research groups reported the first successful alteration of a mouse gene by homologous recombination or targeting [9, 10, 67, 92]. Early efforts with this technology essentially created null mutations or 'knockouts'.

The discovery that many such mutations led to embryonic lethality instigated the development of targeting technology that makes it possible to determine tissue and temporal specificity using conditional mutation systems. The first developed was the *Cre–Lox* system. Mice carrying a transgene containing the gene for the prokaryotic Cre recombinase enzyme linked to a tissue-specific promotor are mated with mice carrying insertions of the LoxP target sequence flanking the gene to be removed [93, 94]. Tissue-specific expression of the Cre enzyme enhances recombination between the loxP sites

and deletes the targeted gene's function in that tissue. A similar system can be created with the Flp recombinase [95, 96]. Temporal control is achieved using tetracycline-inducible mutations [97, 98]. Finally, it is possible to replace an endogenous gene with another functional gene [99]. We can now go from gene to phenotype (reverse genetics) as well as from phenotype to gene (forward genetics). Nevertheless, it should be noted that being able to manipulate specific genes is still a long way from being able to generate specific phenotypes; frequently, targeted mutations cause an unexpected phenotype or, sometimes, no detectable phenotype at all.

In 1998 Cumulina the mouse joined Dolly the sheep as a mammal that can be cloned from somatic cells [100], making possible the creation of mice from somatic mutations. This technology was never exploited to its full potential because the efficiency and success rate of whole-mouse cloning never equalled or exceeded that of mutating ES cells and turning them into mice.

High-throughput mutagenesis increased the mutation rate and allowed screening for subtle phenotypes and the identification of novel genes. The widespread use of the powerful mutagen ethylnitrosurea (ENU) resulted from the research of William Russell's group at Oak Ridge National Laboratory, Tennessee (USA) [101]. Several large-scale mutagenesis centres or multicentre programmes were established in Europe and North America during the latter part of the 1990s (see e.g. references 102-105).

Insertional mutagenesis was used to randomly mutate genes by insertion of a DNA sequence that could subsequently be used to identify the mutated gene [106]. The first of these was developed by Rick Woychik, then at Oak Ridge, using insertion of transgenes carrying a selectable construct [107]. This approach was replaced by gene-trapping technology using sequences that integrate only into gene-specific genomic regions such as promoters [108] or other gene-specific sequences in ES cells [106, 109].

Biomedical research

In the 1980s and 1990s molecular technology advances made it possible to (i) more rapidly identify mutated genes and (ii) genetically manipulate the mouse genome to alter genes shown to be mutated in human diseases. For example, the transgenic strain overexpressing the promoter region and exon 1 of the human Huntington's disease gene, B6CBA-TgN(HDexon1)62Gpb/J, causes disease symptoms that mimic the human condition, beginning at 9-11 weeks of age [110]. A transgenic mouse carrying the human superoxide dismutase 1 gene with the mutation associated with amyotrophic lateral sclerosis (ALS, Lou Gehrig's disease) provides a good model for that disease [111]. One of the early targeted mutations created was in the gene encoding the transmembrane protein mutated in human cystic fibrosis (*Cftr*) [67], but the mutant mice died early in life because of intestinal abnormalities. Combining the *Cftr* knockout with a functional human *CFTR* transgene expressed in the intestine allowed cystic fibrosis null mice to survive long enough to provide a model to study and test therapies for the debilitating lung phenotype that affects human patients [112]. Conditional mutagenesis allowed control of the tissue specificity of the mutation or onset of gene expression (temporal control) [93, 94, 98].

2001–2011: the mouse genome sequence and beyond

Sequencing the mouse genome

The beginning of the 21st century in mouse genetics saw the completion of the mouse genome sequence, the ultimate physical map. In 1999 three major sequencing centres, the Wellcome Trust Sanger Institute (Cambridgeshire, UK), the Whitehead Center for Genome Research (Cambridge, MA, USA) and Washington University Genome Institute (St Louis, MO, USA), had combined efforts to form the Mouse Genome Sequencing Consortium (MGSC) to sequence the mouse genome. C57BL/6J was chosen by the MGSC as the first inbred strain to be sequenced. Celera, a private company, won the mouse race, announcing the completion of

the first pass sequence in May of 2001 of a mixed mouse genome, including 129S1/SvImJ, 129X1/SvJ, A/J, C57BL/6 and DBA/2J inbred strains [81, 82]. In December 2002 MGSC published the complete draft sequence of C57BL/6J [7].

One hundred years after Mendel's principles were shown to operate in the mouse, we can relate the genetic map to the cytological map to the ultimate physical map (sequence) of the genome. Yet, genetically mapping traits to locate their positions in the sequence and identifying candidate genes remain crucial in linking the mutation to the phenotype.

A century of generating inbred and mutant strains around the world has resulted in a plethora of research resources available for unravelling basic biological processes and inherited and acquired human diseases. Specialized mutant resources have been developed and consolidated into repositories, databases and information processing systems and have been developed and linked to handle the distribution and sharing of data. In 2005 the Federation of International Mouse Resources (www.fimre.org) was established as an umbrella organization of all the repositories [113]. High-throughput platforms to rapidly sequence individual genomes, group efforts to knock out every gene in the genome (e.g. the International Mouse Knockout Consortium, www.knockoutmouse.org) and new technologies and tools to find an explanation for phenotype beyond the central dogma, including the role of epigenetics and microRNAs in regulating gene expression, have emerged during the last decade.

The whole mouse continues to be the final testbed for determining how genes function, the science of functional genomics and biomedical research. Thus, while other model organisms, such as *Drosophila*, yeast, worms (*C. elegans*) and zebrafish may be easier to manipulate and allow analyses that require hundreds or thousands of animals, the mouse is likely to continue to be the premier mammalian model for understanding human inherited diseases.

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Strains, Stocks and Mutant Mice

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Introduction

The laboratory mouse is an ideal model organism for the study of human physiology and pathophysiology. Humans and mice may not look very much alike, but genes from mice and humans are approximately 95% identical in their coding regions and function in virtually the same way in a biological context. Using mice in research also has numerous practical advantages, including their small size, short gestation times (3 weeks), ease of maintenance and production of experimental cohorts of animals in the form of litters averaging 5–10 pups. Many studies that are developmental in nature or invasive are not possible or ethical with human subjects, but can

be done under very controlled conditions with mice. As a preclinical model of disease, new therapies can be tested in mice for both safety and efficacy prior to use in human patients. Perhaps one of the most salient features of the mouse is the ability to create and maintain inbred strains. Laboratory mice are arguably the most inbred of domestic mammals, so much so that individual mice within a strain are near clones of one another. This uniformity allows for reproducibility of experiments across time among laboratories and the ability to efficiently study the effects of genetic mutations while minimizing phenotypic variance. The relative ease by which mice can be selectively bred and inbred has allowed for the propagation of numerous spontaneous and genetically engineered mutations, as

well as the generation of panels and sets of strains that have been workhorses used in genetic mapping and complex trait analysis over the years. These genetic mapping tools, in turn, led to the identification of numerous causative genes and the discovery of genetic interactions related to disease resistance and susceptibility. In the last three decades technological advances in genetic engineering and embryonic stem (ES) cell technology have provided researchers with the ability to create even more mouse models. These advances in genetic engineering, along with large consortium-based mutagenesis and gene knockout projects, have led to an overwhelming number of mouse models. These valuable genetic resources are preserved and available to the scientific community from a number of mouse repositories around the world.

Stocks and strains

The terminology surrounding genetic crosses, strains and stocks of mice can sometimes be confusing, especially if misused. Thus, it is worth defining some terminology right from the start. A *stock* is defined as an isolated, interrelated breeding population. The term 'stock' is often confused with an inbred strain, but the terms should not be used synonymously. The most common example of a stock is the classical outbreds, such as CD-1 and Swiss Webster. Another example is in the creation and maintenance of a genetically engineered mouse, where two or more strains of mice have been bred together, but not to the point of inbreeding. It is often the case that researchers will characterize and publish data on genetically engineered stocks of mice—those that arise from a common lineage. Although this is a reasonable approach for assessing the general impact of a genetic manipulation, the phenotype of the mouse is very much a result of the genetic contribution of the entire genome and can change as alleles become fixed during the process of breeding or through the maintenance of selected lineages. Likewise, the term *strain* is often used as shorthand to refer to an inbred strain but is frequently misused to refer to a breeding population—which

may or may not be inbred. An *inbred strain* is a line of mice that has been propagated by a single lineage of sister × brother mating for 20 or more generations. One should never assume that the term 'strain' refers to an inbred strain. A *line* refers to a pedigreed stock with a known lineage to a single breeding pair.

A brief word on nomenclature

A stock or strain's generation number provides additional important information on the genetic background and breeding history of mouse strains that cannot be captured in the mouse strain names. The letter 'F' stands for filial or inbreeding (sister × brother) generations. Examples: F1, first filial generation; F2, second filial generation. 'N' represents the number of backcross generations; a backcross is defined as the mating of a mouse from a stock or strain with one of its parents or with an individual genetically identical to one. For example, N1, first backcross generation; N2, second backcross generation; etc. A strain generation of N8F15 would indicate that a strain has been backcrossed 8 generations to the strain designated by the strain's name and then sister × brother mated for 15 generations. Mice that have been backcrossed to a common inbred line for five or more generations will have achieved a relatively uniform genetic background (see section on congenic strains). When maintaining mouse colonies, one should meticulously record the pedigree and generation numbers of the breeding colony.

Inbred strains

Inbred strains are produced by at least 20 generations of sister × brother mating. However, one should be aware that even after 40 generations of inbreeding there can be residual heterozygosity that is essentially eliminated by F60 [1, 2]. Continual inbreeding produces mice that are genetically uniform, being homozygous at virtually all of their loci. The most important practical consequence of inbreeding is that there should be virtually no genetic segregation within the strain and, as such, every mouse is essentially a genetically identical clone of its parents and

siblings, allowing for the perpetual propagation of genetically identical animals. As there is no genetic variation within a fully inbred strain, the observable characteristics and traits, or phenotype, tend to be more uniform within a gender. The only variation between individuals will be due to non-genetic causes.

As noted earlier, the success of the laboratory mouse as a model organism is partly attributable to its ability to overcome *inbreeding depression*. Inbreeding depression is the loss of viability or function resulting from excess inbreeding, as noted in many populations including plant, dog and human where the negative consequences of inbreeding have been most commonly noted. The most frequently cited signs of inbreeding depression are reproductive failures, but it also manifests as poor health. Inbreeding suppression is most prominent in initial filial generations, where the homozygous state of the alleles is rapidly increasing [3]. Inbred strains can be very sensitive to changes in environment and, while some inbred strains breed quite well, others may continue to struggle with poor reproductive performance and small litter sizes, making them vulnerable to extinction. However, once a strain becomes fully inbred, further inbreeding will have no effect. Thus, the phenotype of an inbred strain will only change as a result of the fixation of new mutations or as a result of environmental changes. New mutations are relatively rare, and only a quarter of them will normally be fixed with continued full sibling mating. However, many of these mutations, which can lead to genetic drift, will show no obvious phenotype.

A number of inbred strains were developed from fancy mice in the first third of the 20th century (see Chapter 1.2, Historical Foundations). These are considered the 'classical' inbred strains and among them are BALB/c, the C57 series, C3H, DBA and 129 parent strains, all of which have become the standards for research in most areas of mouse biology. Most commonly used inbred strains have been inbred for 200–300 generations. Individual inbred strains exhibit specific characteristics, passed on from generation to generation, that make them ideally suited for specific types of research and as models for exploring genetic variation and human biology

(see Table 1.3.1). For example, FVB mice are noted for their large litters and females generally tend to take exceptional care of their pups. Fertilized eggs contain large and prominent pronuclei that facilitate the microinjection of DNA, and thus this inbred strain has been used extensively for transgenic research over the years. For example, ageing DBA/2J mice develop progressive eye abnormalities that model human hereditary glaucoma [4]. DBA/2J mice are also known to show an extreme intolerance to alcohol and morphine [5, 6].

Although strain characteristics can be the main focal point of a research project, the characteristics of some of the classical inbred strains are often overlooked during experimental design or data analysis and can compromise experimental results. For example, mice of several strains (e.g. C3H, FVB/N and SJL/J) are blind due to homozygosity for the retinal degeneration 1 mutation, $Pde6b^{rd1}$ [4]; some strains (e.g. 129, A/J, C57BLKS/J, DBA/2J) exhibit early hearing loss [7]; DBA/2J mice are prone to audiogenic seizures prior to their early hearing loss [8]. Genetic differences among 129 strains can substantially impact their value as background strains for targeted mutation experiments [9, 10]. In addition, many inbred strains carry recessive genes affecting coat colour (e.g. BALB/c mice are homozygous for both the brown, $Tyrp1^b$, and albino, Tyr^c, loci). When designing experiments it is critical to thoroughly research strain characteristics to determine whether mice of the considered strain have any characteristics that might benefit or confound the experimental results. The characteristics of many inbred strains of mice (and rats) have been meticulously researched over the years and this information has been nicely compiled and summarized by Michael Festing [11] in an online searchable database currently housed at the Mouse Genome Informatics website, http://www.informatics.jax.org/external/festing/search_form.cgi. The Mouse Phenome database is a more recent effort designed to collect phenotypic data on a defined set of genetically diverse inbred strains of mice: http://phenome.jax.org [12].

Once fully inbred the only way that inbred strains can change is as a result of the accumulation of new mutations or genetic contamination. A breeding programme should be designed to

TABLE 1.3.1: Commonly used inbred lines, origins and research applications

Parent strain	Strain abbreviation	Research applications
129P3/J	129P	Spontaneous testicular teratomas, targeted mutagenesis
129/S1/SvlmJ	129S	Spontaneous testicular teratomas, targeted mutagenesis
A/J	A	Widely used in cancer and immunology research; low-incidence cleft palate
AKR/J	AK	High incidence of leukaemia
BALB/c	Cby	General purpose immunology
C3H/HeJ	C3	General purpose strain in a wide variety of research areas including cancer, infectious disease, sensorineural and cardiovascular biology research
C57BL/6J	B6	General purpose, cardiovascular biology research, background strain for most mice carrying transgenes, spontaneous or targeted mutations
C57BL10/J	B10	General purpose
DBA/1J	D1	Widely used as a model for rheumatoid arthritis; in response to challenge, mice develop immune-mediated nephritis
DBA/2J	D2	General purpose, show low susceptibility to developing atherosclerotic aortic lesions; used in glaucoma research
NZW/LacJ	NZW	Type 1 diabetes
NZB/B1NJ	NZB	Autoimmunity
SJL		Cancer (reticulum cell sarcomas), autoimmunity (experimental allergic encephalomyelitis, EAE)
SWR	SW	General purpose; ageing mice exhibit a high incidence of lung and mammary gland tumours. Highly susceptible to experimental allergic encephalomyelitis

minimize the chance that new mutations will become fixed in the colony. Where large numbers of animals are needed for research purposes, an appropriate breeding scheme is to maintain a small 'foundation' colony, with an expansion colony of sufficient size to provide all the required experimental animals. The expansion colony is used only to produce experimental animals and does not contribute to the long-term survival of the strain [13]. Inbred strains should be checked periodically for gross contamination. Such genetic quality control is relatively easy to perform and can be achieved with as few as 27 single nucleotide polymorphisms (SNPs) [14]. Controlling for genetic drift and testing for genetic contamination can help prevent the establishment of unwanted substrains. From a breeding perspective, substrains are strains of mice that have diverged from their parent strain for 20 or more generations (10 generations each from a common ancestor). Numerous substrains for the classical inbreds exist as a result of isolated breeding and these strains can become quite divergent with time. For example, C57BL/6J, C57BL/6N and C57BL/C substrains are significantly different in their performance on various behavioural tests, a finding which could greatly impact studies in mouse models of autism and other neuropsychiatric disorders [15]. Substrains can also occur as the result of genetic contamination. For example, the '129' family is particularly diverse, with numerous substrains across four separate genetic lineages, and with known phenotypic differences [9, 10, 16]. So, while we often use shorthand such as 'B6', '129' and 'BALB' to describe the

strains we use in experiments, genotypic and phenotypic differences do exist and it is important to note the substrain and vendor information. In one's own research colony the small amount of genetic drift due to new mutations can also be eliminated by preserving frozen embryos or replacing breeders from established commercial vendors. For the most used standard inbred strains, The Jackson Laboratory has established a genetic stability program in which strains are reconstituted from a bank of frozen embryos every five generations [13].

As opposed to outbred stocks, inbred strains have traditionally been the animals of choice for genetic research. Fewer inbred animals will be needed in an experiment to achieve a given level of statistical precision than if genetically segregating or outbred animals had been used. An inbred strain represents a single genotype that can be propagated indefinitely. The sequencing of multiple mouse genomes and increased density of SNP panels is rapidly increasing our understanding of the relationship between strains and our understanding of the genetic basis for these phenotypic differences among strains and substrains [17-19].

A brief word on nomenclature

Inbred strain nomenclature is a combination of parent strain and substrain designations. A parent strain is designated by a brief symbol made up of upper-case letters or numbers or combination of letters and numbers. Substrains include a number and Laboratory Registration Code (lab code). Inbred strain names may be derived from their coat colour, origin or a defining characteristic. For example, C. C. Little's first inbred strain, DBA, originally called dba, is named for its coat colour genes: DBA mice are homozygous for dilute ($Myo5a^d/Myo5a^d$), brown ($Tyrp1^b/Tyrp1^b$) and non-agouti (a/a) recessive mutations. C57BL and C57BR parent strains were derived from inbreeding black (BL) and brown (BR) progeny from a mating of female #57 to male #52 in Little's Line C [20]. Inbred strains also may be named for more application-based phenotypes like the non-obese diabetic (NOD) strain and the strains with high (BPH/J), normal (BPN/J) and low (BPL/J) blood pressure. Laboratory Registration Codes

following the forward slash identify substrains and indicate who created and who currently maintains that substrain, as well as the substrain's history. These Laboratory Registration Codes are issued by the Institute for Laboratory Animal Research (ILAR; http://dels.nas.edu/ilar/).

Hybrid mice

An F1 hybrid results from mating mice of two inbred strains. F1 hybrids (e.g. progeny of C57BL/6J × DBA/2J yield B6D2F1 mice) are similar to inbred strains in that they are genetically and phenotypically uniform. As long as the parental strains exist, they can be repeatedly produced. F1 hybrids are heterozygous at all of the loci at which the parental strains differ, and will therefore not breed true. The mating of two F1 hybrid mice together yields an F2 hybrid, a genetically segregating generation. F2 hybrids are widely used for genetic mapping studies, but are of less value as general research animals.

In contrast to most inbred strains, F1 hybrids display an overall hybrid vigour (i.e. increased disease resistance, better survival under stress, greater natural longevity, larger litters). Thus, they provide the advantage of genetic uniformity with more robustness than the average inbred strain. They are useful as hosts for tissue transplants from mice of either parental strain. Because of the combination of hybrid vigour with genetic and phenotypic uniformity, F1 hybrid mice are often preferred over random bred or outbred mice in a wide variety of research endeavours, including radiation research; behavioural research; and bioassays for nutrients, drugs, pathogens and hormones. They are particularly valuable as foster mothers. F1 hybrids also sometimes possess useful characteristics not normally found in the parental strains. For example, NZBNZWF1 mice are widely studied as a model of autoimmune systemic lupus erythematosus, which is not found in the parental strains [21, 22].

Some deleterious mutations (e.g. the osteopetrosis mutation, Csf^{op}) cause non-viability on an inbred background but can be maintained and provided for research by breeding mice carrying the mutation to an F1 hybrid. The progeny from

this cross are not F1 hybrids, but rather are segregating for any alleles that differ between the parental strains; a mouse may be heterozygous or homozygous for either parental allele. It has also been noted that the expression of some transgenes can be suppressed with inbreeding [23]. In these instances it is beneficial to maintain a colony by breeding to an F1 hybrid. F1 hybrids are generally less sensitive to adverse environmental conditions than inbred strains. Generally speaking, strains carrying mutations on an inbred background that produce mice with a failure to thrive phenotype may perform better when bred back to an F1 hybrid. Since F1 mice are created by mating two inbred strains, a steady supply of F1 hybrids requires the maintenance of the progenitor inbred strains.

A brief word on nomenclature

To name F1 hybrids, the standard strain abbreviations used for inbred lines listed in Table 1.3.1 are used. As is the case in representing all mouse genetic crosses, the 'ladies first' rule applies; the abbreviation of the female parent is listed first and the male parent second. Thus, B6D2F1/J mice are the offspring of a C57BL/6J female mated to a DBA/2J male, and D2B6F1/J mice are offspring of the reciprocal mating; a DBA/2J female mated to a C57BL/6J male. It is important to note the contribution of the Y chromosome carried by the males and the maternally derived mitochondrial genome contributed by the female. Thus, the same gender progeny from reciprocal F1 hybrids (D2B6F1/J vs B6D2F1/J) should not be considered genetically identical.

Traditional outbred stocks

A large number of researchers have used outbred mice in experiments when the precise genetic makeup is not considered crucial. Commonly used stocks include CD-1, Swiss Webster, Black Swiss, ICR and NIH Swiss. Most outbred stocks of mice exhibit hybrid vigour similar to or exceeding that of F1 hybrids. Compared to inbred strains, they have longer

lifespans, higher disease resistance, earlier fertility, higher overall reproductive performance and lower neonatal mortality; they are also considerably less expensive. Each animal is genetically unique and thus there is no information on the genotypes of individuals unless each animal is specifically genotyped. Phenotypic variation of outbred stocks is usually greater than that seen for inbred strains, as individuals differ due to both genetic and non-genetic factors. This means that a larger number of outbred animals are typically needed to achieve statistical validity, as opposed to using inbred animals. Using outbred mice for any experiment, including test treatments that may lead to future genetic studies (e.g. susceptibility vs resistance), can potentially decrease the value of the results and may not be cost-effective in the long term [24].

Most commercial mouse suppliers use breeding schemes that avoid crosses between closely related individuals in order to maintain a maximal level of heterozygosity in progeny of outbred stocks. However, it is a common misconception that outbred stocks of mice are more representative of the genomes of human populations. Outbred stocks are essentially closed colonies and many were originally derived from a very limited gene pool. Most outbred stocks, such as CD-1, were derived from a small number of mice and are thus more reflective of a human founder population than of outbred human populations [25]. Outbred stocks, which typically breed well, are ideally suited as stud males or foster mothers and are widely used in toxicological testing.

A brief word on nomenclature

For outbreds, the common strain root is preceded by the Laboratory Code of the institution holding the stock. For example, Tac:ICR is the ICR outbred stock maintained by Taconic Farms, Inc.

Wild-derived inbred strains

Wild-derived inbred mice are descendants of mice captured in wild populations during the

mid to late 20th century and represent several different *Mus* species from around the world. Many such strains were created as genetic mapping tools by Thomas Roderick and Eva Eicher at The Jackson Laboratory, Jean-Louis Guénet at the Institut Pasteur and Verne Chapman at the Roswell Park Cancer Institute, as well as several Japanese investigators. Examples of frequently used wild-derived strains include *Mus musculus castaneus* (CASA/Rk and CAST/Ei, from Thailand), *M. m. molossinus* (MOLC/Rk, MOLD/Rk, MOLF/Ei, from Japan), *Mus caroli* and *M. pahari* (both from Thailand), *M. hortulanus* (PANCEVO/Ei, from Serbia) and *M. spretus* (SPRET/Ei, from Spain). Inbreeding of wild mice often was commenced from one pair or trio, so these strains are not representative of genetic diversity in the wild populations. The large number of genetic differences in progeny from interspecific crosses with common inbred laboratory mice makes wild-derived inbred mice valuable tools for gene mapping, evolution and systems research [26].

Several of the wild-derived inbred strains (e.g. RBF/Dn, TIRANO/Ei and ZALENDE/Ei) naturally carry multiple robertsonian chromosomes, a fusion of two non-homologous telocentric chromosomes to form a single metacentric chromosome. Robertsonian chromosomes are useful as tissue or cell markers for chimera and transplantation studies, for producing chromosome-specific aneuploidy, and for mapping genes by fluorescent *in situ* hybridization (FISH) of gene probes.

Wild-derived mice are generally more aggressive and quicker-moving than traditional inbreds, which can make them challenging to care for; they are sometimes referred to as 'popcorn mice'. Their wild nature can be quite intimidating to animal care staff, and mice frequently escape during the cage changing process. Animal care technicians should be well trained to handle wild-derived mice and allowed enough time to work with them effectively. The majority of wild-derived mice are extremely sensitive to variations in their environment, making them challenging breeders. The key to breeding wild-derived mice successfully is to provide them with a quiet location in the mouse room, disturb them as little as possible and provide sufficient nesting material. Many new breeders can require up to 8–12 weeks after mating before producing their first litter and pups may require fostering.

A brief word on nomenclature

Wild-derived inbred strains are often given symbols that identify the species, e.g. SPRET/Ei is an inbred strain of *M. spretus* created by Eva Eicher (Ei).

Mice with chromosomal aberrations

The diploid chromosomal complement of standard inbred laboratory strains is $2N = 40$: 19 autosomes, X and Y sex chromosomes. The autosomes and the X chromosomes are telocentric (i.e. the centromere is at one end of a single-armed chromosome) while the Y chromosome is acrocentric (i.e. it has a short p arm as well as the longer q arm, the use of 'p' and 'q' being patterned on human chromosomal nomenclature). The sex-determining genes reside in the short arm of the Y chromosome.

Strains of mice whose chromosomal complement deviates from the normal chromosomal makeup are designated chromosomal aberration strains. Chromosomal aberrations can include intra- and interchromosomal rearrangements or aneuploidy. These include (i) inversions and tanspositions, rearrangements of DNA segments within chromosomes; (ii) reciprocal translocations, robertsonian chromosomes and insertions, exchanges of DNA segments between chromosomes and (iii) aneuploidy, deviations from the normal diploid number of chromosomal arms in somatic cells (e.g. trisomies). Some chromosomal deletions and duplications also may be cytologically detectable.

The B6EiC3Sn *a/A*-Ts(17[16])65Dn trisomy mouse is a widely used mouse model for studying Down's syndrome. Ts65Dn mice have three copies of most of the genes on mouse chromosome 16 that are homologues of human chromosome 21 genes implicated in Down's syndrome.

These extra genes, along with the centromere and about 5-10% of proximal chromosome 17, are contained in a small extra chromosome derived from a reciprocal translocation. The translocation was induced by irradiation of male mice of the strain DBA/2J and isolated by screening progeny for translocations involving chromosome 16 [27]. Ts65Dn mice are aneuploid descendants of females with this translocation and carry the translocation chromosome [16, 17] as a freely segregating supernumerary chromosome.

The ability to detect chromosomal aberrations cytologically makes them useful as dominant markers for linkage studies and for marking tissues in chimera and transplantation experiments. Many chromosomal aberrations are useful in FISH gene mapping and meiotic nondisjunction studies.

A brief word on nomenclature

A chromosome anomaly designation begins with a prefix that denotes the type of anomaly. Each prefix begins with a capital letter, with any subsequent letters being lower case: for example, Ts for trisomy, Del for deletion, Dp for duplication, In for inversion, etc. The chromosome(s) involved in the anomaly are indicated by adding the appropriate numbers in parentheses, between the anomaly prefix and the series symbol. The first and each successive anomaly from a particular laboratory or institution is distinguished by a series symbol, consisting of a serial number followed by the ILAR code of the person or laboratory who discovered the anomaly. For example, T(4;X)37H is the nomenclature to indicate a translocation involving chromosome 4 and the X chromosome, the 37th chromosome anomaly from Harwell.

Congenic strains

A strain is considered congenic when it differs from a particular inbred strain by a specified gene, locus or genetic region. Congenic strains were first developed in the 1940s by George Snell at The Jackson Laboratory to study the genetics of tissue graft rejection. Through continuous backcrossing, Snell was able to identify the 'resistant regions' responsible for tumor graft rejection between inbred strains [28]. Discovery of one of these genetic regions, now known as the major histocompatibility complex (MHC), also designated the *H2* locus or complex in mice, earned Snell the Nobel Prize for medicine in 1980.

Derivation of a congenic strain involves the transfer of a gene or locus from one genetic background on to the defined background of an inbred strain. Traditionally, this is done by successive backcrosses. A congenic state is achieved by backcrossing the donor allele to the recipient inbred for at least 10 generations. The donor allele is usually selected for by PCR genotyping or by phenotypic analysis. Although the generation terminology can sometimes be confusing, the creation of a congenic is straightforward in concept. The first outcross is equivalent to a F1 generation, and the contribution of each genome is 50% from the donor strain and the recipient strain. Each subsequent backcross decreases the heterogeneity of this F1 cross and increases the homogeneity of the recipient inbred genome. The fraction of loci that are still heterozygous at the Nth generation can be calculated as $[(1/2)^{N-1}]$, with the remaining fraction $[1 - (1/2)^{N-1}]$ homozygous for the inbred strain allele [3] (see Figure 1.3.1). At N5, the strain is considered to be an incipient congenic, with 94% of the genome represented by the recipient inbred. After 10 generations, the genetic background of the strain is statistically considered to be 99.8% identical to that of the recipient inbred. It is important to note that the amount of donor genome linked to the gene or locus of interest will not be reduced at a similarly statistical rate. In most congenic strains performed by traditional backcrossing, there exists some residual 'passenger' genomic segment linked to the gene transferred from the donor strain.

Many of the genetic engineering techniques used to develop new mouse models utilize ES cells of a mixed genetic background or incorporate breeding schemes that result in animals with a segregating genetic background. As a segregating genetic background often introduces phenotypic variability, it is desirable to reduce this genetic variability by backcrossing to create a congenic line. The time frame for development
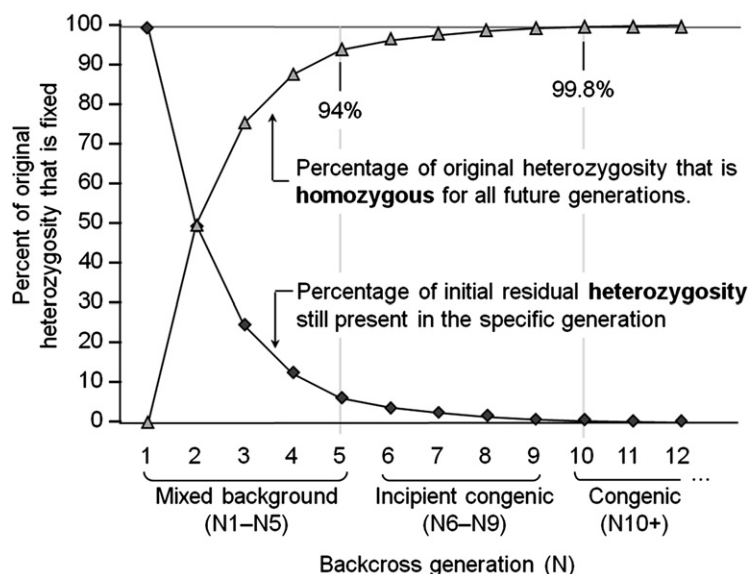
Figure 1.3.1 The effects of backcrossing on homozygosity and heterozygosity throughout the process of creating congenic mice. The symbols represent residual heterozygosity at any given generation, expressed as the percentage of the original heterozygosity in the original N1 generation [13].

of a congenic strain achieved through traditional backcrossing of 10 generations is 2.5–3 years, which is usually a major impediment to the progress of a research project. However, this time can be significantly reduced through the use of marker-assisted backcrossing, also known as the speed congenic approach [30]. By screening backcross offspring with DNA markers that are polymorphic between the donor and recipient strain, one can select for breeders at each generation that have greater homozygosity than might be expected from random selection of breeders. The speed congenic approach takes advantage of the fact that progeny following the second backcross generation (N2) have a range of genomic identities. Progeny that contain the highest percentage of the recipient genome are selected for the next round of backcrossing. A fully congenic line can usually be achieved in four to five generations, taking about 1.5 years. A simple breeding strategy accomplishes fixation of the recipient sex chromosomes. The use of at least one heterozygous female to a recipient inbred male followed by a subsequent backcross using a male carrier to a recipient inbred female ensures that both the X and Y chromosomes are 100% recipient genome.

Consomic (or chromosome substitution) strains are a variation of congenic strains, where an entire chromosome is transferred to a new recipient background by repeated backcrossing. Traditionally, most consomic strains involved transferring the Y chromosome from one strain to another (e.g. BALB/cByJ-Y^{B6By}). However, complete sets of chromosome substitution strains (CSS panels) have been generated in the mouse [31–33]. A CSS panel includes individual recipient strains that have had each of the 19 autosomes and the X and Y chromosomes replaced by that chromosome from a donor strain. CSS panels facilitate quantitative trait loci (QTL) mapping of polygenic traits that differ between two progenitor strains, such as body weight, blood pressure, etc. First, the CSS panel is screened for a phenotype of interest. If a CSS strain differs from the recipient strain, this indicates there must be at least one QTL located on the donor chromosome. In contrast, traditional QTL mapping requires an initial large-scale two-generation cross and the production of perhaps thousands of recombinant progeny, followed by development of multiple congenic strains carrying QTLs of interest. These first steps are avoided by using CSS panels. However, like traditional QTL analysis, finer structure mapping to delineate genes/loci of interest requires additional crosses. CSS panels between strains that demonstrate numerous polygenic trait differences (e.g. A/J and C57BL/6J, C57BL/6J and PWD) have widespread utility.

A brief word on nomenclature

Many strains are maintained on a mixture of C57BL/6 and 129 genetic backgrounds (e.g. B6;129-$Trp53^{tm1Tyj}$) because 129-derived ES cell lines are commonly used in gene targeting and chimeric mice are mated to C57BL/6 to determine germline transmission. Mutations transferred from a mixed to an inbred background by repeated backcrossing are designated using congenic nomenclature. For example, B6.129S2-$Trp53^{tm1Tyj}$ indicates that the $Trp53^{tm1Tyj}$ mutation induced in the D3 ES cell line derived from the 129S2 strain and was subsequently backcrossed to the C57BL/6J inbred strain for at least five generations. Care must be taken in deciphering symbols used in strain nomenclature; the semicolon used to denote a mixed background versus a period used to denote a congenic background is a subtle but critical distinction. In the former, the strain background is a mix of alleles from the two designated parental strains; in the latter, the genetic background contains primarily host strain alleles.

If the donor strain is not inbred, or the genetic difference is complex, the symbol Cg should be used to denote the donor strain, e.g. B6.Cg-$Foxn1^{nu}$/J, the $Foxn1^{nu}$ mutation was first recorded by Dr Grist at the Virus Laboratory, Ruchill Hospital, Glasgow, Scotland. The mutation arose in a mouse stock that was closed but not inbred. The first seven backcrosses onto BALB were made using BALB/cN. It was bred brother (nu/nu) by sister ($nu/+$) for three generations before adopting the pattern of crossing a nu/nu male to a C57BL/6J female every other generation. The genetic complexity of the donor strain warrants the Cg designation. The use of Cg indicates that alleles in the strain name came from more than one source. Parentheses may also be used to show that an inbred, or congenic strain may have a minor contribution from one other strain, e.g. C.129P(B6)-$Il2^{tm1Hor}$, a targeted mutation created in a 129 ES cell line and transferred from a B6;129P mixed background to BALB/c (C).

The generic designation for consomic strains is HOST STRAIN-Chr #[DONOR STRAIN]. For example, C57BL/6J-Chr 7[PWD/Ph]/ForeJ. In this consomic mouse strain, chromosome 7 from the PWD/Ph strain has been backcrossed onto C57BL/6J.

Recombinant inbred strains, recombinant congenic strains and advanced intercross lines

Recombinant inbred (RI) strains are derived by systematic inbreeding from a cross of two distinct inbred strains. Donald Bailey and Ben Taylor first developed and characterized them at The Jackson Laboratory [34, 35]. Most RI strain sets result from randomly mated pairs of F2 mice followed by at least 20 generations of inbreeding. Each strain within a RI set is equally likely to have inherited either the maternal or paternal progenitor strain allele at each autosomal locus. Since alleles of unlinked loci are randomized in the F2 generation, parental and recombinant allelic combinations of unlinked loci should be fixed with equal probability in RI strains (see Figure 1.3.2). Linked genes will tend to remain linked and will become fixed in parental combinations in the strains of an RI set at frequencies

Recombinant inbred (RI) lines
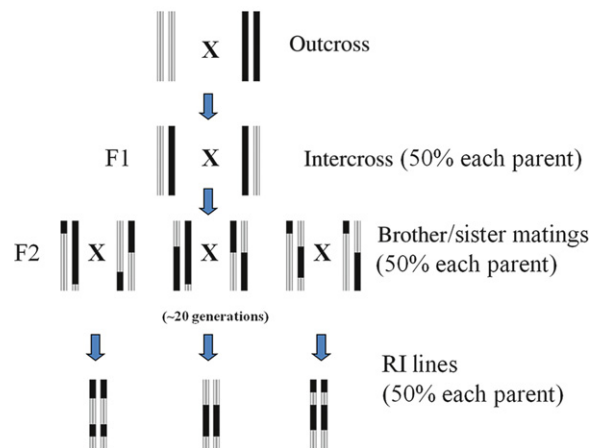
Figure 1.3.2 Creation of a recombinant inbred (RI) line. An RI line begins with the outcross of two inbred lines to create an F1 hybrid. Sister × brother mating creates an F2 generation of segregating alleles. Further inbreeding up to 20 generations creates a panel of genetically unique combinations of alleles from the parental genomes.

directly proportional to the genetic distance between them. Recombinant congenic (RC) strains are a variation on RI strains. Following the initial outcross of mice of two inbred strains, F1 hybrid progeny mice are backcrossed to mice of one of the parental strains for 1 or 2 generations prior to sibling mating for at least 14 generations [36]. In contrast to RI strains that are an approximately 50:50 mixture of the progenitor strain genomes, the genomes of RC strains will be derived predominantly from one parent (the proportion depending upon the number of backcross generations before inbreeding). Sets of RC strains (e.g. NONcNZO1–NONcNZO10) have unique characteristics and are valuable for dissecting polygenic diseases such as type 2 diabetes [37].

RI strains are important resources for genetic mapping of both Mendelian and quantitative traits in the mouse. Since RI strains are inbred and each strain has a unique genotype, RI strains have a number of advantages over F2 or backcross mouse populations as tools for mapping genes or QTL. Genetic and phenotypic data acquired for an RI strain set are both cumulative and comparable, enhancing the value of the strain set for further studies. Phenotypic data can be collected in different laboratories, at different ages, and under different environmental conditions and stored in databases for comparison. The progenitor strains of RI lines often differ in key physiological traits. For example, the 'large' (LG/J) and 'small' (SM/J) inbred mouse strains differ for a wide variety of traits related to body size and obesity. The LGXSM RI strain panel consists of 19 RI lines and provides resource for mapping the genetic basis of complex traits related to obesity and diabetes [38]. The ILSXISS recombinant inbred strains is a significantly larger RI set consisting of more than 60 lines, which are used to study the genetics of neurogenetic, neuropharmacological and behavioural phenotypes involved in alcohol-related traits [39]. Most RI panels, including the widely used original BXD RI set developed by Taylor, typically consist of fewer than 30 lines. A small number of RI strains in a mapping panel reduces the power and precision of mapping quantitative trait loci. Recently, Williams et al. set out to address these limitations for the BXD set by creating a BXD RI mapping panel from two independent advanced intercross

lines (AIL) [40]. In general, AILs are initiated in the same way as RI strain sets; however, mice of the F2 generation and each subsequent generation are intercrossed by avoiding sibling matings [41]. In the BXD AIL, progeny were intercrossed for 9-14 generations before initiating inbreeding. Since the starting AIL population is highly recombinant, the 46 advanced recombinant inbred strains incorporate approximately twice as many additional recombination events as standard RI strains, significantly adding to the power of the BXD RI lines as a whole. A major advantage of expanding the BXD strain set is that both progenitors have been sequenced, and approximately 1.8 million SNPs have been characterized [40].

A brief word on nomenclature

RI strains are written similarly to F1 hybrids but are distinguished from hybrids by the inclusion of an 'X' in the symbol and single-letter strain abbreviations, e.g. BXD1/Ty is the first of a series of RI strains created from C57BL/6J and DBA/2J by Benjamin Taylor (Ty). RC strains are designated like RI strains except that a lower-case 'c' is inserted between the strain abbreviations. The host strain symbol is followed by the donor strain symbol, e.g. NONcNZO1 is the first in a series of RC strains in which NON × NZO F1 mice were backcrossed to NON mice prior to inbreeding [37].

The Collaborative Cross

Both the BXD RI lines and the ILSXISS recombinant inbred strains have been made available to the scientific community through The Jackson Laboratory. One shortcoming of the RI lines is their lack of genetic diversity. AXB/BXA and BXD, the two most commonly used mouse RI panels, capture only 13% of the known genetic variation in laboratory mice, a problem for system genetics that is addressed in the Collaborative Cross (CC) [42]. The CC represents an ongoing effort by the Complex Trait Consortium to develop a common RI reference panel specifically designed for the integrative analysis of

complex systems [42, 43]. The CC is intended to create over 1000 inbred lines and is designed to maximize genetic diversity by combining genomes of eight genetically diverse founder strains: A/J, C57BL/6J, 129S1/SvImJ, NOD/LtJ, NZO/HlLtJ, CAST/EiJ, PWK/PhJ and WSB/EiJ. The progenitors are first crossed pairwise to make all 56 possible parents. A set of possible four-way crosses was performed, keeping Y chromosome and mitochondrial balance. Finally, all eight genomes are brought together, and the offspring of this cross are inbred [44]. The autosomal genomes of each line will theoretically have equal contributions from each founder strain, and the recombination that accumulates during the breeding process will be independent between lines. The average distance between CC recombinations is estimated to be approximately 12 Mb, with a QTL mapping resolution of 1 Mb, which is a much greater mapping resolution than seen today in conventional mouse crosses [45]. The founder strains for the CC include three wild-derived strains: CAST/EiJ, PWK/PhJ and WSB/EiJ. The wild strains contribute 75% of the genetic diversity of the CC and, along with five other inbreds, the CC captures nearly 90% of the known variation present in laboratory mice [43].

The CC is being developed in three locations around the world. The original strains first developed in the United States at Oak Ridge National Laboratory have now been relocated to the University of North Carolina. The other two locations are Tel Aviv University in Israel and the Western Australian Institute for Medical Research. It is estimated that 40 CC lines will be fully inbred and available by the end of 2011 and an additional 100 CC strains will be completed by the end of 2012. The initial research applications demonstrating the utility of the CC are just starting to be published [1, 46, 47].

The CC lines, prior to being inbred, are also the starting material for the Diversity Outbred (DO) mice currently being developed at The Jackson Laboratory. The DO are a heterogeneous stock of mice which was produced by a novel outbreeding strategy that maintains a balance of founder genomes and avoids allelic loss and inbreeding [48]. A drawback of the DO, as with all outbreds, is that each animal is genetically unique and thus not reproducible. However,

combinations of genetic loci that are discovered in the DO mice using the high-density SNP platforms may be able to be replicated in CC strains or in their reproducible F1 progeny. The Mouse Diversity Array described by Churchill, Fernando Pardo-Manuel de Villena and colleagues was designed to capture the full spectrum of genetic diversity present in current stocks of laboratory mice, including classical and wild-derived inbred strains contained in the CC. The array-based hybridization platforms allow simultaneous genotyping of many SNPs [49]. In this regard, the CC and DO populations can be used together and may prove to be a powerful mapping tool for gene discovery and complex trait analysis.

Mutant mice: spontaneous mutations, transgenes and targeted mutations

Spontaneous mutations

A large number of mouse models are the result of spontaneous single gene mutations. Many of these occurred within The Jackson Laboratory's large production breeding colonies and have been developed into models by the laboratory's Mouse Mutant Resource Program or by members of the research staff [50]. Many mouse mutations (both spontaneous and induced) have come from the radiation/chemical risk assessment programmes at the Oak Ridge National Laboratory in the USA and the Medical Research Council Genetics Programme at Harwell in the UK.

Traditionally, the detection of spontaneous mutations in an animal colony has been limited to alterations of observable phenotypes. These include mutations that cause changes in coat colour (e.g. yellow, leaden), growth defects (e.g. dwarf, pigmy), abnormal morphology (e.g. limb deformity, legless), or alterations in behaviour or motor coordination (e.g. ataxia, circling). Large-scale phenotypic screening for desired

traits that are not easily observed or measured is time-consuming and not cost-effective, given the rarity of spontaneous mutations.

Mice carrying spontaneous mutations provide a rich source of animal models for human genetic diseases. Spontaneous mutations have a unique advantage over targeted mutations in that they are often not a complete loss-of-function mutation but rather mimic the subtler missense mutations in naturally occurring human inherited diseases [51]. Spontaneous mutations are identified on the basis of a biomedically relevant phenotype first and the gene identified by reverse genetics later, whereas the phenotype of targeted mutations cannot be accurately predicted. Table 1.3.2 lists some examples of mouse models of human disease developed and/or currently maintained at The Jackson Laboratory whose underlying genes have been identified. Because of the high degree of gene conservation between the mouse and human genomes, such models are valuable for identifying human disease genes.

A brief word on nomenclature

Spontaneous mutations are alleles of initially unknown genes and are given allele names and symbols based on their phenotype (e.g. diabetes, *db*). Recessive mutations (i.e. requiring two copies of the mutated allele to manifest the phenotype) are represented by all lower-case letters while dominant (i.e. one or two copies of the mutated allele produces the phenotype) and semidominant (i.e. one mutant allele produces an intermediate phenotype) spontaneous mutations are represented by an upper-case first letter, followed by lower-case letters. Once the gene responsible for the mutant phenotype has been identified, the allele symbol is superscripted to an approved gene symbol (e.g. the diabetes mutation is a point mutation in the leptin receptor gene, $Lepr^{db}$). The Mouse Genomic Database Nomenclature Committee approves and assigns gene names and symbols, which may be registered online (http://www.informatics.jax.org) or requested by email (nomen@informatics.jax.org). Gene names and symbols may change as the function of a gene is better understood or to better correspond with gene symbols of other species (primarily human).

Induced mutations

The generation and use of mice carrying induced or genetically engineered mutations has increased over the past decade. Random mutagenesis protocols, such as treating mouse gametes or ES cells with chemical mutagens [52] and gene trapping [53, 54], are frequently used to drive high-throughput mutagenesis screens [55-57]. Random mutagenesis produces both dominant and recessive mutations. To obtain maximum value from random mutagenesis approaches, rapid and systematized protocols for phenotypic screening, such as the SHIRPA system, were developed [58]. Increasing the mutation frequency by chemical mutagenesis, such as ethylnitrosourea (ENU), when coupled with screening protocols, enables the detection of mutations that cause subtle phenotypes to model specific categories of diseases [59]. Several large-scale ENU mutagenesis projects have been completed [60-62].

Transgenes and targeted mutations

Two broad areas of technology—transgenesis and targeted mutagenesis using homologous recombination—are currently used to create genetically engineered strains of mice (see Chapter 1.5, 'Generation of Mouse Mutants by Genotype-Driven Mutagenesis', for more information on these technologies).

Transgenic mice have genetic material randomly added to their genomes [63]. Thousands of transgenic strains have been used to study gene function and expression and have resulted in many important disease models. Since transgene insertion is a random event, the phenotype of the mouse may vary depending on the site of integration and/or the copy number of transgenes integrated. Transgene integration may cause disruption in an endogenous gene (insertional mutation), creating an inherited phenotype (usually recessive) unrelated to transgene expression. In these cases the transgenic animal provides a vehicle for gene discovery through the mapping and subsequent cloning of the disrupted gene (e.g. the pygmy locus was identified as an allele of the high-mobility group AT-hook 2 (*Hmga2*) gene as

TABLE 1.3.2: Selected cloned mouse genes with homologous human disorders

Gene/Allele symbol	Chr	Allele name	Gene name	Reference	Human orthologue	Human map location	Human disorder (OMIM number[a])
Ar<Tfm>	X	Testicular feminization	Androgen receptor	Charest et al. 1991	AR	X (q11.2–q12)	Androgen insensitivity syndrome (AIS) #300068
Galc<twi>	12	Twitcher	Galactosylcer amidase	Sakai et al. 1996	GALC	14 q31	Krabbe's disease #245200
Ghrhr<lit>	6	Little	Growth hormone releasing hormone receptor	Godfrey et al. 1993	GHRHR	7(p15–p14)	Growth hormone deficiency, isolated *139191
Gus<mps>	5	Mucopoly-saccharidosis VII	Beta-glucuronidase	Sands and Birkenmeier 1993	GUSB	7 q22	Mucopolysacc-haridosis type VI *253220
Hps4<le>	5	Light ear	Hermansky–Pudlak syndrome 4 homolog	Suzuki et al. 2002	HPS4	22 q11.2–q12.2	Hermansky–Pudlak syndrome (HPS) *606682; #203300
Lep<ob>	6	Obese	Leptin	Zhang et al. 1994	LEP	7 q32.1	Obesity, leptin deficiency, hypogonadism *164160
Lepr<db>	4	Diabetes	Leptin receptor	Chen et al. 1996	LEPR	1 p31	Obesity, morbid, with hypogonadism *601007

Pit1<dw>	16	Dwarf	Pituitary specific transcription factor 1	Li et al. 1990	POU1F1	3p11	Pituitary hormone deficiency (CPHD) #173110
Pou4f3<ddl>	18	Dreidel	POU domain, class 4, transcription factor 3	Frankel et al. 1999	POU4F3	5 q31	Deafness, autosomal dominant non-syndromic sensorineural 15 (DFNA15) *602460, #602459
Rab27a<ash>	9	Ashen	RAB27A, member RAS oncogene family	Wilson et al. 2000	RAB27A	15 (q15−q21.1)	Griscelli syndrome #214450
Tgn<cog>	15	Congenital goiter	Thyroglobulin	Kim et al. 1998	TG	8 (q24.2−q24.3)	Goitre, familial, with hypothyroidism AR *188450
Tnfsf6<gld>	1	Generalized lymphoproli-ferative disease	Tumor necrosis factor (ligand) superfamily, member 6	Takahashi et al. 1994	TNFSF6	1 q23	Autoimmune Lymphoproliferative syndrome (ALPS), type 1B *134638, #601859

[a] An asterisk (*) before an OMIM number means that the phenotype determined by the gene at the given locus is separate from those represented by other asterisked entries and that the mode of inheritance of the phenotype has been proved (in the judgment of the authors and editors). In general, an attempt has been made to create only one asterisked entry per gene locus.

a result of an insertional mutation caused by a human globin transgene [64].

A brief word on nomenclature

Transgenes are designated by Tg, followed by a designation for the DNA insert in parentheses (preferably the gene symbol), then a number indicating the founder line and finally a lab code. Transgene symbols are not italicized. For example, Tg(CD8)lJwg is a transgene containing the human *CD8* gene, the first transgenic line using this construct, described by the laboratory of Jon W. Gordon (Jwg). The promoter also may be designated within the parentheses to clarify the transgene expression pattern: Tg(Zp3-cre)3Mrt designates the cre transgene with a *Zp3* promoter, the third transgenic line from the laboratory of Gail Martin (Mrt).

Targeted mutations are created using homologous recombination to alter or replace a specific locus or gene [65-67]. Many of the earlier strains developed by gene targeting were engineered to carry a null mutation. Increasingly, however, conditional targeted mutations are created that allow control of both the tissue specificity of the mutation [68-70] and the temporal onset of gene expression [71, 72]. Gene targeting produces strains used to study gene function and to create models for human genetic diseases for which the offending gene is known. Transgenesis and targeted mutagenesis technologies often produce unexpected results, creating mice with either no observable phenotype or an unexpected phenotype, one outside the researcher's area of expertise or interest. Thus, this gene-based approach may lead to the discovery of novel pathways for an already-known gene. The various techniques of gene targeting and the mice they are capable of producing fall beyond the scope of this chapter. However, large-scale gene targeting projects such as the Knockout Mouse Project (KOMP) REF and the International Knockout Mouse Consortium (IKMC), along with the high-throughput phenotyping that will accompany these mice, will create a number of conditional and knockout lines that will no doubt prove to be an invaluable resource [73].

Targeted alleles of genes are designated by the approved gene symbol followed by a superscript containing tm (for targeted mutation), an allele number, and the lab code (all in italics). For example, *Apoa1^{tm1Unc}* represents the first targeted mutation in the apolipoprotein AI (*Apoa1*) gene made in the laboratory of Nobuyo Maeda at the University of North Carolina at Chapel Hill (UNC) [74]. Other more complex forms of gene replacement, such as partial knockins and Cre-Lox recombination events are not conveniently abbreviated and should be given a conventional tm#Labcode superscript. Although some alterations made in a gene appear to lend themselves to a simple naming convention, details of the targeted locus should be given in associated publications and database entries. For a more detailed review of strain nomenclature, see 'Guidelines for Nomenclature of Genes, Genetic Markers, Alleles, and Mutations in Mouse and Rat' (http://www.informatics.jax.org/mgihome/nomen/gene.shtml#gtm).

Conclusion

Tremendous accomplishments in mouse genetics were made during the 20th century [75, 76]. The major advances (see 'Historical Foundations', Chapter 1.2) were accompanied by a rapidly increasing number of mouse strains, stocks and mutants available to the biomedical research community. Beginning with the creation of the first inbred strain in 1909, the number and different types of mouse models has increased from numbering in the dozens to the current exponential growth and generation of thousands in the last 20 years. Global information regarding the phenotypes of spontaneous and genetically engineered mice can be found at Mouse Genome Informatics (MGI) (http://www.informatics.jax.org). MGI is the most comprehensive source for information on the laboratory mouse, providing integrated data on genes, phenotypes, gene expression, gene function, biological pathways, strain and SNP data, as well as information on orthology.

The large number of genetic resources continuously being generated by coordinated efforts has created a need for centralized repositories for the purpose of both archiving and distribution. A number of individual and consortium-based groups generate and distribute mice

TABLE 1.3.3: Listing of consortiums and repositories for generating and distributing mice and genetic resources

International Mouse Strain Resource (IMSR)*	http://www.findmice.org
The Jackson Laboratory Mouse Repository (JAX)	http://jaxmice.jax.org
Mutant Mouse Regional Resource Centers (MMRRC)	http://www.mmrrc.org
Mouse Models of Human Cancer Consortium (MMHCC)	http://web.ncifcrf.gov/researchresources/mmhcc/
International Knockout Mouse Consortium (IKMC)	http://www.knockoutmouse.org
Knockout Mouse Project (KOMP) Repository	http://www.komp.org
Canadian Mouse Mutant Repository (CMMR)	http://www.cmmr.ca
European Mouse Mutant Archive (EMMA)	http://www.emmanet.org
European Conditional Mouse Mutagenesis Program (EUCOMM)	http://www.eucomm.org
Texas A&M Institute of Genomic Medicine (TIGM)	http://www.tigm.org
German Gene Trap Consortium (GGTC)	http://genetrap.helmholtz-muenchen.de
International Gene Trap Consortium (IGTC)	http://www.genetrap.org
Japan Mouse/Rat Strain Resources Database	http://www.shigen.nig.ac.jp.ezproxy.jax.org/mouse/jmsr/top.jsp
RIKEN BioResource Center	http://www2.brc.riken.jp/lab/animal/search.php
Taconic Knockout Repository	http://kodatabase.taconic.com/database.php

* The IMSR is a database for publicly available holding across individual repositories. The goal of the IMSR is to assist the international scientific community in locating and obtaining mouse resources.

(see Table 1.3.3). The majority of these mouse distributors register their holdings with the International Mouse Strain Resource (IMSR). The IMSR is a searchable online catalogue of mouse strains and stocks available worldwide, including inbred, mutant and genetically engineered mice maintained as breeding stock, cryopreserved embryos and gametes, and ES cell lines. The goal of the IMSR is to assist the international scientific community in locating and obtaining these resources from individual repositories.

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Mouse Genomics

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Introduction

The laboratory mouse has been used as a privileged model organism since the early days of genetics, more than a century ago. Over the past decade our knowledge of mouse genetics has changed dramatically, particularly with the recent availability of the complete genome sequence and the development of high-throughput mutagenesis programmes (both gene- and phenotype-driven). Partial or complete genome sequences from several inbred strains are now available, allowing for the discovery of millions of single-nucleotide polymorphisms (SNPs) that will contribute to the development of modern quantitative genetics, especially when used in combination with large panels of recombinant inbred strains like the Collaborative Cross. Similarly, the massive production of point mutations by use of chemical mutagenesis associated with comprehensive and standardized

phenotyping of mutant mice will certainly help with the annotation of many mouse genes. Next-generation sequencing systems should considerably accelerate the identification of the genes responsible for these phenotypes by use of positional cloning strategies. As part of the gene-driven approach, large-scale projects like the International Knockout Mouse Consortium (IKMC) envision generating mutant alleles for all protein-coding genes in the mouse, using a combination of gene targeting and gene trapping in embryonic stem cells. This endeavour will be complemented by other systems, such as gene-driven chemical mutagenesis. The availability of these new mutant alleles will also facilitate the comprehensive analysis of gene functions. This chapter is an overview of the past achievements and recent progress in mouse genomics, with a focus on the current knowledge of the structure of the mouse genome and the functional annotation of mouse genes.

Structure of the mouse genome

The mouse genome sequence

The mouse genome was the second mammalian genome to be entirely sequenced [1]: a draft sequence was released only a few months after the draft sequence of the human genome was made public [2, 3] and just a couple of years before the publication of the rat genome sequence [4]. Making this sequence publicly and freely available to the community through the internet must be regarded as a major event in the history of mouse genetics because it provided direct access to the blueprint of a living creature that is relatively close to our own species, allowing for the identification of similarities and differences. In turn, it became possible to gather a wealth of invaluable information about genome evolution and gene function at the molecular level. As Professor Waterston and his colleagues said in the conclusion of their seminal paper:

> The mouse provides a unique lens through which we can view ourselves [...]. With the availability of [its] sequence, it now provides a model and informs the study of our genome as well [1].

The sequencing of the rat genome has also been very important because it allowed making three-way comparisons with the human and mouse genomes, providing details about mammalian evolution on a relatively short time-scale (around 13 million years). In this first section of the chapter we focus on the structural characteristics of the mouse genome with frequent references to the human and rat genomes.

How was the mouse genome sequenced?

A mammalian genome is considerably bigger than a bacterial genome. Just to give an idea, the genome of the bacterium *Escherichia coli* K-12, the favourite organism for bacterial geneticists, consists of 4 639 221 base pairs (bp). In contrast, the mouse haploid genome is composed of around 2.7 billion bp, according to recent estimates (i.e. approximately 585 times bigger than that of *E. coli* K-12). To give a more tangible idea of the size of the mouse genome, we computed that, if its sequence were printed in a single line using the 12-point Courier font, the length of this line would be roughly equivalent to the distance from Paris to Montreal!

Mammalian genomes are also very complex entities. For example, if we take into account the fact that there are around 25 000 genes in a mouse genome (this estimate will be discussed in detail later) and 4400 genes in *E. coli*, this indicates that the gene density in the mouse is much lower than in the bacterium. This also means that a large amount of mammalian DNA is not 'genic', and, accordingly, sequencing it might appear a waste of time.

In addition to its large size and low gene density, geneticists have observed that repeated sequences of various types are extremely numerous in mammalian genomes, as we will explain later in this chapter. Considering all these issues, one can imagine how difficult, not to say ambitious, it was to embark on the sequencing of an entire mammalian genome. In spite of these difficulties, the decision was taken to systematically and comprehensively sequence the mouse genome and this, we think, was a very wise decision. An accurate knowledge of the genome of this species is so important for the progress of biology that it is likely that it would have been sequenced sooner or later, although probably in a rather disorganized manner, in small sections, with many redundancies and gaps, and at a greater cost for the community. It was also a very democratic decision because laboratories that do not have easy access to sequencing facilities can now use this public resource for designing optimized experiments. Finally, if we consider the number of scientific papers that have been published since the release of the initial draft sequence, there is no doubt that the community has greatly benefited from the mouse genome sequencing effort.

There are basically two strategies for sequencing an entire mammalian genome. The first one, known as hierarchical shotgun sequencing (HSS), makes use of cloned DNA sequences, with large inserts such as bacterial artificial chromosomes (BACs), P1 phages or, less

frequently, yeast artificial chromosomes (YACs). These clones need to be assembled in a series of overlapping DNA segments, known as contigs (from contiguous DNA segments), which altogether make up a high-resolution physical map of a chromosomal segment. The cloned DNAs are chosen from those that have been thoroughly checked for integrity, rejecting those that are chimeric or carry deletions (a situation that is quite common in YACs but less common with BACs). The assembly of these cloned DNAs is achieved by careful fingerprinting, as explained elsewhere [5]. When the contigs are established, in general from several individual clones ranging from 200 to 1000 kbp, a subset of minimally overlapping clones is chosen and each of its elements is sequenced several times to minimize errors (this minimal set is sometimes known as the 'Golden Path'). The primary sequence is called a 'read' and the released genome sequence results from the integration of several independent reads (in general six or seven, sometimes more). With this number of independent reads the percentage of sequencing error is very low, in general less than one error per 10^5 bp. This feature is very important, as we will discuss later.

The HSS strategy is slow but it is systematic and reliable. The use of clones with large DNA inserts is also a way to bypass, at least to a certain extent, the problems associated with the repeats and copy number variations. The HSS strategy has the disadvantage that only cloned DNA can be sequenced.

A second strategy called whole-genome shotgun (WGS) is radically different from HSS. The first step of this strategy consists of the mechanical fragmentation (e.g. by sonication) of the mammalian DNA in segments measuring 100–400 bp, which are then sequenced from both ends using the chain termination method. Multiple reads for the target DNA are obtained by performing several independent rounds of this fragmentation, each followed by sequencing. Computer programs are then used to put the pieces of the puzzle together, arranging the individual fragments into contigs, then in super or hypercontigs and finally in ultracontigs based on the overlapping sequences of the different reads (Figure 1.4.1). The WGS method is fast because it does not require the pre-existence of a physical map. Unfortunately, it does not allow the sequencing of certain genomic segments

Figure 1.4.1 Whole-genome sequencing strategy. An illustration of the whole-genome sequencing strategy (WGS) that has been used for sequencing the mouse genome. The first step consists of the mechanical fragmentation of nuclear DNA samples to obtain a mixture of independent, randomly cut, 100–400 bp-long stretches. These stretches are then cloned, using adaptors, labelled, and then sequenced end-to-end several times to minimize the sequencing errors. In the third step sequences overlapping are looked for by using appropriate computer software and the clones are then arranged in a head-to-tail manner to form contigs of non-redundant top-level sequences, whose sizes are constantly growing as new sequence becomes available. In a final step the contigs are aligned to the specific chromosome they belong to. The process is generally repeated several times to minimize the number and size of the unsequenced regions. Even if it is generally necessary to use another strategy to complete the sequence (see text for explanations), the WGS strategy is well adapted to the rapid sequencing of a genome, especially when it makes use of the new sequencing methods.

such as highly repeated regions. Combining the two strategies, HSS and WGS, allows the correction of almost all these imperfections. In short, the two strategies are to some extent complementary: WGS provides a rapid and better coverage early in a project, while HSS is more systematic and more efficient for the sequencing of regions with repeated sequences. The human genome was sequenced by using mostly the HSS strategy, while the mouse and all other mammalian genomes were sequenced mostly by using the WGS strategy with the help of HSS for some regions [1, 4, 6, 7].

The latest assembly released by the Mouse Genome Sequencing Consortium (MGSC) (NCBI build 37-27, May 2011) has a length of 2 716 965 481 bp, of which about 99% is finished with less than one sequencing error per 10^5 bp. All the chromosomes are entirely sequenced including X, Y and even the mitochondrial DNA, allowing comparisons with homologous regions of the human and other mammalian genomes to be performed at a very high resolution. Such comparisons, revealing similarities and differences, are a rich source of information. Similarities, as we shall discuss later, allow us to detect regions that are under selective pressure and which, for this reason, have remained unchanged or nearly so for several millions of years, indicating that they are presumably genetically important and, accordingly, have resisted random drift. Differences at the sequence level may be even more interesting a priori, because they may contain keys explaining how speciation proceeds. Some genes are present in one species but absent in others. It is obviously interesting to know which mechanisms led to this situation and what are the consequences of this difference. For example, no homologous genes have been identified in the mouse and rat genomes for human interleukin 8 (*IL8*) (Figure 1.4.2).

The mouse sequencing project was undertaken by the MGSC, an organization that consisted originally of three laboratories: the Whitehead Institute for Biomedical Research Massachusetts Institute of Technology (USA), the Washington University Genome Sequencing Center (USA) and the Wellcome Trust Sanger Institute (UK). The project is almost finished, even if molecular biologists in these laboratories keep working at its integral completion in some

regions [8]. Based on discussions with the scientific community at large, MGSC investigators decided to sequence first the genome of a female mouse from the C57BL/6 inbred strain. At almost the same time four other inbred strains (A/J, DBA/2J, 129X1/SvJ and 129S1/SvImJ) were included by Celera in another independent WGS project [9-11]. Here again, interstrain comparisons have been of great interest when matched with particular phenotypes (see 'Mouse Phenome Project'). The Mouse Genomes Project from The Wellcome Trust Sanger Institute is currently sequencing the genomes of an additional 17 inbred mouse strains: 129P2, 129S1/SvImJ, 129S5, A/J, AKR/J, BALB/cJ, C3H/HeJ, C57BL/6NJ, CAST/EiJ, CBA/J, DBA/2J, LP/J, NOD/ShiLtJ, NZO/HiLtJ, PWK/PhJ, SPRETUS/EiJ and WSB/EiJ.

All future genome sequencing projects will benefit from the new, ultra-efficient sequencing technologies [12], even if the development of bioinformatics capabilities for the interpretation of the overwhelming amount of data remains a major challenge. In the future it is likely that many individual mouse genome sequences from the offspring of some specific crosses will be available, contributing efficiently to the analysis of complex traits. As a proof, the first complete genome of an individual (none other than James D. Watson himself!) was completed in 2008 [13], and complete genomes from several individuals (including a family of four) from different ancestries are now available [14-16].

What is the mouse genome made of?

We mentioned above that the size of the genome of a C57BL/6 inbred mouse is close to 2.7 Gbp (the genome of the inbred rat strain BN has a similar size), roughly 14% smaller than the human genome (approximately 3.1 Gbp). The explanation generally offered to account for this relatively important difference is a higher rate of deletion in the mouse lineage [1]. Again, this indicates that the mammalian genomic DNA is probably made up of a variety of sequences of varying importance. This observation had already been made 25 years ago by

Figure 1.4.2 The interleukin-8 (*Il8*) gene is missing in the mouse and rat genomes. The figure shows a representation of the region of human chromosome 4 where the *IL8* gene is located, along with the homologous regions in mouse chromosome 5 and rat chromosome 14. The absence of a homologous gene for human *IL8* in the mouse and rat genomes can be clearly seen. It is also interesting to note the inversion of this region in rat chromosome 14, when contrasted with the human and mouse homologous regions. The images are from the *Ensembl* Genome Browser database (August 2011).

cytogeneticists who found that some large chromosomal deletions (i.e. visible through the optical microscope) were still compatible with a normal phenotype in homozygous mice. Below we briefly review the different kinds of DNA sequences that are found in a mouse genome.

Genes, gene families and pseudogenes

Among the motivations for sequencing the entire genome of a given species, the idea of making a complete inventory of its genes is of course top ranked. This is a real challenge, however, because, as mentioned earlier, these genes are dispersed in an ocean of puzzling DNA sequences. In spite of all these difficulties, this inventory has been undertaken and its

completion is in progress in many laboratories. It is typically achieved in two successive steps. First, candidate genes are searched by looking for DNA sequences with a very high degree of preservation across species and containing open reading frames. The rationale is that such sequences are preserved because they are transcribed and translated into functional proteins. In another words, similar structural features imply similar functions and accordingly a tendency to be preserved through evolution. Second, once identified through their sequence, these candidate genes are then validated by all possible means. This second step, known as 'gene annotation', is the assignment of known or predicted biological functions. This is much more difficult than the previous step and the reason why gene characterization is far from

being completed in any mammalian species. Nonetheless, the number of fully annotated genes is slowly but steadily increasing.

Using sophisticated computer software, it has been established that approximately 5% of the mouse genomic DNA can be classified as highly preserved sequences. Of this 5%, the protein-coding sequences represent no more than 1.5% of the total DNA. The more recent estimates are 1.27% for the mouse genome and 1.0% for the human genome [8]. The other 3.5% consists of sequences whose function is only partially known (Figure 1.4.3). An important proportion of these sequences is involved in regulation of gene expression (e.g. DNA binding sites), chromosome architecture and folding and binding to the mitotic spindle. An example of such highly conserved DNA sequences is the so-called TATA box, which is part of the promoter sequence of most eukaryotic genes. Still, a large proportion of the genome has sequences with unknown function, but these regions are now being investigated carefully. Interestingly, some of these sequences have been eliminated completely in mice, yielding no significant phenotypic differences [17].

Up to July 2011, with the above-described strategy, geneticists have identified 29 190 genes with nucleotide sequence data, of which 24 840 are validated with protein sequence data. This information is reliable and makes sense when compared with other species. Of these genes, only 14 463 have been functionally annotated either by the existence of (at least) one mutant allele or by an expression assay. Annotation of the mouse genome is proceeding (although slowly), thanks in part to the thousands of mutations that have been induced randomly, either with the powerful chemical mutagen *N*-ethyl-*N*-nitrosourea (ENU) or by targeted mutagenesis in embryonic stem (ES) cells (see below). All these mutations, associated with sophisticated phenotyping protocols, have turned out to be of great help. Since gene annotation is universal, we now understand from a different point of view why sequencing at least three mammalian genomes (human, mouse and rat) was so important. Once fully annotated in one species, a gene has a great chance of being validated in the list of genes of other species. For example, we know that about 99% of mouse genes have a homologue (in this case an orthologue) in the human genome. There
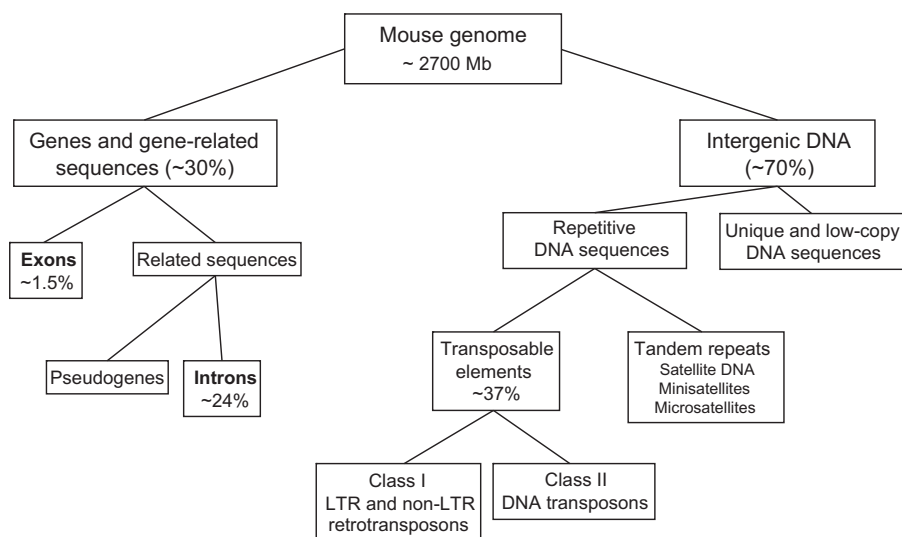
Figure 1.4.3 Types of DNA in the mouse genome. The graphic shows the different types of DNA sequences present in all mammalian genomes. It is estimated that only around 30% of the genome is represented by genes (protein-coding sequences) and gene-related sequences (e.g. introns, regulatory sequences, and pseudogenes). On the other hand, the so-called intergenic DNA constitutes up to 70% of the genome. This non-coding DNA corresponds to different categories of repetitive and transposable sequences, together with single-copy and low-copy-number sequences (see text for details). To a great extent, this DNA (inaccurately referred to as 'junk' DNA) has no known biological function; however, many non-coding DNA sequences are highly conserved between mammals, most likely because they have important biological functions. At the same time, genetic variations in non-coding sequences have been widely used as tools in mouse genetics.

are many other examples to further justify the 'comparative genomics' approach [18-22].

Mouse genes have a common architecture with other mammalian genes and are frequently (but not always) made out of coding (exons) and non-coding (introns) sequences with some other canonical sequences in the flanking regions, either upstream or downstream. The average size for a mouse gene is approximately 30 kbp. The smallest known gene is 0.1 kbp and encodes the t-RNA^{Tyr}. The biggest gene is *Titin* (*Ttn*) with 2.8 Mbp of genomic sequence and a cDNA of 82 kbp. The coding and non-coding regions are also of various sizes, ranging from 0.5 kbp for the shortest intron to 30 kbp for the biggest (dystrophin, muscular dystrophy-*Dmd*), with an average intron size of 4.7 kbp. For the exons, the shortest consists of only 9 bp (exon 2 of *Myo-VIa*), and the largest is 7.6 kbp long (exon 26 of *Apob*), with an average exon size of approximately 300 bp. The number of exons per gene varies from 1 to 314 with an average of 7.5 [23]. About 4000 genes have only one exon.

As in other species, mouse genes are alternatively spliced, which means that not all exons of a given gene are systematically represented in a given transcript (mRNA) or protein. Alternative splicing is indeed a very clever way—retained by evolution—to encode more specific proteins within the same number of genes, simply by assembling different exons (in general coding for peptides with different domains) in separate units. It also means that the number of genes can in no way reflect the degree of genetic complexity of a given species; the total number of exons is a much better piece of information. The most recent estimate indicates that there are about 220 000 exons in the mouse genome. Interestingly, interspecific comparisons point out that, whereas most exons in the mouse and human genomes are strongly conserved, exons that are only included in alternatively spliced forms (as opposed to the constitutive or major transcripts) are mostly not conserved and thus are the product of recent exon creation or loss events [24].

When interspecific comparisons are made, it is interesting to note that most mouse genes are conserved in blocks, with the same linear arrangement in the human or rat genomes. For example, when a hypothetical gene G_2 is found in the mouse genome flanked by genes G_1 and G_3, there is a very high probability that the same linear order G_1-G_2-G_3 is preserved in the other two species. This conservation of *synteny* (from the Greek, meaning 'on the same ribbon') is very important because it helps validating candidate genes. It also allows for the discovery of duplications or deletions among species. For example, about 90% of the mouse and human genomes can be partitioned into regions of conserved synteny, reflecting the structural organization of the chromosome in the common ancestor. These genomes share about 350 segments of conserved synteny, with sizes ranging from 300 kbp to 65 Mbp. The cluster of genes encoding oligoadenylate synthetase (*OAS*) is a good example of the interspecific conservation of synteny. In human chromosome 12 there are three such genes in the linear order centromere–*OAS1*–*OAS3*–*OAS2*–telomere. These three genes are transcribed in the same direction. In the mouse chromosome 5 there are 10 such genes in the linear order centromere–*Oas2*–*Oas3*–*Oas1e*–*Oas1c*–*Oas1b*–*Oas1f*–*Oas1h*–*Oas1g*–*Oas1a*–*Oas1d*–telomere. The structure and organization of the rat cluster is very similar to that of the mouse (no surprise), with eight *Oas1* genes. The orthologues of mouse *Oas1a* and *Oas1e* are missing in the rat, while two additional isoforms are present: *Oas1k* and *Oas1l*. This cluster of genes encoding OAS, which are molecules with similar functions, altogether represent a gene family. Such gene families are very common in all mammalian genomes and include the globin, myosin, HOX and G-protein-coupled receptor gene families.

Another interesting feature of the mouse genome is the presence of rodent-specific and even mouse-specific genes. The majority of these genes belong to gene families associated with reproductive functions, exhibiting spermatid- or oocyte-specific expression, or with vomeronasal receptors [8, 25]. Some of these new genes originated from relatively recent duplications (expansions) that occurred on the mouse linage since the time of its divergence from the rat, around 12-16 million years ago. On the other hand, the human genome (the primate lineage) has experienced losses of genes coding for olfactory and vomeronasal receptors [26].

The mammalian genome contains a great number of sequences that look like protein-coding

genes but are not. These sequences are called *pseudogenes* and are basically of two kinds: processed and unprocessed. Processed pseudogenes originate from the retrotranscription of messenger RNAs back into the genomic DNA in more or less random locations. They have no introns and exhibit mutations in their sequences (including frame shifts and stop codons) indicating that they are not transcribed. Unprocessed pseudogenes arise either from the tandem duplication of a gene during DNA replication or are degenerated genes that have become inactive and are no longer under selection. Among the different genes of the mouse *Oas* cluster discussed above, geneticists noted that although transcribed, some exons of these genes have molecular changes conflicting with a normal function (stop codons, for example) and concluded that they were most likely unprocessed pseudogenes. There are roughly 5000 pseudogenes in the mouse genome assembly (build 37), but their identification is often difficult. Synonymous mutations, those that will not modify the amino acid sequence, occur at the same frequency in genes and pseudogenes, while non-synonymous mutations are rare in functional genes. The ratio of the number of non-synonymous substitutions to the number of synonymous substitutions in orthologous genes is strong evidence for deciding whether a 'gene' is a true gene or a pseudogene. Most mouse pseudogenes do not have a corresponding homologous gene in the same syntenic position in the human or rat genomes, whereas active genes generally do.

Non-coding DNA

As we have already mentioned, the great majority of the mammalian genome is made out of non-coding sequences. However, part of these non-coding sequences is highly conserved between humans and mice, likely because they have important biological functions [27]. The function(s) of these conserved non-coding sequences is the subject of intense research at the moment, and it has been suggested that they could even be associated with some diseases [28]. However, a significant portion of the non-coding DNA is not under selective pressure or conserved, and exhibits a higher degree of genetic variation

(polymorphism). The following is an outline of these genetic variations.

Repetitive DNA sequences

These are non-coding sequences that are found in multiple copies within the mammalian genomes. Depending on the number of repeats, they are classified as moderately or highly repetitive DNA sequences. Among the latter, we find tandem and interspersed repeats [29]. Interspersed repeats are considered transposable elements and will be explained below. Tandem repeats take place when a motif of two or more nucleotides are repeated adjacent to each other in the genome. Depending on the number of nucleotides on the motif, these repeats are known as *satellite* DNA (120–250 nucleotides), *minisatellites* (10–60 nucleotides) and *microsatellites* (2–6 nucleotides). In this type of genetic variant, the polymorphisms (alleles) are due to variations in the number of tandem repeats within a locus. In the mouse, satellite DNA comprises about 5% of the genome and is divided into major satellite repeats (6 Mb long and located pericentrically) and minor satellite repeats (from 500 kb to 1.2 Mb in size and located in the centromere) [30]. Minisatellite loci (also known as variable number tandem repeats, VNTRs) are around 5–10 kb in size, extremely abundant, and distributed throughout the mammalian genome [31]. These highly polymorphic loci were used as genetic markers in the late 1980s, particularly in human studies. They are also the basis of the DNA fingerprinting that famously revolutionized forensic science [32]. This was the individual-specific band pattern resulting from the hybridization (by use of Southern blot) of restriction endonuclease-digested DNA with probes directed against extremely polymorphic minisatellite loci [33]. Even though it was used in a few mouse linkage studies and in genetic monitoring of inbred strains (isogenic individuals within an inbred strain share the same band pattern) [34-37], the use of DNA fingerprinting in the mouse was short-lived because it was quickly surpassed by microsatellite markers.

Microsatellites, also known as short tandem repeats (STRs) and simple sequence length polymorphisms (SSLPs), are very abundant (hundreds of thousands of copies per genome), extremely

polymorphic and widely distributed throughout the genomes of animal and plant species. Since the early 1990s microsatellites have been the genetic marker of choice in mouse genetics because their analysis is very simple, affordable and highly reliable [29] (see Box 1.4.1 and Figure 1.4.4). Microsatellites are very valuable for genome scans in linkage studies and background characterization (including the development of congenic strains by marker-assisted selection) as well as in genetic monitoring of mouse and rat inbred strains [38–40]. Routine analysis of DNA samples with microsatellite markers will confirm isogenicity and, provided the markers have been carefully selected, strain authenticity [41]. With

the enormous number of microsatellite loci available, it is generally not a problem to find a set of markers such that, when amplified, their PCR (polymerase chain reaction) products define a strain-specific pattern (Table 1.4.1).

One of the great advantages of microsatellites is the fact that one locus can display several alleles (multiallelic marker), allowing the evaluation of several inbred backgrounds with fewer markers. The other advantage of these markers is that thousands of primer pairs (each one amplifying a particular locus) are readily available for the mouse and the rat. In addition, individual primer pairs as well as panels of markers are commercially available. For detailed information

BOX 1.4.1
G e n e t i c   m a r k e r s

Genetic markers can be defined as specific DNA sequences with a known location on a chromosome and are essential tools for linkage and association studies. These studies are just not feasible without the possibility of differentiating the origin of the recombinant chromosomes on the DNAs obtained from mapping crosses. The availability of genetic markers is directly related to the presence of polymorphisms. Genetic polymorphism is the occurrence of alternative DNA sequences (alleles) at a locus among individuals, groups or populations, at a frequency greater than 1%. In the laboratory mouse the analysis of these genetic variations has been evolving with the need for genetic markers in linkage studies. There are several different techniques for detecting genetic variations in the mouse, most of them shared with forensic DNA profiling. The history of these techniques, along with their advantages and disadvantages, has been reviewed elsewhere [165–167]. The availability of an enormous number of genetic markers polymorphic between inbred strains has been instrumental in the development of genetic maps and the subsequent identification of genes by positional cloning [29]. As we explain in this chapter, many polymorphisms have been described in the mouse, but only two are now widely used as genetic markers in linkage analysis: microsatellites and SNPs.

The PCR amplification of microsatellites, in general dinucleotides of the type $(CA)_n$ or $(TA)_n$, is achieved using flanking primers that are specific for a particular locus. The PCR products, typically around 100–300 bp in size, are then analysed using 4%

agarose or polyacrylamide gel electrophoresis. The use of fluorescently labelled primers for microsatellite loci combined with capillary electrophoresis represents a new, fast, automated system for genetic monitoring and linkage analysis [40, 168, 169]. With this method, the resulting PCR products can be distinguished from one another by both their size and by the fluorescent dye associated with them. The availability of different dyes allows the possibility of developing multiplex PCR (i.e. the combination of primers for multiple loci in one reaction) and pooling several PCR products in one capillary. Another advantage of this methodology is the ability to differentiate alleles (PCR products) that differ only by 2 bp.

SNP genotyping is inexpensive and can be done in most research institutions. Genotyping is currently available based on allele-specific PCR [170], real-time PCR (TaqMan®) [171], DNA microarrays of variable density [80, 172] and KASPar system (a competitive allele-specific PCR coupled with fluorescent resonance energy transfer technology) [173]. However, sequencing a few short DNA stretches looking for SNPs is still an alternative approach for small-scale projects. Another option, and a clever idea, is to exploit the occurrence of SNPs that create a RFLP. For those interested in this approach, a web-based tool is available (see list of URLs at the end of the chapter) that can help extract region-specific SNPs from the dbSNP database and identify those that create an RFLP in a pair of selected inbred strains, and even help in the design of a suitable pair of PCR primers flanking the SNP [174].
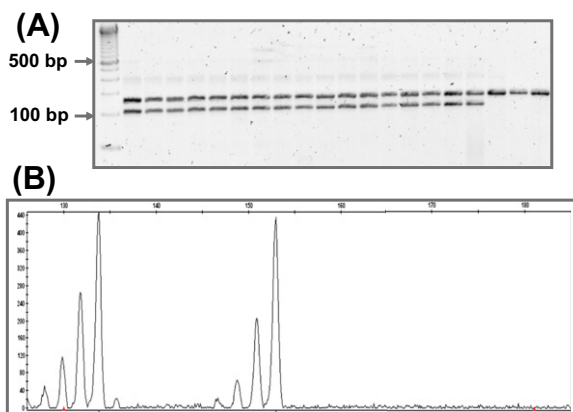
Figure 1.4.4 Microsatellite markers. (A) An ethidium bromide-stained 4% agarose gel of PCR-amplified microsatellites (negative image). These PCR products are obtained using species-specific and locus-specific primers for a microsatellite marker along with genomic DNA. The first 17 samples on the gel are heterozygous (2 bands) for the same alleles and the last 3 samples are homozygous (1 band) for the upper allele (approximately 170 bp). The first lane shows the 100 bp ladder. (B) Representative data output for analysis of mouse DNA amplified with a microsatellite marker using fluorescent primers (6-FAM) and separated by capillary electrophoresis. The sizes in bp are indicated on the top of the x axis (with smaller alleles to the left and larger alleles to the right). The values on the y axis indicate fluorescent signal intensity (relative fluorescent units). The mouse on this example is heterozygous 132 bp/152 bp for this marker (only the highest fluorescence intensity is considered).

on the expected alleles from different mouse inbred strains and primer sequences, visit the Mouse Genome Informatics (MGI) webpage. One of the disadvantages of the microsatellites is the occasional occurrence of spontaneous mutations that change the length of the allele (e.g. a small insertion or a deletion within the repeat). Typing DNA samples from the BXD set of recombinant inbred strains (RIS) between the parental strains C57BL/6 and DBA/2, Dallas and co-workers found that several amplification products had a size different from the parental strains and were then considered as 'mutants'. This occurred at a rate of 10^{-2} to 10^{-4}, a frequency that is not trivial [42].

Copy number variations, insertions and deletions

Although deletions, insertions and other large genomic rearrangements have been known since the 1980s, during the late 2000s there was increasing interest in the study of segmental duplications and copy number variations (CNVs) in the human and mouse genomes. CNVs are structural variants that result in copy number changes in a specific chromosomal region. As a consequence, large DNA segments (from 1 kb to several Mb), with more than 90% sequence conservation, are found at variable copy numbers when compared with a reference genome or within individuals of the same species (or between inbred strains). Though less abundant than SNPs, CNVs are estimated to account for at least 10% of the human genome, because of the large DNA sequences involved [43]. Most importantly, these CNVs are now thought to be affecting gene expression (altering transcript dosage) and phenotypic variability in genetic diseases (e.g. affecting the penetrance of the trait) [44]. This can be particularly relevant if we consider that two randomly selected individuals may differ by at least 1% of their genome sequences, mainly due to CNVs and SNPs. Like SNPs, CNVs are widely distributed across the genome. In the mouse around 100 genomic regions have been shown to harbour CNVs across the 19 autosomes, ranging in size from 20 kb to 2 Mb [45–48]. A striking aspect of the CNVs is that most of them contain annotated genes, mostly associated with the immune response (e.g. antigen binding) and environmental sensing (e.g. odorant and pheromone binding) [48]. The change in gene dosage associated with these CNVs could easily explain their involvement in phenotypic variation. Curiously, the laboratory of William Pavan has reported two CNVs located in chromosome 19 within the C57BL/6J inbred strain, something unexpected for supposedly isogenic animals. Moreover, these CNVs cause duplication and increased expression of the *Ide* (insulin degrading enzyme) and *Fgfbp3* (fibroblast growth factor binding protein 3) genes in a high proportion of C57BL/6J mice [49]. It will be important to discover if such intrastrain CNVs also occur within other inbred strains.

Almost 2 million small insertions and deletions (*indels*), ranging from 1 bp to 10 kb, have been reported in humans, with around 40% of these mapping to known genes, including coding exons. Clearly, this type of variation is likely to influence the phenotype of human genetic

TABLE 1.4.1: Mouse microsatellites

This is just a selection of 15 microsatellite markers (representing 13 chromosomes) that are polymorphic between a group of popular classical inbred strains like AKR, BALB/c, C57BL/6, C3H and DBA/2. This list could be used for genetic monitoring of these strains, if we carefully select the markers for each strain combination. The values represent the size of the allele in base pairs (we can observe this after PCR amplification from genomic DNA). The nomenclature for microsatellites is as follows: D [chromosome number] [Lab code] [marker ID], for example, D18Mit202 is a marker located on chromosome 18, identified at the Massachusetts Institute of Technology (MIT) with ID #202

Microsatellite	BALB/c	C57BL/6	DBA	C3H	AKR
D1Mit24	202	202	218	202	218
D2Mit59	120	146	134	120	146
D3Mit200	127	131	107	131	115
D4Mit32	184	148	142	184	182
D5Mit222	104	104	89	104	89
D6Mit150	140	140	150	150	150
D8Mit155	139	115	151	151	151
D9Mit179	147	147	149	149	151
D11Mit78	80	106	80	80	122
D11Mit228	124	134	114	114	120
D13Mit67	140	152	162	160	162
D13Mit185	152	146	148	152	146
D16Mit139	174	148	174	172	148
D17Mit123	137	133	155	155	155
D18Mit202	143	111	143	143	133

diseases [50]. In the mouse, indels were found to comprise close to 10% of all polymorphisms (excluding microsatellites), with deletions being more frequent than insertions [51-53]. An integrated database resource (MouseIndelDB) containing thousands of mouse indel polymorphisms (ranging from 100 bp to 10 kb) is now available [54].

Transposable elements

Transposable elements (TE) are DNA sequences that move from one location in the genome to another and are found in virtually all eukaryotes. These interspersed repetitive DNA sequences can be copied to a different location through DNA recombination, and after many generations the repeat could spread over various regions. There are two classes of TEs: class I, long terminal repeat (LTR) and non-LTR retrotransposons, which transpose via an RNA intermediate in a 'copy and paste' fashion; and class II, DNA transposons, subdivided into subclasses 1 and 2, which use

a 'cut and paste' mechanism that does not involve an RNA intermediate [55-57]. LINEs (long interspersed nuclear elements) and SINEs (short interspersed nuclear elements) are among the most studied class I non-LTR retrotransposons.

LINEs are considered autonomous retrotransposons and include the family of LINE-1 (L1) sequences, the most active non-LTR element identified in mammalian genomes, with around 100 000 copies per haploid genome. The mouse genome shows some L1 activity, with a full-length L1 mRNA of 7.5 kb, but a higher level of LTR retrotransposition [58]. SINEs are non-autonomous retrotransposons that exhibit repeat motives of around a few hundred base pairs. The more common examples are the Alu sequences in humans and the B1 and B2 sequences in the mouse, the latter with around 100 000 to 150 000 copies and a repeat size of 150–200 bp [59]. In evolutionary terms, these interspersed sequences are classified as lineage-specific (added to the mouse genome after the divergence from a common ancestor with other rodents), and

ancestral (before the divergence). It has been estimated that, in the mouse, these lineage-specific sequences contribute roughly 32% of the genome, compared with 24% in the human genome. On the other hand, the ancestral sequences represent around 5% of the mouse genome, in contrast to 22% in the human genome [60]. The low number of ancestral sequences may explain why the number of interspersed sequences is only 37% in the mouse versus 46% in the human genome.

The nature of the TE–host relationship (parasitism, symbiosis or commensalism?) as well as the role of TEs in disease and evolution has been extensively debated in recent decades. There are several reports of human diseases caused by L1-driven insertional mutagenesis [59], though LINE- and SINE-related pathologies are less common in the mouse compared to insertions by endogenous retrovirus [61]. Overall, the mouse genome was reported to have higher rates of spontaneous mutations caused by LTR elements, compared to the human genome where non-LTR elements are the primary source of disease-causing insertions [58]. Even though the role of TEs in the evolution of vertebrate genomes remains controversial, it was shown that these mobile elements can facilitate sequence-mediated chromosomal rearrangements that can potentially generate new gene regulatory sites. This was proposed for some human SINE sequences believed to be involved in generating new enhancers implicated in brain formation [62, 63]. Finally, it is interesting to note that these TE have opened the possibility for new germline mutagenesis systems in the mouse and other mammals, such as Sleeping Beauty and PiggyBac [64, 65].

Finally, this section would not be complete without mentioning the endogenous retroviruses. Retroviral infections in the mouse have led to germline integrations that contributed to shaping the mouse genome. The expression of these endogenous retroviruses has been associated with physiological functions and disease [66]. A classic example of the role of endogenous retroviruses as mutagens is the hairless (*hr*) allele of the hairless (*Hr*) gene [67]. This recessive mutation is the result of a retroviral insertion of murine leukaemia proviral sequences into intron 6 of *Hr* on chromosome 14, resulting in aberrant splicing of the gene [68]. Another example of this type of insertional mutagenesis is the recessive mutation digitation anormale (now *Lrp4^{dan}* on chromosome 2), which causes polysyndactyly in the affected mice [69]. As a final note, retroviral sequences have been used in the mouse as a tool in the identification of oncogenic mutations through retroviral insertion mutagenesis screens [70].

Single-nucleotide polymorphisms

Although the existence of SNPs within the genomes has been known for many years, the use of these single-nucleotide variations in linkage and genome-wide association studies increased greatly in the last decade. A SNP (pronounced 'snip') is a single-nucleotide change found in a DNA sequence, in a comparison between individuals of the same species or between inbred strains (Figure 1.4.5). SNPs are

Mouse Strains	129S1	129X1	A/J	C3H	C57BL	BALB	DBA/2	FVB/N	NOD	CAST	NZW
C/T SNP	C	C	C	C	C	C	T	C	C	T	C
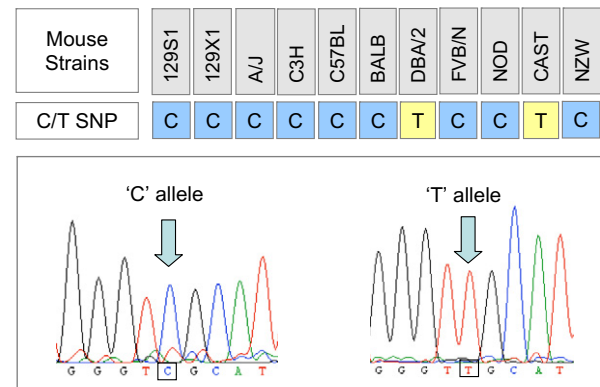
Figure 1.4.5 Single-nucleotide polymorphisms (SNPs). SNPs are discrete DNA variations occurring when a single nucleotide in the genome differs between members of the same species (or inbred strains in the case of the laboratory mouse). They result from random point mutations occurring at a constant rate during evolution, either in the coding regions or intergenic DNA, and are scattered throughout the genome. In the mouse genome they are not uniformly distributed along the chromosomes, with 'SNP-rich' and 'SNP-poor' regions, depending on the phylogenetic origin of the chromosomal segment. This allows the determination of a SNP pattern, which is unique to a given strain and accordingly can be used for assessing strain purity or as genetic markers for linkage analysis. In the figure the upper panel represents a C/T SNP that is polymorphic between DBA/2 and CAST (homozygous for the 'T' allele) and other common inbred strains (homozygous for the 'C' allele). The lower panel presents DNA sequencing electropherograms showing the SNP (arrow).

the most common type of genetic variation and are found in both coding and non-coding regions. When localized in coding sequences, if the variant leads to an amino acid change the SNP is said to be *non-synonymous*; if the SNP does not change the protein sequence it is considered *synonymous*. Those variants introducing a premature stop codon are known as nonsense SNPs. Almost all SNPs are biallelic, presenting one of only two possible nucleotides (e.g. homozygous G/G or T/T) or both (e.g. heterozygous G/T) in an individual. In humans we can find variations in the frequency of certain nucleotides (alleles) between populations; that is, a SNP allele can be common in one geographical or ethnic group and atypical in another [71].

Petkov and co-workers from The Jackson Laboratory (Maine, USA) have described the allelic distribution of 235 SNPs in 48 mouse strains and selected a panel of 28 such SNPs, enough to characterize most of the almost 300 inbred, wild-derived, congenic, consomic and RIS strains maintained at The Jackson Laboratory [72]. This set of SNPs, encompassing all mouse chromosomes, is an excellent tool for detecting genetic contamination in mouse facilities by way of automated PCR systems. The same laboratory developed a new set of 1638 informative SNPs selected from the publicly available databases and tested 102 inbred strains using Amplifluor genotyping [73, 74]. The selected SNPs are distributed approximately 1.5 Mb apart across the mouse genome and, on average, 37% will be polymorphic between any two inbred strains. This new SNP set is an excellent tool for performing quantitative trait loci (QTL) analysis and association studies in the mouse [75, 76]. Interestingly, these markers revealed subtle differences between closely related inbred strains and substrains, something that was independently confirmed for the most popular C57BL/6 substrains: C57BL/6J from The Jackson Laboratory and C57BL/6N from the National Institutes of Health [77, 78]. For those interested in the allele distribution of SNPs in different inbred strains, the Mouse Phenome Database presents a comprehensive collection of SNPs, with more than 8 million unique loci and numerous inbred strains genotyped (allele tables are provided by

investigators or retrieved from public resources). All the SNPs are mapped to the NCBI mouse genome build 37.1 reference assembly (C57BL/6J) and data was gathered from 22 different sources, including data sets from Perlegen, Celera, Wellcome Trust and The Jackson Laboratory (TJL1, TJL2 and TJL3 panels). Another option is to search the NCBI mouse dbSNP database, with the possibility of selecting among several inbred strain combinations and different types of SNPs (e.g., synonymous vs non-synonymous).

As mentioned in chapters 1.1 and 1.3, the availability of unprecedented numbers of informative SNPs (in the order of 10 million) allowed the study of the fine structure of genomic variation in the laboratory mouse. One of the early findings, right after the first draft genome was made available, was the presence of long segments of either extremely high (~40 SNPs per 10 kb) or extremely low (~0.5 SNPs per 10 kb) polymorphism rates, when comparing inbred strains [60]. Later on, in a comparison involving five inbred strains and a large set of SNPs (~70 000) covering the entire chromosome 16, it was confirmed that the SNPs are not evenly distributed, with SNP-poor and SNP-rich segments [79]. Several SNP panels, with markers evenly distributed across the mouse genome, have been developed in recent years and used to generate high-resolution genetic maps [53, 80–82]. One of the most recent variation maps includes the impressive number of 8.27 million SNPs, and was obtained by partial resequencing of the genomes of 15 inbred strains, 4 wild-derived and 11 classical [10]. All these new maps confirmed that, in the mouse, the SNP distribution exhibits a mosaic pattern of inheritance. The availability of the SNPs was also instrumental in the elucidation of the origin and relationships of the classical inbred strains. Data gathered using SNPs suggested that the genomes of inbred strains are mosaics of a handful of haplotype blocks (contiguous SNPs on a chromosome) present in the founder population of 'fancy' mice, with genetic contributions from several *Mus musculus* subspecies, including *M. m. domesticus* (predominantly), *M. m. musculus*, *M. m. castaneus*, and the hybrid *M. m. molossinus* [10, 60, 83–88].

Functional annotation of the mouse genome

Connecting biological information to sequences

As discussed earlier, the mammalian genome is difficult to analyse because it is enormous in size and heterogeneous in structure. Some elements are repeated, some are unique and some are present but not essential. It is clear, however, that enough information is encrypted in any genome for the making of a new individual, with all the characteristics (anatomical, physiological, immunological, etc.) of its species. Two main questions remain: (i) How to sort out the essential elements from those that are superfluous in the genomes? (ii) How is it possible for the genome to work in a spatiotemporal manner? At least two independent strategies can be used to answer these questions.

The first of these strategies would be to collect and characterize a large amount (ideally all) of the transcripts (e.g. in the form of cDNAs), from all the tissues and at all stages of development. This is obviously a huge amount of work, since we can expect at least three to four times as many cDNA molecules as the actual number of protein-encoding genes. Japanese scientists from the FANTOM consortium (Functional Annotation of the Mammalian Genome) at RIKEN Yokohama undertook this ambitious project several years ago and have collected and sequenced around 103 000 full-length mouse cDNAs [89]. This project turned out to be fundamental, because it improved the previous estimates concerning the total number of genes (and their alternative transcript isoforms) in the mouse, and because it expanded our knowledge of the gene families and revealed that a large fraction of the *transcriptome* is non-coding (a real surprise). Nowadays, the tissue-specific expression of many genes is being unravelled and DNA chips are progressively being released to help with this characterization. It is already possible, for example, to make the exhaustive inventory of the genes that are expressed in the

brain at embryonic day 14.5 [90] (see the Eurexpress Atlas webpage). It is also possible to identify the genes that are turned on (or off) when a mouse is experimentally infected with a specific pathogen. Obviously, many important findings are expected from this sort of research.

Another very efficient strategy would be to collect mutations resulting in the inactivation of each and every unit in the mouse genome, then to compare the phenotypes of the mutants with the wild-type mice (non-mutant genotype). Several projects of that kind have been undertaken over these last years and some are still in progress, including the IKMC. They require that, in parallel, a rigorous phenotyping programme be available for the scientists to be able to characterize even subtle phenotypic changes (possibly associated with genotypic alterations). We will not discuss further the great value of the cDNA resources that have been established, in particular by the RIKEN-FANTOM consortium, because it is beyond the scope of the present chapter. However, we will discuss in some detail the different sorts of genetic alterations that are potentially available in the mouse, either as the result of rare spontaneous events or as a consequence of a programme of systematic mutagenesis or genetic manipulation. We then discuss their advantages and limitations in the perspective of genome annotation.

Spontaneous mutations: their allelic interactions and their frequency

Every scientist who has been in charge of a colony of inbred mice or rats, even if only for a few years, has almost certainly discovered a mutation segregating in one of its breeding nuclei. Dominant spotting, for example, a dominant allele at the locus encoding the oncogene *Kit* (formerly W, now Kit^W, on chromosome 5), is very common and easy to discover when it occurs on a C57BL/6, C3H or CBA background because it lightens the coat colour, the tail in particular, and often induces a blaze and a belly spot. In fact, 135 Kit^W alleles have already been identified at this locus, with similar but not completely identical phenotypes, among which 66 are spontaneous events. Mutations at this locus are so common that new

occurrences are no longer kept, unless they have specific or unique phenotypic characteristics. Other mutations are also quite common, especially those with an obvious viable phenotype (e.g. skeletal anomalies, cerebellar defects, neuromuscular syndromes, anaemia, skin defects and inner ear defects). All these mutations generally fall into one of two categories: they are either recessive or dominant. Recessive mutations occur randomly, in any kind of cells and at any time of development, resulting in a mosaic mouse with a mixture of mutant and normal cells in various proportions. At this stage, the mutation has generally no phenotypic expression (it is cryptic). It is transmitted to the next generation only when the clone of cells affected by the mutation participates in the formation of the germline (oocytes and sperm cells). When this happens around 50% of the gametes in this 'next generation' mouse will carry the new mutant allele, in other words, this founder mouse will be heterozygous ($+/mut$) for the mutation, but with normal phenotype. When two chromosomes carrying the same mutation converge in a zygote (e.g. if we cross two heterozygous mice), then the animal becomes homozygous (mut/mut) and exhibits the phenotypic characteristics of the new mutation. Since inbreeding increases the level of homozygosity in the population, it also enhances the probability of discovering mutant phenotypes; however, inbreeding does not primarily increase the frequency of mutations.

Dominant mutations appear in the same way as recessive mutations, but they are expressed immediately (in approximately 50% of the offspring) when they are transmitted through the germline. A mutation is said to have complete dominance when the phenotype of the heterozygous mice ($Mut/+$) is indistinguishable from that of the homozygous mutant (Mut/Mut). If we observe three distinctive phenotypes, one for the $Mut/+$, another (more severe) for the Mut/Mut, plus the normal phenotype ($+/+$), then we consider the mutation to be semi-dominant. This is the case for the old spontaneous mutation Naked (N) on chromosome 15. In some cases the so-called 'dominant' mutations have a certain phenotype when heterozygous, and thus appear dominant, but they are lethal when homozygous. In this case they are recessive lethals with a heterozygous dominant

phenotype. It is also important to know the classification based on the effect of the mutation on the gene activity. For example an *amorphic* allele (null or loss-of-function) mutation will completely eliminate the activity, while a *hypomorphic* mutation will have less activity than the wild-type allele. In the same way, a *hypermorphic* allele will have increased gene activity, a *neomorphic* allele will show a new function, and an *antimorphic* allele will have a dominant negative function.

Many mutations, dominant or recessive, frequently exhibit variations in their phenotypic expression. For example, mice heterozygous for the brachyury mutation ($T/+$; chromosome 17) exhibit a shortening of the tail, but although this shortening is extreme in some individuals it is sometimes limited to a small kink in other individuals, and may escape identification (Figure 1.4.6). These phenotypic variations are frequent in mammals, including humans, and are generally qualified by geneticists with reference to two concepts: penetrance and expressivity. *Penetrance* refers to the proportion of individuals with the same genotype that actually express the expected phenotypic trait. When all mutant genotypes express the expected phenotypic trait, the penetrance is said to be complete or absolute. Variations in the penetrance are known to be influenced by the genetic background; however, these variations are also observed within inbred mice (that share the same background), indicating that other factors (e.g. environment and epigenetics) may play a role. *Expressivity* refers to phenotypic variations among individuals carrying a particular genotype. For example, mice affected by the piebald mutation (formerly *s*, now *Ednrb^s*, on chromosome 14) have a white spotted coat but the pattern of spotting and the size of the spots varies among mutant mice (Figure 1.4.6). Mutations said to have variable expressivity show a relatively large amount of phenotypic variation among individuals having the same genotype. Expressivity, of course, is estimated only when the mutation exhibits complete penetrance.

Spontaneous mutations occur, in general, at a low frequency but this varies greatly among loci. Geneticists at The Jackson Laboratory [91, 92] have estimated the mutation rates at five classical coat-colour loci (*a*, *Tyrp1^b*, *Tyr^c*, *Myo5a^d*

All mice $T/+$

All mice $Ednrb^s/Ednrb^s$

Penetrance

Expressivity

T: brachyury

$Ednrb^s$: endothelin receptor type B
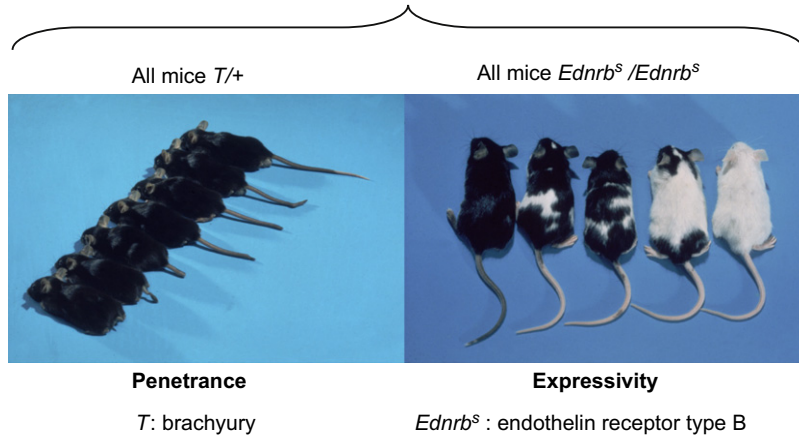
Figure 1.4.6 Penetrance/Expressivity. The picture illustrates two major characteristics of the phenotypic expression of mutant alleles in mammalian species. In the present case, all seven mice on the left panel are affected by the same mutation *Brachyury* (*T*), and share the same inbred background, but they exhibit great variations in the phenotypic expression (expressivity), with some mice (top of the picture) indistinguishable from a normal phenotype (lack of penetrance). On the right, all mice exhibit a spotted coat with wide variations in expressivity (mutation *Ednrb^s*). The penetrance characterizes the fraction of individuals of a given genotype that actually show a particular phenotype, irrespective of the degree of its expression. The expressivity characterizes the phenotypic variation among individuals having the same genotype. It is now well established that modifier genes influence the phenotypic expression but these genes cannot explain all sort of variations since phenotypic variations are also observed in inbred strains.

and $Mlph^{ln}$), by computing the number of new mutational events on 7 million inbred mice, and found it to be, on average, 11×10^{-6} per locus per gamete for mutations towards a recessive allele. This mutation rate was confirmed by other scientists, in particular W. Russell and co-workers [93], who computed 28 spontaneous mutations at the same five loci in a population of 531 500 mice (10.5×10^{-6}). A comparison of the rates at the five specific loci with other loci for which mutations were also recovered (among the same 7 million mice) showed that the five specific loci had, on average, sixfold higher mutation rates. Finally, the mutation rate toward a dominant allele was estimated by the same scientists to be around $1.0\text{-}8.2 \times 10^{-7}$ per gamete (i.e. about 10 times lower). These mutation rates must be considered with caution and only for guidance. The mutation rates toward a dominant allele are certainly underestimated because such mutations occur only once and escape identification in many cases. The dominant mutation Extra-toes ($Gli3^{Xt}$ on chromosome 15) is a good example of this situation because the phenotype of affected mice consists exclusively of a tiny 'extra toe' at the

inner edge of the hind leg which requires very close examination to be detected. Mutation rates towards dominant alleles are also underestimated because mutations with late-onset phenotype (say after 18 months or so) are in general not taken into account. Recessive mutations do not have these drawbacks since they are, in general, occurring repeatedly in the breeding nucleus; however, some of them—with specific anatomical defects—are difficult to identify. For example, any mutation inducing a cleft palate is totally incompatible with life, for the simple reason that pups cannot suckle milk from their mother and accordingly die shortly after birth. Similarly, all mutations with a phenotype leading to death *in utero* are identified only if a special protocol is used for their identification.

Spontaneous mutations have some advantages. The first, and probably the most important, is that they are produced at virtually no cost and are in general freely available. Another advantage is that they have, in general, an obvious phenotype given that they are identified on the basis of observation. Also, spontaneous mutations represent a great variety of molecular events, like deletions, insertions, and

point mutations, generating not only loss-of-function alleles, but also hypomorphic and hypermorphic ones. In many cases the phenotype of these mutations can help to establish better animal models than those produced by knockout models [94, 95].

Unfortunately, spontaneous mutations also have drawbacks and a major one is that the primary molecular defect they result from is, in most instances, totally unknown. In these conditions, spontaneous mutations are of unpredictable value for gene annotation as long as this molecular defect is not clearly established. The spontaneous recessive mutation oligotriche (*olt*) is a good example to illustrate the situation. As the name suggests, oligotriche mice have an abnormal coat, and in addition they exhibit male sterility through defective spermatogenesis. It was demonstrated by positional cloning (an approach that will be explained in detail later) that the oligotriche phenotype was the consequence of a 234 kbp deletion involving no less than six contiguous genes on mouse chromosome 9 (*Plcd1*, phospholipase C delta 1; *Vill*, villin-like; *Dlec1*, deleted in lung and oesophageal cancer 1; *Acaa1b*, acetyl-coenzyme A acyltransferase 1B, synonym thiolase B; *Ctdspl*, C-terminal domain RNA polymerase II polypeptide A small phosphatase-like; and *Slc22a14*, solute carrier family 22 member 14) [96]. This structural change made it impossible to establish a link between gene, protein and phenotype and of course complicated the use of this mutation for the annotation of the genes involved. Spontaneous mutations have another serious drawback: they show up at very low frequency. While the number of collected mutant alleles increases steadily, the percentage of new mutations (i.e. occurring for the first time at previously unknown loci) remains low, indicating that many genes do not have yet a mutant allele. Given that the number of genes in a mouse genome has been estimated to be in the range of 22 000–25 000, geneticists realized that the number of 'missing' mutant alleles is not negligible. For this reason, several programmes aiming to produce large numbers of new mutations (in general using mutagenic treatments) are now in progress, in order to compensate for the shortage. We now review some of these approaches.

Mutagenesis in the mouse: the mass production of new mutations

In all the species used as models in genetics, for example *Drosophila melanogaster*, *Cænorhabditis elegans*, *Saccharomyces* spp. and *Arabidopsis thaliana*, the possibility of increasing the spontaneous mutation rate has been a major concern for the simple reason that new mutant alleles are, by definition, the fuel for genetic research. Accordingly, in all cases mutagens and mutagenic treatments have been developed successfully. In the mouse the situation was historically slightly different. Many mutations were collected after the Second World War, especially in the USA (Oak Ridge National Laboratory) and in the UK (Medical Research Council at Harwell) as by-products of the intensive research on the risks associated with the use of nuclear energy, X-rays and gamma-rays. Some of these mutations have been used as models for human genetic diseases or as genetic markers (for the establishment of genetic maps), but since they frequently turned out to be associated with small chromosomal rearrangements, sometimes involving several contiguous genes, they did not prove very useful for genome annotation.

Chemical mutagens have also been developed, some of them as by-products of the chemical or pharmaceutical industry, but many of the molecules that proved to be active in other model species turned out to be inactive in the mouse (e.g. nitrosoguanidine) or proved to be active only on the postmeiotic germ cells (haploid phase) producing only a transitory mutagenesis in treated male mice. Most of these traditional mutagens (ethyl methane sulphonate for example) had a limited efficacy and accordingly were abandoned. It was a breakthrough when W. Russell, from Oak Ridge National Laboratory [97], reported that the alkylating agent ENU was 'the most potent mutagen in the mouse'. Indeed, this discovery can be considered another crucial step in the development of mouse genetics. Experimental results indicate that with ENU as a mutagen and an appropriate protocol of mutagenesis (a single injection of 150–250 mg/kg body weight or three injections of 80–100 mg/kg at weekly

intervals in male mice aged over 8 weeks), the basic mutation rates can be multiplied by a factor of at least 120-150. The advantages of ENU as a mutagen are numerous and its mode of action has been extensively studied [98-103]. ENU is an alkylating agent producing mostly base-pair changes (point mutations). Adenine, and to a lesser extent thymine, are the favourite targets of ENU but the other two bases are occasionally affected too, as demonstrated by Takahasi [104]. After an optimal treatment one can expect ENU to induce, on average, 0.7-1.9 nucleotide substitutions per Mbp of DNA, which translates in one mutation at a specific locus in every 670-1000 mice of a G3 generation (Figure 1.4.7).
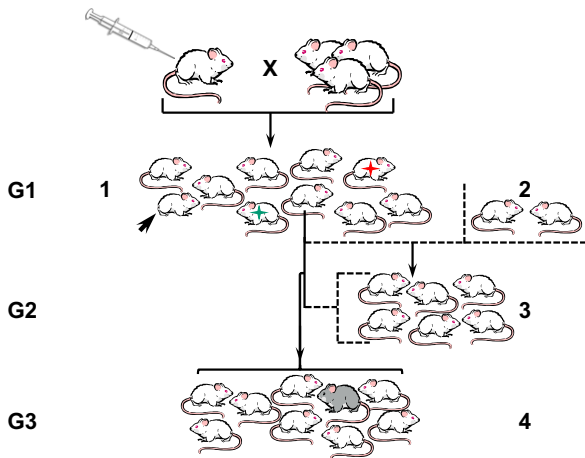
Figure 1.4.7 Phenotype-driven mutagenesis with ENU. Phenotype-driven mutagenesis consists of four successive steps. In the first step males are treated with the powerful mutagen ENU (see text for doses) and mated with females when they have recovered from a 10–13 week sterility period. Dominant mutations are looked for among the G1 offspring (arrow). In a second step males of the G1 generation (which are potential heterozygous carriers of recessive mutations of all kinds) are selected for the establishment of micro-pedigrees. First, they are mated with female of either the same or from a different strain, and 4–6 female offspring (G2) are backcrossed to their G1 father. Finally, the progenies of the G1 males × G2 female offspring are submitted to careful phenotypic examination (for example in a 'mouse clinic'). Micro-pedigrees producing mutant phenotypes are then isolated for in-depth analysis. The number of G2 females and of their G3 offspring are established after statistical computation to optimize the possibility of detection of new phenotypes.

Several collaborative projects aimed at the mass production of new mutant alleles were launched in the late 1990s, particularly in Europe, Japan and North America [105-108]. In most instances these projects have been associated with downstream phenotypic screens to detect some specific types of mutations (e.g. leading to neuromuscular diseases or to deafness [109]). Other interesting projects, making use of the phenotype-driven mutagenesis strategy, have also been undertaken to assess, for example, the number of genes that are involved in the innate defence mechanisms of the mouse after infection with specific viruses. These projects have proved to be extremely rewarding, allowing the discovery of new genes [110]. In conclusion, it is clear that chemical mutagenesis is an efficient and interesting strategy for the induction of new mutant alleles in the mouse, especially when associated with a phenotypic screen. It has also the enormous advantage of generating mostly point mutations (base-pair substitutions), which, by definition, affect only a single gene. Nevertheless, the production and phenotyping of spontaneous and chemically induced mutations alone does not contribute to the identification and characterization of genes at the molecular level. This characterization, which is the essence of gene annotation, requires other experiments that we now review.

Positional cloning of mouse mutations

Positional cloning or *forward genetics* is one of the strategies used by geneticists to identify the gene(s) responsible for a particular phenotype or a biological process. It is a bottom-up approach since it proceeds from the phenotype to the genotype. This strategy uses spontaneous or induced mutations with a phenotype of interest as raw material, and requires the availability of a genetic map with as many informative genetic markers as possible. In fact, the best tool for positional cloning is a high-density/high-resolution molecular map [80-82]. Positional cloning is the process of identifying a gene based on its position in the genome, without any prior idea of its function. A good historical example of positional cloning is the identification of the gene responsible for

the obese mutation (*ob*, now *Lep^{ob}*, on chromosome 6) [111]. Mice affected by the *ob* mutation grow very fat from the age of 14 days, and in many instances they are also affected by severe type 2 diabetes. It was obviously interesting to characterize this gene at the molecular level because the mouse syndrome clearly resembles morbid obesity in humans. Scientists at the Rockefeller University in New York embarked on a positional cloning project by setting up an intercross between a classical inbred strain of mice (C57BL/6), segregating for the *ob* mutation, and highly unrelated wild-derived mice (*Mus m. castaneus*), segregating for the wild-type allele of *ob*. Using a large number of molecular markers and DNA samples from *ob/ob* hybrid F2 mice, the researchers were able to restrict the genetic localization of the *ob* locus to a relatively small interval on chromosome 6. (At that time not many molecular markers were available for positional cloning. They were mostly RFLPs—restriction fragment length polymorphisms—plus a few microsatellites. The Rockefeller team generated new DNA probes, allowing the identification of RFLPs, by mechanically scratching the region of chromosome 6 supposed to contain the *ob* locus. A real challenge!) After identification of the genes in the interval, they came to the conclusion that the most likely candidate for the *ob* mutation was a gene encoding a cytokine now known as leptin [111]. This discovery, which must be considered a great achievement, opened the way to many other similar experiments. Many genes have been cloned following this protocol, and presumably many more will be cloned in the years to come given that the strategy has been greatly facilitated since the sequencing of the genome. In addition to this, and after the massive production of new alleles, many interesting phenotypes suggestive of a human pathology will become available.

The essential steps in the positional cloning process are always the same. First, a high-resolution map is achieved using molecular markers to define the shortest possible interval containing the mutant allele. In order to complete this first step, a specific cross must be set up involving two strains: the first strain segregates for the mutant allele that is to be cloned while the other segregates for the wild-type allele. The two strains involved in the cross are selected based on the greatest possible differences in terms of genetic polymorphisms. Strains recently derived from wild progenitors (wild-derived inbred strains, often abbreviated WDIS) of either the *Mus m. musculus* or *Mus m. castaneus* subspecies, which are now available from most mouse suppliers, are ideal tools for that sort of cross because they are so remotely related to the classical laboratory strains that they allow thousands of polymorphisms to be used [112] (see also Chapter 1.1). A large number of F2 or backcross mice must then be bred from this initial cross (see Box 1.4.2 and Figure 1.4.8). Of course, the larger the number of offspring, the greater the resolution of the genetic map. In general, 100–500 F2 mice are bred and processed for DNA genotyping.

A rapid (low-resolution) map can then be achieved using a subset of 60–80 DNA samples and a set of molecular markers (microsatellites or SNPs) evenly distributed across the genetic map (also known as a whole-genome scan). This is in general sufficient to identify a non-recombinant interval containing the mutant allele (linkage analysis). From now on consultation of the reference molecular map of the mouse is necessary to identify another set of molecular markers located inside the critical (non-recombinant) interval on the same chromosome. Only these markers are then assayed on the rest of the DNA samples looking for recombination, in the hope of defining an even smaller interval. When the non-recombinant interval is smaller than 1 Mbp (ideally less than 500 kbp) there is in general no need to continue the mapping programme, and the candidate gene analysis can start. With the genetic and phenotypic data in hand, the geneticist can now look at the available databases (genome browsers) accessible online, checking the genes that have been localized in the critical interval by the sequencing projects (these are commonly designated 'positional candidate genes'). The number of genes in a candidate region can be very variable, but in most cases will be between 10 and 30 genes for a 1 Mbp region (Figure 1.4.9). When the identification of potential candidate genes has been achieved, it is advisable to check the expression databases to see if the available phenotypic data are in agreement with the expression data. Expression studies based on RT-PCR or

BOX 1.4.2
Mapping crosses

The use of laboratory mice for gene mapping offers some advantages: (i) it is possible to set up crosses between inbred strains at will, (ii) inbred strains produce only one type of gametes and (iii) an almost unlimited number of mice can be produced. We should keep in mind that the occurrence of variant forms (alleles) of genetic markers in the parental strains is a requisite. The classical breeding schemes used for linkage analysis are the backcross and the intercross (using classic or wild-derived inbred strains).

Backcrossing is a two-generation breeding protocol that starts by generating hybrid F1 mice between two inbred strains (preferably distantly related), one of them carrying the mutation of interest. Then, F1 mice are mated with a member of one of the parental inbred strains to generate N2 mice. All F1 mice will be heterozygous for all the genetic markers that are polymorphic between the parental strains. However, since traceable genetic recombinations are present only in the gametes from the F1 parent, approximately 50% of the N2 mice will be heterozygous and 50% will be homozygous for a given informative marker. The association between the mutant phenotype and certain

genetic markers along a chromosome will determine linkage, and a putative location for the mutant gene. Genetic markers located far apart from the mutant gene will show no linkage. Backcrosses can be used for the study of dominant mutant alleles, but also for recessive mutations, if homozygous mutant mice are fertile [29]. In this case the advantage is that 100% of N2 mice (*mut/mut* and +/*mut*) will be informative. One negative aspect of the backcross is that only one meiotic event is analysed (the one from the F1 gamete) in each N2 mouse.

Intercrossing is also a two-generation breeding protocol that starts by generating hybrid F1 mice between two inbred strains (one carrying the mutation). Then, F1 mice are intercrossed in order to generate F2 mice. In this case informative meiotic events will take place in both parents, giving twice as much recombination information per mouse (1 mouse = 2 meioses), compared with backcross mice. Another advantage of this approach is that it can be used to map recessive mutations that are not viable in the homozygous state [29]. Regardless of the breeding scheme chosen, an ideal number of meioses to be analysed for a positional cloning project should be in the order of 500−1000.

quantitative real-time RT-PCR will also be helpful in this context.

When all the preceding steps have been completed and strong candidate genes are on hand, it is time to detect the primary genetic defect. This is typically achieved by sequencing the candidate genes, using either cDNA or genomic DNA. In the former case primers for direct sequencing are designed based on the RNA transcript. For genomic DNA it is advisable to design primers to sequence the complete coding region (all the exons) and the intron-exon boundaries. Comparisons with the reference mouse sequence available online will, in general, point out differences that will require a second, careful examination, particularly to discriminate SNPs from the causative mutation. At present the new sequencing technologies make it possible to sequence the whole candidate region, even if it is 2–4 Mbp in size [113]. This will definitely save a lot of time. Finally, once the primary genetic alteration is identified, and even if it seems totally obvious, a confirmation of a causal relationship between the mutated protein and the mutant

phenotype is in general necessary, for example by means of phenotypic rescue [114] or the availability of other mutations for the same gene (e.g. ENU-induced alleles or knockout mice) [115]. These topics of linkage analysis, genetic mapping, and positional cloning can be found in several books and many review articles [29, 116–119].

Even though the identification of genes accountable for single-gene phenotypes is very important, in particular in the context of gene annotation, most of the pathologies that affect human patients are not 'monogenic' but, on the contrary, are influenced by multiple genes with additive or synergistic effects. In the same way, most mutations accounting for a deleterious phenotype have been found to affect the coding regions of a gene (base-pair substitutions, deletions, insertions, splicing abnormalities, etc.), but mutations with an effect on the quantitative or spatiotemporal expression of a gene are not well known, although they are probably quite common. Finally, genes with a modifier effect, for example increasing the severity of a phenotype or making a certain inbred strain more or less susceptible to

(A)

Strain A (+/+) ✕ Strain B (*m/m*)

Backcross

F₁ (+/m) ✕ Strain B (*m/m*)

N₂

(+/m)   (*m/m*)

Genome-scan

(B)

(C)

Strain A (+/+) ✕ Strain B (*m/m*)

Intercross

F₁ (+/m) ✕ F₁ (+/m)

F₂

(+/+)   (+/m)   (+/m)   (*m/m*)

Genome-scan

(D)
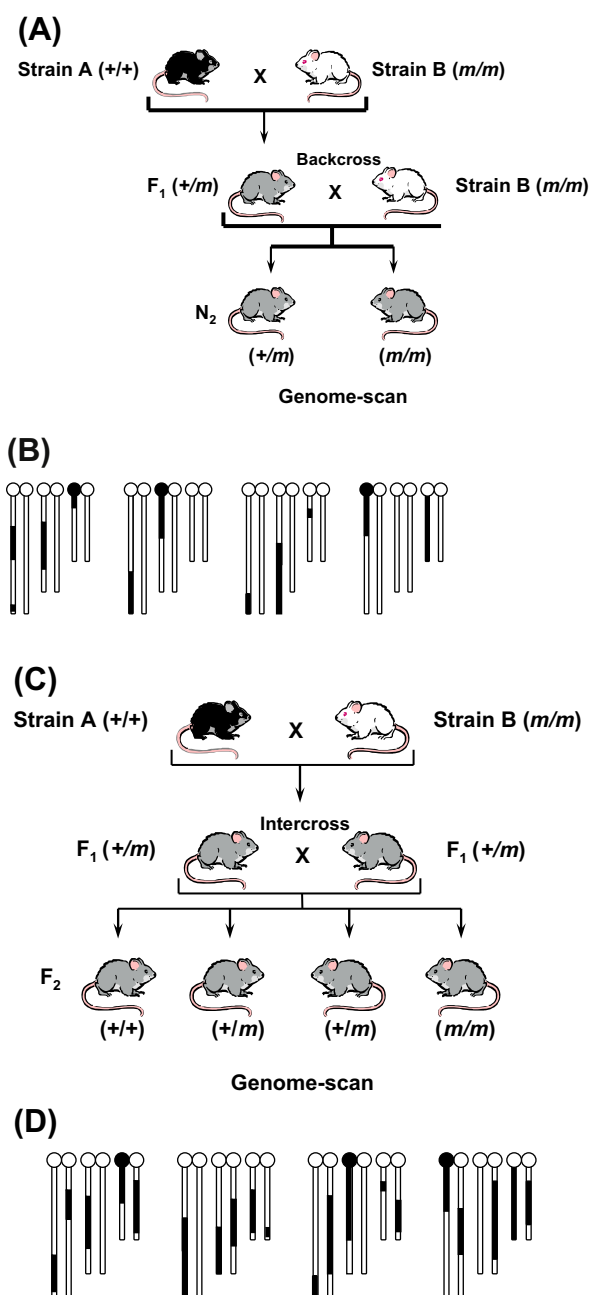
Figure 1.4.8 Mapping crosses. (A) The figure shows a backcross scheme where strain B is homozygous for a recessive mutation (*m*) that is viable and fertile, and strain A is wild-type for this mutation. After producing hybrid F1 mice (100% heterozygous), these are backcrossed onto the strain carrying the mutation, to produce N2 mice. These mice will be on average 50% heterozygous for the mutation (normal phenotype) and 50% homozygous (mutant phenotype). All mice in the N2 generation can be genotyped for a linkage analysis. (B) Three chromosome pairs representing large (180 Mbs), medium (120 Mbs) and small (60 Mbs) sizes from four independent N2 mice. Note that each N2 mouse always receives a non-recombinant (all white) chromosome from the parental strain B.

an infectious disease, have been identified only exceptionally. In fact, in our analysis of the genotype–phenotype relationships so far, we have probably investigated only the tip of the iceberg, because we did not have enough specific tools for assessing the genetic analysis of complex traits. This situation is now changing, as we will explain.

The Complex Trait Consortium (CTC) was created in 2002 by a group of scientists who decided to identify tools to tackle the problems related to quantitative inheritance in the mouse [120–123]. Among the strategies suggested by the CTC, the most innovative was the implementation of a resource known as the Collaborative Cross (CC) [122, 124]. In the end, the CC will consist of a total of around 1000 RIS (see Box 1.4.3), each derived from an initial eight-way cross involving very different and unrelated inbred strains (Figure 1.4.10). Theoretical computations indicate that the genome of each RIS in such a cross will capture around 135 unique recombination events (135 000 for the whole set of RIS) and each of these RIS will then have a unique genomic constitution representing a patchwork of elements with, roughly, an equal proportion of the eight founder genotypes. Once fully inbred, each line will display a fine-grained homozygous mosaic of the founder haplotypes, capturing an abundance of polymorphisms that will be sufficient to drive phenotypic diversity in almost any trait of interest, provided it segregates among the eight parental strains. Even if the eight strains that have been selected as founders of the eight-way cross represent only a sample of the polymorphisms that may segregate in the mouse, this will

(C) Intercross mapping protocol where strain B is homozygous for a recessive mutation (*m*) that is viable and fertile. After producing hybrid F1 mice, these are intercrossed to produce F2 mice. On average, 75% of the F2 mice will exhibit normal phenotype and 25% will exhibit a mutant phenotype. This latter group is typically chosen for a first genome scan with genetic markers, although all the F2 mice could be informative. (D) Three chromosome pairs, representing large, medium and small sizes, from four independent F2 mice. Note that F2 mice can receive recombinant (black and white) chromosomes from both F1 parents. Note: The genes governing the coat colours of the mice in these crosses are independent from those responsible for the mutant phenotype. The black and white colours on the parental strains were chosen only to relate with the recombination of the chromosomes.

Figure 1.4.9 Identification of candidate genes during positional cloning. In the final stages of a mapping project, the number of genes in a candidate region can be very variable. The figure shows a 1 Mbp candidate region from hypothetical positional cloning projects comprising three independent 'classic' spontaneous mouse mutations: nude (*Foxn1*^{nu}) on chromosome 11, obese (*Lep*^{ob}) on chromosome 6, and scid (*Prkdc*^{scid}) on chromosome 16. This 1 Mbp region will carry more than 40 genes in the case of the nude mutation, around 20 genes for the obese mutation, and fewer than 10 genes for the scid mutation. Sequencing candidate genes one by one using conventional methods will require different effort levels for each hypothetical project. The images are from the Ensembl Genome Browser database (August 2011).

probably be more than enough to allow unravelling of at least some mechanisms of quantitative inheritance in mammals. The first reports analysing CC lines (still not yet at the 12th generation of inbreeding) already support the use of the CC for dissecting complex traits [125-127].

Gene-driven chemical mutagenesis: an alternative source of mutant alleles

Spontaneous and ENU-induced mutations, as we have said, are generally identified through the

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BOX 1.4.3

Mapping panels

Mouse mapping panels are established sets of DNA obtained from animals that carry random recombination events in their chromosomes. Two of the most popular DNA panels have been created using RIS and interspecific backcrosses (a description of RIS, recombinant congenic strains and advanced intercross lines can be found in Chapter 1.3). One of the advantages of using these DNA panels is that the need to breed mice is circumvented; however, these panels are obviously not suitable for mapping mouse mutations known only by their phenotype. Another benefit is that the genotyping information coming from a large number of previously typed genetic markers and loci is already available, and keeps growing (i.e. data is cumulative and

comparable, and the resources renewable). RIS have proved very helpful when used for gene mapping, in particular for the rapid regional assignment of microsatellites. They have also been used for the mapping of chromosomal regions (QTLs) involved in the genetic determinism of some behavioural characteristics and immunological responses, and they will very likely be of help in future experiments where the phenotype is measured on a group of animals rather than on individuals [175]. Mapping DNA panels represent a valuable resource when looking for a set of precisely ordered markers that can be used in any cross where they exhibit polymorphisms. The CC described in this chapter is a good example of the value of these panels of RIS.
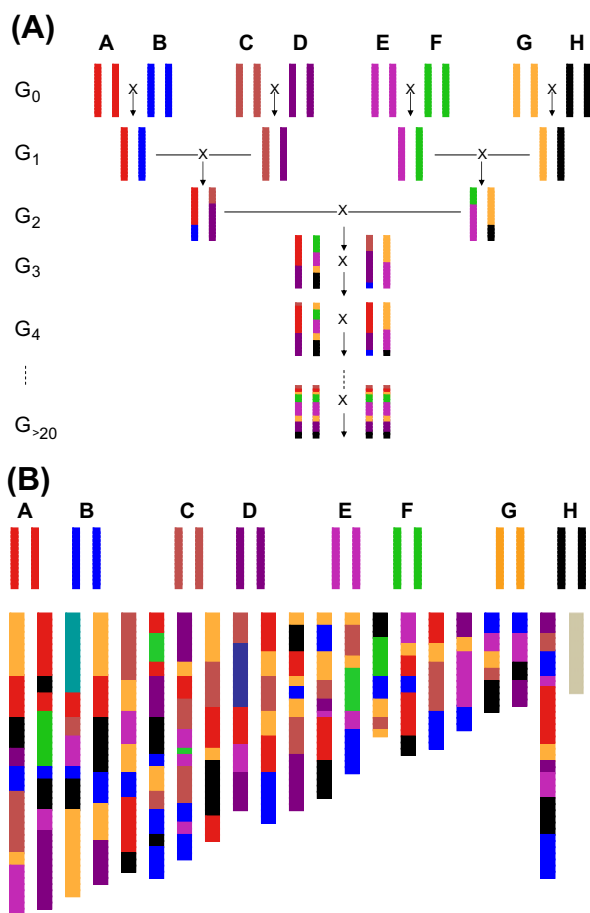
Figure 1.4.10 The Collaborative Cross. (A) A randomized cross of eight unrelated mouse inbred strains designed by members of the CTC. The lines are first crossed pairwise to make all 56 possible G1 parents. A set of possible four-way crosses is performed, keeping Y chromosome and mitochondrial balance. Finally, all eight genomes are brought together in G2:F1 and the offspring of this cross are inbred. The Collaborative Cross is a community resource that was initially designed for the purpose of mapping complex traits. (B) The initial plan was to breed around 1000 inbred strains where all the alleles of the initial inbred strains would be associated in a wide and unique variety of combinations. Only one strain is represented (19 autosomes plus X and Y chromosomes) in this illustration; other strains would be similar but with a different pattern of parental strain distribution.

observation of an abnormal phenotype. This is sometimes advantageous when an investigator wishes to make an inventory of the genes that are involved in a particular phenotype or behaviour. The problem is that not all mutant genes have an obvious phenotype [128] or, conversely, the phenotype of some mutant alleles is sometimes so severe that offspring die *in utero* and

escape identification. In these cases gene annotation is impossible by means of forward genetics. When mutant forms of a gene are not readily available, the only possible approach for gene annotation is 'top-down', trying to address what is (are) the function(s) of a gene of interest by generating *de novo* mutations in it. For questions like this, geneticists have several answers, some of which will be explained in Chapter 1.5 of this book. Here we would just like to mention that ENU can also be used to perform the so-called gene-driven mutagenesis, which is an interesting alternative to the phenotype-driven mutagenesis explained above.

As mentioned earlier, treatment of male mice with ENU generates many new point mutations in the genome of their G1 offspring. If we assume that several inbred G1 mice are produced and their sperm cells deep-frozen, a very large number of mutations are then potentially available in this resource that are more or less evenly distributed across the mouse genome [103] (Figure 1.4.11). With 2.7×10^9 bp in the mouse genome and around 0.7–1.9 nucleotide changes per Mbp (on average), after ENU treatment, one can expect about 1900 *de novo* substitutions per G1 mouse. If we assume that these mutations are randomly distributed, one can expect about 30 nucleotide changes in the coding DNA (1.5%) of which perhaps 23 will generate an amino acid change ($\sim$77%). If we consider that the splicing sites are also potential targets for ENU mutagenesis then a minimum of 30 *de novo* mutations are produced by ENU in each G1 offspring. In a repository containing 30 000 G1 independent sperm samples a total of 900 000 new mutations are potentially available. Given that the total number of genes in the mouse is 22 000–25 000, this means that several mutations per locus are available. Statistical computations indicate that any gene in the genome could carry at least seven mutations at the 5% risk level. Even if only a (small) fraction of these mutations result in a detectable phenotype, this still represents a very interesting approach [129].

A major issue is that even if these mutations are 'virtually' present somewhere in the sperm cell repository, they have nonetheless to be detected. Then the relevant sperm cell sample, once identified, must be thawed and finally used to restore a breeding nucleus segregating
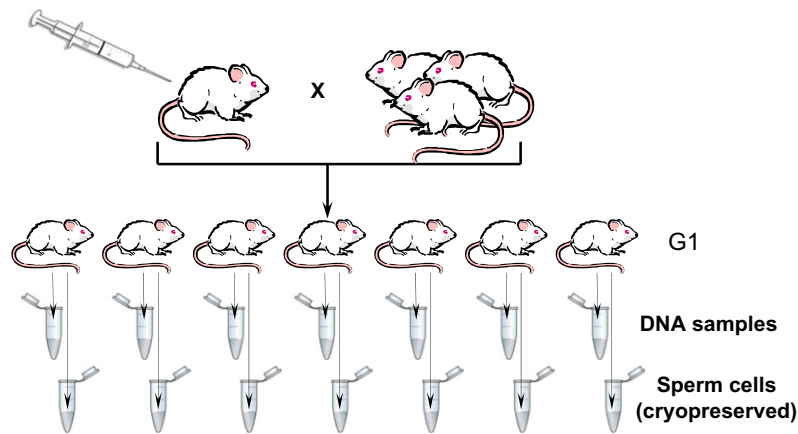
Figure 1.4.11 Genotype-driven mutagenesis with ENU. Male mice of the same inbred strain are treated with ENU and mated to females (preferably of the same inbred strain) once they have recovered from a 10–13 week sterility period. A large number of G1 males are then bred, which all are heterozygous carriers of a great number of independent point mutations (base-pair changes). Sperm samples from each G1 mice are collected and preserved deep-frozen while DNA samples of the same mice are processed and stored. Identification of the mutations generated by the ENU treatment in a specific target (a gene or any other specific sequence) is achieved by molecular techniques identifying DNA mismatches or directly by sequencing. Once the base-pair changes are identified and considered potentially interesting (stop codons, missense, etc.), the corresponding sperm cells are thawed and heterozygous mice are produced by *in vitro* fertilization with oocytes of the same background strain. A major advantage of this method is that it produces all types of point mutations, not only knockouts. A drawback is the difficulty of and time required for identifying the mutations in the targeted region. With the rapid expansion of the new sequencing techniques, the identification step should be somewhat alleviated.

for the mutant allele. All this is like finding the proverbial needle in a haystack. In most instances, it is achieved by establishing two parallel cross-referenced repositories, the first consisting of the deep-frozen sperm samples of individual G1 mice, the second of genomic DNA samples from the same males. Identification of ENU-induced mutations at a given locus is performed on the genomic DNA by amplifying with primers specific for the gene of interest, followed by mutation detection methods based on the recognition of base-pair mismatches (e.g. single strand conformational polymorphism, denaturing high-performance liquid chromatography, etc.). For example, by screening the DNA samples of the UK ENU programme discussed above, mutations in the gene encoding for connexin 26 have been identified, and mutant mice produced by IVF using the corresponding archived sperm cells [130]. Similar experiments have been performed in other laboratories and gene-driven mutagenesis has even been efficiently applied to the rat for the production of a missense mutation in the sodium channel gene *Scn1a* [131, 132] as well as the *Brca1* and *Brca2* tumour suppressor

genes [133]. In the future, using next-generation sequencing techniques, it should probably become possible to catalogue all the mutations available in the different repositories, for a relatively affordable cost, by directly sequencing all the stored genomes. Gene-driven ENU mutagenesis might then become an efficient way to generate point mutations (null alleles, missense alleles, mutations in splicing sites) in the mouse genome. Finally, it is interesting to note that the analysis of the distribution of ENU-induced mutations in the mouse genome revealed that these mutations occur randomly with no hot or cold spots [103].

Engineering genetic alterations in embryonic stem cells

The observation of a particularly high frequency of testicular teratocarcinomas in the inbred strain 129 [134] and the *in vitro* culture of cell lines derived from these tumours [135], which was for almost a decade a material of choice for

investigating the processes at work in tissue differentiation [136], undoubtedly opened the way to the establishment of ES cells by Evans and Kaufman [137], and simultaneously by Martin [138]. ES cells are undifferentiated pluripotent embryonic cells derived from the inner cell mass of blastocysts. They are cultured *in vitro*, in general on feeder layers of fibroblasts, in supplemented tissue culture media. To prevent them from differentiating, low concentrations of leukaemia inhibitory factor (LIF) are added to the culture medium and the cells are transplanted at a relatively rapid pace. ES cells represent a material of choice because they can be manipulated *in vitro* like ordinary somatic cells, and they retain their developmental potential when injected into the cavity of a blastocyst; however, long-term culture of ES cells can lead to decreased pluripotency and the gain of chromosomal abnormalities. More importantly, ES cells are capable of participating in the formation of the germ-cell lineage of chimeric mice [139], an indispensable step in generating founder mice carrying the targeted mutation. The first

experiments of genetic engineering with ES cells were achieved by Gossler and co-workers [140] and Robertson and co-workers [141] and constituted a real breakthrough. Following these pioneering experiments, thousands of knockout and knockin mice have been created, including the more recent conditional models (Box 1.4.4). All these techniques are reviewed in Chapter 1.5.

Influence of genetic background on mutant phenotypes

It is increasingly recognized that the genetic background (i.e. all genomic sequences other than the gene(s) of interest) can have profound influences on the phenotype of an animal model. It has been shown that mutations (spontaneous and induced), transgenes and targeted alleles (knockouts and knockins) that are 'moved' onto a different background) can show a change in phenotype [142-144] (see also the online *Genetic Background Resource Manual* from The Jackson Laboratory).

BOX 1.4.4
Where to get mouse mutations

Several major genetic repositories are established worldwide where mutant alleles or strains are stored, generally in the form of deep-frozen embryos or sperm cells, less frequently in the form of 'breathing' animals. These mutants and strains are available to the community, at a reasonable cost, but require a Material Transfer Agreement (MTA). The Federation of International Mouse Resources (FIMRe) is a collaborating group of mouse repository and resource centres worldwide whose collective goal is to archive and provide strains of mice as cryopreserved embryos and gametes, ES cell lines and live breeding stock to the research community. Members of this federation are:

1. In North America:
   a. The Jackson Laboratory, Bar Harbor, ME, includes the Mouse Mutant Resource (MMR) as the primary repository of strains and stocks carrying spontaneous mutations
   b. Mutant Mouse Regional Resource Centers (MMRRC)
   c. Mouse Models of Human Cancer Consortium (MMHCC), Frederick, MD
   d. Canadian Mouse Consortium (CMC)

   e. Canadian Mouse Mutant Repository (CMMR), Toronto, Ontario.
2. In Europe:
   a. European Mouse Mutant Archive (EMMA).
3. In Japan:
   a. RIKEN BioResource Center, Tsukuba (RBRC)
   b. Center for Animal Resources and Development (CARD), Kumamoto University, Kumamoto.
4. In Australia:
   a. Australian Phenomics Network (APN).

The URLs of some of these major centres are provided at the end of the chapter.

Other resources for mouse researchers are:

1. search IMSR (International Mouse Strain Resource)
2. search MGI (Mouse Genome Informatics)
3. post a request on the Mouse Genome Informatics (MGI) e-mail list service (mgi-list).

In all cases it is recommended to order deep-frozen embryos or sperm cells because they are generally available at relatively short notice, they are somewhat cheaper than live animals and they eliminate the risk of transmission of infectious diseases.

A classic historical example involves the mutations obese (Lep^{ob}) and diabetes ($Lepr^{db}$), well-described mouse models of human type 2 diabetes. On a C57BL/6 background, these two mutations cause obesity and mild or transient diabetes, while on the C57BLKS/J background they cause obesity and overt diabetes [145, 146]. Interestingly, the C57BLKS/J strain was recently recognized as a 'contaminated' substrain, with 70% of its genome coming from the original C57BL/6J strain, 20% from strain DBA/2J and the remaining 10% from an unidentified origin. These fortuitous observations indicate that background-unique modifier genes, derived from either strain DBA/2 or from the unknown strain, are influencing the phenotypic expression of the *Lep* and *Lepr* mutations, making the 'diabetes' phenotype mild or severe [147]. In parallel to this observation, recent investigations on the transcription level in pancreatic beta cells revealed contrasting differences between the C57BLKS/J-$Lepr^{db}/Lepr^{db}$ and C57BL/6J-$Lepr^{db}/Lepr^{db}$ strains for two genes: nicotinamide nucleotide transhydrogenase (*Nnt*) and pleiomorphic adenoma gene like 1 (*Plagl1*) [148].

Another interesting example to illustrate the importance of the genetic background on the phenotype involves the multiple intestinal neoplasia (*Min*) mutation at the adenomatosis polyposis coli (*Apc*) gene (the new symbol for the mutation is Apc^{Min}), a popular mouse model for human colorectal cancer [149]. This dominant mutation, which generates a premature stop codon, was ENU-induced and was recognized because the heterozygous mutant mice were anaemic from intestinal bleeding due to the presence of intestinal polyps. Homozygous mutant mice are not viable. On the C57BL/6J genetic background heterozygous mutant mice have severe chronic anaemia and die before 120 days of age, exhibiting multiple adenomas in the intestinal tract at the time of necropsy. Interestingly, when the point mutation Apc^{Min} is transferred onto the AKR/J, MA/MyJ or CAST (*Mus m. castaneus*) inbred backgrounds, by performing a series of backcrosses, affected mice show a significant reduction in the number of polyps [150]. This observation suggests that certain inbred strains, at least the three mentioned above, carry modifier genes that affect polyp multiplicity in these mice. The first of these modifier genes to be identified was *Mom1* (modifier of *Min* 1) [151]. We now know that *Mom1* is a semi-dominant

modifier of both polyp size and multiplicity, and that it encodes for the secretory type 2 non-pancreatic phospholipase A2 (*Pla2g2a*) [152–155]. Other modifiers of Apc^{Min} (*Mom2–Mom7*) have been discovered in the last few years [156–158] using the same strategy of backcrossing the mutant allele onto different backgrounds. Modifiers are a very interesting kind of gene because, while they display (apparently) no phenotype of their own, they have an enhancing, reducing or even suppressing effect when associated with some mutant alleles with a deleterious effect.

Similar situations have been observed repeatedly in transgenic and knockout mice when used as models of human pathologies. In 1995 Threadgill and colleagues reported one of the first cases of a strong influence of the genetic background on the phenotype of a knockout mouse. Mice homozygous for a null allele at the epidermal growth factor receptor (*Egfr*) died *in utero* when on a 129/Sv (now 129X1) background, but survived until 3 weeks of age when moved to a CD-1 (outbred) background [159]. A few other examples are: dramatically different phenotypes in transgenic mice overexpressing amyloid precursor protein depending on the genetic background [160]; changes in tumour types on *Trp53* knockout mice between C57BL/6 and BALB/c backgrounds [161]; changes in incidence and spectrum of tumours on *Pten* (heterozygous) knockout mice among several inbred strains [162, 163]; and changes in metabolic phenotypes between C57BL/6, 129S2, C3H and BALB/c strains [164]. This indicates that the genetic background may be a reservoir of genes with confounding effect on the phenotype of study, and an important source of variation that scientists can use to uncover mechanisms of pathogenesis. Obviously, more has to be learned concerning the role of these modifiers, but this can only be achieved using animal models.

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Relevant URLs for mouse genomics

Ensembl Genome Browser. http://useast.ensembl.org/index.html

Eurexpress Atlas. http://www.eurexpress.org/ee/

European Mouse Mutant Archive (EMMA). http://www.emmanet.org

Federation of International Mouse Resources. http://www.fimre.org

Gene Dictionary of the Mouse Genome. http://www.nervenet.org/main/dictionary.html

The Jackson Laboratory Genetic Background Resource Manual. http://jaxmice.jax.org/geneticquality/background.html

International Knockout Mouse Consortium (IKMC). http://www.knockoutmouse.org

International Mouse Strain Resources (IMSR). http://www.findmice.org

Mouse Atlas of Gene Expression. www.mouseatlas.org

L.M. Silver. Mouse Genetics: Concepts, and Applications. www.informatics.jax.org/silver

Mouse Genome Informatics (MGI). http://www.informatics.jax.org

Mouse Genome Project at Baylor College of Medicine. http://www.mouse-genome.bcm.tmc.edu

MouseIndelDB database. http://variation.osu.edu

Mutant Mouse Regional Resource Centers (MMRRC). http://www.mmrrc.org/index.html

Mouse Phenome Database. http://phenome.jax.org

NCBI Mouse Genome Resources. http://www.ncbi.nlm.nih.gov/genome/guide/mouse/

NCBI mouse dbSNP database. http://www.ncbi.nlm.nih.gov/SNP/MouseSNP.cgi

Online Mendelian Inheritance in Man (OMIM). http://www.ncbi.nlm.nih.gov/omim

RIKEN Mouse Mutagenesis Program. http://www.yokohama.riken.jp/english/index.html

FANTOM consortium. http://fantom.gsc.riken.jp/4/

SNP2RFLP. http://genetics.bwh.harvard.edu/snp2rflp

Tennessee Mouse Genome Consortium. http://www.tnmouse.org

UCSC Genome Browser. http://genome.ucsc.edu

Generation of Mouse Mutants by Genotype-Driven Mutagenesis

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Introduction

The publication of the mouse genome initial sequence in 2002 was a milestone in biomedical research and further strengthened the importance of the laboratory mouse as the leading mammalian model organism. It became possible to make comprehensive comparisons between the mouse genome and the previously published human genome. Currently, the genomes of both species are estimated to have about 25 000 genes [1]. Although the lineages of mice and humans diverged about 65–75 million years ago, more than 99% of mouse genes have a homologous sequence in the human genome and about 80% have a strict orthologue [2]. This similarity in genomic sequences presumably reflects an underlying similarity in gene function between humans and rodents, and validates the pivotal role of laboratory mice as experimental organisms for human biology.

The challenge is to understand the role of newfound genes in normal and perturbed biological processes. For this, the molecular analysis of the genome needs to be supplemented by investigations on the *in vivo* level. To date, the most practical approach to studying the function and regulation of newly discovered genes is to use mutations. Human genes can only be studied on the basis of naturally occurring mutations. Experimental manipulation of the human genome to study the potential function of human genes is unthinkable for ethical reasons. The mouse genome is a viable alternative since it encodes an experimentally controllable and tractable organism with great genetic and physiological similarities to humans.

During the first decades of mouse genetics, progress was dependent on spontaneous mutations. There are many examples of randomly discovered mutations that resulted in very important animal models such as the nude mouse [3]. However, spontaneous mutations only occur with a low frequency of about 5×10^{-6} per locus and are only detectable if they result in a visible phenotype. Accordingly, the discovery of new genes and their functions was very rare.

To accelerate the generation of novel mutants for genetic studies, researchers tested strategies to experimentally induce heritable mutations in mice. One approach was the treatment with known chemical mutagenes. N'-ethyl-N'-nitrosourea (ENU) was identified as a powerful mutagen that induces single base-pair changes in mouse spermatogonia germ cells and has since then been used in conjunction with large-scale 'phenotype-driven' mutagenesis programmes [4, 5]. ENU mutagenesis in mice has helped in the discovery of many novel genes involved in human diseases. However, the initial event is random. Extensive phenotype screening and efficient positional cloning strategies are necessary to identify physiological alterations and their corresponding mutated genes.

In parallel to ENU mutagenesis, several 'genotype-driven' techniques were developed in the 1980s to manipulate the mouse genome by stable integration of *in vitro* recombined transgenic DNA sequences. Since the creation of transgenic mice by pronuclear DNA injection [6], the field of genetically engineered animal models has been undergoing constant development. This chapter addresses the most important aspects concerning the generation of transgenic mice, taking the advantages and limitations of routinely used methods and tools into particular consideration.

Transgenic animals— a definition

The term 'transgenic' was used for the first time by Gordon and Ruddle [7] to describe genetically transformed mice produced by pronuclear DNA injection. According to the

TABLE 1.5.1: Developmental stages of mouse embryos routinely used for different methods of transgenesis

	Sperm Oocyte	• SMGT/ICSI-mediated gene transfer
	Zygote	• pronuclear DNA injection • transposon-mediated gene transfer • sub-zonal injection of lentiviral vectors
	2-cell / 4-cell stage	
	Morula	• ES cell injection and aggregation • co-culture with lentiviral vectors
	Blastula	• ES cell injection

ES cell, embryonic stem cell; ICSI, intracytoplasmic sperm injection; SMGT, sperm-mediated gene transfer.

Guidelines for Nomenclature of Mutations in Mice and Rat, the application of the term has been extended to any rodent with a stable integration of an experimentally introduced foreign DNA sequence into the germline [8]. The term is applicable irrespective of the activity, functionality, phenotypic relevance or transfection route of the transgene (Table 1.5.1). However, transgenic animals can generally be separated into two categories according to the type of chromosomal integration of the transgenic sequence: (i) by an illegitimate recombination process designated as non-homologous DNA end-joining (NHEJ) or (ii) as a targeted event at a selected locus by homologous recombination (HR) between introduced and endogenous sequences. Once integrated into the germline, transgenes are maintained and propagated as normal endogenous loci through meiotic and mitotic divisions.

Methods for additive transgenesis by random integration

Pronuclear DNA injection

The most routinely used method to generate transgenic rodents with a randomly inserted

foreign DNA sequence is the microinjection of transgenes into the pronucleus of a zygote [9]. Transgenes are usually injected into the bigger and better-visible male pronucleus about 24 h after fertilization of the oocyte. In mice the procedure often yields more than 20% transgenic founders among the offspring. Transgenes are generally separated from the prokaryotic sequence of the cloning vector by restriction digest and injected as linearized molecules.

DNA molecules of nearly 500 kb have been successfully transmitted by this method [10]. When two different transgenes are coinjected they typically cointegrate at the same integration site. Alternatively, transgenes can be designed as bicistronic constructs with a promoter and an internal ribosomal entry site (IRES) or a 2A-peptide sequence between two coding sequences. The resulting mRNA transcripts allow for the expression of two distinct proteins [11-14].

The genetic background of zygote donors is of great importance for the efficient application of pronuclear injection [15]. In spite of potent hormone treatments, the number of zygotes obtained per donor is quite low in most inbred strains and their embryos are generally less resistant to the different steps of *in vitro* manipulation as compared to hybrids or outbred stocks. Nevertheless, the use of defined inbred backgrounds for the generation of genetically modified mice is strongly recommended in order to avoid the laborious backcross procedure necessary to generate a congenic strain with defined genetic background [16].

Considering the low frequency of chromosomal insertions for injected DNA, founder animals generated by pronuclear injection are theoretically expected to be hemizygous, i.e. with a transgenic integration on one chromosome of a homologous pair. However, exceptions frequently occur, resulting in a variety of genotypes for founder animals. In principle, scientists should be aware that founders are unique organisms and therefore inappropriate for initial expression analysis of the transgene.

Chromosomal integration of pronuclearly injected DNA molecules depends on double-strand breaks occurring both naturally and as side effect of the injection procedure [17]. The resulting genetic environment is unique for each transgenic locus and can cause phenotypic variation referred to as the 'position effect variegation'. The transgene can either be silenced or display an ectopic expression pattern. The position effect might be caused by transcription interference between the transgene and a neighbouring gene, by the integration into imprinted or heterochromatic regions and by integration into sex chromosomes. Due to random inactivation of one X chromosome in somatic cells, hemizygous females display a mosaic expression pattern for transgenes integrated into an X chromosome.

To protect the transgenic construct from genomic position effects, specific DNA sequences known as insulators have been successfully used to block the interaction with *cis*-acting regulatory elements. Such locus control regions (LCRs) and matrix attachment regions (MARs) are shown to function in combination with heterologous sequences [18-20].

Transgenes constructed from complementary DNA are especially sensitive to position effects. It is usually impossible to recreate a functional gene with all the regulatory elements that are a prerequisite for an independent and physiologically normal expression. Moreover, cDNA constructs have lost the intron/exon structure of the coding region which is important for the chromatin structure of the transgenic locus [21]. Therefore, genomic sequences should be the preferred source for transgenic constructs in order to provide natural conditions for transcription and a physiological expression pattern [22]. However, large genomic fragments usually overcharge the capacity of plasmid-based vectors and have to be cloned in P1 artificial chromosomes (PACs), bacterial artificial chromosomes (BACs) or yeast artificial chromosomes (YACs) [23-25].

Due to the small injection volume (approximately 1 pl), only a restricted amount (about 2 fg) of DNA is added to the haploid set of the mouse zygote's genetic material. Consequently, the number of copies injected into a pronucleus is dependent on the size of the transgenic construct. For transgenes with a common size of about 10 kb, this equals approximately 180 injected molecules. Even if a large number of DNA constructs is injected, multiple integration sites per genome are infrequent. The rare founders with more than one integration site are usually detected by significantly more than

the expected 50% of transgenic offspring. Unlinked transgenic loci will segregate and thereby generate offspring with different genotypes. Therefore, it is important to divide multiple integration sites into separate mutant lines, each with a single transgenic locus.

The injection of numerous copies of the transgene per zygote results in transgenic loci with multicopy tandem arrays. The typical head-to-tail arrays are thought to be assembled via HR between transgenic molecules immediately after microinjection but before transgene integration. This HR is very efficient and is harnessed to transfer large (50 kb) constructs into the pronucleus. The transgenic sequence is separated into smaller constituent fragments with overlapping regions of about 2.5 kb. After these fragments are injected they will recombine to reconstitute the original sequence prior to chromosomal integration [26].

Integrated arrays are highly variable in copy number among the founders. This may of course result in different expression levels for the transgene. However, there is no linear relationship between copy number and expression level. In contrast, transgenes of large multicopy arrays mimic repetitive elements and are often found to be silenced by assembling repressive chromatin structures [27].

Transgenic arrays have been found as non-integrated DNA molecules until the blastocyst stage. Due to an uneven distribution of the concatemeres during cell cleavage, probably not all cells of the developing embryo will receive injected transgenes. Consequently, the chromosomal integration of the unevenly distributed transgene arrays can occur in single blastomeres during all preimplantation stages of the embryo [28]. This, however, may result in a genetic mosaic animal consisting of both transgenic and non-transgenic cells [29]. Each mosaic is unique and mosaic founders will transmit the mutation less frequently to the offspring than expected.

Pronuclear injection is accompanied by complex chromosomal rearrangements at the transgene integration site [30]. This might potentially affect endogenous genes by insertional mutations and may therefore collaterally affect the phenotype of the transgenic line. The best way to find out if an insertional mutation was induced by the integration of the transgene is to breed homozygous transgenic animals. This way, the animals will also become homozygous for the insertional mutation (which are mostly recessive) and consequently may display a new phenotype.

Bearing all this in mind, it becomes clear that each transgenic line produced by pronuclear injection and illegitimate DNA recombination is unique and not reproducible. To exclude unknown side effects of the random integration side, several independent founder lines are required to validate an observed phenotype as the exclusive result of the expression of the transgene. Therefore, several lines of each transgenic construct have to be produced and characterized as a basis for selection of the appropriate lines for further experimental use.

The broad application of the pronuclear injection technique is attributed to its relatively simple practicality, adequate efficiency and applicability to various mouse strains. However, the specific characteristics and unpredictability of the induced mutations require great effort to correctly breed and characterize each transgenic line for proper experimental use.

Vector-mediated transgenesis

Several alternative methods have been developed in order to increase the frequency of transgenic founders per research project and to overcome some of the described disadvantages of pronuclear injection. Vectors of different origin have been used as shuttles for transgenes. As in pronuclear microinjection, vector-mediated techniques have an unpredictable integration site. However, as part of a vector, the transgenic sequence is usually protected against cellular nucleases and the integration is conducted by vector-specific mechanisms. It has to be kept in mind that different vector-mediated mutagenesis strategies alter the genome in different ways, which may affect the resulting phenotype.

A few years before successful pronuclear injection of purified DNA was accomplished, *retroviral vectors* constructed from oncoretroviruses had been used to stably introduce foreign genes into mouse embryos [31, 32]. This method had several disadvantages and did not become accepted as routine until the recent development

of lentiviral vectors based on HIV [33]. Transgenes integrated via infection of retroviral vectors derived from the Moloney murine leukemia virus (MMLV) often became silenced. The long terminal repeats (LTR), a sequence repeated at both ends of the vector that is required for chromosomal insertion, is thought to be detected as a target for *de novo* methylation and gene silencing [34]. In comparison to the original retroviruses, the new lentiviral vectors are not identified as parasitic elements and are therefore not silenced by the defence mechanisms of the host cell. Furthermore, the infection process is not restricted to dividing cells.

Lentiviral vectors are delivered by simple injection under the zona pellucida (into the perivitelline space) of zygotes or by temporary coculture of denuded embryos in a lentiviral suspension. The highly efficient integration events occur without significant alteration of the endogenous sequence at the integration site. Up to 80% of the offspring were found to be transgenic [35]. However, unwanted multiple integration sites per genome are found much more frequently than in pronuclear injection. Therefore, increased effort to separate transgenic loci is necessary to establish non-segregating transgenic lines. Moreover, it has been shown that HIV and SIV favour the integration in transcription units, thereby increasing the risk of insertional mutagenesis in endogenous genes [36, 37]. The low cargo capacity of less than 10 kb and the reduction of the virus titre by specific transgene elements (such as splice and polyadenylation signals) further restrict the use of lentiviral vectors in mouse transgenesis to specific applications.

Sperm-mediated gene transfer combined with intracytoplasmic sperm injection

Sperm-mediated gene transfer (SMGT) combined with intracytoplasmic sperm injection (ICSI) is comparable to pronuclear DNA injection, or even better in certain respects [17]. There is improved frequency of transgenic offspring, especially for large constructs cloned by BACs and YACs [38], and it produces far fewer genetic mosaics. However, only spermatozoa subjected to disruption of the head membrane by shock freezing or treatment with Triton X-100 have

been reproducibly used for transgenesis by coinjection with transgenes into unfertilized oocytes [39]. Original attempts using simple cocultures of fresh spermatozoa from the caudal epididymis and DNA molecules to generate transgenic mice via *in vitro* fertilization were not consistently reproducible [40-43]. The pretreatment process, however, results in dead sperm cells which are only usable as transgene vectors through the demanding ICSI procedure. As this technique is only established in a few laboratories around the world, SMGT has not become a routine method for mouse transgenesis. Moreover, there are major concerns regarding ICSI-mediated gene transfer, such as the impact of the pretreatment on the sperm genetic material, the circumvention of naturally occurring sperm selection before fertilization, and inconsistent activation of the injected oocyte [44-46].

Recent attempts to improve transgenesis by ICSI have used a coinjection of fresh sperm cells with single-stranded transgenic DNA (ssDNA) coated with *E. coli* RecA bacterial recombinase [47]. RecA-complexed ssDNA is protected from degradation by nucleases and therefore can generally improve transgenesis in mammals [48]. RecA-coated DNA was successfully used for ICSI-mediated transgenesis in combination with fresh, untreated spermatozoa, consequently avoiding treatment-induced DNA damage. Unfortunately, this approach resulted in a very high percentage of undesired mosaic founders [49, 50].

Transposon-mediated gene transfer

A transposon-mediated technique has been successfully applied in lower metazoan models such as *Drosophila melanogaster*. Recent developments make DNA-based transposable elements (TEs) interesting as a toolkit for genetic modifications in vertebrates. Similar to viral vectors, transposons integrate randomly into host chromosomal sites as a single copy and without rearranging the endogenous sequences. But in contrast to viral vectors, TEs are not infectious and no reverse transcription is needed before chromosomal integration. Several TE systems are known and are applicable to transposition-based genome modification [51]. Two of them have been successfully adapted for the efficient

generation of transgenic mammals: (i) the *piggy-Bac* system derived from the moth *Trichoplusia ni* and (ii) the *Sleeping Beauty* system, a synthetic transposable element that has been generated from an ancestral Tc1/*mariner*-like transposon found in salmonid fish [52, 53]. New variants of hyperactive transposases have been developed for both systems and will expand the application of TEs in mammalian transgenesis [54, 55].

The procedure generally includes two components: the transposon carrying the transgene and the corresponding transposase provided by transient expression of its mRNA sequence. Excision of the transgene from the transposon and its insertion into a random chromosomal site occur as a conservative cut-and-paste mechanism. Providing that the transposase is only transiently expressed, the transposon-mediated chromosomal integration of the transgene is stable. For the delivery of transposon and transposase, the pronuclear injection technique is used and both components are co-injected in one step.

Sleeping Beauty and *piggyBac* transposons are very efficient in mouse transgenesis with more than 60% transgenic offspring (own unpublished results; Figure 1.5.1A, B). Genetic mosaics are rare among founders generated by transposon-mediated transgenesis. However, as for viral vectors, undesired multiple integration sites per genome are a typical feature of TEs. Furthermore, the maximum size of the transgenic insert while maintaining efficient transposition still needs to be investigated for both systems in order to be used as routine applications.

Transgenesis by targeted mutation using embryonic stem cells

While it is a very efficient method for additive transgenesis, random integration by illegitimate DNA recombination cannot be used to alter a selected endogenous gene of interest. The ability to induce targeted mutations in the mouse genome became feasible for the first time through the successful isolation and long-term culture of pluripotent embryonic stem (ES) cells [56, 57]. Immortal ES cells can be exclusively derived from the epiblast, an embryonic lineage of the inner cell mass of late blastocyst embryos, and have the capacity to differentiate into several
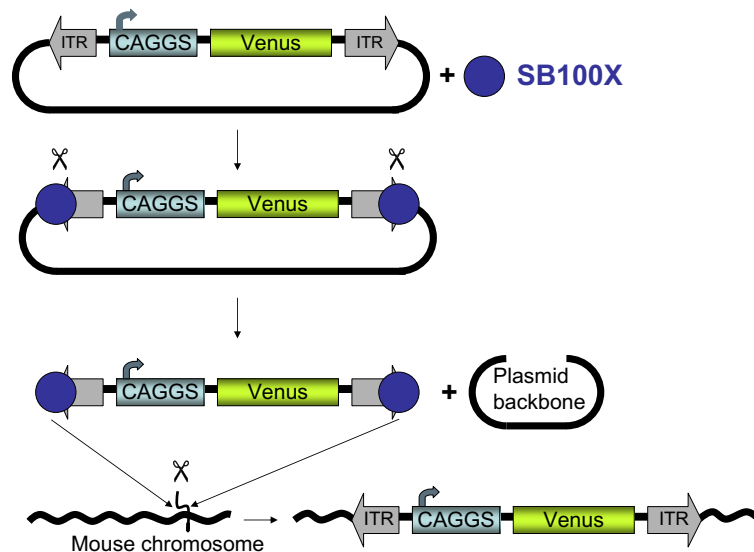
Figure 1.5.1A Transposon-mediated transgenesis. Schematic representation of *Sleeping Beauty* (SB) transposition. A Venus reporter (or any gene of interest) is positioned between two inverted terminal repeats (ITR) on a circular donor plasmid. The ITR-flanked gene can be mobilized by the SB transposase *in trans*. The transposase protein binds to the ITRs and catalyses the transgene's excision from the plasmid and random integration into the host genome.

(A)

(B)
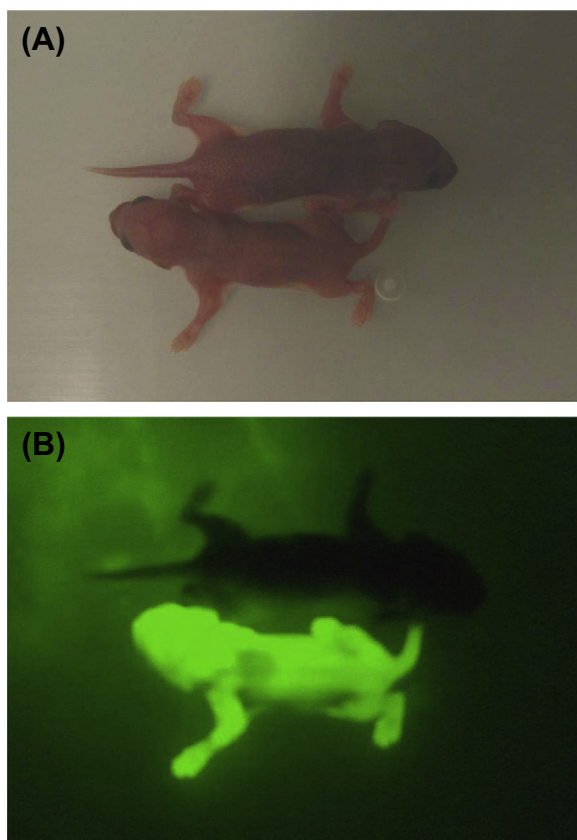
Figure 1.5.1B Generation of transgenic mice using the SB transposon system. According to the method described in [54] the circular pCAGGS-Venus plasmid was coinjected with the transposase mRNA into the pronucleus and the cytoplasm of mouse zygotes. On average, more than 60% of the F0 generation born gave rise to transgenic founders as detected by genomic PCR and through mostly uniform expression of the Venus reporter in the skin of the newborns as shown for transgenic and non-transgenic littermates. (A) Bright field image; (B) fluorescence image (FSH/LS light source with Venus filter, BLS, Hungary). *Photo: Katharina Katter.*

derivatives (usually all fetal and a subset of extra-embryonic lineages) [58]. Given the appropriate conditions during culture, they are able to self-renew *in vitro* and to repopulate the body and the germline of an embryo after retransplantation into a host blastocyst [59]. Several genes have been associated with self-renewal in cultured mouse ES cells. In particular, the transcription factors encoded by *Oct4*, *Sox2*, *Stat3* and *Nanog* are crucial for sustaining pluripotency [60-63].

Attempts to isolate and culture pluripotent ES cells from different inbred strains of mice revealed that the genetic background of the donor strain is of eminent importance. Most ES cell lines used for gene targeting during the last two decades are descended from 129 substrains [64, 65]. Unfortunately, neither 129 mice nor undefined hybrids with mixed genetic background are optimal for most experimental studies. However, recent developments in stem cell research have paved the way to routine use of ES cell lines of more appropriate inbred strains instead of being restricted to derivatives of 129 substrains [66]. It is suggested that the variable ability to establish cultured ES cell lines is due to strain-specific differences in the auto-inductive stimulation of the mitogen-activated protein kinase (ERK) pathway. The normal activation of ERK signalling during early embryo-genesis impairs self-renewal to keep ES cells pluripotent [67]. Therefore, a new culture regime for murine ES cells includes two inhibitors ('2i'-culture): one for the inhibition of the prodifferentiation ERK pathway and a second for the inhibition of the glycogen synthase kinase 3 (*Gsk3*) to suppress residual differentiation [68].

To avoid time- (and animal-)consuming back-crossing to generate a congenic strain with appropriate background for the induced mutation, ES cells originating from C57BL/6 inbred mice are now predominantly used for gene targeting. Therefore, targeted ES cells available from the International Knockout Mouse Consortium (IKMC), a group of four organizations working together to provide freely accessible loss-of-function mutations of all protein-coding genes in the mouse genome, exclusively uses ES cells of C57BL/6N origin [69].

Nevertheless, the general experience of most transgenic laboratories is that more effort is necessary to generate mutants with C57BL/6 ES cells. Instability of the karyotype and loss of pluripotency may be responsible for reduced germline contribution of the produced chimeras (Figure 1.5.2). Optimal culture conditions and appropriate strains to produce host blastocysts are therefore essential for successful gene targeting projects.

Since cultured ES cells are pluripotent, the most common technique to produce a mouse from a targeted ES cell clone is injection into a host blastocyst. Alternatively, injection of ES cells into the morula stages and even into the perivitelline space of mouse zygotes has been

Figure 1.5.2 A male germline chimera and his F1 offspring after mating with a C57BL/6N female. The chimera was generated by injection of XY (male) ES cells of C57BL/6N origin into BALB/c host blastocysts. The extent of chimerism can be evaluated by the coat colour. All offspring of the chimera have a black coat colour, suggesting an injection of the ES cells into an XX (female) embryo. Due to a high contribution of the injected XY cells to the gonads of the developing embryo, the chimera developed as a male that will exclusively produce sperm cells of ES cell origin.

used to efficiently produce germline chimeras [70, 71]. Another simple method to generate mouse mutants from ES cells is aggregation with denuded eight-cell embryos after removing the zona pellucida [72].

Several techniques to alter ES cells genetically have been developed and have made the mouse uniquely approachable as a mammalian organism for genetic studies. The primary application of gene targeting was the generation of null mutations to evaluate the function and regulation of a gene during ontogenesis. However, the genetic tractability of ES cells allows the induction of several other kinds of mutations including the induction of precise point mutations in endogenous genes and structural chromosomal aberrations. In contrast to methods using gametes and embryos for

transgenesis, mutations induced in ES cells can be verified *in vitro* before starting to produce the mouse mutants.

Mutation of endogenous genes induced by gene targeting

In contrast to different methods based on random transgene integration, gene targeting allows both the predetermination of the integration site of the transgene and precise insertion without the extensive sequence rearrangements that are common for random integration of naked transgenes. The procedure is based on HR and can be used for the targeted mutation of endogenous genes but also for the integration

of transgenes at predefined and appropriate loci in the mouse genome.

HR is a replication-dependent and conserved process in the S phase of cell division and important for genomic integrity. The accurate repair of double-strand breaks (DSB) poses a particular concern for the cellular repair machinery. In ES cells DSB are repaired by both HR and non-homologous end-joining [73]. A comparison of both reactions in unfertilized and fertilized oocytes of the African clawed frog *Xenopus laevis* revealed a dramatic change from predominantly HR before fertilization to prevalently NHEJ after it [74]. These results are also echoed in transfected ES cells, where the frequency for targeted mutations is two or three orders of magnitudes lower than random integrations of the targeting construct. Both the introduction of a selection marker into targeting vectors and efficient screening strategies using the polymerase chain reaction (PCR) and Southern analysis are necessary to identify rare homologous integration events.

The first strategy to enrich ES cells that have undergone HR in non-selectable genes was termed *positive/negative selection* [75]. The enrichment of cells with an integration of the targeting vector is based on the expression of a drug resistance gene. A collection of antibiotic resistance genes are available as positive selection markers. Most widely used is the neomycin transferase gene (*Neo*). Other markers routinely used for positive selection are hygromycin, puromycin, blasticidin and Zeocin™ resistance.

As all ES cells with the targeting vector either randomly or homologously integrated are antibiotic resistant, a negative selection step can be additionally used for further enrichment of clones that underwent HR. The thymidine kinase (TK) gene of the herpes simplex virus and the diphtheria toxin A-fragment gene (DT-A) of *Corynebacterium diphtheriae* are the most commonly used negative selection markers. Both markers act as cell autonomous toxins. The negative selection marker is not recombined into the genome in the case of HR and therefore only cells with a correct targeting event will survive the negative selection step.

Selection markers are generally driven by strong promoters to facilitate efficient enrichment. Therefore, the marker has to be removed after detection and isolation of mutated clones to prevent its regulatory elements from interacting with neighbouring genes [76, 77]. This is usually done by site-specific recombination systems either during the ES cell culture or by crossing the generated mutants with mice expressing a site-specific recombinase.

Another source of confounding effects for phenotype analysis of targeted mutations is the targeting vector design. Dramatic differences in the phenotype have been observed as a consequence of the 'neighbourhood effect' among mice generated using different knockout strategies of the same gene [77-80]. For example, null alleles generated by large sequence deletions can also eliminate regulatory elements of neighbouring genes.

The most common type of a gene-targeting vector is the replacement vector, typically consisting of two arms of homology flanking the positive selection marker [81]. The challenge of vector design is to create the desired mutation without affecting the activity of adjacent genes. Complementation testing to restore the phenotype of a gene knockout can be used to identify neighbouring uncertainties. However, the reintroduction of the sequence of the targeted gene to recapitulate authentic wild-type conditions is often challenging [82]. A novel method developed for high flexibility in engineering targeted mutations can also be used as a complementation test to identify possible artefacts of a targeted mutation. The approach is based on a recombinase-mediated conditional inversion of an essential segment of the target gene. Site-specific recombinases like Cre induce an inversion of a sequence between opposing *loxP* recognition sites. The strategy is therefore designated as COIN (COnditional by INversion) [83, 84]. Depending on the orientation of the gene segment, the inversion will induce a knockout or convert a null allele to a phenotypical wild-type allele (see also 'Conditional control of genetic modifications' later in this chapter).

In principle, it is important that the researcher is knowledgeable not only about the target gene but also about adjacent genes and their regulatory elements. Nowadays, the necessary data are available from public resources providing detailed information about single genes. Comparison of sequences from the mouse

and the human genome databases can also provide information about highly conserved sections in introns, which may be of regulatory relevance. In general, the targeting vector has to be designed to induce a specific mutation by the smallest possible genetic alteration.

Initial attempts to target foreign DNA into specific chromosomal sites used pronuclear microinjection. However, only 1 of about 500 transgenic lines was identified with a targeting event [85]. Routine targeting of foreign DNA into the mouse genome first became possible in pluripotent ES cell lines in combination with efficient procedures for the selection of targeted cell clones [86]. Gene targeting by HR is achievable whether or not the target gene is actively transcribed. Therefore, the approach is applicable for any murine gene. The most efficient method for delivering the targeting construct into ES cells is by electroporation.

An important practical question is whether one needs to repeat a targeted mutation with another mouse generated from a second targeted, independent ES cell clone. To exclude random rearrangements in the targeting vector and in the targeted locus, the screening strategy must be sufficiently rigorous to identify false-positive clones and makes a collaborating mutant line unnecessary.

It appears more important to consider that ES cells are prone to collecting random mutations. For example, karyotyping of targeted clones is routinely used as a quality check before the generation of chimeras. Different types of aneuploidy are frequently observed and are thought to be responsible for germline transmission failure. In contrast, ES cell clones with small deletions and duplications have been found to be germline competent. Moreover, copy number variation seem to occur frequently in routinely cultured ES cells, resulting in genetic variants without affecting the germline competence of the cells [87]. Mutations in ES cells which do not interfere with germline transmission can be cotransmitted with the targeted mutation into the mouse germline. Until now, researchers have paid little attention to this source of confounding experimental results. Therefore, unexpected and varying phenotypes of a novel targeted mutation on a defined genetic background should be carefully analysed for the impact of spontaneous mutations transmitted via cultured ES cell lines.

Gene trapping in embryonic stem cells

Gene trapping is a strategy for random insertional mutagenesis. The aim is to mutagenize an endogenous gene of the mouse genome at the integration site by insertion of a specific designed trap vector. Trap vectors in combination with ES cells provide a valuable tool to discover unknown genes and to elucidate the regulation of their activity [88]. Usually, a trap vector consists of a promoterless reporter gene (usually *lacZ* for expression of beta-galactosidase) that will be exclusively expressed after insertion into an endogenous gene. A large proportion of the mouse genome is expressed in undifferentiated pluripotent ES cells. Since most gene trap vectors are dependent on gene expression, only the proportion of endogenous genes that are expressed in ES cells can be efficiently detected.

The discovery of sequences untrappable by conventional strategies could be facilitated through the addition of the neomycin transferase gene as selectable marker or by using the beta-galactosidase-Neo^R (β-GEO) fusion marker in the trap vector. The β-GEO marker is a fusion protein encoded by *lacZ* and the neomycin resistance gene. To prevent the secretion of the selection marker when integrated into a secretory pathway gene, a transmembrane domain is added to the trap vector anchoring the secretory (marker) protein to the cell membrane [89].

Gene trapping and promoter trapping usually depend on integrations within a gene. Since the reporter gene of those vectors is flanked downstream by a polyadenylation signal, transcription of the trapped gene is terminated prematurely. This simultaneously inactivates the trapped gene and reports its expression pattern. Moreover, the vector can be used as a sequence tag to efficiently identify the disrupted gene by RACE PCR (rapid amplification of cDNA ends).

Gene trapping in ES cells produces not only null mutations but also allelic series of the same gene. Such a panel of multiple mutations within the same gene often causes phenotypes that are of varying severity or completely different.

They can also allow for detection of alternative splicing and different transcription start sites of a trapped gene. Newly designed trap vectors include binding sequences for routinely used recombinases to allow for postinsertional modification to create additional alleles for further analysis of the trapped genes.

Trap vectors can be delivered by electroporation or viral infection into ES cells [90]. Both approaches have advantages and disadvantages [91, 92]: Electroporated plasmid-based constructs can form transgenic arrays before chromosomal integration and are exposed to partial digestion by endonucleases, making the identification of the trapped genes problematic, whereas viral vectors have only limited cargo capacity and are biased towards integration into actively expressed loci.

A novel tool for gene trapping might be sequentially activated TEs containing a trap vector. The application of gene trapping approaches is a concerted activity of large-scale mutagenesis centres under the auspices of the International Gene Trap Consortium (IGTC). Thousands of mutagenized ES cell clones are currently archived in frozen libraries and freely accessible to the scientific community.

Advanced methods of transgenesis

Targeted mutagenesis in mouse zygotes using nucleases

For a long time, gene targeting via HR in ES cells was restricted to laboratory mice. With the recent successful development of rat ES cells it became possible to extend gene targeting technology to another species [93-95]. In spite of tremendous efforts in many laboratories around the world, the brown or Norway rat (*Rattus norvegicus*) is the only species apart from the mouse where germ-line competent ES cells have been successfully established. Gene targeting in other species is conducted in differentiated cells followed by reproductive cloning via nuclear transfer. Although there have been occasional successes,

the nuclear transfer approach often fails due to poor perinatal viability of cloned mammals [96, 97]. Therefore, most other mammalian species are still lacking feasible tools for targeted mutagenesis. Recently, the use of zinc finger nucleases (ZFN) has been successfully tested as an ES cell-independent approach for genome editing applicable in mice and in other mammalian zygotes [98, 99].

ZFN are designed by combining zinc fingers (DNA-binding domains of eukaryotic transcription factors) with the nuclease domain of the *Fok*I restriction enzyme [100]. The robust cleavage activity of *Fok*I is restricted to a DNA-binding event and the binding specificity of a ZFN is provided by linking 3-6 triplet specific zinc fingers to bind a 9-18 bp target sequence. These multifinger peptides can be modularly assembled from fingers developed for most triplet sequences [101-103]. Because *Fok*I monomers must dimerize to cleave, two ZFNs are needed which bind specific sequences downstream and upstream of the cleavage site.

ZFN are used to induce a DSB at a specific point of a target gene. Repairing a DSB by the error-prone NHEJ pathway occasionally includes small alterations of the sequence at the site of the break, possibly resulting in disruption of the target gene. Targeting events after pronuclear and intracytoplasmic injection of ZNFs as mRNA into C57BL/6 and FVB mouse zygotes resulted in 20-75% offspring with a targeted mutation in the form of specific deletions ranging from several bps to more than 1000 bp in length [104, 105].

Interestingly, ZFN can also be used to target a foreign DNA into a specific chromosomal site. This strategy is based on the observation that the induction of DSBs in mammalian cells increases the rate of HR by several orders of magnitude [106]. After pronuclear or intracytoplamic coinjection of a *Rosa26* specific ZFN with a targeting construct containing the beta-galactosidase gene, 1 out of 22 offspring was found to have a homologically directed integration of the transgene [99].

Compared to the unusable low frequency after simple pronuclear injection of a targeting construct [85], the use of ZFN seems to be a suitable approach for ES cell-independent gene targeting. This strategy could expand further in

the future by utilizing bacterial transcription activator-like effector nucleases (TALENs) [107, 108]. Similar to the molecular architecture of ZFNs, sequence-specific TALENs are fused to the catalytic domain of the *Fok*I endonuclease and have been successfully tested to direct DNA DSBs to specific target sites [105].

The application of engineered nucleases for genome editing in the mouse provides several advantages in comparison to gene targeting in ES cells: (i) it is applicable in zygotes by microinjection, which is a well-established technique; (ii) positive and negative selection markers are dispensable; and (iii) the frequency of animals with targeted mutations is high enough for routine use. Therefore, targeted mutagenesis using sequence-specific nucleases is a promising tool for genetic studies in a great variety of scientific model systems. Nevertheless, the risk of insertional mutagenesis by off-target cleavage still has to be taken into account. Comprehensive genotyping of the resulting mutants combined with a bioinformatic approach will be indispensable in permitting detection of undesirable mutation events.

The knockdown approach by RNA interference

RNA interference (RNAi) is a new tool that can be used as a fast alternative to the conventional gene targeting approach. The strategy is based on the post-transcriptional silencing of an endogenous gene by induced fragmentation of the corresponding mRNA. The resulting animals are usually described as 'knockdown' because the expression is only downregulated and not completely abolished as it is for a knockout mutation. In contrast to the conventional gene targeting procedure, a knockdown is induced without modifying the chromosomal locus. The target molecule of RNAi is the mRNA of a gene of interest.

RNAi is an endogenous pathway for gene regulation found in many eukaryotes including mammals [109]. RNAi-mediated gene silencing in transgenic mice is usually triggered by short hairpin RNAs (shRNA, small RNAs that form hairpins) which are expressed from a transgene. The RNAi pathway starts with the cleavage of

the hairpin structure into small fragments by a cellular mechanism. These fragments are subsequently integrated into the RNA-induced silencing complex (RISC). The RISC binds to the complementary sequence of an mRNA which will then be cleaved by the catalytic component of the RISC complex. By leaving the genomic sequence unchanged, the conditional expression of shRNA allows for a tissue-specific and reversible alteration of the target gene [110, 111]. Furthermore, the RNAi approach usually affects all RNA molecules produced from a gene by alternative splicing.

Although the knockdown strategy will not replace gene targeting, it is a powerful alternative tool for mammalian genetics. However, RNAi is currently not widely used as a routine method for mouse transgenesis. Transgenic animal models expressing shRNA are usually generated by pronuclear injection or via viral vectors. The resulting animals display highly variable degrees of gene silencing, as is characteristic for mutants produced by random integration of the transgene [112]. Targeting the shRNA expression vector into an appropriate genomic locus as *Rosa26 or ColA1* would circumvent the unreliability of random transgenesis but depends on rare HR events. (*Rosa26* is a constitutively transcribed locus in the mouse genome originally identified by gene trapping and has been used for ubiquitous transgene expression. The locus was named according to the trap vector design—reverse orientation splice acceptor) [113]. To overcome these drawbacks, a new and efficient strategy has been developed that is applicable to ES cells for robust and controllable gene silencing in mice [114]. It uses the recombinase-mediated cassette exchange approach (RMCE) instead of HR to reproducibly introduce the shRNA expression vector into the preselected chromosomal site [115, 116]. Furthermore, new strategies have been developed for conditional gene knockdown using RMCE in combination with site-specific recombinases or transcription initiation [117].

Current expression vectors for shRNA use a natural microRNA (miRNA) backbone and are therefore transcribed by RNA polymerase II [118]. This is important to avoid toxic effects of the transgene potentially induced by an interferon response to polymerase III-driven shRNA

expression [119]. Furthermore, a successful shRNA vector design should result in the exclusive knockdown of the target gene. An unwanted off-target effect could, however, be possible if the shRNA shares sequence homology with other genes of the genome.

Conditional control of genetic modifications

The study of gene function and regulation by simple inactivation is often limited by the pleiotropic nature of many genes. Pleiotropic genes can be responsible for several distinct and unrelated phenotypic effects. An embryonic or perinatal lethal phenotype of an induced knockout would only reveal the first non-redundant function of a targeted gene without detecting any role of the gene during later developmental stages. Even if the mutation is non-lethal, compensatory mechanisms can mask a future phenotype when it is induced constantly during ontogenesis.

A genetic modification may also interfere with the viability and fertility of the mutant and render the breeding of the affected animals difficult or impossible. Finally, the mutation can cause a strong phenotype even in the absence of experimental treatment. The resulting animal welfare problems may impair the maintenance and breeding of the mutant line. Most of these issues can be resolved by using a conditional mutation and a variety of systems have been developed to be applied in transgenic mammals.

The activation or silencing of a (*trans-*) gene can be controlled temporally or in a tissue-specific manner and some systems are reversible. Conditional gene targeting is consequently used by the members of the IKMC and has enormously expanded the versatility of mouse models.

The routinely used approach for conditional gene expression is a binary system consisting of two transgenic mouse lines: one contains the effector construct and the other contains the responder (target) gene. Crossing both lines allows the two components of the system to interact in the resulting conditional line [120].

Widely used systems for conditional control of gene expression are based on site-specific recombinases (SSRs). Currently used examples of SSRs for genome engineering are the Cre recombinase from bacteriophage P1 (recognizes and binds to specific sequence sites called *loxP*) [121], the Flp recombinases from *Saccharomyces cerevisiae* (binds to *Frt* sites) [122] and the Dre recombinase from a P1-related phage (binds to *Rox* sites) [122, 123]. It should be noted that the wild-type Flp recombinase becomes unstable at a temperature above 37 °C resulting in mosaic recombination [124]. Therefore, the thermo-stable and codon-optimized variant Flpo should be used for *in vivo* applications in mice [125, 126]. Also a codon-improved Cre recombinase (iCre) was developed and successfully tested to reduce its occasionally observed epigenetic silencing in mammalian cells [127]. Since the different SSRs are heterospecific they can be used to act simultaneously in the same genome. Moreover, several mutant *loxP* or *Frt* sites are available, which cannot recombine with each other or with the wild-type site. This significantly increases the control and flexibility of SSRs [128, 129]. Currently used targeting or trap vectors can therefore be designed to allow different kinds of postintegration site-specific recombinations in parallel in a genome.

Along with improving the recombination efficiency of existing SSRs, a fourth system, the PhiC31 integrase, has been successfully tested in the mammalian genome and might become a routinely used tool for genetic engineering [126, 130, 131]. In contrast to the *Cre/loxP*, *Flp/Frt* and *Dre/Rox* systems, PhiC31 naturally recombines between two heterotypic recognition sites (*attB* and *attP*), which are altered by the recombination. The remaining *attL* and *attR* sites are no longer substrates for the recombinase. Unlike the other systems, PhiC31 mediates irreversible recombinations. Therefore, the PhiC31 system could be an ideal tool for site-specific integration of transgenes [132].

All currently used systems mediate a conservative recombination between their corresponding recognition sites. Depending on the orientation of the recognition sites, SSRs mediate deletions or inversions of flanked DNA fragments in cis. Furthermore, the *Cre/loxP* system has been used to generate chromosomal rearrangements in trans [133, 134]. SSRs can be used for many other applications in addition to

conditional gene expression. Most important is the deletion of so called 'floxed' or 'flrted' selectable markers after gene targeting in ES cells.

Since the first demonstration of a transgene activated by Cre-mediated deletion of a stop cassette [135], a large collection of effector mouse lines with specific expression patterns for the Cre recombinase have been produced (see database of Cre transgenic lines, http://nagy.mshri.on.ca/cre_new/index.php). This currently qualifies Cre as the most essential tool for conditional mutagenesis. However, prolonged Cre expression can be detrimental in cultured cells and in mice [136, 137]. Therefore, Cre should be eliminated as soon as possible after completion of the site-specific recombination.

SSRs can also be used as a ligand inducible fusion protein consisting of the recombinase and the oestrogen receptor (ER) or progesterone binding domain (PBD). The CreERT2 fusion protein has become the favoured system for inducible Cre recombination through treatment of the mice with tamoxifen, a synthetic ER antagonist [138].

Another widely used system for conditional transactivation is the tetracycline-dependent (TetRepressor based) system [139]. Two variants are available according to the ability of the transactivator to bind DNA. The tetracycline transactivator (tTA) cannot bind the operon sequence (*tetO*) when the inducer (doxycyclin) is present (tet-off). In contrast, the reverse tetracycline transactivator (rtTA) will bind DNA and thereby activate the responder gene after application of doxycyclin (tet-on).

The system can be used to reversibly activate and silence a transgene. Many impressive experiments have been conducted in which reversibility of the target gene expression was demonstrated [140-142]. However, it has to be taken into account that the kinetics of doxycyclin in the organism is slow and therefore this system is inappropriate for inducing fast phenotypic changes. Another limitation is the variable drug bioavailability in a whole organism. Since the inducer is not equally available in all tissues, the doxycyclin response will vary according to the local drug concentration.

While these conditional systems can be extremely useful, care must be taken during the generation of the required mouse lines. When the transgenic lines are produced using illegitimate DNA recombination and random integration as it is currently done, the transgenes are accordingly subject to position effects that affect their expression. Differences in expression level and expression pattern can cause leakiness of the system before induction, or somatic mosaics due to incomplete activation/inactivation of the target genes. These limitations are usually overcome by producing several transgenic lines to test the appropriate combination of effector and responder lines, or by producing the lines using targeted integration of the transgenes.

Fluorescence reporter in transgenic mice

Genetic markers are powerful tools for phenotypic analysis of transgenic mice. The bacterial *lacZ* gene, encoding beta-galactosidase has been the marker of choice for some time. Beta-galactosidase cleaves a variety of substrates to produce a colorimetric manifestation of the expression pattern of a (*trans*-)gene. It is still popular for histological analysis of distinct cell populations and for reporting the function of regulatory elements of specific genes [143]. Since beta-galactosidase staining requires fixation of the tissue, it cannot be used to mark living cells. Therefore, the development of vital imaging based on genetically encoded fluorescent markers provides an important new tool for functional mouse genomics.

Today, several fluorescent markers cloned from jellyfish (*Aequorea victoria*), sea anemone (*Discosoma* sp.) and other marine organisms are available and can be selected for specific applications. The green fluorescent protein (GFP) was the first fluorescent marker [144]. The original GFP was then sequence-optimized by mutagenesis to meet the requirements for enhanced and stable expression in living mammalian cells and this new enhanced version (eGFP) is now the most popular fluorescent marker [145]. Further mutagenesis of GFP has not only improved its applicability in terms of thermostability,

maturation kinetics and brightness; researchers were also able to create new variants with distinct spectral properties. Venus, named after the bright planet, is currently one of the most brilliant fluorescent proteins derived from GFP. Although its excitation and emission maxima are further red-shifted, Venus can be detected with standard GFP filters. Due to its enhanced brightness, it is easily detectable even at lower concentrations using current imaging systems. This might be an important advantage since we and others have shown that fluorescent protein (eGFP in particular) is toxic in high concentrations because it can induce heart failure during embryogenesis [146]. Furthermore, most if not all GFP-specific antibodies cross-react with Venus and can be used for immunohistological studies.

After the cloning of GFP and the success of eGFP in biological research applications, much effort was directed towards generating red fluorescent proteins (RFP). A second group of fluorescent markers with sufficient brightness and spectral separation from eGFP allows dual labelling applications. Additionally, the emission wavelength of RFP has clear advantages for deep imaging of animal tissues due to better tissue penetration of red light and reduced autofluorescence. Therefore, red fluorescence labelling would enable non-invasive whole-body imaging for the analysis of transgene activity, cell tracking and other experimental applications in living organisms [147].

The original RFP (DsRed) functions as an obligate tetramer [148]. However, for the construction of fluorescent fusion proteins a monomeric active marker would be the first choice. mCherry was the first monomeric RFP widely used as a marker for *in vitro* and *in vivo* applications [149]. Several other RFPs with improved photostability and brightness are also currently applied in mouse transgenesis. Well-defined examples are the tandem dimer tdTomato [149] and the dimeric Katushka [150]. The improved monomeric form of Katushka, mKate2, is currently under investigation as a fluorescent marker in mice [151]. All available RFPs can be easily detected with standard RFP filter sets designed for DsRed or Texas Red. Since GFP and RFP are of distinct origin, antibodies do not cross-react between the two fluorescent proteins.

Because of the wide range of possible applications, there is increasing interest in using fluorescence proteins in biotechnology. Their use in transgenic animals provides a valuable tool for advanced phenotyping (Figure 1.5.3).

Perspective

Since the first transgenic mice were produced about three decades ago, new and improved methods have been developed, enhancing the power of transgenic technologies. Transgenesis
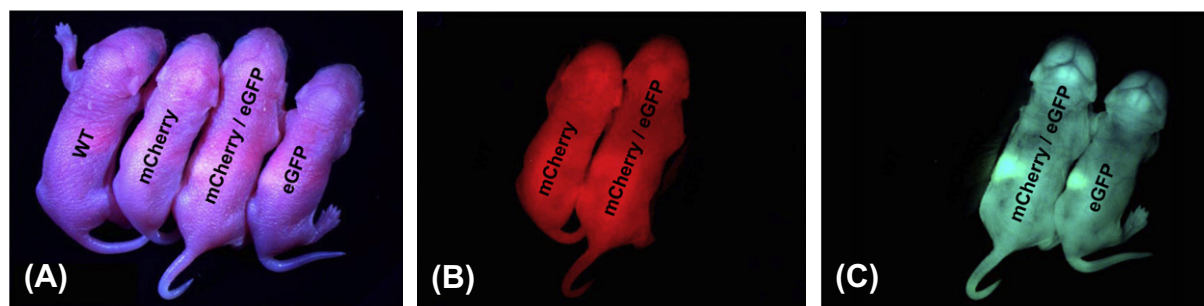
Figure 1.5.3 Expression of green and red fluorescence reporter proteins in transgenic mice. Whole-body expression of eGFP and mCherry in single and double transgenic neonates is shown. Double transgenic (hemizygous) offspring expressing eGFP and mCherry were generated by mating animals of the single transgenic lines. Due to the perfect spectral separation both reporters can be combined for fluorescence labelling applications. (A) Bright field image of littermates: transgenic for only eGFP, mCherry or both fluorescence reporters; (B) mCherry fluorescence signal (TXR filter, Leica, Germany); (C) eGFP fluorescence signal (GFP3 filter, Leica, Germany). *Photo: Dieter Fink.*

in mice is a multistep procedure starting with the design and construction of the transgene, followed by a variety of methods to stably integrate it into a mouse chromosome. Moreover, several strategies are available to alter the structure and the activity of a (trans-) gene after it has been incorporated into the mouse genome. Both techniques to engineer the mouse genome and tools to precisely characterize the phenotype of the resulting mutants have multiplied in the previous two decades. There are two advancements with great impact on functional mouse genomics. The first is the phage-based recombineering system in *Escherichia coli* to produce transgenes and gene targeting constructs in a high-throughput manner [84, 152]. The second is the development of different classes of SSRs and nucleases to manipulate the mouse genome.

In addition to the numerous technical developments, coordinated international projects are currently in progress to generate mouse mutants in a standardized and cost-efficient manner. The IKMC aims to generate conditional knockout alleles for nearly all genes of the mouse genome in ES cells. At the same time, the IGTC generates further experimental mutations in addition to the loss-of-function alleles generated by gene targeting. The mouse will therefore be the first mammal with both a sequenced genome and a full description of the corresponding gene functions.

The methods for gene targeting and gene trapping have been adapted for high-throughput use, making *in vivo* mouse genetics available in a faster form and to a much wider research community than ever before. However, scientists who have recently turned to the mouse as their research model are sometimes unaware of specific factors influencing the outcome of animal experiments. It is generally accepted that the phenotype of a mouse mutant can be seriously affected by the genetic background. The decision of the IKMC partners to consistently use C57BL/6N as donor strain for ES cells was therefore an important step to ameliorate the comparability and reproducibility of experimental results and followed the general recommendations concerning the genetic background of mutant mice [16]. However, genetic differences between substrains of C57BL/6 have been detected and should also be considered for breeding of the mutant line and selection of the wild-type control animals [153, 154].

Moving a mutation from a mixed genetic to a defined (inbred) background or between various inbred strains to control background effects is accomplished by congenic breeding strategies. The standard procedure calls for at least 10 backcross generations or about 3 years breeding effort. Marker-assisted selection protocols have reduced the number of required generations to less than half of that [155].

The extreme increase in the number of mouse mutants has caused serious problems in maintenance and administration of the animals. Because most of the mutant lines are not commercially available, they are exchanged between research laboratories with unequally hygienic conditions. To reduce the risk of a hygienic contamination of the animal facility and to make new animal models available to the scientific community, central repositories have been founded. The task of the European Mouse Mutant Archive (EMMA) and other mouse resources is the collection, archiving and distribution of valuable mouse mutants [156]. The animal models are available as frozen embryos or germ cells and can be ordered as mice in SPF (Specific Pathogen Free) quality.

The direct availability of mouse mutants relevant in biomedical research will save time and money for competitive research projects. Furthermore, the central publication of distributable mutants in databases can avoid unnecessary repetitions of a mouse mutant and thereby reduce the number of experimental animals. The correct application of the standardized nomenclature for rodent strains, genes and mutations when naming a new transgenic line facilitates its incorporation and retrieval from databases. Moreover, a rigorous identification of each mutation is indispensable for the maintenance and administration of great numbers of mouse mutants in animal facilities, archives and databases [157]. However, it should always be appreciated by the scientist that a correct designation of transgenic rodents also enables precise communication of published experimental results.

Our ability to genetically modify the mouse genome has become very sophisticated in recent years. However, the high-throughput and reliable

generation of mouse mutants for each gene is not adequate to maximize the potential of engineered mouse models in biomedical research. So far, most scientists have concentrated their investigations on phenotypic changes expected in the transgenic line according to their knowledge about the induced mutation. However, the traditional assumption of a direct link from gene to function is insufficient for complex biological traits. Due to pleiotropism, a gene may produce multiple effects in different organs and at different time points during development. Moreover, overlapping functions of pleitropic genes cause partial redundancy, frequently observed in knockout mouse models [158]. Therefore, the restricted analysis of a mutant mouse in specialized research laboratories needs to be supplemented by a systematic phenotype analysis. A first step toward this goal is the establishment of centres for comprehensive mouse phenotyping according to standardized protocols [159-161]. Furthermore, animal models offer the opportunity for systematic exposure to specific environmental challenges. Currently, mouse mutants are mostly analysed under highly standardized conditions that may not expose the animal's capacity to react to environmental changes. A systematic analysis that reflects the complexity of a mammalian organism will not only reveal new functions of investigated genes but also provide a better mouse model for the dissection of human disease pathways.

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Early Mouse Development

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Introduction

Mouse embryonic development has been studied for a long time, and over the past three decades enormous progress has been made in the analysis of cell fates, developmental potential of cells in the early embryo, and the understanding of molecular mechanisms regulating patterning and differentiation. Mouse embryonic development takes 18-20 days depending on the strain (Figure 2.1.1). The first two differentiated cell types are present in the blastocyst 3.5 days after fertilization, and implantation takes place 1 day later. Development proceeds rapidly after implantation with the formation of the egg cylinder. With the onset of gastrulation around embryonic day 6.5, dramatic morphological changes occur: the three embryonic germ layers are laid down, and the basic body plan with its major axes is established. Organogenesis commences with the formation of a functional cardiovascular system at embryonic day 8.5; by day 11.5 of embryonic development, primordia of most organs are established. During subsequent stages until birth, organs are further elaborated and the embryo continues a massive growth programme.

The aim of this chapter is to provide a basic introduction to mouse development until the early postgastrulation period. The intention is to give an overview rather than being comprehensive, in order to help newcomers working with mice to better understand more detailed and specialized descriptions and illustrations [1-4] as well as instructions for various kinds of manipulations of pre- and postimplantation embryos and embryonic stem cells [5-7].

Fertilization and preimplantation development

Fertilization

Fertilization, the fusion of the female (oocyte) and male (sperm) gametes, activates the egg to commence embryonic development. The specialized structures of oocyte and sperm and the unique genetic complement carried by each
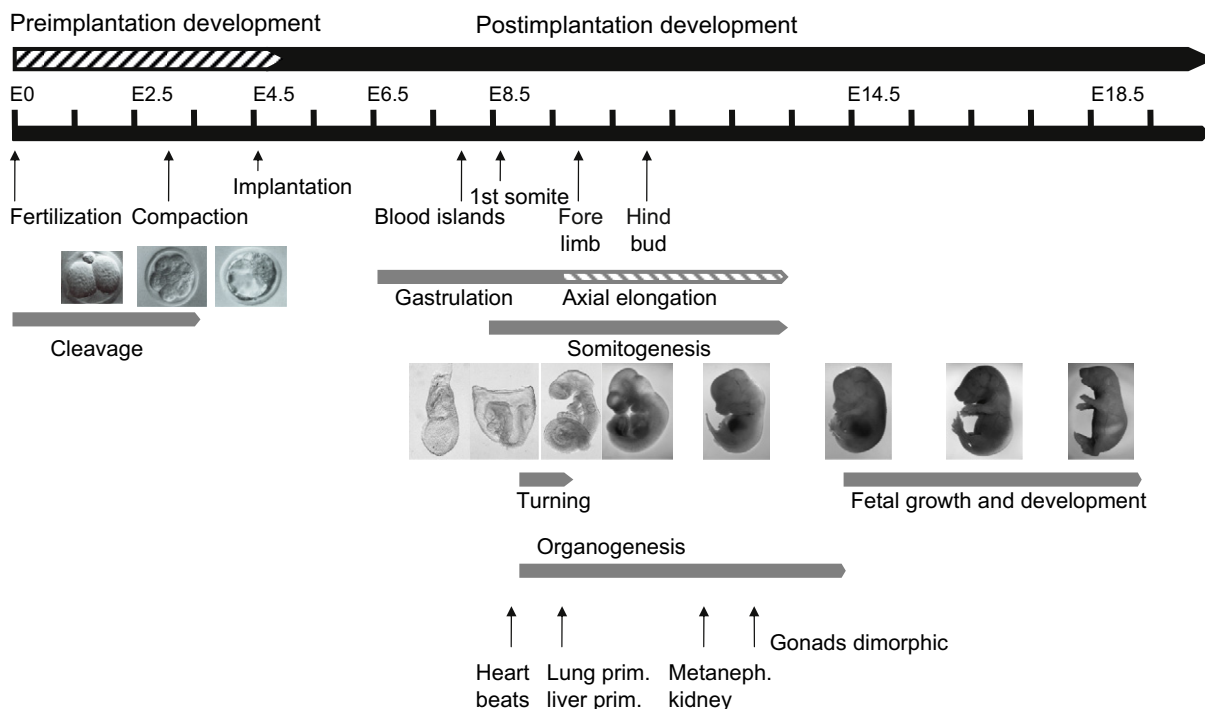
Figure 2.1.1 Overview of mouse development. Mouse embryonic development takes approximately 19 days. The pre- and postimplantation phases are indicated above the timeline, critical events and processes are indicated below the time line. For details see text.

gamete as a consequence of meiotic recombination are generated during gametogenesis. In mature sperm the genetic material is reduced to a haploid set of chromosomes, whereas in the oocyte reduction to haploidy is achieved only after fertilization (see below). A fully grown mouse oocyte ready to undergo the final maturation steps measures about 85 μm in diameter and is surrounded by a thick extracellular envelope, the zona pellucida, which in turn is embedded in multiple layers of follicle or granulosa (cumulus) cells. The large nucleus, called the germinal vesicle, contains the chromosomes in the prophase of the first meiotic division. In each hormonal cycle, which takes about 4–5 days in the mouse, only a few follicles respond to an increase in the level of follicle stimulating hormone (FSH), mature, accumulate fluid and move towards the periphery of the ovary. Shortly before ovulation the level of luteinizing hormone (LH) surges and the nuclear maturation of the egg commences with the disintegration of the nuclear membrane (germinal vesicle breakdown) and resumption of meiosis. One set of chromosomes is removed from the egg together with some cytoplasmic material as the first polar body. Nuclear

maturation is arrested in the metaphase stage of the second meiotic division and only proceeds after fertilization. Upon ovulation eggs are transported into the ampulla, the most anterior part of the oviduct, by the movements of the cilia of the epithelial cells that line the opening of the oviduct facing the ovary (infundibulum). Each egg is surrounded by its zona pellucida and cumulus cells, the latter being embedded in an extracellular matrix of proteins and hyaluronic acid. Freshly ejaculated sperm is initially not able to fertilize the egg; it becomes competent to do so in the course of its sojourn in the female reproductive tract. In this capacitation process, which involves various signalling events, the sperm surface is modified by removal of decapacitation factors acquired in the epididymis [8]. After arrival in the ampulla, the sperm locate to the cumulus cell-oocyte complex in a chemotactic manner [9] and penetrate the viscous matrix of hyaluronic acid surrounding the cumulus cells and the egg to associate with the surface of the zona pellucida. The major components of the zona pellucida are three glyocosylated proteins: Zp1, Zp2 and Zp3 [10]. Various reports indicate that Zp3 functions as the primary sperm receptor,

but Zp2 plays an important role in preventing polyspermy once proteolytically converted by an oocyte-secreted enzyme, and Zp1 crosslinks Zp2/Zp3 to create the filamentous structure of the zona pellucida [11]. The acrosome is a Golgi-derived exocytotic organelle that covers the tip of the sperm head. Binding between egg-binding proteins of the sperm head and the zona component Zp3 elicits (at least *in vitro*) acrosomal exocytosis, the so-called acrosome reaction, and releases proteolytic and glycolytic enzymes that allow the sperm to penetrate the zona pellucida after limited proteolysis [12, 13]. While earlier *in vitro* studies implicated sperm galactosyltransferase as a Zp receptor [14, 15], knockout studies did not support such a role but suggested the membrane-anchored sperm surface protein with integrin and metallopeptidase domain 3 (Adam3, cyritestin) to be the major Zp binding activity on the sperm head [16–18].

After penetrating the zona pellucida, acrosome-reacted sperm immediately meet and fuse with the egg plasma membrane and trigger a cascade of events, which prevent polyspermy and ultimately lead to the formation of the diploid zygote. Sperm–egg fusion requires the Ig superfamily type I membrane protein Izumo1 on the sperm and the tetraspanin protein Cd9 on the egg cell surface, although direct physical interaction between the two has not yet been reported [19–22]. See recent reviews [23, 24] for a more detailed discussion of the molecular factors regulating fertilization.

Fertilization activates the egg and triggers the completion of meiosis. This results in the extrusion of the second polar body and leaves behind a haploid set of maternal chromosomes in the egg's female pronucleus. The nuclear membrane of the sperm nucleus breaks down, the chromatin decondenses and is reorganized, and a new nuclear membrane is formed around the male pronucleus. Then the two pronuclei move towards each other and DNA replication takes place. Upon meeting, the two pronuclei do not fuse but their nuclear membranes break down, the chromosomes assemble on the metaphase plate and cleavage commences with the first cell division. Mature oocytes are transcriptionally silent. An initial burst of zygotic transcription occurs at the end of the one-cell stage, followed by a second larger burst at the two-cell stage [25, 26]. This second burst is accompanied by degradation of maternal mRNAs, most likely by microRNA-mediated mechanisms [27]. Thus, the final steps of egg maturation, early postfertilization events and the first cell division are controlled by stored maternal proteins and mRNA. The mechanisms that control the transcriptional activity in the mature egg and after fertilization are not entirely clear and appear to be multifactorial. However, recent data point to an important role of epigenetic mechanisms encompassing DNA methylation, post-translational histone modification, chromatin remodelling and alterations in nuclear architecture in these processes [28].

Cleavage divisions and formation of a blastocyst

The zygote undergoes a series of early cleavage divisions that produce an increasing number of progressively smaller cells, known as *blastomeres*, without changing the overall size of the embryo. Cleavage divisions in the mouse, as in other mammals, are slow. The first cell division occurs about 20 h after fertilization (Figure 2.1.2). The next divisions follow at approximately 12 h intervals but are not truly synchronous between the different blastomeres. Up to the eight-cell stage, blastomeres are spherical cells, which are loosely attached at their sites of contact. At the eight-cell stage, blastomeres alter their adhesive behaviour and the embryo, now called the *morula*, undergoes a dramatic morphological change. Blastomeres flatten towards each other and maximize cell-to-cell contacts, and the former grape-like structure is transformed into a compact aggregate of cells. This phenomenon is called *compaction* and is a prerequisite for the formation of the blastocyst. Compaction is associated with the formation of adherens junctions, and later tight junctions between the cells [29]. One major component of the compaction process is E-cadherin, a Ca^{2+}-dependent cell adhesion protein that becomes localized to regions of cell contact at the eight-cell stage [30–32]. Compaction does not require *de novo* transcription but is regulated by post-transcriptional mechanisms including the modification and intracellular redistribution of E-cadherin [33, 34].
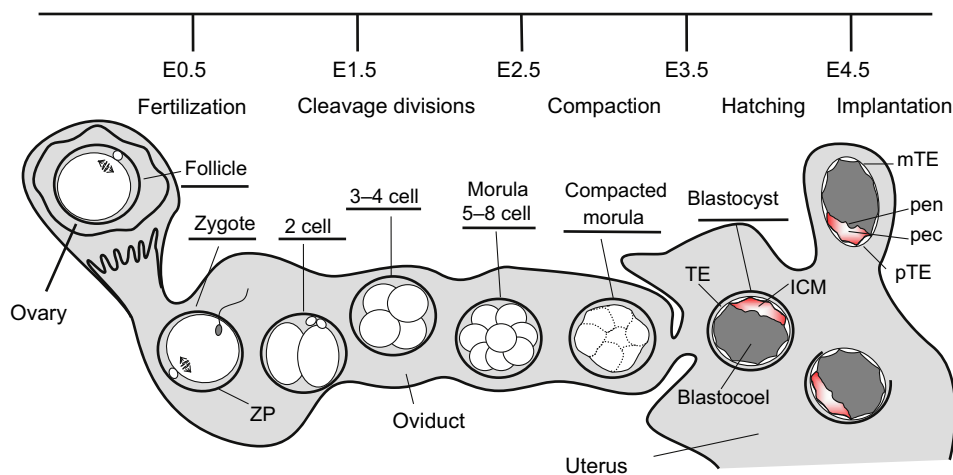
Figure 2.1.2 Schematic presentation of mouse preimplantation development. After fertilization, which occurs in the proximal part of the oviduct, the zygotes undergo cleavage while migrating through the oviduct towards the uterus. Compaction occurs at the eight-cell stage as a prerequisite for blastocyst formation, which occurs around day 3.5 in the uterus. Hatching frees the blastocyst from its zona pellucida and is required for implantation. ICM, inner cell mass; pec, primitive ectoderm; pen, primitive endoderm; TE, trophectoderm; pTE, polar trophectoderm; mTE, mural trophectoderm; ZP, zona pellucida.

Shortly before compaction individual blastomeres develop polarity with distinct apical and basolateral surfaces [35]. Concomitant with changes in adhesiveness, alterations in cytoskeletal architecture, lectin binding properties and distribution of membrane and cytoplasmic components occur [36–38]. It is currently unclear how apical–basal cell polarity is established at the eight-cell stage but it seems that cell contact is partially responsible and may be aided by interactions between the nucleus, microtubules and cortex [39, 40]. Irrespective of the precise molecular mechanisms of initiating polarity, it is accompanied like in other epithelial cells by the differential distribution of polarity complexes including atypical protein kinase C (Pkc) and partition-defective (Par) to the apical and lethal giant larva homologue (Lgl) to the basolateral side [41].

After compaction and polarization two further rounds of cell division double the number of blastomeres from 8 to 16 and finally to 32. Since blastomeres are polarized along their apical–basal axis, the plane of division will affect the distribution of the cellular content to the daughter cells. Divisions parallel to the apical–basal axis result in two daughter cells both of which have inner (basolateral) and outer (apical) surfaces, while division perpendicular to this axis generates one polarized

daughter cell on the outside and one apolarized daughter cell in the inside [42, 43]. In this manner two distinct groups of cells emerge at the late morula stage: polar cells on the outside and apolar cells in the inside. Position and polarity are mutually dependent at this stage, since changing position of a blastomere will alter its polarity and changing the polarity will affect its position [44–46]. Importantly, however, from the 32-cell stage onwards the two cell populations will take different developmental routes. 'Inner' cells generated during cell divisions in the late morula give rise to the inner cell mass (ICM) cells of the blastocyst while outer cells predominantly form trophectodermal cells [47–49]. However, this allocation of cells reflects developmental fate rather than developmental potential, since when cells isolated from the 'inside' of late morulae are reaggregated and cultured *in vitro*, they can form normal blastocysts with ICM and trophectoderm, as is also true for 'outer' cells [44, 50], and the significance of polarization for the differentiation of ICM versus trophectoderm remains unclear.

Around the 32-cell stage a fluid-filled cavity, the *blastocoel*, becomes evident. Outer trophectodermal cells pump fluid into the nascent cavity, which rapidly expands, and seal the cavity by tight junctions [51]. Blastocoel formation shifts

the ICM to one side of the inner surface of the trophectoderm, generating a clear asymmetry along the embryonic (ICM)–abembryonic (blastocoel) axis of the blastocyst. The timing of cavitation seems to depend on the nucleocytoplasmic ratio of DNA or chromosomal replication, but does not depend on the absolute number of cells or cell divisions in the zygote. When the number of cells in the embryo was experimentally reduced or enlarged, or cell divisions were suppressed with cytochalasin-B (which does not affect DNA replication), the time of blastocyst formation was not affected [52].

After formation of the blastocoel at embryonic day 3.5, the blastocyst matures for another 24 h and becomes ready to implant in the uterine wall. Shortly before implantation, at embryonic day 4.5, some of the ICM cells differentiate into a second epithelial cell type, the primitive endoderm. This tissue, which arises on the free surface of the ICM facing the blastocoel, will give rise to the embryonic membranes, i.e. the endodermal component of the visceral yolk sac and parietal yolk sac. The remaining ICM cells, the primitive ectoderm or epiblast, will give rise to the embryo proper and to the extraembryonic mesoderm. The trophectodermal cells give rise exclusively to extraembryonic tissue (Figure 2.1.3).

Up to the eight-cell stage blastomeres have a remarkable regulative ability. Single blastomeres of two- and four-cell embryos can form blastocysts *in vitro* [53], and blastocysts formed *in vitro* from single blastomeres of two-cell embryos can develop into normal mice after transfer into foster mothers [54]. In contrast, individual blastomeres from four- and eight-cell embryos cannot generate a mouse by themselves [55], which is probably due to the small number of cells in the resulting experimental
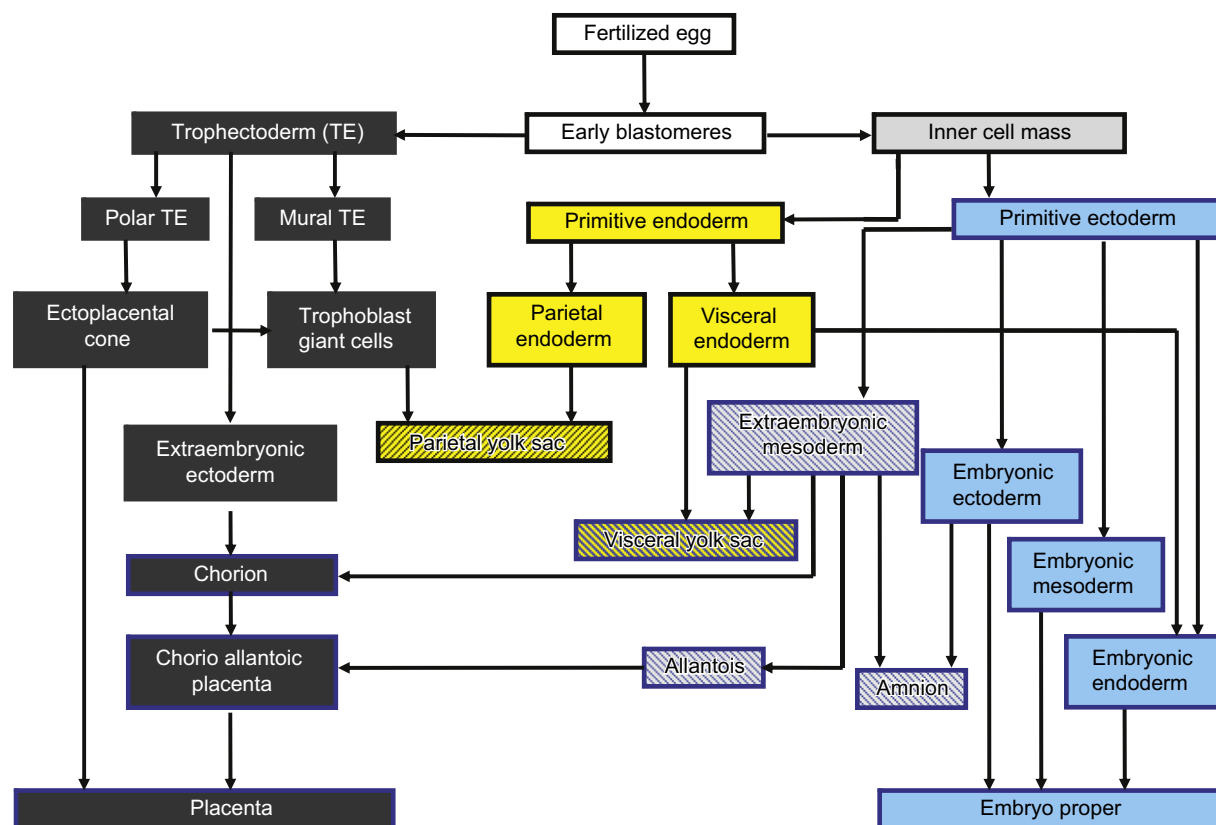
Figure 2.1.3 Cell lineages in the early mouse embryo. The developmental potential of cells in the early embryo becomes progressively restricted. The first two distinct cell lineages, the trophectoderm and inner cell mass, are present in the blastocyst. The primitive endoderm and primitive ectoderm lineages are established from the inner cell mass. Both trophectoderm and primitive endoderm contribute to extraembryonic tissues, the primitive ectoderm gives rise to the germ layers of the embryo and to extraembryonic mesoderm which contributes to the extraembryonic tissues (amnion, yolk sac and allantois).

blastocyst. However, this does not reflect a lack of developmental potency, since when single, isolated blastomeres are combined with (genetically) marked eight-cell embryos they can form normal chimaeric embryos and contribute to a broad range of embryonic and extra-embryonic tissues [56]. The position of the 'single' blastomere during the aggregation seems to have a strong influence on its developmental fate: labelled blastomeres which were placed on the outside of aggregates of other blastomeres developed predominantly into trophectoderm cells in resulting blastocysts and were mainly found in trophoblast tissue at day 10 of development. When labelled blastomeres were surrounded by other blastomeres they contributed large numbers of daughter cells to the ICM and formed parts of the embryo rather than extraembryonic tissues at later stages of development [57]. The remarkable regulative capacity of individual blastomeres greatly declines when the three lineages of the blastocyst are established: the trophectoderm at embryonic day 3.5, and the primitive endoderm and epiblast from the ICM at embryonic day 4.5.

The mechanisms that trigger these cell lineage decisions, particularly the first one between trophectoderm and ICM, are still under debate. For some time it was suggested that pre-patterning at the stage of the zygote (along the animal-vegetal axis, the animal pole being defined by the location of the polar body or localized maternal components) or even physical constraints provided by the zona pellucida trigger the first lineage segregation as well as polarity in the blastocyst. A series of experiments have recently questioned the claims for early asymmetry, and have favoured the concept that cues from position (outside vs inside) and/or apical–basal cell polarity of the blastomeres translate into fate and symmetry in the preimplantation embryo (for a summary of this debate see [58–61]).

Irrespective of the precise trigger, recent years have identified the POU-domain transcription factor Pou5f1 (also known as Oct4) and the caudal-type homeobox 2 (Cdx2) as crucial mediators of the first binary fate decision in the embryo [62]. Cdx2 specifies the trophectoderm, and Oct4 together with two other transcription factors, Sox2 and Nanog, maintains the pluripotency of ICM cells. Oct4 and Cdx2 are initially coexpressed in all blastomeres of the compacted morula but then establish a mutually exclusive expression pattern in inner and outer cells, respectively, by autoactivation and mutual repression [62, 63]. Initial upregulation of Cdx2 in outer cells might depend on polarity cues and differential cell adhesion that act via inhibition of the Hippo signalling pathway [64, 65]. The second lineage decision between primitive ectoderm and epiblast in the ICM also employs a reciprocal feedback mechanism between two transcription factors, the Gata-type zinc finger protein Gata6 and homeobox protein Nanog. Gata6 and Nanog are initially expressed in a random and mosaic pattern in the blastocyst, but then Gata6-expressing cells start to segregate to the free surface of the ICM [66, 67] Gata6 drives endoderm differentiation, whereas Nanog together with Sal-like4 (Sall4) maintains the pluripotency of epiblast precursors [68–70] (see [59, 60, 71, 72] for excellent reviews on the molecular control of cleavage divisions and blastocyst formation in the mouse).

Implantation

Implantation is the process by which the blastocyst comes into intimate physical and physiological contact with the uterine endometrium. Implantation absolutely depends on the synchronized development of the blastocyst to a stage when it is competent to implant, and of the uterus to a stage when it is receptive to blastocyst growth and implantation. The receptive character of the uterus is the result of profound changes of its tissue architecture that are triggered by ovulation and culminate with implantation on the fifth day of development. These tissue changes are regulated by the concerted action of two ovarian steroid hormones, progesterone and oestrogen. On the first day of pregnancy uterine epithelial cells proliferate under the influence of preovulatory oestrogen secretion. Rising levels of progesterone secreted from freshly formed corpora lutea initiate proliferation of the underlying fibrous stromal cells from embryonic day 3 onwards. Stromal cell proliferation is enhanced by a small increase

in oestrogen levels secreted on the morning of day 4 of pregnancy, making the uterus receptive (for recent reviews on hormonal control of uterine differentiation in pregnancy and implantation see [73, 74]).

Before the blastocyst can implant, it has to shed its zona pellucida. This process is called *hatching* and is brought about by localized proteolysis of the zona and contractions and expansion of the blastocyst. Once freed from the zona the blastocyst attaches to the epithelium of one of the lateral uterine walls with the mural trophectoderm (the trophectoderm opposite to and not facing the ICM). The uterine wall attached to the blastocyst responds by bulging into the lumen, orienting the ICM either to the anterior or posterior end of the uterine horn. This and the following reorganization result in an invariable orientation of the early embryo. The axis through the ICM towards the opposite pole of the blastocyst parallels the dorsoventral axis of the mother, the ICM always facing the dorsal side. The future anterior–posterior axis of the embryo, which becomes evident around day 6.5 of development with the onset of gastrulation, is more or less perpendicular to the anterior–posterior (longitudinal) axis of the uterine horn. However, the significance of this invariant orientation of the embryo with respect to the uterus in the determination of the embryonic axes is not clear, since embryos can also develop normally *in vitro* from preimplantation stages up to the limb bud stage [75]. A detailed analysis of the orientation of mouse embryos during implantation and a discussion of how this might be achieved and be related to embryonic axis formation is given by Smith [76, 77]. After attachment to the uterine wall, the trophectodermal cells invade the degenerating uterine epithelium and penetrate into the endometrium (stroma) of the uterus. The mesenchymal stromal cells respond with increased proliferation, resulting in the formation of a thick layer of mesenchymal tissue, the *decidua*, which encloses the embryo. The implantation sites are readily visible within 1 day after implantation by the decidual swellings of the uterus. Embryo–uterine interactions are controlled by a large number of signalling molecules and pathways, which are reviewed in [73, 74, 78].

Early postimplantation development

Formation of the egg cylinder and cell type diversification

Preimplantation development results in the formation of the blastocyst, which contains approximately 200 cells around the time of implantation. Three distinct cell types are present in the blastocyst at implantation: the trophectoderm, the ICM with the epiblast or primitive/embryonic ectoderm, and the primitive endoderm. These cell types will rapidly diverge further (for a review see [79]). Cells of the primitive ectoderm and overlying trophectoderm proliferate and form an elongated structure, the *egg cylinder*, that projects into the blastocoel. The egg cylinder shows a distinct junction between the distally located embryonic ectoderm and the proximal, trophectoderm-derived extraembryonic ectoderm. Proximal to the extraembryonic ectoderm some trophectodermal cells stop proliferating, undergo endoreduplication and form trophoblastic giant cells. Other trophectoderm cells proliferate and form the ectoplacental cone, which together with the extraembryonic ectoderm will form most of the fetal part of the placenta. The primitive endoderm cells will give rise to the parietal and visceral yolk sac endoderm. The primitive or embryonic ectoderm will give rise to the three definitive germ layers of the embryo during gastrulation, and will also contribute to extraembryonic tissues. Below, the development during early postimplantation stages of the three cell types present in the embryo at implantation will be described.

Trophectoderm and its derivatives

The epithelial layer of trophectodermal cells present in the late preimplantation blastocyst does not consist of a homogeneous cell population. The cells overlaying the ICM constitute the

polar trophectoderm, the cells without contact to the ICM the mural trophectoderm. Mural trophectoderm cells stop proliferating and by endoreduplication (DNA synthesis without mitosis) become large polyploid cells, the primary trophoblastic *giant cells*. In contrast, polar trophectoderm cells remain diploid, continue to proliferate and give rise to the ectoplacental cone and the extraembryonic ectoderm [80-84]. Polar trophectoderm cells that move away from the embryonic pole differentiate into mural trophectoderm. Contact or proximity to the ICM or its derivatives control whether trophectoderm cells continue to proliferate or cease cell divisions and become polyploid giant cells. When trophoblast cells (these are the trophectodermal cells after implantation) were isolated from contact with ICM derivatives, they ceased proliferation and transformed into giant cells [81] while contact with ICM cells appears to prevent endoreduplication of trophectoderm cells [85, 86].

Recent studies suggest that the ICM stimulation of polar trophectoderm proliferation and inhibition of differentiation is mediated by fibroblast growth factor (Fgf) signalling [87-89]. Fgf4 expression in the ICM is maintained by Nodal signalling, which also directly acts on the extraembryonic ectoderm to prevent differentiation [90]. In contrast, absence of Fgf4 and Nodal, and the basic helix–loop–helix transcription factor Mash2, and presence of the transcription factor Hand1 and the retinoic acid signalling target gene *Stra13* favour differentiation of trophoblast giant cells [91, 92]. The polar trophectoderm gives rise to both the extraembryonic ectoderm and ectoplacental cone. The extraembryonic ectoderm projects into the blastocystic cavity while the ectoplacental cone extends in the opposite direction (Figure 2.1.4). Cells from the periphery of the ectoplacental cone form additional (secondary) trophoblastic giant cells that further differentiate into different subtypes [83, 93]. Ectoplacental cone and extraembryonic ectoderm give rise to the majority of cells in the fetal part of the placenta. The extraembryonic ectoderm becomes epithelial, moves back towards the ectoplacental cone and together with extraembryonic mesoderm cells constitutes the *chorion*. The chorion together with the allantois, another mesodermal tissue (which gives rise to the umbilical cord, see below), forms the chorioallantoic placenta, or labyrinthine region
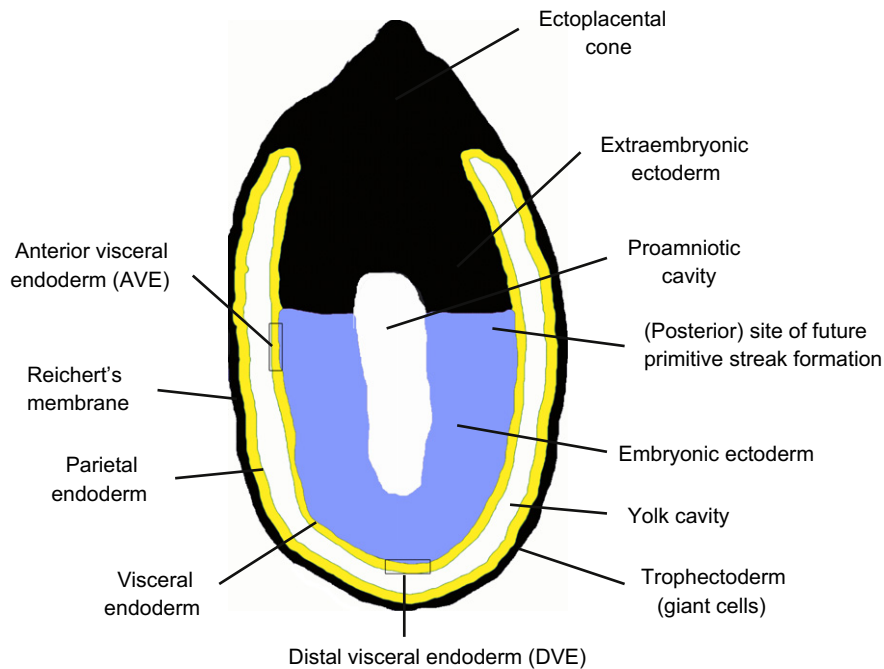
Figure 2.1.4 Schematic presentation of an early egg cylinder embryo. The early egg cylinder consists of the trophectoderm-derived ectoplacental cone and extraembryonic ectoderm, the embryonic ectoderm, and the visceral and parietal endoderm. The visceral endoderm covers the embryonic and extraembryonic ectoderm, the parietal endoderm the inner surface of the trophectoderm. For details see text.

of the placenta, where exchange of metabolites and gases occurs between fetal and maternal blood (for recent reviews on trophoblast development see [93, 94]).

Primitive endoderm and its derivatives

Prior to implantation primitive endoderm cells differentiate on the surface of the ICM facing the blastocystic cavity (Figure 2.1.4). As the egg cylinder forms, primitive endoderm cells undergo further differentiation into two morphologically and biochemically distinct cell types. The cells remaining in contact with and covering the egg cylinder constitute the visceral endoderm, cells that grow out and migrate onto the inner surface of the mural trophectoderm constitute the parietal endoderm (Figure 2.1.4). Visceral and parietal endoderm cells are part of the extraembryonic membranes, the visceral and parietal yolk sac, respectively (see below).

Parietal endoderm cells start to grow and migrate onto the inner surface of the trophectoderm shortly after implantation, and from day 6 on they cover the inner surface of the trophectoderm as a lawn of evenly spaced individual cells [95]. These cells produce and secrete large amounts of extracellular matrix material as laminin, entactin, type IV collagen and heparan sulfate proteoglycan [96, 97] and lay down a thick basement membrane, known as *Reichert's membrane*, between the parietal endoderm and the underlying trophoblastic giant cells. Parietal endoderm, Reichert's membrane and trophoblastic giant cells together constitute the parietal (outer) yolk sac of the embryo (for a review see [98]). Until it starts to break down around day 16 of gestation, Reichert's membrane may serve as a major barrier and coarse filter between maternal and fetal environments. Nutrients from the mother can pass through this barrier, while penetration of maternal cells is prevented [99].

The visceral endoderm cells become organized into a distinct epithelium, the apical surface covered by microvilli facing the former blastocoel, which is now called the *yolk cavity* [100]. On the basal side visceral endoderm cells are separated from the underlying embryonic and extraembryonic ectoderm cells by a thin basement membrane [101]. The visceral endoderm secretes signals that control patterning and differentiation of the epiblast [72]. It also contributes a small number of cells to the definitive endoderm [102]. Together with extraembryonic mesoderm cells, the visceral endoderm constitutes the visceral yolk sac [103]. The visceral yolk sac has important absorptive and secretory functions in maternal-embryonic exchange of nutrients as well as waste products [104].

Embryonic ectoderm

The cell types described thus far only give rise or contribute to extraembryonic tissues, which serve essential supportive functions for the developing embryo. The embryo proper is almost exclusively formed by descendants of primitive ectoderm cells (Figure 2.1.3), which in addition give rise to the germ line and extraembryonic mesoderm [105-107]. Around implantation primitive ectoderm cells form a core of about 30-40 cells surrounded by primitive endoderm cells on the side facing the blastocoel, and juxtaposed to extraembryonic ectoderm cells on the other side. Shortly after implantation primitive ectoderm cells start to proliferate rapidly [108] and form the distal part (tip) of the egg cylinder. Between days 5.5 and 6 of embryonic development a small lumen called the *proamniotic cavity* forms in the centre of the primitive ectoderm and the cells form a columnar epithelium. The apical side faces the lumen of the cavity and cells are joined apically by junctional complexes. The basal surface is attached to the thin basal membrane separating primitive ectoderm from visceral embryonic endoderm. Around day 6 post coitum (d p.c.) the central cavity extends more dorsally into the extraembryonic ectoderm, resulting in a small lumen throughout the egg cylinder.

Gastrulation and development of the germ layers

At 6.5 d p.c. the stage is set for a morphogenetic process which will completely reshape the embryo: *gastrulation*. As a result, a three-germ-layered embryo composed of mesoderm, endoderm and ectoderm is generated. The rotational symmetry of the egg cylinder is transformed
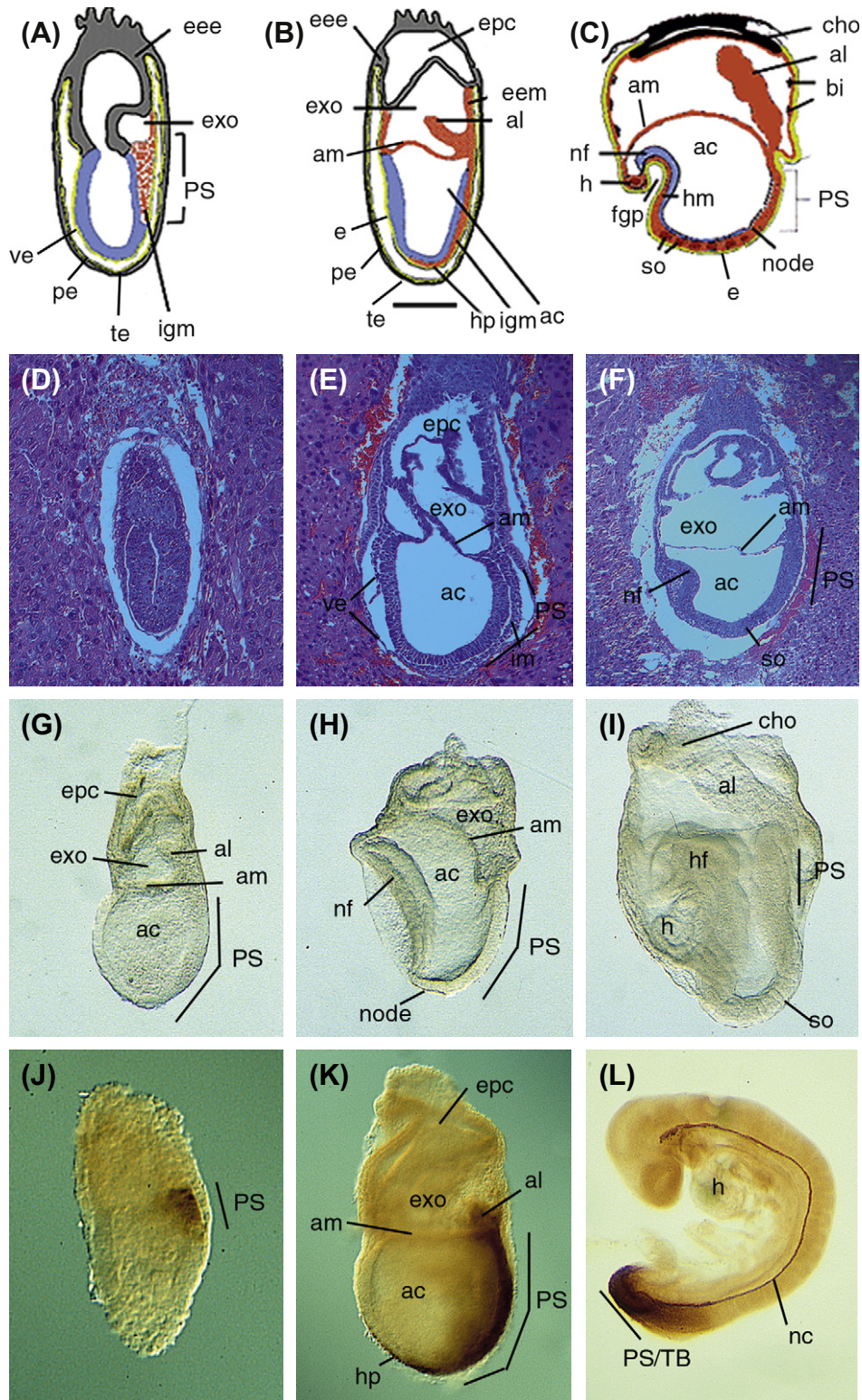
Figure 2.1.5 Mouse development from early primitive streak to early organogenesis stages. (A–C) Schematic representation of early postimplantation development: (A) 6.75 d p.c. embryo; (B) 7.5 d p.c. embryo; (C) 8.25 d p.c. embryo. Colour code: yellow, (visceral, parietal, definitive) endoderm; red, mesoderm; grey, extraembryonic ectoderm; blue, embryonic ectoderm/epiblast; black, chorion. (D–F) Histological sections of early gastrulation stage mouse embryos with surrounding uterine tissue. 10 μm paraffin sections stained with haematoxylin and eosin. (D) 6.25 d p.c. embryo; (E) 7.0 d p.c. embryo; (F) 8.0–8.25 d p.c. embryo. (G–I) Whole

into a bilaterally symmetrical organization with distinct anterior–posterior and dorsal–ventral axes. The left–right axis is determined after onset of gastrulation and becomes morphologically obvious with the looping of the heart tube at 8.5 d p.c. (for recent reviews on mouse gastrulation see [72, 109]).

Morphogenetic movements and the generation of the germ layers

At the initiation of gastrulation epiblast cells at the future posterior end of the embryonic portion of the egg cylinder undergo an epithelial–mesenchymal transition and leave the epithelial continuity of the primitive ectoderm. This region is called the primitive streak and extends soon after its appearance distally towards the tip of the egg cylinder. Epithelial–mesenchymal transition involves the disruption of cell contacts and reorganization of the cytoskeleton. Downregulation of expression of the adhesion molecule E-cadherin is a crucial prerequisite that is controlled by Fgf-induced expression of the transcriptional repressor Snail (reviewed in [110]). The epiblast-derived mesenchymal cells move as a new tissue layer between the visceral endoderm, the epiblast and the extraembryonic ectoderm, and differentiate into mesodermal cells (Figure 2.1.5). Primitive streak induction and mesoderm formation is controlled by bone morphogenetic protein (Bmp) signals from the extraembryonic ectoderm, and Nodal and Wnt signals from the epiblast that interact in a reinforcing loop (see references in [72]). Wnt and Fgf signals converge to activate transcription of the T-box transcription factors T (Brachyury) and Tbx6 that maintain

mesendoderm formation and patterning in the primitive streak [111–113].

The first mesenchymal cells to emerge from the primitive streak migrate towards the extraembryonic ectoderm and will give rise to the extraembryonic mesoderm. At the margin of the embryonic part of the egg cylinder, ectodermal cells bulge into the lumen of the egg cylinder together with the underlying extraembryonic mesoderm and form the amniotic folds. Formation of the amniotic folds progresses from posterior to anterior, leading to a continuous constriction of the central cavity that is most advanced at the posterior end. The amniotic folds grow towards each other, and finally meet and fuse. Concomitantly, the mesoderm within the folds develops a central cavity, the *exocoelom*, pushes the extraembryonic ectoderm towards the ectoplacental cone and separates it from the embryonic ectoderm. On day 7.5 p.c., after the amniotic folds have fused, the proamniotic cavity of the egg cylinder has been divided into the amniotic, exocoelomic and ectoplacental cavities (Figure 2.1.5A–D, G, H, J, K). Extraembryonic ectoderm with the underlying extraembryonic mesoderm constitutes the chorion, visceral endoderm with the attached extraembryonic mesoderm forms the visceral yolk sac. The amnion consists of an ectodermal cell layer covered by extraembryonic mesoderm. At the posterior end of the embryo extraembryonic mesoderm cells give rise to a finger-like structure, which grows through the exocoelom towards the chorion. This tissue is called *allantois* and will later fuse with the chorion, linking the embryo with the ectoplacental cone. The allantois will form the umbilical cord and together with the chorion will give rise to the chorioallantoic placenta (Figure 2.1.5C, F, I, K; for reviews see [114, 115]).

gastrulation stage embryos. (G) 7.5 d p.c. embryo; (H) 7.75 d p.c. embryo; (I) 8.5 d p.c. embryo. The black bar represents 200 μm. (J–L) Gastrulation stage embryos marked for the primitive streak/tail bud region. Immunohistological detection of Brachyury protein expression in whole embryos [199]. (J) 6.5 d p.c. embryo. Brachyury expression marks the primitive streak, which has formed at the future posterior end of the embryo proper. (K) 7.5 d p.c. embryo. The primitive streak has extended right to the distal tip of the egg cylinder. Brachyury protein expression also indicates the extraembryonic mesoderm (allantois) and the head process. (L) 9.0 d p.c. embryo. The primitive streak or tail bud marks the posterior pole of the embryo. Brachyury protein is detected all along the notochord. Anterior is to the left of the picture and proximal to the top. ac, amniotic cavity; al, allantois; am, amnion; bi, blood islands; cho, chorion; e, (definitive) endoderm; eee, extraembryonic ectoderm; eem, extraembryonic mesoderm; epc, ectoplacental cavity; exo, exocoelom; fgp, foregut pocket; h, heart; hp, head process; igm, ingressing mesoderm; nc, notochord; nf, neural folds; pe, parietal endoderm; PS, primitive streak; so, somites; TB, tail bud; te, trophectoderm; ve, visceral endoderm.

During 6.5 and 7.5 d p.c. the primitive streak extends from the posterior end of the embryo proper to the distalmost part of the egg cylinder (Figure 2.1.5A, B, J, K). During all that time mesodermal cells continuously form and move laterally and anteriorly away from the primitive streak [116]. In addition, cells originating from the anterior region of the primitive streak displace part of the visceral embryonic endoderm cells into the yolk sac and intermingle with another part to form the definitive endoderm [102]. They will colonize the midline region of the embryo and will eventually form the midgut [117, 118]. The mechanisms that guide separation of endodermal and mesodermal precursors are not well known, but the T-box transcription factor eomesodermin and upstream Nodal signalling may be involved [72, 119].

Around day 7 p.c. an ectodermal thickening emerges at the anterior end of the streak representing the node (*Hensen's node*, as this structure is called in birds). Cells migrating through this area move anteriorly to form a transient embryonic structure lying in the midline of the embryo: the notochord (Figure 2.1.5B, K) [117, 120, 121]. Endodermal cells from this region contribute to trunk endoderm [117]. The embryonic ectoderm cells overlying the notochord and its anterior extension, the prechordal plate, form the neural plate that folds in the midline to form the *neural groove*.

From day 7.5 p.c. onwards, extensive anterior growth and regression of the primitive streak extend the neural plate posteriorly. Concomitantly, the primitive node moves back and cells migrating through the regressing node form more posterior parts of the notochord. Ingression of cells through the primitive streak persists up to and through day 10 p.c. (midgestation) leading to posterior elongation of the embryo. Between 9.5 and 10.5 d p.c. the primitive streak gradually loses its identity and is then referred to as the *tail bud* instead. Gastrulation continues in the tail bud at the posterior end of the embryo until 13.5 d p.c., generating posterior trunk and tail tissue (Figure 2.1.5C, I).

Cells ingressing at different positions along the primitive streak (and the tail bud) have distinct developmental fates and give rise to different prospective mesodermal and endodermal tissues. Cells emerging from the posterior part of the streak move mainly into the extraembryonic mesoderm. Cells from the middle region of the streak give rise to lateral mesoderm (mesoderm located laterally to the paraxial mesoderm). Cells emerging anteriolaterally to the streak form paraxial mesoderm (giving rise to somites and head mesenchyme), and cells emerging from the anterior part of the primitive streak mainly contribute to notochord and gut [114, 122]. In addition to the position-dependent allocation of cells to different mesodermal tissues, which is at least partially controlled by dose-dependent Nodal signalling [123], the streak has a stage-dependent potential to form different mesodermal cell types. While the early primitive streak (day 6.5-8) produces both embryonic and extraembryonic mesoderm, the older primitive streak (from day 8.5 onwards) continues to produce embryonic mesoderm but ceases to contribute to extraembryonic mesoderm [122]. Hence, there is a time- and space-dependent translation of anterior–posterior positional values in the primitive streak into an axial-lateral, i.e. dorsal–ventral, patterning of the mesoderm.

Similarly, the fate of embryonic ectoderm cells in the day 7.5 embryo seems to depend on the position along the anterior-posterior axis. Cells from the anterior regions give rise to neuroectoderm of the prosencephalon and mesencephalon, cells flanking the anterior end of the streak give rise to neuroectoderm of the rhombencephalon, and cells flanking the anterior and middle region of the streak give rise to the spinal cord. The future dorsoventral orientation of neuroectodermal cells in the neural tube seems to be already established at this stage of gastrulation. Cells closer to the midline end up in more ventral positions than cells which are located laterally. Ectoderm from the most posterior regions gives rise to surface ectoderm and cells from positions most lateral to the midline are the presumptive neural crest cell precursors [106, 107, 114]. These cell fates, however, do not imply that cells are committed to specific lineages prior to gastrulation, since there is little regional restriction in the developmental potency of embryonic ectoderm cells [106].

Anterior–posterior patterning and early organizing centres

The first morphological indication of breaking the rotational symmetry of the egg cylinder is

the emergence of the primitive streak. The position of the primitive streak defines the posterior pole of the anterior-posterior axis of the emerging bilaterally symmetrical embryo. The pregastrulation epiblast is characterized by extensive cell mixing, making it very unlikely that positional information for anterior-posterior axis specification can be maintained within the epiblast [124]. However, extraembryonic tissues like the visceral endoderm and the extraembryonic ectoderm grow coherently and could instruct the underlying epiblast with positional information [125]. In fact, it has turned out that reciprocal interaction between the visceral endoderm, the extraembryonic ectoderm and the epiblast by secreted growth factors of different families leads to regionalized gene expression in these tissues and to establishment of a proximal-distal axis in the epiblast. Soon afterwards the radial symmetry is broken and the anterior and posterior molecular identities emerge (for a review see [61, 72]). Evidence for these early patterning events is first apparent by local thickening of the visceral endoderm at the distal end at embryonic day 5.0-5.25 and later at embryonic day 5.5-6.0 on one side (the future anterior) side of the egg cylinder [126, 127]. The primitive streak forms opposite the thickening of the anterior visceral endoderm (AVE), identifying this tissue as a reliable landmark for the anterior pole of the body axis. The distal visceral endoderm (DVE) is established by Nodal signalling stating from embryonic day 5.0. Nodal induces the expression of inhibitors of Nodal and Wnt signalling in the DVE which then acts back to inhibit Nodal and Wnt signalling pathways in the overlying epiblast, thus, generating a proximal-distal gradient of activity of these pathways [128, 129]. As mentioned before, Nodal signalling also maintains the progenitor character of cells in the extraembryonic ectoderm [90, 129]. In turn, signals from this tissue, including Bmps, pattern the proximal epiblast and prevent the visceral endoderm from acquiring a distal character [130]. Lineage tracing showed that visceral endoderm cells from the distal tip of the egg cylinder directionally migrate to the future anterior side of the proximal region [131]. The direct descent of the AVE from DVE cells has recently been questioned [132]. In any case, this global migration of DVE repositions a source of Nodal and

Wnt antagonists, and, thus, inhibits activation of these pathways and formation of primitive streaks at the (now) anterior end of the embryo [133]. The AVE plays an additional role as a signalling centre for the underlying (future anterior neural plate) embryonic ectoderm [134].

Molecular and minor morphological asymmetries are already present in the preimplantation as well as in the pregastrulation embryo. Although no experimental proof exists, it is conceivable that there is a flow of information originating in the zygote or even the oocyte to the blastocyst, or from the blastocyst to the egg cylinder, that is translated into anterior-posterior polarity in the egg cylinder embryo [58, 59, 61, 132].

While the molecular mechanisms governing establishment of the anterior-posterior axial polarity of the gastrulation stage embryo are just being unravelled, a great deal of evidence has accumulated to highlight the role of the node as an embryonic tissue to function as an organizer of anterior-posterior polarity within the embryo itself (for reviews see [135, 136]). The node only becomes morphologically visible as an indentation at the distal tip of the 7.75 d p.c. embryo [137] (Figure 2.1.5H). However, lineage studies have shown that the precursors of the node can be traced back to the anterior end of the primitive streak in the 6.5 d p.c. embryo [124, 138]. Cells of this early node as well as the late node contribute to axial mesendoderm, the prechordal plate or head process, the notochord and the gut endoderm. Lengthening of the anterior-posterior axis of the embryo results in a posterior displacement of the node from 8.0 d p.c. on, leaving behind the mesodermal cells that undergo a convergent extension movement to form the notochord (Figure 2.1.5I).

Transplantation experiments have shown that the anterior streak of the 6.5 d p.c. embryo and the node of the 7.5 d p.c embryo respectively not only give rise to axial mesendoderm but provide a patterning system that induces and organizes neighbouring tissues into an anterior-posterior axis [139, 140]. The late node and the early anterior streak behave as late and early gastrula organizers (EGO), respectively. They are functional homologues of the dorsal blastopore lip region of the amphibian gastrula, the

paradigm for such an organizing centre [136]. The gastrula organizer progressively induces more posterior cell fates. At present it seems that it never induces very anterior neural fate, suggesting the existence of a separate head organizing activity. Alternatively, EGO and AVE activities may synergize in inducing anterior cell fates [141, 142].

Molecular analysis of organizer gene expression and function in the *Xenopus* and the mouse embryo have shown that EGO and node rather than working by secretion of inducing factors are a rich source of inhibitory molecules of the Wnt and Bmp signalling families. Locally restricted inhibition of Bmp and Wnt pathways generates gradients of these signals important in patterning the anterior-posterior and dorsoventral axes (for review see [143, 144]).

Left–right asymmetry

The external appearance of the mouse body, like that of every mammal, is of overt bilateral symmetry. However, most internal organs are asymmetric in shape or in position. This asymmetry is generated by different mechanisms. In some cases a differential looping or turning programme is involved (e.g. heart, guts), in other cases differential growth is the driving force (e.g. lungs), and finally, differential remodelling of originally identical sides may lead to asymmetry, as seen in the vascular system.

This asymmetry along the left–right axis is the last of the embryonic asymmetries to be established. Symmetry breaking occurs during gastrulation in embryogenesis, the first morphological sign being asymmetric looping of the heart tube at 8.5 d p.c. (for reviews see [145, 146]). Genetic studies in the mouse as well as embryological manipulations in the chick have established a series of events that mediate left–right asymmetry. A major player in establishing this asymmetry is the node, the structure which also organizes dorsoventral and anterior-posterior patterning of the embryo. Ventral node cells harbour monocilia with a 9+0 microtubule arrangement, which rotate in a clockwise fashion [147]. Disruption of nodal cilia or perturbation of ciliary rotation leads to randomization of left–right asymmetry of internal organs,

suggesting that the directed rotation of nodal cilia creates a flow of the extraembryonic fluid from right to left [148, 149]. In turn, this might result in the excitation of sensory cilia at the periphery of the node. Alternatively, it may generate a differential transport of a signalling molecule across the nodal epithelium between 7.0 and 7.5 d p.c [149, 150]. Signalling molecules including retinoic acid and Sonic hedgehog (*Shh*) are loaded on membrane-sheathed lipoprotein particles, so-called nodal vesicular parcels (NVP), that are launched from microvillar extensions of nodal cells. NVPs are transported to the left nodal side where they are fragmented and absorbed by nodal cells [151]. This leads to a transient rise in Ca^{2+} in these cells which propagates to the left lateral plate mesoderm where new molecular signals including the paired-like homeodomain transcription factor 2 (*Pitx2*), the signalling molecules Nodal and the left–right determination factor 2 (*Lefty2*) become asymmetrically expressed. They finally induce the asymmetric morphogenesis of the visceral organs starting from 8.5 d p.c.

Embryonic turning

The arrangement of ectoderm inside and endoderm on the outside of the embryo, which is found prior to and during early gastrulation, is known as inversion of the germ layers and is common to mouse, rat, rabbit, guinea-pig and other closely related rodents. In sagittal section the embryo is U-shaped, with the midgut endoderm lining the outer curvature of the U and fore- and hindgut following at either end. At 8.25-8.5 d p.c., when the first six to eight somites have formed (see below), the inversion of the germ layers is reversed by a process known as *turning*. During turning the embryo rotates anti-clockwise around its anterior-posterior axis. As a consequence, the curvature of the U is reversed and the ectoderm comes to lie at the outer aspect of the embryo. The embryo becomes surrounded by the extraembryonic membranes, the amnion and the visceral yolk sac, since they are attached to the embryo along the boundary of the body wall and the future site of attachment of the umbilical cord. For a more detailed and illustrated description of turning, see [2].

Differentiation of mesoderm and early development of mesodermally derived organs

Various types of mesoderm, which contribute to different tissues of the embryo, are generated during gastrulation [152]. Mesoderm along the midline of the embryo (axial mesoderm) forms the prechordal plate and notochord. The notochord is a transient embryonic tissue that disintegrates after embryonic day 12.5 and is taken up into the intervertebral discs to form their central nuclei pulposi. Unlike in lower vertebrates where the notochord also fulfils structural tasks, the murine notochord is a thin rod that merely acts as an important signalling centre for patterning of surrounding tissues [153]. Signals from the prechordal plate/notochord induce the overlying ectoderm to form the neural tube. Shh from the notochord subsequently induces ventral fates including the floor plate in the neural tube. Other targets of notochordal Shh activity include the underlying endoderm as well as the adjacent paraxial mesoderm, which flanks the notochord and the neural tube laterally on both sides as thick tissue bands.

Beginning at around day 7.75 p.c., balls of mesenchymal cells condense from the posterior unsegmented region of paraxial mesoderm, the so-called *presomitic mesoderm*, undergo an mesenchymal–epithelial transition and form somites, vesicles of a single-layered epithelium that enclose the *somitocoel*, a cavity containing few mesenchymal cells [154, 155]. The first somites form in the posterior head fold region of the embryo; somite condensation then progresses posteriorly, while new mesoderm continues to be generated from the primitive streak at the caudal end of the embryo (Figure 2.1.6). The total number of about 65 somite pairs is formed at around day 13 of development. The metameric somites can be considered as a primary segmental subdivision along the anterior–posterior body axis. The highly synchronized sequential formation of somite pairs is under the control of multiple signalling pathways including Wnt, Fgf and retinoic acid that exhibit graded distribution of signalling activity within the paraxial mesoderm. While Wnt and Fgf signalling show highest activity in the posterior presomitic mesoderm, retinoic
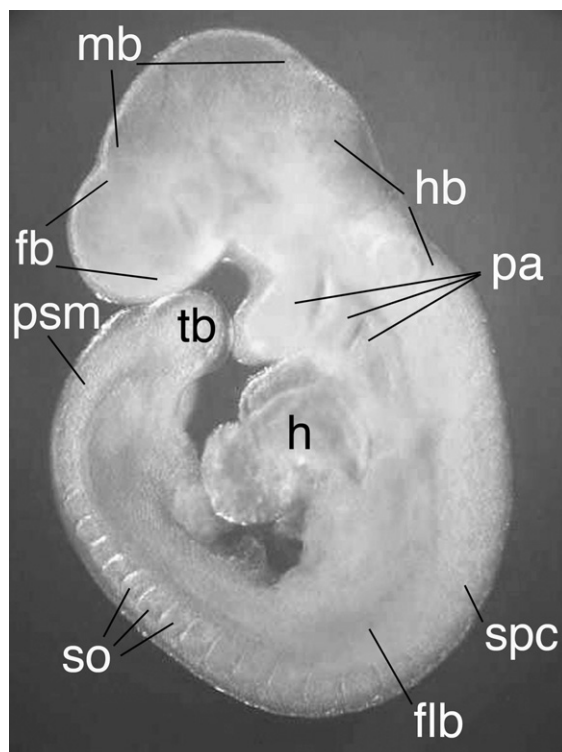
Figure 2.1.6 Formation and differentiation of the mesoderm and onset of organogenesis. Lateral view of a whole 9.5 d p.c. embryo. fb, forebrain; flb, fore limb bud; h, heart; hb, hindbrain; mb, midbrain; pa, pharyngeal arches; psm, presomitic mesoderm; so, somites; spc, spinal cord; tb, tailbud.

acid signalling establishes a counter-gradient with the highest activity in the somites. In addition, high levels of Wnt and Fgf signalling together with Notch signalling are required for activity of a molecular oscillator in the PSM [156]. Of note, the specification of anterior–posterior positional identities along the body axis occurs in the presomitic mesoderm by controlling the combinatorial and sequential expression of genes from the four murine Hox clusters [157].

Shortly after their formation somites differentiate further (for a review see [154, 158]). Cells from the ventral part of the somites become mesenchymal again and migrate towards the notochord and the neural tube to enclose them. These cells, called *sclerotome*, give rise to the axial skeleton (vertebrae, ribs). The cells left behind form a bilayered structure called *dermomyotome*. The dorsal dermatome cells give rise to the connective tissue of the skin. The dorsal myotome will form the skeletal muscles of the back (hypaxial musculature), whereas ventral myotomal cells form the muscles

of the body wall and limbs (epaxial musculature). Correct segmentation of the axial skeleton relies on a second patterning process which subdivides somites along the anterior–posterior axis into two halves. Cells from anterior and posterior somite halves differentially contribute to the vertebrae, and impose a segmental mode of migration and trajection onto the neural crest cells and spinal nerves, respectively, to ensure the segmental arrangement of the peripheral nervous system. While dorsoventral patterning of somites is regulated by extrinsic signals including Shh from the notochord/floor plate and Wnts from the dorsal neural tube and ectoderm, anterior–posterior somite polarization depends on an intrinsic mechanism involving the Notch/Delta signalling pathway and Mesp2 transcription factor activity in the presomitic mesoderm, and subsequent antagonistic action of the two transcriptional repressors Uncx4.1 and Tbx18 in the somites and the lateral sclerotome [159, 160].

Mesodermal cells immediately flanking the somites, the intermediate mesoderm, form the urogenital system comprising the gonads, the sex ducts and the kidneys. Specification of the intermediate mesoderm similar to the somitic mesoderm is controlled by expression of specific transcription factors (for a review see [161]). Development of the excretory system is unusual, since three embryonic kidneys are sequentially laid down in this tissue from anterior to posterior: the pronephros at the levels of the fore limbs, the mesonephros between fore and hindlimbs, and the metanephros at the levels of the future hindlimbs. Metanephric development is controlled by reciprocal signalling between the epithelial Wolffian duct and its derivative, the ureter, and the surrounding metanephric mesenchyme. While the Wolffian duct and the ureter will form the collecting duct system of the kidney and male sex ducts, respectively, the latter will form stroma and the nephrons (for a review see [162]). Both sex ducts and gonads are initially sexually indifferent, but upon genetic and subsequent hormonal cues the somatic aspects of the gonads will differentiate into testes and ovaries, while the two sex duct systems that are initially established in both sexes (the Müllerian duct and the Wolffian duct) will selectively degenerate (the Müllerian duct in the male and the Wolffian duct in the female) and differentiate (the

Müllerian duct to the vagina, the uterus and the oviduct in the female, and the Wolffian duct to the vas deferens and the epididymis in the male), respectively (for a review see [163, 164]).

Further laterally lies the lateral plate mesoderm. Lateral plate mesoderm splits into a dorsal (or somatic) aspect underlying the ectoderm and a ventral (splanchnic) region underlying the endoderm. Limbs arise from the somatic lateral plate mesoderm by the local formation of two pairs of tissue protrusions. Outgrowth and patterning of these limb buds are regulated by various signalling centres including an epithelial thickening at the distal outline of the surface ectoderm, the apical ectodermal ridge (AER), and the zone of polarizing activity (ZPA) in the posterior limb bud mesenchyme. Signals from these two centres (Shh from the ZPA and Fgfs from the AER) interact to drive outgrowth and proximal–distal and anterior–posterior patterning of the bud. The mesenchyme of the limb bud will contribute to the formation of tendons and skeletal elements, whereas the nerves and the muscles derive from the spinal cord and the somitic mesoderm, thus migrating into the developing limb (for a review see [165]).

Bilateral subregions of the anterior splanchnic lateral plate (the so-called *first heart field*) are destined to a cardiac fate, and fuse at the midline to form a cardiac crescent from which a simple peristaltically active tube arises around embryonic day 8.25 (for a recent review on heart development see [166]). The tube is further elongated by recruitment and delayed myocardial differentiation of precursor cells from the pharyngeal mesoderm (the *second heart field*) at the two poles. Concomitantly with the morphogenetic process of looping that repositions the venous pole anteriorly, chamber formation is induced in discrete zones along the outer curvature of the growing heart tube at embryonic day 8.5–9.0. The regions flanking and separating the chamber myocardium, the inflow tract (IFT) and outflow tract (OFT), and the atrioventricular canal (AVC), respectively, retain a less differentiated myocardial phenotype and contribute to the free left ventricular wall and to the conduction system, but also induce the overlying endothelium to delaminate and, as mesenchymal cells, populate the extracellular matrix of the cardiac jelly to form cushions from which the valves will develop.

While the cardiac tube only consists of an inner epithelial layer of endothelial cells and a surrounding myocardium, the outer layer, the epicardium, derives from an extracardiac cell population, the proepicardium, at the posterior pole of the heart. Cardiac specification, differentiation and morphogenesis are driven by the tight interplay between signalling pathways, most notably Bmps and Wnts, and transcription factors including Nkx2.5, Gata proteins and T-box factors.

Concomitant with heart development, the initially bilateral inflow and outflow vessels are reshaped to attain a clear asymmetry. Between the splanchnic and somatic layers of the lateral plate mesoderm, the coelom forms; this will later be subdivided into the separate pleural, pericardial and peritoneal cavities.

Extraembryonic mesoderm of the visceral yolk sac is the first site of haematopoiesis in the developing embryo [167, 168]. From the 7th day of embryonic development onwards blood islands appear on the inner side of the visceral yolk sac. These are condensations of mesenchymal cells, which form an irregular girdle around the exocoelom. The inner cells of these condensations become embryonic red blood cells (which are nucleated cells, in contrast to the adult erythrocyte), the peripheral cells differentiate and form the endothelium of blood vessels of the yolk sac. From embryonic day 8.0, specific haematopoietic cell generation is seen in the allantois and subsequently in the placenta. Between embryonic day 9 and 10 haematopoiesis shifts into a region derived from the intermediate and lateral plate mesoderm referred to as AGM, which contributes to the formation of the aorta, the gonads and the mesonephros [167, 169]. Around day 12 of development the fetal liver takes over this function (for a review see [170]). See Figure 2.1.6 for a 9.5 d p.c. embryo with mesodermal and ectodermal differentiation at the onset of organogenesis.

Differentiation of ectoderm and early organogenesis of the nervous system

The nervous system develops from neural plate ectoderm, which gives rise to the neural tube and the neural crest, which in turn form all parts of the central and peripheral nervous system (for a review see [171]). Starting around day 7.5 the neural groove begins to form along the midline of the neural plate. While the primitive streak is regressing and the neural plate extending posteriorly, the neural groove deepens and the neural folds develop. In the cranial third of the embryo the head folds emerge rapidly and bulge deeply into the amniotic cavity due to rapid growth and the indentation of the foregut pocket, which pushes the overlying neuroectoderm ahead of itself. As the folds become higher, the edges start to approach each other and finally meet and fuse to form the neural tube, which underlies the surface ectoderm. Closure of the neural tube starts around day 8.25 at the position of the fourth to fifth somite and progresses anteriorly and posteriorly. The open ends of the neural tube are called the *anterior and posterior neuropore*. Development of the neural tube progresses more rapidly in the cranial region. The anterior neuropore is closed around day 9 while closure of the posterior neuropore is not complete until day 10 p.c. Cells from the edge of the neural folds between neuroectoderm and surface ectoderm give rise to the neural crest; this is a transient structure which is present only shortly after closure of the neural tube. The neural crest cells disperse rapidly and migrate through the embryo. Depending on which part along the anterior–posterior axis they originated from and where they finally settle, they give rise to a variety of cell types. Among these are the neurons and glial cells of the spinal ganglia, the peripheral nervous system and the adrenal medulla, the melanocytes of the epidermis and most of the mesenchymal cells of the head (skeletal and connective tissue) (for reviews on neural crest development see [172–174]).

Differentiation of endoderm and organogenesis of the gut and its derivatives

The definitive endoderm derives, together with the mesoderm, from epiblast cells that ingress through the node and the anterior primitive streak during early gastrulation. While the mesodermal cell layer moves anteriorly and laterally, cells fated to become definitive endoderm

migrate ventrally and intercalate with the visceral endoderm cell layer to generate an epithelial sheet of definitive endoderm [102]. Endoderm is specified from mesendodermal precursors by high levels of Nodal signalling, whereas low levels induce mesodermal fates [175]. Nodal signals direct a network of transcription factors including Mix-like proteins, Foxa2, Sox17, eomesodermin and Gata4-6 that further direct this lineage. By the head fold stage (7.5-8 d p.c.), the definitive endoderm consists of some 500 cells, which are organized in a single cup-like sheet. At this time deep invaginations occur at the anterior and posterior end of the embryo to form the foregut and hindgut pockets. They will later make contact with the definitive endoderm of the midgut region. Concomitantly with embryonic turning (8.25-9.0 d p.c.), the lateral walls of the endoderm sheets are brought into juxtaposition and fuse, generating a continuous gut tube [176]. During midgestation stages, the growth of the gut tube exceeds that of the body cavity leaving parts of the midgut herniating outside the ventral body wall (for review of endoderm development see [177, 178]).

A broad anterior–posterior patterning of the gut has already occurred at the late primitive streak stage, possibly by node- and streak-derived signals. Extensive morphogenetic movements between the late streak stage and midgestation juxtaposes the gut endoderm with various mesodermal cell types. Epithelial–mesenchymal interactions between gut endoderm and the surrounding splanchnic mesoderm result in a progressively refined anterior–posterior patterning of the gut tube and induction of gut organ appendages. The anterior gut tube differentiates into oesophagus, the midgut into stomach and small intestine (duodenum, jejunum, ileum), and the hindgut into the large intestine (caecum, colon, rectum), with highly specialized endodermal cell types and distinct radial patterns of the splanchnic mesoderm differentiating into fibrous tissue, smooth muscle layers and vascular endothelium. Signalling factors that regulate anterior–posterior patterning of the endoderm include Fgfs, Wnts, Bmp and retinoic acid. They converge onto different transcriptional regulators that broadly specify identity of the foregut (*Hhex, Sox2*), the midgut (*Pdx1*) and the hindgut (*Cdx2*). Along the anterior–posterior axis of the gut, endodermal

organs form by budding: the thyroid at 8.5 d p.c. [179], the lung at 9.5 d p.c. [180], the liver from 9 d p.c. [181] and the pancreas (from a ventral and a dorsal bud of the foregut endoderm) from embryonic day 9.5 [182]. Again this is under the control of dose-dependent and combinatorial action of a number of mesodermal signals, including Fgfs, Bmps and Wnts, which induce expression of transcription factors that further specify the respective lineages. Lung and pancreas development can be considered as typical for branching morphogenesis. The foregut endoderm buds out, proliferates and undergoes extensive branching while interacting with the surrounding mesenchymal cell layer. Perhaps not surprisingly at this point, reciprocal signalling between the epithelial and mesenchymal tissue compartments has been recognized as a driving force of these morphogenetic programmes. For references on the molecular control of endoderm patterning and organ formation, see the recent review [177].

Primordial germ cells

Primordial germ cells (PGCs), the ancestors of the gametes, originate in the mouse at least as early as on day 7 of embryonic development (for a review see [183]). They arise from a population of pluripotent somatic cells in the proximal epiblast near the extraembryonic ectoderm [184]. Bmp signals from this tissue have been shown to select PGCs from their somatic neighbours dose-dependently via the Smad pathway [185, 186]. PGCs actively suppress differentiation programmes of somatic cells and acquire pluripotency, functions that are mediated downstream of Bmp signalling by the two zinc finger transcriptional repressors Blimp1 and Prdm14 [187, 188]. PGCs pass through the posterior primitive streak and are found first in the posterior part of the embryo at the base of the allantois [189, 190]. They are large, round cells which contain a high level of alkaline phosphatase activity [191]. More recently, a pluripotency marker, the POU transcription factor Oct4, was found as a PGC marker [192]. A truncated *Oct4* promotor was used to drive expression of green fluorescent protein in transgenic animals. Thus, migration of PGCs was visualized in a living embryo [193]. From day 8.5 onwards PGCs migrate through the hindgut and mesentery wall and colonize the genital ridges. The genital

ridges, which give rise to the gonads, are a paired mesodermal tissue that lies beneath the dorsal mesentery of the body. By day 12.5 of development PGCs are largely confined to the developing gonads. *Invitro* studies suggest that colonization of the genital ridges is brought about by active movement of the PGCs, and that PGCs lose their invasive motility after entering the gonad anlagen [194]. Directed migration of PGC is regulated by cues from their somatic environment, including chemotactic signals presumably from the gonad, as well as gradients formed by proteins in the extracellular matrix. Again depending on environmental cues, PGCs proliferate during their migration, and the population of about 10–100 PGCs present around day 7–8 p.c. in the extraembryonic mesoderm increases to more than 20 000 in the colonized genital ridges around day 14 p.c. [195]. Once within the genital ridges massive epigenetic changes occur in PGCs including random X-chromosome inactivation in female PGCs.

At 12 d p.c. differences between male and female genital ridges become apparent and male and female germ cells embark on their specific developmental programmes. This is not cell-autonomous but relies on the somatic environment of the PGCs. Male PGCs enter mitotic arrest around day 13 p.c. and continue development only after birth. In contrast, female mouse PGCs enter meiosis from day 13 of development onwards and by about 3–5 days after birth all germ cells have undergone oogonial development and are in the diplotene stage of meiosis (for recent reviews see [183, 196]).

Late embryonic development: completion of organogenesis and fetal growth

By midgestation (day 11 of development) the basic body plan has essentially been established. The three embryonic axes have been laid down and patterning and cellular differentiation along these axes has progressed considerably. Whereas at the anterior end of the embryo tissue differentiation and organogenesis has already progressed and accelerated considerably, the axial elongation is only gradually coming to an end in the tail bud region. In the nervous system separation of the four brain vesicles proceeds and the major divisions of the brain are now clearly visible. The cellular differentiation of the nervous system, which begins around day 9 p.c., continues. Proliferating neuroblasts are found in the walls of the entire central nervous system and the spinal ganglia are well formed. The major elements of the circulatory system have developed and are functional to supply the growing embryo with nutrients and oxygen-enriched blood. Fore- and hindlimb buds are present, the anterior limbs, because they arise first, are more developed than the posterior ones. In the trunk region the development of the vertebrae commences and in this region somites start to become less discernible. All major organ anlagen are present or emerge within the next few days.

Generally, organs functional in the embryo are laid down more anteriorly and mature more quickly. Organs dispensable for the embryo are established more posteriorly and mature more slowly. Hence, organs like the kidneys and the lung only become functional at or after birth. As briefly discussed above, development of all organs is characterized by a highly coordinated programme of cell and tissue interactions, cell and tissue movements (morphogenesis) and locally controlled cellular differentiation pathways. Detailed discussions of these developmental programs are beyond the scope of this introductory chapter and can be found in [197].

Besides locally controlled proliferation rates leading to directional outgrowth of organs and appendages like limbs, jaws or external genitalia, a massive increase in size occurs between day 6.5 of development and birth. Global growth control occurs concomitantly with gastrulation and organogenesis but can genetically be uncoupled, leading to small newborn pups [198]. Global growth control is mediated by a cocktail of systemic factors, which are under hormonal control at some point during development.

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CHAPTER 2.2

Gross Anatomy

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Introduction

This chapter presents illustrations likely to be of practical importance to those working with laboratory mice. They include the body regions, a simple demonstration of the skeleton, the muscles and a dissection of the body cavities with description of major organs. More detailed information is provided in several publications [1–5]. The terminology used here is based on the international veterinary anatomical nomenclature [6]. In the figure captions, XY denotes male and XX female.
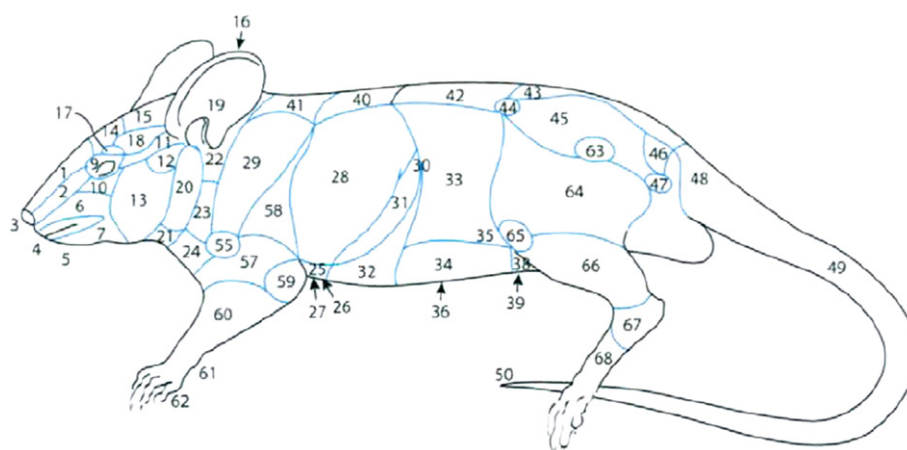
Figure 2.2.1 The regions of the body (regiones corporis), lateral view. *Regions of the face (regiones faciei),* 1. regio dorsalis nasi, 2. regio lateralis nasi, 3. regio naris et apex nasi, 4. regio oralis, 5. regio mentalis, 6. regio buccalis, 7. regio mandibularis, 8. regio intermandibularis, 9. regio orbitalis, 10. regio infraorbitalis, 11. regio zygomatica, 12. regio articulationis temporomandibularis, 13. regio masseterica. *Regions of the skull (regiones cranii),* 14. regio frontalis, 15. regio parietalis, 16. regio occipitalis, 17. regio supraorbitalis, 18. regio temporalis, 19. regio auricularis et auricula. *Regions of the neck (regiones colli),* 20. regio parotidea, 21. regio subhyoidea, 22. regio colli dorsalis, 23. regio colli ventralis, 24. regio trachealis. *Regions of the chest (regiones pectoris),* 25. regio presternalis, 26. regio sternalis, 27. regio mammaria thoracica (see Figure 2.2.4), 28. regio costalis, 29. regio scapularis, 30. arcus costalis. *Regions of the cranial abdomen (regiones abdominis craniales),* 31. regio hypochondriaca, 32. regio xiphoidea. *Regions of the middle abdomen (regiones abdominis mediae),* 33. regio abdominis lateralis, 34. regio umbilicalis, 35. regio plicae genus, 36. regio mammaria abdominalis
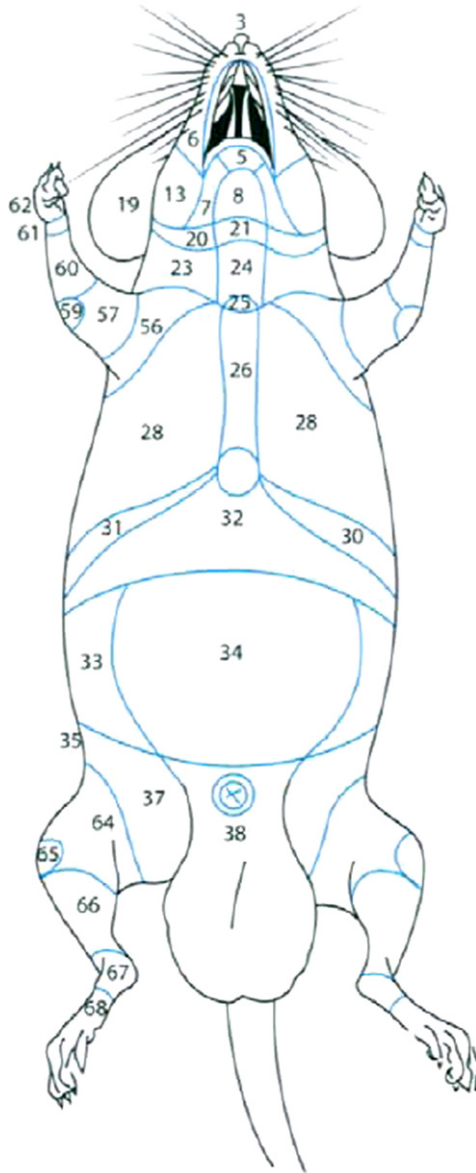
Figure 2.2.2 The regions of the body (regiones corporis), ventral view XY (for labelling see Figure 2.2.1).
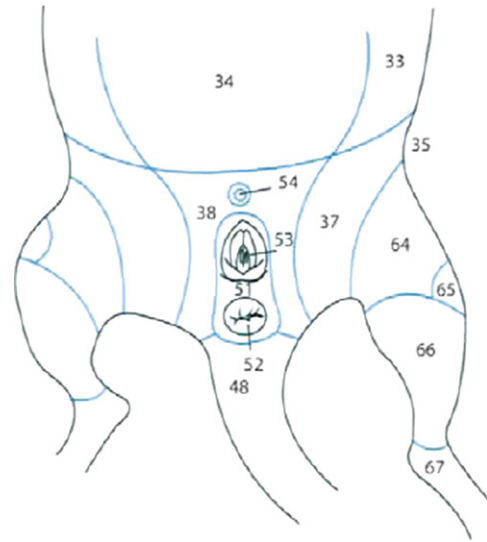
Figure 2.2.3 The regions of the body (regiones corporis), ventral view XX (for labelling see Figure 2.2.1).
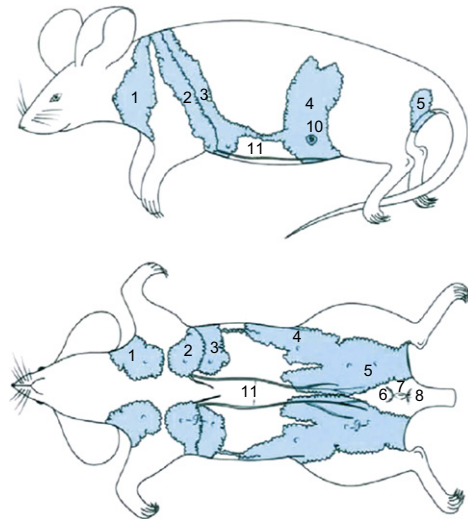
Figure 2.2.4 The mammary gland (XX). 1. Pars cervicalis, 2. pars thoracica cranialis, 3. pars thoracica caudalis, 4. pars abdominalis, 5. pars inguinalis, 6. clitoris et orificium urethrae externum, 7. introitus vaginae, 8. anus, 9. papillae mammae, 10. lymphonodus subiliacus, 11. vena epigastrica cranialis superficialis.

(see Figure 2.2.4). *Regions of the caudal abdomen (regiones abdominis caudales),* 37. regio inguinalis, 38. regio pubica (scrotalis et preputialis in XY), 39. regio mammaria inguinalis (see Figure 2.2.4). *Regions of the back (regiones dorsi),* 40. regio vertebralis thoracis, 41. regio interscapularis, 42. regio lumbalis. *Regions of the pelvis (regiones pelvis),* 43. regio sacralis 44. regio tuberis coxae, 45. regio glutea, 46. regio clunis, 47. regio tuberis ischiadici, 48. regio radicis caudae, 49. regio corporis caudae, 50. regio apicis caudae, 51. regio perinealis, 52. regio analis, 53. regio vulvae, 54. regio clitoridis. *Regions of the forelimb (regiones membri thoracici),* 55. regio articulationis humeri, 56. regio axillaris, 57. regio brachii, 58. regio tricipitalis, 59. regio cubiti, 60. regio antebrachii (cranialis, lateralis, caudalis, medialis), 61. regio carpi (cranialis, lateralis, caudalis, medialis), 62. regio manus (metacarpi et digiti, cranialis, lateralis, volaris/palmaris, medialis). *Regions of the hindlimb (regiones membri pelvini),* 63. regio articulationis coxae, 64. regio femoris (cranialis, lateralis, caudalis, medialis), 65. regio genus, 66. regio cruris (cranialis, lateralis, caudalis, medialis), 67. regio tarsi (cranialis, lateralis, caudalis, medialis), 68. regio pedis (metatarsi et digiti, dorsalis, lateralis, plantaris, medialis).
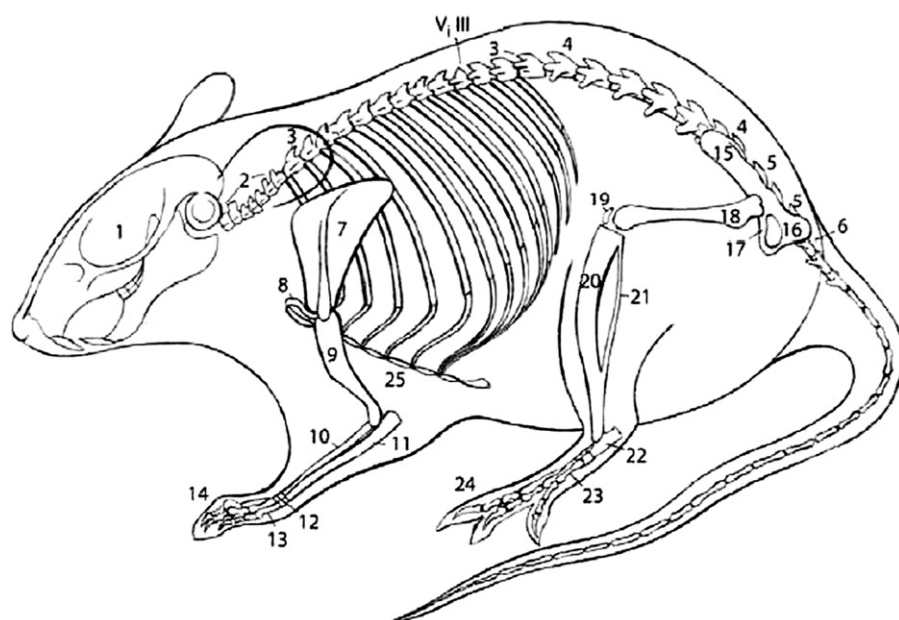
Figure 2.2.5 The skeleton. 1. Skeleton capitis, 2. vertebrae cervicales (7), 3. vertebrae thoracicae (about 13, 12–14), 4. vertebrae lumbales (5–7), 5. vertebrae sacrales (3–4), 6. vertebrae caudales (about 28, 27–30), 7. scapula, 8. clavicula, 9. humerus, 10. radius, 11. ulna, 12. ossa carpi, 13. ossa metacarpi, 14. phalanges digitorum, 15. os ilium, 16. os ischii, 17. os pubis (15, 16 and 17 considered to form 'innominate bone' of the pelvic girdle), 18. femur, 19. patella, 20. tibia, 21. fibula, 22. calcaneus et ossa tarsi, 23. ossa metatarsi, 24. phalanges digitorum, 25. sternum, V$_i$III vertebra inflexa III sive vertebra anticlinalis.
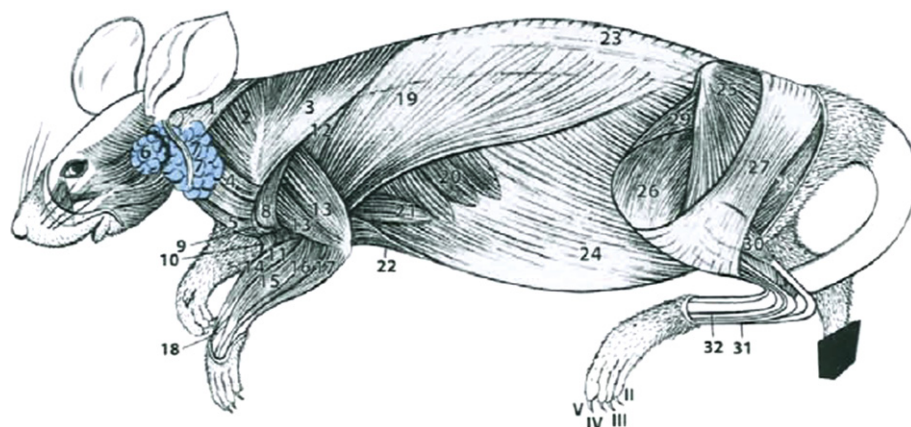
Figure 2.2.6 The muscles of the body (m., musculus). 1. M. sphincter colli superficialis, 2. m. trapezius, pars cervicalis, 3. m. trapezius, pars thoracica, 4. m. cleidocephalicus, 5. pars scapularis musculi deltoidei, 6. glandula lacrimalis extraorbitalis, 7. m. paritidoauricularis et glandula parotidea, 8. m. sternooccipitalis, 9. pars clavicularis musculi deltoidei, 10. m. biceps brachii, 11. pars acromialis musculi deltoidei, 12. m. teres major, 13. m. triceps brachii, caput longum; 13′. caput laterale, 14. m. extensor carpi radialis longus, 15. m. extensor digitorum communis, 16. m. extensor digitorum lateralis, 17. m. extensor carpi ulnaris, 18. m. abductor digiti l. (pollicis) longus, 19. m. latissimus dorsi, 20. m. serratus ventralis, 21. and 22. pars abdominalis m. pectoralis majoris, 23. fascia thoracolumbalis, 24. m. obliquus externus abdominis, 25. m. gluteus superficialis, 26. m. rectus femorism—quadriceps, 27. m. biceps femoris, 28. m. semitendinosus, 29. m. tensor fasciae latae, 30. caput laterale musculi gastrocnemii, 31. m. extensor digitorum lateralis, 32. m. extensor digitorum longus, II – V digitus secundus, tertius, quartus, quintus.
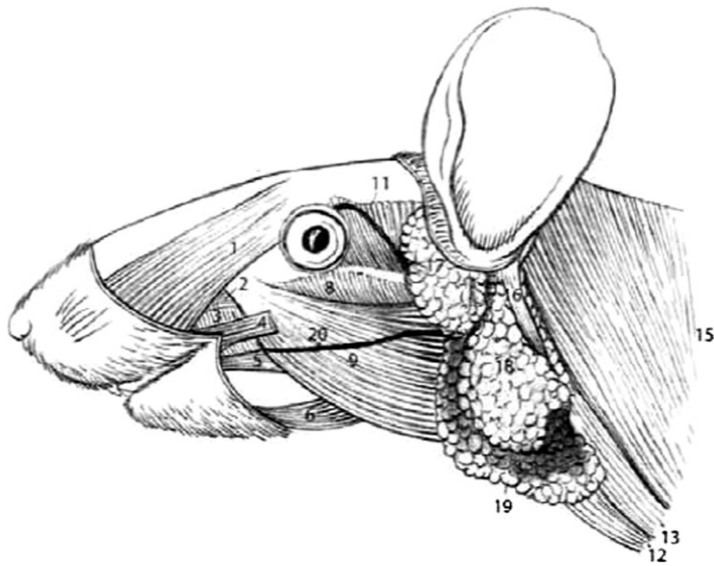
Figure 2.2.7 The muscles of the head. 1. M. levator nasolabialis, 2. m. levator labii superioris proprius, 3. m. buccinatorius, pars buccalis, 4. m. zygomaticus, 5. m. depressor labii inferioris, 6. m. digastricus, venter rostralis, 7. m. digastricus, venter caudalis, 8. m. masseter, pars profunda, 9. m. masseter, pars superficialis, 10. m. buccinatorius, pars molaris, 11. m. temporalis, 12. m. sternooccipitalis, 13. m. cleidooccipitalis, 14. m. sterno-hyoideus, 15. m. trapezius, pars cervicalis, 16. m. parotidoauricularis, 17. glandula lacrimalis extraorbitalis et eius ductus, 18. glandula parotidea, 19. glandula submandibularis, 20. ductus parotideus.
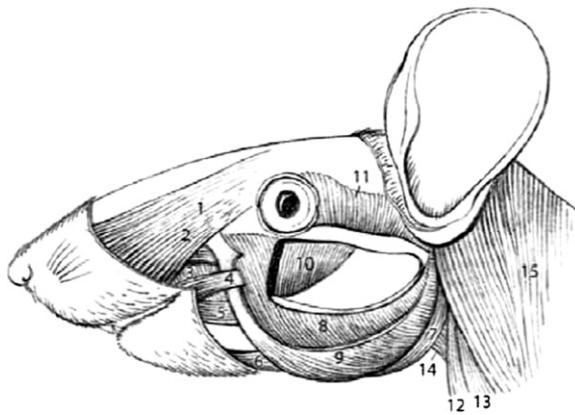
Figure 2.2.8 The muscles of the head (for labelling see Figure 2.2.7).
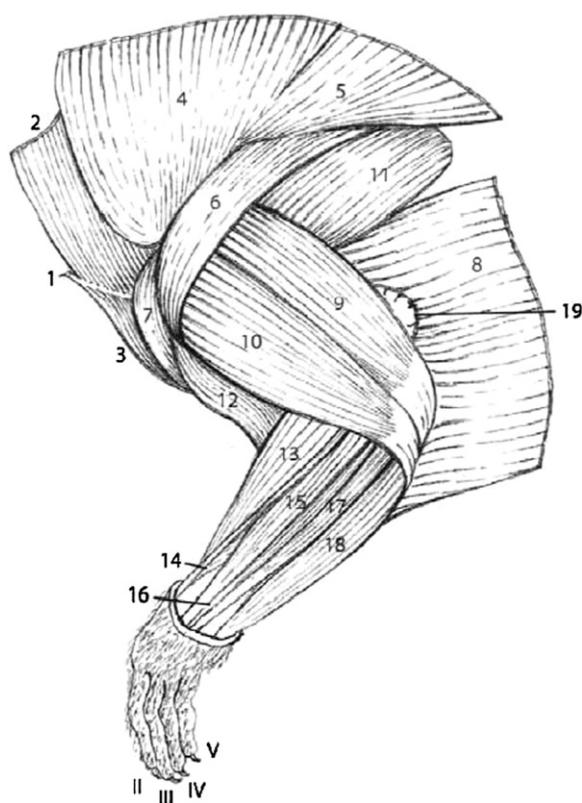
Figure 2.2.9 The muscles of the forelimb, lateral view. 1. Clavicula, 2. m. cleidocephalicus, 3. m. leidobrachialis, 4. m. trapezius, pars cervicalis, 5. m. trapezius, pars thoracica, 6. m. deltoideus, pars scapularis, 7. m. deltoideus, pars acromialis, 8. m. cutaneus trunci, 9. m. triceps brachii, caput longum, 10. m. triceps brachii, caput laterale, 11. m. infraspinatus, 12. m. biceps brachii, 13. m. extensor carpi radialis longus, 14. m. abductor digiti l. (pollicis) longus, 15. and 16. m. extensor digitorum communis, 17. m. extensor digitorum lateralis, 18. m. extensor carpi ulnaris, 19. lymphonodus axillaris accessorius, II–V digitus secundus, tertius, quartus, quintus.
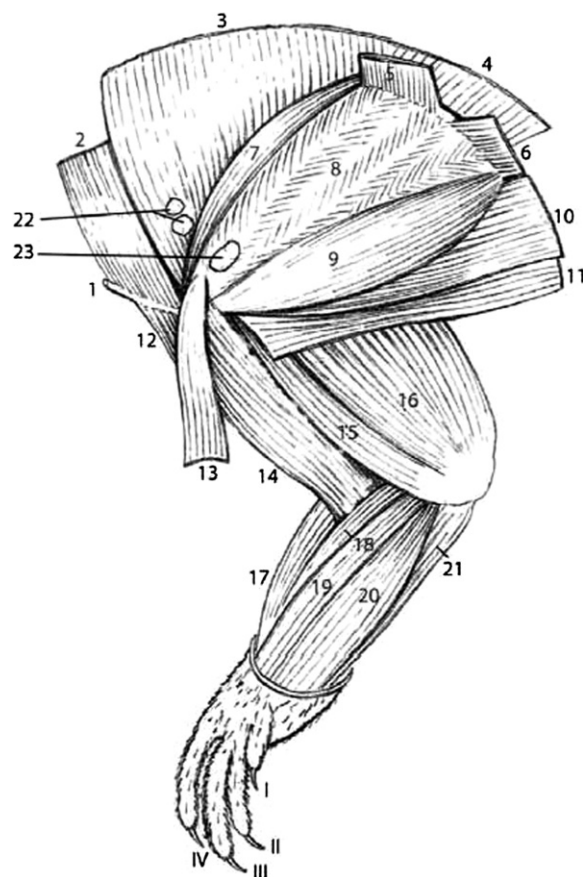
Figure 2.2.10 The muscles of the forelimb, medial view. 1. clavicula, 2. m. cleidocephalicus, 3. m. trapezius, pars cervicalis, 4. m. trapezius, pars thoracica, 5. m. rhomboideus, pars cervicalis, 6. m. rhomboideus, pars thoracica, 7. m. supraspinatus, 8. m. subscapularis, 9. m. teres major, 10. m. latisimus dorsi, 11. m. cutaneus trunci, 12. m. cleidobrachialis, 13. m. pectoralis ascendens, 14. m. biceps brachii, 15. m. triceps brachii, caput mediale, 16. m. triceps brachii, caput longum, 17. m. extensor carpi radialis, 18. m. pronator teres, 19. m. flexor carpi radialis, 20. m. flexor digitorum profundus, 21. m. flexor carpi ulnaris 22. lymphonodi cervicales superficiales, 23. lymphonodus axillary proprius, I–IV digitus primus, secundus, tertius, quartus.
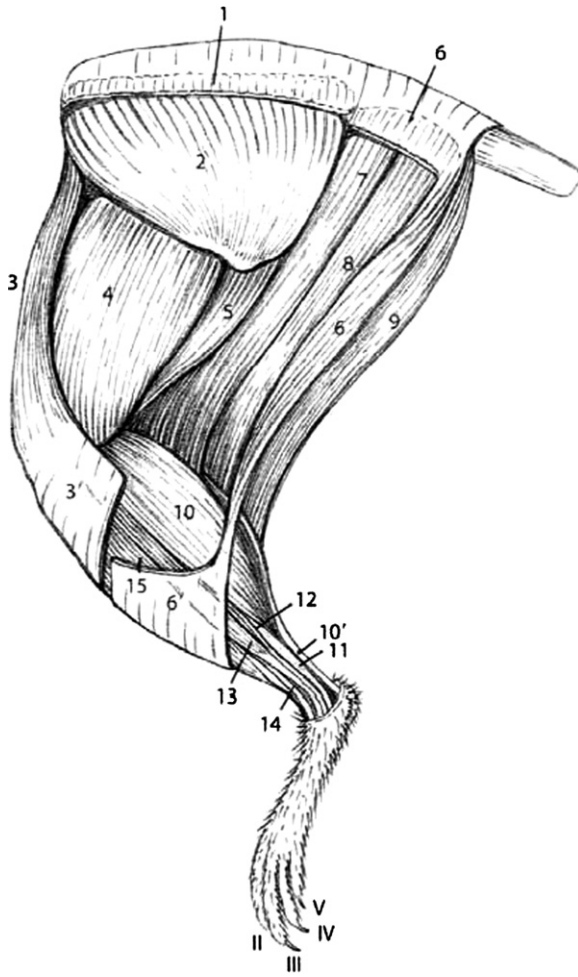
Figure 2.2.11 The muscles of the hindlimb, lateral view. 1. M. gluteus superficialis, 2. m. gluteus medius, 3. m. tensor fasciae latae, 3′. fascia lata, 4. m. rectus femoris, 5. m. vastus lateralis, 6. m. biceps femoris, 6′. fascia cruris, 7. m. adductor, 8. m. semimebranosus, 9. m. semitendinosus, 10. caput laterale musculi gastrocnemii, 10′. tendo musculi tricipitis surae, 11. m. flexor digiti I, (hallucis) longus, 12. m. extensor digitorum lateralis, 13. m. extensor digitorum longus, 14. tendo musculi peronei longi, 15. m. tibialis cranialis, II–V digitus secundus, tertius, quartus, quintus.
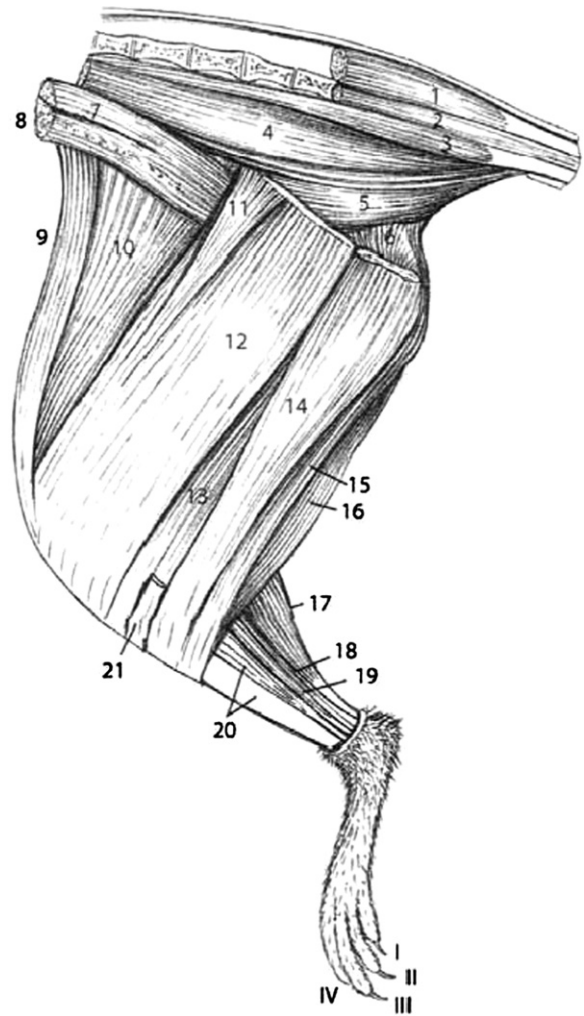
Figure 2.2.12 The muscles of the hindlimb, medial view. 1. M. lumbosacrocaudalis dorsalis lateralis, 2. musculi intertransversarii, 3. m. lumbosacrocaudalis ventralis lateralis, 4. m. coccygeus dorsalis, 5. m. coccygeus ventralis, 6. m. obturator externus, pars intrapelvina, 7. m. psoas minor, 8. m. psoas major, 9. m. tensor fasciae latae, 10. m. rectus femoris, 11. m. pectineus, 12. m. vastus medialis, 13. m. adductor, 14. m. gracilis, 15. m. semimebranosus, 16. m. semitendinosus, 17. caput mediale musculi gastrocnemii, 18. m. tibialis caudalis, 19. m. flexor digiti I (hallucis) longus, 20. m. flexor digitorum longus et tibia, 21. insertio musculi sartorii, I–IV digitus primus, secundus, tertius, quartus.

(A)

(B)

(C)
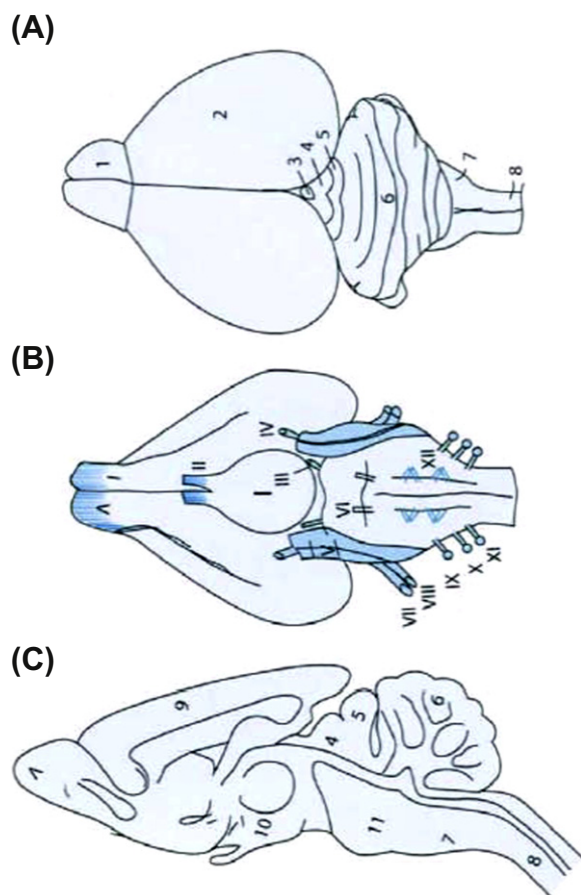
Figure 2.2.13 The brain. (A) Dorsal view, (B) ventral view, (C) midline section, 1. bulbus olfactorius, 2. hemispherium cerebri, 3. corpus pineale, 4. colliculi rostrales (tectum mesencephali), 5. colliculi caudales (tectum mesencephali), 6. cerebellum, 7. medulla oblongata, 8. medulla spinalis, 9. cortex telencephali, 10. hypothalamus, 11. pons, I n. (= nervus) olfactorius (termination in the bulbus), II n. opticus, III n. oculomotorius, IV n. trochlearis, V n. trigeminus, VI n. abducens, VII n. facialis, VIII n. vestibulocochlearis, IX n. glossopharyngeus, X n. vagus, XI n. accesorius, XII n. hypoglossus.
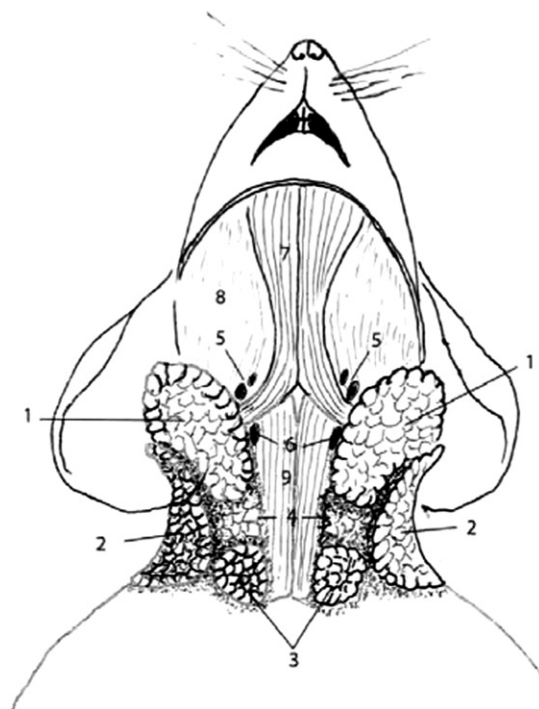
Figure 2.2.14 The salivary glands. 1. Glandula submandibularis, 2. glandula parotidea, 3. pars cervicalis thymi, 4. glandula sublingualis, 5. lymphonodi mandibulares, 6. lymphonodus retropharyngeus lateralis, 7. m. digastricus, 8. m. masseter, 9. m. sternohyoideus et m. sternothyroideus.
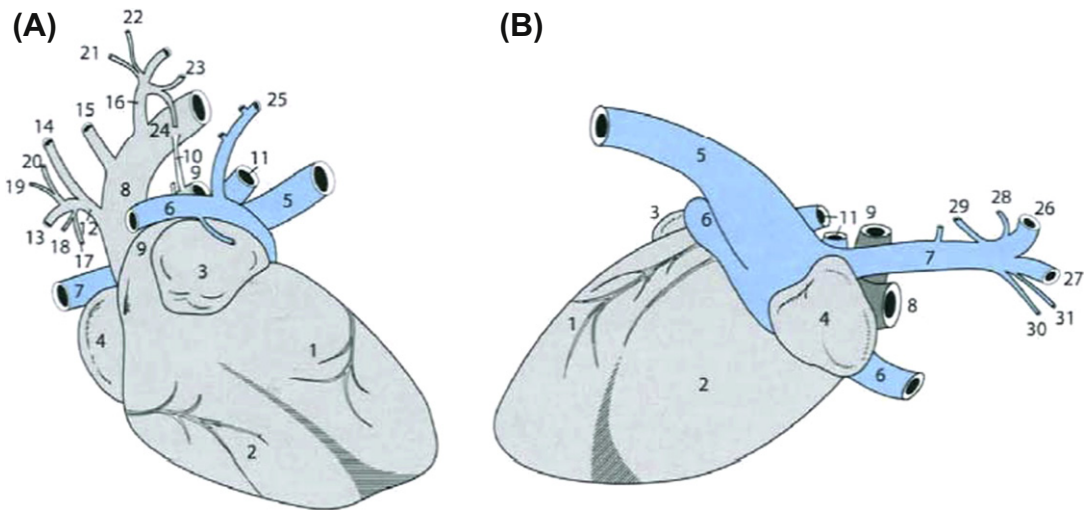
Figure 2.2.15 The heart. (A) Left lateral view, branching of the aorta, (B) right lateral view, branching of the vena cava cranialis dextra, 1. ventriculus sinister, 2. ventriculus dexter, 3. auricula sinistra, 4. auricula dextra, 5. vena cava caudalis, 6. vena cava cranialis sinistra, 7. vena cava cranialis dextra, 8. arcus aortae, 9. truncus pulmonalis, 10. ligamentum arteriosum (Botalli), 11. venae pulmonales, 12. truncus brachiocephalicus, 13. arteria subclavia sinistra, 14. arteria carotis communis sinistra, 15. arteria carotis communis dextra, 16. arteria subclavia dextra, 17. arteria thoracica interna sinistra, 18. ramus thymicus sinister, 19. arteria cervicalis superficialis sinistra, 20. arteria vertebralis sinistra, 21. arteria vertebralis dextra, 22. arteria cervicalis superficialis dextra, 23. ramus thymicus dexter, 24. arteria thoracica interna dextra, 25. vena azygos sinistra, 26. vena subclavia dextra, 27. vena jugularis externa dextra, 28. vena thymica dextra, 29. vena thoracica interna dextra, 30. vena jugularis interna dextra, 31. vena cervicalis superficialis dextra.
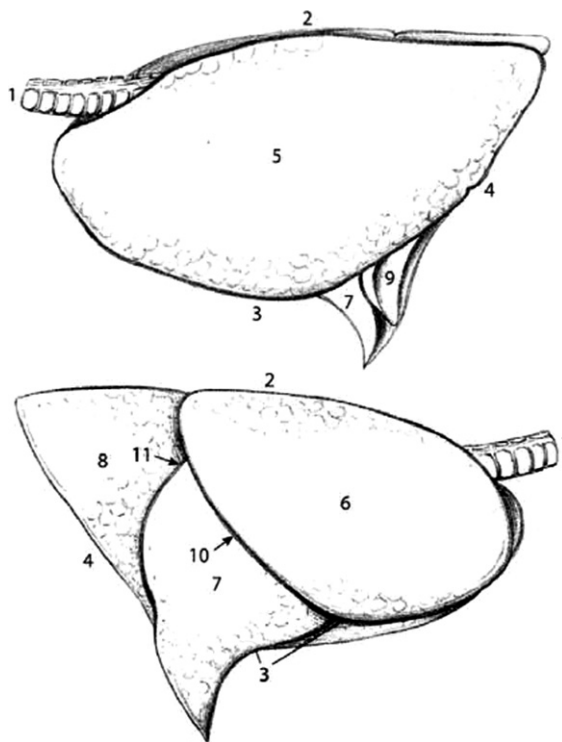
Figure 2.2.16 The lung, lateral (costal) view. 1. Trachea, 2. margo obtusus, 3. margo acutus, 4. margo basalis, 5. pulmo sinister, 6. lobus cranialis pulmonis dextri, 7. lobus medius pulmonis dextri, 8. lobus caudalis pulmonis dextri, 9. lobus accessorius pulmonis dextri, 10. incisura cardiaca, 11. fissura interlobaris.
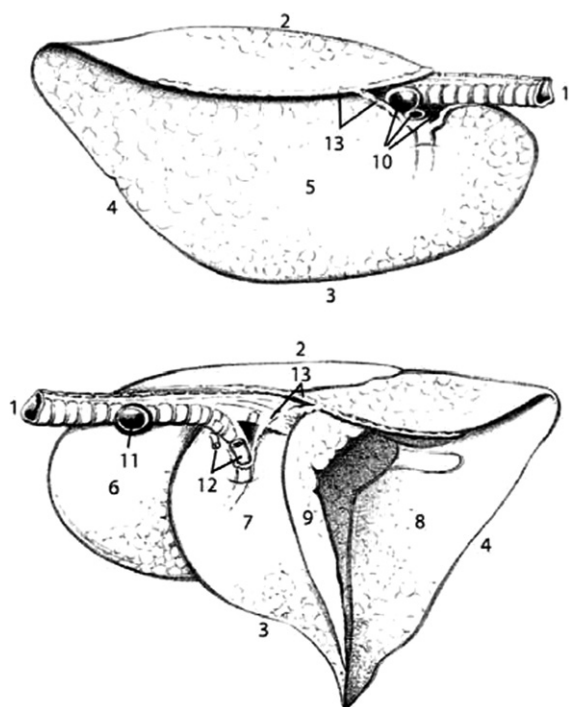
Figure 2.2.17 The lung, medial (mediastinal) view.
1. Trachea, 2. margo obtusus, 3. margo acutus, 4. margo basalis, 5. pulmo sinister, 6. lobus cranialis pulmonis dextri, 7. lobus medius pulmonis dextri, 8. lobus caudalis pulmonis dextri, 9. lobus accessorius pulmonis dextri, 10. bronchus principalis dexter et rami arteriae et venae pulmonalis, 11. bronchus principalis sinister, 12. rami arteriae et venae pulmonalis, 13. ligamentum pulmonale.
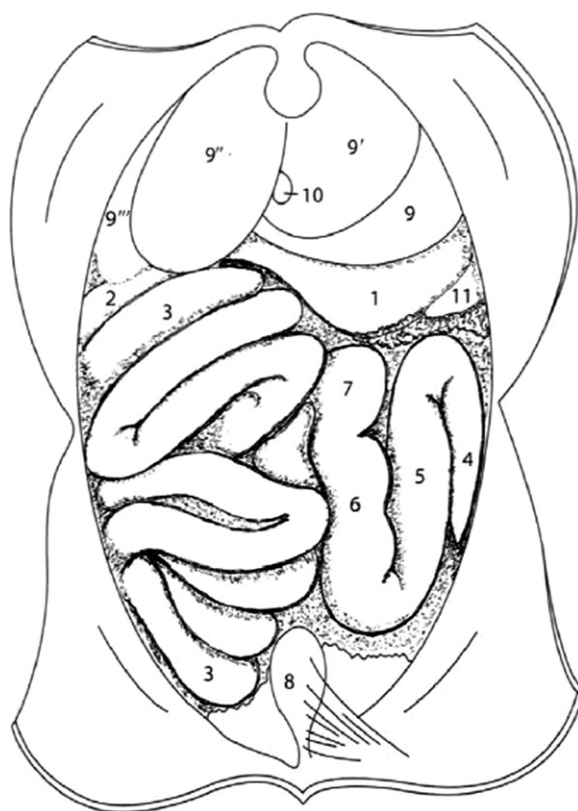
Figure 2.2.18 The abdominal situs viscerum.
1. Gaster, 2. duodenum ascendens, 3. jejunum, 4. apex ceci, 5. corpus ceci, 6. ampulla coli, 7. colon ascendens, 8. vesica urinaria et ligamenta, 9. lobus sinister hepatis lateralis, 9′. lobus sinister hepatis medialis, 9″. lobus dexter hepatis medialis, 9‴. lobus dexter hepatis lateralis, 10. vesica fellea, 11. lien.
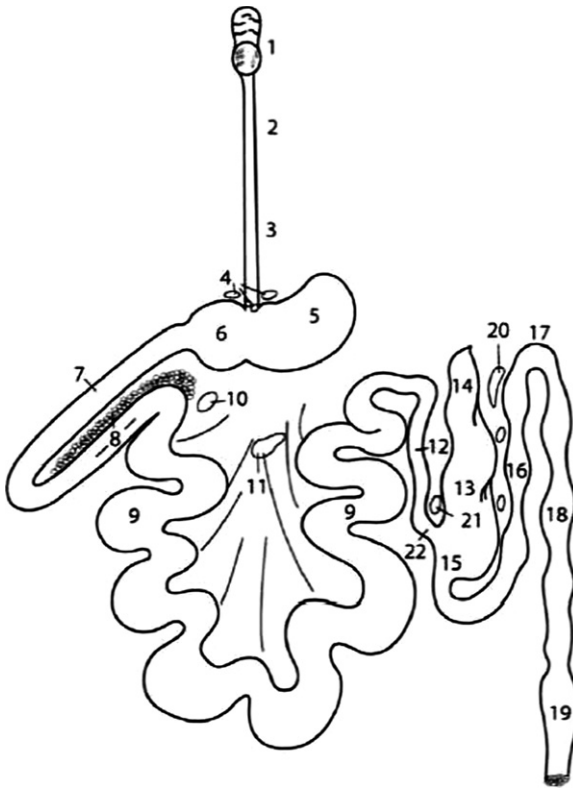
Figure 2.2.19 The digestive tract. 1. Lingua, 2. pars cervicalis esophagei, 3. pars thoracica esophagei, 4. pars abdominalis esophagei et lymphonodi gastrici, 5. pars cardiaca ventriculi (saccus cecus, forestomach), 6. pars fundica et pylorica ventriculi (glandular stomach), 7. pars descendens duodeni, 8. pars ascendens duodeni et pancreas, 9. jejunum, 10. lymphonodus pancreaticuduodenalis, 11. lymphonodus jejunalis, 12. ileum, 13. corpus ceci, 14. apex ceci, 15. ampulla coli, 16. colon ascendens, 17. colon transversum, 18. colon descendens, 19. rectum, 20. lymphonodi colici, 21. lymphonodus ileocolicus, 22. sacculus rotundus.

(A)

(B)
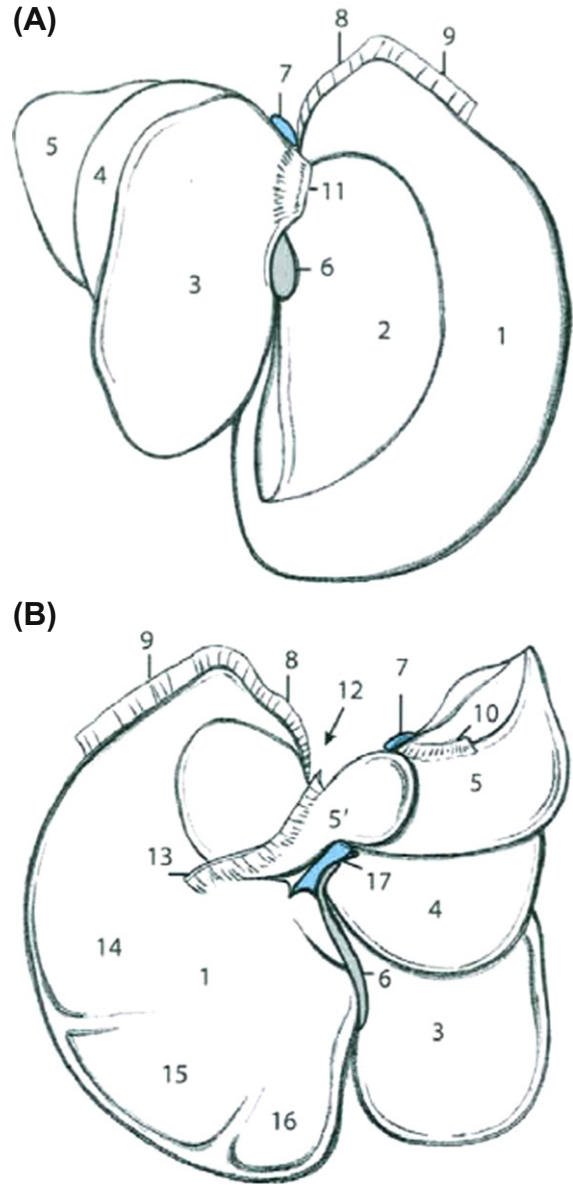
Figure 2.2.20 The liver. (A) Facies diaphragmatica (B) facies visceralis, 1. lobus sinister lateralis hepatis, 2. lobus sinister medialis hepatis, 3. lobus dexter medialis hepatis, 4. lobus dexter lateralis hepatis, 5. lobus caudatus hepatis, 5′. processus papillaris hepatis, 6. vesica fellea, 7. vena cava caudalis, 8. ligamentum coronarium sinistrum, 9. ligamentum triangulare sinistrum, 10. ligamentum hepatorenale, 11. ligamentum falciforme et ligamentum teres hepatis, 12. impressio esophagica, 13. omentum minus, 14. impressio ventricularis, 15. impressio duodenalis, 16. impressio jejunalis, 17. vena portae, arteria hepatica, ductus choledochus.
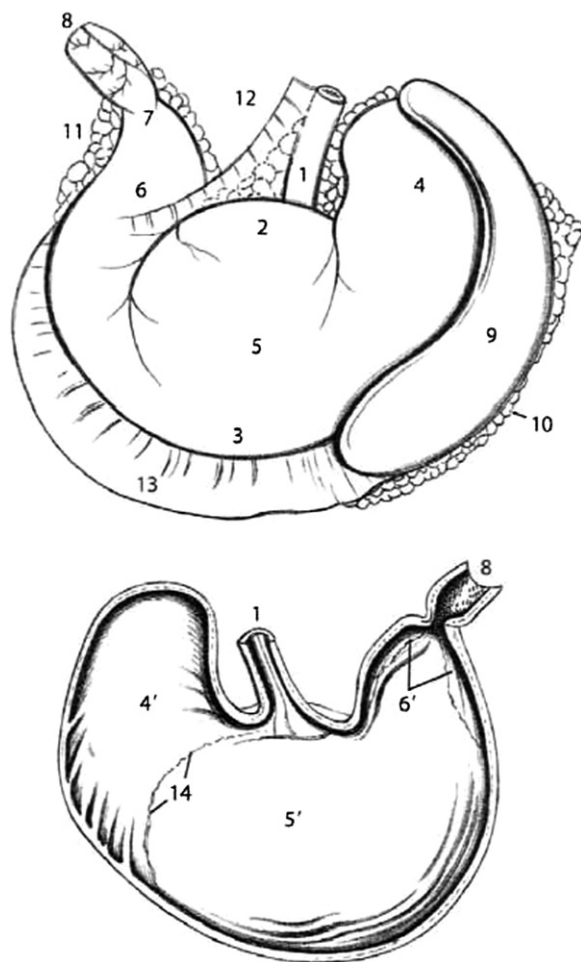
Figure 2.2.21 The stomach. 1. Oesophagus, 2. curvatura minor, 3. curvatura major, 4. saccus cecus ventriculi, 4′. pars cardiaca tunicae mucosae, 5. fundus ventriculi, 5′. pars fundica tunicae mucosae, 6. pars pylorica ventriculi, 6′. pars pylorica tunicae mucosae, 7. pylorus, 8. duodenum, 9. lien, 10. and 11. pancreas, 12. omentum minus, 13. omentum majus, 14. margo plicatus.
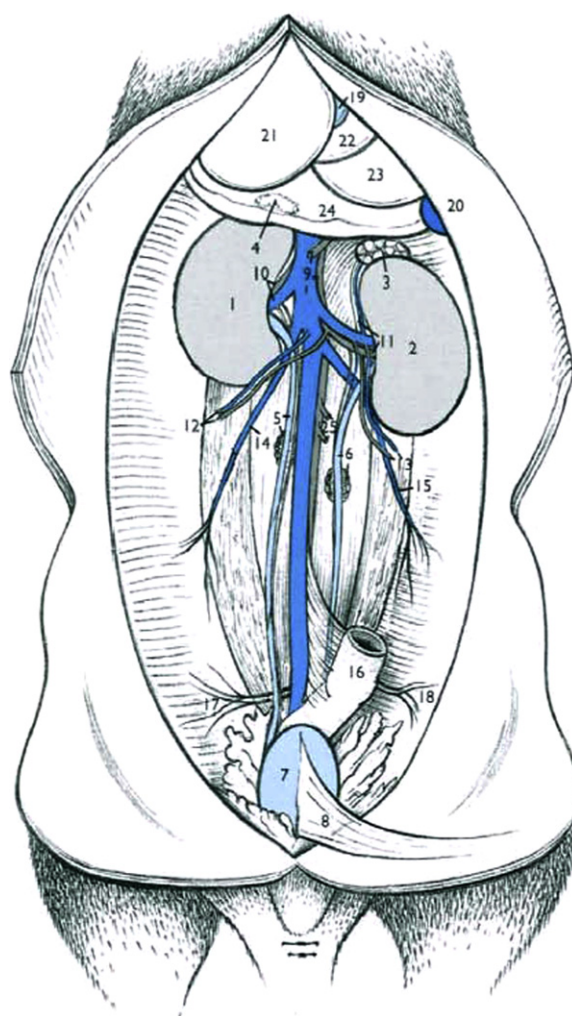
Figure 2.2.22 The kidneys *in situ.* 1. Ren dexter, 2. ren sinister, 3. glandula adrenalis sinistra, 4. glandula adrenalis dextra (under the stomach), 5. ureter dexter et lymphonodus lumbalis aorticus dexter, 6. ureter sinister et lymphonodus lumbalis aorticus sinister, 7. vesica urinaria, 8. ligamentum vesicae medianum, 9. vena cava caudalis, aorta abdominalis, arteria mesenterica cranialis, 10. arteria et vena renalis dextra, 11. arteria et vena renalis sinistra, arteria et vena adrenalis sinistra, 12. arteria et vena ovarica dextra (in XX), 13. arteria et vena ovarica sinistra (in XX), 14. ramus muscularis dorsalis dexter, 15. ramus muscularis dorsalis sinister, 16. colon descendens, 17. arteria et vena circumflexa ilium dextra, 18. arteria et vena circumflexa ilium sinistra, 19. vesica fellea, 20. lien, 21. lobus dexter medialis hepatis, 22. lobus sinister medialis hepatis, 23. lobus sinister lateralis hepatis, 24. curvatura major ventriculi, 25. arteriae mesentericae caudales.
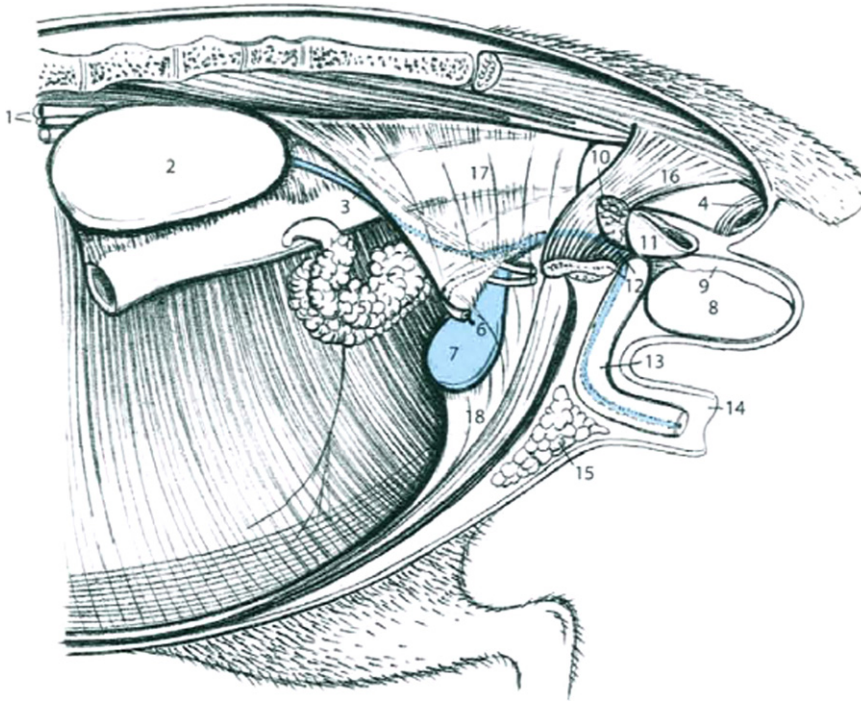
Figure 2.2.23 The male genital organs *in situ.* 1. Aorta descendens et vena cava caudalis, 2. ren sinister, 3. ureter sinister, 4. rectum et m. sphincter ani externus, 5. glandula vesiculosa, 6. vas deferens sinister, 7. vesica urinaria, 8. testis sinister, 9. epididymis, 10. glandula bulbourethralis (partim resecta), 11. diverticulum glandulae bulbourethralis, 12. symphysis pelvina et m.urethralis et radix penis, 13. penis, 14. preputium, 15. glandula preputialis, 16. m. coccygeus, 17. ligamentum vesicae laterale, 18. ligamentum vesicae medianum.
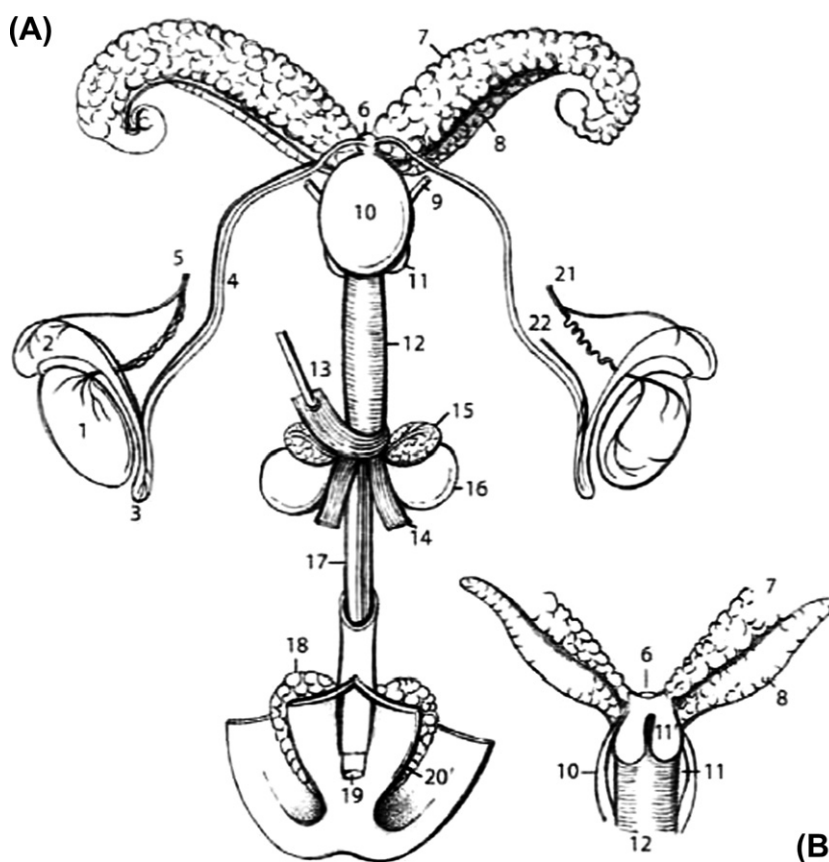
Figure 2.2.24 The male genital organs. (A) Ventral view, (B) dorsal view, 1. testis dexter, 2. caput epididymidis, 3. cauda epididymidis, 4. vas deferens dexter, 5. vena testicularis dextra, 6. glandula ampullaris, 7. glandula vesiculosa, 8. pars anterior prostatae (glandula coagulationis), 9. ureter sinister, 10. vesica urinaria, 11. prostata (pars ventralis), 11'. prostata (pars dorsalis), 12. pars membranacea urethrae et m. urethralis, 13. m. bulbo-glandularis, 14. m. ischiocavernosus, 15. glandula bulbourethralis, 16. diverticulum glandulae bulbourethralis, 17. penis, 18. glandula preputialis, 19. glans penis, 20. preputium, 21. arteria testicularis sinistra, 22. arteria vas deferentis sinistra.
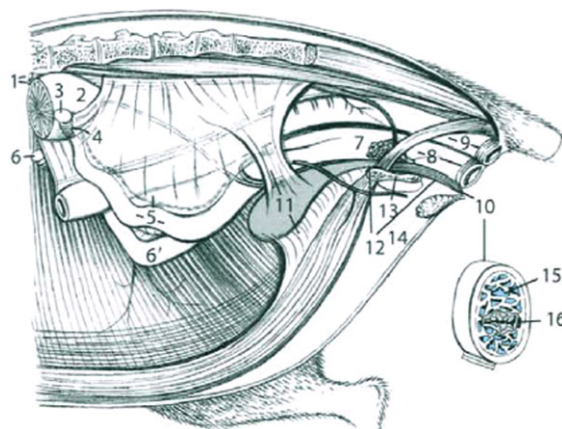
Figure 2.2.25 The female genital organs *in situ*. 1. Aorta abdominalis et vena cava caudalis, 2. ren sinister, 3. ovarium sinistrum, 4. oviductus sinister et mesosalpinx sinister, 5. cornu uteri sinistrum, ligamentum latum uteri, 6. ovarium dextrum, 6'. cornu uteri dextrum, 7. cervix uteri, 8. vagina et m.constrictor vulvae, 9. anus et m. sphincter ani externus, 10. clitoris et seccio transversalis clitoridis, 11. vesica urinaria et ligamentum vesicae laterale et ligamentum vesicae medianum, 12. urethra, 13. symphysis pelvina, 14. glandula clitoridis (preputialis feminina), 15. corpus cavernosum clitoridis, 16. urethra.
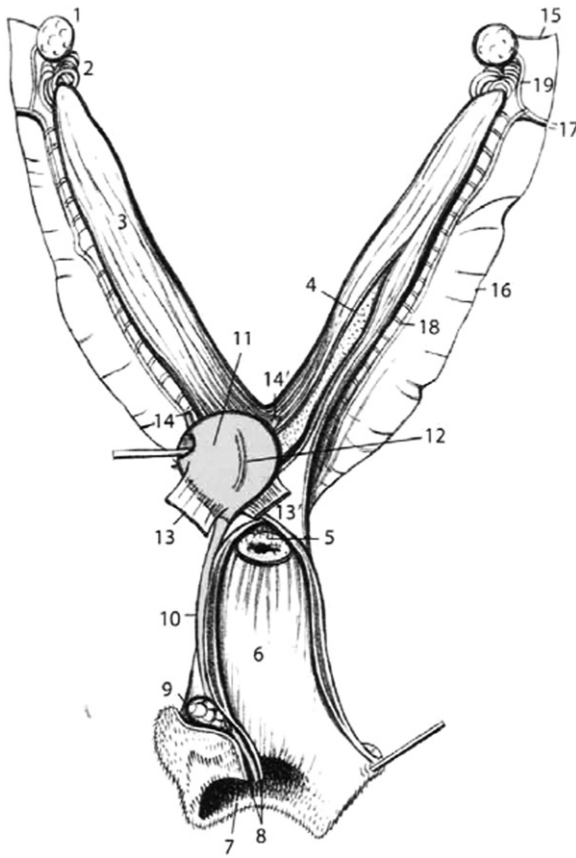
Figure 2.2.26 The female genital organs, ventral view. 1. Ovarium dextrum, 2. oviductus dexter, 3. cornu uteri dextrum, 4. cornu uteri sinistrum (partim resectum), 5. portio vaginalis uteri, cervix, 6. fornix vaginae, 7. vestibulum vaginae, 8. clitoris et preputium clitoridis, 9. glandula clitoridis (preputialis feminina), 10. urethra, 11. vesica urinaria, 12. ligamentum vesicae medianum, 13. ligamentum vesicae laterale dextrum, 13′. ligamentum vesicae laterale sinistrum, 14. and 14′. ureter dexter et sinister, 15. mesovarium, 16. mesometrium, 17. arteria et vena ovarica sinistra, 18. ramus uterinus arteriae et venae ovaricae sinistrae, 19. ramus ovaricus arteriae et venae ovaricae sinistrae.
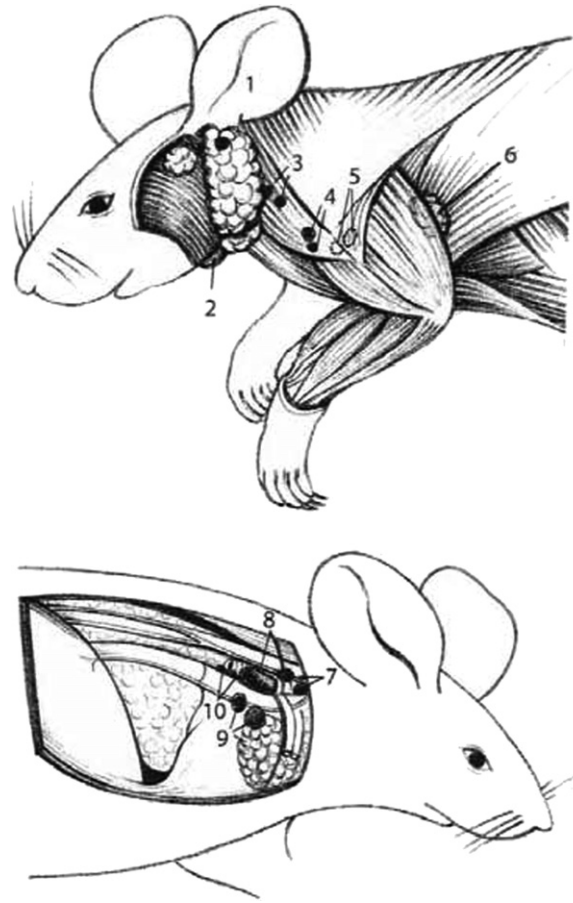
Figure 2.2.27 The lymph nodes of the head, neck and thorax (ln., lymphonodus; lnn., lymphonodi). 1. ln. parotideus, 2. ln. madibularis, 3. lnn. cervicales profundi, 4. lnn. cervicales superficiales, 5. ln. axillaris proprius, 6. ln. axillaris accessorius, 7. ln. mediastinalis cranialis, 8. lnn. tracheobronchiales, 9. lnn. mediastinales medii et pars thoracica thymi, 10. lnn. mediastinales caudales.
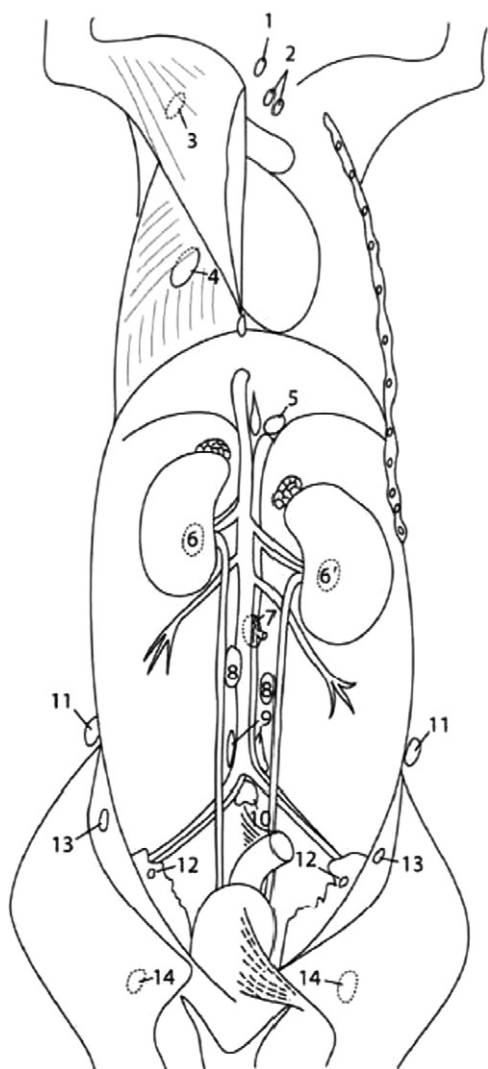
Figure 2.2.28 The lymph nodes of the body. 1. ln. cervicalis profundus caudalis, 2. lnn. mediastinales craniales, 3. ln. axillaris proprius, 4. ln. axillaris accessorius, 5. ln. aorticus, 6. and 6' lnn. renales, 7. ln. mesentericus caudalis, 8. lnn. lumbales aortici, 9. lnn. iliaci externi, 10. ln. iliacus internus, 11. ln. subiliacus, 12. ln. iliofemoralis, 13. ln. inguinalis superficialis, 14. ln. popliteus.

Acknowledgement

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Histology

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Introduction

The 'normative' descriptions and microscopic illustrations of the organs focus on features characteristic for the mouse in general. The abundance of mouse strains, spontaneous mutants and genetically engineered models does not allow excursions to their detailed features. Some strain differences are briefly commented on.

The following rules were applied. For bilateral organs the word 'paired' is consistently used. Bilaterally symmetrical organs such as the teeth, or the organs located in the body midline, such as the brain or the nasal cavity, are not considered as 'paired'. The description of hollow organs generally adheres to a concept of three-layered wall: inner lining, such as intima or mucosa; middle layer, such as muscularis; and outer covering, such as adventitia. Adventitia exposed to the abdominal cavity is called serosa. For descriptions of the glands consisting of smaller units whose excretory ducts progressively join to form main ducts, the term 'compound' is used.

The morphological features described here are generally those of healthy young adult individuals (Table 2.3.1). Characteristic changes occurring with advanced age are briefly mentioned. Further details can be found in specialized publications [1–5]. Various research projects, mostly devoted to 'phenotyping' and correlation of genotypic with phenotypic features, are published on the internet: a selection is presented in the References section.

Spontaneous diseases, including hyperplastic and neoplastic changes, are dealt with in a number of sources [6–10].

Except where noted otherwise, all microphotographs in this chapter show haematoxylin- and eosin-stained paraffin sections from young adult mice of the CD-1 strain.

Cardiovascular system

Aorta

The wall of the aorta is composed of the intima with an endothelial lining, the thick media formed

TABLE 2.3.1: Some particular morphological features of the laboratory mouse
Motto: the mouse is not a miniature rat

Organ	Mouse	Rat
Adrenal gland	Subcapsular fusiform cells (type A) proliferate spontaneously	There are no subcapsular fusiform cells
	The cortical zona reticularis is not recognizable	There is cortical zona reticularis
	At the junction of cortex and medulla there is the X zone, which regresses after weaning and in females undergoes lipid vacuolation	There is no X zone
Bone	The cortical bone does not have distinct haversian systems	Haversian systems are present
	Growth of long bones is complete by 26 weeks	The cartilaginous growth plates are not totally resorbed
Gallbladder	Present	Absent
Kidney	The parietal epithelial cells of the Bowman's capsule are cuboidal in males and flattened in females	There is no such sexual dimorphism
Liver	Anisocytosis and anisokaryosis are regular features	These features are not characteristic
Lung	The lymphoid tissue (BALT) is rarely seen in the healthy mouse lung	BALT is seen
Spinal cord	Motor nerve fibres are located in the ventral and lateral columns	Motor nerve fibres are partially located in the deep portion of dorsal columns
Testes	Time for spermatogonia to develop into spermatozoa is 35 days and 12 stages of the cycle of the seminiferous epithelium are recognized	Spermatogenesis takes 56 days and the cycle is divided into 14 stages
Urinary bladder	In the lamina propria aggregates of lymphoid tissues occur, especially in ageing animals	No such aggregates of lymphoid tissue occur
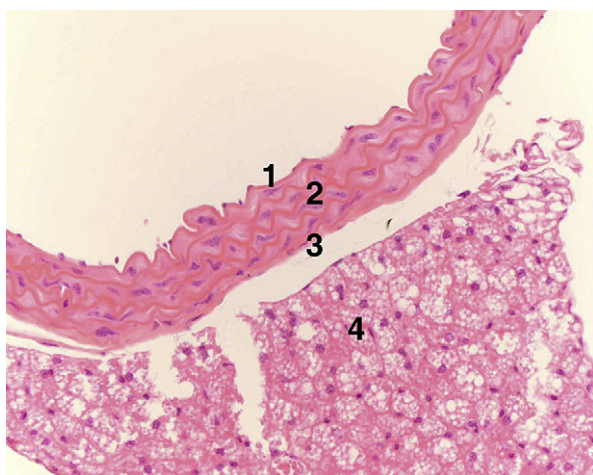
Figure 2.3.1 Aorta. 1. Intima, 2. media, with prominent wavy elastic fibres, 3. adventitia, 4. mediastinal brown fat (the cytoplasm contains multiple small lipid droplets).

predominantly by elastic fibres with smooth muscle fibres, and the adventitia (Figure 2.3.1).

Heart

The heart is located in the thoracic cavity, surrounded by the pericardium. It is a hollow muscular organ containing a left and right atrium and a left and right ventricle (Figure 2.3.2). The heart wall consists of the endocardium, myocardium and epicardium. The myocardium has striated fibres with centrally located nuclei (Figure 2.3.3). At the base of the heart there is a supportive 'skeleton' formed by fibrous connective tissue. The valves between the atria and ventricles (right tricuspid and left bicuspid) are
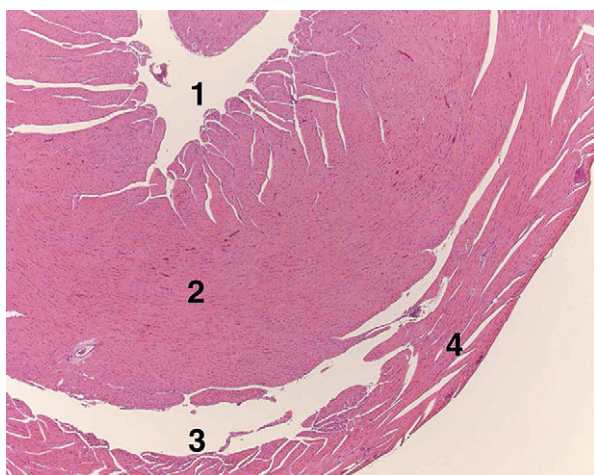
Figure 2.3.2 Heart. 1. Left ventricle, 2. interventricular septum, 3. right ventricle, 4. wall of the right ventricle.
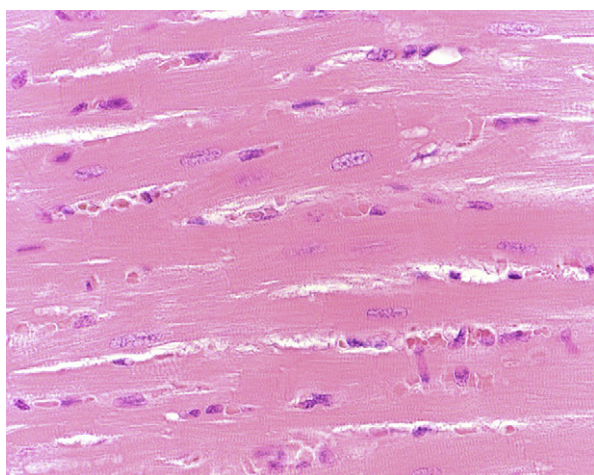
Figure 2.3.3 Heart. Myocardial fibres exhibit cross-striation and centrally located nuclei.

formed by connective tissue and covered by the endocardium. They have a pale, myxomatous appearance. Certain mouse strains are genetically predisposed to develop spontaneous myocardial calcification [11].

Digestive system

Oesophagus

The oesophagus is located dorsally to the trachea, slightly to the left of the medial level. It is lined by stratified squamous epithelium and the muscular wall is formed by longitudinal and circular striated muscle fibres (Figure 2.3.4).
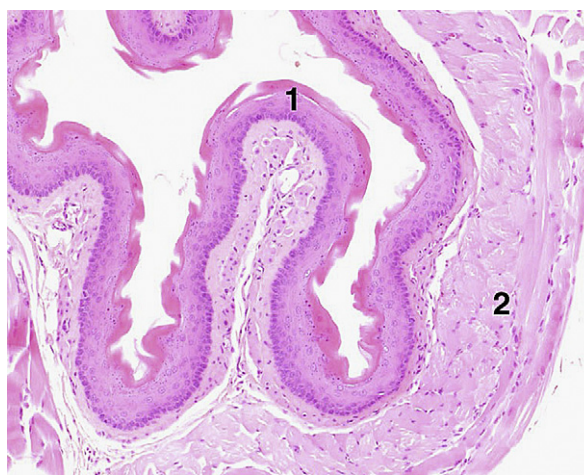
Figure 2.3.4 Oesophagus. 1. Stratified squamous epithelium, 2. striated muscle arranged in a longitudinal and circular direction.

Gallbladder

The mouse gallbladder is located at the base of the deep bifurcation of the median lobe of the liver. It consists of a fundus, a body and a neck, which continues into the cystic duct. The cystic duct unites with the hepatic duct to form the common bile duct, which opens at the duodenal papilla. The wall of gallbladder is formed by mucous membrane, thin smooth muscle and serosa (Figure 2.3.5). The mucosa is lined by cuboidal epithelium and is folded when the bladder is empty.
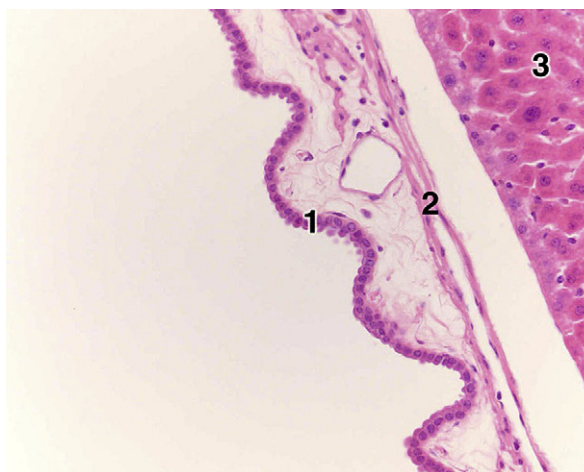
Figure 2.3.5 Gallbladder. 1. Mucosa, 2. muscularis and serosa, 3. liver.

Intestine

The intestine is divided into the small and large intestine. Throughout its length the intestine has three principal layers: the mucosa with submucosa, the muscularis and the serosa. The mucosa has an epithelial lining and fibrovascular stroma called the *lamina propria mucosae*. The lamina propria is separated from the submucosa by the *lamina muscularis mucosae*, a thin layer of smooth muscle. The submucosa consists of connective tissue surrounding blood and lymphatic vessels and nerves. The muscularis consists of an outer longitudinal layer and an inner circular layer of smooth muscle. The serosa is formed by a thin layer of visceral peritoneum. The lymphoid tissue (GALT, gut-associated lymphatic tissue) forms nodules scattered in the submucosa and the lamina propria. The larger aggregates form 'Peyer's patches' (Figure 2.3.6), which in the small intestine are located opposite the mesenteric attachment, in an antimesenteric position. In the large intestine they are not strictly antimesenteric. The most common cell type of the mucosal epithelium are the absorptive cells with a luminal cell membrane forming microvilli. The mucous goblet cells are scattered between other cell types. The Paneth cells (Figure 2.3.7) contain brightly eosinophilic cytoplasmic granules, especially large in the mouse, which contain lysozyme and antimicrobial peptides. They occur in the small intestine, especially the jejunum, and become conspicuous after several hours of

Figure 2.3.7 Intestine: Paneth cells. Paneth cells contain large eosinophilic granules and are located at the base of intestinal crypts.

fasting. The enteroendocrine cells are polypeptide-producing endocrine cells, diffusely distributed along the gastrointestinal tract. On the surface of the Peyer's patches the epithelium forms *M cells*, which serve to sample antigens. *Caveolated cells* are considered to represent intestinal chemoreceptors.

The *small intestine* is formed by the *duodenum* (Figure 2.3.8), *jejunum* (Figure 2.3.9) and *ileum* (Figure 2.3.10). The mucosal surface of the small intestine of the mouse lacks the folds (plicae) that are found in larger species. The mucosa forms villi consisting of epithelium and lamina propria, which project into the intestinal lumen. Each villus contains a central lymph vessel, the lacteal. The length of the villi decreases from

Figure 2.3.6 Intestine: Peyer's patch. The Peyer's patch consists of lymphoid tissue located between the muscularis and the mucosal epithelium.
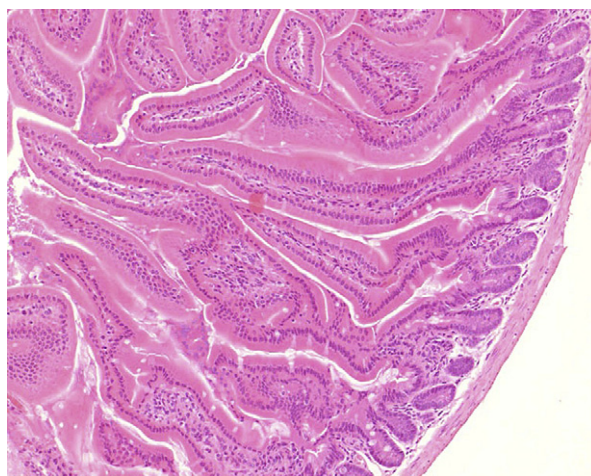
Figure 2.3.8 Intestine: duodenum.
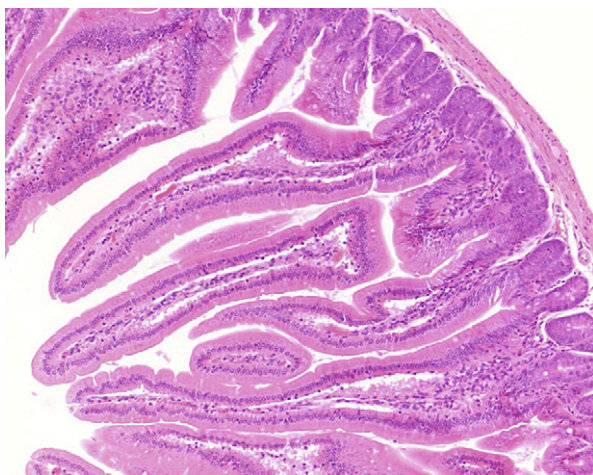
Figure 2.3.9 Intestine: jejunum.
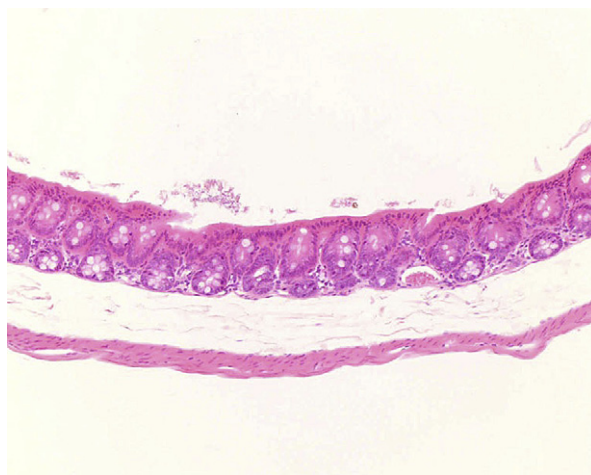
Figure 2.3.11 Intestine: caecum.
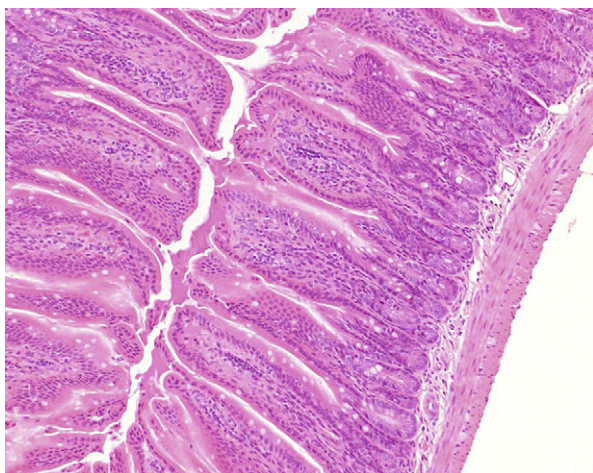
Figure 2.3.10 Intestine: ileum.
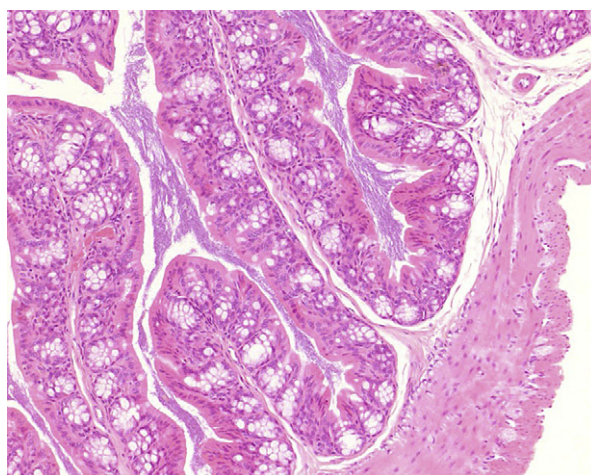
Figure 2.3.12 Intestine: colon.

the duodenum to the ileum. Among the villi are mucosal protrusions in the opposite direction, beneath the mucosal surface, forming so-called crypts or intestinal glands. The initial portion of duodenum is equipped with special tubuloalveolar duodenal glands, the Brunner's glands. One or more main pancreatic ducts and the common bile duct open at the duodenal papilla.

The *large intestine* consists of the *caecum* (Figure 2.3.11), *colon* (Figure 2.3.12) and *rectum* (Figure 2.3.13). The mucous membrane of the large intestine contains a larger proportion of goblet cells than that of the small intestine. It forms crypts, but no villi. The mouse caecum has a corpus and apex. The entrance of the ileum forms the sacculus rotundus, and the exit of the colon the ampulla coli. The caecal mucosa forms
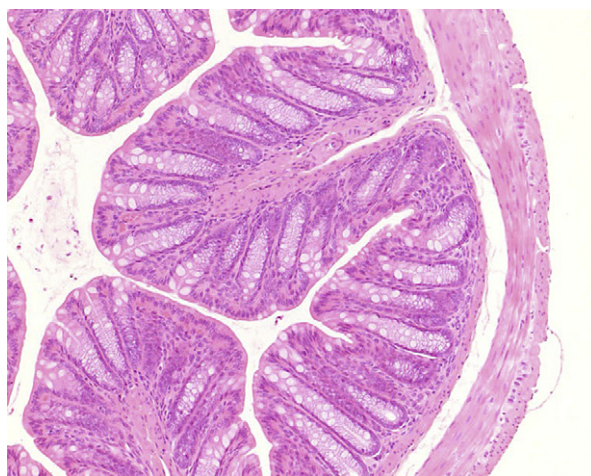
Figure 2.3.13 Intestine: rectum.

transverse folds. The colon has an ascending, a transverse and a descending part. The mucosa of the ascending and transverse colon forms transverse folds, whereas the descending colon and the rectum have prominent longitudinal folds, protruding into the lumen, formed by mucosa and submucosa. The muscularis mucosae is more prominent in the rectum than in the colon. At the transition of rectum to anus the surface epithelium becomes stratified squamous. Around the anus are the modified sebaceous circumanal glands.

Liver

The mouse liver consists of the left lateral lobe, the median lobe subdivided into left and right portions, the right lateral lobe subdivided horizontally into anterior and posterior portions, and the caudal lobe subdivided to two portions, located dorsally and ventrally to the oesophagus. The posterior surface of the caudal lobe forms the papillary process. This particular pattern of hepatic lobulation is most frequent; however, at least 13 different patterns have been described [1]. The surface of the liver is covered by a fibrous capsule, forming connective tissue septa within the liver tissue. The liver tissue is arranged in lobules with portal triads at the periphery and the central vein in the middle. The portal triads consist of branches of the hepatic artery and portal vein, as well as intrahepatic bile ducts (Figure 2.3.14). The blood flows from the
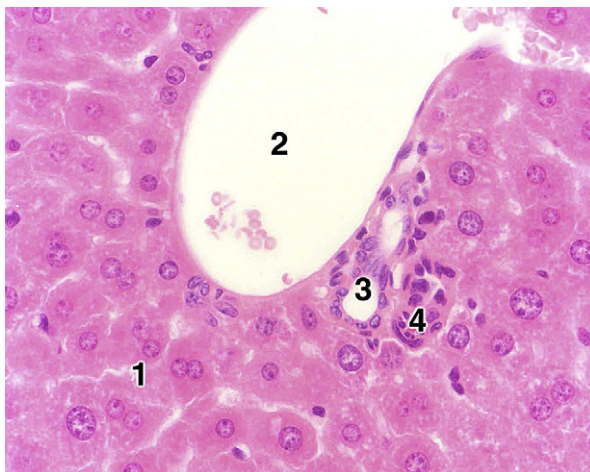
Figure 2.3.15 Liver: anisocytosis and anisokaryosis. Anisocytosis, an uneven size of hepatocytes, and anisokaryosis, an uneven size of hepatocellular nuclei, are characteristic features of the mouse liver.

perilobular area towards the central vein from where it is conducted over large hepatic veins to the vena cava. The liver cells (hepatocytes) are arranged in plates radiating from the central vein towards the lobular periphery. A characteristic feature of the mouse liver is normally occurring anisocytosis and anisokaryosis, e.g. great variation in size of the liver cells and their nuclei (Figure 2.3.15). The hepatocytes have a bile canalicular surface, which together with the surfaces of other hepatocytes forms the bile canaliculus, and a perisinusoidal surface, which is separated by the space of Disse from the sinusoidal wall formed by fenestrated endothelial cells. Specialized hepatic cells are the Kupffer cells, fixed macrophages attached to the sinusoidal wall, Ito cells containing cytoplasmic lipid droplets and storing vitamin A, and pit cells which are large granular lymphocytes with activity of natural killer cells. During the first few weeks of postnatal life megakaryocytes can be seen in the mouse liver. In contrast to the anatomical hepatic lobules, the functional units, defined as acini, have their centre at the portal triads and the periphery at the central vein. Development of the hepatobiliary system in the mouse embryo has been described in a histology atlas [12].

Oral cavity

The upper lip is split, exposing the two upper incisor teeth. The lip folds close the space

Figure 2.3.14 Liver: portal triad. 1. Hepatocytes, some of them binucleated, 2. portal triad: vein, 3. portal triad: bile duct, 4. portal triad: artery.

between the incisor and the molar teeth (the diastema). The hard palate has eight rows of ridges formed by dense connective tissue. The mucosa is formed by keratinizing stratified squamous epithelium. There is no distinct submucosa. The soft palate forms the roof of the posterior oral cavity and the floor of the nasopharynx.

Pancreas

The pancreas is located in the mesentery of the duodenal loop and the transverse colon and in the greater omentum close to the stomach and spleen. In other species the organ is subdivided into left lobe (tail), body, and right lobe (head), but such subdivision is not apparent in mice. The exocrine pancreas is a compound acinar gland (Figure 2.3.16). The acinar cells are pyramidal in shape and in haematoxylin- and eosin-stained sections have basophilic cytoplasm and large nuclei in the basal portion and acidophilic zymogen granules in the apical portion. The cell shape and granule content depend on the secretory activity. The acini are connected to intercalated ducts leading to intralobular and then to interlobular ducts which open to the main excretory ducts. One or more main excretory pancreatic ducts lead to the duodenal papilla.

Salivary glands

The mouse has three pairs of major salivary glands: the parotid gland, the submandibular (submaxillar, mandibular) gland and the sublingual gland. There are also minor glands within the tongue, palate, pharynx and larynx. The major salivary glands are located ventrally in the subcutaneous tissue of the neck. The parotid gland extends laterally to the base of the ear and lies adjacent to the exorbital lacrimal gland. All three major salivary glands are compound tubuloalveolar, the parotid is serous and the sublingual mucous. The submandibular gland is mostly described as mixed, serous and mucous. However, some authors describe it as serous [1], indeed, the microscopic appearance of the acinar cells is usually serous and distinctly different from the mucous acini of the sublingual gland. However, occasionally glands with mucous acini can be encountered, so that the appearance of acini may depend on their physiological state. The excretory ducts of the submandibular and sublingual glands open caudally to the level of incisor teeth, and those of the parotid opposite the lower molars. The submandibular gland exhibits prominent sexual dimorphism (Figures 2.3.17 and 2.3.18). In male mice the acinar cells, and especially the cells of convoluted (granular) ducts, are larger than in females. The convoluted (granular) ducts occur only in the submandibular gland and produce biologically active polypeptides including nerve growth factor and epidermal growth factor. In all three glands there are intercalated, intralobular and interlobular excretory ducts. The serous acinar cells (Figure 2.3.19) have cytoplasm which is basophilic at the
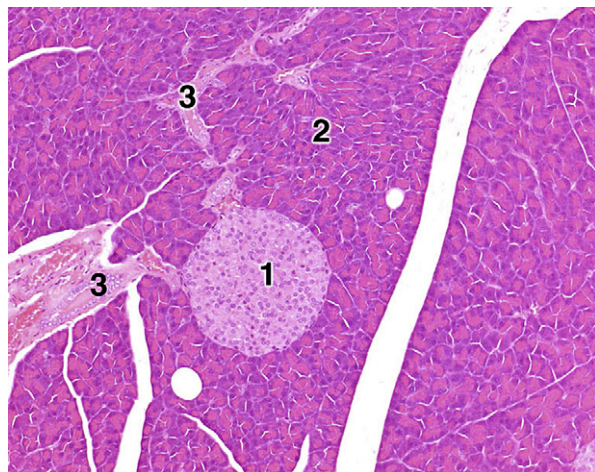
Figure 2.3.16 Pancreas. 1. Pancreatic islet, 2. exocrine acini, 3. intralobular duct.
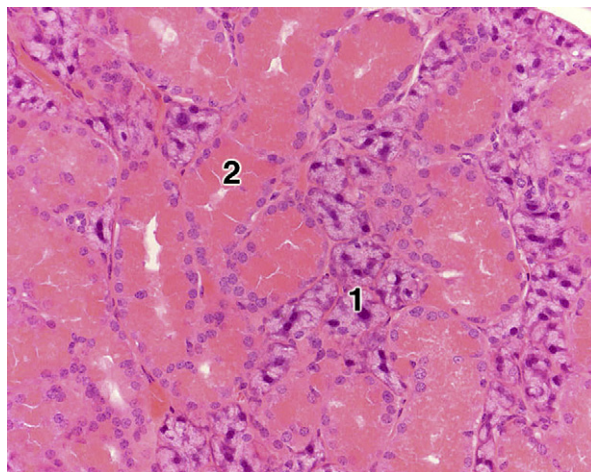
Figure 2.3.17 Salivary glands: male submandibular gland. 1. Acini, 2. convoluted ducts.
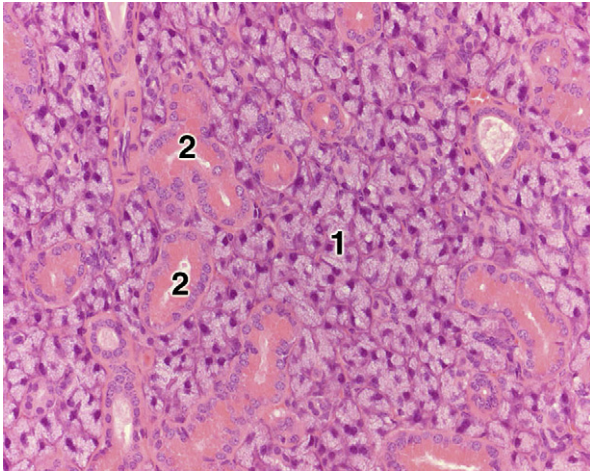
Figure 2.3.18 Salivary glands: female submandibular gland. 1. Acini, 2. convoluted ducts.
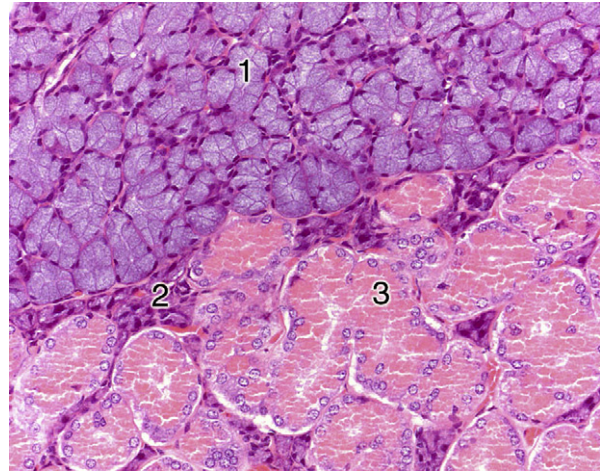
Figure 2.3.20 Salivary glands. 1. Sublingual gland, mucous acini, 2. acini of submandibular gland, 3. convoluted ducts of submandibular gland.
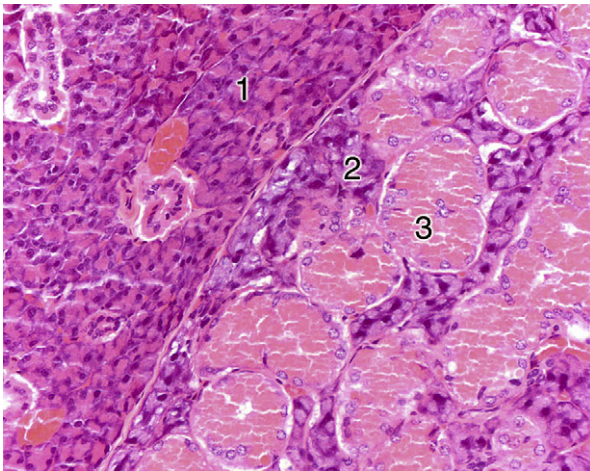
Figure 2.3.19 Salivary glands. 1. Parotid gland, serous acini, 2. acini of submandibular gland, 3. convoluted ducts of submandibular gland.

Figure 2.3.21 Stomach: gastric wall and mucosa. 1. Stratified squamous epithelium of the forestomach, 2. limiting ridge (margo plicatus), 3. mucosa of the glandular stomach, 4. muscularis.

base and granular eosinophilic in the apical portion. The mucous acinar cells (Figure 2.3.20) have basally located nuclei and pale, slightly basophilic cytoplasm. The acinar cells are surrounded by myoepithelial cells. In the mouse parotid gland, foci of basophilic hypertrophic acinar cells may occur spontaneously [13].

Stomach

The stomach is located in the left cranial part of the abdominal cavity, partially covered by the left lateral hepatic lobe. Its left half is formed by the forestomach (pars cardiaca, saccus cecus)

(Figure 2.3.21), the right half by the glandular stomach (pars fundica, pars pylorica) (Figure 2.3.22). The gastric wall consists of the mucous membrane, the smooth muscle muscularis and the serosa. The mucosa of the forestomach is lined by stratified squamous epithelium, that of the glandular stomach by epithelium forming gastric glands. The border of both kinds of epithelium is called the limiting ridge, or margo plicatus. The gastric glands are lined by single columnar epithelium and form gastric pits or foveolae gastricae, which are perpendicular to the gastric wall. Close to the limiting ridge they
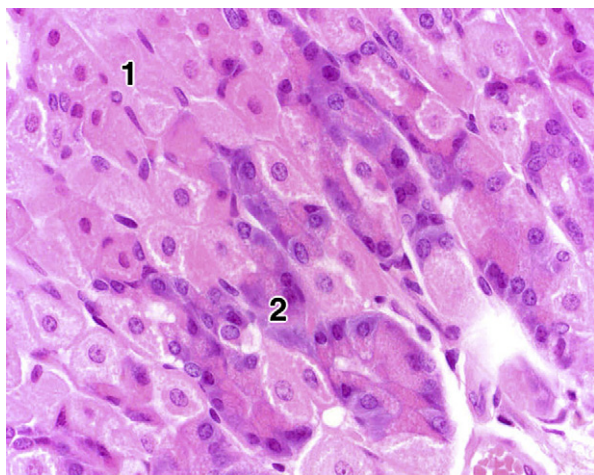
Figure 2.3.22 Stomach: glandular. 1. Parietal cells, 2. chief cells.

contain mucous cells and are called cardiac glands. The major part of the glandular stomach has the fundic glands with granular eosinophilic parietal cells producing hydrochloric acid and, at the base of the glands, the basophilic chief cells, producing zymogen. The pyloric region of the glandular stomach contains mucous pyloric glands. The enteroendocrine (entero-chromaffin-like) cells are scattered between the gastric glands. The muscularis has an inner oblique, a middle circular and an outer longitudinal layer.

Tongue

The tongue is attached to the floor of oral cavity and its portion anterior to the molars is free. The dorsal surface is rough (Figure 2.3.23); at the tip is a median dorsal groove and in the posterior part an elevated median intermolar eminence and the postmolar vallate papilla. The surface is covered by stratified squamous epithelium forming on the dorsal surface keratinized papillae. The tongue contains prominent striated muscles and connective tissue with minor salivary glands.

Teeth

The dental formula in mice is I 1/1, C 0/0, PM 0/0, M 3/3, i.e. an incisor and three molars on each side of the jaws, so that the total number of teeth
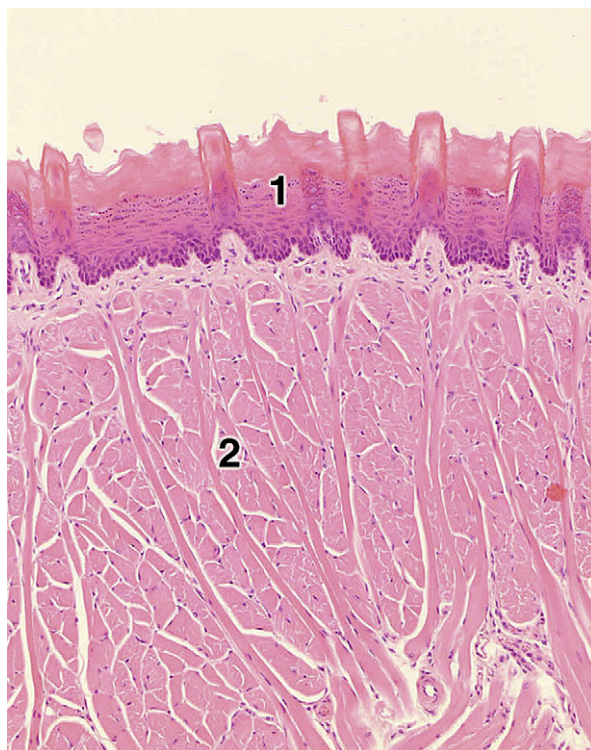
Figure 2.3.23 Tongue: dorsal surface. 1. Keratinizing stratified squamous epithelium, 2. striated muscle.

is 16. Mice have only one set of teeth—there are no temporary deciduous teeth. The histological layers of a tooth are the enamel, produced by the ameloblasts; the dentin, produced by the odontoblasts; and the cementum, produced by the cells of periodontal ligament (Figure 2.3.24).
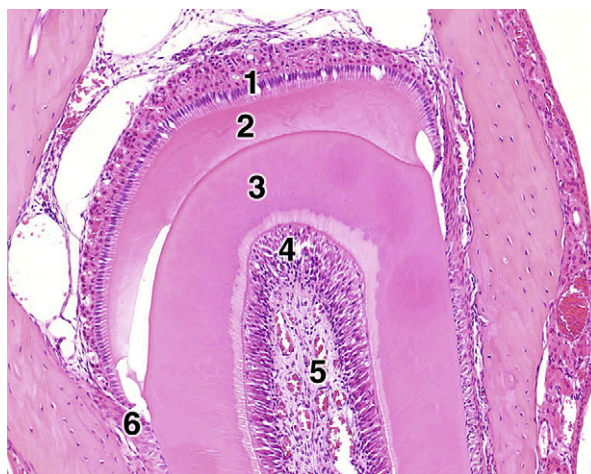
Figure 2.3.24 Tooth: incisor. 1. Ameloblasts, 2. enamel, 3. dentin, 4. odontoblasts, 5. pulp, 6. periodontal ligament.

Inside the tooth is a cavity with dental pulp, consisting of the connective tissue, blood and lymphatic vessels and nerves. The incisors grow and are worn down continuously, so that their apical foramina remain open. Spontaneously occurring malformations of mouse maxillary incisors have been reported [14].

Endocrine system

Adrenal gland

The paired adrenal glands are located cranially to the kidneys. The gland is composed of the cortex, derived from the coelomic epithelium, and the medulla, derived from the neural crest. In the mouse, accessory adrenal cortical nodules are commonly found attached to the cortex or dispersed in the retroperitoneal fat. Mouse adrenal cortex is capable of regenerating by downgrowth from the subcapsular area. Spontaneously proliferating subcapsular cells may be fusiform, so-called A cells, or more rounded B cells, resembling normal cortical cells. The cortical cells contain lipid droplets and produce steroid hormones such as mineralocorticoids, glucocorticoids and sex hormones. The cortex is composed of superficial zona glomerulosa and deep zona fasciculate (Figure 2.3.25). The zona reticularis, which occurs in other species, is not recognizable in mice. At the junction of the cortex and medulla there is the so-called X zone, which represents a specific feature of the mouse adrenal gland (Figure 2.3.26). This zone is fully developed during the first postnatal weeks, until weaning, and regresses in adult life. The function of the X zone is not well understood. In males the zone disappears by the age of puberty (about 5 weeks) without undergoing lipid vacuolization, whereas in females it continues to increase in size until the age of about 9 weeks and then regresses rapidly during the first pregnancy. In virgin females, however, it regresses slowly and undergoes lipid vacuolization, which in some cases may persist until advanced age. Sexual dimorphism of the mouse adrenal gland is expressed by a difference in size: female glands are generally larger than male. The medulla produces the biogenic amines noradrenaline (norepinephrine) and adrenaline (epinephrine), and a number of regulatory peptides. The medullary cells contain dense core vesicles for storage of biogenic amines. Adrenal medullary cells are often referred to as 'chromaffin' since the oxidation of their biogenic amines by chromate solutions results in red-brown coloration. The cells react positively with antibody
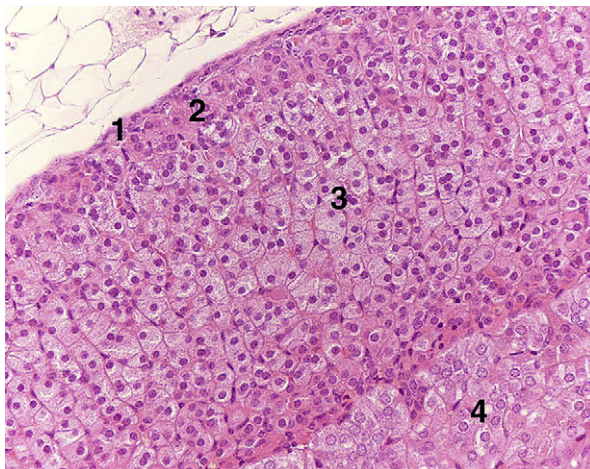
Figure 2.3.25 Adrenal gland (male). 1. Capsule, 2. zona glomerulosa, 3. zona fasciculata, 4. medulla.
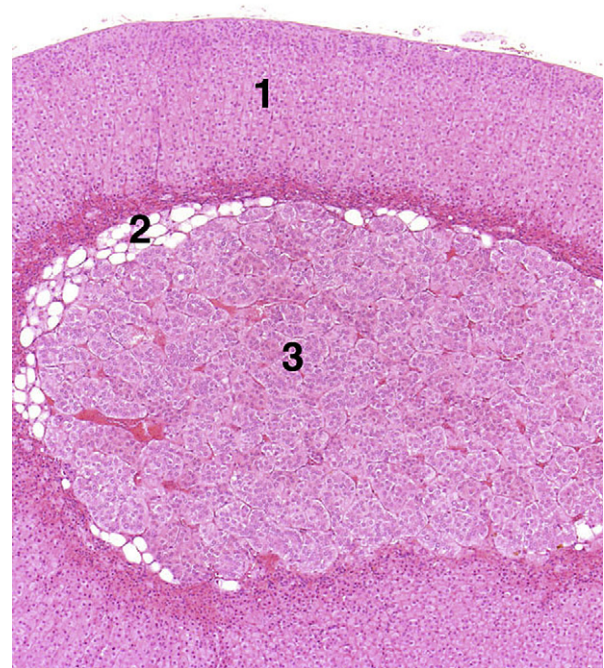
Figure 2.3.26 Adrenal gland (female). 1. Cortex, 2. X zone (showing lipid vacuolation), 3. medulla.

for tyrosine hydroxylase, chromogranin and synaptophysin.

Brown fat

This type of adipose tissue is found especially between the scapulae (so-called 'hibernating gland'), in the axillae, along the jugular veins, adjacent to the thymus, along the aorta (see Figure 2.3.1), at the renal hilum and along the urethra. The tissue is composed of polygonal cells with multiple lipid droplets in the cytoplasm and centrally located nuclei.

Oestrous cycle

See 'Uterus' and 'Vagina'. Female mice kept in groups usually synchronize their oestrous cycle; this is known as the Whitten effect [15]. (A similar effect in women who live together is known as menstrual synchrony, the dormitory effect or the McClintock effect.)

Leydig cells

The endocrine Leydig cells are located between the seminiferous tubules of the paired testes (Figure 2.3.27). They are also called interstitial cells. Their cytoplasm is abundant, eosinophilic, and can be finely vacuolated. The cells produce testosterone under the regulation of pituitary luteinizing hormone (LH).

Pancreatic islets

The pancreatic islets (islets of Langerhans) form the endocrine pancreas (see Figure 2.3.16). The islets consist of pale-staining polygonal cells and are well capillarized. The cell types include the glucagon-producing alpha cells, the somatostatin-producing delta cells, and the pancreatic polypeptide-producing PP cells, all three types being located at the insular periphery. The insulin-producing beta cells are located in the centre of the islets.

Parathyroid gland

The paired parathyroid glands are located bilaterally at the surface of the thyroid gland (Figure 2.3.28). Occasionally they may lie deep within the thyroid, or there may be more than two. They consist of cords of polygonal cells, which are either active, dark chief cells, or inactive light cells. Between the cells are numerous blood capillaries and sinusoids. The product of the parathyroid gland is parathormone (PTH).

Pineal body

The pineal body lies on the dorsal surface of the brain, in the midline, between the cerebral hemispheres and the cerebellum.

Figure 2.3.27 Testis. 1. Spermatogonia, 2. spermatocytes, 3. round spermatids, 4. elongated spermatids, 5. Sertoli cells, 6. Leydig cells.
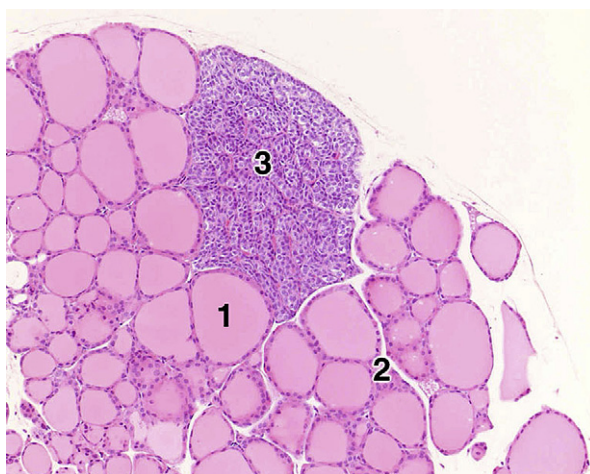
Figure 2.3.28 Thyroid gland (with parathyroid gland). 1. Thyroid follicles, 2. parafollicular C cells, 3. parathyroid gland.
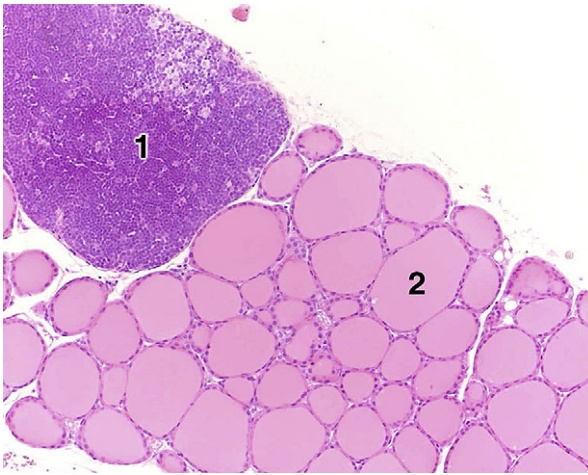
Figure 2.3.29 Thyroid gland (with ectopic thymus). 1. Ectopic thymic tissue, 2. thyroid follicles.

Figure 2.3.30 Pituitary gland. 1. Neurohypophysis, 2. pars intermedia, 3. hypophyseal cleft, 4. pars distalis.

Thyroid gland

The thyroid gland consists of two lobes connected by a thin, ventral isthmus. The gland is located at the posterior part of the larynx and initial part of the trachea. In the mouse there are usually two parathyroid glands located bilaterally at the surface of the thyroid lobes. Portions of ectopic thymic tissue can be sometimes found at this position and can be mistaken for the parathyroid gland (Figure 2.3.29). The thyroid gland contains follicles filled with eosinophilic colloid and lined by epithelial cells which, depending on their secretory activity, may be inactive, flattened, to highly active, high columnar. The products of follicular cells are thyroid hormones T_3 and T_4 (thyroxin). Between the thyroid follicles there are calcitonin-producing parafollicular C cells.

Pituitary gland

The gland is located on the ventral surface of the brain, in the midline, attached to the hypothalamus. In the mouse the gland is larger in females than in males. It consists of the adenohypophysis and the neurohypophysis (Figure 2.3.30). The adenohypophysis is made up of the pars tuberalis, pars distalis and pars intermedia. Between the pars distalis and the pars intermedia there is a hypophyseal cleft lined by a layer of epithelial cells. Traditional classification of the secretory cells of the adenohypophysis is based on their appearance in haematoxylin- and eosin-stained sections. In this stain the cells are acidophils, basophils or chromophobes. Immunohistochemical markers enable more detailed classification to be made according to secretory products. The acidophils include growth hormone (GH)-producing somatotrophs, and prolactin (PRL)-producing mammotrophs. The basophilic gonadotrophs produce the FSH (follicle stimulating hormone) and LH. Also the thyrotrophs producing thyroid stimulating hormone (TSH) are basophils. The pars intermedia contains chromophobes, especially corticotrophs producing adrenocorticotropic hormone (ACTH) and melanotrophs producing melanocyte stimulating hormone (MSH). The neurohypophysis contains terminal axons of the hypothalamic neurosecretory neurons producing vasopressin, oxytocin and antidiuretic hormone (ADH), and modified astroglia, so-called pituicytes.

Genital system

Ampullary gland

The paired ampullary glands are male accessory genital glands. They form groups of branched tubular glands lined by low cuboidal epithelium with large, oval nuclei. The glands open into the ampullae, near the seminal collicle.

Bulbourethral gland

The paired bulbourethral glands are male accessory genital glands (also called Cowper's glands). They are located at the base of the penis, and consist of the body and tail (Figures 2.3.31 and 2.3.32). The body is buried in the bulbocavernosus muscle, the tail is covered by ischiocavernosus muscle. The excretory ducts open into the urethra immediately cranially to the urethral diverticulum. The mucosal epithelium has abundant foamy cytoplasm, considered a secretory state, and eosinophilic, fine granular cytoplasm, considered a resting state. The 'resting' cells occur in the tail area.
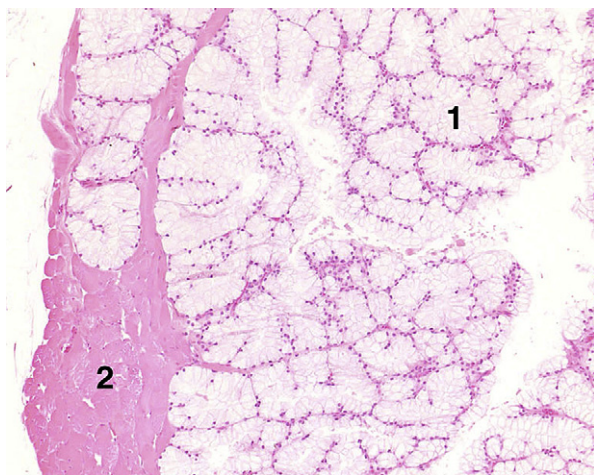
Figure 2.3.31 Bulbourethral gland: body. 1. Epithelium in secretory state (foamy), 2. skeletal muscle.
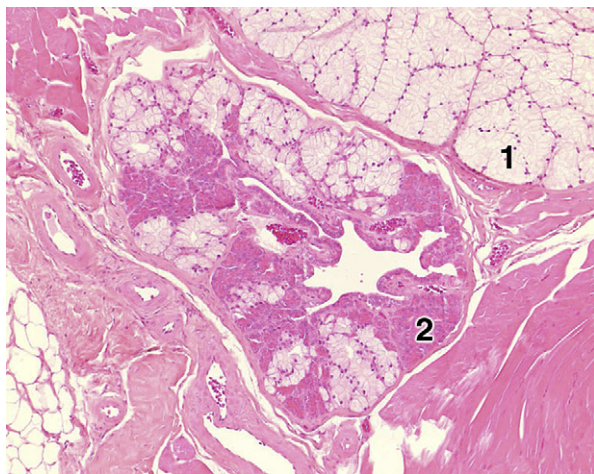
Figure 2.3.32 Bulbourethral gland: tail. 1. Epithelium in secretory state (foamy), 2. epithelium in resting state (eosinophilic, fine granular).

Clitoral gland

Female preputial gland (paired)—see 'Preputial gland'.

Coagulating gland

Anterior prostate—see 'Prostate gland'.

Epididymis

The paired epididymides consist of the head (caput), body (isthmus) and tail (cauda) (Figure 2.3.33). The organ contains ducts lined by columnar to cuboidal epithelium and occasional specialized clear cells which contain lysosomal bodies and exert enzymatic activity. The head receives testicular ductuli efferentes, and the vas deferens begins in the tail. The ductal wall contains smooth muscle which becomes more prominent at the transition to vas deferens.

Ovary

The paired ovaries lie caudally and laterally to the kidneys. They are surrounded by the ovarian bursa and connected to the uterine horns by the convoluted oviducts (Figure 2.3.34). The ovary is covered by simple cuboidal to columnar epithelium and consists of numerous follicles, yellow

Figure 2.3.33 Epididymis: tail. 1. Spermatozoa in the lumen, 2. epididymal ducts formed by epithelial lining and smooth muscle.
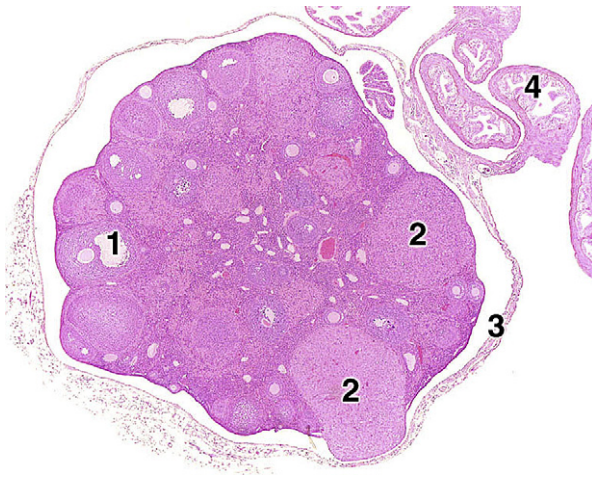
Figure 2.3.34 Ovary. 1. Antral follicle, 2. corpus luteum, 3. ovarian bursa, 4. oviduct.

bodies and clusters of polygonal interstitial cells. Rudiments of the rete ovarii may persist near the hilum as blind tubules or cords of epithelial cells. The follicles contain eggs and develop from small primordial follicles to larger growing follicles and large preovulatory Graafian follicles, which, owing to the presence of a cavity, belong to antral or vesicular follicles. Mature follicles contain an egg (oocyte) surrounded by granulosa cells and an outer layer of fusiform theca cells. Polyovular follicles may occur in young mice or in certain strains. During the lifetime only about 20% of the available follicles ovulate; the majority undergo follicular atresia characterized by cell death (apoptosis) of granulosa cells. The mature follicles produce hormones such as oestradiol, inhibin, progesterone and androgens. Follicular growth is regulated by pituitary FSH and LH and ovulation is stimulated by release of LH. After ovulation the granulosa and theca cells form a progesterone-producing yellow body (corpus luteum). The presence of a regular number of oocytes and follicles in different stages of development is considered an indicator of intact fertility and is tested in special experimental procedures. A classification scheme for mouse ovarian follicles has been proposed [16] which discriminates 10 types or subtypes in all. For practical applications, a simplified classification has been developed, categorizing the follicles as small, growing and antral [17, 18].

Oviduct

The paired oviducts are convoluted tubes connecting the ovaries to the uterine horns (see Figure 2.3.34). The oviduct is lined by a layer of cuboidal to tall columnar epithelium. The wall is formed by smooth muscle and adventitia.

Penis

The penis consists of the root, body and glans. It contains the distal part of the urethra, cavernous bodies and a small os penis (Figure 2.3.35). The glans is enclosed in a fold of modified skin, the prepuce.

Preputial gland

The paired preputial glands are modified sebaceous glands, located in the subcutaneous adipose tissue lateral to the penis (in females to the clitoris). The excretory ducts are lined by stratified squamous epithelium, and have wide lumina (Figure 2.3.36). The gland opens at the border of prepuce and skin.

Prostate gland

The prostate gland is a male accessory genital gland. It consists of the anterior, dorsal and ventral lobes (Figure 2.3.37). The anterior lobes

Figure 2.3.35 Penis. 1. Dorsal vein and nerves, 2. os penis (cartilaginous part), 3. corpora cavernosa, 4. urethra.
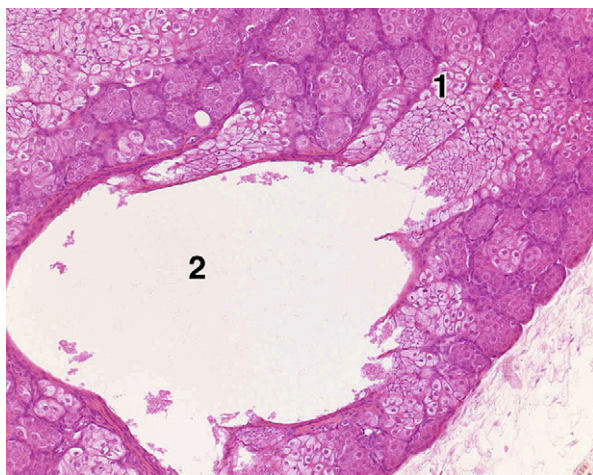
Figure 2.3.36 Preputial gland. 1. Sebacious cells, 2. excretory duct.

Figure 2.3.37 Prostate gland: ventral lobe. 1. Alveolus containing secretions, 2. alveolar wall lined by epithelium of variable height.

are known as the coagulating gland and are attached to the seminal vesicles. The dorsal lobes surround the urethra as a single body, the ventral lobes are between the urethra and the urinary bladder. All three lobes open into the urethra at the seminal collicle. The gland is tubuloalveolar, lined by epithelium which may be flattened to columnar, depending on the secretory activity.

Seminal vesicle

The paired seminal vesicles are male accessory genital glands. They are relatively large, located dorsolaterally to the urinary bladder and attached to the anterior prostate (coagulating gland). The ducts of the seminal vesicles open at

the seminal collicle, together with those of the prostatic lobes. The combined secretions of the seminal vesicles, the prostate and the bulbourethral glands form a copulatory plug which prevents outflow of semen from the vagina after ejaculation. The seminal vesicle has a wall composed of smooth muscle and tall columnar epithelium forming branching mucosal folds (Figure 2.3.38). When the gland is distended owing to a large content of secretory material, the folds stretch and become short (Figure 2.3.39).

Figure 2.3.38 Seminal vesicle (non-distended). 1. Smooth muscle wall, 2. tall columnar epithelium lining branching mucosal folds, 3. secretory material (brightly eosinophilic).
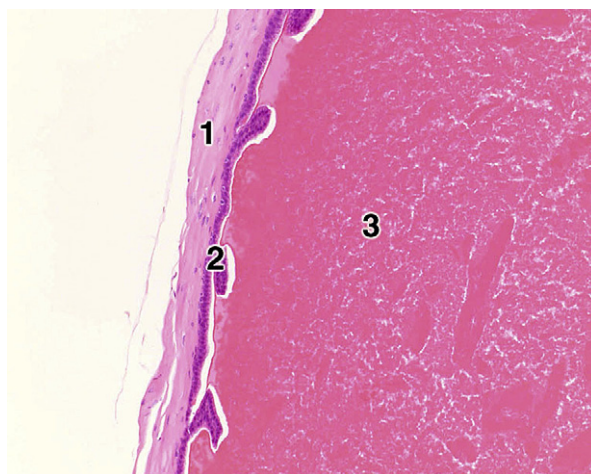
Figure 2.3.39 Seminal vesicle (distended). 1. Smooth muscle wall, 2. epithelium forming only short mucosal folds, owing to its distension by large amounts of secretory material, 3. secretory material (brightly eosinophilic).

Testis

The paired testes are located in the scrotum. They are covered by tunica albuginea and tunica vaginalis, which also covers the inner surface of the scrotum. The testis contains convoluted seminiferous tubules. The germinal epithelium is arranged in layers, the early stages of maturation at the tubular basis, the most advanced stages at the lumen. The cell types from the periphery towards the lumen are spermatogonia, spermatocytes, and round and elongated spermatids (see Figure 2.3.27). The Sertoli cells reach from the basal lamina to the tubular lumen. Maturing elongated spermatids are attached to their cell membrane. The mature spermatids become spermatozoa and are released. This process is called *spermiation*. In the mouse the approximate time for spermatogonia to develop into spermatozoa is 35 days and 12 stages of the cycle of the seminiferous epithelium are recognized. The methodology of spermatogenic staging (determination of the stages of spermatogenic cycle) has been established [19, 20]. The seminiferous tubules end in tubuli recti, which lead to rete testis and then over a collecting chamber to efferent ducts and the head of the epididymis. The rete testis is a small area under the tunica albuginea consisting of tubules lined by simple epithelium: these must not be mistaken for abnormal, atrophic seminiferous tubules. Among the seminiferous tubules are the endocrine Leydig cells, together with other peritubular cells, interstitial macrophages and interstitial vasculature.

Uterus

The mouse uterus consists of two long horns which join together in a single body that is connected by the cervix to the vagina. The uterine horns lie in the dorsal abdominal cavity, beginning at the oviducts, and the body and vagina lie ventrally to the rectum and dorsally to the urinary bladder. The wall is composed of mucosa (endometrium), an inner circular and an outer longitudinal smooth muscle layer (myometrium), and the adventitia. The endometrial mucosa is formed by simple columnar epithelium, which extends tubular endometrial glands into the endometrial stroma (lamina propria). The morphology of endometrium is influenced by
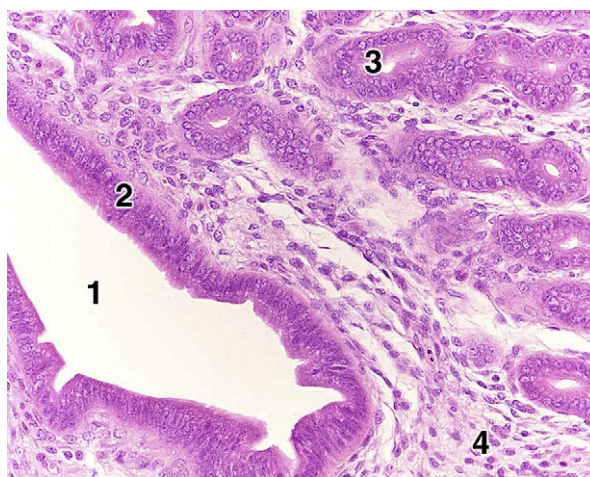
Figure 2.3.40 Uterus (proestrus). 1. Lumen, 2. endometrial epithelium, 3. endometrial gland, 4. endometrial stroma.

the oestrous cycle. During proestrus and oestrus the lumen is distended, and stroma hyperaemic (Figures 2.3.40 and 2.3.41). In metoestrus the epithelium shows vacuolar degeneration (with apoptotic bodies) (Figure 2.3.42), and during dioestrus the epithelium regenerates (Figure 2.3.43). The reactivity of the uterus to oestrogenic stimuli is used in the *mouse uterotropic assay*, a procedure designed for detecting potential oestrogenic effects of synthetic chemicals. The stimulated uterus increases the weight and the height of endometrial epithelium [21]. The uterus of ageing mice frequently develops spontaneous adenomyosis (growth of endometrium into and beyond the myometrium). This has been demonstrated to result from increased plasma level of prolactin [22].
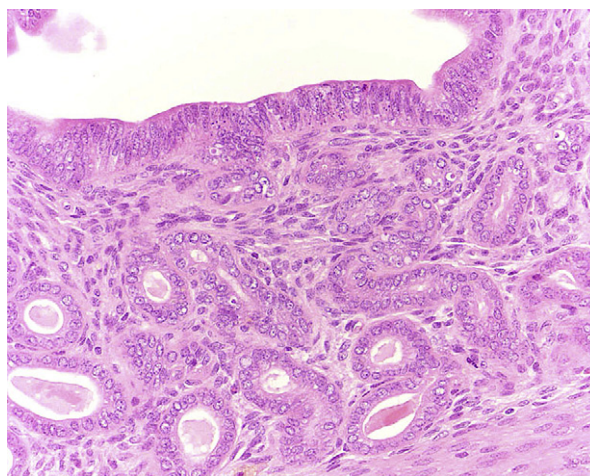
Figure 2.3.41 Uterus (oestrus).
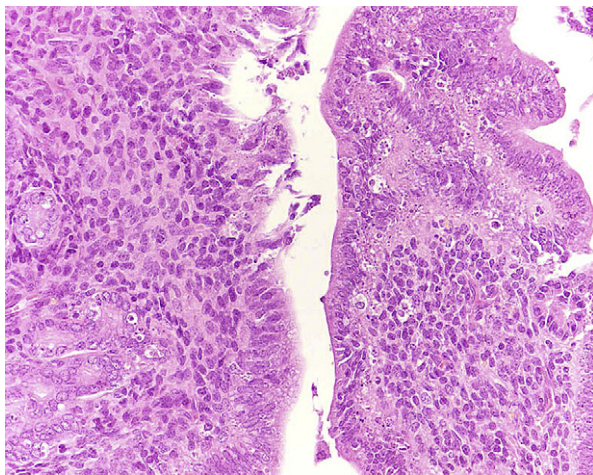
Figure 2.3.42 Uterus (metoestrus).

Figure 2.3.44 Vagina (proestrus). 1. Mucous change of the superficial layer, 2. layer of cornified cells, 3. thick stratified squamous epithelium.
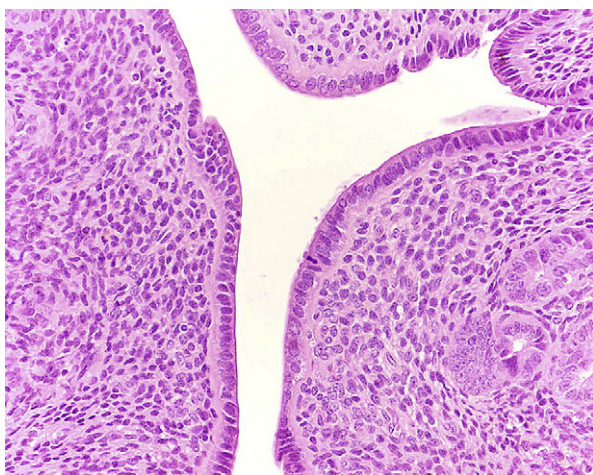
Figure 2.3.43 Uterus (dioestrus).
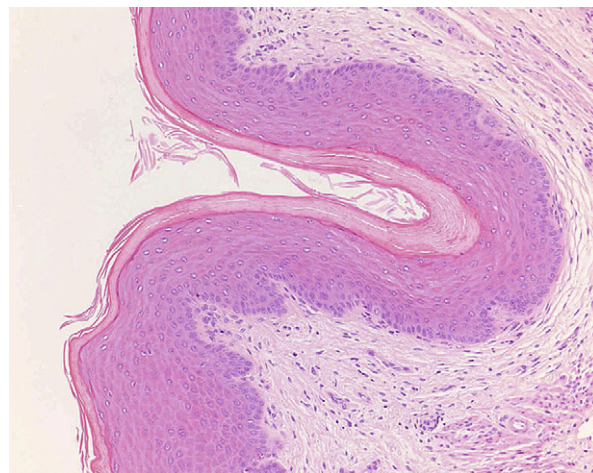
Figure 2.3.45 Vagina (oestrus).

Vagina

The wall of the vagina has a mucous membrane formed by prominent stratified squamous epithelium, and thin muscularis. During the oestrous cycle the vaginal epithelium undergoes characteristic changes. In proestrus the superficial layers show mucous change and a layer of cornified cells develops underneath (Figure 2.3.44). The stratified squamous epithelium is thick. Oestrus is characterized by thick stratified squamous epithelium with a distinct layer of cornified cells at the surface (Figure 2.3.45). In metoestrus the cornified cells become detached and may still be present in the lumen (Figure 2.3.46), and in dioestrus the stratified squamous epithelium becomes thinner and is
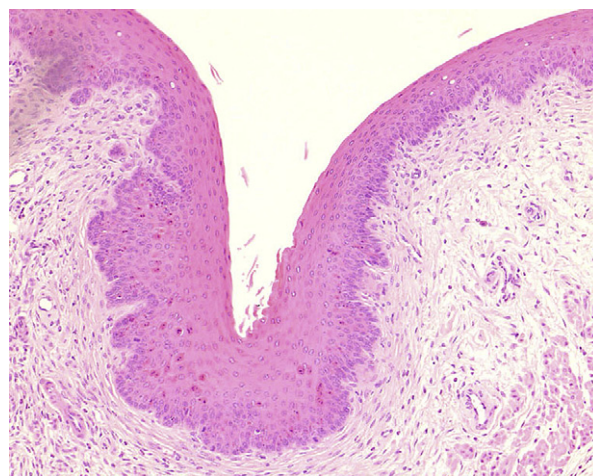
Figure 2.3.46 Vagina (metoestrus).
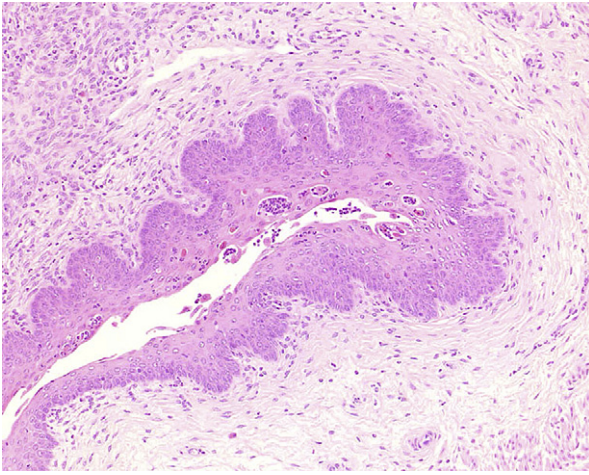
Figure 2.3.47 Vagina (dioestrus).

infiltrated by polymorphonuclear leucocytes (Figure 2.3.47).

Vas deferens

The paired vas deferens begin at the tail of the epididymis and end at the seminal collicle. The wall is formed by ciliated columnar epithelium, thick middle circular and inner and outer

Figure 2.3.48 Vas deferens. 1. A layer of sperm, 2. epithelium, 3. smooth muscle.

longitudinal smooth muscle layer, and adventitia (Figure 2.3.48).

Haematopoietic and lymphoreticular system

Bone marrow

The bone marrow consists of a highly vascular, loose connective tissue stroma and the haematopoietic cells (Figure 2.3.49). In the mouse nearly all bony cavities are filled with active marrow, leaving little reserve space for extending haematopoietic activity. This lack of marrow reserve is compensated for by extramedullary haematopoietic activity, especially in the spleen. In decalcified haematoxylin- and eosin-stained paraffin sections an estimate of general haematopoietic activity (cellularity) and myeloid/erythroid ratio can be made. Finer differentiation requires special stains and preparation techniques.

Lymph nodes

The lymph nodes are connected with the lymphatic system and distributed through the
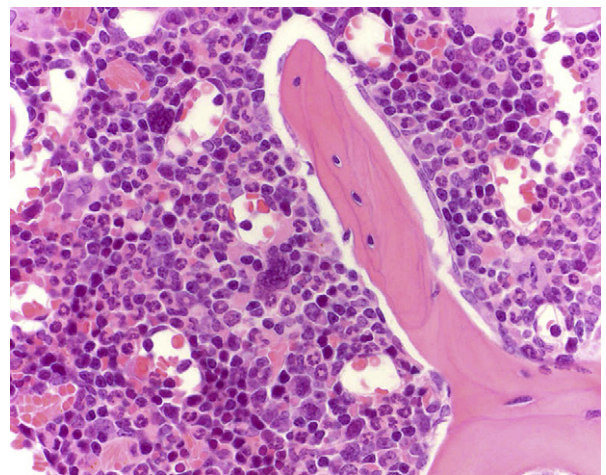
Figure 2.3.49 Bone marrow (sternum). Bone marrow exhibits large megakaryocytes with multilobulated nuclei, erythroid elements with deeply basophilic nuclei, and myeloid elements with larger pale nuclei which differentiate to doughnut form and then to segmental form. The mature erythrocytes are red.
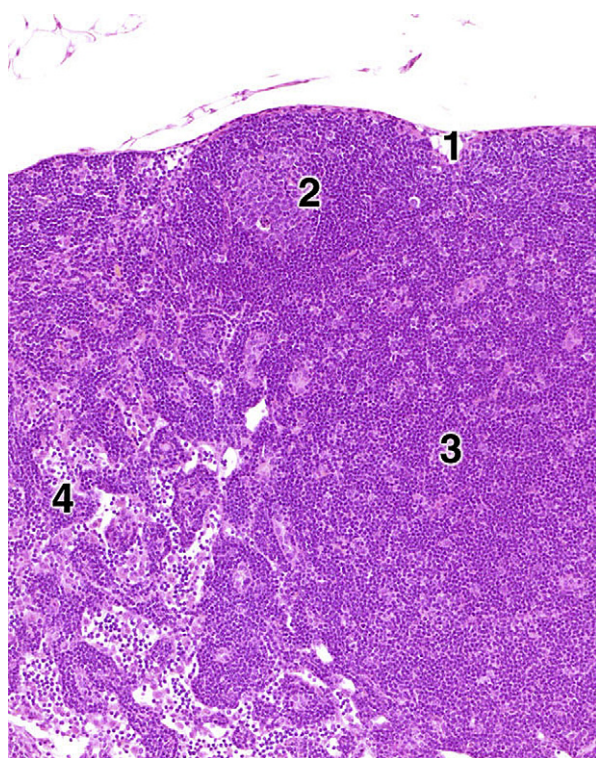
Figure 2.3.50 Lymph node (mesenteric). 1. Subcapsular sinus, 2. lymphoid follicle, 3. paracortex, 4. medullary cords.

whole body. Some are paired (e.g. axillary) but most of the visceral nodes are not (e.g. mesenteric) (Figure 2.3.50). Lymphatic tissue incorporated in selected organs is commonly known as NALT (nose-associated lymphatic tissue), BALT (bronchial-associated lymphatic tissue) in the lung and GALT (gut-associated lymphatic tissue) in the intestine. The lymph nodes are lymphatic structures separate from the organs. Each lymph node is covered by a connective tissue capsule which can form septa within the node. The lymph nodes consist of numerous endothelial sinuses and reticular tissue, arranged in a meshwork filled with lymphatic cells. The lymph enters the lymph node through vasa afferentia, which penetrate the capsule, and reaches the subcapsular sinus, which is connected to paratrabecular and medullary sinuses. It exits through the vas efferents in the hilus. The superficial lymphatic tissue forms the cortex, which contains lymphatic follicles mainly composed of B cells. Primary follicles are non-stimulated and contain dense aggregates of small lymphocytes. Secondary follicles are produced in response to immune stimulation. They are larger and have germinal centres with numerous large pale lymphoblasts and some macrophages with cell debris. The follicles are surrounded by the paracortex, composed mainly of T cells. The periphery of the paracortex has the highest concentration of specialized high endothelial venules. The deep lymphatic tissue forms the medulla which is arranged in medullary cords spreading towards the hilus.

Spleen

The spleen lies in the left dorsocranial part of the abdominal cavity, along the greater curvature of the stomach. It is elongated in shape and triangular in transverse section. The spleen has a connective tissue capsule which spreads in to the parenchyma forming splenic trabeculae. The parenchyma consists of white and red pulp (Figure 2.3.51). The white pulp is organized into periarteriolar lymphoid sheaths (PALS, representing mainly T cells) and lymphatic follicles, which become prominent in response to stimulation. The periphery of white pulp is formed by a less densely cellular marginal zone. The red pulp consists of reticular tissue and venous sinuses and is the site of extramedullary haematopoiesis, which normally occurs in the mouse spleen.
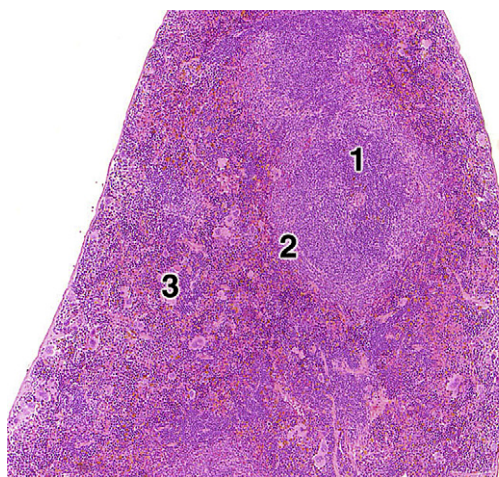
Figure 2.3.51 Spleen. 1. White pulp: periarteriolar lymphoid sheath (PALS), 2. white pulp: marginal zone, 3. red pulp with prominent extramedullary haematopoietic activity.
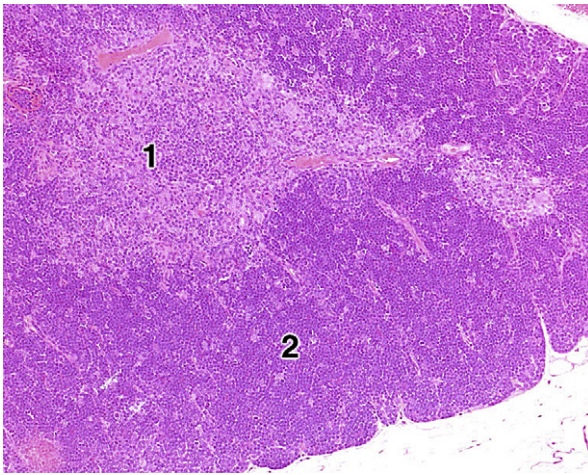
Figure 2.3.52 Thymus. 1. Medulla, 2. cortex.

Figure 2.3.53 Long tubular bone (femur). 1. Epiphysis, 2. epiphyseal growth plate, 3. metaphysis, 4. trabecular (cancellous) bone, 5. cortical (compact) bone, 6. bone marrow.

Thymus

The thymus consists of two lobes. It is located partly in the cervical but mostly the thoracic area, between the larynx cranially and the heart caudally. It is covered by a connective tissue capsule and, in the thoracic cavity, surrounded by mediastinal brown fat. The thymus consists of the cortex and the medulla and is divided into distinct lobules (Figure 2.3.52). The medulla is rich in epithelial cells occasionally arranged in Hassall's body-like formations. The cortex is densely filled with lymphocytes, especially the differentiating T cells. The thymus retains its size until the young adult age and regresses thereafter by atrophy. The thymus does not develop in the so-called 'nude mouse', which is homozygous for the *nu* gene. These mice are hairless and lack T lymphocytes.

Musculoskeletal system

Bone

The mouse skeleton is composed of bones which, according to their shape and structure, are tubular (extremities) or flat (cranium, scapula, ribs). Some bones, such as the vertebrae, have both tubular and flat portions. Long tubular bones have a shaft (diaphysis), terminating at both ends as metaphysis (Figure 2.3.53). The

metaphysis is connected by epiphyseal cartilage (the so-called growth plate) to the most peripheral part, the epiphysis. The surface of the bones is covered by a connective tissue membrane, the periost. The bones grow either by *endochondral ossification*, in which precursor cartilage such as the epiphyseal growth plate is converted to bone, or from the periost by *periosteal ossification*. Most of the diaphysis is formed by compact cortical bone, the inside of the metaphysis and the epiphysis, as well as of short tubular and flat bones contains trabecular (cancellous) bone tissue. The bones form cavities in which the haematopoietic tissue, bone marrow, is located. The bone tissue contains bone lining cells, osteoblasts, osteocytes and osteoclasts. The bone lining cells are resting preosteoblasts, lining the bone surface as flattened cells. The osteoblasts are also located at the bone surface, but are larger and polyhedral. They actively deposit osteoid and progressively become incorporated in osteoid matrix and differentiate to osteocytes. The osteocytes are completely surrounded by mineralized bone. The osteoclasts are multinucleated macrophages which resorb bone and enable bone remodelling. The bone contains collagen fibres which may be arranged in parallel layers (lamellar bone) or in a random pattern (nonlamellar, woven bone). The woven bone is considered to represent immature bone, whereas the lamellar bone is more differentiated and forms both the cortical (compact) and the trabecular

(cancellous) bone tissue. In the mouse the cortical bone does not have the distinct haversian systems that occur in other species. Growth and modelling of various long bones in mice is complete by the age of about 26 weeks, and further remodelling serves for maintenance or occurs in response to changing external forces acting on the bones, or to a disease.

Joints

The bones are fixed to each other by various kinds of joints. Some joints are quite rigid, fibrous (such as sutures in the skull) or cartilaginous (such as between the vertebrae or the sternebrae). Synovial joints connect bones more loosely, allowing for movement. The bones in synovial joints are equipped with articular cartilage, sometimes with additional cartilaginous menisci, ligaments and capsules. The inside of the synovial joints is covered by a synovial membrane and filled with synovial fluid.

Skeletal muscle

As in other species, the skeletal muscle consists of striated, extrafusal and intrafusal muscle fibres, connective tissue, blood vessels, nerve fibres and motor and sensory nerve endings. Each muscle fibre is a multinucleated cell with nuclei located at the periphery and cytoplasm containing contractile myofibrils (Figures 2.3.54 and 2.3.55).
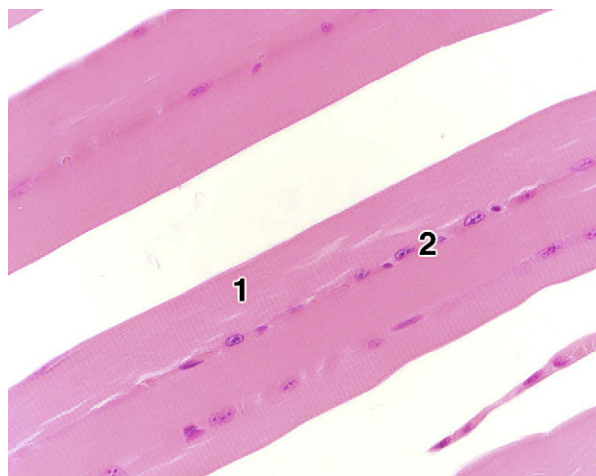
Figure 2.3.54 Skeletal muscle (longitudinal section). 1. Cross-striated cytoplasm of muscle fibres, 2. peripherally located nuclei.
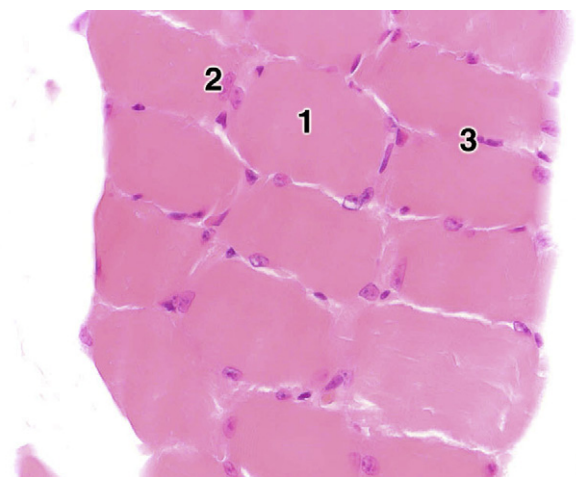
Figure 2.3.55 Skeletal muscle (transverse section). 1. Cytoplasm, 2. nuclei of muscle fibres (pale, oval), 3. endomysial nuclei (dark, elongated).

Nervous system

Brain

The mouse brain is lissencephalic, since the surface of the cerebral hemispheres is devoid of gyri and sulci. The caudate nucleus and the putamen form a continuous structure, the caudatoputamen. The major parts of the brain are the forebrain (including the cerebral cortex, hippocampus and olfactory bulbs) (Figure 2.3.56), the upper brainstem (including the basal ganglia, septum, epithalamus, thalamus and hypothalamus), the midbrain (including the tectum, tegmentum and pedunculi cerebri), the cerebellum with the pons (Figure 2.3.57) and the medulla oblongata. Atlases showing selected brain parts or the complete mouse brain are available [23-27]. The brain tissue consists of functional cells (nerve cells, neurons) and the supporting cells, macroglia and microglia. The macroglia are oligodendrocytes, which are the central myelin-forming cells, and astrocytes, which occur both in the grey and the white matter. The ependymal cells line the walls of brain ventricles, are ciliated and may react positively with astrocytic markers such as GFAP. The choroid plexus epithelium forms microvilli and reacts positively with epithelial markers. The central nervous system is covered by meninges (leptomeninx,
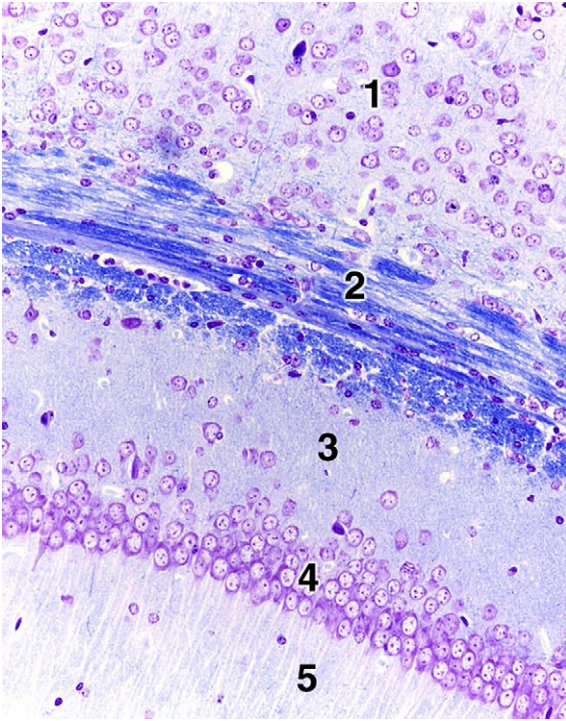
Figure 2.3.56 Brain: forebrain (cresyl violet and luxol blue). 1. Neocortex (grey matter), 2. neocortex, white matter (corpus callosum), 3. molecular layer of hippocampus, 4. pyramidal layer of hippocampus, 5. polymorphic layer of hippocampus.
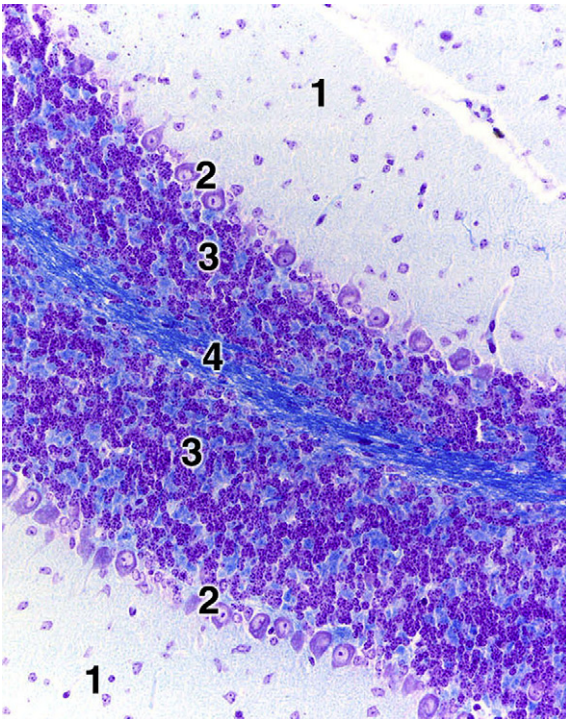
Figure 2.3.57 Brain: cerebellar cortex (cresyl violet and luxol blue). 1. Molecular layer, 2. Purkinje cell layer, 3. granular cell layer, 4. white matter.

pachymeninx) and surrounded by cerebrospinal fluid.

Peripheral nerves

As in other species, the peripheral nerves consist of unmyelinated and myelinated nerve fibres and connective tissue sheaths. A variety of mouse mutant strains serve as models of genetic diseases of human peripheral nerves [28].

Spinal cord

The spinal cord consists of central grey matter surrounded by columns of white matter (Figure 2.3.58). The dorsal columns contain ascending sensory nerve fibres, the ventral columns descending motor nerve fibres, and the lateral columns both. The grey matter has sensory dorsal horns and ventral motor horns. In the middle is the central spinal canal. The
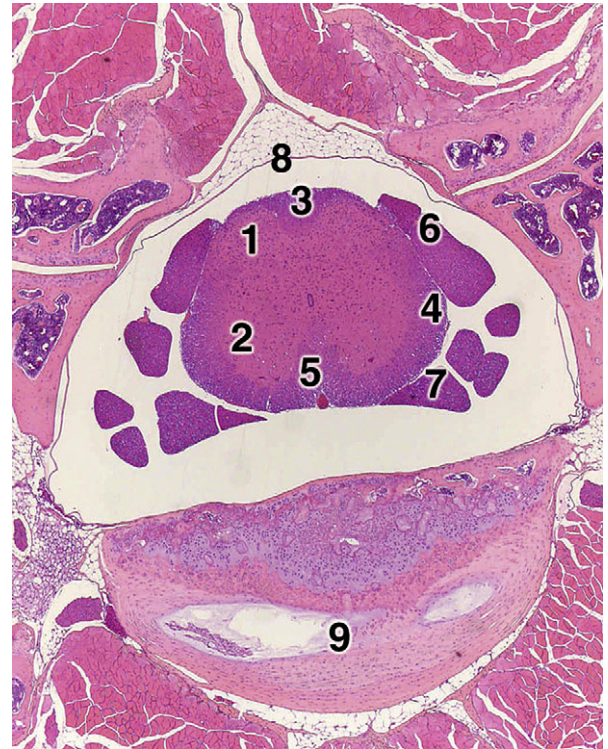
Figure 2.3.58 Spinal cord: lumbar segment. 1. Grey matter (dorsal horn), 2. grey matter (ventral horn), 3. white matter (dorsal columns), 4. white matter (lateral columns), 5. white matter (ventral columns), 6. dorsal spinal root, 7. ventral spinal root, 8. pachymeninx, 9. intervertebral disk.

spinal cord has segmental organization and it forms a pair of spinal nerve roots in each segment. In the mouse the roots of the fourth to eighth cervical and first and second thoracic segments contribute to the brachial plexus supplying the forelimbs, and the roots of the third to sixth lumbar segment contribute to the lumbosacral plexus supplying the hindlimbs.

Respiratory system

Larynx

The larynx is located between the pharynx and the trachea. The entrance of the larynx is bordered by the epiglottis. The laryngeal wall is formed by three layers: the epithelial lining, the cartilage with striated muscles and vocal cords, and the outer loose connective tissue. The epithelial lining varies from stratified squamous epithelium cranially, on the epiglottis, to pseudostratified ciliated columnar respiratory epithelium caudally, at the transition to the trachea. At the base of epiglottis there are subepithelial seromucous glands. Specific areas of the laryngeal wall have intermediate types of epithelium. The vocal folds and vocal processes are covered by low cuboidal to squamous epithelium, the ventral laryngeal poach (diverticulum) is lined by a mixture of ciliated columnar and cuboidal cells. Recommendations about the appropriate processing for detailed histological examination of the mouse larynx have been published [29, 30].

Lung

The lungs are located in the thoracic cavity, covered and surrounded by pleura. The left lung has a single lobe, whereas the right lung is subdivided to cranial, middle, caudal and accessory lobes. The lung is entered by main bronchi produced by tracheal bifurcation. The main bronchi branch to form intrapulmonary bronchi to terminate as bronchioles. The larger airways are lined by columnar epithelial cells, mainly non-ciliated Clara cells, ciliated cells, and some neuroendocrine cells, mucous cells and brush cells. The mucous cells are of small granule type; goblet cells with prominent mucous
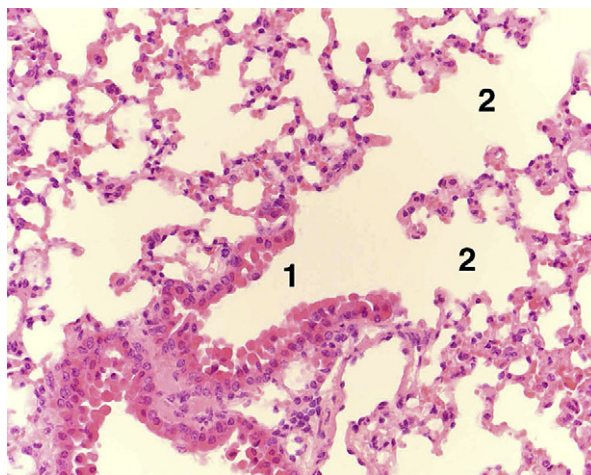
Figure 2.3.59 Lung. 1. Terminal bronchiole, 2. alveolar duct.

cytoplasm do not occur in the mouse lung. The smallest airway of the mouse lung is the terminal bronchiole, which opens to the alveolar ducts (Figure 2.3.59). The alveolar ducts lead to alveolar sacs and alveoli. The alveolar epithelium consists of thin pulmonary type I cells and cuboidal type II cells. Lymphoid tissue (BALT) is rarely seen in the healthy mouse lung.

Nasal cavity

The nasal cavity is separated by a cartilaginous septum. From the walls the turbinates (conchae) project into the lumen; these are formed by bone and covered by mucous membrane. In the anterior nasal cavity there are nasoturbinates and maxilloturbinates (Figure 2.3.60), whereas the posterior nasal cavity contains ethmoturbinates. Among the turbinates are the air passages, the dorsal, middle and ventral meatus. The vomeronasal organ, which is an organ of chemoreception for pheromones and food flavour, occurs medioventrally in the nasal cavity. The paired nasolacrimal ducts connect the medial canthus of the eye with the nasal cavity and pass through the bony nasolacrimal canal. The anterior portion of the nasal cavity (vestibule) is lined by stratified squamous epithelium which extends through the ventral meatus into the pharynx. The nasoturbinates, maxilloturbinates, cranioventral portion of ethmoturbinates and most of the nasal septum are covered by respiratory epithelium which contains ciliated and unciliated columnar cells,

Figure 2.3.60 Nasal cavity. 1. Nasoturbinate, 2. maxilloturbinate, 3. nasolacrimal duct, 4. incisor tooth, 5. nasal septum, 6. vomeronasal organ.

Figure 2.3.61 Nasal cavity: pseudostratified columnar respiratory epithelium.
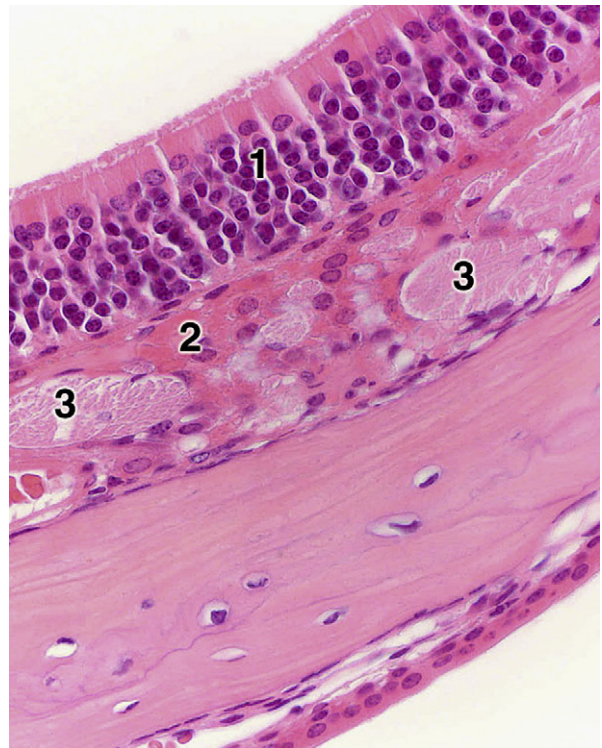
Figure 2.3.62 Nasal cavity. 1. Olfactory epithelium, 2. lamina propria with Bowman's glands, 3. bundles of nerve fibres.

cuboidal cells, goblet cells, brush cells and basal cells (Figure 2.3.61). The lamina propria of the respiratory epithelium contains serous glands at the anterior nasal septum and mucous glands at the posterior septum. The lateral walls of air passages and the naso- and maxilloturbinates are lined by 'transitional respiratory epithelium' consisting of cuboidal cells, unciliated columnar cells, brush cells and basal cells. The dorsal wall of the nasal cavity and the ethmoturbinates are covered by olfactory epithelium. This epithelium is pseudostratified columnar and consists of specialized bipolar olfactory neurons, sustentacular (supporting) cells and basal cells . The axons of olfactory neurons form bundles of unmyelinated nerve fibres, which synapse with the neurons in the olfactory bulb. The lamina propria of olfactory epithelium contains tubuloalveolar Bowman's glands (Figure 2.3.62). 'Nasal diagrams' are available which demonstrate the nasal topography at various section levels and serve as a basis for assessment of histopathological findings [31]. The nasal septum of mice usually contains an eosinophilic substance consisting of collagen and amorphous material. This is probably related to the vomeronasal organ [32].

Pharynx

The pharynx is the site behind the nasal and oral cavities where the respiratory and digestive

Figure 2.3.63 Trachea. 1. Smooth muscle connecting the ends of cartilage, 2. mucous membrane, 3. cartilage, 4. isthmus of the thyroid gland.

Figure 2.3.64 Ear: cochlea with organ of Corti. 1. Scala media, 2. scala tympani, 3. limbus, 4. tectorial membrane, 5. hair cells, 6. spiral ganglion, 7. cochlear nerve.

passages cross. It is lined by stratified squamous epithelium. Its dorsal part (oropharynx) receives the openings of the eustachian tubes.

Trachea

The trachea connects the larynx to the left and right principal bronchi. It is located ventrally to the oesophagus in the cervical area. It is formed by 15-18 C-shaped hyaline cartilages, with smooth muscle joining the ends (Figure 2.3.63). The mucosa is lined by pseudostratified columnar epithelium consisting of Clara cells, goblet cells, ciliated cells and basal cells.

Sensory organs and adnexa

Ear

The paired ears are composed of three parts: the external ear, the middle ear and the inner ear. The external ear is formed by concha auriculae (the pinna) and the external auditory canal (meatus). The pinna is an elastic cartilaginous structure covered by skin on both sides. In the mouse the lateral portion exhibits hair and some sebaceous glands, the medial part has much less hair and more sebaceous glands. The middle ear consists

of the tympanic cavity with the tympanic membrane and the eustachian tube, connecting the middle ear to the pharynx. Within the tympanic cavity lie the auditory ossicles—malleus, incus and stapes. The inner ear consists of the labyrinth (organ of equilibrium) and the cochlea (organ of audition). In the cochlea there is the organ of Corti with sensory hair cells which are connected to spiral ganglion cells (Figure 2.3.64). The mouse cochlea has one and a half turns, but its length varies among some strains [33].

Eye

The paired eyes are nearly spherical in shape. The lens is also spherical and relatively large. Because of the animal's nocturnal way of life, the mouse retina does not have areas of increased visual acuity such as a central round area or horizontal streak. The mouse eye is atapetal, i.e. the tapetum lucidum is not developed. The sclera and the cornea form the outer fibrous tunic. The cornea consists of the external layer of stratified squamous epithelium and of the stroma formed by collagen fibres, fibroblasts and a few elastic fibres (Figure 2.3.65). Bowman's membrane is not recognizable in mice. Descemet's membrane lines the inner surface of the cornea. The uvea consists of the iris, ciliary body and choroid. Except in albino mice, these layers are pigmented, as is the retinal
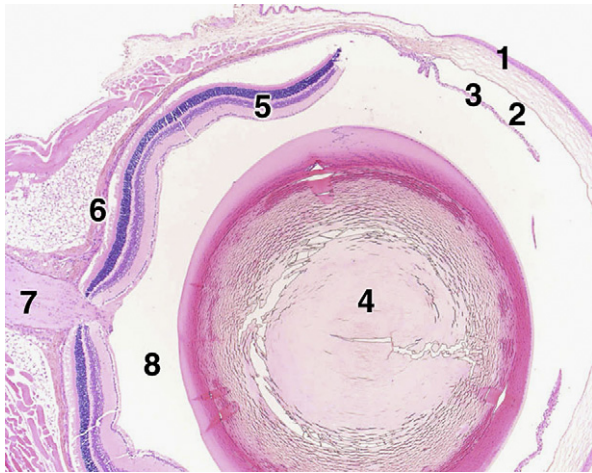
Figure 2.3.65 Eye. 1. Cornea, 2. anterior chamber, 3. iris, 4. lens, 5. retina, 6. choroid and sclera, 7. optic nerve, 8. vitreous body.
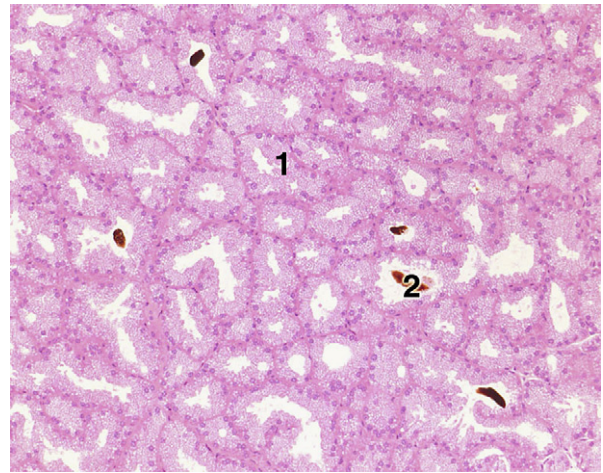
Figure 2.3.66 Harderian gland. 1. Secretory cells with finely vacuolated cytoplasm, 2. occasional porphyrin accretions.

pigment epithelium. The lens consists of laminated fibres formed by modified epithelial cells, enclosed by a capsule. The retina is formed by photoreceptor cells, predominantly the rods, lined by retinal pigment epithelium. There are three layers of cell nuclei arranged in the outer and inner nuclear layer and the innermost ganglion cell layer. The outer nuclear layer is formed by photoreceptors, the inner nuclear layer contains specialized bipolar, horizontal and amacrine cells. The glial cells of the retina are the astrocyte-like Müller cells, which traverse all retinal layers and have the nuclei located in the inner nuclear layer. The ganglion cells form the axons of the optic nerve, conducting the visual impulses towards the brain.

Harderian gland

The paired Harderian glands are located deep within the orbit, surrounding the optic nerve and several external ocular muscles from the dorsal, medial and ventral direction. The gland has a tubuloalveolar structure. On each side a single excretory duct resulting from the connection of alveolar, lobular and lobar lumina opens at the base of the outer surface of the nictitating membrane. There are no intralobular or interlobular ducts. The excretory duct is lined by columnar epithelium except at the opening on the nictitating membrane where stratified squamous epithelium occurs. The cytoplasm of secretory cells appears finely vacuolated owing to the presence of lipid droplets containing mainly glyceryl ester diesters and phospholipids (Figure 2.3.66). Major secretory products are porphyrins, controlling the amount and quality of light reaching the retina and providing photoprotection to the eye. Occasional porphyrin accretions occur in the glandular lumina. In many mouse strains the amount of porphyrins is significantly higher in the female than in the male. Between the secretory cells and the basement membrane are located the myoepithelial cells, which enable the release of secretions in response to nervous, especially cholinergic, stimuli [34].

Lacrimal gland

The mouse possesses two pairs of lacrimal glands. The exorbital glands are located subcutaneously, ventral and anterior to the external ear (Figure 2.3.67). The intraorbital glands are located at the outer canthus, where the joint excretory ducts of both ipsilateral glands open. The lacrimal glands are tubuloacinar, consisting of lobes and lobules. The serous secretory cells have basophilic cytoplasm near the basally located nuclei and more pale cytoplasm at the lumen. Myoepithelial cells are found between the epithelium and the basement membrane. The intralobular ducts are lined by cuboidal cells

Figure 2.3.67 Lacrimal gland (exorbital). 1. Secretory cells, 2. intralobular duct.

and the excretory ducts by stratified columnar epithelium [34].

Optic nerve

The paired optic nerves are formed by the processes of retinal ganglion cells connecting the eyes to the brain (see Figure 2.3.65). The optic nerve tissue belongs to the central nervous tissue, the myelinating cells are oligodendrocytes and the outer sheaths investing the optic nerve are continuations of cerebral meninges. The mouse optic nerve contains about 65 000 nerve fibres.

Skin and mammary glands

Mammary glands

Female mice have five pairs of mammary glands, three pairs in the cervicothoracic region and two pairs in the inguinoabdominal region. Male mice have only four pairs of glands and no nipples. The mammary gland is a compound tubuloalveolar gland. The branched system of lactiferous ducts is embedded in adipose tissue (Figure 2.3.68), and, in developed glands, it leads to lobules of secretory alveoli. The lactiferous ducts are lined by pseudostratified low columnar or cuboidal epithelium, the alveoli by low cuboidal epithelium. The secretory cells are surrounded by
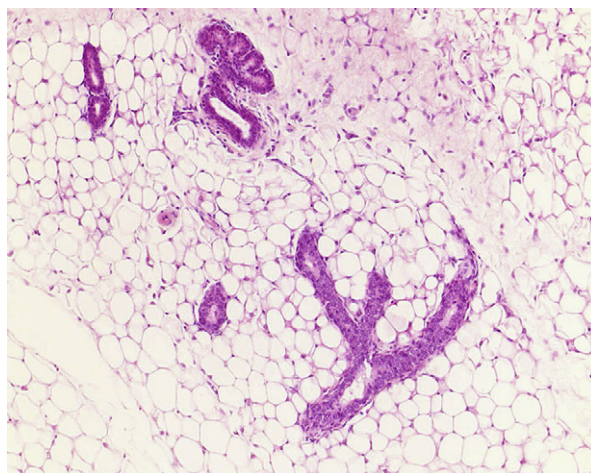
Figure 2.3.68 Mammary gland (virgin female). The resting mammary gland in a virgin female consists essentially of lactiferous ducts embedded in adipose tissue.

myoepithelial cells. Details of mouse mammary gland biology have been reviewed [35].

Skin

The skin is composed of the epidermis, dermis and subcutis (hypodermis) (Figure 2.3.69). Skin
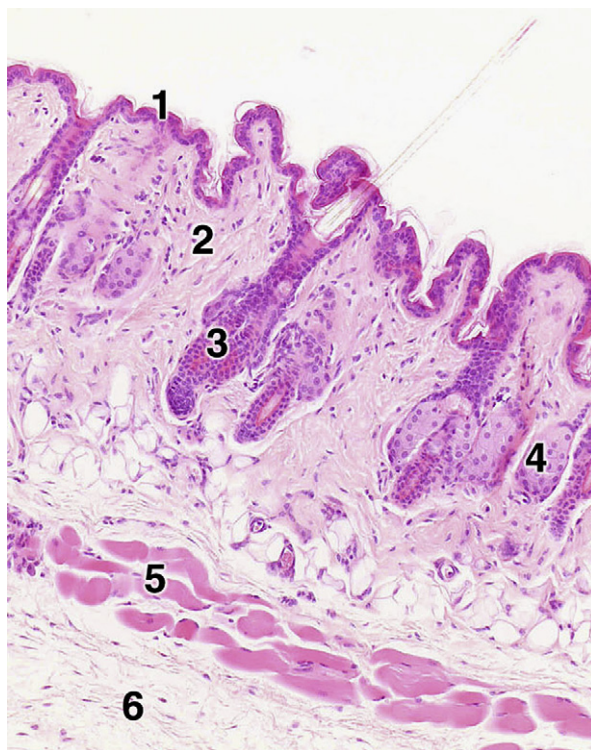
Figure 2.3.69 Skin. 1. Epidermis, 2. dermis, 3. hair follicle, 4. sebaceous gland, 5. striated muscle (panniculus carnosus), 6. subcutis.

adnexa are hair follicles and sebaceous glands. The epidermis consists of four layers: basal cell layer (stratum basale), prickle cell layer (stratum spinosum), the granular cell layer (stratum granulosum) and a horny layer (stratum corneum). In pigmented (non-albino) strains melanin pigment occurs in the cells of the basal layer, the hair follicles and hairs. It is produced by melanocytes. The dermis (corium) consists of fibrous connective tissue and has a subepidermal papillary layer and deeper reticular layer. The subcutis is formed by loose connective tissue with a moderate amount of fat tissue. A sheet of striated skeletal muscle lies between the dermis and the subcutaneous tissue and is prominent especially in the regions of neck, thorax and abdomen. The hair coat of the mouse (pelage) is formed by short hairs. Among the pelage hairs are scattered longer guard hairs, which have a tactile function. Very large tactile hairs, the vibrissae (whiskers), occur on the nose. The hairs are keratohyaline products of epithelial hair follicles which protrude from the epidermis into the dermis. The hair follicles are associated with sebaceous glands. In the mouse, sweat glands occur only on the footpads.

Zymbal's gland

The paired Zymbal's glands are auditory sebaceous glands. The gland consists of acinar sebaceous cells and excretory ducts lined by stratified squamous epithelium (Figure 2.3.70). It opens into the external ear canal.
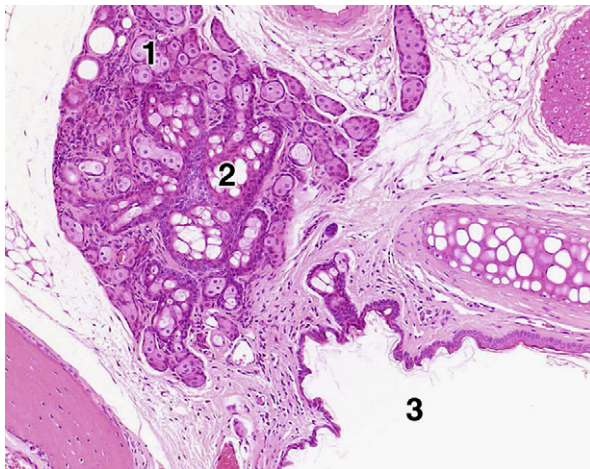
Figure 2.3.70 Zymbal's gland. 1. Sebaceous cells, 2. excretory ducts, 3. external ear canal.

Urinary system

Kidney

The paired kidneys are located in the dorsal part of abdominal cavity, the right kidney slightly more cranially then the left kidney. The mouse kidney is unilobar with a single papilla. It consists of the cortex and the medulla (Figure 2.3.71). The cortex contains cortical tubular labyrinths (mainly proximal convoluted tubules), and medullary rays extending from the outer medulla. The medulla is subdivided into outer and inner zones. The outer zone has outer and inner stripes, and the inner zone forms the papilla (Figure 2.3.72).

The functional unit is the nephron, consisting of the glomerulus, convoluted and straight portions of the proximal tubule, the descending and ascending portions of the loop of Henle, and the strait and convoluted portions of the distal tubule (Figure 2.3.73). The nephrons are connected to the collecting ducts, which run
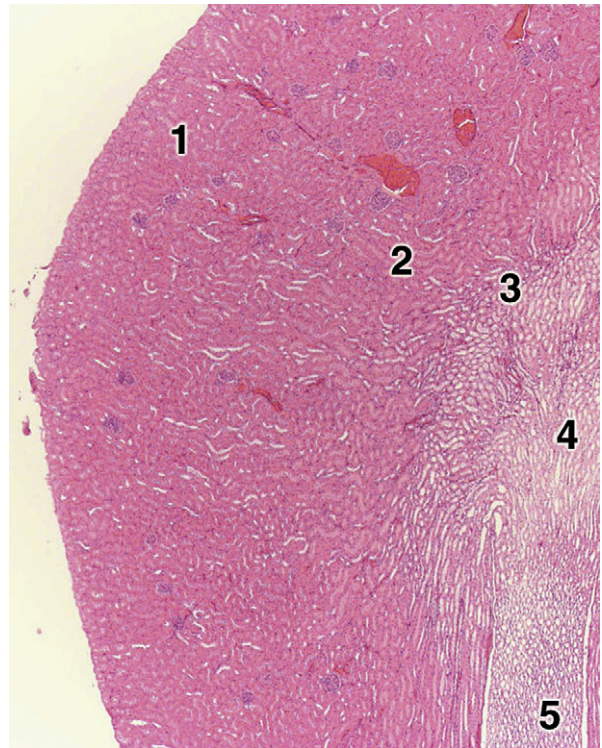
Figure 2.3.71 Kidney: cortex and medulla. 1. Cortex, 2. outer stripe of outer medulla, 3. inner stripe of outer medulla, 4. inner medulla, 5. papilla.

Figure 2.3.72 Kidney: pelvis and papilla. 1. Papilla, 2. pelvis, 3. cortex.
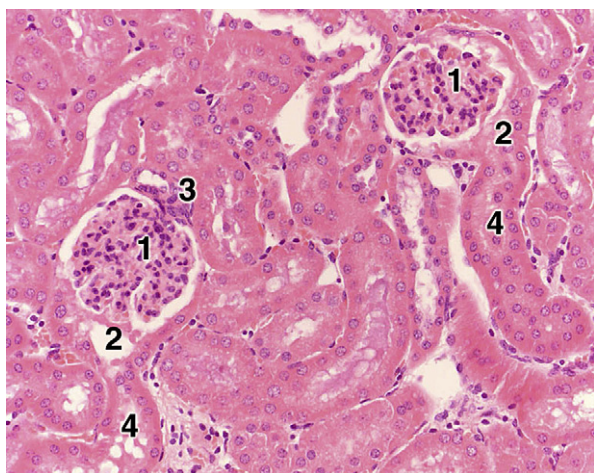
Figure 2.3.73 Kidney: cortex. 1. Glomerulus, 2. urinary pole, 3. vascular pole with macula densa, 4. proximal convoluted tubule.

into papillary ducts. The papillary ducts open at the tip of renal papilla into the renal pelvis. The renal pelvis is lined by transitional cell epithelium and its continuation forms the ureter. The mouse renal papilla may be very long and protrude into the initial portion of the ureter. The glomerulus is surrounded by the Bowman's capsule, which in most mouse strains is considered to exhibit sexual dimorphism: the parietal epithelial cells are cuboidal in males and flattened in females. This difference could not be demonstrated in the sections of CD-1 mouse kidneys used to illustrate this text. Regardless of the gender, the parietal cells of Bowman's capsule were flattened at the vascular pole and cuboidal at the urinary pole of glomeruli. Reportedly the sexual dimorphism occurs under influence of testosterone only in mature males. The proximal tubules are found mainly in the cortex. They have cuboidal cells with prominent brush border (microvilli). The descending and ascending loop of Henle is found in the medulla. They are lined by flattened epithelium resembling endothelium of blood vessels. The distal tubules re-enter the cortex and have cuboidal epithelium similar to proximal tubules, but devoid of brush border. The straight portion of the distal tubules leads to the macula densa at the vascular pole of glomerulus, where renin is produced by specialized cells.

The mouse renal vasculature is similar to other species. The branches of the renal artery form the arcuate arteries at the corticomedullary border. Interlobular branches of the arcuate arteries supply the afferent arterioles of glomeruli. The efferent arterioles supply the cortex and form descending vasa recta to supply the medulla. The venous blood collects in ascending vasa recta and interlobular and arcuate veins. Spontaneously occurring vacuolation, probably of lysosomal origin, in renal tubular epithelium of the outer medulla in CD-1 mice has been reported [36].

Ureter

The paired ureters connect the kidneys to the urinary bladder. The wall of the ureter consists of transitional epithelium, muscularis with inner circular and outer longitudinal layers of smooth muscle fibres, and adventitia.

Urethra

The urethra connects the urinary bladder to the body surface. In males the urethra is divided into the membranous and penile parts

(see Figure 2.3.35). The membranous urethra contains the colliculus seminalis and receives there the openings of both vas deferens, prostate, seminal vesicles and ampullary glands. The area of seminal collicle is lined by columnar epithelium, the remaining area by transitional epithelium. Into the lumen of membraneous urethra open small mucous urethral glands (glands of Littré). Before its transition to the penile part the urethra forms a diverticulum and this area receives the openings of bulbourethral glands. The lumen of the penile urethra is lined by transitional epithelium and the external opening (orificium) by stratified squamous epithelium. In females the urethra opens independently of the vagina—it empties into the clitoral fossa, cranially to the vaginal opening.

Urinary bladder

The bladder is located in the dorsocaudal abdominal cavity, ventrally to the colon. The wall is formed by transitional epithelium with well–vascularized lamina propria, smooth muscle muscularis and adventitia (Figure 2.3.74). In the lamina propria nodules of lymphoid tissue may occur, especially in ageing mice: they must not be mistaken for an inflammatory or neoplastic lesion. The thickness of the bladder wall depends on the degree of distention by the content. Distended transitional epithelium is about two to three cell layers thick. The empty bladder has thick folds of transitional epithelium and lamina propria. The superficial cells of the transitional epithelium (also called 'umbrella cells') are large, may be binucleated and polyploid.
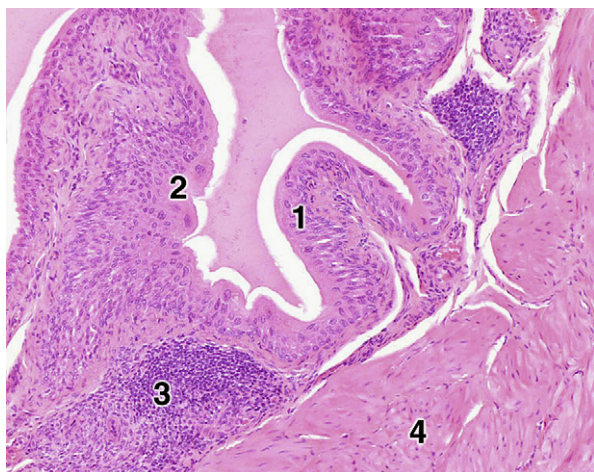
Figure 2.3.74 Urinary bladder. 1. Transitional epithelium, 2. superficial cells of transitional epithelium are large and may be polyploid, 3. nodules of lymphoid tissue may occur in the lamina propria, 4. muscularis.

Most common strain differences in the occurrence of age-related changes

At Harlan Laboratories Ltd, Switzerland, histological changes occurring in control animals (untreated ageing animals) obtained from 104-week oncogenicity studies were compared in three commonly used mouse strains: NMRI, CD-1 and B6C3F1. The main differences related to mortality rates as well as neoplastic and non-neoplastic lesions included the following.

NMRI and CD-1 mice revealed the highest mortality rate (mean NRMI: males 56.9%, females 76.8%; CD-1 males 52.2%, females 61.1%). In contrast, B6C3F1 mice survived significantly longer (mean mortality rate: males 17.7%, females 26.3%).

Regarding non-neoplastic lesions, liver cell necrosis, Kupffer's cell proliferation and dermatitis were encountered at high incidences in NMRI mice, whereas in CD-1 mice the most common non-neoplastic lesion was amyloidosis (depending on its incidence in the source colony), followed by periarteritis, chronic progressive nephropathy and glomerulosclerosis, diffuse hyperplasia of the glandular stomach mucosa, and cardiomyopathy, dilation of the preputial/clitoral gland, dermatitis, granulopoiesis in the bone marrow and deposition of an eosinophilic substance in the nasal cavities. In contrast, the most common non-neoplastic lesions in B6C3F1 mice when compared to other mouse strains consisted of uterine cystic endometrial hyperplasia, fibro-osseous lesion in the sternum and foci of mineralization in the brainstem (thalamus).

Among the neoplastic lesions, the highest incidences for pulmonary alveolar/bronchiolar

adenoma, Harderian gland adenoma and systemic neoplasia (such as haemoproliferative lesions) were encountered in NMRI mice. In contrast, along with foci of hepatocellular alteration, hepatocellular neoplasms were most common in B6C3F1 mice.

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Skin and Adnexa of the Laboratory Mouse

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Introduction

The skin is the largest of the intermediate-sized organs [1]. Dermatology, anatomy and histology textbooks assign simple functions to this organ system which, in reality, is as complicated as any organ in the body. More importantly, it is integrated with every organ of the body, not simply a wrapping to hold things together. The list of functions of the skin is constantly expanding, and Box 2.4.1 presents a summary from a published debate on this topic [2].

Spontaneous and genetically engineered mutations in laboratory mice have changed the basis of our knowledge of the function of the skin and how gene expression in the skin may be a reflection of similar expression in different organs [3, 4]. For example, for a long time it was generally thought that mice without hair (alopecia) have some form of immunodeficiency. This was largely based on observations relating to the nude mouse. These mutant mice appear to lack hair at the gross level and lack a cell-mediated immune system because of failure of the thymus to develop normally. In fact, these mice have hair follicles that cycle normally and produce hair shafts but the hair shafts are defective due to the role of the mutated forkhead box N1 (*Foxn1*) gene that codes for the nude

phenotype (*Foxn1^{nu}*) and acts as a transcription factor to downregulate hard keratin production [5–7]. This gene also plays a role in terminal differentiation of keratinocytes at other anatomical sites [8]. Hairless (*Hr*), another mutant mouse [9], also has a minor abnormality in its immune system [10]. The advent of the severe combined immunodeficiency mutant mice (*Prkdc^{scid}*) with normal pelage and hair cycle changed the limited correlation between immunodeficiences and hair loss [11]. We now know that each skin defect can be unique and may or may not be associated with visceral lesions [3].

Numerous mutations have occurred spontaneously in laboratory mice, induced by radiation or various chemical mutagens, or created using transgenesis or targeted mutagenesis [3, 12–23]. It is beyond the scope of this chapter to cover the large number and variety of mutant mice currently available. However, this information is available online where it can be, and is, constantly updated. The best general resource for information on the allelic mutations of all known genes is through the Mouse Genome Informatics web site (www.informatics.jax.org). Images of the specific lesions are available in the Mouse Tumor Biology Database, a part of Mouse Genome Informatics (http://tumor.informatics.jax.org) [24–26]. In addition, histopathology of spontaneous and genetically engineered mice in general is to be found on the European Mouse Pathology Consortium Pathbase site (http://www.pathbase. net) [27–29]. There are numerous books and

website resources available that provide overviews or detailed information on the skin as well as all organ systems in the laboratory mouse [30].

This chapter provides an overview and references as sources for more specific information on normal anatomy, development and cycling of the skin and its adnexa [30, 31]. It also provides information on routine methods to prepare specimens for analysis. General, systematic descriptions of necropsy procedures evaluating all organ systems can be found in Chapter 5.7 of this book.

Clinical evaluation, tissue collection, and preservation of the skin

Clinical evaluation

The normal mouse is completely covered with hair. Although the tail and ears appear to be free of hair, close examination reveals that they too are covered by very fine specialized hairs. Careful examination reveals at least two hair types, as is the case with most domestic mammals. A fine, short hair coat covers most of the body (truncal hairs) while long hairs are evident around the head (vibrissae, incorrectly called whiskers by many investigators since there is no anatomical similarity to androgen-responsive facial hair in humans) and distal limbs. On studying the mouse hair more carefully, however, one will note that there are actually many hair types present. Within the pelage hairs covering the body there are classically four types: (i) *guard hairs* are long, straight, thick, and protrude above the level of most hairs; (ii) *auchene hairs* are nearly as long as guard hairs with a gradual bend at the distal end; (iii) *awl hairs* are also straight with a bend at the distal end but are short and thin; and lastly (iv) *zigzag hairs* are the underhairs, which have two bends giving them a 'Z' shape (Figure 2.4.1). These hair types are best differentiated in plucked samples mounted on glass slides with mounting media and a coverslip, and then examined microscopically. This approach forces the hairs to lie in one

Figure 2.4.1 Scanning electron micrograph of telogen-stage plucked hairs from the dorsal truncal skin of an adult mouse. (A) Guard hairs, (B) awl hairs, (C) zigzag hairs.

plane. The hairs can be examined with a microscope, photographed and a variety of light sources used that can provide diagnostic information [32–34]. This is superior to the historical approach in which the hair was attached with double-sided sticky tape to a glass microscope slide.

In addition to the pelage or truncal hairs, there are many other specialized hair types in the mouse. The tail is covered with very short, broad fibres. Ears have a variety of very short, fine hair shafts (Figure 2.4.2). Eyes have vibrissae above the eyelids and a network of long hair shafts protruding from the lid margins called *cilia*. Vibrissae are also found around the mouth, eyelids, and near the foot pads on the lower legs (Figure 2.4.3). Perianal hairs are large, thin structures that form a network above the opening of the anus (Figure 2.4.4). Hairs also change around nipples and the base of the ear. These differences can often only be seen using a hand lens, dissection microscope, scanning electron microscopy or other means of magnification.

Hairs are usually thin and straight, with a uniform distribution pattern within a strain. Variations, especially hair loss, may suggest that the mice have a mutant phenotype but only after simple diagnostic methods rule out infectious causes or infestations. Ectoparasites remain common in many animal facilities and will result in alopecia, often mistaken by the novice for a mutant phenotype (Figure 2.4.5). Mites are easily diagnosed by placing a piece of haired skin in a closed petri dish into a refrigerator then examining it after an hour or so with a hand lens. Mites migrate to the tips looking for another host. They can also be easily identified histologically if hairs are not shaved during preparation of the skin
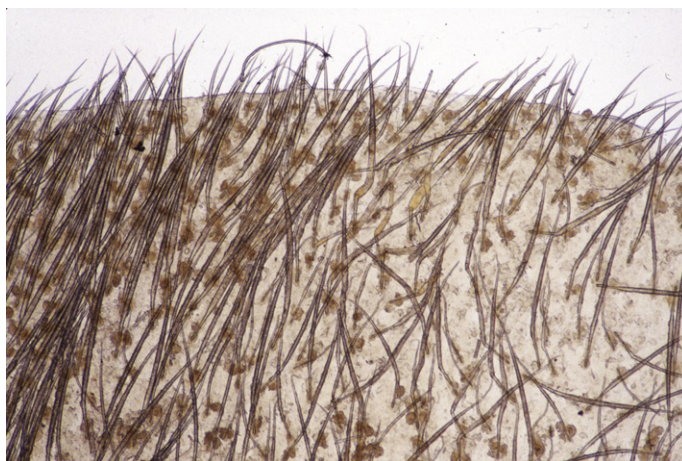
Figure 2.4.2 Subgross photograph of pilosebaceous units (hair follicles with the sebaceous gland at its base) in cleared skin from the ear of an adult mouse.
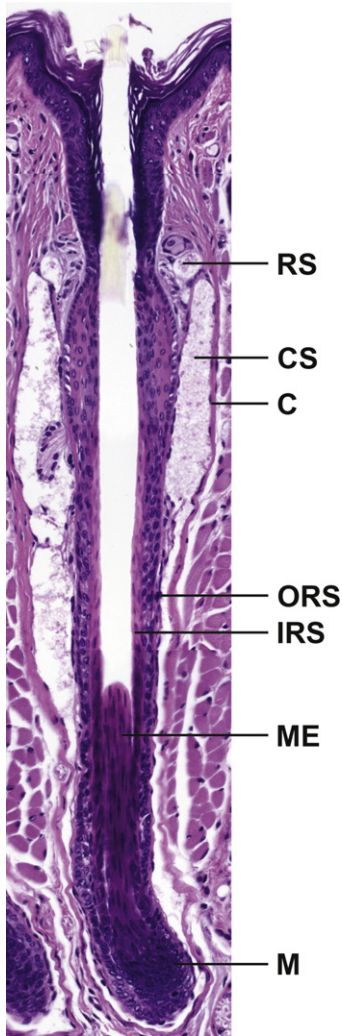
Figure 2.4.3 Anagen-stage vibrissae hair follicle from the muzzle of an adult mouse. Ring sinus (RS), cavernous sinous (CS), capsule (C), outer root sheath (ORS), inner root sheath (IRS), medulla (ME), matrix (M).

(Figure 2.4.5). Other infectious diseases require the assistance of a trained veterinary pathologist for correct diagnosis.

Tissue collection and preservation for histologic evaluation of the skin

Every pathologist has his or her own preference for fixation of tissues. It is always best to work with the pathologist who will be evaluating the tissues before proceeding. Neutral buffered formalin solution is the most universal fixative used. Tissues are often left in formalin for long periods, however, which causes many epitopes to be modified due to cross-linking of amino groups by the aldehydes, making immunohistochemistry difficult or impossible [35]. Fekete's acid alcohol formalin minimizes this problem, especially when tissues are transferred to 70% ethanol after overnight fixation. Commercially available zinc-based preservatives are claimed to maintain epitopes and optimize immunohistochemical results while maintaining some degree of the histological quality that pathologists are used to with paraffin sections. Bouin's solution is popular as a general fixative but it hyalinizes collagen fibres, so fine detail of the skin can be difficult to interpret. Also, use of Bouin's solution requires washing in tap water and transfer to ethanol. Failure to do so results in major artefacts, often making the tissue unusable [35]. These and other fixatives are
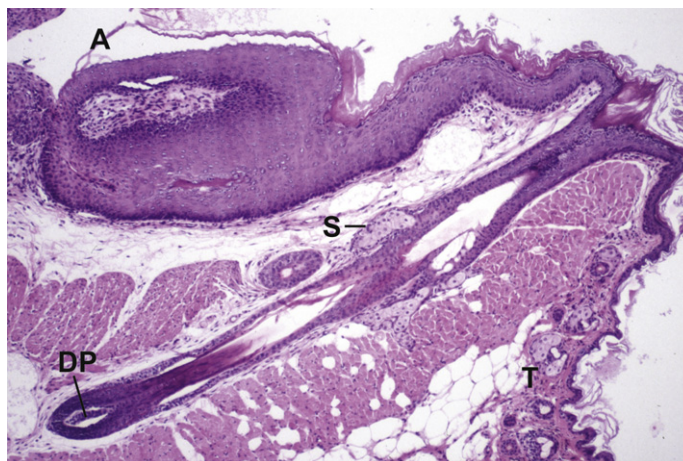
Figure 2.4.4 Anagen-stage perianal hair. Anus (A), dermal or follicular papilla (DP) within the bulb, sebaceous gland (S), telogen-stage truncal hair follicles (T).

Figure 2.4.5 (A) Photomicrograph of an egg (nit) anchored to a hair fibre, (B) Scanning electron microscopy reveals the mite (*Myocoptes musculinus*) holding on to a hair fibre, (C) Ectoparasites (mites) above the epidermis in a histologic section.

discussed in Chapter 5.7 on necropsy methods, which includes formulations for their preparation.

Since skin and hair follicles vary dramatically by location, several locations should be sampled in order to evaluate potential changes. In fact, transcriptome studies utilizing skin collected from various anatomical sites suggest that each site might be better considered as a separate organ [36]. Collection of tissue consistently throughout a study will make specimens comparable. Dorsal skin can be collected over the thorax, making sure to label cranial and caudal orientations so the tissue will be trimmed correctly. Ventral skin covering the thorax is also taken. Both dorsal and ventral skin are very similar histologically so they should be placed in separate cassettes and labelled (e.g. D for dorsal and V for ventral) or placed into separate cassettes, each with other skin that has distinct histological features. Vibrissae on the head are collected by removing all the skin on the head as a complete unit. Vibrissae on the muzzle are trimmed as one piece. Eyelids are sectioned from this piece of skin as well, to include upper and lower lids. Ears and tail are removed from the body and fixed by immersion. Tail skin can be removed from the bone and muscle or collected together. If the latter is done, the bone must be decalcified. Footpads are also collected. Details are provided in Chapter 5.7 and elsewhere [33, 37]. Nails are collected attached to the feet and digits. Distal limbs can be disarticulated and fixed *in toto*. If the paw is to be examined, it can be fixed under weight to lay it flat then sectioned horizontally to include all the joints after decalcification. Sagittal sections are the most useful for evaluation of the nail unit. Digits are processed *in toto* and serially sectioned lengthwise after decalcification.

Scanning electron microscopy of the skin and hair fibres

Scanning electron microscopy provides a detailed three-dimensional view of structures at various magnifications. X-ray microanalysis can determine the relative element content of a specimen, which may be useful for evaluation of some mutant mice. Hairs are made up of the high-sulfur keratins (hard keratins or hair keratins) and keratin-associated proteins. The low-sulfur keratin proteins are found in the flexible areas of skin, the interfollicular epidermis and foot pads. Changes in sulfur levels can be detected by element analysis and suggest abnormalities are present in these hairs, at least in their cuticles. Such is the case with the ichthyosis (Lbr^{ic-J}), nude ($Foxn1^{nu}$) and many other mutant mice that have forms of trichothiodystrophy, all of which show evidence of low sulfur levels in the hair shafts (Figure 2.4.6) [6, 38]. Mutant mice with defects in their hair shaft medullas may not have detectable decreases in sulfur levels [39–41]. Toxic agents, especially heavy metals, can also be identified using this method [42–44].

Whole mounts of skin or nails can be easily made by removing tissues at the time of necropsy, spreading soft tissues out on a firm nylon membrane to fix them flat, and placing them in buffered glutaraldehyde using standard methods. Electron microscopists will critically point dry the specimen, coat it with gold and then examine it with the investigator [45].

Hairs can be examined in whole mounts or manually removed and examined individually. Adult mouse hair follicles are in telogen for prolonged periods so the hairs can be easily removed manually from lightly anaesthetized animals without causing pain; damage to fibres is rare since
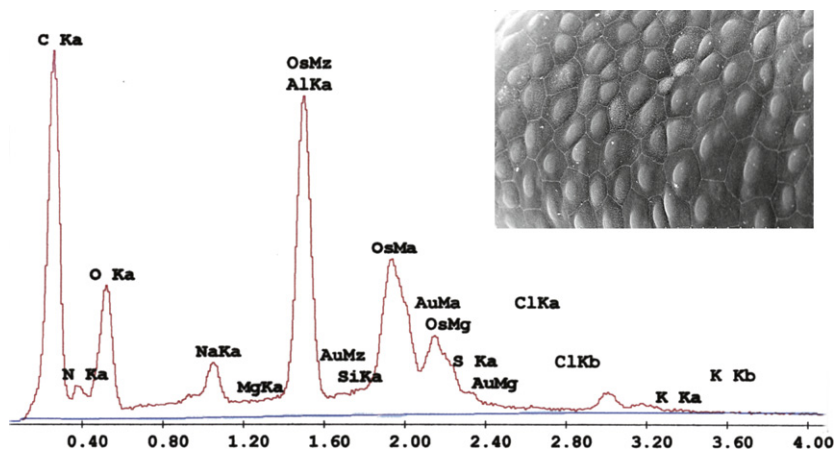
Figure 2.4.6 X-ray microanalysis of skin of the tip of a digit from a mouse embryo. The surface of the skin (keratinocytes) is evident. High element peaks represent the specimen preparation materials. Sulfur levels (S) can be quantitated.

they come out so easily [31, 46, 47]. Hairs are placed in a dry vial and processed routinely. Shipments we receive from collaborating laboratories for evaluation are routinely disinfected on the external surface and are then filled with 70% ethanol and stored for a week or more before processing because many research colonies are commonly infested with mites. This approach kills the mites, thus avoiding their introduction into our local colonies.

Transmission electron microscopy of the skin and hair fibres

Transmission electron microscopy can provide a great deal of information but is technically difficult and labour intensive. Tissue is removed during necropsy but should be finely minced into $1\,mm^3$ pieces, as glutaraldehyde fixatives do not penetrate tissues deeply. Cacodylate or phosphate buffered glutaraldehyde are commonly used but other fixatives are available and described in Chapter 5.6. Tissues should be stored refrigerated and embedded soon after collection to minimize artefacts [45].

Other methods

Many different methods have been developed to evaluate skin. We have tested a thermal imaging device that measures infrared radiated from mice under general anaesthesia (Thermogenic Imaging, Billerica, MA). This appeared to be a useful device for determining response to treatment for mutant mice with thick, scaly, neovascularized skin or those with various forms of alopecia (Figure 2.4.7). Longitudinal studies revealed that thermal changes over time reflected the hair cycle in both mutant and control mice since the hypodermal fat layer, and therefore the insulation value of the skin, varied dramatically throughout the hair cycle.

Transepidermal water loss is an important measurement in mice with abnormalities in the cutaneous water barrier. Mice are first sedated with 100 mg/kg ketamine HCl plus 0.5 mg/kg xylazine intraperitoneally. Dorsal hair is removed with electric clippers and then depilated for 5 min with a chemical agent such as Neet (Reckitt and Coleman, Wayne, NJ). Transepidermal water loss is measured 24 h later by placing a Servo Med Evaporimeter EPI probe (Servomed AB, Stockholm, Sweden) on the bald area [48-50].

Surface lipids can be collected by dipping euthanized mice into 40 ml of acetone 10 times and drying the acetone under argon gas. The residue is dissolved in toluene and plated in separate lanes on silica gel G chromatographic plates (Merck, Rahway, NJ). The plates are developed to 19 cm in hexane–ether–acetic acid (80:20:1). Following drying of the plate it is sprayed with 50% sulfuric acid [49, 51].

Kinetic studies can be easily done if considered at the time of necropsy. Mice can be injected with bromodeoxyuridine (50 µg/g body weight) 1 h before necropsy [52]. A consistent time
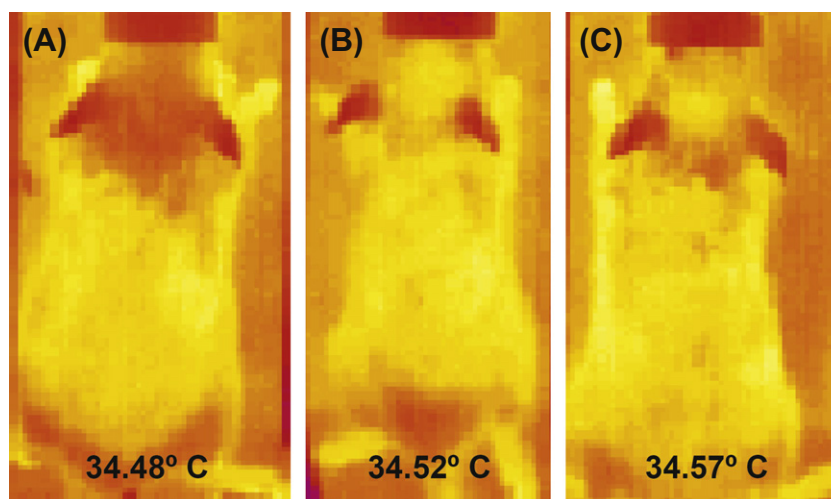
Figure 2.4.7 Thermal images of (A) a normal C3H/HeJ mouse, (B) one with focal alopecia areata and (C) one with diffuse alopecia areata. There is a quantifiable increase in heat loss associated with increased hair loss.

interval between injection and necropsy is critical since it will determine the rates at which this compound is incorporated into DNA currently being synthesized. Unstained sections are processed routinely for immunohistochemistry [53] and an anti-bromodeoxyuridine antibody used. Positive cells in S phase, the DNA synthesis phase of the cell cycle, will have nuclei that are brown or red depending upon the chromogen and enzyme system used [52, 53]. An alternative is to use tritiated thymidine. This radionuclide requires special safety precautions, takes 3-6 weeks for development and can be difficult to interpret, so it is less commonly used today [54]. Interpretation is complicated and can depend upon what types of proliferation rates are needed for evaluation of a particular mutant. Standard approaches are described for interfollicular skin such as counting the number of positive nuclei per 1000 basal cell nuclei or per linear millimetre of skin, if it lies flat [55-59]. Some mutant mice have marked proliferation of the infundibulum, which requires modifications and special adaptation of counting criteria [54].

Gene arrays for transcript analyses are now a stable technology that is commonly used, especially with a variety of gene network analysis software, including The Database for Annotation, Visualization, and Integration Discovery (DAVID) v6.7 hosted by The National Center for Biotechnology Information (NCBI; http://david.abcc.ncicrf.gov/home.jsp), Ingenuity Pathway Analysis

Software (http://ingenuity.com) or Ariadne Pathway Studio (http://ariadnegenomics.com). Each provides various tools for analysing gene expression data to identify potential pathway involvement as well as interactions between or among genes or proteins and their relationship to various cell processes and diseases. In each case, however, the critical starting material is high-quality RNA. What tissue to select and how to prepare it are controversial topics, developing as the technology evolves. We have used the entire skin of mice that develop a generalized cutaneous phenotype. The advantage is that an adequate volume can be obtained to provide enough RNA for many experiments. The disadvantage is that hair follicles in various stages are obtained, anatomically discrete areas are mixed and not all areas are affected. Assuming similar anatomical defects are found in age- and gender-matched controls, the differences in gene expression profiles should represent those related to the disease under investigation. More specific sites or time points in hair follicle morphogenesis and cycling may be chosen later as the disease is better understood [36]. The main advantage of gene arrays is that complex pathways that can take a great deal of time to analyse using traditional methods can be screened with a small group of animals in a matter of days. However, often these methods also generate large amounts of data that may likewise take weeks to months to analyse. For example, the chronic proliferative dermatitis

mutant mouse (*Sharpin^{cpdm}*) develops a psoriasiform dermatitis that closely resembles a form of human idiopathic hypereosinophilic syndrome [60]. By studying multiple time points, from clinically inapparent disease through severe scaly skin disease with ulceration, it was possible to work through the pathogenesis involving multiple complex molecular pathways [60–62]. These time course studies, as opposed to single observations, help to explain variations in therapeutic responses as diseases progress.

Tissue arrays (sometimes called tissue microarrays) are another technology with direct application to many research projects. Tissue arrays are built on traditional histology methods whereby paraffin blocks are systematically punched at prescribed sizes and the cores placed into pre-drilled holes in a new paraffin block [63]. Large numbers of tissues from many different organs or different case materials of similar lesions from the same organs can be used. This provides a tool to specifically evaluate the cells producing proteins from the up- and downregulated transcripts detected using gene arrays. Custom or predesigned arrays are commercially available and described in detail elsewhere (http://www.origene.com; http://www.imgenex.com; http://www.tissue-array.net; http://www.biomax.us). Tissue arrays can be made using skin from several strains of mice, different allelic mutations of the same gene or skin from model systems affecting many different species (we call these *phylogenetic disease arrays*). These provide the advantage that the same antibody or special stain can be used on multiple tissues from different individuals at the same time on the same slide, thus saving time and money and providing uniform staining/labelling.

Development of the normal skin and adnexa

Each hair follicle type starts to develop at different time points during embryogenesis. Therefore, it is not surprising to find clusters of hair follicles in a section at different stages of development. The large vibrissae develop earliest and are nearly fully developed by birth. Other hair types do not fully develop until several days later. In spite of this, all hair follicles develop in a similar anatomical fashion. This developmental scheme is detailed both historically and anatomically elsewhere [64] and serves as a guide for the summary below.

The sequential stages of hair follicle development begin with the pregerm stage, which is hard to recognize histologically but can be defined with various immunohistochemical markers. It consists of a sharply demarcated plaque of basal and suprabasal epidermal keratinocytes. In stage 1 the pregerm develops into an histologically evident epidermal thickening where the keratinocytes display a vertically polarized orientation compared with the more cuboidal appearance of adjacent basal cells. Concurrently, dermal fibroblasts increase in number immediately below this structure, forming what will become the dermal (follicular) papilla. Stages 2–4 produce a column of epidermal keratinocytes that develop a cap, invagination of the dermal papilla and formation of the basic hair follicle structure. The root sheaths begin to form and differentiate. Stage 5 is the bulbous peg stage with elongation of the inner root sheath and development of the bulge and first sebocytes. Melanin begins to form at this stage in pigmented mice. At stage 6 the follicle begins to extend below the level of the dermis into the hypodermal fat layer. The hair canal can now be identified. In stage 7 the tip of the hair fibre leaves the inner root sheath and enters the hair canal at the level of the infundibulum of the forming sebaceous gland. Stage 8 is the maximum length of the hair follicle where it extends down to the panniculus carnosus muscle and the hair fibre emerges through the epidermis. This process begins *in utero* and is completed for all follicle types by 5 days post-partum when the hair is evident on the skin of most strains of normal mice.

The epidermis develops from a single layer into a multilayered structure. In newborn mice it is thick at all anatomical sites and keratinocytes follow a classical differentiation scheme for stratified squamous epithelium (Figure 2.4.8). Cuboidal basal cells (keratins 5 and 14 positive) are located on the basal lamina [65]. Above this layer the cells differentiate into the statum spinosum or prickle-cell layer. Here the cells begin to
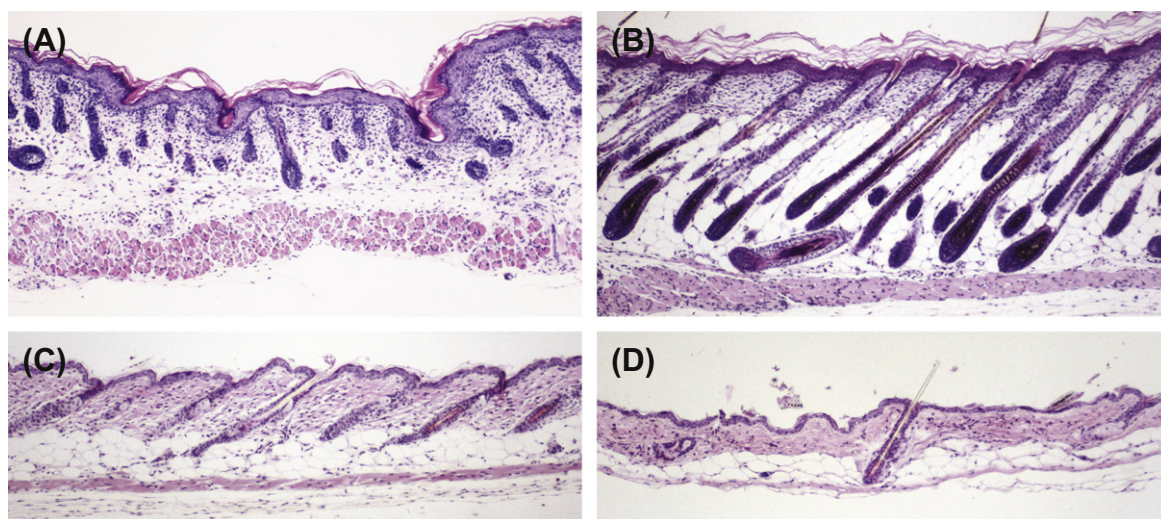
Figure 2.4.8 Hair cycle of the mouse. (A) Newborn mouse skin has a thick epidermis with incomplete development of hair follicles. (B) By 1 week of age hair follicles are fully developed in anagen and producing hair fibres that emerged at 5 days of age. (C) At 14 days of age the follicles enter catagen and begin to regress, undergoing apoptosis. (D) Within 3–5 days the follicles are in the resting (telogen) phase. Note that the epidermis thins and remains thin for life under normal circumstances by 2 weeks of age (C).

elongate along the axis of the skin and have prominent intercellular bridges (desmosomes) that are evident under high magnification. These spine-like structures are due to artefactual shrinkage of the tissues during preparation. This layer can be identified by the presence of keratins 1 and 10. The next layer, the stratum granulosum, has cells that are flattened along the axis of the skin and contain prominent basophilic granules (keratohyalin granules). Two types of granules are present in the mouse, profilaggrin (P) and loricrin (L) granules. The larger profilaggrin granules are blue structures visible by light microscopy [66, 67]. The most superficial layer, the stratum corneum, is brightly eosinophilic and consists of compacted, flattened keratinocytes. This is the critical portion of the skin that provides a strong aqueous barrier due to the presence of lamellar bodies, small lipid-based structures only detectable by special staining and transmission electron microscopy [68].

The epidermis of a newborn mouse is relatively thick, but as the mouse ages (within 2 weeks) the truncal epidermis thins to only about two cell layers with the stratum granulosum and corneum becoming very thin and often hard to visualize by light microscopy. Other anatomical sites do not change. The tail skin remains thick throughout the mouse's life. The muzzle skin is thinner than at birth but thicker than truncal skin. Foot pads remain thick once formed *in utero*.

Normal anatomy of the skin and the hair cycle

Histology of the normal skin

The anatomy of the skin and hair follicles are illustrated in Figures 2.4.4 and 2.4.8. The top layer of epithelial cells is called the *epidermis*. This layer differentiates from the cuboidal basal cells in the stratum basale into the polygonal cells of the stratum spinosum, then more flattened cells with fine blue granules in the stratum granulosum, and ultimately into the flat cells that lack a nucleus and become very eosinophilic at the surface in the stratum corneum. The outermost layer of cells separating from the surface are sometimes called the stratum dysjunctum. The hair follicle is a very complicated structure that invaginates into the dermis and hypodermal fat undergoing major changes on a regular basis with the hair cycle (see below). A large gland

protrudes from its side that consists of swollen pale cells with fine uniform vacuoles. These are sebaceous glands that produce oils to coat the surface of the skin and hair fibre. The oils can be visualized in frozen sections stained with oil red O, sudan black or other histological means to follow how the lipids spread out over the surface of the skin in normal compared with mutant mice [54]. The dermis consists of dense irregular collagenous connective tissue, elastic connective tissue, blood vessels, nerves, smooth muscle (arrector pili muscles that lift hair follicles and fibres; Figure 2.4.9) [69], and includes a variety of individual cell types including fibroblasts, mast cells and small numbers of cells from the immune system. One important feature of skin that is characteristically found in many rodents, especially laboratory mice, is that apocrine sweat glands are not present. Modified apocrine glands, mammary glands, are abundant, however, because of the large litter size most mice have [70]. The mouse does not normally have rete ridges as are seen in human skin [71] where the lower aspect of the epidermis forms ridges of cells that extend into the dermis. The dermis between such ridges is commonly called the *dermal papillae*, a term also used by hair biologists for the specialized fibroblasts that populate the base of an anagen hair follicle called the bulb. Because of this, the fibroblasts within the bulb are also called the follicular papilla. Rete ridge-like structures do become prominent when mouse skin heals following ulceration. These changes resemble those found in neoplasms of the epidermis such that the changes are referred to as pseudoepitheliomatous or pseudocarcinomatous hyperplasia. Below the dermis is a layer of fat, the hypodermal fat layer.

The thickness of this fat layer changes, with the hair cycle being thickest during anagen when follicles need a great deal of energy to produce a hair fibre. The panniculus muscle separates the hypodermal fat layer from the adventitia, loose collagenous connective tissue that attaches the skin to the underlying musculature and fat. Mammary glands are found in the fat below this skeletal muscle layer.

Hair cycle in the mouse

Mouse skin undergoes significant changes during the first 2 weeks of life (see Figure 2.4.8). Hair follicles continue to develop and enter late-stage *anagen* 5 days postpartum when hair fibres emerge through the epidermis. The truncal epidermis is relatively thick at birth and thins to normal by 2-3 weeks of age. Hair follicles produce fibres over the thorax until around 14 days of age at which time the lower portion undergoes apoptosis, a phase commonly referred to as *catagen*. During this regression the dermal papilla is retracted by actin filaments (see Figure 2.4.9) and will reside just below the isthmus during the resting or *telogen* stage until the hair cycle is reinitiated. This usually lasts about 3 days in young mice. The follicle develops into a new anagen-stage follicle, pushing the old follicle laterally. The new fibre emerges adjacent to the old one. At some point the old fibre is lost in what is now called the *exogen* stage [72]. The general features of the different stages of the hair cycle are illustrated in Figure 2.4.8. Hair follicle morphogenesis and cycling has been studied anatomically and with molecular and immunological markers to differentiate numerous stages within each major portion of the hair cycle that have been detailed and
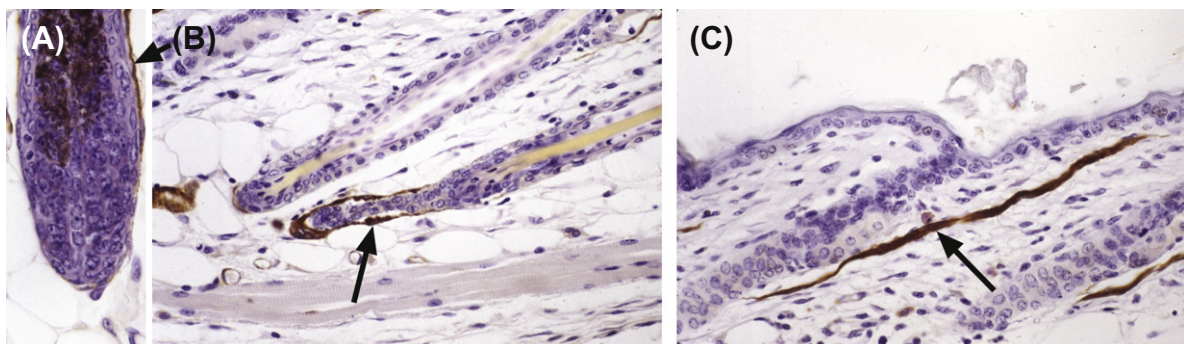
Figure 2.4.9 Smooth muscle actin expression. (A, B) Expression (grey, arrows) is located around the outer root sheath of anagen and catagen follicles. (C) It is also present in the arrector pili muscles.

reviewed elsewhere [46, 64, 73]. What is commonly called the second hair cycle, or the first real hair cycle after embryogenesis, has a short anagen stage and prolonged telogen stage. The hair cycles progress in a cranial to caudal pattern that can be easily seen in pigmented mice. Unlike humans, pigment in the mouse skin is limited to the bulb of anagen follicles and hair fibres. Interfollicular epidermis rarely contains pigment and, when it does, it is usually only in mutant mice [74]. If the mice are shaved, irregular pigmented areas will be seen [47]. These are areas containing anagen follicles. If mice are followed daily, these pigmented patches will migrate caudally following the hair cycle progression. This feature is dramatic in mutant mice such as hairless (Hr^{hr}). These mice have normal hair 5 days after birth but no subsequent hair cycles. Beginning at 2 weeks of age their hair is shed from head to tail [12]. Other hair follicle types have different hair cycles, as evidenced by hairless mice appearing to retain vibrissae while being otherwise completely bald. The length of the hair cycle determines the length of the hair fibre. Thus, hairless mice have long, persistent vibrissae while short pelage hairs are lost. This feature was also demonstrated with angora ($Fgf5^{go}$) mutant mice that have a 3 day prolongation of their truncal hair cycle and as a result often have long, shaggy hair compared with normal littermates [75].

Numerous genes regulate development and cycling of the hair follicles [73, 76-82]. Classic work done half a century ago detailed changes in the skin and hair follicles as they cycle, not just the changes in the follicles but also changes in

sebaceous gland size and shape as well as the thickness of the hypodermal fat layer [83-91]. Furthermore, hormones cause changes as well [92]. These are important to understand when comparing differences between wild-type, normal mice and mutant mice. Not only should the mice be age and gender matched in such studies but it is critical to match the stage of the hair cycle as well.

Nails

The mouse has nails or claws on each digit, just like most other mammals, including humans. The term 'claw' suggests these structures are different from human nails, which may be why little attention has been paid to them. (As an aside, 'claws' found on many invertebrates are very different, such as claws found on crabs and lobsters.) In fact, anatomically, mouse and human nails are very similar at the gross and histologic levels, the primary difference being that human nails are dorsoventrally flattened to form a plate while rodent nails are laterally flattened. These differences, not restricted to humans since similar refinements are found in many non-human primates, are associated with the function of the nails in primates as a refined tool associated with manual dexterity rather than as a weapon or digging tool. Sagittal sections illustrate that mice have a nail matrix, nail plate, nail bed, hyponychium and other structures (Figure 2.4.10) identical to but smaller than the human nail [71]. To veterinarians this is
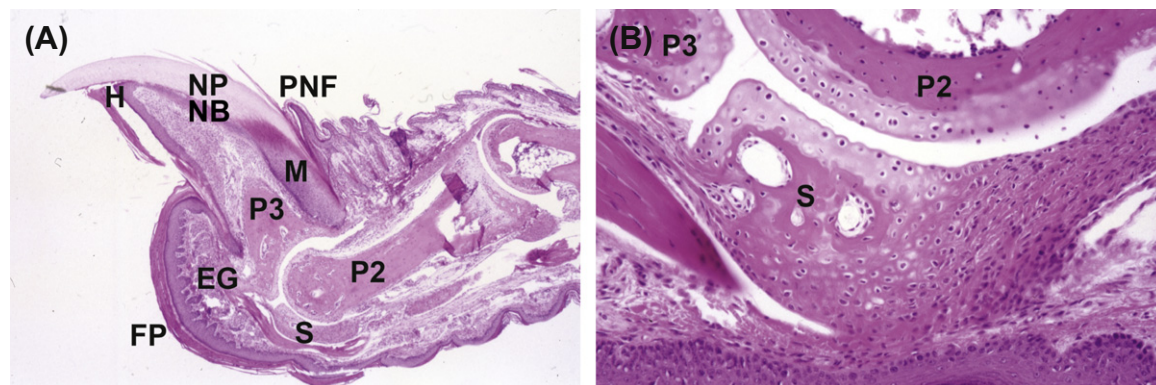
Figure 2.4.10 (A) Normal sagittal section of the nail from an adult mouse. Hyponychium (H), nail plate (NP), nail bed (NB), proximal nail fold (PNF), matrix (M), phalanx 3 (P3), phalanx 2 (P2), sesamoid bone (S), eccrine gland (EG), foot pad (FP). (B) High magnification of a sesamoid bone under P2.

not at all surprising since all mammals have nails (or claws) that are variations on this general theme. Nails can be extremely difficult to prepare and interpret histologically. However, dramatic changes in mutant mice can be seen when these structures are magnified with a dissection microscope or by scanning electron microscopy [3, 6].

Other specialized glands

Mammary glands are specialized forms of apocrine sweat glands with a complex developmental and lactation cycle that will not be discussed here. Other glands found at specific anatomical sites are modified sebaceous glands, a type of holocrine gland. These include the preputial and clitoral glands around the genitals, meibomian glands in the eyelid and Zymbal's or auditory glands within the outer ear. All are large glands with a structure similar to that found in the sebaceous glands associated with hair follicles. The major difference is that each has a duct lined with stratified squamous epithelial that empties directly onto the

structure where it is located [3]. Hair follicles are specialized and have sebaceous glands associated with them that vary in size. The most notable are the perianal hairs, which have large sebaceous glands. Salivary, lacrimal and Harderian glands are very different and are described in chapters dealing with the organs they are associated with.

Skin and adnexal mutant phenotypes

It is beyond the scope of this chapter to describe or even list all mutant mice with skin and/or hair/nail phenotypes. As a general starting point we have grouped phenotypes into 10 classes (Box 2.4.2). Detailed lists, descriptions, references and illustrations are published elsewhere [3, 15-20, 22, 23].

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Development and Disease of Mouse Muscular and Skeletal Systems

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PART A. MUSCLE SYSTEMS
Introduction

In animals a highly conserved series of temporally and spatially programmed gene expression profiles is responsible for muscle development and differentiation. These programmes allow for the initial derivation of embryonic myogenic precursors; their differentiation into myoblasts; fusion of fetal myoblasts into multinucleated myofibres; the assembly and proper functioning of the contractile structures, the sarcomeres; and adult muscle growth and regeneration following injury. Body movement involves a complex cascade transforming neural signals to depolarization of myofibres, binding of individual myosin and actin filaments in the sarcomeres leading to myofibre contraction, and myofibre cross-linking transmitting force throughout muscle groups and into the skeletal system via their tendinous attachments to bone. The accurate coordination of these processes requires exquisite structural integrity and fluidity. This is obtained through proper alignment of all the requisite components during development.

The muscular dystrophies represent a wide range of inherited muscle disorders that can arise either from defects in muscle development, or from defects of muscle maintenance and repair. Although the age of onset, muscle types affected and severity of disease vary widely, they are all characterized by an imbalance between muscle wasting and the ability of muscle to repair itself. Much of our understanding of muscle development has been illuminated through studies of what goes awry in these disorders. The laboratory

mouse, *Mus musculus*, has historically been an invaluable tool for studying both muscle development and muscle disease, and continues to play a central role in our understanding of how muscle becomes damaged in dystrophies, through disuse atrophy, and through the ageing process. In this chapter we address the current state of knowledge of how mammalian, and specifically mouse, muscle develops, and we highlight the key mouse models for human muscular dystrophies and how genetic defects lead to muscle disease in these animals.

Cellular and molecular development

Somite formation and embryonic muscle development

Gastrulation of the early embryo creates the mesoderm, ectoderm and endoderm germ layers, with the mesoderm being the source for blood, blood vessels, bones, cartilage and muscles [1]. The mesoderm subsequently divides into the axial mesoderm (notochord), intermediate mesoderm, paraxial (or presomitic) mesoderm (PSM) and lateral plate mesoderm [1]. Skeletal muscle of the trunk and limbs derives from the somites, which are part of the PSM forming on either side of the neural tube. In the mouse somites form between embryonic day E7.5 and E8, with mesenchymal precursors moving into the PSM and compacting into segmented spheres surrounded by epithelial cells that evolve into the somites in a rostral to caudal gradient [2]. One pair of somites is produced in the mouse embryo approximately every 2 h, resulting in 60 somite pairs [3]. Pairs of somites pinch off from the anterior tip of the PSM in a regular pattern. The rostral to caudal development of the somites is regulated by the segmentation clock, an oscillator that interacts with a maturation wave to produce the development of the somites through the paired cycling of Notch, fibroblast growth factor (FGF) and Wnt pathways [4]. This oscillation

creates alternating permissive/non-permissive states of the presomitic mesoderm complemented by a caudally advancing maturation wavefront, with somites forming where the wavefront meets cells in a permissive state [5]. Each somite differentiates into a ventral sclerotome (giving rise to the axial skeleton), and a dorsal epithelium, known as the dermomyotome (Figure 2.5.1).

The dermomyotome is the source of the myogenic progenitors, and is divided into two major polarized compartments, the epaxial and hypaxial domains [2]. This dermomyotome gives rise to dermal, endothelial and smooth muscle cells, along with myogenic cell lineages [6, 7]. The borders of the dermomyotome undergo an epithelial to mesenchyme transition and form the myotome with the first differentiated myofibres. By E8.75 in the mouse some myogenic precursors progress to terminally differentiated mononucleated myocytes [8] in the first wave of myogenesis. During this phase muscles consist of small numbers of myotubes that grow and have a round shape, and primarily express both embryonic fast and slow myosin heavy chain isoforms (MyHC) [8]. In later (secondary) myogenesis secondary fibres are made by fusion of fetal myoblasts and express primarily fast embryonic and perinatal myosin isoforms, with no slow isoforms [8]. Progenitors from the central dermomyotome become mesenchymal tissues, while the epaxial domain nearest the neural tube will differentiate into the muscles of the back, and myogenic precursors of the hypaxial domain will migrate to form the musculature of the diaphragm, body wall and limbs [2]. This process starts at E9.25 in the forelimbs and completes by E11.0 in the hindlimbs [9]. By E11.0 myoblasts fuse into multinucleated primary myotubes through incorporating mononucleated myocytes [8].

Myogenic regulatory factors

Myogenesis is regulated by four basic helix–loop–helix (bHLH) transcription myogenic regulatory factors (MRFs; Myf5, Mrf4(Myf6), MyoD and Myogenin). Dorsally located muscle progenitors respond to Wnts and Shh from the adjacent neural tube, notochord and ectoderm, and directly activate Myf5 to commit precursors to
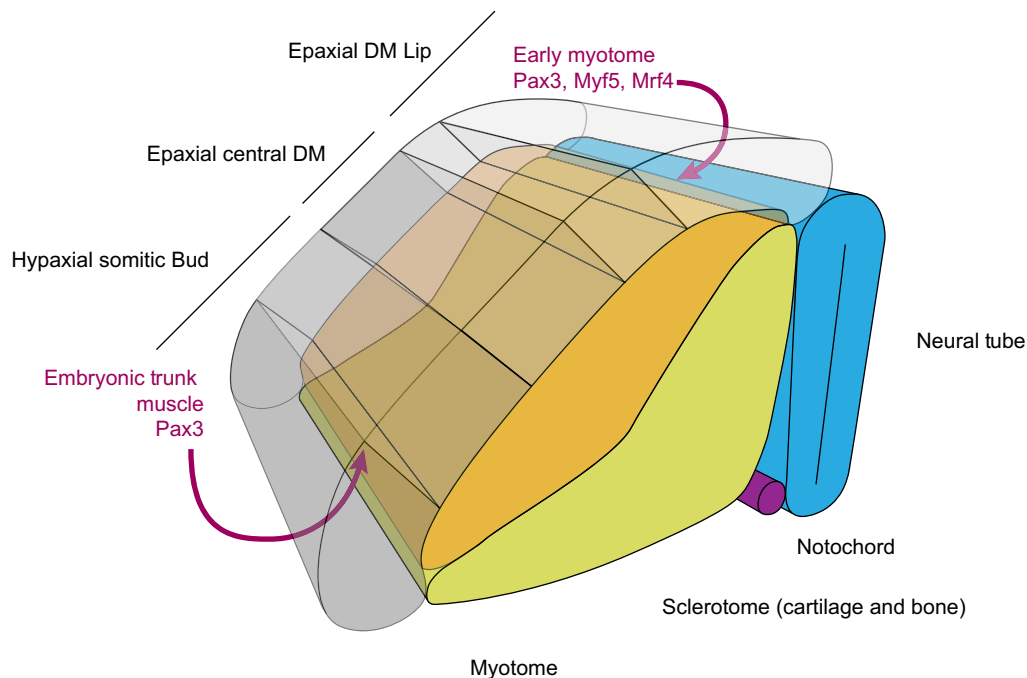
Figure 2.5.1 Amniote dermomyotome.

Labels in figure:
- Epaxial DM Lip
- Early myotome Pax3, Myf5, Mrf4
- Epaxial central DM
- Hypaxial somitic Bud
- Neural tube
- Embryonic trunk muscle Pax3
- Notochord
- Sclerotome (cartilage and bone)
- Myotome

enter myogenesis [10]. Myogenin and Mrf4 are not involved in primary myogenesis, but are rather factors involved in muscle differentiation, with myogenin required for mononucleated myoblast fusion into myotubes. Hypaxial muscles do not require Shh signalling from the neural tube or notochord, but require signals instead from the dorsal ectoderm [8]. In addition to the Notch signalling pathway, combinations of homeobox (*HoxA* and *HoxC*) genes are also involved in the proper timing and location of myogenic differentiation and patterning in adult muscle [11].

Myostatin, a transforming growth factor beta (TGFβ) signalling molecule, prevents excess muscle growth by limiting the proliferation of Pax-positive progenitor cells during embryonic and fetal development [12]. Myostatin is produced in an inactive form containing a propeptide inhibitory domain, and two cleavages are required for its activation [13]. Once released into the extracellular matrix and serum, it can be inactivated by binding to the inhibitory propeptide or to its inhibitor follistatin [13]. In adults myostatin circulates in the blood and inhibits muscle growth. Deletion of murine myostatin results in a wide increase in adult muscle mass through a combination of increased muscle cell numbers (hyperplasia) and increased fibre size (hypertrophy) [14]. This activity appears to be through inhibition of both proliferation and differentiation of satellite cells, as well as through effects on protein synthesis in differentiated myotubes [15].

Pax3 and Pax7

Pax3 and Pax7 are paired homeobox transcription factors and are both expressed in myogenic precursors as the somites develop. As the muscle precursor cells move from the dermomyotome to the myotome, they activate the myogenic determination genes *Myf5* and *MyoD*, which initiate skeletal muscle development. Pax3 is necessary for delamination and migration of the muscle progenitors out of the dermomyotome and into the limb buds [16]. Migration of muscle progenitors requires coordinated action between the migrating cells and the targets where they are fated to arrive through chemokine receptors and their ligands [17]. Migrating muscle cell progenitors express the chemokine receptor CXCR4, while its ligand, SDF1, is expressed in the limb and brachial arch mesenchyme, which forms the route of travel and target of the migrating progenitors [17].

Pax3 is necessary for all embryonic myogenesis, while Pax7 is required for limb fetal

myogenesis and the development of satellite cells (see below) [9]. In muscle progenitors Pax3 and Pax7 maintain expression of MRFs and allow for population expansion and maintainance of the myogenic lineage commitment that allows for differentiation of muscle precursors [6]. Pax3 is expressed in the PSM prior to segmentation, and then is restricted to the dorsal dermomyotome in the epaxial and hypaxial extremities, while Pax7 expression is concentrated in the central domain of the dermomyotome [6]. Embryonic myogenic cells which are Pax3+/Pax7− eventually contribute to muscle and endothelium, are required for embryonic myogenesis and subsequently give rise to Pax7+ cells, which themselves are required for fetal myogenesis and satellite cell formation [18]. Pax7-deficient mice develop normal numbers of satellite cells during development, but they are rapidly lost postnatally due to cell death, indicating an anti-apoptotic role of Pax7 [7]. Using conditional Pax7 deletion in mice, it has been shown that the requirement for Pax3 and Pax7 in satellite cell maintenance exists for up to 3 weeks after birth, but is lost after that, and that adult satellite cells with *Pax7* deleted are still fully functional and muscle regeneration is not compromised [19].

Fetal muscle development

Between E14.5 and E17.5, secondary myogenesis occurs, with fusion of fetal myoblasts with each other and with primary fibres, and during this phase satellite cells (adult muscle stem cells) can be seen as mononucleated cells between the basal lamina and the sarcolemmal membrane [8]. Fetal myoblasts and satellite cell differentiation are inhibited by TGFβ and/or bone morphogenic proteins (BMP), but embryonic myoblasts are not [20]. Therefore, embryonic myoblasts can undergo differentiation and fuse into primary fibres, which then stimulate fetal myoblasts to expand and form secondary fibres [8]. Each developing multinucleated muscle fibre is innervated at a neuromuscular junction (NMJ), initially by axons from multiple motor neurons with cell bodies in

the ventral horn of the spinal cord, and subsequently during early postnatal development by a single axon through axon elimination [21].

Insulin-like growth factor (IGF-1) is differentially spliced in developing skeletal muscle in response to mechanical stretch signals, and the two isoforms direct different actions in myoblasts and satellite cell precursors. Mechano growth factor isoform (MGF) is upregulated by the type of cyclical loading seen during early development with newly formed spasmodic contractions, and directs proliferation of mononucleated myoblasts and the establishment of the satellite cell pool, while IGF-IEa is upregulated by single-stretch and endogenous tension, like that generated by traction of bone growth, and directs myotube formation and muscle hypertrophy [22]. In adult muscle MGF is expressed by mechanically overloaded muscle and aids in activation of satellite cells, with MGF upregulation impaired in aged muscle fibres, thus contributing to decreased muscle repair during ageing [23].

Head muscles do not derive from the somites, but rather from the prechordal and pharyngeal head mesoderm, and these are reviewed in [9, 24]. *Pax3/Myf5* double mutants do not form trunk and limb muscles, but head muscles are normal; Wnt signalling is myogenic in the somites, but inhibits myogenesis in the head musculature [25], thus illustrating the different developmental routes for these two main muscle forms.

Satellite cells

Adult skeletal muscle does not normally have rapid turnover of its postmitotic cell population, but regular use of muscle results in normal damage that needs regular repair. The majority of this repair process comes from satellite cells, which are mononucleated progenitor cells residing between the basal lamina and the sarcolemma of each multinucleated myofibre (Figures 2.5.2 and 2.5.3). Satellite cells were first reported 'wedged' between the plasma membrane of the muscle fibre and the basement membrane in the frog [26]. In immature fibres most satellite cells are dividing and differentiating into new myofibres. Pax3/7-positive cells in the central dermomyotome give rise to satellite cells of the trunk, while Pax3 progenitors that migrate from the hypaxial dermomyotome give rise to
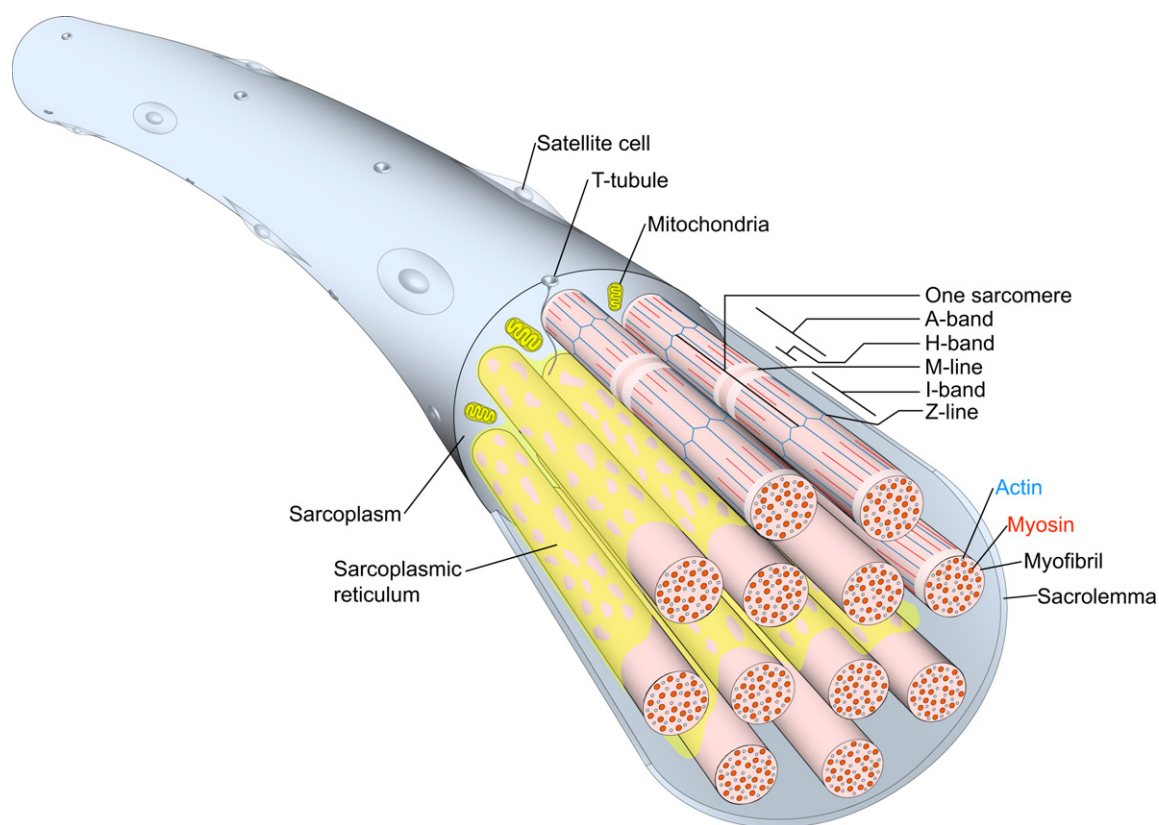
Figure 2.5.2 A schematic overview of striated skeletal muscle fibre.

satellite cells of the limbs [27, 28]. During peri- and postnatal development, satellite cells divide and differentiate into primary myoblasts, which fuse with myotubes to continue to grow the musculature [8]. As muscle ages satellite cells are generally quiescent until damage to the myofibre results in their activation, entry into cell proliferation and differentiation. CD34, Myf5 and M-cadherin are markers that define the majority of quiescent adult satellite cells [29].

Notch/Numb

Satellite cell activation results in proliferation into myogenic precursors, and this cell division activates Notch-1, at the same time asymmetrically dividing Numb, the antagonist of Notch-1, in the divided daughter cells, therefore creating a heterogeneous population of precursor cells with respect to Notch-1 responsiveness for myogenic differentiation [30]. This allows a subpopulation to remain undifferentiated for return to the stem cell pool for future activation. In old muscle the Notch ligand, Delta, fails to upregulate in satellite cells, and therefore the regenerative potential of

satellite cells is limited, but by increasing Notch activity, either through exogenous promotion or through exposure of old satellite cells to a young muscle environment, the regenerative potential of old satellite cells can be restored [31].

Niche

Satellite cells are surrounded by M-cadherin, which is deposited around the satellite cell next to the basement membrane [32]. Mechanical stretching of single muscle fibres has been shown to activate quiescent satellite cells, but the population is heterogeneous in its activation potential [33]. Mechanical stretch of damaged muscle fibres stimulates NO synthesis, which activates matrix metalloproteases that then release hepatocyte growth factor (HGF) from its tethering in the ECM, allowing it to bind c-met receptors on quiescent satellite cells and inducing activation [34]. As satellite cells age and their regenerative potential declines, they convert from a myogenic to a fibrogenic lineage, mediated by increased canonical Wnt signalling in their immediate aged muscle environment [35].
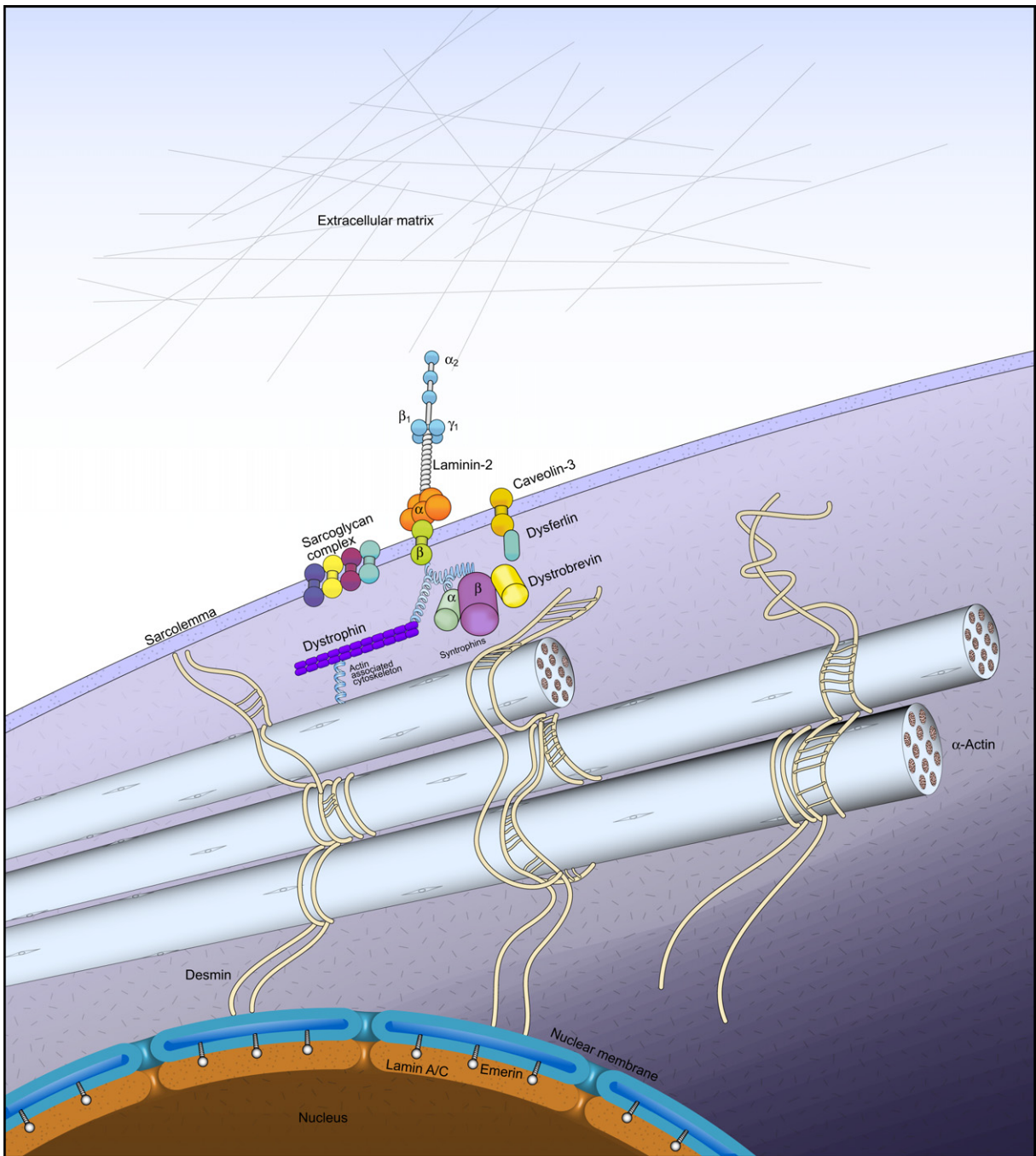
Figure 2.5.3 Structure of skeletal muscle, including extracellular matrix, linkage proteins, myofibrils and intermediate filaments.

Quiescent versus activated

As quiescent satellite cells are activated, they express Pax7 and MyoD, and most proliferate, downregulate Pax7 and differentiate into myofibres [36]. A subset, however, maintain Pax7 and lose MyoD and return to the quiescent state, again maintaining the satellite cell pool in a self-renewing manner [36]. Pax7 is involved in maintaining proliferative capability and preventing differentiation, but does not itself initiate quiescence [37]. The plane of satellite cell division seems to determine the fate of the daughter cells, with cells carrying out planar division generating daughter cells with symmetric Myf5 expression, and cells dividing along the apical–basal plane

generating daughters with asymmetric Myf5 expression [38]. This in turn leads to two distinct populations of daughter cells, with Myf5+ cells going on to differentiate and fuse into myotubes, and Myf5− cells maintaining a pool of uncommitted satellite cells for future use. Sphingomyelin levels are high in the plasma membrane of quiescent satellite cells but fall as they are activated, indicating changes in the organization of the plasma membrane and generation of signals involved in activation [39]. Sphingomyelin is metabolized to sphingosine-1-phosphate, which can directly induce activation of satellite cells [40].

Additional adult muscle stem cell populations

In addition to the satellite stem cell population, several other cell types with myogenic potential reside in or around muscle fibres. These include pericytes associated with the microvascular walls in skeletal muscle, which unlike satellite cells do not express Pax7, Myf5 or MyoD until after they are induced to myogenic differentiation [41]. Another population includes embryonic mesoangioblasts, a vessel-associated stem cell able to differentiate into myoblasts under the direction of Pax3 [42]. A third population of myogenically potential stem cells includes interstitial cells, bone marrow cells, and neural stem cells [43]. Mesenchymal stem cells are located in the muscle interstitium and are the source of ectopic fat cells in skeletal muscle [44]. These PDGFRa$^+$ mesenchymal progenitors are separate from satellite cells, and there is an inhibition of adipogenesis in the presence of satellite cell-derived muscle fibres, indicating that adipose deposition seen during many muscular disorders is due not to plasticity of satellite cells into fat, but rather to a misregulation of adipose inhibition by diseased muscle [44].

Adult muscle function

Fibre types

Skeletal muscle is the most abundant tissue in the body of vertebrates, comprising about 50% of total mass. Most mammalian muscle fibres are composed of various proportions of fast and slow fibre types, which determine contractile force and duration. The contractile properties of skeletal muscle depend on fibre size, fibre properties and arrangement and number of fibres in a muscle [45]. Fibre type composition is dependent on embryonic cues, but neuronal signalling is central to the eventual development of fibre type, and activity patterning causes different members of the nuclear factor of activated T cell (NFAT) transcription factor family to translocate to the nucleus and contribute to the transcription of fibre type-specific genes, such as myosin heavy chain (MyHC) genes [46]. The classification of adult muscle fibres is based on their speed of contraction by their ATPase activity, which depends on the proportion of fast and slow MyHC isoforms. In mice a single slow *MyHC* gene is subject to multiple post-translational modifications, with embryonic and postnatal MyHC isoforms replaced with three adult fast MyHCs—IIa, IIx(d) and IIB [47]. Adult muscle fibres are divided into four major classes based on their speed of contraction and predominant expression of MyHC isoforms: type I, type IIA, type IIX/D and type IIB [48]. Type I are slow twitch/fatigue resistant, type IIA and IIX/D are fast twitch and moderately fatigue resistant, and type IIB are fast twitch and not fatigue resistant. Muscle fibres can, however, change type in response to stimuli, with endurance training converting fast fibres to slow phenotype, and strength training resulting in fibre hypertrophy and conversion from slow to fast phenotype [49].

In addition to the extrafusal (main) fibre types, there are also intrafusal fibres, which although they do not participate in force generation do help to control contraction by monitoring muscle length and transmitting this prioceptive signal through the sensory neurons into the central nervous system [8]. Intermediate filaments (IFs) (desmin being the most abundant IF protein; Figure 2.5.3) are localized around the Z-disk and link the contractile apparatus of striated skeletal muscle with mitochondria, myonuclei and the sarcolemma to aid in force transmission as well as coordination of energy demands, gene expression and protein/lipid targeting [50]. Lack of desmin

results in abnormal subsarcolemmal clumping of mitochondria, potentially by the loss of a proper linkage for mitochondrial placement, or loss of proper mitochondrial membrane protein/lipid targeting [50].

Sarcoplasmic reticulum

The sarcoplasmic reticulum (SR) is a form of smooth muscle endoplasmic reticulum (ER) found in skeletal muscle that functions as a regulator of Ca^{2+} storage and release homeostasis during and after muscle contraction [51]. The SR is a series of tubules and terminal cisternae that share a common lumen and a single continuous membrane, including both longitudinal and junctional domains with characteristic protein localization. Longitudinal SR is involved in Ca^{2+} reuptake through the sarco/endoplasmid reticulum Ca^{2+}-ATPase (SERCA) pumps. The longitudinal SR merge into terminal cisternae at the junctional SR, where the ryanodine receptor type 1 (RyR1) Ca^{2+} release channels of skeletal muscles are localized [52]. The dihydropyridine receptors (DHPRs), which are voltage-gated Ca^{2+} channels, are organized into tetrads on the plasma membrane facing the junctional SR [52]. Calcium is stored in the SR through the Ca^{2+}-binding proteins calsequestrin, histidine-rich Ca^{2+}-binding protein, junctate and sarcalumenin [53]. Excitation–contraction (E–C) coupling occurs at the triads, which are intercellular junctions of the transverse tubules and the SR terminal cisternae. Two terminal cisternae and one T-tubule form triads. E–C coupling occurs through conformational changes in the DHPRs, which then directly transmit the conformational changes to the RyRs in the SR terminal cisternae [54] resulting in Ca^{2+} release. Tetrads are formed by groups of four DHPRs that form arrays directly facing the RyRs, thus forming an appositional junctional domain for Ca^{2+} release [55]. Muscle contraction is initiated with depolarization of the cell membrane by binding of acetylcholine released from nerve terminals to acetylcholine receptors on the muscle side of the neuromuscular junction. The action potential travels into the T-tubule, activating DHPRs and opening the RyR1 channels, releasing Ca^{2+} from the SR, which then binds to troponin C on the actin (thin) filaments, changing the conformation of tropomyosin and allowing the myosin (thick) filament cross-bridges to alternatively attach to and detach from actin under the influence of ATP, thus pulling along actin and shortening the sarcomere. As the action potential ends, SERCA pumps remove Ca^{2+} back into the SR, changing tropomyosin so that it again blocks myosin binding, thereby releasing the contraction. Mutations in RyR1 result in several human muscle diseases including malignant hyperthermia (MH), central core disease (CCD) and multi-minicore disease, with a mouse knockin of a human RyR1 mutation (Y522S) recapitulating MH with muscle contracture and hypermetabolic crisis resulting from exposure to halogenated surgical anaesthetics [56, 57].

Mitochondria

Mitochondria-associated membranes (MAMs) are subdomains of the ER/SR that are tightly linked to mitochondria and assist in phospholipid synthesis and transfer [58]. Mitochondria are located immediately adjacent to the Ca^{2+} stores in the SR, and entry of Ca^{2+} into the mitochondria stimulates the respiratory chain, increasing cellular ATP to support muscle activity [59]. Mitochondria are integrated with muscle Ca^{2+} release by being tethered by their outer mitochondrial membrane to the intracellular Ca^{2+} muscle stores. This tethering is developmentally regulated, shifting from general longitudinal formation at birth to an adult-specific Ca^{2+} release unit-coupled transverse orientation with mitochondria packed into a narrow space in the I band between the Z-line and the triad [60]. This tight coupling allows for bidirectional signalling, with contraction of muscle fibres resulting in increased ATP production, and mitochondrial production of reactive oxygen species (ROS) and detoxification regulating the local redox environment of the Ca^{2+} release unit (CRU) and inhibiting SR Ca^{2+} release [61].

Costameres

Costameres are regions associated with the sarcolemma of skeletal muscles that aid in

transmitting force from the contractile apparatus to the extracellular matrix at the Z- and M-lines, thus transferring force to adjoining muscle fibres and providing mechanical stability to muscles during contraction [62]. They consist of proteins of both the dystroglycan–glycoprotein complex (DGC) along with the vinculin–talin–integrin system [62]. They are localized to different regions depending on metabolic fibre type (fast vs slow), being positioned above the I-bands in some tissues and above the A-bands in others [62].

Muscle cytoskeleton and extracellular matrix connections

Adult muscle fibres transform axonal signals into mechanical contraction that transmits force to the tendon and the skeletal structures [63]. The muscle fibre cytoskeleton has several main domains, including the contractile sarcomeric cytoskeleton containing the thin (actin) and thick (myosin) myofilaments, the intra-sarcomeric region containing titin, nebulin alpha-actinin and others which anchor the myofilaments, the intermyofibrillar cytoskeleton consisting of desmin, which links adjacent myofibrils, and the subsarcolemmal cytoskeleton, which links the actin cytoskeleton to the extracellular matrix (ECM) and stabilizes the sarcolemmal membrane [63]. The DGC is a skeletal muscle protein complex that links the actin cytoskeleton to the extracellular matrix [64], and provides stabilization of the sarcolemmal membrane during contraction. The DGC is composed of a variety of proteins, including dystrophin; alpha- and beta-dystroglycan; alpha, beta, gamma and delta sarcoglycans; sarcospan, alpha1, beta1, and beta2 syntrophins; and alpha-dystrobrevin. The proper regeneration of damaged skeletal muscle depends not only on the muscle fibres themselves, but also on the proper linkage to the extracellular matrix. In addition to the DGC, heparan sulfate proteoglycans (HSPGs), which are present in ECMs, interact with a range of heparin-binding growth factors important in muscle development and repair such as FGF, HGF, and TGFβ [65].

Degenerative diseases and genetic models

In this section we address the major mouse models of human muscular disorders. Several of these models exhibit different muscle phenotypes from those seen in humans, and several models exist which have not been directly attributed to human genetic mutations but are involved in similar pathways. In some muscular dystrophies (e.g. dystrophin, sarcoglycans) the mutated gene is not normally expressed in satellite cells, and the muscle disease is therefore not directly attributable to satellite cell failure, while in other dystrophies (e.g. lamins, laminin, emerin) the gene is expressed in satellite cells as well as general muscle fibres, and therefore a direct effect on satellite cells may be at least partially responsible for muscle wasting [66].

Sarcolemmal maintenance and repair/dystrophin–glycoprotein complex
Dystrophin

Duchenne muscular dystrophy (DMD) is the most common X-linked birth defect (1/3500 male births), and usually leads to the need for a wheelchair and death by the early thirties (http://www.mdausa.org/disease/dmd.html). Loss of the dystrophin protein results in the disruption of the DGC, resulting in impaired connection of the sarcolemmal membrane to the extracellular matrix and a loss of stability of the muscle fibre membrane. This leads to increased damage to the membrane and influx of Ca^{2+} ions into the muscle fibre. In the *mdx* mouse model of DMD, inflammatory cells induce expression of inducible nitric oxide synthase (iNOS), which binds to RyRs in the SR, resulting in increasingly leaky Ca^{2+} channels, leading to activation of Ca^{2+}-dependent proteases (calpains) that cause muscle damage and wasting [67]. While aberrant calcium homeostasis has been a main hypothesis for muscle cell death in DGC mutations, signalling

has also been implicated, as some models show no sarcolemmal instability (and therefore little to no calcium leaking) but still exhibit cell death [68]. In dystrophin-null muscles structural proteins, including those necessary for costamere formation, are upregulated to compensate for the loss of stabilization resulting from the lack of dystrophin [69].

Ankyrin-repeat molecules have high affinity for other ankyrin repeats, and are extremely useful in skeletal muscle because of their high resistance to mechanical forces [70]. Ankyrins are crucial for proper muscle development and function through proper localization of ankyrin-binding proteins such as dystrophin and dystroglycan to the costameres and neuromuscular junctions in skeletal muscle sarcolemma [70]. Mutations in dystrophin that affect binding to ankyrin B are involved in the pathogenesis of Becker muscular dystrophy [71].

Dystroglycan

The myodystrophic *Large* mouse (*Large*^*myd*^) has a lethal muscular dystrophy [72] caused by incomplete glycosylation of alpha-dystroglycan due to a frameshift mutation in the catalytic domain of the glycosyltransferase Large [73]. A human mutation (and recapitulated in a mouse model) in dystroglycan leads to muscular dystrophy by interfering with LARGE-dependent maturation of phosphorylated *O*-mannosyl glycans on alpha-dystroglycan, causing defective binding to laminin [74]. Mutations in other human glycosyltransferases have been found in Fukuyama muscular dystrophy, muscle–eye–brain disease, and Walker–Warburg syndrome [75], with mouse mutations in Fukutin-related protein (Fkrp) [76] and protein *O*-mannose beta-1,2-*N*-acetyglucosaminyltransferase 1 (POMGnT1) [77] resulting in models of Fukuyama muscular dystrophy and muscle–eye–brain disease, respectively.

Sarcoglycans

A variety of human limb girdle muscular dystrophies (C, D, E, F) are caused by mutations in sarcoglycans (gamma, alpha, beta and delta, respectively), with targeted deletion of alpha- [78], beta- [79], and gamma-sarcoglycans [80] leading to muscular dystrophy in mouse models.

Alpha-dystrobrevin

Alpha-dystrobrevin is a component of the DGC, having both structural and signalling roles in muscle, and binds directly to dystrophin, syntrophin, and sarcoglycans. Alpha-dystrobrevin also binds to the intermediate filaments [68]. Mice with knockout of alpha-dystrobrevin have severe muscle degeneration and neuromuscular junction abnormalities [68]. Loss of alpha-dystrobrevin results in disorder of the linkage between dystrophin and beta-dystroglycan, potentially due to the loss of both structural and functional aspects of alpha-dystrobrevin [68].

Dysferlin

Dysferlin is a type II membrane protein involved in repair of the sarcolemmal membrane in skeletal muscle. Repair occurs through patch formation by fusion of subsarcolemmal vesicles to the membrane at regions of disruption [81], and involves several fusogenic muscle proteins along with various lipid components [82]. Dysferlin itself appears to be stored in an available pool immediately below the cell surface through interactions with tubulin and microtubules [82]. Mutations in dysferlin result in limb girdle muscular dystrophy type 2B, Miyoshi myopathy and distal anterior compartment myopathy in humans. Mouse models of dysferlinopathies include the A/J [83] and SJL [84] strains, which carry an intron 4 retrotransposon insertion and a 171 bp in-frame deletion in the dysferlin gene, respectively.

Sarcomeric structural proteins
Titin

The giant muscle protein titin spans one complete half sarcomere from the Z-disc to the M-line. It provides passive elasticity and muscle signalling during stretch, and is involved in muscle assembly. The formation of the sarcomere involves the coordinated polymeric assembly of a large group of proteins. Initially, MURF2-associated microtubules colocalize with myosin, and then titin-associated actin molecules coalign with the MURF2–microtubule–myosin complex, leading to maturation of the complete

sarcomere [85]. Variations in the pattern of titin isoforms can adjust the passive stiffness of striated muscle fibres [86]. The titin molecule has an elastic region with two spring elements: the tandemly arranged immunoglobulin (Ig)-like domains, and the PEVK region (rich in proline, glutamate, valine and lysine) [87]. Each titin molecule extends from the Z-line at its N-terminus to the M-line at its C-terminus. The C-terminus is bound to the thick filament (myosin) in the A-band, and the N-terminus contains the elastic region between the thick filament and the Z-line. The Z-line is the anchoring site for the thin, thick and titin filaments, and is the major conduit for force generated during contraction. Arrays of actin (thin) and myosin (thick) filaments slide past one another during contraction. The elastic properties of titin add to the sliding force and velocity, allowing sarcomeres to return to their normal shape after contraction [88].

The muscular dystrophy with myositis (*mdm*) mouse is the result of a recessive mutation that results in a deletion and LINE insertion in the titin gene [89]. This results in an 83-amino-acid deletion from the N2A region of TTN, losing binding to calpain-3. Mutations in TTN have been found to be the cause of human tibial muscular dystrophy [90].

The shrunken head mouse (*shru*) is an ENU-induced mutation in titin that results in cardiovascular defects [91].

Titin regulates sarcomere assembly through interaction at the M-line with its binding partner titin cap (T-cap or telethonin). Mutations in T-cap underlie human limb girdle muscular dystrophy type 2G [92], with disease arising from disruption of the sarcomere–T-tubule interaction [93]. An M-line deficient mouse shows that initial assembly of the sarcomere proceeds normally, but proper titin filament formation and stability of the embryonic sarcomere is disrupted, with sarcomere growth failure and disassembly [94].

The *kyphoscoliotic (ky)* mouse is a spontaneous muscular dystrophy mouse model eventually leading to spinal deformity, with a GC deletion creating a premature stop codon resulting in total loss of the KY protein, a cytoskeletal-associated cysteine protease/transglutaminase-like protein [95]. The loss of the KY protein results in the constitutive upregulation of a series of titin-associated stretch response signalling proteins, leading to signalling instability [95].

Calpain-3

Calpain-3 is a muscle-specific calcium-dependent cysteine protease that binds to titin at the N2A line and the M-line. Its loss leads to defects in sarcomeric remodelling in human limb girdle muscular dystrophy 2A [96]. Deletion of Calpain-3 in mice also leads to muscular dystrophy [97].

Integrins

Mutations in the alpha-7 integrin subunit cause congenital muscular dystrophy in humans [98], and deletion of either alpha-5 or alpha-7 in mice causes muscular dystrophy [99, 100]. Talin 1 and Talin 2 connect integrins to the actin cytoskeleton, and regulate the stability of the myotendinous junction (MTJ), with loss of Talin 1 or Talin 2 resulting in defects in myoblast fusion and sarcomere assembly [101].

Phospholipid synthesis, microRNAs, and other functional systems

Choline kinase beta

The rostrocaudal dystrophy mouse (*rmd*) is a mouse model of muscular dystrophy that bears a rostral to caudal gradient of severity with enormously enlarged mitochondria, with hindlimb muscles most severely affected [102]. The mutation is caused by a genomic deletion in the choline kinase beta (*Chkb*) gene which results in impairment of the production of phosphatidylcholine. Partial compensation in non-hindlimb tissues by another isoform, Chk-alpha, explains the observed rostrocaudal nature of the disorder [103, 104]. Human populations with *CHKB* mutations leading to a similar muscle/mitochondrial phenotype have been identified in Turkey, Japan and the UK [105].

MicroRNAs

In a range of human muscular dystrophies, microRNA expression is altered, indicating a possible

TABLE 2.5.1: Additional genes with mouse models of human muscular dystrophies

Human disease	Gene	Cellular function affected	Reference	OMIM
Limb-girdle muscular dystrophy type 2H (LGMD2H)/sarcotubular myopathy	Tripartite motif-containing protein-32 (Trim32)	Sarcomeric structural proteins	[188]	254110
Facioscapulohumeral muscular dystrophy 1A (FSHD1A)	D4Z4 repeats on 4q35	Pre-mRNA splicing	[189]	158900
Oculopharyngeal muscular dystrophy (OPMD)	Poly(A)-binding protein-2 (PABPN1)	Polyalanine expansion nuclear aggregates	[190]	164300
Bethlem myopathy/Ullrich congenital muscular dystrophy	Collagen, type VI, alpha 1 (Col6a1)	Extracellular matrix	[191]	120220
Autosomal dominant Emery—Dreifuss muscular dystrophy (AD-EDMD)/limb girdle muscular dystrophy type 1B (LGMD1B)	Lamin-A	Nuclear membrane	[192]	150330
Selenoprotein N myopathies/rigid spine muscular dystrophy 1	SEPN1	Satellite cell function	[193, 194]	606210
X-linked Emery—Dreifuss muscular dystrophy-1 (EDMD1)	Emerin	Nuclear membrane	[195, 196]	310300
Congenital muscular dystrophy	Laminin alpha 2 chain (Lama2)	Extracellular matrix	[197]	156225

functional role in muscle pathologies generated by genetic mutations in muscle protein genes [106]. Several muscle-specific miRNAs (miR-1, miR-206 and miR-133) have been found to be activated by MRFs such as Myf5, myogenin and MyoD [107], with miRNA-206 directly promoting differentiation [108]. Bicistronic gene clusters of these miRNAs (miR-1-1/133-a-2, miR-1-2/133a-1, and miR206/133b) contain cis-regulatory elements bound by SRF, MEF2 and MyoD [106]. When Dicer is eliminated specifically from the embryonic myogenic compartment, mice die perinatally with decreased skeletal muscle mass and abnormal myofibre morphologies [109].

Additional mouse models of muscular dystrophies

Table 2.5.1 provides a list of additional mouse models of human muscular dystrophies, along with their associated genes and human OMIM disease categories.

PART B. SKELETAL SYSTEMS
Introduction

Bone is a mineralized connective tissue that serves two main physiological roles: structural and metabolic. First, bone provides mechanical support for the organism and serves to protect the vital internal organs. Second, bone serves a metabolic function as it acts as a reservoir for ions [110]. Bone is a dynamic tissue that is constantly formed and destroyed, and thus is able to adapt to the changing needs of the organism. The mouse has been an invaluable

tool for understanding both the mechanism by which the bone is remodelled, and also for the study of the regulation of bone remodelling. Both spontaneous mutants and transgenic models have been developed that demonstrate the role of key genes in the development and regulation of bone, several of which are described below. Furthermore, classical inbred strains of mice have been very valuable for studies of the genetic regulation of bone mass.

Bone anatomy and composition

At the tissue level bone is composed of an organic phase (or osteoid) and a mineral phase, with 28–30% of bone weight consisting of organic material, 60% inorganic matter and the remainder being water. Of the organic phase or osteoid, approximately 90% of the protein is type I collagen (COL1A1 and COL1A2), which is arranged in a highly ordered collagen fibrous network [111]. Other non-collagen proteins are also found in the osteoid, including growth factors, proteoglycans and glycoproteins [112]. The osteoid is mineralized to form a rigid structure. Mineral in the form of hydroxyapatite is found in tight association with the collagen fibrils. These hydroxyapatite crystals are usually imperfect and other ions such as potassium, strontium, magnesium and sodium are found as substitutions within the crystal [111, 112].

The mineralized collagen fibrils can be arranged in either a haphazard fashion, forming so-called *woven bone* or in a more organized sheet-like structure called *lamellar bone*. Woven bone is often referred to as immature bone and is structurally weaker than lamellar bone. Woven bone can be formed quickly and is later remodelled into lamellar bone. The exception is in certain pathological conditions in which woven bone persists. In non-rodent species lamellar bone is organized into haversian systems or secondary osteons in which lamellar bone is arranged in concentric rings around a central cavity [111]. In mice a haversian system of organization is not seen.

At the subanatomical level, bone can be divided into cortical (compact) bone and trabecular (cancellous or spongy) bone. Trabecular bone is found inside the cortical shell and is composed of a spongy lattice type of network. Haematopoietic bone marrow fills the internal space of the long bones including the small spaces between individual trabeculae. The internal surface of the cortical bone is referred to as the endosteal surface and the external surface the periosteal surface. Each of these surfaces is lined with osteogenic cells. The role of cortical bone is essentially to provide structural support whereas the role of trabecular bone is more metabolic.

Bone cells

Bone is a dynamic tissue such that it is constantly remodelled. Formation of new bone is accomplished by the osteoblast and resorption of bone tissue is the domain of the osteoclast. Osteoclasts differentiate from marrow-derived haematopoietic/monocytic lineages and are considered specialized macrophage-like cells [113]. Mesenchymal stem cells (MSC) are pluripotent cells capable of differentiating into osteoblasts, as well as chondrocytes, adipocytes and myocytes [114]. A third cell type that is important for maintaining bone mass is the osteocyte. These cells are osteoblasts that have become embedded in the mineralized bone matrix. Osteocytes appear to function as 'mechanosensors', and may control the formation of new bone, as well as bone resorption.

Osteoblasts

Osteoblasts are terminally differentiated (Figure 2.5.4) mononuclear cuboidal cells which line the bone matrix at sites of active bone formation [115]. These cells stain strongly for alkaline phosphatase, a characteristic which is used both in cell culture and in histological studies to identify osteoblast-like cells. The primary functions of the osteoblasts are to make the protein matrix of the osteoid and to participate in the mineralization of this matrix [112]; thus, like many secretory cells, osteoblasts have a large Golgi apparatus and well-developed ER. The maturation of MSC into mature osteoblasts is tightly
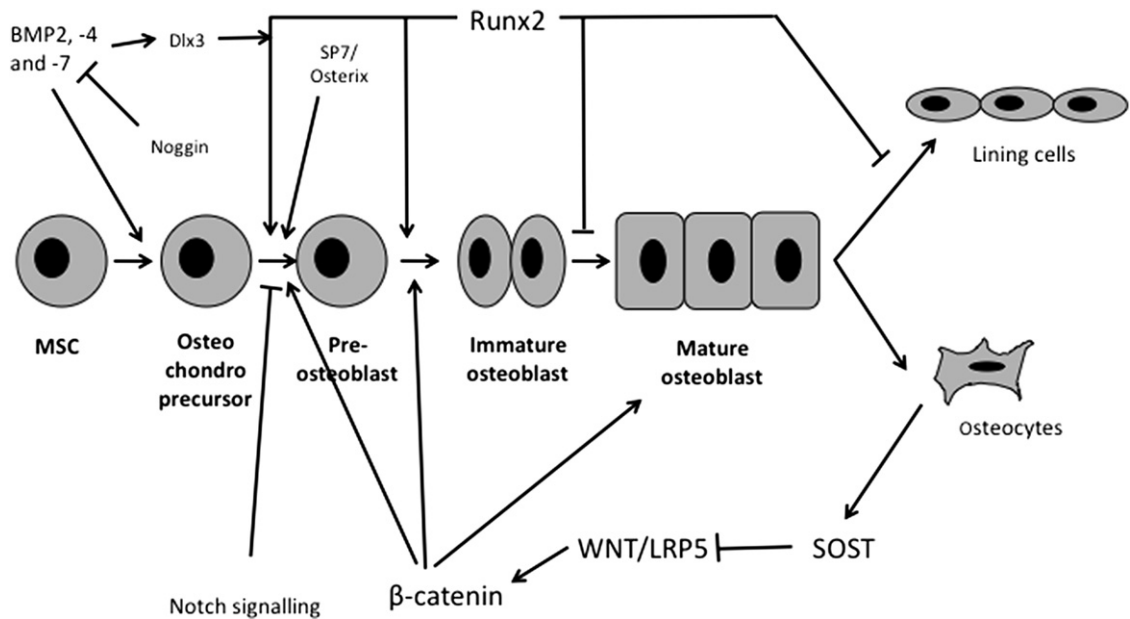
Figure 2.5.4 Osteoblast maturation. Mesenchymal stem cells (MSC) are able to terminally differentiate into the bone-forming osteoblasts. The maturation and function of the osteoblast is controlled by a variety of pro-differentiation factors such as RUNX2 and beta-catenin as a well as inhibitory factors such as SOST. Mature osteoblasts are able to further differentiate into osteocyte or lining cells.

controlled by a variety of factors, several of which are discussed in greater detail below. Morphologically, these cells go through four main stages of development: preosteoblasts, osteoblasts, osteocytes and lining cells [115, 116]. The preosteoblast cells are located at least one cell layer away from the mineralized bone matrix. These cells will stain positive for alkaline phosphatase and resemble the osteoblast cell histologically. However, unlike the mature osteoblast, these cells may still retain the ability to divide. The osteocyte is an osteoblast cell that has become embedded in the bone extracellular matrix. These cells are morphologically and functionally distinct from the osteoblast cell; a more thorough discussion of this cell type can be found below. Lining cells are postproliferative, flat, elongated cells that can be found adjacent to the bone matrix. It was traditionally thought that these cells represent quiescent or inactive osteoblasts [115], but newer research suggests that these cells play a key role in preparing the bone surface for remodelling. In general, osteoblastogenesis is controlled by a careful balance of prodifferentiation and antidifferentiation factors (Figure 2.5.4). Several of these factors are described in greater detail below. However, a pool of MSC must be maintained.

ID, TWIST and DERMO have been shown to maintain MSC in a proliferative and undifferentiated state and these factors must be repressed for maturation of osteoblasts to begin [117].

Bone morphogenic proteins

The BMPs are secreted factors and are members of the TGFβ superfamily. Transgenic mouse models have demonstrated that several of the BMPs play key roles in embryonic development and organogenesis [118]. Generally, BMPs are considered pro-osteoblastogenesis factors [118] and it has been demonstrated that BMP2, -4 and -7 are required for the commitment and differentiation of MSC down the osteoblastic linage [119]. Inappropriate BMP2 and -4 signalling is thought to be a key mechanism by which ectopic bone is formed in fibrodysplasia ossificans progressiva [120]. In contrast to the actions of the other BMPs though, mice lacking *Bmp3* or osteogenin have increased bone mass and *in vitro* studies have suggested that BMP3 can inhibit the pro-osteogenesis actions of BMP2 [118].

The BMPs bind as dimers to heterodimeric type I and type II serine/threonine kinase receptors. This, in turn, results in the phosphorylation of SMAD1, -5 and -8. These three SMADs then

form a complex with Co-SMAD and SMAD4 and translocate to the nucleus, resulting in the induction of gene transcription (reviewed in [120]). BMP2 induces expression of *Dlx3*, which in turn induces the expression of *Runx2* [119]. RUNX2 protein, though, can bind with both SMAD1 and -5 to induce BMP-mediated gene transcription events [116] and both the *Bmp2* and *Bmp4* genes contain RUNX2 binding elements, suggesting the existence of a positive feedback regulatory loop [118]. In addition, BMP2 and BMP4 can bind with Noggin, which acts as an inhibitor of these two cytokines [121].

Runx2

Runt-related transcription factor 2 (*Runx2*)/core-binding factor alpha 1 (*Cbfa1*)/polyoma-enhancer binding protein 2 alpha A (*Pebpa2a*) is considered the master control gene for osteoblastogenesis and will herein be referred to as *Runx2*. This transcription factor, which was originally identified by its ability to bind to the *Bglap* (Osteocalcin) promoter, is a member of the runt domain gene family [122, 123]. The *Runx2* gene has two separate promoters, resulting in two isoforms of the RUNX2 protein. Both isoforms have similar function in skeletal development [124]. *Runx2* is highly expressed in osteoblasts as well as prehypertrophic and hypertrophic chondrocytes [123, 124]. Mice lacking *Runx2* lack a mineralized skeleton and thus die within minutes of birth as a result of an inability to breathe [123]. These mice do form a cartilaginous skeleton, but the extracellular matrix of cartilaginous skeleton is undermineralized and chondrocyte maturation is inhibited [122-125]. RUNX2 forms a heterodimer with CBFβ1 and this complex in turn associates with a large number of coactivator proteins including TAZ, LEF1 and members of the SMAD family [116, 126]. This complex induces the expression of a variety of key bone matrix proteins including *Col1a1*, *Col1a2*, *Spp1* (Osteopontin) and *Bglap* [127-129] and has been shown to repress expression of *Ibsp* (bone sialoprotein, [130]). Interestingly, overexpression of *Runx2* in terminally differentiated osteoblasts leads to a decrease in bone mass, associated with a brittle, woven bone phenotype, and osteoblast maturation appears inhibited in these mice [131]. It is currently thought that *Runx2* is required for MSC to mature into preosteoblastic like cells, but that *Runx2* expression must cease or be decreased in order for these cell to completely mature into functional osteoblast cells. Low levels of expression of *Runx2* have been observed in mature osteoblast cells, where it may be required for continued expression of both *Col1a1* and *Bglap* [124].

Osterix

Osterix (*Osx*) or Sp7 transcription factor 7 (*Sp7*) is a C2H2-type transcription factor containing a zinc finger motif, belonging to the SP/Krüppel like factor family of transcription factors. Like *Runx2* null mice, mice lacking *Sp7* do not form a mineralized skeleton and die shortly after birth [125]. Multiple studies have demonstrated that this transcription factor is required for MSC differentiation into osteoblasts [125, 132]. The *Sp7*-null mice do express *Runx2* in osteogenic cells and there are phenotypic differences when comparing the $Runx2^{-/-}$ and $Sp7^{-/-}$ mice, which collectively suggests that *Sp7* acts downstream of *Runx2* [125]. Studies using conditional deletion mutants have shown that loss of *Sp7* is also required for osteocyte maturation and function [132].

Notch signalling

The Notch signalling pathway is a key pathway in development in a variety of different organisms. In mice four receptors have been described, *Notch1*, *-2*, *-3* and *-4*, all of which are large single-pass transmembrane proteins. In addition, 12 Notch ligands have been identified: the DSL/DOS ligands (*Dll1*, *Jag1* and *Jag2*), the DLS-only ligands (*Dll3* and *Dll4*), the DOS coligands (*Dlk1*, *Dlk2/Egfl9*) and the non-canonical ligands (*Dner*, *Mfap2/Magp*, *Mfap5/Magp2*, *Cntn1* and *Cntn6/NB-3*). Like the Notch receptors, the Notch ligands are also transmembrane proteins, and thus Notch signalling requires cell-to-cell contact. Binding of ligand to the Notch receptor initiates a cascade of proteolytic cleavage events. First the ligand is cleaved by the disintegrin and metalloprotease (ADAM) proteases and then by the gamma-secretases. This serial proteolytic cleavage releases the Notch intracellular domain (NICD), which translocates to the nucleus. Once

in the nucleus the NICD forms a complex with the CSL DNA binding protein (RBPJ/LAG-1/CBF1), LAG-3 and the MED8 mediator transcription activation complex, leading to increased expression of Notch target genes (reviewed in [133]).

A number of studies have demonstrated that Notch signalling plays a key role in osteoblastogenesis. Specifically, mice lacking *Psen1* and *Psen2*, both gamma-secretases, lack signalling from all four Notch receptors and have an increase in bone mass and a decrease in MSC number. Similarly, mice lacking *Notch1* and *Notch2* also have increased bone mass. In contrast, mice in which expression of the NICD was driven by the *Col1a1_3.6Kb* promoter, a preosteoblast-specific promoter, had decreased bone mass and a decrease in osteoblast number [134]. Studies in osteoblast-like cell lines suggest that Notch signalling results in the upregulation of *Hey1*, which in turn suppresses *Runx2* transcriptional activity [135]. Together, this data suggests that Notch signalling prevents the early stages of osteoblastogenesis and may be required to maintain MSC pools [134, 135]. However, an increase in osteoblast number was observed in mice in which expression of NICD was driven by the *Col1a1_2.3kb* promoter, an osteoblast-specific promoter which drives expression in late osteoblastogenesis. This suggests that Notch signalling plays different roles at different stages of osteoblast maturation [134].

Fibroblast growth factor signalling

The FGFs play an important role in development and metabolism. In mice the *Fgf* gene family consists of 22 members, and four FGF receptors have been described [136, 137]. Many of the FGFs have a role in skeletal development and in the maintenance of bone mass (reviewed in [138]) and FGF signalling generally induces proliferation of immature osteoblasts [120]. In particular, *Fgf2* appears to be expressed at all stages of osteoblastogenesis, and mice lacking *Fgf2* have reduced bone mass. In the osteoblast *Fgf2* appears to both regulate and be regulated by *Bmp2* [139]. Studies of activating mutations in *Fgf2r* have shown that this receptor plays an important role in osteoblast differentiation from MSCs via signalling through both the ERk1/2 and protein kinase C alpha signalling pathways [140]. Mutations in *Fgf2r* cause a variety of congenital skeletal disorders in humans [137].

Wnt signalling

The Wnts are secreted glycoproteins that were first discovered in Drosophila and are responsible for the 'wingless' mutation [141]. Wnts can be divided into two classes depending on the signalling pathway used: canonical signalling via beta-catenin or the beta-catenin-independent non-classical pathway [142]. In canonical WNT signalling, signalling is mediated via the transduction of beta-catenin to the nucleus where it forms a complex with the TCF/LEF family of transcription factors and is able to induce gene transcription. Control of WNT signalling can be accomplished by regulating the abundance of beta-catenin and its stability [143]. In the absence of WNT ligand, beta-catenin is phosphorylated [144] and is degraded via the ubiquitin pathway [143]. Phosphorylation of beta-catenin accomplished by casein kinase 1 (CK1) and GSK3, which bind to beta-catenin as part of a complex involving Axin and adenomatous polyosis coli (APC). When WNT ligand is present, it binds to a receptor complex consisting of one of the frizzled receptors and either LDL receptor related protein 5 (LRP5) or LRP6. The binding of ligand to the frizzled receptors results in the phosphorylation of Disheveled (DSH). This results in the inhibition of GSK3 and the dissolution of the CK1–GSK3–Axin–APC complex, thus preventing the phosphorylation of beta-catenin [143]. If beta-catenin is not phosphorylated it will accumulate in the cytoplasm and then translocate to the nucleus [145]. The soluble frizzled-related proteins (SFRP) act as pseudoreceptors for WNT and can inhibit binding of WNT to the frizzled receptors [142]. Furthermore, Dickkopf (DKK) can bind to LPR5 or LRP6 and prevent the formation of the frizzled receptor–LPR complex, thus inhibiting canonical WNT signalling [146].

The WNT ligands can also signal through the so-called non-canonical pathways, which are beta-catenin independent. Several non-canonical pathways have been identified, but these are less well characterized. In non-canonical signalling WNT still binds to the frizzled receptor, but

LRP5 and LRP6 do not form a complex with the frizzled receptors. Signalling is mediated via a number of mechanisms including the Ca^{2+}-CAMKII−PKC pathway and RHO and RAC activation of JNK (reviewed in [147]).

Both canonical and non-canonical WNT signalling play an important role in inducing commitment of MSC down the osteoblast linage [146, 147]. Canonical WNT signalling through beta-catenin plays an important role in regulating osteoblast function and can prevent apoptosis of mature osteoblasts. Mice lacking the canonical WNT signalling coreceptor *Lpr5* have low bone mass, and loss-of-function mutations in this gene in humans cause pseudoglioma syndrome. In contrast, mutations in the extracellular domain of *LRP5* are associated with high bone mass in humans. Both SOST and DKK bind to the extracellular domain of LRP5 and act as suppressors of canonical WNT signalling. Loss of binding of SOST or DKK to LRP5 in osteoblasts results in an increase in bone mass due to the inability to suppress WNT signalling [146].

Parathyroid hormone and parathyroid hormone related peptide

Parathyroid hormone (PTH) is secreted by the parathyroid gland and plays a key role in regulating serum Ca^{2+} and phosphate levels. Parathyroid hormone related peptide (PTHrP) shares significant homology to PTH in amino acids 1–13 at the N-terminus of the peptide and both PTH and PTHrP are able to bind to the G-protein-coupled PTH 1 receptor (PTH1R). While PTH acts in an endocrine fashion, the actions of PTHrP are autocrine/paracrine and PTHrP is expressed in a wide variety of tissue types including bone and kidney. PTH acts in both an anabolic and a catabolic fashion. Chronic administration of PTH results in bone loss, whereas intermittent administration of either PTH or PTHrP results in increases in bone mass. Intermittent administration of either of these compounds results in an increase in both osteoblast proliferation and survival. This is thought to in part be mediated by the actions of PTH on WNT signalling and by regulating *Sost* expression [148, 149]. Continual administration of PTH results in bone loss through increases in osteoclast number and subsequent increases in

bone resorption [148]. Specifically, PTH or PTHrP signalling increases level of RANKL and suppresses secretion of the anti-osteoclastogenesis factor OPG [150].

Insulin-like growth factor system

IGF-1 is a hormone in that it can be transported via the circulation and act on a distant location, but it is expressed in virtually every tissue and has demonstrated autocrine and paracrine actions as well. The liver is the primary source of circulating IGF-1. In circulation, IGF-1 is bound as part of a complex involving IGF-binding protein 3 (IGFBP3) as well as the acid-labile subunit (ALS). The purpose of this complex appears to be to increase the serum half-life of IGF-1. In addition to IGFBP3, five other IGF-binding proteins have been described which modulate the bioavailability of IGF-1 [151]. IGF-1 is the most abundant growth factor found in bone [152] and has been shown to mediate the effects of growth hormone on long bone growth [151]. In mice in which IGF-1 expression was ablated in osteoblasts, severe decreases in mineralization were observed, as well as significant decreases in body length [153]. Mice in which expression of the *Igf1* in the liver has been abolished have near-normal long bone growth, but do have decreased bone density, despite normal expression of IGF-1 in bone [154].

The IGFBPs modulate the actions of IGF-1 but also have functions that are independent of IGF-1 [151]. All but *Igfbp1* are expressed in bone, with the most abundant IGF-binding proteins in bone being IGFBP4 and -5 [155]. IGFBP4 is well described as inhibiting the actions of IGF-1 in bone [156] whereas the role of IGFBP5 in bone remains unresolved [155]. IGFBP2 affects both cortical and trabecular bone in a gender-specific fashion. Female mice lacking *Igfbp2* have increased cortical bone, whereas male *Igfbp2*-null mice have decreased cortical and trabecular bone volume. In addition, IGFBP2 regulates *Pten* expression in both osteoblasts and osteoclasts in an IGF-1 independent fashion [157]. Transgenic mice that overexpress *Igfbp3* have increased osteoclast number and increased bone resorption, which results in a decrease in bone mass [158]. While *Igfbp6* is expressed in osteoblasts, its function remains unknown.

Classically, it was thought that *Igf2* is only expressed during embryonic development in mice and that expression ceases in all tissues except the choroid plexus and leptomeninges before weaning. Newer studies have suggested that *Igf2* is expressed at low levels in selected stem cells in bone in adult mice and that IGF-2 may play a role in maintaining pools of these adult bone stem cells. Furthermore, it has been postulated that in adult mice, IGF-2 is required for both osteoblast and osteoclast maturation and that IGF-2 regulates both bone formation and resorption in the adult mouse [159].

Osteoclasts

Osteoclast cells are polarized, multinucleated cells that are formed by the fusion of multiple haematopoietic/monocytic precursor cells [111, 112]. These cells are generally considered to be members of the monocyte/macrophage family of cells [113]. Compared to the osteoblast and osteocyte, the osteoclast is a relatively rare cell in bone and is substantially shorter-lived. Their primary function is bone resorption, although new data suggests additional functions for these cells [160]. Osteoclasts have an abundance of acid-containing vesicles and mitochondria, but do not contain extensive ER.

Development of the osteoclasts can be divided into three stages. In the first stage the monocytic/macrophage precursor cells become activated and differentiate into preosteoclast cells. These express tartrate resistance acid phosphatase (TRAP) and calcitonin receptor. Staining for TRAP is commonly used in both cell culture and in histology to mark osteoclast-like cells. In the second stage preosteoclast cells fuse into large, multinucleated, unpolarized and non-functional osteoclast-like cells. In the third and final stage the osteoclasts attach to the bone surface, polarize and are able to resorb bone [161]. Upon recognition of the bone matrix, the osteoclast forms an actin-rich sealing ring around the perimeter of the cell where it contacts the bone, creating sealed-off space under the osteoclast. The formation of the sealing zone results in a rearrangement of the osteoclast cytoskeleton and the formation of the F-actin ring. Within the actin ring, the cell membrane increases in surface area and forms a highly folded structure

known as the ruffled border. The osteoclast pumps protons into the space between the ruffled border and the bone, making a highly acidified microenvironment that dissolves the mineral component of the bone. Lysosomal enzymes such as Cathepsin K and TRAP, as well as metalloproteinases, are then secreted into space and degrade the protein matrix [111, 113, 162]. Protons and acid proteases are also secreted into the space between the ruffled border and the bone surface, and the mineralized compartment of bone located beneath this space is dissolved and the organic component is degraded. These degraded fragments are then endocytosed through the ruffled border, transcytosed through the osteoclast and secreted from the cell.

Macrophage colony stimulating factor

In the marrow space early monocytic cells differentiate into late monocytic cells upon activation by PU.1, an ETS protein domain containing transcription factor. Upon activation by macrophage colony stimulating factor 1 (M-CSF, *Csf1*) and members of the MITF family of proteins, these cells then undergo commitment to the osteoclast lineage. M-CSF is secreted by the osteoblast and stimulates the proliferation and migration of the late monocytic cell/early osteoclast precursor cell [113]. Mice homozygous for the *osteopetrotic* (*op*) mutation, a frameshift mutation in the *Csf1* gene, lack functional M-CSF and exhibit severe osteopetrosis due to a severe reduction in osteoclast number [163, 164]. M-CSF binds to the tyrosine kinase receptor colony stimulating factor 1 receptor (*Cfs1r*), which is also known as *c-fms*, and which in turn signals through the MAP kinase and ERK pathways [160]. Like mice carrying the *op/op* mutation, mice lacking *Csf1r* are also severely osteopetrotic and have a considerable reduction in osteoclast number [165].

RANK, RANKL and OPG

Once activated by M-CSF, osteoclast precursor cells then express tumour necrosis factor receptor superfamily, member 11 (*Tnfrsf11a*) on the cell surface. *Tnfrsf11a,* which is more commonly known as receptor activator of nuclear factor kappa-B (RANK), is a type I membrane protein that contains four

extracellular cytosine-rich domains [166]. RANK is the receptor for the pro-osteoclastogeneis cytokine RANKL (also known as *TNFRSF11* or TRANCE), which is expressed on the surface of the osteoblast cells and binding of RANKL to the RANK is required for the commitment and differentiation of the preosteoclast [166]. The binding of RANKL to its receptor activates NF-kappaB, c-Fos, phospholipase C-gamma and NFATc1 signalling [160] via TRAF6 [167]. The mature osteoclast secretes a dummy receptor for RANK called Osteoprotegerin (OPG or TNFRSF11b). OPG is structurally similar to RANKL, but lacks a transmembrane domain. OPG is able to bind to RANKL, prevent the binding of RANK and thus inhibit the maturation of additional osteoclasts [166]. Mice lacking either *Tnfrsf11a* or *Tnfrsf11* exhibit a lack of osteoclasts and have severe osteopetrosis, suggesting that RANKL and RANK are fundamentally required for osteoclast maturation [168, 169]. In contrast, mice lacking the *Tnfrsf11b* gene are severely osteoporotic due to increased osteoclast activity [166]. In the kidney PTH increases production of 1,25-dihydroxyvitamin D, the active form of vitamin D; 1,25-Dihydroxyvitamin D can stimulate the production of RANKL and suppresses expression of OPG [150]. In addition to RANKL, a number of other inflammatory cytokines such as IL6 and TNFα are secreted by the osteoblast and can induce osteoclastogenesis [150].

NFATc1 and osteoclast precursor fusion

The last steps of osteoclastogenesis are fusion, binding and polarization [113, 162]. While the mechanism of osteoclast fusion is not completely characterized, several factors have been identified which are required for and can induce fusion. NFATc1 is a transcription factor, expression of which is upregulated by RANKL signalling. Upregulation of *Nfatc1* expression, in the absence of RANKL, is sufficient to induce osteoclast maturation, demonstrating the importance of this factor in osteoclastogenesis. Specifically, NFATc1 induces expression of ATPase, H^+ transporting, lysosomal V0 subunit D2 (*Atp6v0d2*) and transmembrane 7 superfamily member 4 (*Tm7sf4*), which is also known as DC-STAMP and are key factors for mediating cell–cell fusion [161].

Integrins

The integrins are plasma membrane spanning receptors and are involved in cell attachment as well as cell signalling. All integrin are heterodimers consisting of an α and a β subunit. In osteoclasts the $\alpha_v\beta_3$ integrins recognize RGD (Arg-Gly-Asp) motifs in osteopontin, a bone matrix protein, allowing for the attachment of the osteoclast to the bone surface. Mice lacking the β_3 integrin subunit have an osteopetrotic phenotype due to a lack of functioning osteoclasts, demonstrating the importance of attachment to osteoclast maturation. Attachment to the bone stimulates c-SRC, which induces the polarization of the cell and formation of the ruffled border [113].

Osteocytes

Osteocytes are former osteoblasts that are embedded in the bone matrix and reside in so-called osteocyte *lacunae*. Previously it was thought that the transition from osteoblast to osteocyte was a passive process that occurred as the cell became trapped, but new research has suggested that the formation of osteocytes is an active process [170]. These cells are the longest-lived cells in bone and in humans are thought to live 10–20 years. In rodent models, it has been predicted that these cells can live for nearly the whole of the animal's lifespan. Osteocytes are the most numerous of the bone cells, with between 10 000 and 20 000 osteocytes per mm^3 found in humans. Osteocytes are significantly smaller than osteoblasts and comparatively flattened in shape. Unlike osteoblasts, which are active secretory cells, osteocytes contain few intracellular organelles and have low levels of secretory ability. Osteocytes develop cytoplasmic projections that connect with both the lining cell on the surface of the bone as well as other osteocytes, forming a complex network within the calcified matrix of the bone (Figure 2.5.5). These tunnels through the bone matrix are referred to as osteocyte *canaliculi* (reviewed in [171]). Osteocytes appear to have two major functions: they function as the mechanosensor in bone and they play a key role in the regulation of phosphate metabolism [171, 172].
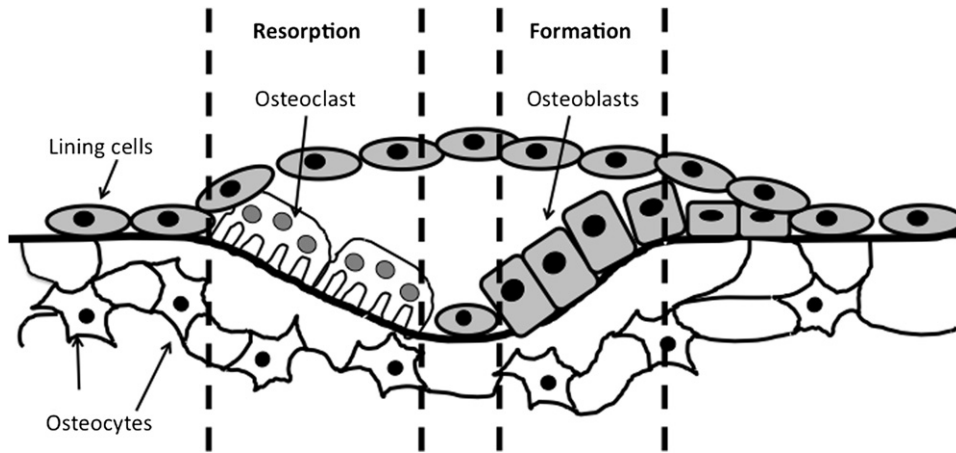
Figure 2.5.5 Bone resorption. The remodelling of bone is accomplished by the basic multicellular units (BMUs), which consist of the osteoclast at the leading edge, the reversal cells and the osteoblast cells at the lagging edge. While a number of factors can trigger remodelling, the osteocytes are thought to sense microdamage in the bone and signal to the lining cells and bone surface osteoblasts via direct contact through cytoplasmic projections which extend through the canaliculi. The lining cells prepare the bone surface for attachment by the osteoclasts, which are the cells that remove or resorb the old bone. Bone formation is accomplished by the osteoblasts, which are much longer-lived than osteoclasts. Resorption can occur in as little as 2 weeks in humans, but bone formation is a much slower process. The BMU is sequestered by a layer of cells, creating a specialized remodelling niche.

The osteocyte canaliculi and lacunae are filled with extracellular fluid. *In vitro* studies have strongly suggested that osteocytes can sense and respond to the shear stresses induced by changes in fluid flow. From this data it is thought that when a compressive mechanical force is applied to bone, movement of the extracellular fluid surrounding the osteocyte occurs and that this is the mechanism by which the osteocyte senses loading [173]. In mouse models where the osteocytes have been ablated, there is an increase in osteoclast activity and a decrease in mineralization by the osteoblasts [171]. The osteocytes in bone are in direct contact with the surface osteoblasts via gap junctions. Fluid flow studies have shown that, when stimulated, the osteocyte can inhibit osteoclastogenesis via suppression of the RANKL production and an increase in OPG production [173]. In addition, mice lacking osteocytes have increased microdamage to the bone and with time develop severe trabecular bone loss. However, in disuse-tail suspension models, mice lacking osteocytes do not lose trabecular bone. Together this data suggests that the osteocyte directly regulates osteoclastogenesis as well as bone mineralization, via its direct contact with the surface osteoblasts [171, 173].

Sclerostin

Sclerostin (SOST), the protein product of the *Sost* gene, is secreted glycoprotein which is preferentially produced by the osteocytes [174]. Numerous lines of evidence suggest that SOST inhibits bone formation. Mice which overexpress human *SOST* have low bone mass whereas mice lacking *Sost* have high bone mass [149]. SOST can weakly bind to BMP, resulting in an inhibition of BMP-induced bone formation by the osteoblast [149, 174]. In addition, SOST can bind to LRP5 and LRP6 and act as a repressor of canonical WNT signalling. Although how *Sost* expression is regulated is not completely understood, new data suggest that PTH signalling through PTH 1 receptor 1 (PTH1R) suppresses *Sost* expression. This is likely one of the key mechanisms by which PTH is able to have an anabolic action on bone, and mediating PTH signalling may be the major function of SOST protein [149].

FGF-23 and DMP-1

The osteocytes produce FGF-23, which is a secreted FGF that an acts in a hormone-like fashion. Expression analyses suggest that bone is the only source of FGF-23 (http://biogps.gnf.org). This FGF plays a major role in phosphate metabolism. Specifically,

FGF-23 suppresses expression of 2a and 2c sodium–phosphate cotransporters in the brush border of the proximal tubule of the kidney and in this manner is able to regulate phosphate reuptake by the kidney. In addition, FGF-23 can indirectly regulate phosphate absorption by the gut via its ability to regulate vitamin D (1,25(OH)$_2$D) metabolism in the kidney. Specifically, FGF-23 can suppress the conversion of 25(OH)D to 1,25(OH)$_2$D in the kidney by suppressing expression of [25(OH)D]-1α-hydroxylase. Mice with increased FGF-23 levels exhibit hypophosphataemia and have a rickets-like bone phenotype (reviewed in [172]).

Dentin matrix protein 1 (DMP-1) is an extracellular matrix protein that is preferentially expressed in odontoblasts and in osteocytes [175]. Like mice overexpressing *Fgf23*, mice lacking *Dmp1* display hypophosphataemia and have a rickets-like bone phenotype characterized by a reduction in bone mass and hyperosteoidosis. Furthermore, mice lacking *Dmp1* have increased levels of FGF-23, suggesting that DMP-1 regulates FGF-23 production [176]. The DMP-1 protein is post-translationally processed into a 37 kDa N-terminal fragment and a 57 kDa C-terminal fragment. Recent experiments have shown that the 57 kDa fragment is able to regulate expression of *Fgf23* in the osteocyte. Furthermore, the 57 kDa fragment of DMP-1 may control osteocyte maturation via regulation of expression of *Sp7* and *Sost* [177].

Embryonic origin of bone

During embryogenesis, bone development begins with a condensation of mesenchymal progenitor cells at the site of future bone development. The craniofacial bones are derived from neural crest cells from the branchial arches; the axial skeleton is derived from the sclerotome compartment of the somites; and the appendicular skeleton is derived from the lateral plate mesoderm [178]. The flat bones, such as the bones of the skull and mandible, develop via intramembranous ossification and axial and appendicular skeleton formed via endochondral ossification [111].

Intramembranous ossification

In intramembranous ossification the mesenchymal progenitor cells proliferate and form compact condensations. A subset of these mesenchymal progenitor cells differentiate into pre-osteoblasts and then osteoblasts. These osteoblasts synthesize woven bone wherein the collagen fibrils are haphazardly arranged and mineralization is irregular. Haematopoietic bone marrow will be formed between layers of woven bone and the woven bone will ultimately be remodelled into mature lamellar bone [111].

Endochondral ossification

Endonchondral ossification differs greatly from intramembranous ossification in that a cartilage template is first formed and this is later replaced with mature lamellar bone. Endochondral ossification can be broken into a series of stages. First, the MSC condense and differentiate into prechondroblasts and then chrondrocytes. Next, the chrondrocytes differentiate into two subpopulations of cells, the low-proliferating distal chondrocytes, which are located at the two ends of the bone, and the highly proliferating chondrocytes in the middle, which are arranged in a columnar fashion. These columnar chondrocytes exit the cell cycle and differentiate into prehypertrophic and then hypertrophic chondrocytes. The hypertrophic chondrocytes form an anlagen extracellular cartilage matrix, which becomes calcified, and then these cells undergo apoptosis. The outside perimeter of the cartilage matrix (the perichondrium) is surrounded by fibroblast-like cells and MSC, which differentiate into osteoblast-like cells. The mineralized matrix surrounding the hypertrophic chondrocytes is invaded by blood cells, which allows infiltration of myloid-derived osteoclasts. The osteoclasts resorb the mineralized cartilage, and the osteoblasts replace this matrix with mineralized bone. Lastly, the mineralized bone is remodelled to form the marrow cavity. Postnatally, secondary ossification centres are formed within the distal chondrocytes. The growth plate, which consists of a band of chondrocytes arranged in columnar fashion, is organized between the secondary and the primary ossification centres. This allows for continued long bone growth [179]. In humans

the epiphyseal growth plates fuse after puberty, preventing continued growth. In mice the epiphyseal growth plates do not fuse at sexual maturity and long bone growth can continue well past sexual maturity.

Bone remodelling

Bone is not a static tissue; it is constantly changing to allow it to adapt to the current needs of the organism. Bone modelling is critical for bone growth and to allow for adaption to changes in load on the bone. In bone modelling new bone is formed and unneeded bone is removed; formation and resorption occur, for the most part, at separate anatomic locations. Bone remodelling serves a slightly different purpose: it removes old or damaged bone and plays a critical role in maintaining mineral homeostasis. When bone remodelling is balanced, i.e. when bone formation equals resorption, there is no net change in bone mass. During growth, formation exceeds bone loss, resulting in a net gain of bone mass. If resorption exceeds formation, as is seen in certain pathologies in humans, there is a net loss of bone mass [180, 181].

Bone remodelling is accomplished by the 'basic multicellular units' (BMUs). The BMUs consist of three main cell types: osteoclasts, reversal cells and osteoblasts (Figure 2.5.5). Traditionally, the BMU is considered to have a leading edge, where the osteoclasts are located, and a tail, where the osteoblasts are located. As described above, the osteoclasts are the cells responsible for bone resorption and the osteoblasts are responsible for forming new bone in the space created after resorption. It is hypothesized that the reversal cells may prepare the newly resorbed bone surface such that the osteoblast can form new bone on it. It is not completely clear at present where the reversal cells are derived from, but the available data suggest that these cells are derived from the osteoblastic lineage [180, 181].

A number of activation signals can lead to bone remodelling. For example, hormones such as PTH and oestrogen have been shown to increase bone remodelling [180]. Mechanical loading or microdamage can also be an initiating factor for bone remodelling [182]. It is not completely clear how the existence of microdamage in the bone leads to remodelling, but it is well understood that osteocytes at the site of microdamage undergo apoptosis and this is linked with bone remodelling. This is supported, in part, from data obtained from mice in which osteocytes have been postnatally ablated. In these mice increased bone resorption and loss of trabecular bone mass is observed in the absence of functioning osteocytes; however, these mice also have increased microdamage compared to age-matched controls. Ikeda observed that RANKL expression was significantly increased after osteocyte ablation, suggesting that, during apoptosis, the osteocyte can signal to the osteoblast to increase osteoclastogenesis [171]. Furthermore, the osteocyte expresses TGFβ, which functions to inhibit the maturation of the osteoclast. In the absence of osteocytes local TGFβ production ceases, which could create a localized osteoclastogenesis-permissive environment [180]. In order for the osteoclast to fuse and attach to the bone surface, the lining cells must first 'prepare' the bone surface [180, 181]. Specifically, these cells digest off the osteoid surface at the site of bone remodelling and expose RGD (Arg-Gly-Asp) adhesion sites on the surface of the mineralized matrix. These RGD sites allow for the osteoclast to adhere to the bone surface via $\alpha_v\alpha_3$ integrin [180] and the osteoclasts are able to resorb the bone. Once bone resorption is complete the reversal cells remove the exposed collagen remnants from the site of bone resorption. Bone formation can then proceed by the osteoblasts. It is not clear what signals the completion of resorption to allow formation to begin, and this is an active topic of ongoing research. During the formation stage the osteoblasts fill in the cavity created by the osteoclasts, and the bone is repaired. Once sufficient new bone has been generated, remodelling is terminated by an as yet unknown mechanism [180].

Bone and energy metabolism

A number of studies have now demonstrated that bone is an endocrine organ. Osteocalcin or

bone-specific Gla protein (BGLAP) is thought to be the most abundant non-collagen protein found in the bone matrix [183]. In addition, more recent evidence suggests that osteocalcin is a bone-derived hormone involved in energy metabolism [184]. In mice two genes, *Bglap* and *Bglap2*, are found immediately adjacent to one another on chromosome 3. Both of these genes code for the osteocalcin protein, and their coding sequence is nearly identical. In bone *Bglap* and *Bglap2* appear to only be expressed by the osteoblast and mice lacking both of these genes have increased bone mass and increased bone formation [183]. The mechanism by which this increase in bone mass occurs is not completely understood. Interestingly, mice lacking *Bglap* and *Bglap2* are obese, have decreased insulin sensitivity and are glucose intolerant [184]. Both RUNX2 and FOXO1 protein appear to negatively regulate expression of osteocalcin in the osteoblast [185, 186] and mice lacking *FoxO1* in just the osteoblasts have increased insulin secretion and increased insulin sensitivity due in part to increased osteocalcin expression [186]. Mice lacking the protein tyrosine phosphatase receptor type V (*Ptprv*, also known as *Esp*) gene have decreased fat mass in conjunction with increased insulin secretion and increased insulin sensitivity. This gene appears only to be expressed in the osteoblasts and Sertoli cells. The OST-PTP protein (the product of the *Ptprv* gene) appears to control the bioavailability of osteocalcin [184]. Furthermore, insulin receptor signalling specifically the osteoblasts also appears to affect osteocalcin bioavailability [187].

Summary

In this chapter we have presented a genetic/functional description of the processes involved in mammalian muscle and bone development, from embryo to adult, and of the disease processes generated through inherited genetic defects. Future questions on vertebrate musculoskeletal development will require an understanding of how bone and muscle development and disease are regulated and influenced by such factors as cell–cell adhesion, microRNAs, and epigenetics and environmental factors. The laboratory mouse will continue to be a vital player in our growing understanding of these processes.

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CHAPTER 2.6

The Cardiovascular System

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Introduction

During the past two decades tremendous strides have been made in assessing the murine cardiovascular system. This has occurred because of the logarithmic increase in opportunities to use transgenic mice and embryonic stem cell and homologous recombination approaches to target gene expression and to ablate/overexpress specific gene products. These gene mutations and targeted genes produce animals that have altered cardiovascular phenotypes. Hence, the need to accurately assess cardiovascular function is vitally important. This chapter considers the known normal state of the cardiovascular system of mice, as well as the changes measured when there is heart pathophysiology such as in myocardial hypertrophy and myocardial ischaemia and reperfusion. No attempt will be made to describe the altered haemodynamic states of the hundreds of relevant transgenic/knockout mice used to study the heart and cardiovascular system; rather, select examples are cited for emphasis. The surgical technique used to induce these altered states—restriction of aortic blood flow and occlusion of a coronary artery, respectively—are outlined. A variety of methods currently used to measure the mouse cardiovascular anatomy and function are summarized. The age-related differences in response to surgically induced pathophysiological states in the mouse are also discussed.

In terms of body weight, a dog is approximately 1000 times the size of a mouse: 0.025 kg for the mouse and 25 kg for the dog. This 1000-fold difference extends to heart weight between the dog and the mouse. There is a corresponding 100-fold difference in area, and a 10-fold difference in linear dimensions. This is illustrated in
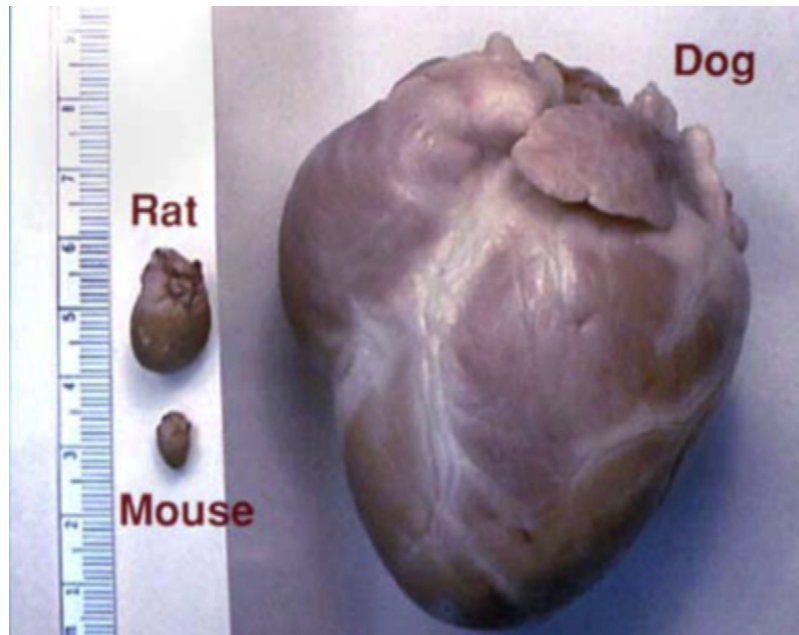
Figure 2.6.1 Dog, rat and mouse hearts. Comparison of heart sizes from a 25 000 g dog, a 250 g rat and a 25 g mouse.

Figure 2.6.1. In order to measure cardiovascular function in such a small system as the mouse heart, many development projects have been undertaken to miniaturize sensors and transducers needed to measure these functions. Besides the size, mice have a very fast resting heart rate of 500-600 bpm, requiring instrumentation with higher signal fidelity and better temporal and spatial resolution than that used for larger species.

Anatomical considerations

The most complete description of the anatomy of the cardiovascular system of the mouse may be found in an article by Cook [1] detailing all aspects of the system anatomy. Gross anatomy of the venous and arterial supply to all regions of the body, including limbs, is diagrammatically illustrated. A brief description of the heart and major blood vessels is also given in a publication from The Jackson Laboratory [2]. The major branches of the mouse aorta are similar to that seen in humans. The right innominate artery leaves the aortic arch and divides into the right

common carotid artery and the right subclavian artery. Approximately 1 mm distal on the aortic arch is the left common carotid artery, followed laterally adjacent by the left subclavian artery. This anatomical arrangement is different from that of an animal species such as the dog where the right innominate artery leads to both right and left common carotid arteries. The striking differences in the mouse heart and vessels appear to be in the arrangement of the venous system of the heart. Cardiac veins are the most prominent structures on the epicardial surface of the left ventricle, far exceeding the visibility of the coronary arteries. Small cardiac veins abut at right angles to the largest coronary vein, the left cardiac vein, which proceeds from the ventral surface of the left ventricle and the apex of the heart toward the dorsum of the heart to drain into the left anterior vena cava at its junction with the right anterior and posterior vena cava connection with the right atrium. This vessel distribution appears similar to that seen in the rat heart by Halpern [3]. In addition, Halpern describes two major veins which drain the conal region of the right ventricle and the ventrocephalic region of the left ventricle. These were called extracoronary cardiac veins because they originated on the heart and terminated in vessels not otherwise associated with the coronary

circulation, i.e. in this case the anterior vena cava. In summary, one is struck by the great abundance as well as size of veins on the surface of the heart. At first glance the venous architecture may be confused with the underlying and more subtle coronary arterial system which is embedded in the musculature of the heart. Observation of the coronary artery system of the mouse heart is much more difficult; a source of intense light and magnification is required to visualize these deeper and more hidden vessels. This discrimination is critical, as occlusion of an epicardial coronary vein, in an effort to produce myocardial ischaemia or frank infarction, is almost uniformly associated with mortality in our hands. The coronary artery anatomy of the mouse is shown in Figure 2.6.2, which illustrates that the coronary ostia originate within 2 mm of the aortic valve. The right coronary artery usually divides into two major branches, one supplying the right ventricle and the second the septal region. The left coronary artery divides generally into a major septal branch and a left anterior descending coronary artery (LAD) supplying the free wall of the left ventricle, part of the septum and the apical region of the left ventricle. The pattern of coronary artery distribution is shown in Figure 2.6.3, which schematically illustrates the various patterns as seen in six separate mice. In all of these animals the left circumflex coronary artery, which is a major branch of the left coronary artery in other animal species and humans, is not clearly a major vessel in these mice, appearing rudimentary. Therefore, the variability in epicardial coronary architecture is a very important consideration even in the same genetic stock.

Histopathological characteristics of the normal mouse heart

The development of transgenic and knockout animals has led to the widespread use of mice in studies investigating the pathophysiology of cardiovascular disease. Mouse models have provided insight into cardiac development [4] and have contributed to our understanding of the pathogenesis of myocardial infarction (MI) [5–7], myocardial hypertrophy [8, 9], myocarditis [10] and atrial fibrillation [11]. Although much smaller in size, the adult mouse heart shares many common pathological features with the hearts of higher mammals.

The murine atria have a very small mass, creating significant problems for investigations
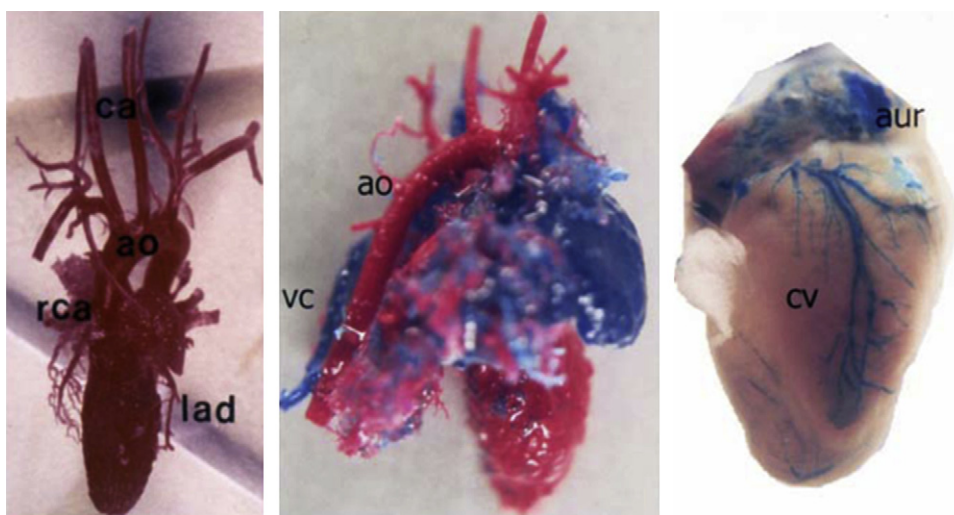
Figure 2.6.2 Arterial and venous systems in the mouse. Left: Photograph of latex cast of mouse arterial system including coronary arteries. ca, carotid artery; lad, left anterior descending coronary artery; ao, aortic arch; rca, right coronary artery. Centre: Latex cast of major arteries and veins in and around heart and lungs; ao, aortic arch; vc, posterior vena cava. Right: Latex cast of major cardiac veins (cv) on the lateral wall of left ventricle; aur, auricle. *Left panel reprinted with permission from* Am. J. Physiol. Heart Circ. Physiol. **269**, *H2147–H2154.*
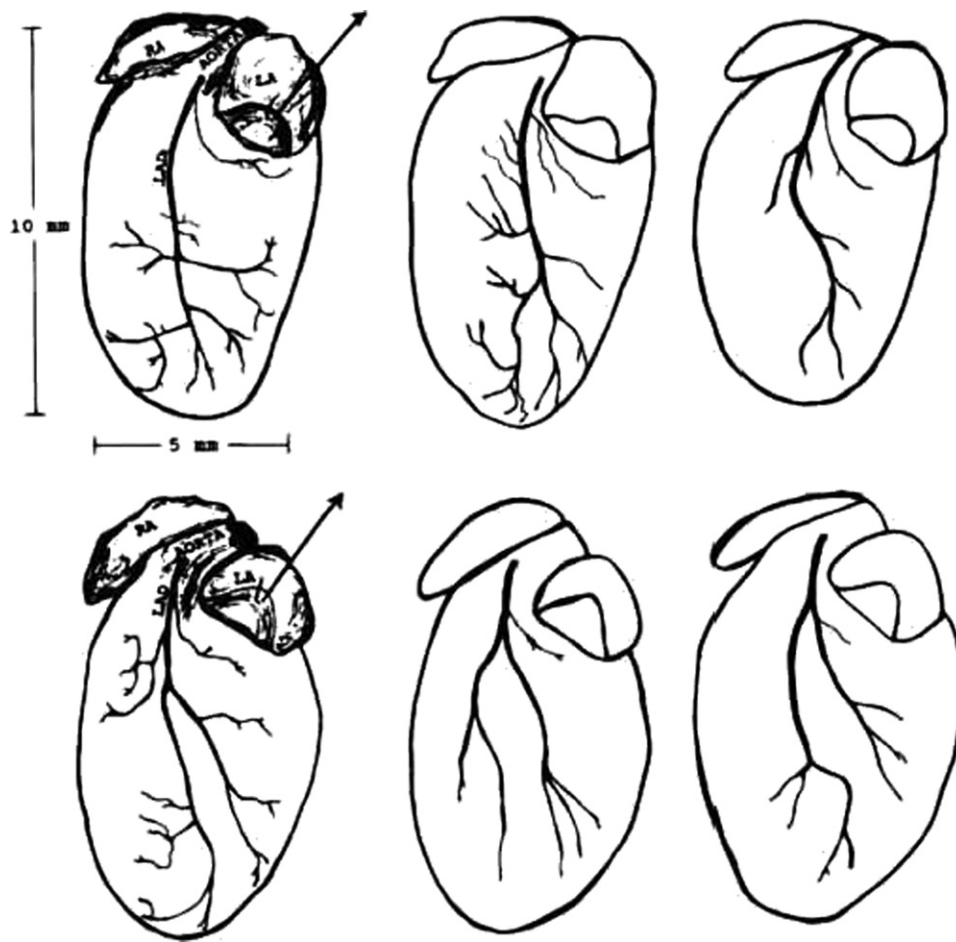
Figure 2.6.3 Schematic of the major coronary arteries of the mouse heart. Coronary artery anatomy of six individual mouse hearts showing patterns of bifurcation. Top row of three hearts shows major singular LAD pattern; bottom row of three hearts shows major bifurcation pattern. *Reprinted with permission from* Am. J. Physiol. Heart Circ. Physiol. **269**, H2147–H2154.

studying the generation of atrial fibrillation [12]. The leaflet tissue of the two murine atrioventricular valves is a continuous veil showing no commissures or clefts. The right atrioventricular valve of the mouse is not morphologically tricuspid. The mitral valve is served by two papillary muscles, which do not become independent from the ventricular wall, resembling trabeculae carnae rather than true papillary muscles [13].

Cardiomyocytes in the murine ventricles can be divided into three layers: myofibres in the middle layer run mainly circumferentially (Figure 2.6.4A), whereas those in the inner and outer layers run parallel or oblique to the apical-basal axis [14]. Among non-cardiomyocytes, fibroblasts appear to be the predominant cell type in the mouse heart and play an

important role in cardiac homeostasis by maintaining the integrity of the extracellular matrix network. The murine myocardium has a rich vascular supply, composed of relatively thin-walled arterioles, venules and a well-organized capillary network (Figure 2.6.4B). Vascular pericytes of the murine myocardium are extensively branched cells that form an incomplete layer around the capillary endothelium and postcapillary venules [15]. Arterioles are easily identified (Figure 2.6.4C). Resident inflammatory cells, such as macrophages and mast cells (Figure 2.6.4D) are relatively rare in normal adult mouse hearts compared with other mammalian species [16], found occasionally in the pericardium and in close proximity to vascular structures. In contrast, murine arterial trunks exhibit a large population of adventitial mast cells that
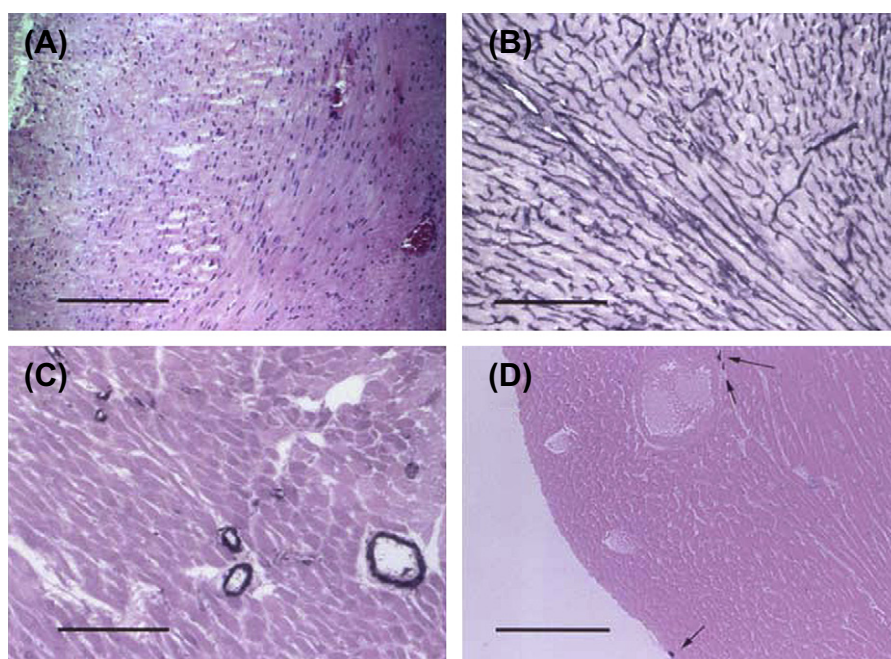
Figure 2.6.4 Murine heart. (A) Haematoxylin/eosin staining of a normal murine heart. Cardiomyocytes in the murine left ventricle can be divided into three layers: myofibres in the middle layer run mainly circumferentially, whereas those in the inner and outer layers run parallel or oblique to the apical-basal axis. (B) Normal mouse heart stained with an antibody to CD31/PECAM-1 identifying the vascular endothelium. The murine myocardium exhibits a rich vascular supply with a dense capillary network. (C) α-smooth muscle actin staining identifies arterioles in the normal mouse heart. (D) The murine heart shows a small number of resident inflammatory cells. Toluidine blue staining identifies occasional mast cells located around vessels and in close proximity to the pericardium (arrows). Black bars in figures are 75 μm in length.

may be involved in regulation of vascular tone. The cellular elements are embedded in a complex network of extracellular matrix [17] that is primarily composed of type I collagen with smaller amounts of type III and type V collagen, fibronectin, proteoglycans and basement membrane components (such as laminin and type IV collagen). In the normal heart the matrix not only serves as a scaffold for muscle fibres and vessels, but also plays an important role in transducing key molecular signals regulating survival and function of both cardiomyocytes and interstitial cells [18–20].

Mouse electrocardiography

A very comprehensive review of mouse electrocardiography (ECG) was published by Wehrens et al. [21] detailing measurement and quantification of the mouse ECG. Goldbarg et al. [22] investigated the ECG characteristic of normal strains of mice and useful definition of Q-T segments. The first mouse ECG was recorded on a string galvonometer in 1929, but current efforts have produced a system that records a 12-lead ECG. Electrophysiological (EP) techniques have been used in a mouse model of *hypertrophic cardiomyopathy* (HCM) [23, 24]. Given the fact that Richards et al. [25] were unable to show a clear T-wave following the QRS complex, he directed experiments to investigate whether the T-wave could be made distinct from the main QRS complex. He was able to show that the notch on the QRS wave, which potentially represented the T-wave, was separated from the main QRS wave on cooling and was accentuated with increases in potassium. Methods have been developed to determine the various components of the ECG during myocardial ischaemia, long QT syndrome mutations and atrial fibrillation. Within any individual study in a longitudinal manner, the ECG data

are substantially reproducible. However, variation in the data between studies is often illustrative of differences in strain, anaesthesia or other factors. Using a multiple electrode catheter, the electrophysiology of the mouse can be shown to be highly dependent upon age and these agents. For example, the sinus node recovery time varied by a factor of three in differing strains and by a factor of five in the same strain with different anaesthetic agents [26].

Genetically modified mice are continually being produced to study cardiac depolarization and repolarization phenomena and to produce the long QT syndrome and atrial fibrillation. Implantable telemetry systems are helpful in monitoring changes in Q-T interval and the generation of arrhythmias in transgenic mice with ion channel defects [27] without the confounding effects of anaesthetic agents. Similarly, placement of pad electrodes in the cage can be used to get non-invasive ECGs, again without anaesthetics [28]. Mutations of the underlying genes controlling the various ion channels and gap junctions offer promise for understanding the origin of various parts of the ECG and abnormalities produced by disease. Our laboratories continuously monitor the ECG of all surgically operated or haemodynamically monitored mice and this has been especially important in studies of myocardial

ischaemia and reperfusion. While the T-wave is only a 'notch' in the ECG on the down-slope of the QRS wave complex, this site on the ECG shows striking changes in voltage when the animal is challenged with myocardial ischaemia by coronary artery occlusion. The notch is increased in height with a plateau (generally named 'ST segment'), which becomes 50–90% of the QRS voltage peak height. When the measured peak voltage in the ST segment produced at the end of a 1 h coronary occlusion is plotted against the infarct damage measured as a percentage of area at risk at 24 h after the ischaemic insult, there is a direct relationship between the peak ST segment and the infarct size. This is shown in Figure 2.6.5 where 25 min into a 60-min coronary artery occlusion there is an ST segment change that was plotted against infarct damage measured at autopsy 24 h later. Clearly, the ECG ST segment is reflective of the damage that occurs in the myocardium with ischaemia. It is also clear that mice do not die of ventricular fibrillation post MI or reperfusion of an occluded vessel. However, re-entry and fibrillation in the mouse heart was produced by sustained burst pacing [29]. In our hands, any ECG tracing interpreted as ventricular fibrillation reverts to normal rhythm when the artificial voltage disturbance is discontinued.
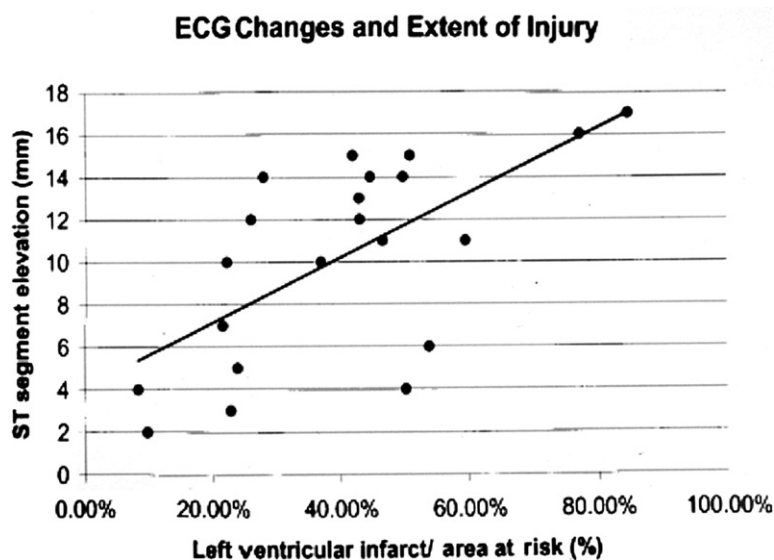
Figure 2.6.5 ECG vs infarct size. The ST segment elevation at 25 min during a 60-min coronary artery occlusion plotted against the infarct weight as percentage of the area at risk, measured 24 h later at autopsy with triphenyltetrazolium stain.

Functional cardiovascular measurements in the mouse—invasive versus non-invasive

Intense efforts have been made to develop instruments that will be able to monitor flow, pressure and dimensions in the mouse heart. When developing instrumentation to monitor the cardiovascular system in mice, it is important to realize the challenges inherent in both invasive and non-invasive measurement methods. Invasive experiments generally involve placing sensors and/or catheters in a vessel or the heart in an anaesthetized open or closed chest state. Generally, the experiment is completed within hours and the animal cannot be used for repeat measurements [30-35]. Conscious-mouse methods, such as telemetry, require great care in surgical dissection to implant the sensors for chronic studies [27, 36, 37]. Several non-invasive methods are reported in this chapter, such as MRI and echocardiography. Even these methods rely on anaesthetizing the animal on the day of the experiment, except in less common conscious-mouse experiments where some degree of stress still may be present. Other studies have attempted to look at conscious non-invasive methods, as exemplified by telemetry studies and use of implanted sensors [27, 37]. In experiments to measure cardiac output, regional blood flow and intravascular volumes in the 'conscious' mouse, injections were made through the femoral artery in animals that had just recovered from anaesthesia but were still restrained. Heart rates in this case were lower than normal. Resting heart rate normally is between 500 and 600 bpm and values lower than 300 might be considered non-physiological, whereas rates above 750 are rare.

Doppler ultrasound system

In order to detect aortic blood flow velocities, and blood flow velocities across the mitral valve as seen in human studies, a pulsed Doppler system was designed for use in mice to serially measure the function of the cardiovascular system including pulse wave velocity [38-41]. The Doppler system uses probes consisting of 1 mm diameter 10 or 20 MHz ultrasonic crystals mounted at the end of 2 mm diameter stainless steel tubes small enough to be oriented parallel to direction of flow from a site at the border of the sternum. The measured Doppler audio signals are acquired and processed with a high-frequency, real-time signal acquisition and analysis system that was developed specifically for use in mice [42]. In this system the best velocity resolution is 5 mm/s and the best temporal resolution is 0.1 ms. The maximum measurable velocity is approximately 9 m/s [43]. This system also monitors the ECG simultaneously throughout the experiment and relates the temporal features of the velocity wave to the ECG.

Doppler aortic flow velocities reflect cardiac systolic function and flow velocities across the mitral valve reflect diastolic function as blood flows into the relaxed left ventricle. Both aortic and peak mitral flow velocities were measured in myocardial ischaemia and reperfusion experiments longitudinally in time [44]. Figure 2.6.6 illustrates the peak aortic velocity (A) and peak early filling velocity (B) in three types of animals: sham, permanent occlusion and occlusion followed by reperfusion. In these experiments Doppler velocities indicated a return to normal values in reperfused animals but not those permanently occluded.

Pulsed Doppler blood velocity signals can be obtained non-invasively from many peripheral vessels as well as from the heart. Figure 2.6.7 shows 20 MHz spectral Doppler signals representing blood velocity versus time from the left and right carotid arteries, the left and right renal arteries, the coeliac artery, and several sites along the aorta in an anaesthetized mouse. Also shown is a coronary flow velocity signal for another anaesthetized mouse. The renal artery signals were obtained with the mouse in the prone position and the others were obtained with the mouse supine. All have high signal quality with magnitudes and waveforms similar to those recorded from the same vessels in humans and larger animals. The
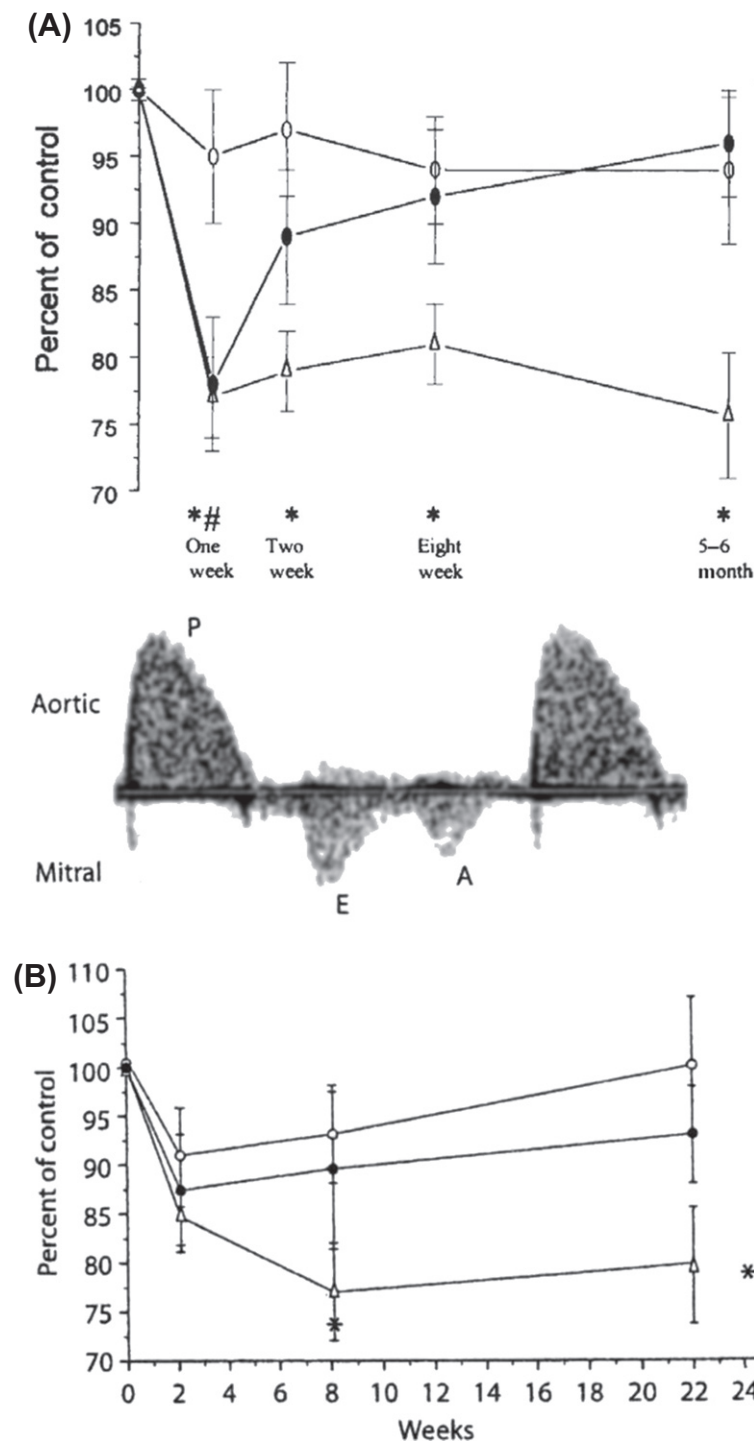
Figure 2.6.6 Peak aortic flow velocity and peak early filling velocity. (A) Peak aortic flow velocity followed for 5–6 months in mice subjected to sham operation (○), 2-h occlusion followed by reperfusion (●), and permanent occlusion (△). Data are % of preoperative values and are expressed means ± SE. Preoperative values: sham (n = 15), 104 ± 20 cm/s; permanent occlusion (n = 24), 111 ± 17 cm/s; reperfusion (n = 13), 102 ± 10 cm/s. *P < 0.05, permanent occlusion vs sham; *P < 0.05, reperfusion vs sham. (B) Peak early filling velocity followed for 5–6 months in mice subjected to sham operation (○), 2-h ischaemia followed by reperfusion (●), and permanent occlusion (△). Data are % of preoperative values and are expressed means ± SE. Preoperative values: sham (n = 15), 69 ± 3.1 cm/s; permanent occlusion (n = 24), 71 ± 1.9 cm/s; reperfusion (n = 13), 66 ± 2.1 cm/s. *P < 0.05 vs sham. The central figure shows representative aortic and mitral Doppler signals taken from the ascending aorta (aortic) and from the mitral valve (mitral). Peak aortic flow velocity (P) was measured
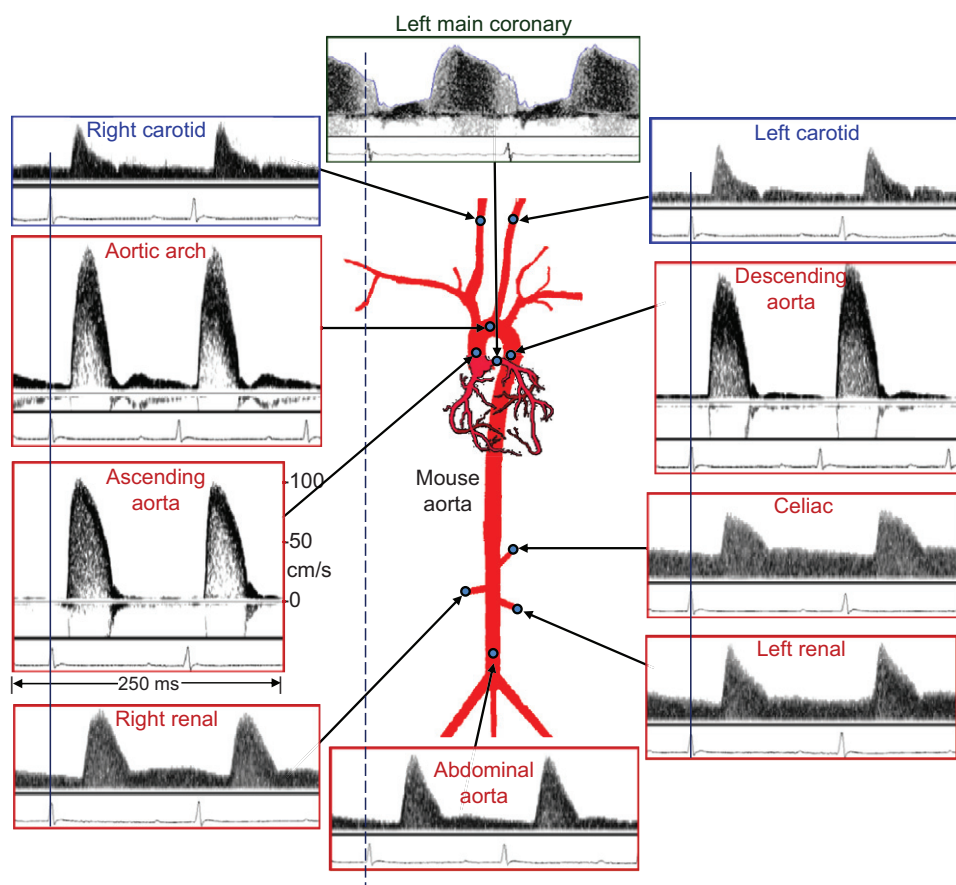
Figure 2.6.7 Doppler flow velocity profiles at various sites on the mouse arterial system. Right and left carotid arteries, left main coronary artery, transverse aorta, ascending and descending aorta, abdominal aorta with both right and left renal arteries. *Reprinted with permission from* ILAR J. **43(3)**, *Institute for Laboratory Animal Research, National Academy of Sciences, 500 Fifth Street NW, Washington, DC, 2001 (www.national-academies.org/ilar).*

variations in magnitude and waveforms are due to differences in peripheral vascular impedance in the arterial bed distal to the measurement site. Many of the murine models and interventions produce alterations in cardiac performance and regional *vascular impedance* which change these signals in characteristic ways. In the aortic banding model described later, which produces pressure overload, there are dramatic alterations in the blood flow waveforms in the right and left carotid arteries with only small changes in the average or mean velocity. Figure 2.6.8 shows right and left carotid artery velocity signals before and immediately after placement of the aortic constriction. The signals are comparable in magnitude and waveform before banding, but after banding the

maximum velocity and *pulsatility index* (PI) are increased in the right and decreased in the left carotid arteries (PI = (max − min)/mean, M = mean). Some of the observed changes in velocity are due to differences in driving pressure, but most are due to adaptive changes in peripheral impedance (resistance and compliance) in order to maintain cerebral perfusion and to minimize the load seen by the heart during systole. Pressure decrease across the aortic band ranges from 30 to 50 mmHg as determined by stenotic jet velocity and approximate Bernoulli's equation ($AP = 4V^2$, where AP is pressure gradient in mmHg and V is stenotic jet velocity in m/s)[45]. Stenotic jet velocity depends on the tightness of the band and can be as high as 4-5 m/s [42] as shown in Figure 2.6.9.

during systole; peak flow velocities across the mitral value were measured during diastole: E, peak early flow velocity; A, peak atrial flow velocity resulting from atrial contraction. *Modified from* Am. J. Physiol. Heart Circ. Physiol. **277**, *H660–H668.*
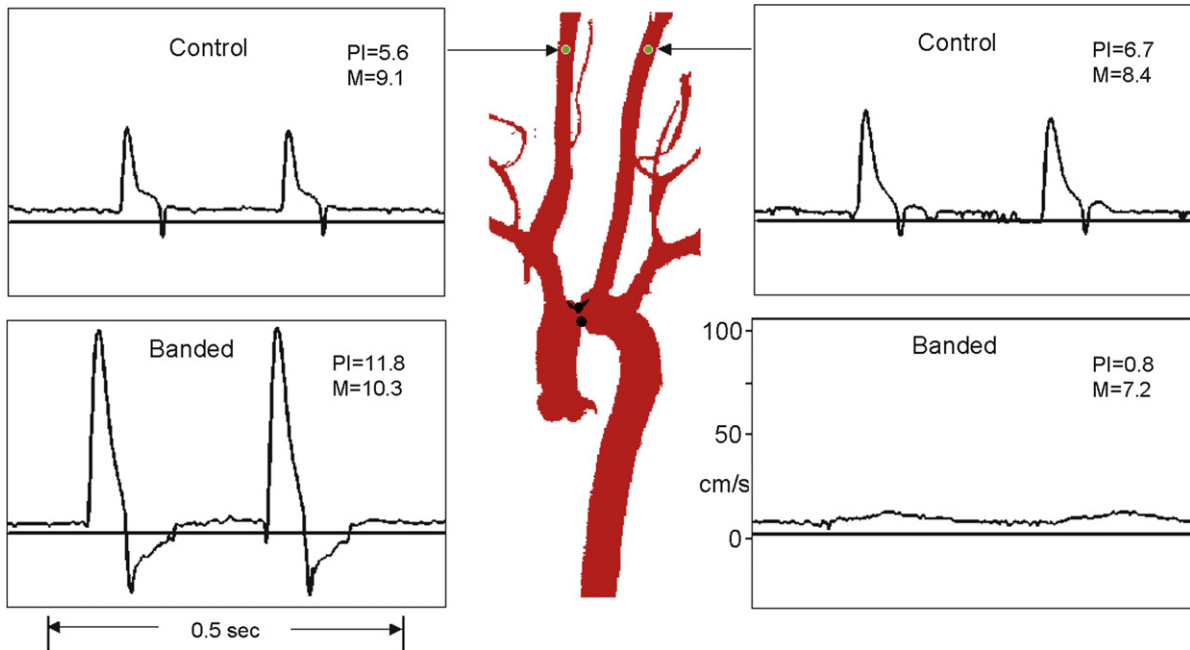
Figure 2.6.8 Schematic diagram of carotid arteries, ascending and descending aorta, and aortic arch with indication of banded site. Doppler signals are shown for right and left carotid arteries. After banding (lower panels) flow velocity is increased in the right carotid artery and decreased in the left carotid artery distal to stenosis. PI, pulsatility index; M , mean; $PI = (max − min)/mean$. The ratio of pulsatility indices (right/left) is a useful indicator of degree of banding.

The apolipoprotein E (ApoE) knockout mouse may be considered as a model of atherosclerosis similar to that seen in humans [46]. ApoE knockout mice have atherosclerotic lesions which cover approximately 50% of the aorta by 1 year of age with elevated cardiac output, elevated pulse wave velocity and cardiac hypertrophy [47]. A unique alteration in aortic arch acceleration was discovered in these mice. In wild-type normal mice velocity in the aortic root and arch during acceleration is smooth and continuous with the maximum slope or peak acceleration occurring early, as shown in Figure 2.6.10. In ApoE knockout mice acceleration in the aortic root appears normal, but at the aortic arch, acceleration occurs in two distinct phases with a slower initial rise followed by a later peak. This unusual waveform is probably caused by alterations in aortic impedance and a strong and early-arriving reflected wave from the stenosed carotid bifurcation [47]. This study also illustrated that pulse wave velocity is an indication of changes in vascular stiffness. Differences in pulse arrival times in the aortic arch and abdominal aorta allowed calculation of pulse wave velocity and
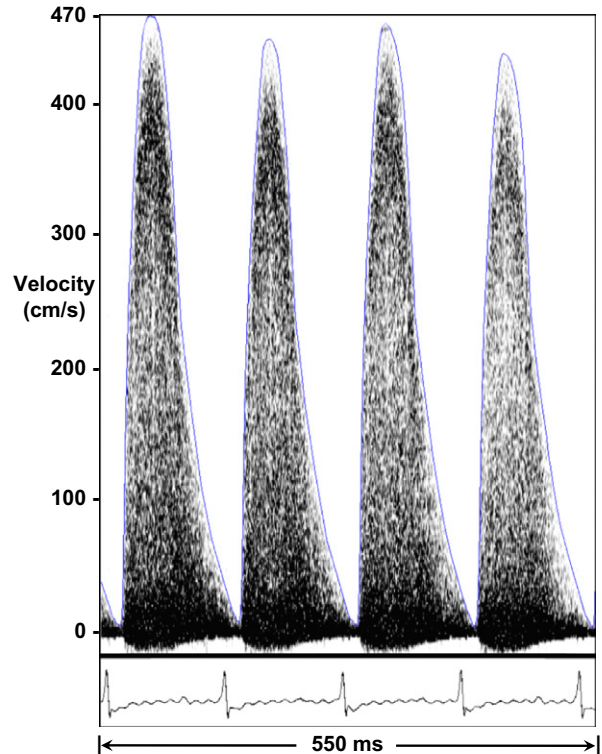
Figure 2.6.9 Jet flow velocity across a transverse aortic stenosis in a mouse. Doppler shifts as high as 475 cm/s can be seen. This signal was obtained using a 20 MHz pulsed Doppler probe.
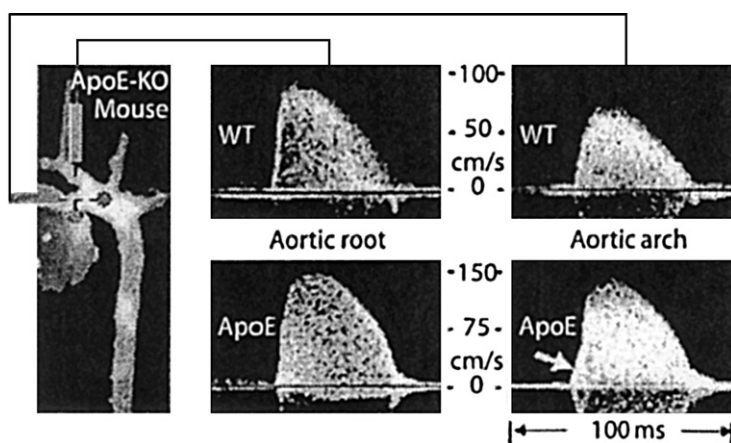
Figure 2.6.10 Schematic diagram of carotid arteries, aortic root, aorta, and aortic arch. Representative signals from aortic root and arch in a wild-type and an ApoE knockout mouse.

demonstrated that ApoE knockout mice had substantially elevated values, reflecting a less compliant arterial system.

Coronary artery flow velocity may be measured non-invasively with relative ease and consistency in the mouse by holding the Doppler probe in a stable position and aligning with the axis of the left main coronary artery [48, 49]. Another useful index may be calculated to underscore the reserve capacity of the mouse heart. When the oxygen demand of the myocardium increases, coronary artery vessels dilate, allowing increased blood flow. The maximum capacity of coronary arteries to supply blood to the heart is termed *coronary flow reserve* (CFR). CFR may be calculated as the ratio of coronary blood flow velocity during maximal vasodilation hyperemia relative to resting coronary blood flow velocity at rest [50–52]. Vasodilation may be accomplished with isofluorane, which is used as an anaesthetic in rodents and acts as a potent coronary vasodilator with a smaller change in heart rate than the standard vasodilator, adenosine [48, 53–55]. Typically, resting coronary flow velocity is measured at 1% isofluorane and hyperaemic coronary velocity is measured at 2.5% isofluorane [48, 56].

Isofluorane-induced CFR (H/B) increased from 2.4 in six-week-old mice to 3.6 in two-year-old mice but was reduced to 2.5 in two-year-old atherosclerotic ApoE knockout mice. The CFRs in control mice are in substantial agreement with the CFRs reported in rats and in humans. It appears that age and resting velocity are the major factors determining H/B in the absence of coronary stenoses [48]. In the pressure overload mouse model created by transverse aortic constriction (TAC), CFR decreased from 3.2 before TAC to 2.2 one day after TAC and progressively decreased to about 1.1 after twenty-one days as the heart adapted and remodelled after TAC [49]. Additionally, the amount of flow during systole progressively increased after TAC and almost equalled flow during diastole, perhaps indicating a redistribution of flow away from contracting myocardium or from endocardium to epicardium as the heart hypertrophied [57–59]. In mice with permanent left anterior descending coronary artery occlusions, CFR decreased from 3.2 to 2.0 and, in mice with chronic infusions of angiotensin, CFR decreased from 3.0 to 1.4 [60]. CFR is relatively easy to measure in mice and appears to be reduced by most forms of heart disease. It appears that it may be used in place of ejection fraction as an index of global cardiac reserve in mice. The coronary systolic/diastolic flow velocity ratio, which does not require the use of a vasodilator, may also be a useful index of myocardial perfusion status.

Echocardiography

Cardiac mass and function are now being evaluated routinely using high-frequency cardiac ultrasound. Figure 2.6.11 shows echocardiographic tracings from normal, hypertrophic and dilated mouse hearts.
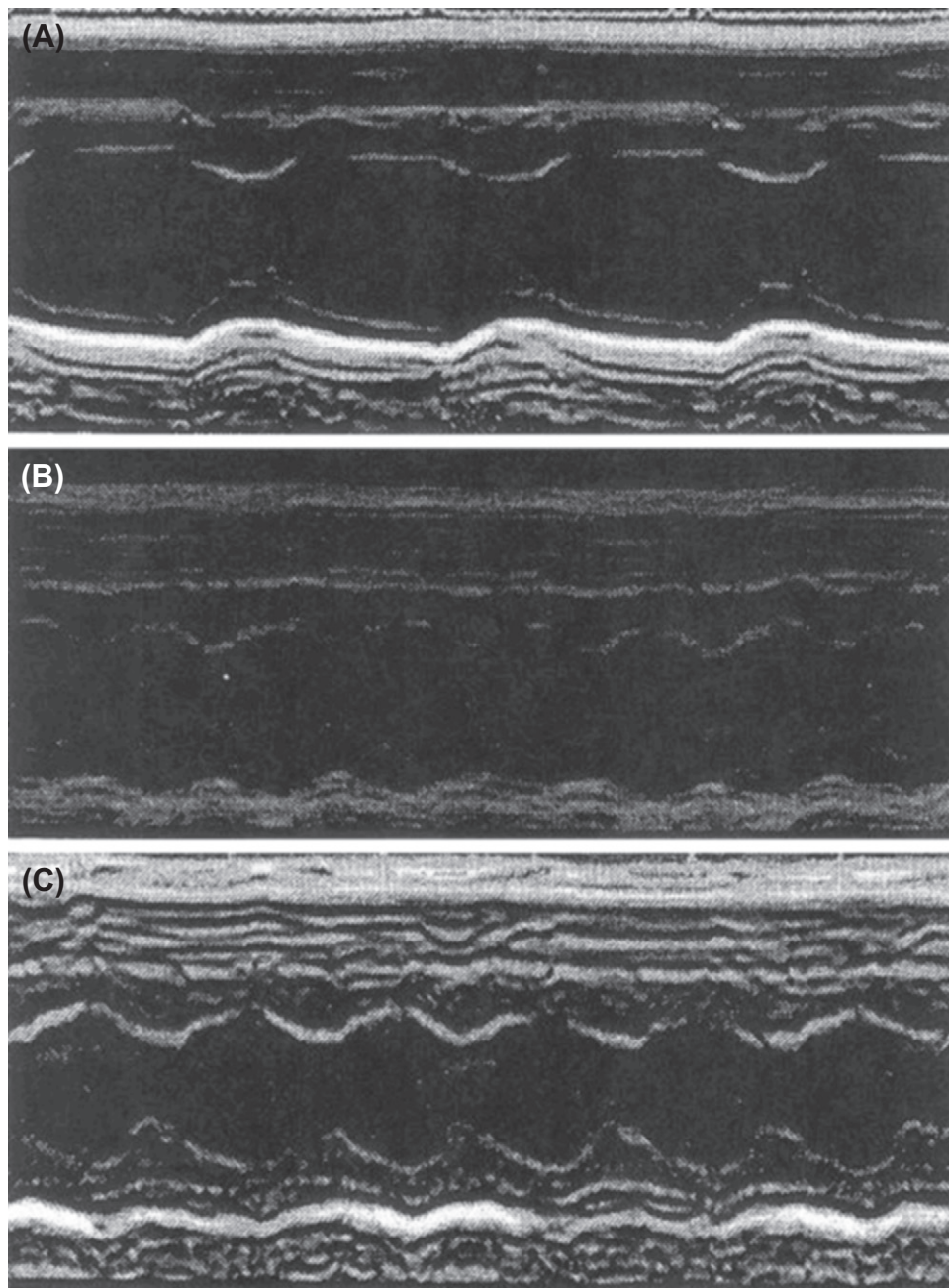
Figure 2.6.11 Representative echocardiograms. (A) Dilated, (B) normal and (C) hypertrophied mouse hearts.

Overcoming the temporal and spatial resolution has been a challenge in using the mouse, with its fast heart rate and small size [61, 62]. Left ventricular imaging by transthoracic echocardiography allowed assessment of left ventricular mass and systolic function [63–66]. A two-dimensionally directed M-mode approach allowed good visualization but should be used cautiously in LV segment wall motion abnormalities or in instances where the left ventricle hypertrophies in a non-homogenous fashion.

Myocardial shortening in these mice was found to be approximately 57% [63]. Tanaka et al. [67] used echocardiography to estimate LV function in normal and transgenic mice including mice that had overexpression of the beta-2 adrenergic receptor and the *H-ras* gene. Good correlation has been shown between M-mode echocardiography and left ventricular mass determined at autopsy [61, 68]. Transoesophageal echocardiography was developed and applied to mice to evaluate right ventricular size and function and can be

used to image left ventricular function [65]. This type of study is particularly important in the pathophysiology of pulmonary hypertension. The use of non-invasive transthoracic echocardiography in concert with a pulsed Doppler system allows assessment of the mouse heart and cardiovascular system [69, 43].

Compared with transthoracic echocardiography, transoesophageal echocardiography requires that the mouse trachea be intubated so that there is no collapse of the airway. Doppler echocardiography has been pushed to new levels by studies showing that embryonic mouse hearts may be analysed. This method was particularly useful to monitor normal mouse embryos and compare them with embryos that might die during gestation and/or have cardiac failure [70]. An additional feature in echocardiography has been the use of contrast medium to monitor myocardial perfusion in humans. This has now been applied to a mouse model of myocardial ischaemia, illustrating that perfusion defects could be imaged in mice to estimate the quantity of non-perfused myocardium [71].

MRI

MRI continues to be a very useful and rapidly developing technology in mouse imaging (see Chapter 5.5). The procedure is relatively non-invasive and suitable for longitudinal studies. There are significant advantages compared with M-mode or two-dimensional echocardiography murine studies. For example, MRI may be used for three-dimensional reconstruction of all chambers and for twisting movement to measure torsion with the cardiac cycle [72]. MRI provides data about the right heart, which is difficult to image otherwise. In some studies an 'average volume' is provided, which is not likely to reflect end-diastolic or end-systolic measurements [73], but as frame rates increase, more time-resolved information may be obtainable. Chacko et al. [74] combined P31 MR spectroscopy with MRI, allowing simultaneous determination of anatomical/functional data and high-energy phosphate status. Using a similar approach and specific probes, simultaneous measurement of function and intracellular hypoxia, pH or other biochemical parameters appear feasible. Others have visualized small structures such as coronary arteries and valves *in vivo* with MRI that have not been visualized by other techniques [75]. MRI can provide reasonable serial estimates of LV and heart mass for developmental studies [76]. Determination of LV ejection fraction does not require many of the assumptions used by the echocardiographers. However, of necessity, the image acquisition in MRI was gated to both cardiac and respiratory cycles by acquiring ECG and respiratory signals in studies to determine hypertrophic changes in mouse heart mass [77]. The scaling issues of MRI are similar to those faced in echocardiography, but the mouse will fit nicely into a relatively small-bore magnet for studies under anaesthesia (see Table 2.6.1).

While MRI equipment is very expensive to purchase, maintain and operate, the quality of images continues to improve. Most investigators use dedicated coils for mouse cardiac imaging.

TABLE 2.6.1: WT parameters (3–4-month-old mice) measured with MRI						
Investigator	LVEDV (μl)	LVESV (μl)	LVEF (%)	Cardiac output (ml/min)	LV mass (mg)	Stroke volume (μl)
Franco, 1998 [141]	51.7 ± 12.8	13.4 ± 6.7	75 ± 8		156 ± 14	38.3 ± 8.8
Franco et al., 1999 [80]	60 ± 9.8	21 ± 2.6	66 ± 2	15.6 ± 0.7		32 ± 2
Ruff et al., 1998 [142]	45.2 ± 9.3	14.6 ± 5.5	68.6 ± 6.6			
Wiesmann et al., 2000 [76]	63.6 ± 6.6	23.5 ± 4.4	65 ± 3.5	14.3 ± 0.5	101.3 ± 8	40.2 ± 2.7
Chacko et al., 2000 [74]			65 ± 7			

LVEDV, left ventricular end-diastolic volume; LVESV, left ventricular end-systolic volume; LVEF, left ventricular ejection fraction.

At best, frame acquisition is still slow, more than 8 ms (which converts to 12.5 frames per 100 ms cardiac cycle). This factor should continue to decrease because frame rates have increased significantly in recent years. Because of the dependence on numerous acquisitions to generate one image, gating using the ECG for timing must be used and another sensor for respiration may be necessary. This will provide challenges in mice with irregular heart rhythms, a property of some phenotypes. Additionally, the animals must remain absolutely immobile for the repeated acquisitions, though algorithms do exist to handle minimal subject movements and anaesthetics can substantially modify findings [78]. MRI provides no real assay of diastolic function, but it may provide the most robust imaging of the right ventricle and right-sided cardiac function [79].

MRI has also been used to document the systolic dysfunction and cardiac dilation induced by tumor necrosis factor (TNF)-alpha expression [80]. It has also been used to show the hypertrophy with relatively maintained systolic function in the GLUT4 null mouse [81].

While by no means a complete list of relevant review articles, several recent reviews detail the latest developments in mouse MRI modalities that are worthy of consideration. Pautler [82] reviewed the concepts and application of MRI to murine models of cardiovascular disease. MRI and MRS methods and application to study a variety of genes and cell targets in normal and pathophysiology are presented in a comprehensive review by Epstein [83]. Chen and Wu [84] have provided an overview of the significant developments in molecular imaging, targeting a variety of molecular or cellular structures in cardiovascular disease entities.

Ventriculography

Radiographic imaging of the mouse heart to obtain ventricular volumes and ejection fractions uses constant fluoroscopy and a non-ionic contrast medium [85]. Both right and left ventricular function was evaluated, but temporal resolution was limited to the equivalent of 60 frames/s. More recent ventriculography has used technetium-labelled red cells, SPECT techniques and the pinhole lens to obtain good magnification for imaging [86]. This use of techniques currently applied to humans has the potential for greater acceptability than the ultra-short-lived tantalum-178 used in the past [40].

Values based on scaling equations

A variety of mouse cardiovascular parameters were calculated by using scaling laws developed by Dawson [87] which relate a measured value to animal body weight. The power law form utilizes best-fit methods of analysis of the measured parameters versus body weight wherein a coefficient (alpha) and exponent are derived (see Table 2.6.2). The values derived for normal mice are usually very close to the actual measured values for mouse parameters. Blood flow velocities and

TABLE 2.6.2: Relationship of measured parameters to animal body weight			
General scaling equation: $Y = \alpha BW^{b}$			
Parameter	**Relationship to BW (kg)**		**Value (BW = 25 g)**
Heart rate	$\alpha BW^{-1/4}$	$230\ BW^{-1/4}$	578 bpm
Heart weight	αBW^{1}	4.3 BW	112 mg
LV volume	αBW^{1}	2.25 BW	56 μl
Stroke volume	αBW^{1}	0.95 BW	24 μl
Cardiac output	$\alpha BW^{3/4}$	$224\ BW^{3/4}$	14 ml/min
Aortic diameter	$\alpha BW^{3/8}$	$3.6\ BW^{3/8}$	0.9 mm
Arterial pressure	αBW^{0}	100	100 mmHg
Peak aortic velocity	αBW^{0}	100	100 cm/s
Mitral E velocity	αBW^{0}	60	60 cm/s
Pulse wave velocity	αBW^{0}	500	500 cm/s

mean blood pressure are independent of body weight, hence the exponent is zero, requiring no correction of measured value. These latter values for mice are strikingly similar to those seen in other normal animals and humans.

Mouse haemodynamic values vary somewhat as result of strain differences and, more so, as a result of experimental conditions such as anaesthetic regimen, invasive versus non-invasive procedures and genetic alteration as seen with transgenic or knockout manoeuvres. Similarities of wave forms of pressure and velocity across species indicate that arterial time constants scale with cardiac period [88].

Experimental models

Myocardial ischaemia and reperfusion

Anaesthesia and surgical technique

In order to study myocardial ischaemic injury, a coronary vessel is selected for occlusion. In the mouse this vessel is the LAD. Other arterial vessels on the left ventricular free wall and septal region are not prominent. The right coronary artery is a major supplier of the septal region and also the right ventricle. It is important to stress that a consistent coronary artery occlusion site must be chosen so that the area at risk is the same in each heart.

The first method to be described is one in which the thorax is opened and the LAD is occluded and/or reperfused acutely [5, 44]. The second method requires placement of an occluding device and the occlusion activation does not occur for several days, a chronic model [89]. In both methods surgical manipulation requires that the animal be placed on a respirator; this may involve anaesthesia using 2% isofluorane or anaesthetizing the animal with an agent such as sodium pentobarbital before placing it on the respirator. There are several important points which allow the models to be successfully utilized. First, the animal needs tracheal insertion of a tube and this is generally more easily accomplished by a slight incision in the skin of the ventral neck over the laryngeal region; careful reflection of the muscles of the neck allows direct visualization of the trachea. At this point, the tongue is extended and polyethylene (PE)-90 tubing with a bevelled point is slid in through the mouth, past the pharyngeal region, and into the trachea just past the laryngeal cords. Second, the animal is ventilated very carefully, so that the lungs are not over-expanded or under-expanded. In some systems this is allowed by having a loose-fitting connection between the respirator and the endotracheal tube, giving the animal sufficient respiratory volume but not over-expansion of the lungs.

Proper lighting and magnification of the field of interest is essential in order to identify the LAD and to place an 8-0 sterile suture underneath and around the vessel in order to secure the occluding device. In the first method a 2 mm piece of PE-10 tubing is placed on the surface of the heart over the LAD and then the suture is brought up alongside the vessel and tied firmly around the PE tubing in order to compress the coronary artery against the undersurface of the PE tubing. This creates a coronary artery occlusion and results in myocardial ischaemia. For reperfusion studies the ligature on top of the PE tubing is cut, allowing the release of the PE tubing and hence restoring flow into the coronary vessel. If a permanent coronary artery occlusion is desired, the ligature placed around the coronary artery is simply tied with a double knot without placing an intermediate piece of PE tubing. Ligatures used in closing the chest wall are generally either 7-0 or 6-0 sutures. As soon as the chest is closed, the animal is allowed to recover by removal from the respirator and, within a few minutes, it begins spontaneous breathing.

The second major way to promote a LAD coronary occlusion is the chronic model of coronary occlusion, specifically for ischaemia followed by reperfusion experiments in a closed-chest mouse several days after surgery. This allows a time period, after implanting the occlusion device, to allow the dissipation of inflammation and the trauma associated with the surgical manipulation. This model is especially helpful in studying cytokines, chemokines and other inflammatory events which are promoted by the myocardial ischaemic state.

The surgical manipulation and resulting release of a variety of these elements are separated in time from the ischaemic event. In this model a median thoracotomy is not performed as for the acute open-chest occlusion model. The chest is opened with a left lateral cut with fine scissors along the sternum cutting through ribs to approximately mid sternum. The chest wall is then retracted and the pericardium gently dissected to see the coronary artery. After passing the needle of the 8-0 suture underneath the LAD, the needle is cut from the suture and the two ends of the 8-0 suture are then placed through a 0.5 mm piece of PE-10 tubing. This forms a loose snare around the LAD. The sutures are then exteriorized through each side of the chest wall and the chest closed. The ends of the 8-0 suture are then placed underneath the skin and the skin closed with 6-0 suture. At this point the animal is removed from the respirator, allowed to recover consciousness and placed in the intensive care unit (ICU) for recovery.

At various later times, days to weeks, the animal is then reanaesthetized and the appropriate occlusion and/or reperfusion protocol performed without opening the chest. This is done by ventilating the animal with isofluorane anaesthesia and proceeding to extract the two ends of the suture carefully from underneath the skin; coronary occlusion occurs by pulling on the ends of the suture laterally, which then creates a compression on the coronary artery as the small piece of PE tubing is forced downward. Reperfusion results when the lateral tension is stopped. A variety of experiments may be designed using this chronic model such as experiments where brief periods of ischaemia (5 or 15 min), followed by reperfusion of minutes to hours or experiments where there are longer occlusion or reperfusion periods. We have investigated the role of several chemokines and cytokines and their release during numerous experimental paradigms using this model [90].

Myocardial hypertrophy

Pressure overload hypertrophy is induced by transaortic banding in mice [9, 85]. The technique involves opening the anterior chest wall sufficiently to expose the transverse aortic arch. This entails dissecting the fatty material immediately juxtaposed to the arch after placing a ligature loosely around the aortic arch at that site and then applying a 3 mm section of a 27-gauge needle. The ligature is tightened around the needle and aorta sufficiently to occlude the vessel completely, and tied in place with two knots. The small section of steel tubing is immediately removed and hence the vessel blood flow will be similar to the diameter of that removed piece of tubing. After this, the animal's chest wall is closed and the skin is sutured. With practice, this technique allows 80–90% occlusion of the blood flow velocity in the aortic arch. This allows left ventricular hypertrophy and heart weight/body weight ratio increases within 10–14 days. This is comparable to the time course of myocardial hypertrophy seen in similar experiments in rats.

Pathology of myocardial infarction in mice

Murine models of MI, patterned after the models previously described, have contributed to our understanding of the pathogenic mechanisms operative in the ischaemic myocardium and are now being used in numerous laboratories. Much as in other mammalian species, mouse cardiomyocytes are highly susceptible to ischaemia, showing evidence of irreversible injury after 20–40 min of severe ischaemia. Occlusion of the LAD coronary artery in the mouse generates an extensive infarction involving the anterior and lateral left ventricular wall. Evidence of irreversible cardiomyocyte injury, such as contraction band necrosis and wavy fibres, is found in the early postinfarction stages [5]. Much like other mammalian hearts, the mouse myocardium has negligible endogenous regenerative capacity. Thus, repair of the infarcted murine heart is dependent on an inflammatory reaction that ultimately results in formation of a collagen-based scar [91]. Healing of the infarcted mouse heart can be divided into three overlapping phases: the inflammatory phase, the proliferative phase and the

maturation phase [92]. During the inflammatory phase, induction of chemokines and cytokines results in recruitment of leukocytes into the infarcted area. Neutrophils and macrophages clear the wound from dead cells and matrix debris. Subsequent suppression of proinflammatory signals and recruitment of reparative mononuclear cells that differentiate into macrophages and produce growth factors mark the transition to the proliferative phase of cardiac repair. At this stage cardiac fibroblasts undergo myofibroblast transdifferentiation, proliferate and secrete extracellular matrix proteins. An extensive microvascular network is formed, providing oxygen and nutrients to the metabolically active healing wound. The maturation phase of infarct healing follows as fibroblasts and vascular cells undergo apoptosis and a collagen-based scar is formed. The inflammatory and reparative response in healing mouse infarcts exhibits a more rapid time course than in large mammalian species [93, 94].

Murine MI is associated with an intense local inflammatory response, which (similar to higher mammalian species) is significantly accentuated with reperfusion of the myocardium [95, 96]. Reperfused murine infarcts exhibit intense leukocyte infiltration, leading to accumulation of myofibroblasts and deposition of collagen, and thence to the rapid formation of thinned, relatively acellular, scars (Figure 2.6.12). Reperfusion of the murine myocardium appears to reduce the degree of infarct expansion, even under circumstances in which infarct size is not altered, inducing more effective ventricular repair and preservation of ventricular function [44] (Figure 2.6.13).

Figure 2.6.14 illustrates the collagen matrix stained with sirius red in cross-sections of a mouse heart which had been subjected to 24 h of LAD occlusion followed by 2 weeks of reperfusion. Use of the murine model of experimental myocardial ischaemia and reperfusion has elucidated important aspects of the reparative response following infarction. Loss-of-function studies have revealed an important role for cytokines and chemokines in postinfarction inflammation [97–99]. Investigations in a murine model of experimental infarction indicated that TNF-alpha may exacerbate myocardial ischaemic injury at an early stage of reperfusion by
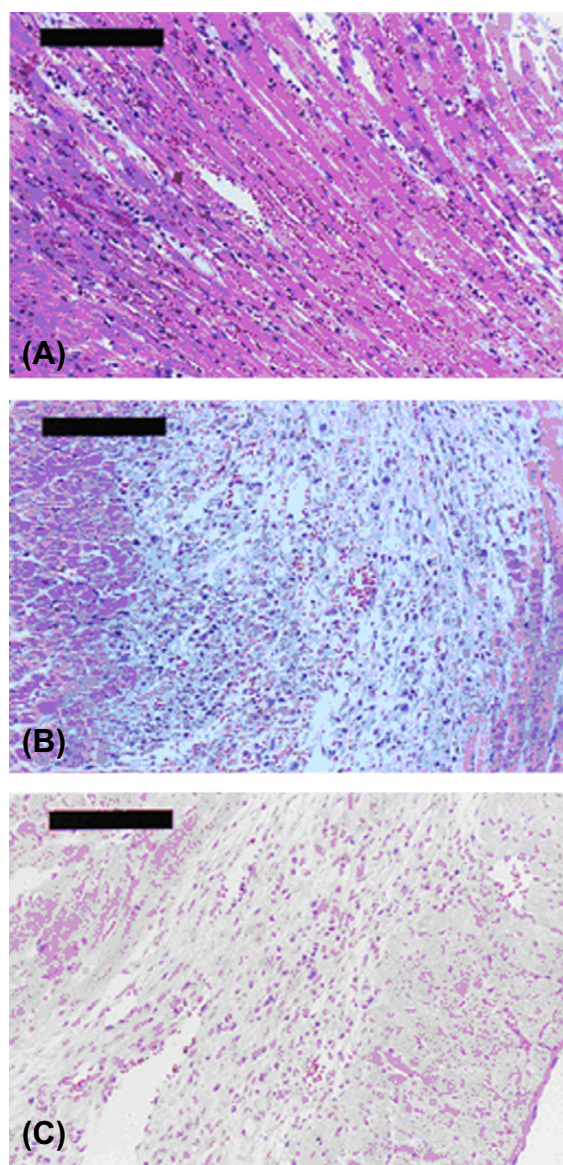
Figure 2.6.12 Haematoxylin and eosin staining of an infarcted murine myocardium. (A) After 1 h of coronary occlusion and 24 h of reperfusion the mouse myocardium exhibits extensive leukocyte infiltration. (B) After 72 h of reperfusion myocyte replacement with granulation tissue is noted. (C) After 7 days of reperfusion the healing infarct has thinned and demonstrates a relatively low cellular content. Black bars are 75 μm in length.

activating NF-kappaB, thereby inducing chemokines and adhesion molecules and facilitating leukocyte infiltration [100]. Other studies, however, indicated that TNF signalling gives rise to one or more cytoprotective signals that prevent and/or delay the development of cardiac myocyte apoptosis after acute ischaemic injury [101], emphasizing the pleiotropic effects

Figure 2.6.13 Transverse sections of representative mouse hearts. Sham, permanent LAD coronary artery occlusion for 8 weeks and LAD coronary artery occlusion for 60 min followed by reperfusion for 8 weeks. *Reprinted with permission from* Am. J. Physiol. Heart Circ. Physiol. ***277,*** *H660–668.*

of cytokines in inflammatory reactions. The importance of adhesion molecules in MI was illustrated using animals deficient in both ICAM-1 and P-selectin and demonstrating impaired neutrophil trafficking without a difference in infarct size [7].

Experiments using mouse models of experimental MI have also documented the importance of inflammatory and profibrotic mediators in postinfarction remodelling. As the infarcted myocardium heals, the ventricle undergoes geometric changes that result in dilation and increased sphericity of the chamber, thinning and expansion of the infarct, and hypertrophy of non-infarcted segments. These alterations are collectively referred to as 'ventricular remodelling'. Adverse postinfarction remodelling is associated with systolic dysfunction, increased mortality and arrhythmogenesis, and is intertwined with the development of chronic heart failure [102]. Alterations in the molecular pathways associated with cardiac repair profoundly affect the remodelling process. Several investigations suggested a critical role for proteases regulating extracellular matrix remodelling in infarct healing: deficiency of urokinase-type plasminogen activator Plau (u-PA) protected against cardiac rupture, whereas lack of gelatinase-B protected against rupture [6]. However, *Plau*-deficient mice showed impaired scar formation and infarct revascularization, even after treatment with vascular endothelial growth factor, and died of cardiac failure due to depressed contractility [6]. In addition, targeted deletion
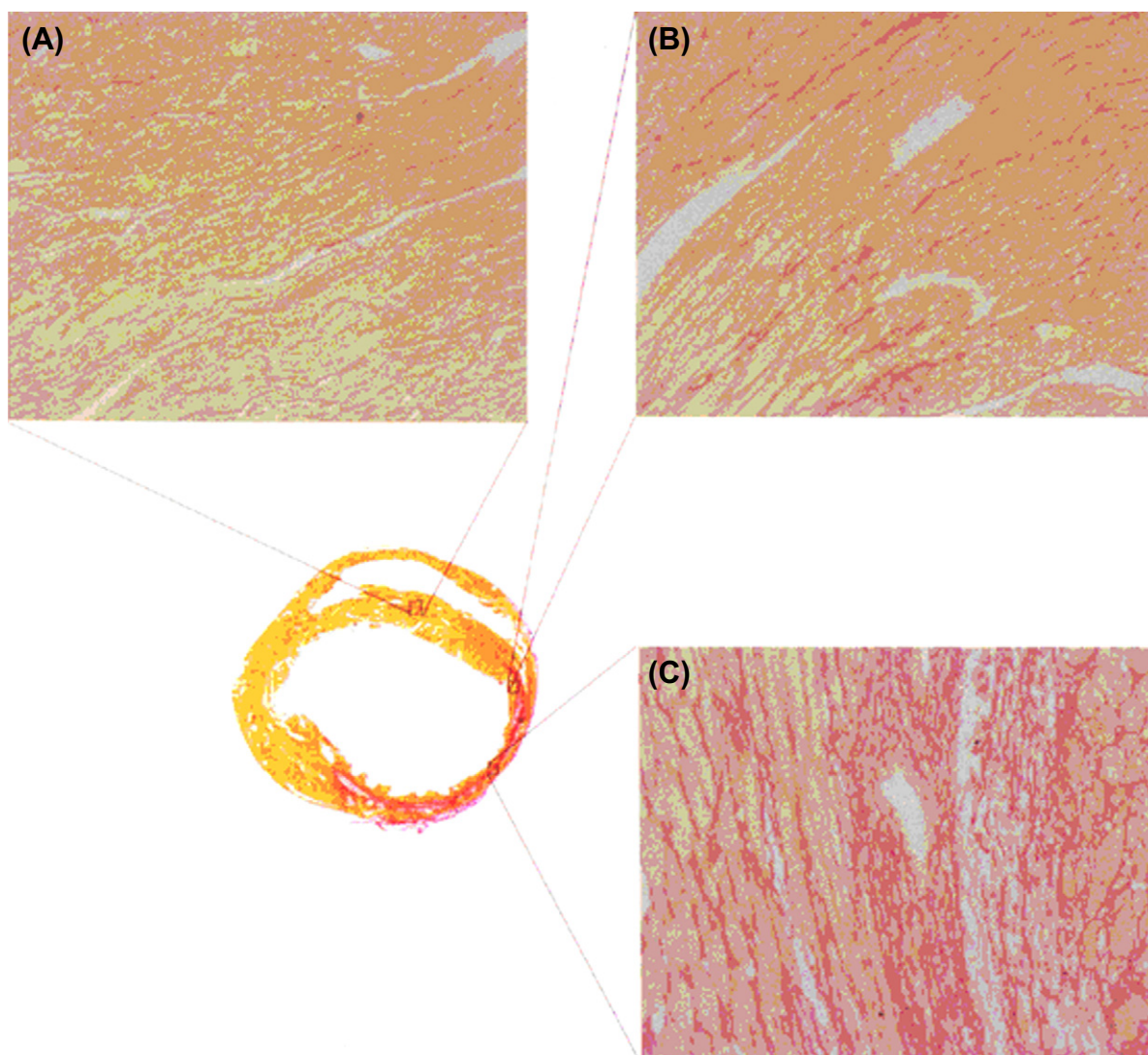
Figure 2.6.14 Cross section of a mouse heart at autopsy after a 2-h coronary artery occlusion followed by 2 weeks of reperfusion. The collagen stain picro sirius red of (A) thinned left ventricular free wall, (B) border zone, (C) septum.

of the matrix metalloproteinase 9 (*MMP9*) gene attenuated left ventricular dilation after experimental MI in mice. The decrease in collagen accumulation and the enhanced expression of other MMPs suggested that *MMP-9* plays a prominent role in postinfarction extracellular matrix remodelling [103]. Investigations using genetically altered mice to study the pathological basis of infarct healing and cardiac remodelling have resulted in an explosion in our knowledge of the cellular and molecular steps involved in myocardial injury and repair. It should be emphasized, however, that significant species differences may exist and should be considered when extrapolating findings derived from

murine studies to the pathogenesis of the human disease process.

Stem cells and cardiac regeneration

Myocardial cell death is inevitable when blood flow ceases to a region of the heart. As adult cardiac myocytes do not proliferate, an exciting new area of cardiovascular research using the mouse relates to the use of a variety of stem cells to promote repair of the irreversibly injured myocardium. The adult mouse heart contains approximately 20-30% myocardial cells

with the remainder composed of endothelial cells, vascular smooth muscle cells and fibroblasts [104]. The potential plasticity of stem cells to form other cells is found in bone marrow, skeletal muscle, cardiac muscle, liver bile ducts, vascular endothelium and other sources. One source of stem cells was an enriched ROSA bone marrow haematopoietic side population of stem cells, which were injected into irradiated mice. After 10-12 weeks the mice were subjected to 1 h of coronary artery occlusion followed by 2-4 weeks of reperfusion. Donor-derived cardiomyocytes as well as endothelial cells were identified in the peri-infarct region [105]. Differentiation of various kinds of stem cells to myocardial cells in the infarcted mouse heart has now been reported by several laboratories [106-108]. While a variety of stem cells appear to offer some promise of partial repair by formation of new myocardial cells, this method of repair may not be the most efficient. Difficulties in cell orientation, integration, and electrical syncytium formation remain as major concerns. The existence of resident cardiac progenitor cells within the heart appears to be a new and promising pathway to form new heart cells. In a recent study progenitor cells differentiate into cardiac muscle when injected intravenously after 1 h of ischaemia followed by 6 h of reperfusion. In this study undifferentiated cells from the adult heart therefore targeted injured myocardium after a systemic injection [109].

Pathology of myocardial hypertrophy

Cardiac muscle hypertrophy and cardiac enlargement include increased myocyte size, sarcomeric formation, reactivation of a fetal gene programme, including upregulation of genes such as beta-myosin heavy chain (MHC), atrial natriuretic factor (ANF), and skeletal alpha actin (SkA) [9, 110-114]. A large number of manipulations of the mouse genome have resulted in murine myocardial hypertrophy. The majority

of the models have employed transgenic overexpression of the candidate gene using the alpha-MHC promoter; fewer studies have attempted to ablate a gene of interest. Although in many cases the level of overexpression of the transgene vastly exceeds that observed in naturally occurring pathological states, useful mechanistic information has been derived from generation and analysis of these genetically engineered mice.

It appears that hypertrophy is not always associated with increased ventricular expression of ANF induction, which has quite often been considered the best general indicator of the transcriptional response [114]. This was deduced using a transgenic mouse model of HCM where hypertrophy occurred in the absence of increased ventricular levels of ANF message and levels of mRNA were absent where cardiac hypertrophy was detected. Localized changes in gene expression, however, did correlate with areas of tissue pathology. This is counter to studies where much greater increases in ANF gene expression occurred in models of acute pressure or volume overload [115-117], which, however, may represent a different pathogenic response. In other transgenic mouse models of HCM *in vivo* expression of the mutant cardiac sarcomeric protein troponin T-Q^{92} led to impaired local cardiac systolic function and increased interstitial collagen [118].

Murine models of cardiac fibrosis

Cardiac fibrosis is characterized by net accumulation of extracellular matrix in the myocardium and is an integral component of most cardiac pathologic conditions. Mouse models have significantly contributed to our understanding of the pathogenesis of cardiac fibrosis. Fibrous tissue deposition in the cardiac interstitium is the end result of many different types of cardiac injury. In MI sudden loss of a large number of cardiomyocytes triggers a reparative response that ultimately leads to replacement of dead cardiomyocytes with

a collagen-based scar ('replacement fibrosis'). Cardiac fibrosis also develops in response to injurious stimuli that do not cause extensive cardiomyocyte loss. Interstitial and perivascular deposition of collagen is associated with a wide variety of cardiac conditions due to haemodynamic, toxic, metabolic and immunological disturbances. Pressure overload induced by hypertension or aortic stenosis results in extensive cardiac fibrosis, associated initially with increased stiffness and diastolic dysfunction, that frequently progresses to ventricular dilation and combined diastolic and systolic heart failure [18]. Volume overload due to valvular regurgitant lesions also results in activation of cardiac fibroblasts leading to fibrosis of the heart [119]. Hypertrophic cardiomyopathy is associated with fibrous tissue deposition in the cardiac interstitium accompanied by alterations in the extracellular matrix scaffold, that may contribute to cardiomyocyte disarray [120]. Hearts with dilated cardiomyopathy often exhibit progressive fibrosis characterized by increased interstitial cellularity and accumulation of extracellular matrix proteins [121]. Finally, cardiac fibrosis is a hallmark of the cardiomyopathic processes associated with metabolic disturbances such as diabetes [122, 123] and obesity [124].

A variety of mouse models that induce cardiac fibrosis, simulating clinically relevant pathophysiological conditions, have been developed (Figure 2.6.15). In experimental models of pressure overload due to TAC, extensive and diffuse fibrotic remodelling of the mouse heart develops after 7 days accompanied by marked cardiomyocyte hypertrophy [125]. Infusion of angiotensin II induces severe perivascular and interstitial fibrosis in the mouse heart [126]. Moreover, brief (15 min) repetitive ischaemia followed by reperfusion results in fibrotic remodelling of the mouse cardiac interstitium in the absence of a completed infarction [127]. Fibrous tissue deposition in this model appears to be dependent on a chemokine-driven inflammatory reaction [128] that mediates recruitment of fibroblast progenitor cells [129].

Genetic screening

In studies where no particular gene was implicated differential screening was attempted in order to identify any and all genes regulated during hypertrophy [130]. Experiments were performed using subtractive hybridization between cDNA from the hearts of aortic-banded compared with sham-operated mice. In these experiments more than 50 genes were identified as being upregulated following the mechanical challenge of pressure overload in mouse hearts. The results revealed similarities between the genetic programmes of the neonatal and pressure overloaded hearts.

Cardiac growth induced by mechanical load requires coupling of extracellular stimuli to gene transcription. Transforming growth factor (TGF)-beta-activated kinase1 (TAK1) is a member of the mitogen activated protein kinase (MAPK) family which is involved in this coupling. TAK1 kinase activity is upregulated after aortic banding in mice and induces hypertrophy and the expression of the TGF-beta [9]. Similar studies, where
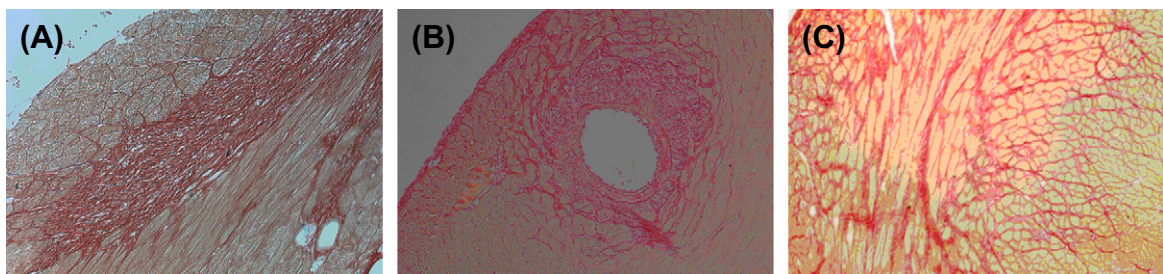
Figure 2.6.15 Sirius red staining identifies the pattern of collagen deposition in three distinct mouse models of cardiac fibrosis. (A) Replacement fibrosis in a model of reperfused infarction (1 h ischaemia/7 days of reperfusion). (B) Perivascular and interstitial fibrosis in a model of pressure overload induced by transverse aortic constriction (7 days). (C) Interstitial fibrosis in a model of brief (15 min) repetitive ischaemia and reperfusion (daily episodes of brief ischaemia for 7 days) induces a fibrotic cardiomyopathy in the absence of a completed infarction.

TAK1 is expressed in the myocardium of transgenic mice, provided sufficient stimulus to produce a P38 MAPK phosphorylation leading to myocardial hypertrophy with various disruptive elements presaging heart failure including interstitial fibrosis and severe myocardial dysfunction.

One of the potential mediators of hypertrophy is a broad-spectrum G protein called Gq. Hence, G-protein-coupled receptors initiate complex MAPKs [131, 132]. One of these MAPKs, MEKK1 (mitogen activated protein kinase/extracellular signal-regulated protein kinase), was activated by specific overexpression of Gq in wild-type mouse cardiac muscle [133]. When MEKK1 was absent, most of the features of hypertrophy induced by Gq (i.e. cardiac mass and myocyte enlargement) were eliminated. Importantly, in the absence of MEKK1 there was protection from negative effects of Gq on heart function.

One of the basic features of myocardial hypertrophy is the increase in RNA and protein per cell. Recently, it was shown that hypertrophy, which may be triggered by signalling proteins such as Gq, calcineurin or chronic mechanical stress, activates cyclin-dependent kinases such as Cdk9 which are required for RNA increase. It appears that the kinases Cdk9 and Cdk7 are downregulated as the heart matures, but both are activated by Gq, calcineurin or chronic mechanical stress to promote cardiac hypertrophic growth. Figure 2.6.16 illustrates that when Cdk9 is increased severalfold in transgenic mice, there is concomitant concentric hypertrophy and substantially increased myocyte size [134].

Inflammatory gene expression

Stress on the mouse heart by banding the aorta and creating mechanical load or acute haemodynamic pressure overload promotes inflammatory cytokine gene expression and several proinflammatory agents such as TNF, interleukin (IL)-1-beta, and IL-6 RNA levels within hours after the mechanical stress [135]. Interestingly it was noted that this was a transient effect and, in fact, after several hours to 3 days, without any change in loading conditions, the proinflammatory cytokines in the heart decreased [136, 137]. This suggests that there may be both load-dependent and -independent mechanisms operative in this period. It should be noted that cytokines may be released as a result of acute surgical intervention and this must always be considered when examining inflammatory mediators [89].

Congestive heart failure

Congestive heart failure (CHF) is a very important condition to model in the mouse. In humans most CHF is the end result of MI, longstanding hypertension or other processes. Significant MIs were produced in young mice (8–12 weeks) by occluding the LAD, anticipating that CHF would develop after infarction. While large infarcts and low perioperative mortality resulted after recovery from the procedure, no clinical manifestations of CHF were noted in these young post-MI mice [44]. There was no mortality beyond the perioperative period associated with respiratory difficulty, weight gain or loss, oedema, or other manifestation of decreased cardiac output or increased filling pressures. There was frequent aneurysmal dilation of the left ventricular free wall, remodelling and hypertrophy of the non-infarcted ventricle, and depressed fractional shortening on echocardiography, but no evidence of CHF. After adding deoxycorticosterone acetate (DOCA) by subcutaneous pellet and 8% saline in lieu of water, no evidence of the CHF syndrome presented and there were no increases in lung weight or lung water in the young mice at necropsy. Therefore, the young mouse, perhaps by using compensatory mechanisms, did not routinely or reproducibly develop CHF post-infarction, even if allowed to live to 16 months.

In contrast, older mice (12–14 months of age) develop CHF postinfarct after LAD occlusion and late mortality associated with respiratory difficulty, decreased grooming and weight loss, and, at necropsy, increases in lung weight [138]. Treatment with an angiotensin-converting enzyme inhibitor, captopril, was associated with
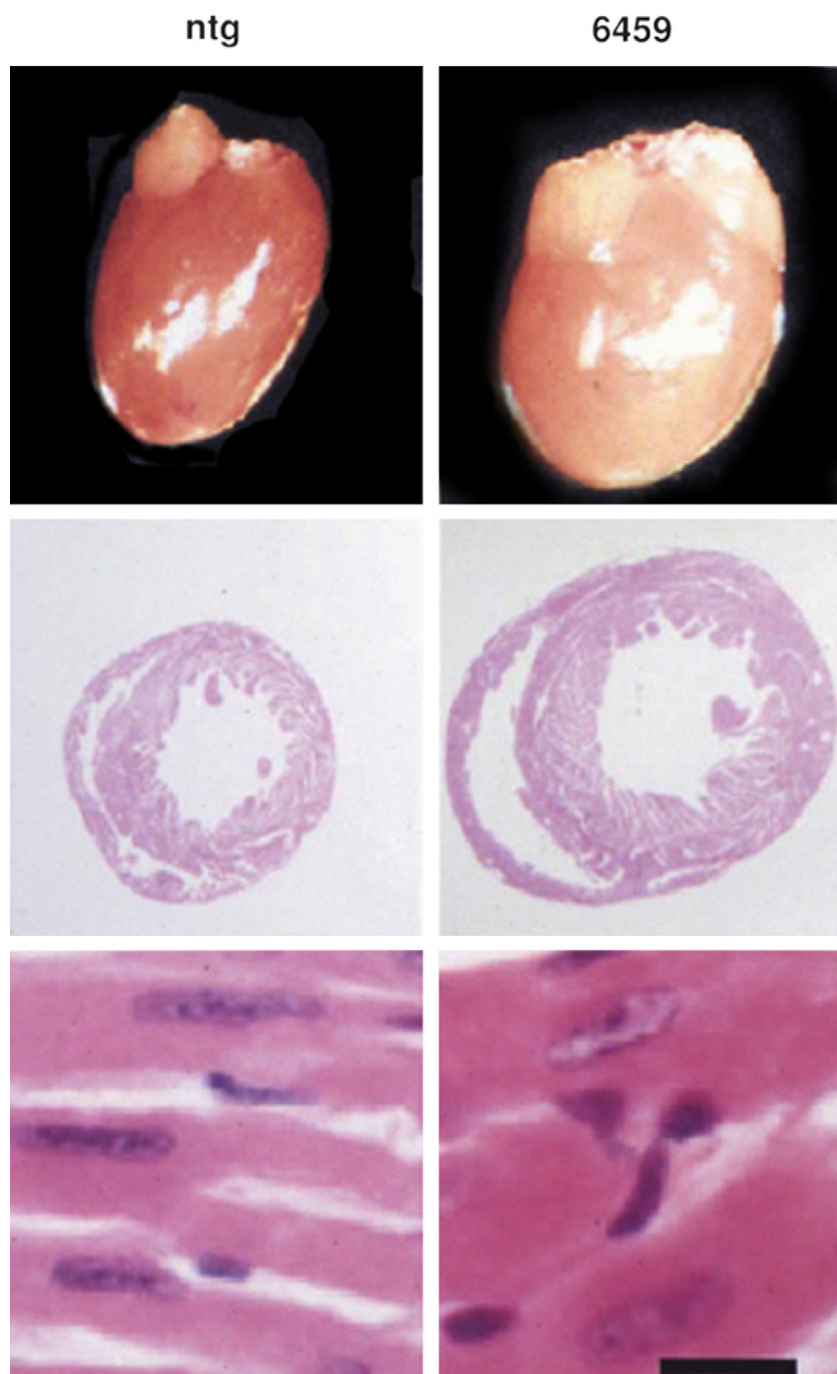
Figure 2.6.16 Transgenic and hypertrophied mouse hearts. Activation of Cdk9 by cyclin T1 in transgenic mice produced increase in heart weight/body weight ratio with 50% increase in myocardial cell size and concentric hypertrophy. *Reprinted with permission from* Nat. Med. *8, 1310–1317.*

improved survival in these older mice. This is one of the most robust findings in trials of CHF in humans and obviously could not be tested in the younger mice, which experienced no mortality. Using a modification of human criteria, we consider heart failure to be present in the mouse when at least one major criterion is present (rales, cardiomegaly, pulmonary oedema determined by elevated lung weight or wet/dry ratio, and decreased survival) as well as several minor criteria (hepatomegaly as determined by elevated liver weight or wet/dry ratio, pleural effusion, weight loss or weight gain, and decreased grooming).

Murine models in aortic disease

Aneurysmal aortic disease is a significant cause of morbidity and mortality in Western societies. Despite intensive research, the pathogenesis of aortic aneurysms remains unclear. Recently, murine studies using genetically altered animals have provided us with valuable insight into the mechanisms involved in aortic aneurysm formation. Periarterial application of calcium chloride and elastase perfusion have been established as convenient and reliable models for creating abdominal aortic aneurysms in mice. Elastase-induced aneurysmal degeneration was suppressed by treatment with a non-selective MMP inhibitor (doxycycline) and by targeted gene disruption of *Mmp9*, but not by isolated deficiency of *Mmp12* [139]. In addition, in a model of abdominal aortic aneurysm induction by abluminal application of calcium chloride, no aneurysm formation was observed after treatment in either the *Mmp9*-deficient or the *Mmp2* knockout mice [140]. Experimental murine aortic aneurysm generation may contribute to our understanding of aortic wall remodelling; however, it should be emphasized that the human disease process is pathogenically complex and may not be adequately simulated by existing experimental models.

Acknowledgements

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CHAPTER 2.7

The Respiratory Tract

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Introduction

The laboratory mouse has developed into the preferred model system for biomedical lung research [1]. Many investigators have turned to murine models of lung disease for several reasons. First, dense genetic and physical maps of the murine genome have been constructed and are in the public domain. Because of similar synteny in the murine and human genomes, knowledge of a defect in a murine gene leading to a disease phenotype may elucidate corresponding human genes responsible for that genotype. Second, the understanding of murine immunology has dramatically increased and inflammatory lung reactions have been analysed in detail. Third, technology has been developed that allows programmed overexpression of target genes or functional ablation of their protein products [2]. In addition, new technologies to construct partly humanized mice have

been developed. Therefore, the murine lung has become the focus of basic research, toxicology and drug development. New measurement techniques for physiological parameters, for example lung function measurements, have been developed. Although mice are genetically very closely related to humans, lung anatomy and function differ significantly (Table 2.7.1). In addition, there are striking differences in the physiology of the lung between mice and humans. For example, murine airway mast cells respond to a variety of stimuli, such as substance P and compound 48/80, with the release of serotonin rather than histamine, whereas these have no effect on human airways.

In spite of these differences, the mouse is now widely used for research on asthma, tumours, and chronic obstructive pulmonary disease (COPD) as well as for toxicological studies. For these models, the standard histological, molecular biological, and immunological tools that are available for the mouse have been adapted to the lung.

TABLE 2.7.1: Comparison between murine and human lungs		
	Mouse	**Human**
Anatomy	Right: 4 lobes, Left: 1 lobe	Right: 3 lobes, Left: 2 lobes
Diameter of main bronchus	1 mm	10–15 mm
Diameter of bronchioli	0.01–0.05 mm	<1 mm
Diameter of terminal bronchioli	0.01 mm	0.6 mm
Diameter of respiratory bronchioli	Not existent	0.5 mm
Diameter of alveoli	0.0039–0.0069 mm	0.2–0.4 mm

In contrast, lung function measurement in the mouse is extremely expensive because of the very small dimensions and the high breathing frequency. In this chapter, we will describe common pathological lesions of the respiratory tract and the state-of-the-art methods of analysing the physiology of the mouse lung and the main applications in biomedical research. The mouse will be compared with the human situation and other available models.

Anatomy and normal structure of the mouse lung

Though the mouse is used as a model for human lung disease, it should be taken into account that a mouse is not the exact equivalent of a human being. There are, in fact, some important differences relevant for the extrapolation from mouse to human. For example, structural differences in the murine respiratory tract compared to the human one might have an impact on the deposition and clearance of inhaled particles and organisms. The discrepancy is already apparent in the nasal cavity, where mice have got a proportionally larger nasal surface area than humans. Furthermore, mice are obligate nose breathers because their epiglottis and soft palate are close together [3]. Following the inhaled air down into the lung, further differences become evident. The tracheobronchial tree possesses smaller and fewer symmetrical branches in mice, with a zero branch angle at the carina. Furthermore, mice lack cartilage in the conducting airways beyond the main-stem bronchi, as well as the respiratory bronchioles that are found in humans [4]. In addition, the tracheobronchial epithelium of mice consists of fewer mucous and serous cells compared to that of humans and includes no submucosal glands [5], a structure commonly found in the human bronchi. Finally, there are approximately 2 000 000 alveoli with a diameter of 50 μm in mice compared to 300 000 000 alveoli with a diameter of 300 μm in humans (see Table 2.7.1). Moreover, there are also physiological differences, for example the differences in innervation which may be the cause of the inability of mice to cough in response to mechanical stimulation of the airway mucosa [6].

It is obvious that a mouse cannot be equated to a human being. However, various mouse strains also exhibit remarkable differences in response to microorganisms in their lungs. Therefore, it is also evident that a particular type of mouse cannot be considered truly representative of all different mouse strains [7]. Nevertheless, there are more similarities than differences when comparing reactions in human and mouse lungs. Although the limitations of mouse models should be taken into account when designing and interpreting experiments, they are very useful tools and results acquired from these models have already provided valuable insights into the biology of lung alterations in humans.

Immunology of the lung

Much of our current understanding of the immune reaction in the human lung has been

derived from studies carried out in mice. Analysis of cytokines, chemokines, growth factors and also cellular differentiation can easily be performed in bronchoalveolar lavage (BAL) fluid. After sacrificing the animals by cervical dislocation or an overdose of anaesthetic, the trachea is cannulated and airways are lavaged twice with 0.8 ml ice-cold saline containing proteinase inhibitor. BAL fluids from each mouse are pooled and the recovered volume and total cell number are determined. The cells can be analysed by flow cytometry and/or by analysing cytospin preparations using haematoxylin and eosin (H&E) staining. They can then be classified by light microscopy according to common morphological criteria. The cell-free supernatants can be stored and analysed for cytokine content. BAL fluid is a very powerful tool for analysing acute inflammatory reactions in infectious diseases and allergic immune reactions. However, it mainly represents the processes in the airways so analysis of the processes in the lung mucosa requires other methods such as measurements in lysates, or enzymatic lung digestion followed by cellular analysis and histological staining [8]. New developments in microscopy, such as two-photon microscopy, allow the high-resolution spatiotemporal characterization of airway immune reactions *in vivo* [9]. In addition, non-invasive technologies such as positron and single-photon emission computed tomography (SPECT) scanners, highly sensitive cameras for bioluminescence and fluorescence imaging and high-magnetic-field MRI scanners can be used to study and monitor lung inflammation in mice [10].

Physiology of the lung

Generation and deposition of aerosol and particles in the murine lung

For toxicological and pharmacological studies, the deposition of aerosols in the lung is critical. Since the murine airway architecture is very different from the human one, aerosol deposition also differs. Humans can inhale all particles in an aerosol with a diameter of up to 7 μm (total respiratory tract deposition). Small laboratory animals would only inhale about 55% of such an aerosol, but inhalability in these animals is predicted to be 95% or greater for particles up to approximately 0.7 μm diameter [11]. In conscious mice for particles with diameters of 1 and 6 μm the total respiratory tract deposition is 28% and 54% respectively, but due to the filter function of the nose, the total lung deposition is only 16% and 1.8% [12]. In contrast, in intubated animals lung deposition can be markedly increased (to >60% for particles typically around 1–3 μm).

Therefore, the technique of aerosol generation is very critical for lung function measurements involving aerosol administration. Aerosols can be generated by various dispersion and condensation processes. Particle generation starts from bulk material which is either liquid or powder. For dispersion, different physical mechanisms for breaking adhesive forces are employed. Nebulization of liquids can be achieved by interaction with a pressurized gas or by focusing ultrasonic energy on to the surface of the liquid [13, 14]. For liquid aerosols it may be reasonable to dry the aerosol to decrease the particle/droplet size to enable better lung deposition.

Methods of measuring lung function

The measurement of pulmonary function in mice is challenging because of the small dimensions and the resulting technical difficulties. Table 2.7.2 gives an idea of the difference in respiratory parameters between mice and humans. For COPD and asthma research, where broncho-obstruction is a cardinal phenomenon, flow limitation and the increase in resistance to airflow in the respiratory tract is a frequently used target parameter. In asthma models the *early airway response* (EAR), which is the specific response during and immediately after allergen exposure, as well as the *late airway response* (LAR) about 4–5 h later can be quantified by lung function measurements. In addition, the *airway hyperresponsiveness* (AHR), defined as an increased unspecific broncho-obstruction in response to

TABLE 2.7.2: Respiratory parameters in mice and humans			
Parameter	Mouse	Human	Unit
Tidal volume	0.16—0.20	500	ml
Respiratory rate	215—230	12	min^{-1}
Minute ventilation	33.5—47.5	6000	ml/min
Total lung capacity	0.9—1.44	6000	ml
Residual volume	0.11—0.14	1500	ml
Lung compliance	0.053—0.13	200	ml/cmH_2O
Airway resistance	1.5	0.0016	$cmH_2O/ml^{-1}\,s^{-1}$
Pa_{CO_2}	34—35	40	mmHg
Pa_{O_2}	78—84	80—100	mmHg
pH	7.37	7.4	

Source: *Adapted from Rao and Verkman, 2000.*

pharmacological stimuli such as methacholine, histamine or serotonin, is widely used [2]. The techniques available for the measurement of airway functions are given in Table 2.7.3.

Depending on which method is used for AHR measurement, different pathways can be distinguished: (i) altered neuronal regulation of airway tone, (ii) increases in muscle content or function and (iii) increased epithelial mucus production and airway oedema (Table 2.7.4; 15).

In vitro *electrical field stimulation*

It has been demonstrated that *in vitro* electrical field stimulation (EFS) of tracheal segments specifically reflects neuronal airway obstruction. Administration of both atropine (disruption of cholinergic pathways) and capsaicin (depletion of sensory neurons) completely blocks responsiveness of tracheal segments to EFS [16, 17].

Airway smooth muscle responsiveness can be assessed by EFS as described in Figure 2.7.1 [18, 19]. Tracheal smooth muscle segments (~ 0.5 cm) are removed and hung between triangular stainless steel wire supports. The contraction in response to EFS stimulus (12 V, 200 mA, 0.5-30 Hz) is measured by means of an isometric force transducer. The frequency that causes 50% of the maximum contraction is calculated from logarithmic plots of the contractile response versus the frequency of EFS and expressed as ES_{50}.

Ex vivo *lung function measurement in the isolated perfused lung*

Perfusion of murine lungs has only rarely been reported and has been restricted mostly to toxicological investigations. Stefan Uhlig's group adapted the technique of the isolated perfused lung to

TABLE 2.7.3: Frequently used methods available for the measurement of airway mechanics in the mouse		
In vitro/ex vivo	Invasive/in vivo	Non-invasive/in vivo
Isolated airway segments, e.g. EFS	Airway pressure measurements during mechanical ventilation	Dual chamber plethysmography
Isolated lung	Flow and pressure measurements during spontaneous respiration, pulmonary resistance, and compliance	Head-out plethysmography (EF_{50})
Precision-cut lung slice (PCLS), e.g. video analysis		Barometric plethysmography (PenH)

Source: *Adapted from Drazen* et al., *1999.*

TABLE 2.7.4: Target cells of different frequently used stimuli in lung function measurements		
Stimulus	Effector cells	Major pathways postulated
EFS	Sensory neurons Motor neurons	Unspecific Depolarization
Methacholine	Smooth muscle cells	M_3 receptors
Histamine	Smooth muscle cells Sensory neurons Motor neurons	H_1 receptors
Serotonin	Sensory neurons Motor neurons	5 HT_1 receptors 5 HT_3 receptors
Capsaicin	Sensory neurons	Vanilloid receptor
Hypotonic H_2O	Sensory neurons	Unspecific

the mouse as an expansion of the technique that had previously been described in detail for the isolated perfused rat lung [20-23]. The experimental setup for the isolated perfused mouse lung has been described in detail by von Bethmann et al. [24]. Use of the perfused murine lung allows several important features of lung physiology to be studied: (i) respiratory mechanics including pulmonary resistance and compliance (thus enabling measurements of

Figure 2.7.1 Schematic drawing of lung function measurement by EFS.

bronchoconstriction and AHR) [23]; (ii) vascular responsiveness [20] and (iii) mediator release, for example of cytokines such as tumour necrosis factor-alpha (TNFα) and interleukin (IL-6) [21, 24].

Ex vivo *precision-cut lung slices in combination with video microscopy*

A recently developed method for studying pulmonary responses is the use of precision-cut lung slices (PCLS), allowing microscopic investigation of the constriction [25, 20]. This method was validated against lung function measurements in mice *in vivo* [26] as well as using isolated perfused mouse lungs [20] and can be used for the characterization of airway and vascular responses in the murine lung. An interspecies comparison of mediator-induced bronchoconstriction was recently published by Seehase et al. In this study PCLS were stimulated with increasing concentrations of representative bronchoconstrictors such as methacholine (MCh), histamine, serotonin, leukotriene D_4 (LTD$_4$), U46619 and endothelin-1. Alterations in the airway calibre were compared between rodents, guinea-pigs, various primates, and humans. Striking differences between humans and mouse were found in response to mast cell mediators such as serotonin and histamine [27].

A major advantage of this method is the reduced use of animals, since up to 30 slices can be obtained from a single lung. In addition, functional pharmacological responses of small and

large airways under cell culture conditions can be observed.

In vivo *invasive lung function measurement*

Invasive measurement is the gold standard for exact determination of lung function, as it enables the measurement of pulmonary resistance and dynamic compliance, which are most specific and sensitive in quantifying airway obstruction (examples are given below). They are derived by calculating breath-by-breath continuous data for tidal airflow, tidal volume and transpulmonary pressure. A widely used technique for invasive measurement of lung function applies mechanical ventilation in paralysed, tracheostomized mice [28]. This method also allows superposition of low-frequency oscillations ('forced oscillation technique') to the test subject to measure input impedance [29]. This yields the advantage of differentiating between central airway resistance and tissue mechanics by determination of tissue resistance/damping and tissue elastance using the constant phase model (for review, see [30]).

Measurements in spontaneously breathing animals after endotracheal intubation via the oral route ('orotracheal intubation') are closer to physiological conditions than in ventilated animals and allow repeated studies. The mouse is placed in the supine position in a plethysmograph and respiratory flow and transpulmonary pressure are measured by means of pressure transducers. This technique has been extensively published for rats [31–33] and was adapted to the mouse between 1999 and 2005 [14, 34, 35] (see Figure 2.7.2). Well-defined aerosol treatments and challenges can be performed. The major advantages are: (i) spontaneously breathing animals, (ii) diagnostic precision, (iii) high deposition and precise dosage of aerosols for drug treatment or challenges, (iv) exclusion of the nasal passages when the lung is the target of interest and (v) repeated measurements are possible. A disadvantage is that measurements of transpulmonary pressure and tidal airflow are technically difficult and require extensive special equipment. In addition, the animals must be anaesthetized. Anaesthetic agents may alter the lung function due to changes in
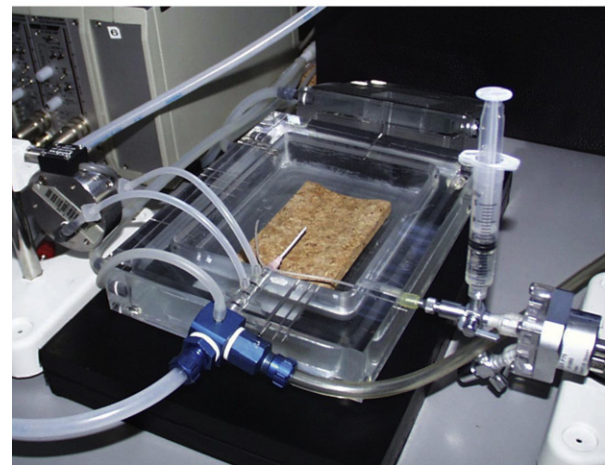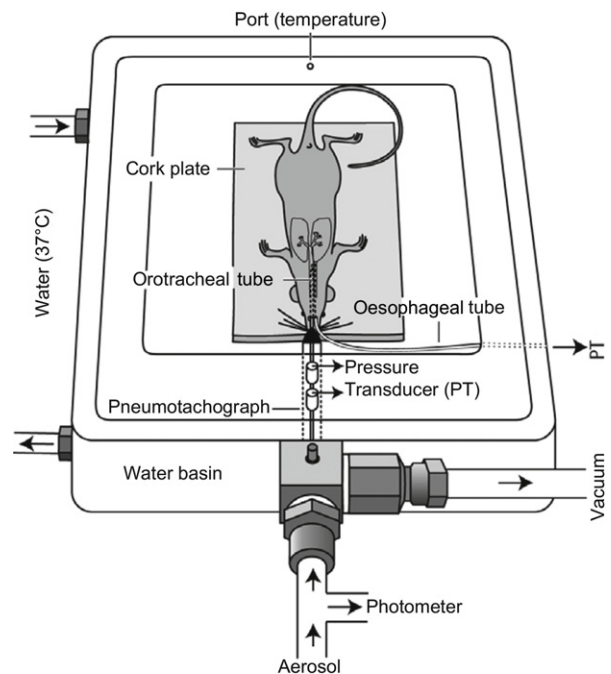
Figure 2.7.2 Schematic drawing and photograph of a plethysmograph (Fraunhofer ITEM, Hannover, Germany) used for pulmonary function testing of anaesthetized, orotracheally intubated mice. A thermostat-controlled water basin (37 °C) built into the plethysmograph chamber ensures a body temperature of 35 °C. For the calculation of lung resistance, transpulmonary pressure was recorded via an oesophageal tube and tidal flow was determined by a pneumotachograph tube attached directly to the orotracheal tube. *Schematic drawing modified from Glaab et al., 2004, with permission of the American Physiological Society.*

neuronal function [2]. Airway responses in the intact animal depend on airway smooth muscle contractility, chest wall compliance, bronchiolar mucus plugging, airway fibrosis and other factors [36].

Using this invasive technique, it has been shown that the EAR can be determined in the orotracheally intubated mouse during and immediately after allergen exposure, and it is quantified as the increase in lung resistance and the decrease in dynamic compliance [37, 38]. An example is given in Figure 2.7.3. Additionally, the AHR in the late allergic phase can be determined in response to inhaled MCh or histamine in increasing dose steps in the same individuals 24 or 48 h later. AHR is assessed, for example, by relating the percentage change in lung resistance to the measured amount of the contractile stimulus (e.g. MCh), plotting a dose–response curve. An effective dose required to achieve a certain contractile response level can be calculated by using the complete dose–response curve data of an animal: $ED_{100}R_L$, for example, gives the MCh dose required for a 100% increase in resistance. This analysis yields the most stable measure for assessing AHR [39].

In vivo *non-invasive lung function: head-out plethysmography*

An alternative way to measure lung function in the mouse is head-out body plethysmography (Figure 2.7.4). Originally developed for toxicological studies [40, 41], the system has been adapted for asthma research [8, 42, 43]. Up to four mice can be placed in four body plethysmographs
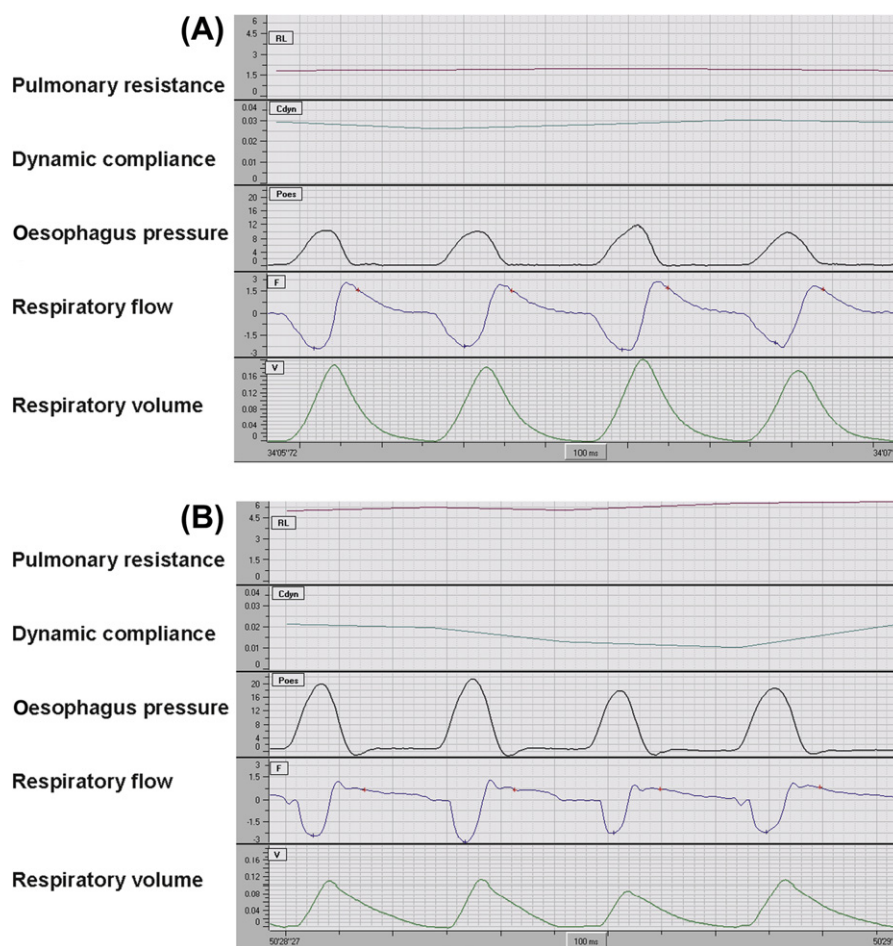
Figure 2.7.3 Invasive lung function measurement in the intubated anaesthetized mouse. (A) Normal respiratory pattern before challenge: base values of lung resistance, dynamic compliance, oesophagus pressure, respiratory flow and volume (from top to bottom); the x-axis shows the time (1 mark corresponds to 1/10 s); (B) signals immediately after inhalation challenge with ovalbumin: increase in resistance and oesophagus pressure as well as decrease in respiratory flow and volume. *Figure by H. G. Hoymann, Fraunhofer ITEM, Hannover, Germany.*
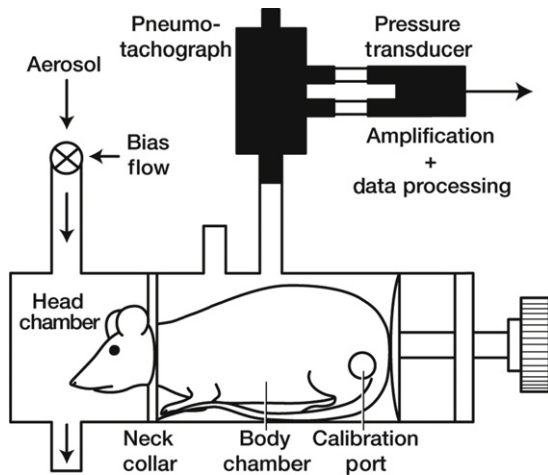
Figure 2.7.4 Schematic drawing of the head-out body plethysmograph and photograph of a complete system (Fraunhofer ITEM, Hannover, Germany). The figure illustrates the exposure system and the equipment used for the measurement of tidal mid-expiratory flow (EF_{50}), tidal volume (VT), expiratory time (TE), inspiratory time (TI) and breathing frequency (f). The conscious animal was placed in a glass plethysmograph that was attached to a head exposure chamber (see photograph: chamber with graph plethysmographs). The output of a jet nebulizer was directed to the inlet of the head exposure chamber that was continuously ventilated with a bias flow of 0.2 l/min. Before data collection, mice were allowed to acclimatize for 15 min in the body plethysmograph. *Schematic drawing adapted from Glaab* et al., *2001.*

attached to an exposure chamber (Figure 2.7.4). Airflow is measured by means of a differential pressure transducer. For the determination of bronchoconstriction, the midexpiratory airflow (EF_{50}), i.e. the expiratory airflow when 50% of the tidal volume is exhaled, is calculated (see Figure 2.7.5 and Table 2.7.5). Changes in EF_{50} in response to bronchoconstricting agonists such as MCh or to allergen delivered by an aerosol generator can be measured during aerosol exposure. The major advantages of this system are: (i) spontaneously breathing animals, (ii) simultaneous analysis of four animals, (iii) no anaesthesia required, (iv) lung function measurement during aerosol challenge and (v) repeated measurements are possible. Disadvantages: the classical lung function parameters such as lung resistance and
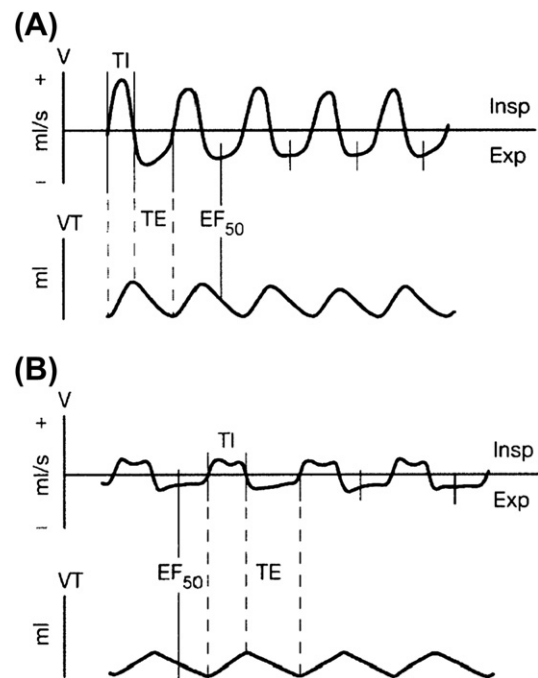
Figure 2.7.5 Head-out plethysmography: characteristic modifications to the normal breathing pattern in unanaesthetized BALB/c mice. (A) Normal breathing pattern of BALB/c mice while breathing room air; (B) characteristic pattern of airway obstruction during aerosol challenge with methacholine, illustrating the decline in EF_{50}. (A) and (B), above: pneumotachograph airflow signals recorded at 40 mm/s. (A) and (B), below: corresponding integrated VT signal as calculated by the computer program from the collected voltage digitalizations. A horizontal line at 0 flow separates inspiratory (Insp; upwards; 1) from expiratory (Exp; downwards; 2) airflow. V, tidal flow. *Adapted from Glaab* et al., *2001.*

TABLE 2.7.5: Lung function measurement by head-out body plethysmography of frequently used mouse strains at 10 weeks of age

	EF5O (ml/min)		Tidal volume (ml)		Frequency (breath/min)	
	Mean	Range	Mean	Range	Mean	Range
CBAJ	1.89	0.91	0.19	0.09	226.40	107.92
C57BL/6J	1.97	0.90	0.17	0.08	258.74	122.08
BALB/c	1.92	0.90	0.14	0.06	267.63	127.00

Source: *R. Bälder, Fraunhofer ITEM, Hannover, Germany.*

compliance are not available, and nasal passage of the inhaled air filters a major part of the delivered aerosols.

In vivo *unrestrained barometric whole-body plethysmography*

Barometric whole-body plethysmography (BWBP), also called unrestrained plethysmography (UP), with its main parameter Penh (the 'enhanced pause'), represents the extreme of non-invasiveness. For barometric plethysmography, unrestrained animals are placed in a chamber and the pressure fluctuations that occur due to breathing are recorded (Figure 2.7.6) [44]. The pressure difference between the main chamber containing the animal and a reference chamber is measured. These box pressure changes are considered to be caused by volume and resultant pressure changes in the main chamber during the respiratory cycle of the animal. From the resulting signal, several parameters such as expiration time or relaxation time can be calculated. For the determination of broncho-obstruction, Penh, a dimensionless variable, is calculated [44]. This method has been widely used for the determination of AHR in asthma models (Figure 2.7.7) [2, 45], but the physiological meaning of Penh is considered controversial (for a short summary see [46]. The advantages of this method are: (i) measurement in conscious, spontaneously breathing animals; (ii) no anaesthesia required; (iii) lung function measurement during aerosol challenge; (iv) repeated measurements are possible and (v) measurements are easy to perform with commercially available equipment (e.g. Buxco). Disadvantages: classical lung function parameters such as lung resistance and compliance or respiratory flow are not available, and the respiratory measures (i.e. Penh) are so tenuously linked to respiratory mechanics that several authors have emphasized the danger of the increasing uncritical use of Penh, with potentially misleading assessment of pulmonary function in animal models of lung
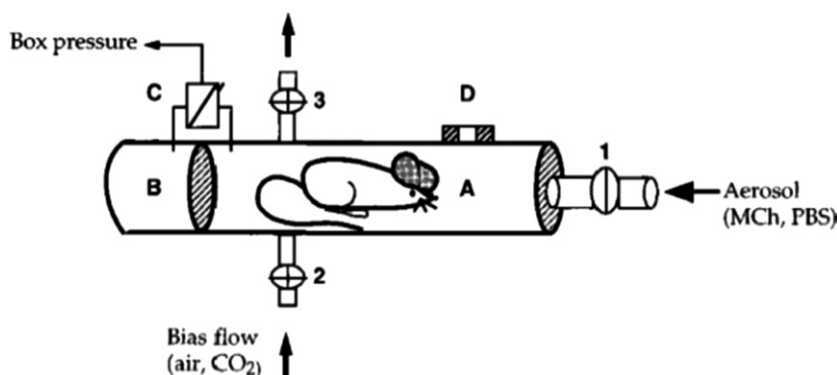
Figure 2.7.6 Schematic diagram of the barometric whole-body plethysmograph. (A) Main chamber containing the mouse, (B) reference chamber, (C) pressure transducer connected to analyser, (D) pneumotachograph, (1) main inlet for aerosol closed by valve, (2) inlet for bias flow with four-way stopcock, (3) outlet for aerosol with four-way stopcock. *Adapted from Hamelmann* et al.*, 1997.*
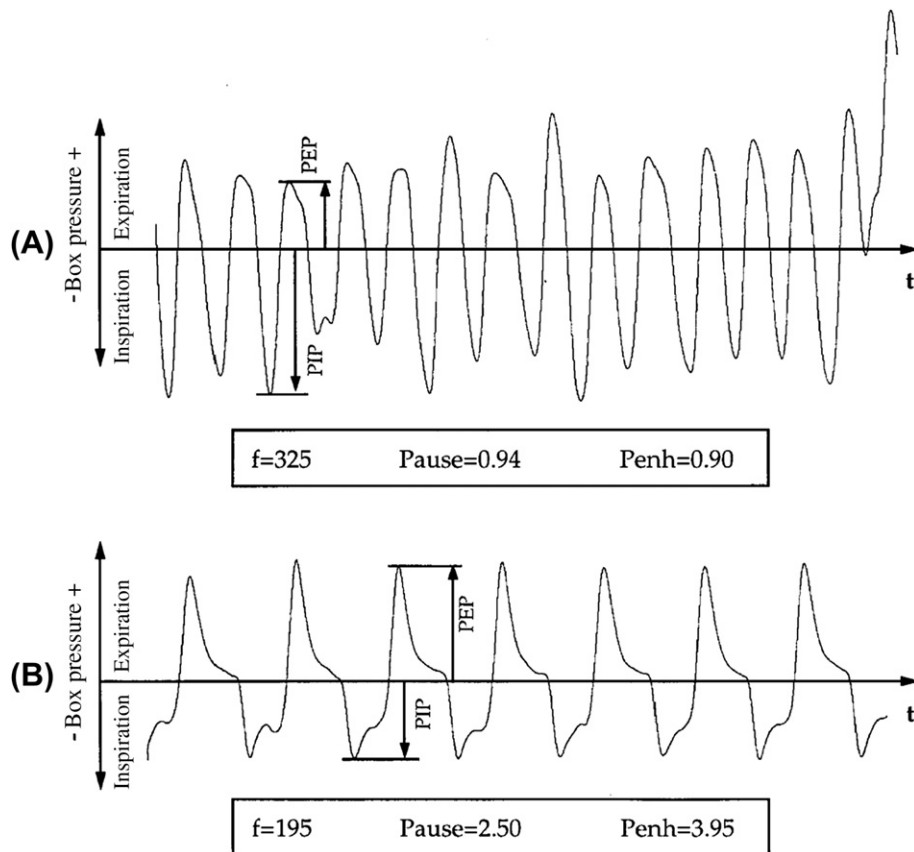
Figure 2.7.7 Barometric whole-body plethysmography: changes in box pressure waveform after methacholine challenge. Waveform of the box pressure signal derived from a normal mouse after 3 min of nebulization with (A) aerosolized PBS or (B) aerosolized methacholine (50 mg/ml in PBS). f, respiratory rate (breaths/min); Pause, Penh (enhanced pause); PIP, peak inspiratory pressure (ml/s), maximal negative box pressure occurring in one breath; PEP, peak expiratory pressure (ml/s), maximal positive box pressure occurring in one breath. *Adapted from Hamelmann* et al.*, 1997.*

disease [46-50]. Penh is an empirical variable which has been shown to be primarily related to ventilatory timing and unrelated to airway resistance [48, 49, 51].

Lung diseases and pathology

Murine models of asthma

Human asthma is characterized by variable airflow obstruction in response to allergen (EAR and LAR) and AHR in the late allergic phase. Structurally, the airways of asthmatics are characterized by the presence of chronic allergic inflammation with intense infiltration of the bronchial mucosa by lymphocytes (especially T cells, type Th2) and eosinophilic granulocytes, accompanied by epithelial desquamation, goblet cell hyperplasia and thickening of the submucosa (airway remodelling) [15]. An animal model of asthma must reproduce these features of the human disease to be credible.

Several murine models that show particular features of asthma have been developed. In standard protocols, inbred mice are first systemically sensitized to an allergen and then challenged with aerosol. The following points are critical for the induction of an asthma-like phenotype with allergic airway inflammation and AHR:

1. *Selection of the antigen:* frequently used allergens are proteins (e.g. ovalbumin and *Aspergillus fumigatus* extract) and microorganisms (e.g. *Aspergillus fumigatus, Schistosoma mansoni* egg). To avoid a mixed form of allergen- and bacterial endotoxin-caused disease, endotoxin-free allergens

should be used (for the influence of endotoxin contamination of ovalbumin, see [52, 53].

2. *Selection of the mouse strain:* this strongly depends on the allergen used. BALB/c mice are often used for ovalbumin protocols.

3. *Selection of the protocol:* the protocol should ensure reproducible sensitization to the allergen (e.g. repeated systemic injection of the allergen associated with an adjuvant such as alum) and induction of a long-lasting allergic airway inflammation (e.g. repeated aerosol challenges over weeks).

An excellent overview of the most commonly used protocols has been given by Lloyd et al. [54]. The acute models are very effective in inducing acute airway inflammation—involving mainly Th2 cells and eosinophils—and AHR [38, 55–57]. In order to induce chronic inflammation, the protocols were developed further. Multiple aerosol challenges and/or live *Aspergillus fumigatus* conidia were used to induce a long-lasting (chronic) inflammation in the airways that was associated with airway remodelling and AHR [8, 58–61]. Recently, a model of asthma exacerbation has been presented. Rhinovirus-induced exacerbation of allergic airway inflammation using minor-group rhinovirus infection of BALB/c mice, or major-group rhinovirus infection of transgenic BALB/c mice expressing a mouse–human ICAM-1 chimera, are examples of this development [62]. Further progress in developing these models (using protocols with repeated allergen challenges) and improvement in lung function measurements have made it possible to acquire early- as well as late-phase responses after allergen provocation in mice [38, 43, 63, 64]. Most of these new therapeutic strategies for asthma, which are designed to neutralize central mediators in asthma pathology, were developed and tested in murine asthma models. Prominent examples are anti-IgE, anti-interleukin(IL)-5, anti-IL-4, anti-IL-13, anti-eotaxin, anti-CD4/8, anti-TNFα and PDE4 inhibitors (for a review, see [54, 65]).

Murine models of chronic obstructive pulmonary disease

The term chronic obstructive pulmonary disease (COPD) summarizes several disease patterns with chronic small airway obstruction which is not fully reversible and with developing lung emphysema as the disease progresses. COPD can be distinguished from chronic bronchitis by the airway obstruction and from asthma by the lack of reversibility of this obstruction, although there may be overlaps in the disease patterns. The main causes of COPD are cigarette smoking and air pollution (e.g. sulfur oxide and particulates). The disease is associated with inflammatory changes and infiltration of neutrophils and macrophages into the lung [66]. Animal models of COPD are extremely difficult to establish, since the human disease develops over decades. Models of the cardinal phenomenon, neutrophil inflammation, have been created by using acute to subacute exposure to cigarette smoke, ozone or LPS aerosol. Exposure to tobacco smoke over as long as 6 months has been shown to induce emphysema. Other possibilities are the use of genetically altered animals. There are some natural mutations, such as the blotchy mouse that develops a connective tissue disorder, or knockout animals. Knockout models of emphysema include those for platelet-derived growth factor A (PDGFA) and the double knockout for fibroblast growth factors 3 and 4 (FGF3 and 4; for review, see [67–69]).

Murine models of lung infection

Unfortunately, there is no one mouse model that shows all facets of a human disease. However, there are some mouse models that mimic certain parts of the compatible human lung infection. For example, the pneumonia virus of mice is used to investigate human respiratory syncytial virus infection [70, 71].

Other mouse models, such as the acute and chronic lung infections with *Pseudomonas aeruginosa* (Figures 2.7.8 and 2.7.9), *Burkholderia cenocepacia*, *Staphylococcus aureus*, and *Haemophilus influenzae*, are used to investigate the molecular mechanism between host defence and pathogen virulence [72–74]. This is of interest especially when additional susceptibility factors such as cystic fibrosis come into play. For this situation, there are a lot of mouse models with a genetically
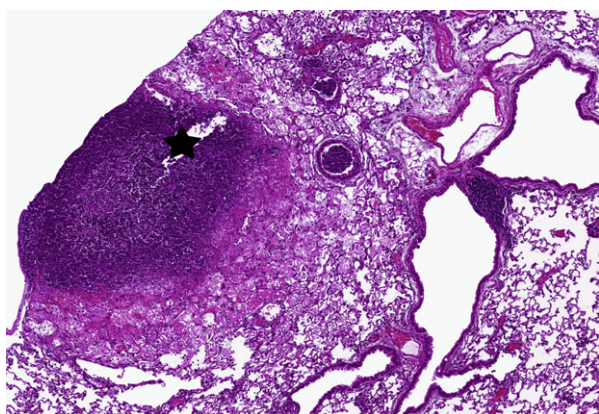
Figure 2.7.8 Mouse after experimental infection with *Pseudomonas aeruginosa* showing an inflammatory reaction with abscess formation (asterisk).
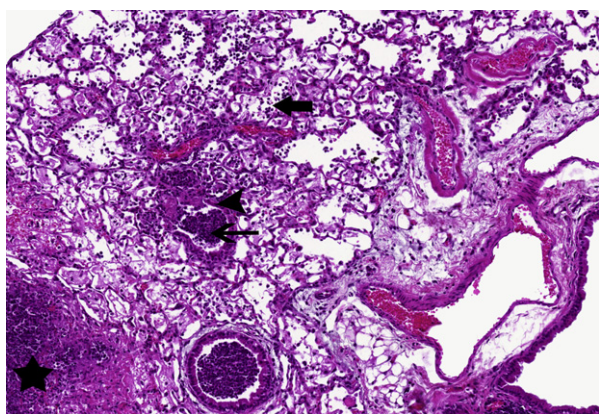
Figure 2.7.9 Higher magnification of Figure 2.7.8. This image details the bronchopneumonia with intra-alveolar (bold arrow) and intrabronchiolar (arrow) neutrophils and degeneration of the epithelium (arrowhead). Margin of abscess formation at the left bottom (asterisk).

engineered *Cftr* gene, which encodes an epithelial chloride channel (for review, see [72]).

Murine models of inhalation toxicology

Changes within the respiratory tract upon inhalation of toxicants depend on the characteristics of the toxicant and on the type of tissue exposed. For example, the olfactory and the ciliated columnar epithelia are most susceptible to inhaled toxicants compared to the more resistant cuboidal and squamous epithelia [75]. In addition, specialized cells such as Clara cells on the one hand are capable of decreasing the toxicity of compounds;

on the other hand, due to their high amount of metabolizing enzymes, they are vulnerable to inhaled or ingested compounds requiring metabolic activation to become cytotoxic [76]. In addition to the different cellular susceptibilities in the respiratory tract, there are specific anatomical sites which represent predilection sites for alterations, such as the ventral pouch of the larynx, the carina or the junctions of terminal bronchioles and alveolar ducts (for more details, see [75]).

Toxicology of particles

As already mentioned above, particle deposition differs between mice and humans and also depends on particle size, with smaller particles being able to reach deeper into the lung, up to the alveoli. The toxicity of these inhaled particles is determined to a large extent by the site where they are deposited in the respiratory tract. However, the respiratory tract has defence mechanisms to clear inhaled particles from the airways. Particles deposited on the ciliated epithelium are transported up to the larynx, where they are either exhaled or swallowed. Smaller particles reaching the alveoli are phagocytized by macrophages and then cleared either via the mucociliar apparatus or via lymphatic drainage from the interstitium of the lungs. Therefore, the interstitium and the lung-associated lymph nodes represent important accumulation sites for many particles. In contrast, single very small particles, for example nanoparticles, are only ineffectively cleared from the alveoli by macrophages and therefore have a longer-lasting contact with the alveoli epithelium. Overall, nanoparticles are able to cause more alterations and more severe changes due to their smaller size and increased specific surface area compared to their bulk.

In recent years, health risks arising from particulate matter in the environment or from nanoparticles to which workers are exposed during manufacturing processes have attracted increasing attention. It is well known that inorganic particles can cause severe lung diseases including pneumoconiosis, COPD and cancer. Consequently, mouse models are needed to further investigate possible alterations and pathogenic mechanisms of inhaled particles.

Diesel exhaust and quartz particles have been used intensively in mouse inhalation studies for

a long time. In a study performed at the Fraunhofer ITEM, NMRI mice after inhalation of $7.5\,mg/m^3$ diesel exhaust, Printex 90® or nano-titanium dioxide (p25) for 19 h per day, 5 days per week over a period of 6 months showed mild degeneration of respiratory and olfactory epithelial cells in combination with an increased incidence of eosinophilic intraepithelial inclusions in the nasal cavity [77, 78]. The lungs of almost all of these animals exhibited peribronchial and intra-alveolar infiltration of particle-laden macrophages and free particulate agglomerates in the alveoli (Figures 2.7.10 and 2.7.11). Some animals showed focal alveolar lipoproteinosis. In a few mice, bronchiolar–alveolar hyperplasia was visible. In a similar study using a lower concentration ($4.5\,mg/m^3$) of diesel
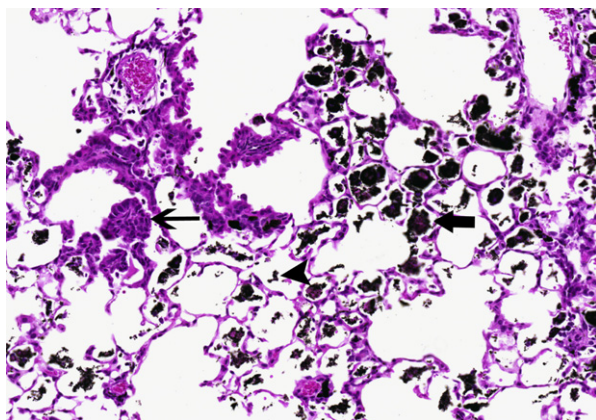
Figure 2.7.10 Mouse after inhalation of diesel exhaust. Particle-laden macrophages in the alveoli (bold arrow) and interstitium, free intra-alveolar particles and bronchiolar hyperplasia (arrow).
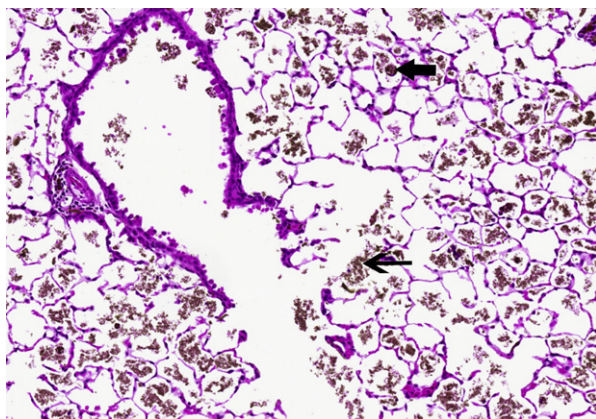
Figure 2.7.11 Mouse after inhalation of titanium dioxide. Particle-laden macrophages within the alveoli (bold arrow), bronchiolus and interstitium as well as free intra-alveolar particles (arrow).

exhaust, C57BL mice exhibited a lower incidence of degenerative changes within the nasal cavity compared to NMRI mice [77, 78]. It should be kept in mind that so-called 'traditional' diesel exhaust is quantitatively and qualitatively very different from today's diesel exhaust, which has been improved by new technologies [79]. Nevertheless, exposure to diesel exhaust particles also has an increasing effect on the sensitization of mice in different mouse models (for review, see [80]). Interestingly, the combination of nanoparticles with different mouse models also leads to an amplifying effect of the particles on the changes normally seen in these models. For example, administration of nano-titanium to the allergic mouse model acquired via administration of ovalbumin further promotes the allergic sensitization [81]. Similarly, exposure to carbon nanotubes exacerbates allergic inflammation of the airways in the same mouse model [82, 83]. Furthermore, inhalation of nickel nanoparticles exacerbates atherosclerosis in the susceptible *ApoE* constitutive knockout mouse model [84].

Comparing the changes in mice caused by inhalation of particles to those in rats, some differences are evident. While rats develop tumours after exposure to diesel exhaust, mice and Syrian hamsters lack this carcinogenic effect [85]. Additionally, a high particulate burden in the lung leads to an overload situation of the lung in rats, whereas mice need a much higher particle concentration to develop a similar condition. Overload of the lung is defined as a particular burden which exceeds the clearance capacity of alveolar macrophages, leading to particle retention in the lung and consequently to accumulation of particle-laden macrophages and persistent inflammation [86].

Non-neoplastic lesions

In this section several non-neoplastic lesions occurring in the respiratory tract of mice are briefly addressed, including some murine models of pulmonary diseases in humans. Most of the described lesions are proliferative and potentially

preneoplastic in nature; they usually develop in response to inhalation of toxicants or particulates, but may also occur spontaneously. Specific infectious diseases are not included in this chapter but are dealt with elsewhere in this book.

Nasal cavity, larynx, trachea

Epithelial hyperplasia

Hyperplastic changes are usually observed as an adaptive response to irritant insults and may involve all cell types that are normally found in the upper respiratory tract, i.e. cells of the squamous, transitional, respiratory and olfactory epithelia as well as basal cells, neuroendocrine cells and cells of the subepithelial glands [75, 87]. Epithelial hyperplasia is often associated with degeneration and inflammation, but is frequently a reversible lesion. Hyperplasia is characterized by a thickening of the epithelium due to a numeric increase in the respective cell type and may result in an undulating rugose surface of the epithelium. In mucous (goblet) cell hyperplasia, which is always non-preneoplastic, mucosal invagination with formation of intraepithelial 'pseudoglands' may occur. Epithelial hyperplasia can also include proliferation of atypical or pleomorphic basal or undifferentiated cells (a potentially preneoplastic lesion), but without disruption of the underlying basal lamina [75].

Squamous cell metaplasia

Squamous cell metaplasia sometimes occurs in association with chronic inflammation or with epithelial regeneration and hyperplasia and characterizes the replacement of the more susceptible respiratory or olfactory epithelium by the more resistant squamous epithelium [88]. Depending on the cause, squamous cell metaplasia may be either fully reversible or preneoplastic, giving rise to squamous cell papilloma or carcinoma. In the larynx, squamous cell metaplasia affects mainly the base of the epiglottis, the medial aspects of the arytenoid projections and the area anterior and lateral to the ventral pouches [75].

Respiratory epithelial metaplasia

Following focal atrophy and degeneration, olfactory epithelium in the nasal cavity may be
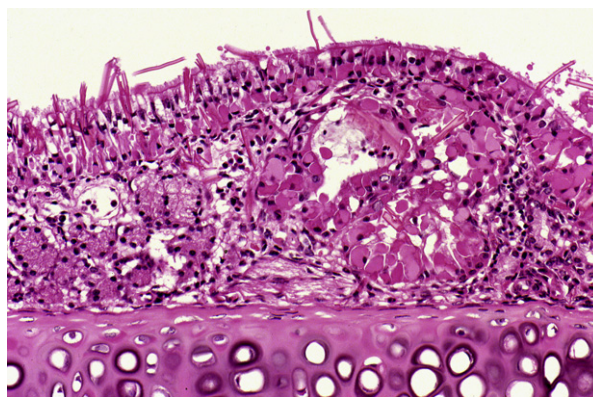
Figure 2.7.12 Eosinophilic globules and crystal formation within olfactory respiratory epithelium and epithelium of submucosal glands in the nasal cavity.

replaced by a columnar epithelium, with or without cilia, that resembles respiratory epithelium. In the area of respiratory epithelial metaplasia, subepithelial glands are also usually replaced by respiratory epithelium. There is no evidence that respiratory epithelial metaplasia is preneoplastic [75].

Eosinophilic globules (droplets)

Eosinophilic globules are occasionally seen in sustentacular cells of the olfactory epithelium, respiratory epithelial cells and epithelial cells of the nasal seromucous glands (Figure 2.7.12). Their occurrence is age-related and considered to represent either a non-specific adaptive response [87] or a degenerative change [75]. In advanced cases these eosinophilic hyaline inclusions may be associated with intra- and/or extracellular crystal formation and are also found in other organs including the lung (see below). Eosinophilic globules have been shown to react with antibodies to carboxylesterase and with antibodies to the Yml sequence of the protein Ym2, a member of the chitinase family [89, 90].

Lung

Alveolar macrophage aggregation (alveolar histiocytosis)

This lesion describes focal intra-alveolar accumulations of macrophages (histiocytes) containing
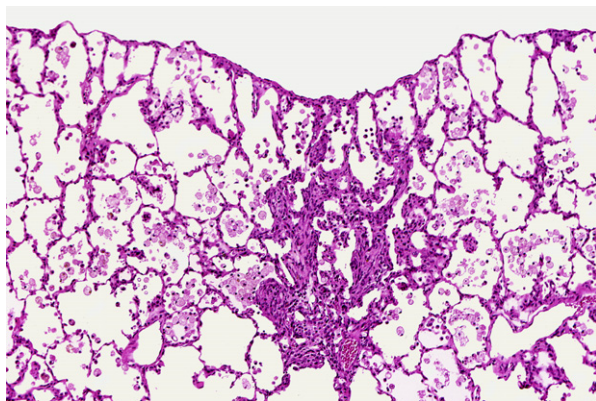
Figure 2.7.13 Subpleural alveolar macrophage aggregation, inflammation and focal interstitial fibrosis.

foamy lipid material [75, 91]. It frequently occurs spontaneously in subpleural areas of aged animals and may be associated with mixed inflammatory cell infiltration, focal fibrosis, and sometimes with cholesterol clefts (Figure 2.7.13). Some macrophages may contain haemosiderin derived from phagocytized erythrocytes from preceding minor haemorrhages. Influx and accumulation of alveolar macrophages is also the first response to inhalation of toxicants or particles due to release of chemotactic factors through complement fixation or following phagocytosis of cytotoxic particles with subsequent death of macrophages [92].

Alveolar lipoproteinosis and related disease models

Alveolar lipoproteinosis is a rare finding in mice, characterized by the presence of intra-alveolar PAS-positive eosinophilic material (lipoprotein). Lipoproteinosis usually also involves accumulation of intra-alveolar foamy macrophages, especially if the pathogenesis includes defective or decreased clearance of the lipoproteinaceous material by alveolar macrophages. In the case of excessive production of surfactant, hypertrophic/hyperplastic alveolar type II cells are observed. Probably due to a defect in surfactant homeostasis, immunodeficient SCID mice (CB.17-*Prkdc*^{scid}/*Prkdc*^{scid}) and SCID-beige (*Prkdc*^{scid}/*Prkdc*^{scid} *Lyst*^{bg}/*Lyst*^{bg}) mice develop spontaneous alveolar lipoproteinosis with marked increases in phosphatidylcholine and surfactant proteins A and B in the alveolar lipoproteinaceous material [93, 94]. Surfactant proteins and phospholipids also accumulate in the alveolar spaces of mice deficient in granulocyte-macrophage colony-stimulating factor (GM-CSF) [95, 96].

A mouse model of Niemann–Pick disease characterized by excessive accumulation of lipoproteins in the reticuloendothelial system and in the lungs has been described in the ASM (acid sphingomyelinase) knockout (ASMKO) mouse [97, 98]. Due to defective catabolism of surfactant lipid by alveolar macrophages (deficient activity of lysosomal acid sphingomyelinase), these mice develop alveolar lipoproteinosis with accumulation of abnormal alveolar macrophages and pulmonary inflammation.

Eosinophilic crystalline inclusions and pneumonia

Endogenously formed eosinophilic crystalline inclusions within the cytoplasm of macrophages and epithelial cells or extracellular crystals have been reported in the respiratory system of several strains of mice. Ultrastructurally, crystals within the cytoplasm of alveolar macrophages were needle-shaped, showed a lattice pattern, and appeared to lie in membrane-bound vacuoles [99]. The crystals stain strongly eosinophilic with H & E, are black-blue with toluidine blue, and Alcian blue- and PAS-negative [100]. Crystal-laden macrophages or free-lying intra-alveolar crystals are often associated with concurrent pulmonary diseases such as parasitic or fungal infections, hypersensitivity reactions or pulmonary neoplasms [89, 90]. In aged mice crystalline deposits may also be seen within gland-like infoldings of the tracheobronchial epithelium.

Eosinophilic crystalline pneumonia (also called 'acidophilic macrophage pneumonia'; Figure 2.7.14) is a sporadic, idiopathic pulmonary disease of certain naturally occurring mouse strains (C57BL/6, 129/SvJae), specific mutant strains such as *Ptpn6*^{me} (motheaten), severe combined immunodeficiency (SCID) mice, or knockout mice such as the CYP1A2 null strain [90, 99, 101]. This type of pneumonia is histologically characterized by the simultaneous presence of crystal-laden macrophages and multinucleate giant cells, alveolar crystalline deposits and other inflammatory cells [99, 100]. Composition of the crystalline material may be very heterogeneous.
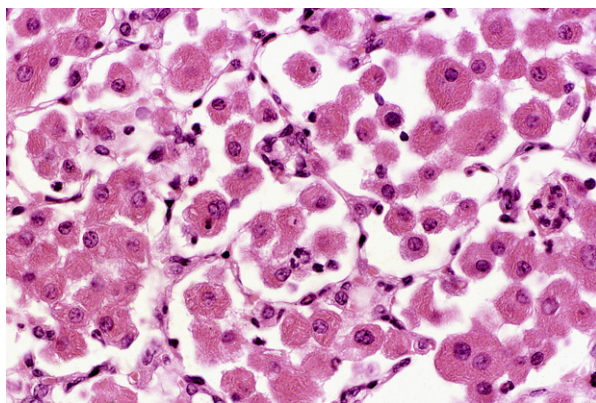
Figure 2.7.14 Eosinophilic crystalline pneumonia. Massive intra-alveolar accumulation of macrophages with eosinophilic crystalline inclusions.

When related to pulmonary haemorrhage, the crystals stain strongly positive for haemoglobin or are morphologically indistinguishable from haematoidin, which results from haemoglobin breakdown following uptake of erythrocytes by alveolar macrophages [102]. Other crystalline inclusions contained various amounts of alpha-1-antitrypsin, IgG and IgA [103]. The crystalloid inclusions of alveolar macrophages in eosinophilic crystalline pneumonia have been proposed to be derived from breakdown products of granulocytes, especially eosinophils [99]. In motheaten ($Ptpn6^{me}$) mice pulmonary crystal formation represents a major feature of the fatal lung inflammation induced by macrophage dysregulation [89, 104]. The alveolar macrophages of these animals, which have a mean lifespan of about 10 weeks, produce significantly increased levels of TNFα [105]. Mass spectrometry analysis of proteins from BAL fluid of motheaten ($Ptpn6^{me}$) mice has identified the crystals as Ym1 protein (T-lymphocyte-derived eosinophil chemotactic factor), which is a member of the chitinase family of proteins [89]. Although the function of Ym1 is largely unknown, the protein is considered to play important roles in haematopoiesis, tissue remodelling, and host immune responses [101]. Ym1 protein has especially been implicated in immune responses to organisms that contain chitin, such as fungi or parasites [89, 90, 106].

Pulmonary fibrosis and related models

The definition of pulmonary fibrosis refers to an increased amount, abnormal location or abnormal nature of collagen in the lung parenchyma (Figure 2.7.13), resulting in disruption of the normal lung architecture. A further differentiation may be used to distinguish potentially reversible fibrogenesis (fibroblast proliferation) with minimal cross-linking of collagen from irreversible pulmonary fibrosis with extensive cross-linking [75, 92, 107]. Pulmonary fibrosis is considered as the end-point of chronic inflammation due to continuous release of fibrogenic cytokines and fibronectin from activated alveolar macrophages and other inflammatory cells following exposure of the lungs to various toxicants, irritant gases or particles. In laboratory mice, however, the fibrotic response, for example to inhalation of mineral fibres or particles such as ultrafine titanium dioxide or carbon black, is generally much less pronounced than in laboratory rats [108, 109].

Several mouse models of pulmonary fibrosis have been developed to identify key cells, mediators and processes which are likely involved in human idiopathic pulmonary fibrosis. The bleomycin (antibiotic isolated from *Streptomyces verticillatus*) model of pulmonary fibrosis is the best-characterized model in use today [110]. Bleomycin is capable of inducing lung injury and fibrosis in a wide variety of laboratory animal species (rodents, dogs, primates) by different routes of application and at different doses. Bleomycin induces degeneration and necrosis of pulmonary endothelial and epithelial cells with leakage of fluid and plasma proteins into the alveoli, formation of hyaline membranes, inflammation and subsequent development of diffuse interstitial fibrosis [110, 111]. The time frame for development of fibrosis in mice is 14–28 days, but the fibrotic response is strain-dependent, probably due to differences in the expression of the inactivating enzyme bleomycin hydrolase [110]. While C57BL/6 and CBA/J mice are highly susceptible to bleomycin, BALB/c mice are very resistant to the drug [111]. Following a single intratracheal application of bleomycin, the fibrotic response has been reported to be self-limiting after 28 days. Bleomycin induces lung injury via its ability to cause DNA strand breaks and oxidant injury with involvement of many chemokines (notably CCL2 and CCL12) and cytokines such as TNFα and TGFβ1 in the inflammatory and fibrogenic cascades [110].

In another murine model of lung fibrosis, intratracheal instillation of fluorescein isothiocyanate (FITC) leads to patchy focal lung destruction, inflammation and fibrosis. Pulmonary fibrosis develops independently of a specific T-cell immune response, strongly depends on CCR2 signalling and persists for at least 6 months. The areas of lung injury can be visualized by the characteristic green FITC fluorescence [110, 112].

Irradiation-induced pulmonary fibrosis develops strain dependently—C3H/HeJ and CBA/J mice are fibrosisresistant, while C57BL/6 mice are fibrosisprone—by 24 weeks after whole-body or thoracic exposure to a dose of 12-15 Gy [110]. Pathogenetically, chronic recruitment, activation and proliferation of bone marrow-derived macrophages and fibroblasts in the areas of lung fibrosis seem to be the key features of this fibrosis model [113, 114].

Inhalation or intratracheal instillation of crystalline silica such as DQl2 induces chronic pulmonary inflammation in several mouse strains—C57BL/6 and MRL/MpJ mice are more sensitive than BALB/c mice—characterized by a strong Th2 response and development of fibrotic nodules resembling those seen in humans exposed to occupational dusts and particulates. This model of fibrosis has the advantage that the fibrotic stimulus persists in the lung, although fibrosis can take 12-16 weeks to develop [110, 115].

In addition, numerous transgenic mouse models of pulmonary fibrosis are available for studying the overexpression of particular molecules involved in interstitial pulmonary remodelling and fibrosis or for delivering fibrotic or antifibrotic mediators via viral vectors (for further details, see [110]).

Airspace enlargement, alveolar emphysema and related models

Alveolar dilation without destruction of alveolar walls is best called 'enlargement of airspaces' rather than emphysema, either in humans [116] or in mice [91]. Enlargement of airspaces characterized by dilated alveolar ducts and flattened alveoli, with corresponding changes in morphometric parameters (increased lung volume, mean linear intercept and total alveolar duct and alveolar volume; decreased internal surface area and elastic fibre length per unit lung volume), but without evidence of alveolar wall destruction has been reported in the senescence-accelerated mouse (SAM) [117]. Enlargement of alveolar airspaces may also develop secondarily due to obstructive and inflammatory lesions in the upper airways. Occasionally, these primary conditions may produce well-circumscribed compensatory emphysematous lesions in the peripheral lung characterized by severely enlarged alveoli, distorted and separated by septa which are partially ruptured and show chronic inflammatory changes and fibrosis (Figure 2.7.15).

Emphysema in humans is defined as an abnormal, permanent enlargement of the air spaces distal to the terminal bronchiole, accompanied by destruction of their walls [116]. Currently, the involvement of three basic processes in the development of emphysema are being discussed: (i) emphysema as a consequence of proteolytic/antiproteolytic imbalance, (ii) emphysema as a result of disruption of the lung's homeostatic maintenance and repair system and (iii) emphysema as an immunological process [118].

Neutrophils and macrophages are usually implicated in emphysema by releasing proteases such as elastase, cathepsins and metalloproteinases (MMP 2, 9 and 12) which are normally inhibited by alpha-1-antitrypsin (AAT) and tissue inhibitors of metalloproteinases (TIMP). In knockout mouse models of the neutrophil elastase [119] or macrophage elastase gene [120], mice are protected against smoke-induced emphysema. In contrast, transgenic mice secreting human MMP 1 develop emphysema [121].
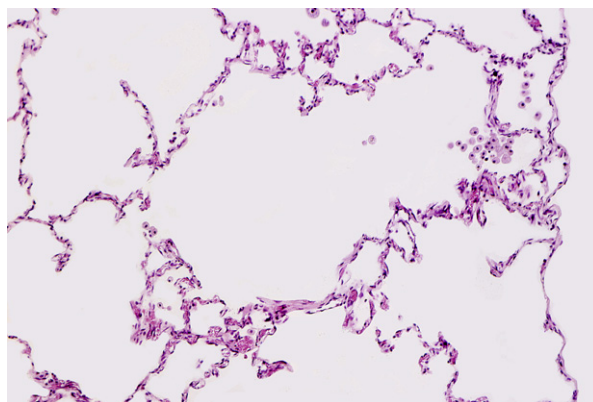
Figure 2.7.15 Alveolar emphysema secondary to airway obstruction. Distorted enlarged alveoli, blunt-ending ruptured septa, and some inflammatory cell infiltration.

The homeostasis of the lung may be disturbed by induction of apoptosis and oxidative stress, for example by application of cigarette smoke. This is normally counteracted by increased phagocytosis of apoptotic cells due to increased secretion of VEGF, which also enhances cell proliferation [122]. VEGF receptor (VEGFR2) blockade or inhibition of the VEGF–VEGFR2 complex may thus result in increased apoptosis, leading to airspace enlargement and emphysema in mice [118].

It is further discussed that autoimmunity plays a role in the pathogenesis of emphysema. Increased numbers of CD4 and CD8 T cells with the Th1 phenotype have been shown in the inflammatory cell infiltrate and were associated with increased MMP 12 release [123]. Furthermore, in human smokers the magnitude of the inflammatory cell infiltrate appears to correlate with emphysema development, and the inflammatory cell infiltrate persists often for many years after smoking cessation [118].

Several genetic models of spontaneous pulmonary emphysema in mice are available, for example the blotchy mouse [124], the tight-skin ($Fbn1^{Tsk}$) mouse [125], the pallid ($Pldn^{pa}$) mouse [126], the $Sftpd^{tm1Jhf}$ mouse [127], the osteopetrotic ($Csf1^{op}$) mouse [128] and the klotho (Kl^{tm1Yin}) mouse [129].

The mutant blotchy mouse develops a progressive panlobular emphysema due to an alteration of copper transport. It was this model which pointed to injury of the elastic fibre of the lung as playing a key role in the development of emphysema [130].

Elastolytic processes with breakdown and loss of interstitial elastic fibres due to a deficiency in serum elastase inhibitory capacity have been shown to be responsible for the emphysema development in the tight-skin ($Fbn1^{Tsk}$) mouse model [131].

In the pallid mouse emphysema develops as a result of an elastolytic process due to a severe inborn deficiency of serum alpha-1-antitrypsin [126].

Ablation of the surfactant protein D gene ($Sftpd$) caused chronic inflammation, emphysema and fibrosis in the lungs of $Sftpd^{tm1Jhf}$ mice. These lesions were associated with increased activity of metalloproteinases and increased hydrogen peroxide production of alveolar macrophages

[127]. Increased secretion of metalloproteinases by alveolar macrophages and abnormal elastin deposition could be related to spontaneous emphysema development in osteopetrotic ($Csf1^{op}$) mice, which have no detectable macrophage colony-stimulating factor (M-CSF) and show macrophage abnormalities also in various other tissues [128]. In the homozygous mutant klotho (Kl^{tm1Yin}) mouse, which is deficient in klotho gene expression, a defect in matrix synthesis and/or in type II pneumocyte function is considered to be involved in the development of pulmonary emphysema [129]. Spontaneous emphysema and pulmonary fibrosis have also been observed in transgenic mice overexpressing human TGFα during the period of postnatal alveolarization [132, 133].

Bronchiolo-alveolar hyperplasia

Bronchiolo-alveolar hyperplasia refers to epithelial hyperplasia distal to terminal bronchioles, affecting alveoli immediately adjacent to the alveolar ducts [75]. It originates from secretory bronchiolar (Clara) cells, alveolar type II cells or a combination of both, and is frequently present in chronically inflamed lungs. In untreated mice the incidence increases with age and correlates well with the prevalence of bronchiolo-alveolar tumours. There are three main histologic types of bronchiolo-alveolar hyperplasia depending on the type of epithelium involved, but the boundaries between the different types are not distinct. The 'bronchiolar type' (bronchiolization) shows alveolar walls lined by cuboidal to tall columnar cells that have bronchiolar epithelial cell differentiation (ciliated cells, Clara cells, mucus cells). This condition often occurs in mice exposed to airborne irritants. The relative contribution of distal growth of pre-existing bronchiolar epithelium and metaplasia (transdifferentiation) of alveolar type II cells is not clear and probably varies according to the causal agent [75]. In the 'alveolar type', alveoli are lined by a single layer of round to oval or cuboidal, sometimes vacuolated, alveolar type II cells with abundant eosinophilic cytoplasm prominently outlining alveolar walls. It is this type of hyperplasia where formation of papillary projections or solid cell clusters marks the transition towards neoplasia
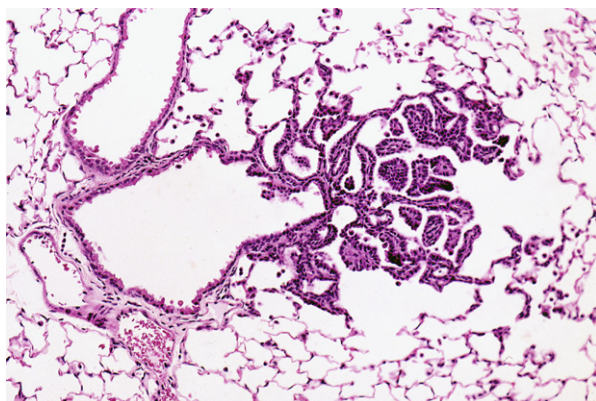
Figure 2.7.16 Preneoplastic bronchiolo-alveolar hyperplasia in the peripheral lung.

Figure 2.7.18 Allergic inflammation. Slight bronchiolar mucus (goblet) cell hyperplasia, interstitial inflammatory cell infiltration, and peribronchiolar fibrosis. Numerous hypertrophic PAS-/Alcian blue-positive mucus cells are the predominant cell type in some areas of the bronchiolar epithelium.

(Figure 2.7.16). This type of hyperplasia may be associated with influx of alveolar macrophages. The third type, i.e. the 'mixed type', of bronchiolo-alveolar hyperplasia, is rarely observed in clean control lungs and shows different proportions of bronchiolar and alveolar types of hyperplasia [75].

Squamous cell metaplasia

Replacement of bronchiolar or alveolar epithelium by squamous cells (Figure 2.7.17) that may contain only keratohyaline granules or be highly keratinized results from transdifferentiation of Clara cells [134] and/or alveolar type II cells or their poorly differentiated precursor cells. Persistent squamous cell metaplasia of bronchiolar and alveolar epithelial cells may occur in mice recovering from chronic infectious pneumonias [75].

Mucus cell hyperplasia/metaplasia

Mucus (goblet) cell hyperplasia in bronchi/bronchioles is characterized by replacement of respiratory epithelial cells by mucus cells (Figure 2.7.18) and is usually associated with inflammation caused by infectious agents or induced by exposure of the airways to irritants [75]. Increased production of PAS-/Alcian blue-positive mucus and occasionally formation of mucus-filled bronchioles may be associated changes. Prominent mucus (goblet) cell hyperplasia has also been induced in CBA/J mice after sensitization and challenge with *Aspergillus fumigatus* conidiae [135] or by ovalbumin sensitization in BALB/c and C57BL/6 mice to mimic late asthmatic responses [136].

Mucus cell metaplasia describes the presence of a single layer of mature mucous cells lining terminal bronchioles and alveoli where normally no mucous cells are found. Pathogenically, mucous cell metaplasia—like squamous cell metaplasia—results from transdifferentiation of Clara cells and/or alveolar type II cells, usually in lungs of mice chronically exposed to airborne irritants [75].

Tumours of the respiratory system

Naturally occurring tumours of the upper respiratory tract are extremely rare in laboratory

Figure 2.7.17 Focal squamous cell metaplasia of bronchiolar epithelium.

mice. However, primary neoplasms of the lung are common in aged animals of many strains of laboratory mice. The most frequent respiratory tract tumours in this species are bronchiolo-alveolar tumours which arise from bronchiolar Clara cells or in distal lung parenchyma. The great majority of lung tumours in mice are observed in animals older than 18 months. In most strains tumour frequency is higher in male mice than in females [137, 138].

The classification and diagnostic criteria of tumours of the respiratory system in mice have been revised by international collaboration of toxicological pathologists working within the global INHAND initiative (International Harmonization of Nomenclature and Diagnostic criteria) [75]. The descriptions are an enhancement of the international classification of rodent tumours in the mouse initiated by WHO/IARC [139, 140], which was used for the previous edition of this book [141].

This recent mouse classification [75] subdivides lesions according to their location either in the larynx, trachea, bronchus, bronchiole or lung alveoli. In addition, the cell of origin or pathogenesis is given. The WHO human classification [142] uses mainly a descriptive morphology term, considering phenotypical features connected to significant prognostic values, for the diagnosis of tumours located in the lung. Usually, tumours are classified and graded by their most well-differentiated and most poorly differentiated components, respectively. In this context, the origin from a particular cell lineage and anatomical structure may also be of value. Frequently, however, it is difficult to identify either of them in advanced neoplasms [143]. The WHO human classification divides adenomas and carcinomas according to their growth pattern, not taking into account their cells of origin. In mouse lung tumour models it is much easier to make a decision for a specific cell type of origin, although it is possible for more inexperienced investigators to summarize diagnostic categories. Tumour progression is frequently associated with changes in respiratory epithelial cell differentiation [144], so it may be difficult to determine the cell of origin in advanced neoplasms.

The diagnostic features [75] for tumours of the respiratory tract are listed in the following sections.

Nasal cavity, nasopharynx, paranasal sinus

Squamous cell papilloma

Nasal squamous cell papillomas may originate from transitional, respiratory or metaplastic olfactory epithelium, or from the squamous epithelium of the nasal vestibule. Usually, papillomas consist of an exophytic mass of uniform, regularly arranged squamous cells that form papillary or filiform structures covering a vascularized connective tissue stalk. Transitional and respiratory epithelial cells may sometimes be included as a minor component. Generally, the basement membrane is intact. Occasionally, squamous cell papillomas grow beneath the mucosal surface.

Adenoma

Nasal adenomas develop from neoplastically transformed transitional, respiratory or glandular epithelial cells. They usually arise in the most anterior part of the nasal cavity, originating from the mucosa of the naso- or maxilloturbinates or from the lateral wall of the anterior nasal cavity. Location of the tumour usually determines the cell type of the adenoma (transitional or respiratory epithelial type, submucosal glandular). Transitional epithelial cell adenomas are found mostly in the lateral meatus of the anterior (proximal) aspect of the nasal cavity. Adenomas grow expansively with occasional protrusion into the nasal or paranasal cavities, but may show endophytic growth as well. Endophytic adenomas of respiratory epithelium or adenomas of submucosal glands may cause compression. Adenomas consist of glandular structures, which may be cystic, or sheets of cells, sometimes with pseudoacinar structures resulting from dropout of dead cells. Secretory activity is mostly visible as mucus production. Usually, adenomas are composed of non-ciliated cuboidal to low columnar cells with basophilic cytoplasm and centrally located nuclei.

Squamous cell carcinoma

Nasal squamous cell carcinomas (Figure 2.7.19) may originate from squamous differentiation of transitional, respiratory or olfactory epithelial cells; epithelial cells of the subepithelial glands; or from malignant transformation of the squamous
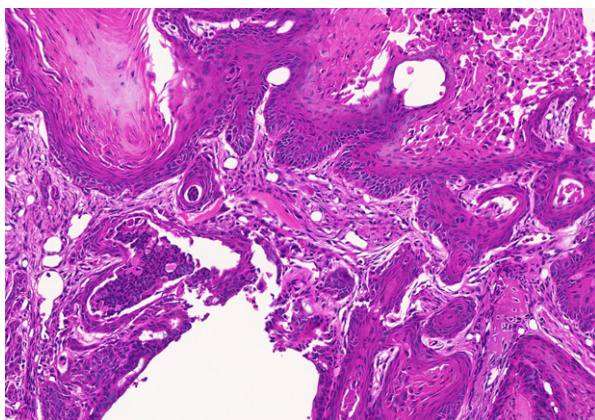
Figure 2.7.19 Squamous cell carcinoma originating in the nasal cavity invading adjacent tissues.

Figure 2.7.20 Adenocarcinoma of the nasal cavity with mucus formation growing in the submucosa.

epithelium of the nasal vestibule. They are observed most often in the anterior nasal cavity, arising from the epithelium of the lateral walls, septum, naso- and maxilloturbinates, and in the ethmoturbinates. Squamous cell carcinomas are composed of solid, often branching cords or masses of cells with various degrees of anaplasia. Shape and size of the cells are irregular. Cells may be large and polygonal, or flattened and stratified. The cytoplasm is eosinophilic and granular to hyalinized as a result of the high keratin content. Squamous cell carcinomas exhibit frequent mitoses, cellular or nuclear atypia or invasion into surrounding tissues.

Adenosquamous carcinoma

Nasal adenosquamous carcinomas are believed to develop by malignant transformation of respiratory epithelium, submucosal gland duct epithelium or basal and sustentacular cells of the olfactory epithelium, or from areas of metaplasia in the epithelia. The tumours have malignant glandular and squamous epithelial components. The squamous components of the tumours may show typical keratin pearl formation. The tumours may contain undifferentiated epithelial cells. Adenosquamous carcinomas show malignant features such as frequent mitoses, cellular or nuclear atypia or invasion into surrounding tissues, lymphatics, vessels or metastases.

Adenocarcinoma

Nasal adenocarcinomas (Figure 2.7.20) evolve from malignant transformation of transitional, respiratory or glandular epithelium, or olfactory Bowman's glands. They are localized in the anterior nasal cavity (originating from subepithelial glands), in the posterior nasal cavity (often originating in the mucosa of the ethmoturbinates) or arise from malignant change occurring in an adenoma. Location of the tumour usually determines the type of adenocarcinoma (transitional or respiratory epithelial). Transitional tumours are found mostly in the lateral meatus of the anterior (proximal) aspect of the nasal cavity. Adenocarcinomas consist of solid, pseudoglandular, papillary, or tubular formations. The lumina may be filled with mucous substances. The cells are large cuboidal to columnar, or anaplastic. There is loss of polarity of the epithelium. The tumours may show only penetration of the basement membrane or additional invasion of the surrounding bone, cribriform plate or olfactory lobes of the brain. Invasion of the cerebrum or metastases to regional lymph nodes or lung may also occur. Areas of squamous differentiation may be present [145].

Neuroepithelial carcinoma

Neuroepithelial carcinomas (Figure 2.7.21) develop from malignantly transformed olfactory epithelium (sustentacular cells, basal cells, immature sensory cells and possibly ductal cells of Bowman's glands). They arise from the olfactory epithelium. Frequently, there is compartmentalization of sheets of neoplastic cells into lobules by fibrovascular septa. Cells are small and round or columnar with poorly defined, pale-staining cytoplasm. They have round to oval, basally located nuclei that do not display marked cytologic
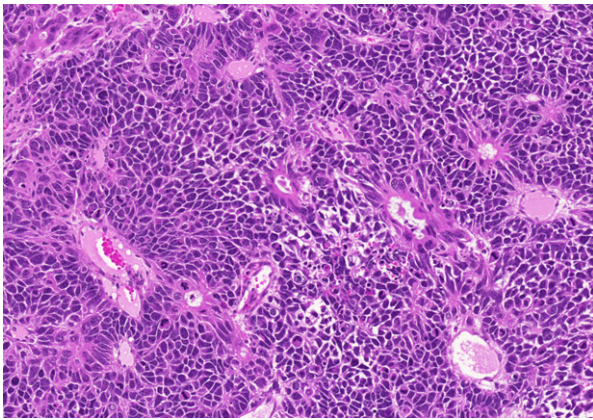
Figure 2.7.21 Neuroepithelial carcinoma showing rosettes and pseudorosettes.

atypia. There is distinct, sharply defined nuclear chromatin. True rosettes or pseudorosettes may be present. Plexiform intercellular fibrils are a diagnostic feature. Areas of anaplastic cells may be present. Neuroepithelial carcinomas often invade the ethmoid bone and brain. Frequency and morphology of rosette structures are highly variable.

Larynx, trachea, bronchi, bronchioles

Papilloma

Papillomas (Figure 2.7.22) of the larynx, trachea, bronchi or bronchioles are neoplastic proliferations of respiratory or squamous epithelium. Histologically, the airway is expanded or distorted by growth of branching papillary structures with a central connective tissue stalk and
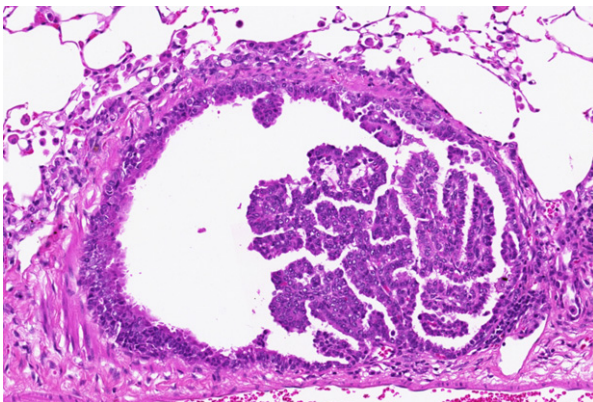
Figure 2.7.22 Papilloma of a bronchiolus.

lined by cuboidal/respiratory epithelial cells. If the papilloma originates in terminal bronchioles, there may be growth by expansion into alveolar parenchyma. The basement membrane is intact and there is no clear evidence of invasion of adjacent structures. The branching connective tissue stalk is usually lined by varying proportions of cuboidal or columnar respiratory epithelium, but may occasionally be lined by squamous cells. Mitotic figures are rare and limited to the basal layers of the epithelium.

Squamous cell carcinoma

Squamous cell carcinomas of the larynx, trachea, bronchi or bronchioles develop by malignant transformation of respiratory epithelium that has undergone squamous metaplasia or of squamous epithelium of the larynx that has progressed to neoplasia. They show a growth pattern in cell clusters or irregular structures with central keratinization (keratin pearls), or without overt keratinization but forming distinct intercellular bridges. Cellular debris and necrosis may be common, as well as the presence of inflammatory cells, in particular, neutrophils. The large cells are polygonal in shape or flattened and stratified. The orientation is irregular. Because of the high keratin content, cytoplasm may frequently be eosinophilic and granular to hyalinized. Squamous cell carcinomas show dysplasia to anaplasia. Mitoses are frequent in some areas. Squamous cell carcinomas exhibit penetration of the basement membrane and invasion of adjacent tissues. A scirrhous response may occur in association with invasion. There are a few reports of experimentally induced squamous cell carcinomas in the airways of mice, but spontaneous development has not been reported.

Adenocarcinoma

Adenocarcinomas of the larynx, trachea, bronchi or bronchioles arise by malignant transformation of respiratory epithelium. These tumours exhibit definitive evidence of origin from a conducting airway. They show evidence of invasion of the basement membrane or adjacent pulmonary structures. Foci of mucinous cell differentiation may be present. Papillary growth may occur in early stages with development of a central connective tissue stalk lined

by cuboidal to columnar or pleomorphic epithelium. Irregular tubular/glandular structures may be present. Cytological features of malignancy and possible evidence of stromal invasion and/or destruction of the airway wall are present. There are a few reports of chemically induced tumours in transgenic mice [146-148].

Terminal bronchioles, alveoli

Bronchiolo-alveolar adenoma

It is generally believed that solid bronchiolo-alveolar adenomas (Figure 2.7.23) are composed of cells expressing alveolar type II cell features and are therefore considered benign alveolar type II cell tumours. Papillary bronchiolo-alveolar adenomas (Figure 2.7.24), however, are considered to be either less well-differentiated type II cell tumours progressing toward a malignant phenotype or of Clara cell origin. Clara cell tumours have been reported in transgenic mice made expressly to target Clara cells. Most papillary tumours in mice arise from alveolar type II cells.

Bronchiolo-alveolar adenomas are frequently located at the lung periphery and are usually small. Microscopically, there are well-circumscribed areas of high epithelial cell density, usually with a strongly convex border. The underlying alveolar architecture is obscured to various degrees. There is frequently sharp demarcation from the surrounding tissue. The neoplastic epithelial cells are relatively uniform. Mitotic figures are rare or absent. Small foci of mild
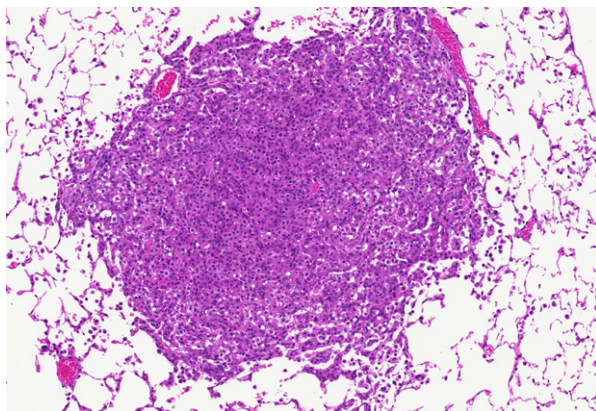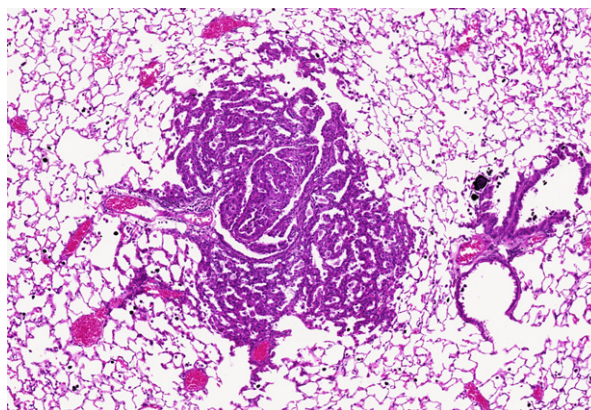
Figure 2.7.24 Papillary bronchiolo-alveolar adenoma extending into a terminal bronchiole in the lung of a female mouse.

atypia may be present. In these foci, cells tend to have a higher degree of pleomorphism, and the number of mitoses is slightly increased. Occasionally, especially papillary bronchiolo-alveolar adenomas extend into adjacent bronchioles.

In solid bronchiolo-alveolar adenomas the alveolar spaces are obliterated by proliferating round to oval cells. Frequently, these solid areas are surrounded by alveoli lined by hyperplastic alveolar type II cells; in other words, there might be no sharp demarcation between the tumour and normal parenchyma. Cells usually have abundant eosinophilic cytoplasm that may appear granular or vacuolated. Cell nuclei are usually round to oval. Mitotic figures are rare or absent. Tumours may extend into bronchioles through the alveolar duct. Compression is frequently observed.

Papillary bronchiolo-alveolar adenomas are composed predominantly of delicate papillary structures lined by cuboidal to columnar cells that may be deeply basophilic. There is a regular pattern, lack of distortion and focal variation in cell appearance. Papillary tumours may show prominent tubular profiles, namely elongated lumina surrounded by cuboidal cells, depending on the orientation of the histologic section. Papillary structures are sharply demarcated from surrounding alveolar parenchyma. The tumours may be associated with peripheral alveolar hyperplasia. Large, sometimes foamy, macrophages may fill spaces between tumour cells. Mitotic rate and the degree of cellular pleomorphism are usually low. There is no invasion and destruction

Figure 2.7.23 Solid bronchiolo-alveolar adenoma composed of uniform epithelial cells in the lung of a female mouse.

of adjacent tissue. Papillary and solid areas may exist in the same neoplasm.

Bronchiolo-alveolar carcinoma

Bronchiolo-alveolar carcinomas (Figures 2.7.25 and 2.7.26) may originate from either alveolar type II or Clara cells, but are generally considered to arise from alveolar type II cells. Histologically, the neoplasms show irregular nodular growth, and they are moderately well to poorly circumscribed. These tumours may occupy an entire lobe. Architectural distortion, variation in appearance and organization of tumour cells from one region to another is visible. Mitotic activity is usually increased.

Bronchiolo-alveolar carcinomas with papillary growth pattern consist of cuboidal to columnar or pleomorphic cells, arranged as papillary structures supported by a connective tissue core.
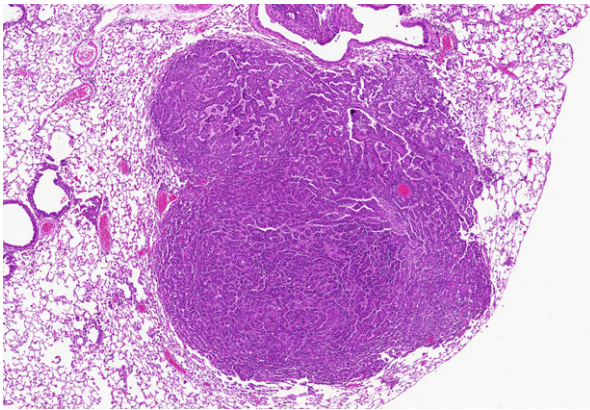
Figure 2.7.25 Bronchiolo-alveolar carcinoma invading a bronchiolus in the lung of a female mouse.
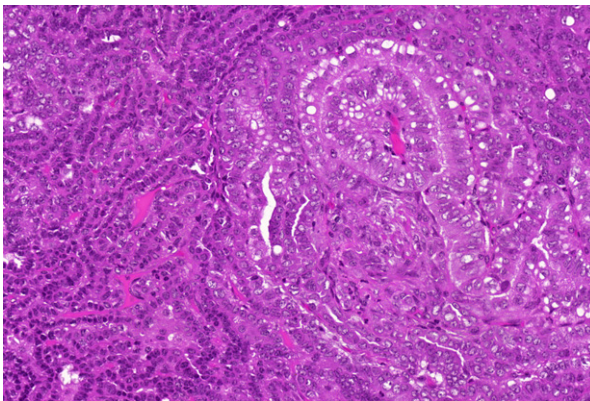
Figure 2.7.26 Higher-power view of a bronchiolo-alveolar carcinoma composed of ribbons of cuboidal and columnar cells.

The cytoplasm of the tumours may contain glycogen or neutral lipids. Bronchiolo-alveolar carcinomas may exhibit areas of increased cytoplasmic basophilia and atypia, indicating local expansion of less differentiated tumour cells. Frequently, there is association with influx of macrophages into the tumour and in adjacent alveoli. Large tumours may have areas of necrosis, haemorrhage, cholesterol clefts, fibrosis (especially in the case of subpleural localization) and obliterating fibrosis of bronchioles. Indication of malignancy is present, such as destruction of parenchyma; invasion of bronchiolar walls, interstitial tissues and/or pleura; and/or dissemination through lymphatics, airspaces and/or distant metastases. Advanced stages of malignancy and invasion (e.g. to pleura) frequently are associated with marked cellular pleomorphism (spindle-shaped to round atypical cells), desmoplasia and increased mitotic rate.

Bronchiolo-alveolar adenomas and carcinomas are the most common naturally occurring and chemically induced lung tumours in rats and mice [137, 138].

Acinar carcinoma

Acinar carcinomas (Figure 2.7.27) are believed to originate directly from bronchiolar epithelial (Clara) cells of the terminal airways by malignant transformation, or to arise from Clara cells populating alveolar walls. Some investigators believe that these tumours arise from parenchymal Clara cells that have migrated from bronchioli to the alveolar epithelium, whereas others consider
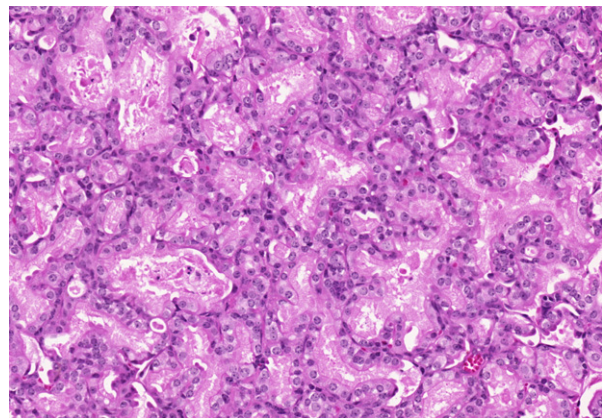
Figure 2.7.27 Pulmonary acinar carcinoma showing a glandular/acinar pattern and composed of ciliated cells.

them to arise from metaplasia of alveolar type II cells. These tumours are diffusely expansile with irregular margins or more circumscribed nodules. In most cases there is a glandular/acinar pattern representing tumour cells that utilize existing alveolar walls. Acinar carcinomas are composed of cuboidal to columnar or pleomorphic cells without distinguishing features or, more commonly, mixed with ciliated cells or mucous cells. Large portions or the entire tumour may show differentiation in favour of a single cell type, for example, mucinous adenocarcinoma. Cells may show variable cytoplasmic eosinophilic globules. These globules are generally considered to represent dilated endoplasmic reticulum containing proteinaceous material [140]. Neoplasms show clear features of malignancy such as penetration of basement membranes and tissue destruction.

Naturally occurring acinar carcinomas are extremely rare. They can be induced by intratracheal instillation of methylcholanthrene or cutaneous treatment with *N*-nitrosobis-(2-chloroethyl) urea in some strains of mice [149].

Adenosquamous carcinoma

Adenosquamous carcinomas (Figure 2.7.28) are believed to derive from acinar carcinomas, or possibly bronchiolo-alveolar carcinomas, with clonal shifts to the malignant squamous cell phenotype. These tumours are nodular or diffusely expansile with irregular margins. They are composed of significant amounts of both adenocarcinomatous and malignant squamous cell components. Squamous cells may show keratinization with formation of central keratin pearls or expansion of acini by desquamated keratinized cells. Squamous cell differentiation may also be recognized by the formation of polygonal cells with prominent intercellular bridges lacking keratinization. Cells may also be greatly enlarged with atypical nuclei. The neoplasms usually show clear indication of malignancy such as penetration of basement membranes and tissue destruction [137, 138, 148, 149].

Squamous cell carcinoma

Pulmonary squamous cell carcinomas (Figure 2.7.29) develop by malignant transformation of squamous cell metaplasia of alveolar epithelium and/or Clara cells. Histologically, they exhibit a growth pattern in cell clusters or irregular nests with central keratinization, or without overt keratinization but forming distinct intercellular bridges. Cells have cytological features of malignancy such as atypia, disorganization and increased mitotic rate. Cells are frequently quite pleomorphic. Squamous cell carcinomas may invade the adjacent lung parenchyma, pleura, vessels and/or bronchi. Frequently, there is a marked scirrhous response.

Non-keratinizing squamous cell carcinomas lack overt keratinization, but cells characteristically display distinct intercellular bridges. The cells are usually pleomorphic and may be small
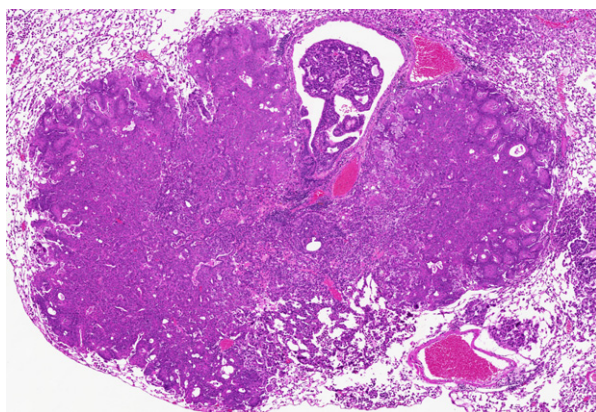
Figure 2.7.28 Adenosquamous cell carcinoma of the lung showing a malignant adenomatous portion with invasion of a bronchus and malignant squamous cell parts.
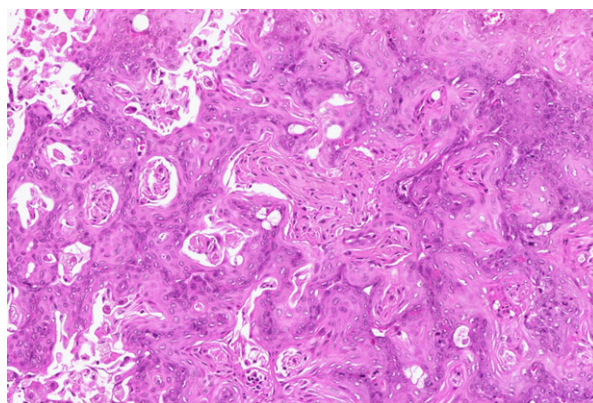
Figure 2.7.29 Well-differentiated squamous cell carcinoma of the lung exhibiting sparse keratinization.

with scant cytoplasm, resembling basal cells of the upper respiratory tract, or may be large and eosinophilic with abundant cytoplasm.

Spontaneously occurring squamous cell carcinomas are extremely rare in the lungs of mice [137, 138].

Malignant neuroendocrine tumour

A histological description based on one case of a spontaneously occurring neuroendocrine tumour has been given by Renne et al. [75]. The tumour cells were separated into lobules and cords by a delicate fibrovascular stroma. Cells were polygonal with distinct cell borders and had abundant pale, finely granular cytoplasm. Mitotic figures were rare. Photographs of neuroendocrine lung tumours from a genetically engineered mouse model carrying *Trp53* and *Rb1* alleles [150] show tumour masses attached to bronchioli and infiltrating lung parenchyma or protruding into the bronchiolar lumen. The cells contain finely dispersed chromatin, scanty featureless cytoplasm and moulding of nuclei of adjacent tumour cells. Immunohistochemical staining was positive for 90% of the cases for calcitonin gene-related peptide (CGRP) and synaptophysin. A small fraction (7-11%) was positive for ASCL1 (human achaete-scute complex homologue 1), which is a key marker in human small-cell lung carcinoma, but negative for the other markers.

Murine models of lung cancer

The incidence of primary lung tumours among inbred strains of mice varies widely. Those strains in which spontaneous lung tumours frequently arise also respond to induction by chemical substances. A wide variety of chemicals can stimulate murine lung tumorigenesis with a range of potencies, including urethane, metals, aflatoxin and constituents of tobacco smoke [151]. Among the most potent single carcinogens are urethane, nitrosamines and polycyclic aromatic hydrocarbons [152]. Lung neoplasia can be additionally enhanced by chronic treatment of mice with the synthetic antioxidant butylated hydroxytoluene, subsequent to carcinogen administration, even though the substance itself is non-tumorigenic [153].

Bronchiolo-alveolar tumours can be induced in mice by a variety of substances and occur spontaneously with high frequencies in some strains of inbred mice such as strain A/J, which was used to detect acceleration of neoplasms, for example by tobacco smoke [154] or welding fumes [155]. In intermediate susceptible strains such as Swiss, CD-1, BALB/c or NMRI, the incidence of lung tumours ranges from 15% to 50% [137, 138]. The most resistant strains include DBA [151] and C57BL/6 [138]. Mice develop primary lung tumours quite similar in structure, molecular characteristics and histogenesis to human adenocarcinomas and to bronchiolo-alveolar carcinoma in particular [152].

A/J mouse-related models

The A/J mouse is frequently used as a model for testing of chemicals by inhalation for lung tumour multiplicities. Belinsky et al. [156] investigated the use of the A/J mouse lung as a model for developing chemointervention strategies by first inducing lung tumours with a single dose of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. Lungs were then staged for tumour development 42 weeks after carcinogen treatment. At this time point an average of seven pulmonary lesions were present on a standard histological section. Mice were treated for 4 or 8 weeks with cis-platinum alone or in combination with either indomethacin, metoclopramide or nifedipine, and the effect on tumour growth was also determined. The most dramatic effects were observed in lungs from mice treated for 8 weeks, where cis-platinum treatment caused a 37% reduction in the size of carcinomas, while tumour mass was reduced by 50-60% with cis-platinum in combination with metoclopramide and/or indomethacin. Although none of the therapeutic combinations affected the size of adenomas, morphological differences were observed among treatment groups. A moderate to marked decrease in cytoplasm was observed in adenomas from mice treated with cis-platinum in combination with indomethacin or metoclopramide, cis-platinum plus metoclopramide and indomethacin, or metoclopramide plus indomethacin.

Witschi [157] compared studies of different laboratories and discussed the complexities of the A/J model. Results obtained in four different laboratories had shown significant increases in lung tumour multiplicities in tobacco smoke-exposed animals. The counting of surface tumours only may occasionally have underestimated the total number of lung tumours and shown false negatives. A major disadvantage of the assay was its low statistical power. While it was easy to detect a 70–100% decrease in lung tumour multiplicity caused by a chemopreventive agent using group sizes of 20–30 animals, detection of smaller reductions (20–50%) would require group sizes in the hundreds.

Stearman et al. [158] examined the gene expression profiles of human and murine (A/J mouse) lung tissues (normal or adenocarcinoma) and compared the datasets of the two species after aligning around 7500 orthologous genes. A list of 409 joint gene classifiers showed significant, positive correlation in expression levels between the two species. The eicosanoid pathway enzymes prostacyclin synthase and inducible prostaglandin E2 synthase were joint classifiers that showed opposite effects in lung adenocarcinoma (prostacyclin synthase downregulated, prostaglandin E2 synthase upregulated). Micro-arrays identified the same protein expression pattern for prostacyclin synthase and prostaglandin E2 synthase in 108 different non-small-cell lung cancer biopsies, and the detection of prostacyclin synthase had statistically significant prognostic value in patient survival. Compared to human lung adenocarcinoma, the A/J mouse-urethane model exhibited similar histological appearance and molecular changes.

Dwyer-Nield et al. [159] employed murine chromosome substitution strains to study how resistance alleles affected sensitive alleles during chemically induced lung carcinogenesis. The C57BL/6J-Chr6$^{A/J}$ strains, created by selectively breeding sensitive A/J and resistant C57BL/6J (B6) mice, each contain one pair of A/J chromosomes within an otherwise B6 genome. Pas1, the major locus responsible for this differential strain response to urethane carcinogenesis, resides on chromosome 6, but C57BL/6J-Chr6$^{A/J}$ mice developed few tumours following a single urethane injection, which demonstrates epistatic interactions with other B6 alleles. C57BL/6J-Chr6$^{A/J}$ mice developed dozens of lung tumours after chronic urethane exposure, however, indicating that these epistatic interactions could be overcome by repeated carcinogen administration. Unlike A/J, but similar to B6, mice, C57BL/6J-Chr6$^{A/J}$ mice were resistant to lung carcinogenesis induced by 3-methylcholanthrene. Tumour multiplicity increased if butylated hydroxytoluene administration followed urethane exposure, showing that a gene on chromosome 6 regulates sensitivity to chemically induced tumour promotion. Unlike A/J tumours, Kras mutations in tumours induced by urethane in C57BL/6J-Chr6$^{A/J}$ mice were similar to B6 tumours. DNA repair genes not located on chromosome 6 may determine the nature of Kras mutations. C57BL/6J-Chr6$^{A/J}$ mice are seen as a valuable resource for testing the ability of candidate genes to modulate lung carcinogenesis [159].

Kras-related models

The probability for a mouse to develop a pulmonary tumour, as well as the structure of that tumour, is dependent on several genes. Three pulmonary adenoma susceptibility (Pas) genes predispose some inbred strains to develop lung tumours, even in the absence of carcinogen exposure, and cause others to be resistant. One is Kras, which may also be overexpressed in these tumours in a mutated form capable of transforming cells. Mice with activated Has transgenes have a high turnover rate of alveolar type II and bronchiolar Clara cells—the cells from which lung tumours arise—than more resistant strains. A high precursor cell turnover rate correlates with a propensity to neoplasia in other animal models as well, possibly due to low concentrations of endogenous growth regulatory molecules. A set of genes other than the Pas genes governs the response to tumour modulation by butylated hydroxytoluene [160].

Chen et al. [145] demonstrated preferential activation of the Kras gene from the susceptible A/J parent in lung tumours of F1 mouse hybrids. Higher levels of expression of the A/J Kras allele were detected in lung adenomas (30 of 30) of the C3A mouse. Higher expression of the A/J allele relative to the C3H allele may be responsible

for the allele-specific activation of the *Kras* gene in lung tumours of F1 hybrid mice [145].

A mouse model for tumorigenesis of lung adenomas and papillary adenocarcinoma-like tumours was developed by Meuwissen et al. [161]. In this model *Kras* can be sporadically activated through *Cre/lox*-mediated somatic recombination. Adenoviral-mediated delivery of *Cre* recombinase in lung epithelial cells gave rise to rapid onset of tumorigenesis, yielding pulmonary adenocarcinomas with 100% incidence after a short latency period. The tumours were immunohistochemically positive for thyroid transcription factor-1, which is a specific marker of thyroid and pulmonary epithelium, for surfactant protein B and surfactant protein C, showing the alveolar type II cell origin of the tumours [161].

To construct a mouse tumour model involving *Kras*, Johnson et al. [162] used a gene targeting procedure to create mouse strains carrying oncogenic alleles of *Kras* that can be activated only by a spontaneous recombination event in the whole animal. They showed that mice carrying these mutations were highly predisposed to early onset of lung cancer. This model was further characterized by examining the effects of germline mutations in the tumour suppressor gene *p53*, which is known to be mutated along with *Kras* in human tumours [162].

A lung tumour model using a regulatable oncogene was established by Fisher et al. [163]. To investigate the role of an activated *Kras* gene in the initiation and maintenance of lung adenocarcinomas, they developed transgenic mice that express murine $Kras^{4bG12D}$ under the control of doxycycline in type II pneumocytes. Focal proliferative lesions of type II pneumocytes were observed as early as 7 days after induction with doxycycline; after 2 months of induction, the lungs contained adenomas and adenocarcinomas, with focal invasion of the pleura at later stages. Removal of doxycycline caused a rapid fall in levels of mutant *Kras* RNA and concomitant apoptotic regression of both the early proliferative lesions and the tumours [163].

Jackson et al. [164] developed a model of lung adenocarcinoma in mice harbouring a conditionally activatable allele of oncogenic *Kras*. They demonstrated that the use of a recombinant adenovirus expressing Cre recombinase to induce $Kras^{G12D}$ expression in the lungs of mice allows control of the timing and multiplicity of tumour initiation. Immunohistochemically, some proliferative lesions were found to be positive for surfactant apoprotein-C and negative for Clara cell antigen, suggesting that these lesions arose from alveolar type II cells or their precursors. Other lesions were found to be negative for surfactant apoprotein-C and positive for Clara cell antigen. In junction areas of bronchioles and alveoli they observed cells which were positive for surfactant apoprotein-C and for Clara cell antigen. Such double-positive cells were not seen in adenovirus-infected wild-type mice and may contribute to the formation of pulmonary adenomas.

Jackson et al. [165] reported the creation of a murine model of spontaneous advanced lung adenocarcinoma. They generated compound conditional knockin mice with mutations in *Kras* combined with one of three *p53* alleles: a contact mutant, a structural mutant or a null allele. Loss of *p53* strongly promoted the progression of *Kras*-induced lung adenocarcinomas. The influence of *p53* loss on malignant progression was observed as early as 6 weeks after tumour initiation.

To determine the effects of expression of mutant *Kras* on lung tumorigenesis, Floyd et al. [166] developed a bitransgenic mouse model that expresses the human $Kras^{G12C}$ allele in alveolar type II and/or Clara cells in a tetracycline-inducible, lung-specific manner. Expression of $Kras^{G12C}$ caused multiple small lung tumours over a 1-year time period. Although tumour multiplicity increased upon continued *Kras* expression, most lung lesions were hyperplasias or well-differentiated adenomas. Expression of $Kras^{G12C}$ was associated with a twofold increase in the activation of the *Ras* and *Ral* signalling pathways and increased phosphorylation of *Ras* downstream effectors, including *Erk*, *p90* ribosomal S6 kinase, ribosomal S6 protein, *p38* and MAP-KAPK-2. Withdrawal of doxycycline for 1 month resulted in an almost complete absence of proliferative pulmonary lesions, suggesting tumour regression in the absence of *Kras* expression. Mutant $Kras^{G12C}$ expression was sufficient for initial lung tumour transformation [166].

To directly assess the requirement for *Rac1* in *Kras*-induced tumorigenesis, Kissil et al. [167]

employed a model of lung cancer in which an oncogenic allele of *K-ras* could be activated by Cre-mediated recombination in the presence or absence of conditional deletion of *Rac1*. They showed that *Rac1* function is required for tumorigenesis in this model. Furthermore, although *Rac1* deletion alone was compatible with cell viability and proliferation, when combined with *K-ras* activation in primary epithelial cells, loss of *Rac1* caused a profound reduction in proliferation, showing a specific requirement for *Rac1* function in cells expressing oncogenic *K-ras*.

Iwanaga et al. [168] used a genetic approach to inactivate *Pten*, a tumour suppressor gene, in the bronchial epithelium of mice. Although, by itself, *Pten* inactivation had no discernible effect on bronchial epithelial histology, it accelerated lung tumorigenesis initiated by oncogenic *K-ras*, causing more rapid lethality than that induced by oncogenic *K-ras* alone (8 weeks vs 24 weeks of median duration of survival, respectively). Lung tumours arose in *K-ras*-mutant, Pten-deficient mice that rapidly obstructed bronchial lumina and replaced alveolar spaces. Relative to *K-ras*-mutant tumours, the *K-ras*-mutant, Pten-deficient tumours exhibited more advanced histologic severity and more prominent inflammation and vascularity. Thus, *Pten* inactivation cooperated with oncogenic *K-ras* in promoting lung tumorigenesis.

SP-C gene-related models

Transgenic mice harbour a chimeric gene comprising the SV40 large T antigen under the control of a transcriptional region derived from the human surfactant protein C (SP-C) gene, which is specific to type II alveolar epithelium cells. Such mice developed pulmonary adenocarcinomas at 4–5 months of age [169].

Yanagi et al. [170] generated a bronchiolo-alveolar epithelium-specific null mutation of *Pten* in mice (SP-C-rtTA/(tetO)$_7$-Cre/Pten$^{flox/flox}$ (SOPten$^{flox/flox}$) mice) which was controlled by doxycycline. Of the SOPten$^{flox/flox}$ mice that received doxycycline *in utero* (SOPten$^{flox/flox}$(E10–16) mice), 90% died of hypoxia soon after birth. Surviving SOPten$^{flox/flox}$(E10–16) mice and mice that received doxycycline postnatally (SOPten$^{flox/flox}$(P21-27) mice) developed spontaneous lung adenocarcinomas. Urethane treatment accelerated both the number and size of lung tumours developing in SOPten$^{flox/flox}$ mice of both ages. Histological and biochemical examinations of the lungs of SOPten$^{flox/flox}$(E10–16) mice revealed hyperplasia of bronchiolo-alveolar epithelial cells and myofibroblast precursors, enlarged alveolar epithelial cells and impaired production of surfactant proteins. Numbers of bronchiolo-alveolar stem cells, putative initiators of lung adenocarcinomas, were increased. Lungs of SOPten$^{flox/flox}$(E10–16) mice showed increased expression of Spry2, which inhibits maturation of alveolar epithelial cells. Levels of Akt, c-Myc, Bcl-2 and Shh were also elevated in SOPten$^{flox/flox}$(E10–16) and SOPten$^{flox/flox}$(P21-27) lungs. Furthermore, *K-ras* was frequently mutated in adenocarcinomas observed in SOPten$^{flox/flox}$(P21-27) lungs. These results indicate that Pten is essential for both normal lung morphogenesis and the prevention of lung carcinogenesis, possibly because this tumour suppressor is required for bronchioloalveolar stem cell homeostasis.

Epidermal growth factor-related models

Transgenic mouse models were established by Ehrhardt et al. [171] to study tumorigenesis of bronchiolo-alveolar adenocarcinomas derived from type II pneumocytes. Transgenic lines expressing the murine oncogene *c-myc* under the control of the lung-specific surfactant protein C promoter developed multifocal bronchiolo-alveolar hyperplasias, adenomas, and carcinomas, whereas transgenic lines expressing a secretable form of the epidermal growth factor (IgEGF), a structural and functional homologue of TGFα, developed hyperplasias of the alveolar epithelium. Since the oncogenes *c-myc* and TGFα are frequently overexpressed in human bronchiolo-alveolar adenocarcinomas, these mouse lines are useful as models of human bronchiolo-alveolar adenocarcinomas. Analyses of double transgenics, hemizygous for both c-myc and IgEGF, show that these mice develop bronchiolo-alveolar adenocarcinomas at the average age of 9 months, indicating that these oncogenes cooperate during lung cancer formation.

To further study the role of these mutations in the initiation and maintenance of lung cancer, Politi et al. [172] developed transgenic mice that express an exon 19 deletion mutant (EGFR$\Delta^{L747-S752}$) or the L858R mutant (EGFRL858R) in type II pneumocytes under the control of doxycycline. Expression of either EGFR mutant led to the development of lung adenocarcinomas. Two weeks after induction with doxycycline, mice that expressed the EGFRL858R allele showed diffuse lung cancer highly reminiscent of human bronchiolo-alveolar carcinoma and later developed interspersed multifocal adenocarcinomas. In contrast, mice expressing EGFR$\Delta^{L747-S752}$ developed multifocal tumours embedded in normal lung parenchyma with a longer latency. In mice carrying either EGFR allele withdrawal of doxycycline (to reduce expression of the transgene) or treatment with erlotinib (to inhibit kinase activity) caused rapid tumour regression, as assessed by MRI and histopathology, demonstrating that mutant EGFR is required for tumour maintenance.

Models for Claracell-derived tumours

Claracell-derived squamous cell neoplasms were observed by Rehm et al. [149] after topical skin application for up to 30 weeks of *N*-nitrosomethyl-bis-chloroethylurea (NMBCU) or *N*-nitroso-tris-chloroethylurea (NTCU) in Swiss mice (Cr:NIH(S)). Overall, 58% of NMBCU-treated and 74% of NTCU-treated mice were diagnosed with lung tumours. Immunohistochemically, the well-differentiated areas of the tumours were positive for Clara cell antigen, suggesting a bronchiolar origin.

Sandmöller et al. [173] used the promoter and 5′-flanking sequences of the rabbit uteroglobin gene to target expression of the SV40 T antigen to the lung of transgenic mice. All transgenic founders as well as the descendants from an established line, UT7.1, developed multifocal bronchiolo-alveolar adenocarcinomas originating from Clara cells. At least three different stages in tumour development with progressive loss of the differentiated phenotype could be distinguished by immunohistochemical data and *in situ* hybridization. Only in the initial stage did bronchiolar cells express both uteroglobin and SV40 T antigen, whereas at later stages, only SV40 T antigen was detected, and the most advanced tumours were negative for both proteins.

In order to explore the impact of keratin 14 (K14) in the pulmonary epithelium, which normally lacks both squamous differentiation and K14 expression, human keratin 14 gene *hK14* was constitutively expressed in mouse airway progenitor cells using a mouse Clara cell-specific 10 kDa protein (CC10) promoter [174]. While the lungs of CC10-hK14-transgenic mice developed normally, Dakir and coworkers detected increased expression of K14 and the molecular markers of the squamous differentiation program such as involucrin, 2Bl. In contrast, wild-type loricrin, small proline-rich protein 1A, transglutaminase 1 and cholesterol sulfotransferase lungs were negative. Aging CC10-hK14 mice revealed multifocal airway cell hyperplasia, occasional squamous metaplasia, and their lung tumours displayed evidence of multidirectional differentiation. They concluded that constitutive expression of hK14 initiates the squamous differentiation programme in the mouse lung, but fails to promote squamous maturation. They provided a model for assessing the mechanisms of pre-malignant lesions *in vivo* by modifying differentiation and proliferation of airway progenitor cells.

Models of squamous cell lung neoplasia

Carraresi et al. [175] generated transgenic mice expressing human papillomavirus type 16 E6/E7 genes under the control of the murine keratin 5 gene promoter, which should confer cell type-specific expression in the basal cells of squamous stratified epithelia. Transgenic mice developed thymic hyperplasia and lung neoplasia with 100% frequency, the thymus showing an increased size at 2 months and reaching its maximum dimension at 6 months, when lung carcinomas appeared. After this time hyperplastic thymus glands decreased in size, while malignant formations invaded the mediastinal area.

A chemically induced model of squamous cell carcinoma of the lung in mice has been described

by Wang et al. [176]. After skin painting of eight strains of mice with *N*-nitroso-tris-chloroethylurea they found lung squamous cell carcinoma in SWR/J, NIH Swiss, A/J, BALB/cJ and FVB/J mice, but not in AKR/J, 129/SvJ and C57BL/6J mice.

Models of adenocarcinoma

To determine if 3p tumour suppressor gene Fhit-deficient mice exhibit increased susceptibility to carcinogen-induced lung cancer, Zanesi et al. [177] treated mice with the pulmonary carcinogen 4-methylnitrosamino-1,3-pyridyl-1-butanone.

Wild-type and Fhit-deficient animals did not exhibit significantly different frequencies of lung lesions, but Fhit knockout mice showed significantly increased average tumour volume and multiplicity in tumour-bearing animals, compared with wild-type mice. Tumours of Fhit knockout mice were all carcinomas, whereas wild-type mice did not develop carcinomas. To determine if Fhit absence, in combination with deficiency of an additional 3p tumour suppressor (*Vhl*), would affect the frequency of tumour induction, Zanesi et al. [177] examined the spontaneous and the dimethylnitrosamine-induced tumour phenotypes of $Fhit^{-/-}Vhl^{+/-}$ mice. While no spontaneous lung tumours were observed in Fhit knockout or Vhl heterozygous mice, 44% of $Fhit^{-/-}Vhl^{+/-}$ mice developed adenocarcinomas by 2 years of age. Dimethylnitrosamine-induced lung adenomas and carcinomas in 100% of $Fhit^{-/-}Vhl^{+/-}$ mice and adenomas were seen in 40% of Fhit knockout mice by 20 months of age. Thus, double deficiency in murine homologues of 3p suppressor genes, including haploinsufficiency of *Vhl*, predisposes to spontaneous and induced lung cancers, showing that Fhit-deficient mice will be useful, in combination with other 3p tumour suppressors, in recapitulating a pattern of lung cancer development similar to the human pattern [177].

Tommasi et al. [178] have derived $Rassf1^{tm1Gpp}$ knockout mice in which exon 1-α of the *Rassf1* gene was deleted, leading to specific loss of *Rassf1a* but not *Rassf1c* transcripts. $Rassf1^{tm1Gpp}$-targeted mice were viable and fertile. $Rassf1^{tm1Gpp}$ knockout mice were prone to spontaneous tumorigenesis in advanced age. While only

2 tumours developed in 48 wild-type mice, 6 tumours were found in 35 $Rassf1^{tm1Gpp}$ heterozygous mice and 13 in 41 *Rassf1a* knockout mice. $Rassf1^{tm1Gpp}$ knockout, heterozygous, and wild-type mice were treated with two chemical carcinogens, benzo*(a)*pyrene and urethane, to induce skin tumours and lung tumours, respectively. $Rassf1^{tm1Gpp}$ knockout and heterozygous mice showed increased tumour multiplicity and tumour size relative to wild-type animals.

Dankort et al. [179] designed *BRafCA* mice which expressed normal *BRaf* prior to Cre-mediated recombination, after which *BRafVE*, a MEK1/2-ERK1/2 pathway activator, was expressed at physiological levels. *BRafCA* mice infected with an adenovirus expressing Cre recombinase developed benign lung tumours that only rarely progressed to adenocarcinoma. Moreover, *BRafVE*-induced lung tumours were prevented by pharmacological inhibition of MEK1/2. *BRafVE* expression initially induced proliferation that was followed by growth arrest. Consistent with *Ink4a/Arf* and *TP53* tumour suppressor function, *BRafVE* expression combined with mutation of either locus led to cancer progression.

Soda et al. [180] have established transgenic mouse lines that express EML4-ALK, a fusion-type protein tyrosine kinase generated in human non-small-cell lung cancer, specifically in lung alveolar epithelial cells. All of the transgenic mice examined developed hundreds of adenocarcinoma nodules in both lungs within a few weeks after birth, confirming the potent oncogenic activity of the fusion kinase.

Xu et al. [181] demonstrated that overexpression of the small ribonucleotide reductase (RNR) subunit potently and selectively induced lung neoplasms in transgenic mice. RNR-induced lung neoplasms histopathologically resemble human papillary adenocarcinomas and arise via a mutagenic mechanism, making RNR-transgenic mice a model of lung cancer.

Murine models of neuroendocrine tumours

Another important issue in humans is small-cell lung carcinoma, which is considered by some working groups to be related to neuroendocrine tumour [150, 182, 183], while others state that there

is no connection [143]. This type of tumour does not occur in untreated mice.

A transgenic mouse model of primary pulmonary neuroendocrine cell (PNEC) hyperplasia/neoplasias using v-Ha-*ras* by the neuroendocrine-specific calcitonin promoter (*ras*cal) was initiated by Sunday et al. [182].

Another model of neuroendocrine lung tumours was developed by Meuwissen et al. [150]. They established a mouse model of neuroendocrine lung tumours by conditional inactivation of Rb1 and Trp53 in mouse lung epithelial cells. Mice carrying conditional alleles for both Rb1 and Trp53 developed aggressive lung tumours with high incidence and with striking morphological and immunophenotypic similarities to small-cell lung carcinoma. Most of these tumours, which they designate as murine small-cell lung carcinoma, diffusely spread through the lung and gave rise to extrapulmonary metastases. In their model, inactivation of both *Rb1* and *p53* was a prerequisite for the pathogenesis of small-cell lung carcinoma [150]. This model utilizes mice carrying Cre-LoxP-based conditional (or 'floxed') alleles of the retinoblastoma (*Rb*) and *p53* tumour suppressor genes. Deletion of these genes in cells of the lung was achieved through intrabronchial injection of a recombinant adenovirus expressing Cre recombinase (Ad-Cre). This method reproducibly resulted in the development of lung tumours with the histological, immunohistochemical and metastatic behaviour of human small-cell lung carcinoma. Tumours invariably showed deletion of the two alleles of *Rb* and *p53*, demonstrating the importance of loss of these two tumour suppressor genes in small-cell lung carcinoma development [184].

Linnoila et al. [185] constitutively expressed the transcription factor ASH1 in non-endocrine airway epithelial cells using transgenic mice which developed progressive airway hyperplasia and metaplasia beginning at 3 weeks of life. ASH1 potently enhanced the tumorigenic effect of SV40 large T antigen in airway epithelium. These double transgenic animals (CC10-hASH1 × CC10-SV40Tag) developed massive neuroendocrine lung tumours. Beginning at 6 days of life double transgenic animals were observed to have generalized distal airway epithelial dysplasia and hyperplasia, but no increase was seen in the relative numbers of neuroendocrine cell foci. Remarkably, by 2–4 months, a high percentage of these proliferative epithelial cells exhibited immunoreactivity for the neuroendocrine markers synaptophysin and CGRP. Such diffuse neuroendocrine marker reactivity was never observed in either the CC10-hASH1 or CC10-SV40Tag strains alone. Neuroendocrine reactivity was confined to distal airway epithelial cells, correlating with transgene expression and focal immunoreactivity for CC10. The degree of this generalized neuroendocrine transdifferentiation was positively correlated with the size of developing tumours in adjacent lungs. Moreover, the resulting adenocarcinomas exhibited frequent neuroendocrine differentiation as well. By immunohistochemistry, tumours were positive for synaptophysin, PGP9.5, CGRP and, to a lesser degree, chromogranin and CC10. Most of the lungs from double transgenic animals had neuroendocrine-positive tumours. In contrast, no tumours found in single transgenic CC10-SV40Tag mice expressed any of the neuroendocrine markers [185].

Other transgenic models

A transgenic mouse model of lung cancer was utilized by Linnerth et al. [186], to identify markers of early lung tumours in humans. DNA microarray analysis of lung tumours arising in MMTV-IGF-II-transgenic mice showed nine genes consistently elevated in the murine lung tumours. Immunohistochemical analyses identified three proteins, microsomal glutathione-*S*-transferase 1 (Mgst1), cathepsin H and syndecan 1, as being consistently elevated in the murine lung tumours compared to non-tumour-bearing transgenic lung tissue and normal lung tissue surrounding the tumour. These three proteins were also elevated in human lung adenocarcinoma and squamous cell carcinomas.

An overview of the molecular carcinogenesis of mouse lung tumour models has been given by Wakamatsu et al. [187]. They state that *p53* mutations are late events in mouse lung carcinogenesis and most likely result from indirect DNA damage or genomic instability. There are many review articles about mouse models of human lung cancer available. In recent papers [188–193] genetically engineered mouse models of human

lung cancer have been described and sorted according to genetic background or phenotype of lung cancer.

Conclusion

Because of the relevance of mice for biomedical research, the mouse lung has become the focus of lung physiologists, pharmacologists and toxicologists. Although the murine lung differs considerably in anatomical and physiological details from the human lung, it is widely used for modelling human lung diseases.

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2.8

The Gastrointestinal System and Metabolism

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Introduction

The gastrointestinal (GI) system of the mouse is quite similar to that of other species in the rodentia family. The mouse, unlike the rat, is a day feeder. The normal mouse will consume the majority of its food during the light period [1, 2]. Exceptions to this feeding pattern have been reported in strains of mice having an obesity phenotype [1]. Hyperphagia is a common feature of mice that have a genetic mutation in the neuroendocrine system that signals hunger and satiety [3-5]. Not all of these mutations are known. In mice with aberrant signals for satiety, the increased food intake has effects on both the GI system and metabolism. These will be reviewed in this chapter.

Digestive system

Anatomy and physiology

The digestive system begins with the mouth and ends with the anus (Figure 2.8.1). In the mouth there are two pairs of incisors, two on the top and two on the bottom, plus cheek teeth. A space called the diastema separates the incisors and cheek teeth. The incisors grow continually. When the diet provided is a powder or unpelleted dry mixture, these incisor teeth must be periodically clipped. The required frequency for this clipping varies depending on the nature of the diet and its nutrient content. In the wild these teeth are ground down by the hard seed coats and other rough-textured edibles in the
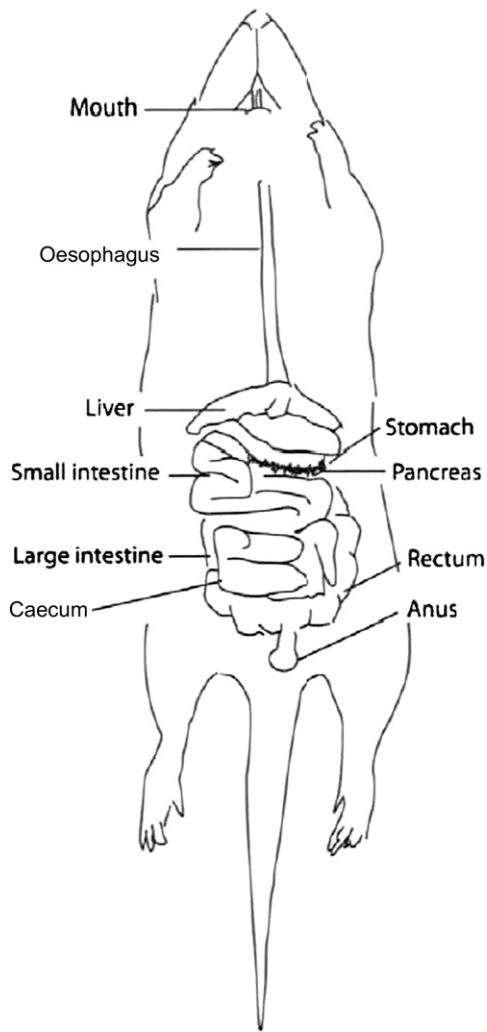
Figure 2.8.1 Anatomy of the mouse digestive system.

environment. In some instances mice will grind their teeth against caging materials or food/water dispensers, thereby shortening them.

Located near the mouth and emptying into it are the salivary glands, the parotid gland lies just behind the ear and extends over the ventrolateral surface of the neck to the shoulder; the mandibular glands are ventral to the parotids and the sublingual glands are under the base of the tongue. These glands contain ascinar cells that drain by way of an interlobular duct. The parotid glands drain into the parotid duct while the mandibular and sublingual glands drain into the mouth via Wharton's duct. This duct terminates in small papillae near the incisors. All three glands produce and release saliva into the mouth. The sublingual secretion is almost entirely mucus while the secretions of the other

two glands vary in the amount of mucus produced. The parotid saliva contains salivary amylase as well as salivary lipase. These two enzymes initiate the digestion of carbohydrate and lipid respectively. However, the amount of digestion that occurs in the mouth and oesophagus is minimal. These two enzymes are inactivated by the low pH of the stomach.

The tongue is long and flexible. It is attached to the floor of the mouth at the rear of the mouth. Taste buds are found on the surface of the tongue. These buds (circumvallate and fungiform papilla) consist of elongated cells arranged around a central lumen that opens to the papillary furrow by a taste pore. It is assumed that salt, sweet and bitter tastes can be detected by these taste buds. However, because there are far fewer of these buds in the mouse than in the human, it is also assumed that the sensory perception of taste is less acute in this species than in the human.

At the back of the mouth is the epiglottis, a raised flap of tissue caudal to the tongue that guards the glottis. Behind this is the soft palate. The soft palate is merely a continuation of the hard palate (roof of the mouth) that in turn gives way to the opening to the pharynx or entryway to the oesophagus.

The oesophagus connects the pharynx to the stomach. It is lined by a thick layer of squamous epithelia cells covered by an acellular layer of cornified tissue. Just internal to the squamous layer is a very thin layer of smooth muscle called the muscularis mucosae and a slightly wider band of connective tissue, the lamina propia.

The stomach consists of the forstomach or entry from the oesophagus, the fundus, and the pyloric region. The fundus is the first true portion of the stomach. It is lined by columnar epithelial cells arranged in deep gastric pits. In these pits are found two types of cells: the chief cells that produce the enzyme pepsin in its precursor form (prepepsin) and the mucin-secreting non-chief cells. On the outer border of the gastric pits are parietal cells. These cells produce a precursor to hydrochloric acid. The pyloric region of the stomach also has an epithelial lining of columnar cells but the cells lining the pits produce only mucin. In the course of digestion, the function of the stomach is to mix the ingested food with both acid and enzymes.

Pepsin, secreted in a precursor form, is activated by the reduced pH that occurs when the stomach contents are mixed with the hydrochloric acid. The muscles of the stomach are arranged such that their contractions and relaxations result in a churning and mixing of the stomach contents, now known as chyme. The stomach is connected to the small intestine by the pyloric valve. This valve functions to regulate the flow of the stomach contents into the duodenum of the small intestine.

The duodenum is the first portion of the small intestine. It is here that the exocrine secretions of the pancreas and Brunner's glands as well as the bile produced by the liver are mixed with the chyme. The digestive enzymes found in these secretions act on the proteins, fats and carbohydrates of the food producing small, easily absorbed molecules (monosaccharides, amino acids and some fatty acids). The digestive enzymes and their functions are listed in Table 2.8.1. Although the mouse has an active lactase at birth, like the rat and some humans, this activity disappears as the neonate develops.

By weaning (at around 21 days) very little lactase activity can be found.

Most maintenance mouse diets have very little fat (<5% by weight) thus a very low lipase activity will be found in the duodenal contents. Low-fat mouse diets have a longer shelf life than diets having a higher percentage of fat. Since the mouse can grow satisfactorily on this level of dietary fat, the typical mouse pelleted ration supplied to mouse production facilities will contain 4–5% fat. Higher-fat diets have been prepared for specific purposes and these will induce a more active lipid digestive system [6]. In addition, those mice produced through transgenics where the mutant gene for one or more of the lipid-carrying proteins has been inserted will become lipaemic when fed high-fat or cholesterol-enriched diets [7].

In general, the mouse maintenance diet contains varying amounts of protein (10–15% by weight) and the remaining part of the diet is a mixture of simple and complex carbohydrates. Mice in the wild may exist on seeds, nuts or scavenged food, widely varying in composition.

TABLE 2.8.1: Digestive enzymes and their substrates

Enzyme	Location	Target or substrate
Pepsin	Stomach	Peptide bonds involving aromatic amino acids
Trypsin	Small intestine	Peptide bonds involving arginine, lysine
Chymotrypsin	Small intestine	Peptide bonds involving tyrosine, tryptophane, phenylalanine, methionine, leucine
Elastase	Small intestine	Peptide bonds involving alanine, serine, glycine
Carboxypeptidase A	Small intestine	Peptide bonds involving valine, leucine, isoleucine, alanine
Carboxypeptidase B	Small intestine	Peptide bonds involving lysine, arginine
Endopeptidases	Enterocytes	di- or tripeptides that enter the enterocytes
Alpha-amylase	Mouth, small intestine	Starch, amylopectin, glycogen
Alpha-glucosidase	Small intestine	α limit dextrin
Lactase	Small intestine	Lactose
Maltase	Small intestine	Maltose
Sucrase	Small intestine	Sucrose
Lingual lipase	Mouth	Triglycerides
Duodenal lipases	Small intestine	Triglycerides
Esterase	Small intestine	Cholesterol esters

The composition of the diet and frequency of feeding will determine the amount and activity of the digestive enzymes released into the duodenum as well as the rate of passage of food from the mouth to the anus. In addition to the release of digestive fluids by both the intestinal enterocytes and the exocrine pancreas, there are also mucus-producing goblet cells intermittently located between the columnar epithelial cells.

The epithelial layer of the small intestine has numerous finger-like projections called villi. These are the absorptive units of the intestine. Amino acids, simple sugars and short-chain fatty acids are absorbed by the villous absorptive cells called enterocytes. The villi are very densely placed in the duodenum. Their density becomes less as the intestinal tract proceeds towards the large intestine. The need for absorptive cells also becomes less as the simple nutrients from the diet are absorbed. Simple sugars and amino acids are the first to disappear from the chyme. Electrolytes disappear rapidly from both stomach and duodenum. Calcium, magnesium, iron and other essential minerals are absorbed slowly all along the intestinal tract. Water-soluble vitamins are absorbed in the duodenum while the fat-soluble vitamins follow the pattern of the long-chain fatty acids and cholesterol. This occurs in the ileum and, to some extent, the jejunum. In general, the essential nutrients (glucose, essential amino acids, vitamins) are absorbed via an active transport system. Some of the essential minerals require dedicated mineral transport proteins while others can share transporters or even compete for these transporters.

The epithelial cells as described above comprise the mucosal layer of the intestinal tract. Beneath the mucosal layer is a relatively thin smooth muscle layer, the muscularis mucosae. These muscles contract and relax rhythmically so as to propel the contents through to the large intestine and anus. Both longitudinal and horizontal contractions occur.

The jejunum is the middle segment of the small intestine, between the duodenum and the ileum. Digestion begun in the duodenum continues here, as does absorption. Generally, the simple sugars and readily available amino acids have already been absorbed but the larger macromolecules are still in need of digestion so as to release more absorbable nutrients. This is also the case for the ileum, the final portion of the small intestine. This portion is characterized by lymph nodules (Peyer's patches) in the submucosa. The villi of the ileum contain thin-walled lymph vessels (lacteals) in the lamina propria that function in the absorption of dietary fat. Dietary fat and vitamins A, D, E and K are absorbed here. The triacylglycerides are hydrolysed and then resynthesized in the process of absorption. With resynthesis they are joined to protein carriers for transport to the peripheral tissues for storage or to the liver for use. Cholesterol likewise is processed. If esterified, the fatty acid is removed and the free cholesterol is joined to a protein carrier and carried via the lymph to the thoracic duct. The lipid–protein complexes so formed at the site of the intestine are called chylomicrons. Lipid–protein transport complexes differ in the ratios of lipid and protein and thus differ in density. The chylomicrons are the least dense. Table 2.8.2 shows these protein carriers and indicates their function. The chylomicrons utilize proteins known as Apo A-I, A-IV, B and C-II. At the fat cell the triglycerides are released through the action of the interstitial lipoprotein lipase. These lipids pass into the fat cell and are stored.

TABLE 2.8.2: Lipid-carrying proteins	
Protein	**Function**
Apo A-II	Protein in the high-density lipoprotein
Apo B-48	Protein in the chylomicrons
Apo A-I	Protein in chylomicrons
Apo C-III	Protein in very low-density lipoproteins
Apo A-IV	Protein in chylomicrons
CETP	Cholesterol transport protein originating in peripheral cells
LCAT	Protein in high-density protein
ApoE	Mediates binding of low-density lipoproteins to receptor
Apo C-I	Protein in low-density lipoprotein
Apo B-100	Protein in the very low-density protein

The loss of the glycerides results in a less dense lipid–protein complex containing primarily cholesterol. The lipid-carrying protein ApoE is added and the complex moves to the liver. Here the cholesterol is taken up and the lipid-carrying proteins released for reuse. Lipids synthesized in the liver are transported to the fat cells using the proteins labelled ApoE, B, B-100, C-I, II and III. These same proteins are used for lipid recycling. In the mouse with an active *de novo* lipogenic system, endogenous lipid transport proteins are more common than those used for the transport of food lipid. Because mice are routinely fed low-fat-high-carbohydrate diets, *de novo* lipogenesis is quite active. If the carbohydrate in the diet is a simple sugar, then lipogenesis becomes very active and indeed, liver lipid, normally 3-4% of the organ by weight, can rise to 5-6%. The flux through the lipogenic pathway in both liver and adipocyte increases as does the activity of the rate-limiting enzyme acetyl CoA carboxylase and enzymes involved in the production of reducing equivalents needed to support lipogenesis. With time the normal mouse will adapt to this high-sugar diet and develop more efficient lipid packaging and export systems. At this point the liver lipid level will return to normal.

The remaining non-absorbed food plus the desquamated epithelial cells and residual digestive enzymes and mucins now leaves the ileum, entering the colon or large intestine. At this juncture is the caecum. In rodents the caecum is a rather large appendage projecting caudally from the ileum–colon juncture; this sac functions as a fermentation vat. In the mouse it can be up to a third of the length of the large intestine. Its size is dependent on the composition of the diet. Diets containing large amounts of complex carbohydrates will result in larger caecums than diets containing very little complex carbohydrate. In this fermentation vat bacteria act on undigested fibres (as well as other undigested materials) and produce metabolically useful products. Short-chain fatty acids are produced here and these can be readily absorbed and used by the body. Some vitamin synthesis occurs here as well as in the colon and this synthesis has a benefit to the animal. In particular, vitamin K is synthesized and will be absorbed by the epithelial cells of the colon. Some of the B vitamins are also produced and absorbed or excreted in the faeces.

If the animal is maintained on a wire mesh floor that prevents *coprophagy* (consumption of faeces), some of this synthesized vitamin will be lost to the animal.

Finally, at the end of the colon is the rectum. Here the last remnants of the intestinal contents are 'stored' prior to defecation. Mucus is produced to lubricate and enhance defecation. The longitudinal layer is thin compared to that of the colon except for two distinct longitudinal bundles called taenia coli. The opening at the end of the rectum is called the anus and consists of keratinized, stratified squamous cells. The lamina propria is very thick and contains circumanal glands that open by short broad ducts to the anal canal. The striated muscle fibres are specialized into internal and external sphincters needed to expel the faeces.

Regulation of food intake

The cells of the GI tract have both an endocrine and an exocrine function. The release of the digestive enzymes has already been described. As such, these cells have a function with respect to nutrient digestion and absorption. They also have an endocrine function in that they can release substances that have hormone activity. The definition of a hormone is a substance that is released by one cell type into the blood and then has an effect on a distant cell. Cholecystokinin and gastrin meet this definition, as does somatostatin (the long form, SS-28). SS-28 serves as a paracrine to inhibit gastrin release and hydrochloric acid release. The GI tract also releases neuromedin B. This neuropeptide counteracts SS-28. Galanin, vasoactive peptide (VIP), gastrointestinal peptide (GIP), motilin, calcitonin gene-regulated peptide (CGRP), endothelin, neurotensin, met-enkephalin, leu-enkephalin, enteroglucagon and bombesin are all 'hormones' produced by cells in the GI tract. Some of these have a role in the generation of a hunger signal while others serve to signal satiety. These substances and their functions are listed in Table 2.8.3. Most of these signals are of very short duration [8] and, although they can stimulate eating or satiety, their effects are not long lasting. Longer-lasting signals to the brain are thought to control the duration of feeding and its cessation.

TABLE 2.8.3: Hormones released by the gastro-intestinal tract

Hormone	Function
Cholecystokinin (CCK)	Stimulates exocrine pancreas to release digestive enzymes found in the brain and thought to signal satiety
Gastrin	Stimulates gastric acid and pepsin release; found in the brain
Secretin	Stimulates bicarbonate release
Enkephalins	Inhibit gut motility, 'may' signal satiety
Enteroglucagon	Trophic factor for intestinal mucosa
GIP	Inhibits gastric acid secretion
Motilin	Stimulates movement of chyme through intestine
Neurotensin	Inhibits insulin release
Pancreatic polypeptides	One of these, Neuropeptide Y, stimulates food intake
Somatostatin	Inhibits growth hormone release
Bombesin	Signals satiety
Tachikinin	Signals satiety?
Endothelin	Vasoconstrictor

These signals include leptin [9], circulating glucose [10] and fatty acids [11]. Some amino acids and proteins may also play a role.

Endocrine aspects of digestion and absorption

In addition to those satiety signals generated by cells of the GI system, there are those released by adipose tissue (leptin) and the central nervous system (CNS) that affect food intake as well as metabolism. Hormones (insulin, somatostatin, glucagon) generated by the endocrine pancreas and the signals (mainly metabolites) generated by the liver affect GI function as well as metabolism. For this reason, the pancreas and the liver are included in the description of the digestive system. The exocrine pancreas produces digestive enzymes as described above and the liver produces the bile needed for fat absorption. In addition, the liver is the first tissue to receive, via the portal vein, the products of digestion and absorption. As such, it is the central integrator of metabolism as it pertains to the use of nutrients derived from food.

The pancreas is both an exocrine and an endocrine gland. As an endocrine gland it produces and releases three hormones that have major regulatory authority over the use of food components by the body: insulin, produced by the beta cells of the islets of Langerhans; glucagon, produced by the alpha cells; and somatostatin (the short form, SS-14) produced by the delta cells of the islets. These three hormones determine the fate of intermediary metabolism, whether it be catabolic or anabolic—that is, whether macromolecules are degraded or synthesized. Somatostatin serves as a regulator of the balance of insulin and glucagon. It inhibits the release of either of these hormones and thus serves to prevent excess levels of either. Insulin acts primarily in the fed animal to facilitate the use of glucose and amino acids while glucagon acts primarily in the starving animal to facilitate the mobilization of energy stores. Glucose from the ingested food is absorbed by the enterocytes and transferred to the blood. Rising blood glucose signals the pancreas to release insulin. Insulin, in turn, stimulates the uptake and metabolism of this glucose by the rest of the body. When the animal ceases to eat and the circulating glucose falls, glucagon is released that in turn facilitates or stimulates the release of glucose from glycogen and also stimulates the synthesis of glucose (*gluconeogenesis*) from metabolites such as pyruvate.

Metabolism

Pathways of intermediary metabolism

The term 'intermediary metabolism' covers all those reactions in the body concerning the

conversion of the products of digestion to useful molecules. It includes the synthesis of macromolecules as well as macromolecule breakdown. As such, metabolism is divided into two parts: *catabolism* (macromolecule breakdown) and *anabolism* (macromolecule synthesis). Catabolic processes include glycolysis, pentose shunt, glycogenolysis, fatty acid oxidation, lipolysis and amino acid catabolism. Anabolic pathways include glycogenesis, protein synthesis, lipogenesis and cholesterogenesis. Each of these pathways functions continuously, yet their activity is determined by the nutritional and hormonal state of the animal. Starving animals are primarily catabolic. Glycogen stores are raided, fat stores are used and some amino acids are oxidized. Glucose is synthesized (gluconeogenesis) to provide this essential fuel to the CNS. Some anabolism does take place during starvation but it is minimal compared with that which takes place in the non-starving animal. Similarly, the fully fed, resting animal is primarily anabolic. Some catabolism takes place to provide the substrates needed to support anabolism but major macromolecule destruction is minimal. In between starvation and full feeding, there is a balance between anabolism and catabolism that occurs such that the physiological state of the animal is optimized. In this respect, there is a constant and steady state of metabolism that is maintained until this state is perturbed. Perturbations can be the result of changes in the environment (food supply, temperature, changes in lighting schedule) or internal processes (chronic disease, pathogens, reproductive activity, age). In any event, normal animals adjust to such perturbations and a new steady state is established to ensure survival. If such perturbations are overwhelming, the animal will not be able to adjust and death will ensue.

Metabolic control

Metabolic processing of carbohydrate, lipid and protein is carefully regulated. Glycolysis, shown in Figure 2.8.2, has three steps that regulate the flux from glucose to pyruvate. The reverse of glycolysis, gluconeogenesis, is shown in part in this figure and again in Figure 2.8.3. Also shown in this figure is the pentose

phosphate shunt that provides reducing equivalents for the support of lipogenesis. When lipogenesis is very active, as in a mouse fed a sugar-rich diet, pentose shunt activity rises. The shunt also produces phosphorylated ribose that is used for DNA and RNA synthesis. The rate-limiting steps for glycolysis, pentose shunt and gluconeogenesis are listed in Table 2.8.4. The end product of glycolysis, pyruvate, enters the citric acid cycle (Figure 2.8.4) for oxidation and produces reducing equivalents that in turn are transferred to the respiratory chain (Figure 2.8.5). The energy developed by the respiratory chain is captured in the high-energy bond of ATP and this high-energy material transfers its energy to those reactions requiring it. ATP synthesis and the respiratory chain are coupled in the process called oxidative phosphorylation (OXPHOS).

Once glucose is phosphorylated it can go into several pathways: glycolysis, the pentose phosphate shunt (Figure 2.8.2) or glycogenesis (minimally shown in Figure 2.8.2). Glucose 6-phosphate is isomerized to glucose 1-phosphate and then converted to UDP glucose. Through the action of active glycogen synthetase, glucose units are added stepwise to pre-existing glycogen molecules. The animal never uses all of its glycogen, even when starving; it always reserves a small amount to serve as a primer for subsequent glycogen synthesis. It might be unmeasurable in quantity but it is always there. Glycogen breakdown (Figure 2.8.6) provides a quick source of energy to working muscle. The release of glucose from glycogen is carefully regulated via a cascade of events initiated by one or more of the catabolic hormones. Epinephrine, for example, stimulates glycogenolysis. This catecholamine stimulates the activation of adenyl cyclase, which catalyses the conversion of ATP to cAMP. This stimulates the activation of cAMP-dependent protein kinase that activates phosphorylase b, which in turn activates glycogen phosphorylase a. Once glycogen phosphorylase a is activated, it catalyses the phosphorylation of glycogen and glucose 1-phosphate is released. After isomerization to glucose 6-phosphate the glucose is available for use. Gluconeogenesis is shown in detail in Figure 2.8.3. The main rate-controlling step is the conversion of malate to phosphoenolpyruvate through the
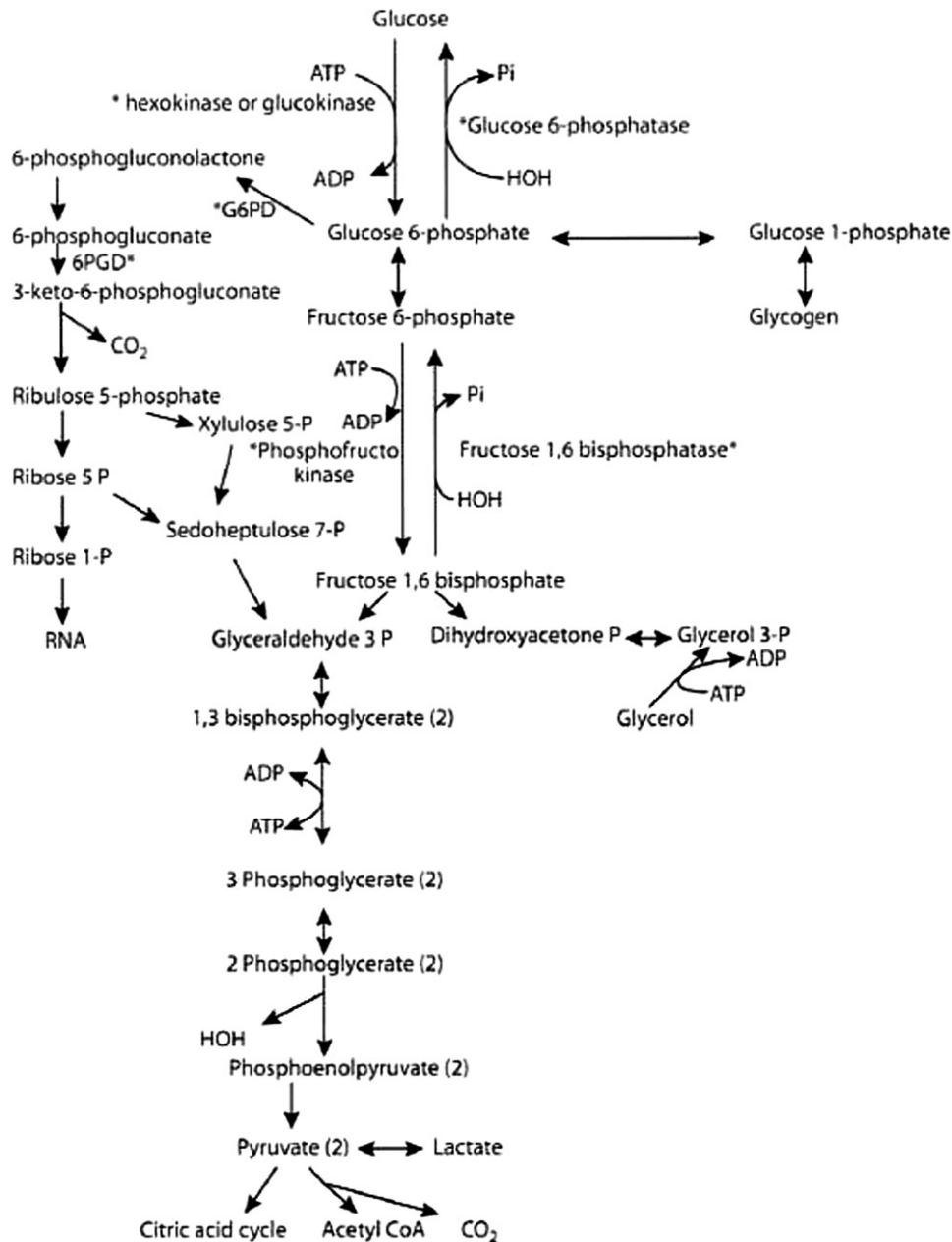
Figure 2.8.2 The glycolytic sequence and the pentose phosphate shunt. Key rate-limiting steps are indicated by an asterisk. Part of the gluconeogenic sequence is also shown through the reverse arrows in the glycolytic sequence. The relationship of the glycolytic sequences to glycogen is indicated.

action of the enzyme phosphoenopyruvate carboxykinase (PEPCK). Malate availability is determined by the outward flow of malate from the mitochondrial compartment into the cytosolic compartment. This flux, known as the malate aspartate shuttle, is in turn influenced by the phosphorylation state of the compartment that is in turn influenced by OXPHOS. PEPCK is very active in starvation and is controlled by

hormonal state [12]. Lipogenesis (Figure 2.8.7) and lipolysis plus fatty acid oxidation (Figures 2.8.8 and 2.8.9) complete the energy storing and releasing processes. Again, the rate-limiting steps are shown in Table 2.8.4. Cholesterol synthesis uses the same starting substrate as fatty acid synthesis, acetyl CoA. Acetyl CoA is joined to acetoacyl CoA to form HMG CoA. This product is converted to melvalonate and it is this step
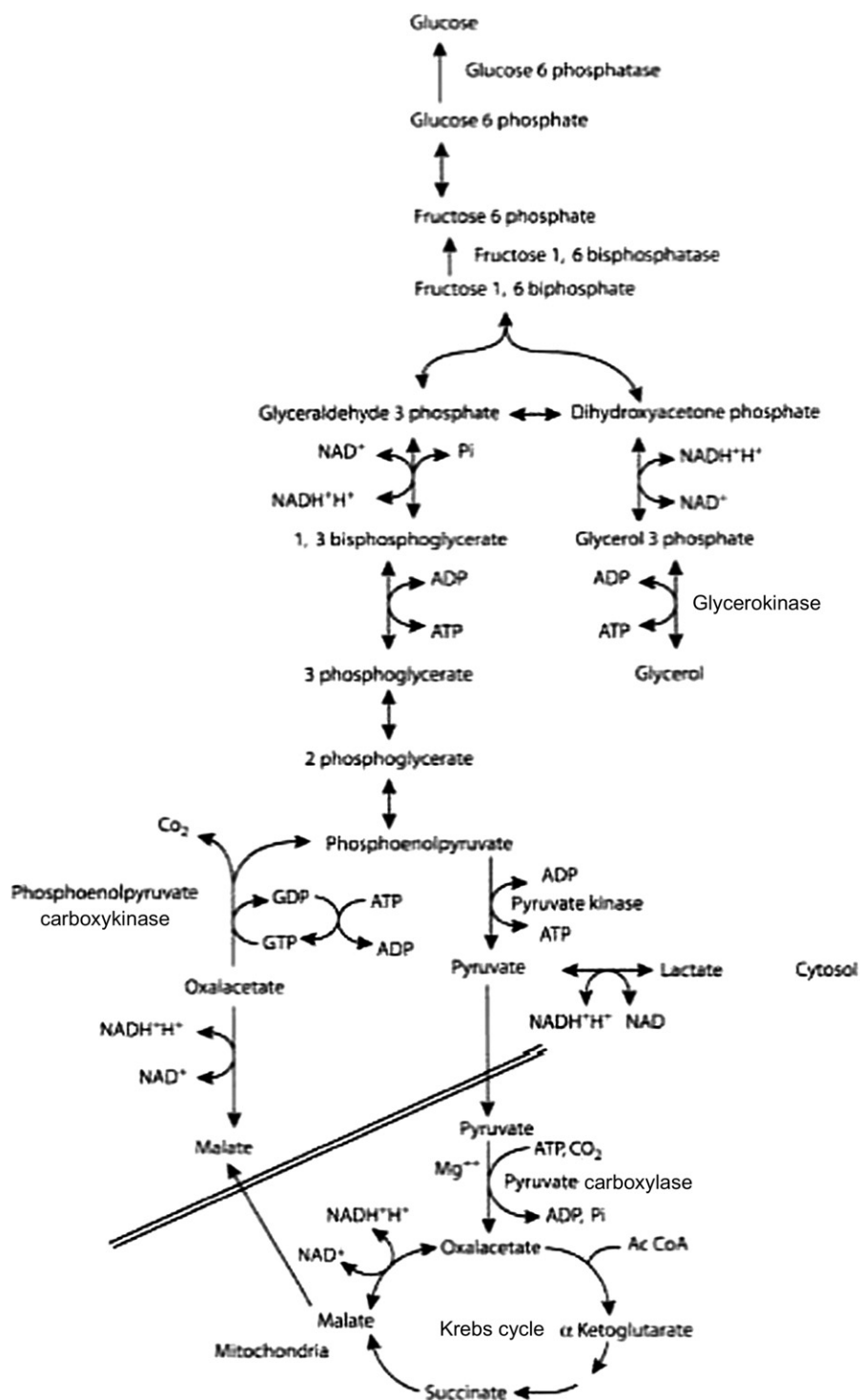
Figure 2.8.3 Gluconeogenesis. This pathway is very expensive with respect to its energy need and is used primarily in the starving animal. Its primary regulatory step is that catalysed by phosphoenolpyruvate carboxykinase.

that is rate limiting. Melvalonate is converted to farnesyl phosphate, then to squalene, then to lanosterol and finally to cholesterol. This cholesterol can be used for the synthesis of the sex hormones, for vitamin D, glucocorticoids and mineral corticoids, and can be esterified to produce cholesterol esters. Protein degradation and amino acid recycling into new protein as

TABLE 2.8.4: Rate-limiting enzymes in metabolic pathways

Pathway	Rate-limiting enzymes
Glycolysis	Hexokinase (glucokinase), phosphofructokinase, pyruvate kinase
Pentose shunt	Glucose 6-phosphate dehydrogenase, 6-phosphogluconate dehydrogenase
Glycogen synthesis	Glycogen synthase
Glycogenolysis	Glycogen phosphorylase (a reaction cascade)
Gluconeogenesis	Phosphoenolpyruvate carboxykinase, glucose 6-phosphatase, glucose 1,6 phosphatase
Fatty acid synthesis	Acetyl CoA carboxylase, fatty acid synthetase
Citric acid cycle	Pyruvate dehydrogenase, pyruvate kinase, ATP-citrate lyase
Lipolysis	Lipoprotein lipase
Fatty acid oxidation	Carnitine palmitoyl transferase
Cholesterol synthesis	HMG CoA reductase

well as amino acid oxidation are sometimes included in the pathways of intermediary metabolism. However, in terms of energy balance, the contribution of amino acids as an energy source is minor compared with that of the lipids and carbohydrates. Protein synthesis and degradation are heat-producing processes and cannot be ignored. These processes will not be discussed in this section.

Heat production (thermogenesis) is a feature of all metabolic processing. The energy lost and gained by metabolism contributes to the energy balance of the animal. The animal is by no means energetically efficient. Most of the energy lost from metabolic processing is lost as heat. This heat is needed to sustain body temperature and optimize enzyme activity. Some energy is used for neural transmissions and vision (chemical energy or electrical energy) and some is used

for the work of the body (mechanical energy). Above and beyond the normal amount of heat energy lost through metabolic processing, additional heat can be generated on demand should environmental conditions so warrant. Thermogenesis by brown fat pads is an important contributor to this extra energy release. A mouse suddenly thrust into a $4\,^{\circ}$C environment will begin adapting to this cold by stimulating brown fat thermogenesis. The catecholamines and the thyroid hormones play an important role in this thermogenic process. Upon exposure to cold the normal mouse will immediately release noradrenaline (norepinephrine) that in turn induces the production of an uncoupling protein that then dissipates the proton gradient generated by the mitochondrial respiratory chain. This results in an uncoupling of oxidative phosphorylation in the brown fat cells and the energy usually trapped in the high-energy bond of ATP is released as heat instead. Cold-exposed genetically obese mice (Lep^{oh}/Lep^{oh} and others) cannot rapidly respond to this cold environment and die from hypothermia. When the temperature is gradually lowered they are able to adapt, so this feature of genetic obesity is characteristic of the phenotype not the cause of its phenotype.

With respect to metabolism in general, the controls of metabolism are similar to those of other mammals. Glycolysis, pentose phosphate shunt, citric acid cycle, oxidative phosphorylation, lipogenesis, lipolysis, ketone formation, glycogen synthesis, gluconeogenesis, glycogenolysis, protein synthesis and degradation are all controlled not only by the availability of the starting substrates as determined by nutritional state but also by certain of the enzymes in the metabolic pathway as well as by the hormonal state of the animal.

Hormonal status can be genetically determined. The mice with mutations that phenotype as diabetes, for example, will have their metabolic pathways perturbed by their developing diabetic state. For example, Roesler and Khandelwal [13] followed Lep^{db} mice and their controls for 16 weeks [13]. Initially, there were no differences in glycogen metabolism between the two groups of mice. However, with age, glycogen synthetase activity rose in the Lep^{db} mouse, reaching a peak at around 8–9 weeks.
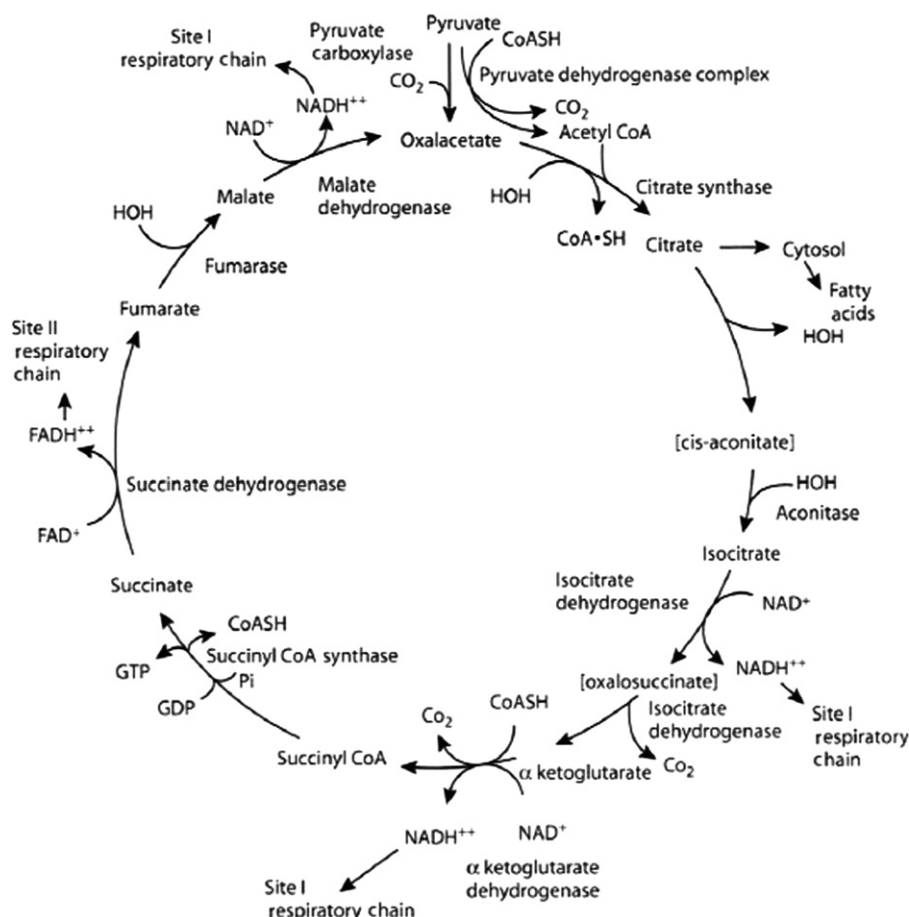
Figure 2.8.4 The citric acid cycle in the mitochondria. This cycle is sometimes referred to as the tricarboxylate cycle or Krebs cycle.
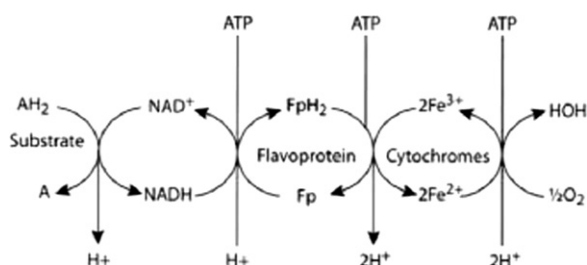
Figure 2.8.5 The respiratory chain. Products of glycolysis and fatty acid oxidation contribute their reducing equivalents to this chain. Pyruvate and alpha-ketoglutarate contribute their reducing equivalents via lipoate at the first entry site of the chain. Proline, 3-hydroxyacyl-CoA, 3-hydroxybutyrate, glutamate, malate and isocitrate contribute reducing equivalents via NAD-linked dehydrogenase, and succinate, choline, glycerol 3-phosphate, acyl CoA, sarcosine and dimethylglycine all contribute via FAD-linked flavoproteins. Each time a proton gradient is developed, ATP is synthesized. A total of three ATPs can be synthesized when reducing equivalents enter via NAD. Two ATPs are synthesized when reducing equivalents are contributed via FAD.

Glycogen synthesis also peaked at this time. Subsequently, synthesis fell as glycogenolysis began to rise in these mice compared with their normal controls. The rise in glycogen breakdown was measured through the activity of phosphorylase a. These changes in glycogen metabolism coincided with the development of the diabetic state in the $Lepr^{db}$ mouse.

Other hormones (in addition to insulin) are also active in the control of intermediary metabolism. The adrenal cortical hormones, the adrenal medullary hormones, the pituitary hormones and the thyroid hormones all have an impact on the flux of substrates through metabolism. Some of these hormonal effects are direct while others are indirect. Nonetheless, all are needed for the integration of metabolism such that nutrients from the food are absorbed, oxidized or used for the synthesis of macromolecules in the body.
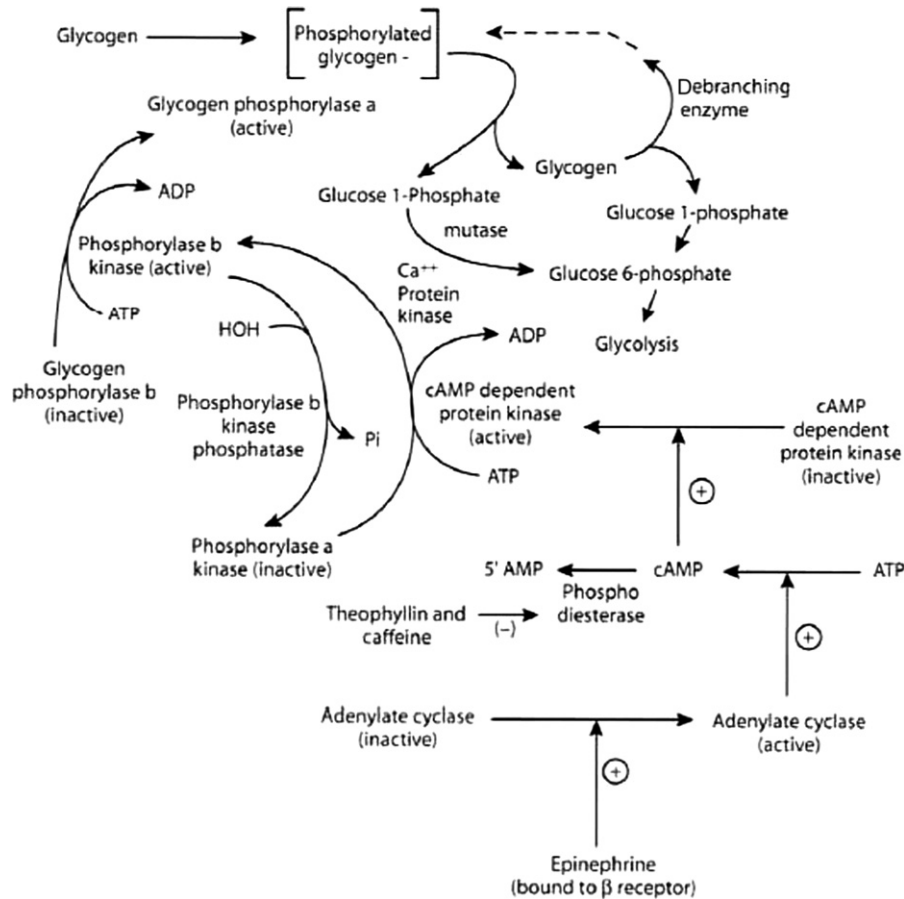
Figure 2.8.6 Glycogen breakdown catalysed by a cascade of reactions initiated by one or more catabolic hormonal signals such as adrenaline (epinephrine).

Differences in metabolism

The regulation of food intake is important to the understanding of metabolism. Where hyperphagia occurs as a result of an error in the production/release/response to satiety factors, the mouse will have an enlarged GI tract due to its increased food consumption and will be more anabolic than catabolic. As hyperphagia means the consumption of more than the needed amount of food to sustain health, this surplus food will provide additional substrates for the anabolic reactions that are part of metabolism. Figure 2.8.10 is an abbreviated outline of metabolic processing of carbohydrate and its conversion to fat. The rate-limiting steps are shown as heavy arrows and indicate that the hyperphagic mouse with the obese phenotype will have a more active reaction sequence than a normal mouse. However, obesity aside, there

are variations in the activities of key regulatory steps in these metabolic pathways. As an example, Table 2.8.5 provides a strain comparison in the activity of hepatic glucokinase [14, 15]. The phosphorylation of glucose via the enzyme glucokinase is the first rate-limiting step in hepatic glycolysis [16]. In the pancreas the glucokinase reaction is the glucose sensor of the endocrine pancreatic beta cell and signals insulin release. Note that considerable variation in hepatic glucokinase activity exists. In part, this variation may be related to genetic variation in the expression of the gene for glucokinase regulatory protein [17]. Glucokinase activity is also regulated by the amount of substrate (glucose) available [18] and the amount of long-chain acyl-CoAs [19, 20]. The latter inhibits enzyme activity while the former stimulates it. However, one should also realize that the amount of enzyme or its activity measured
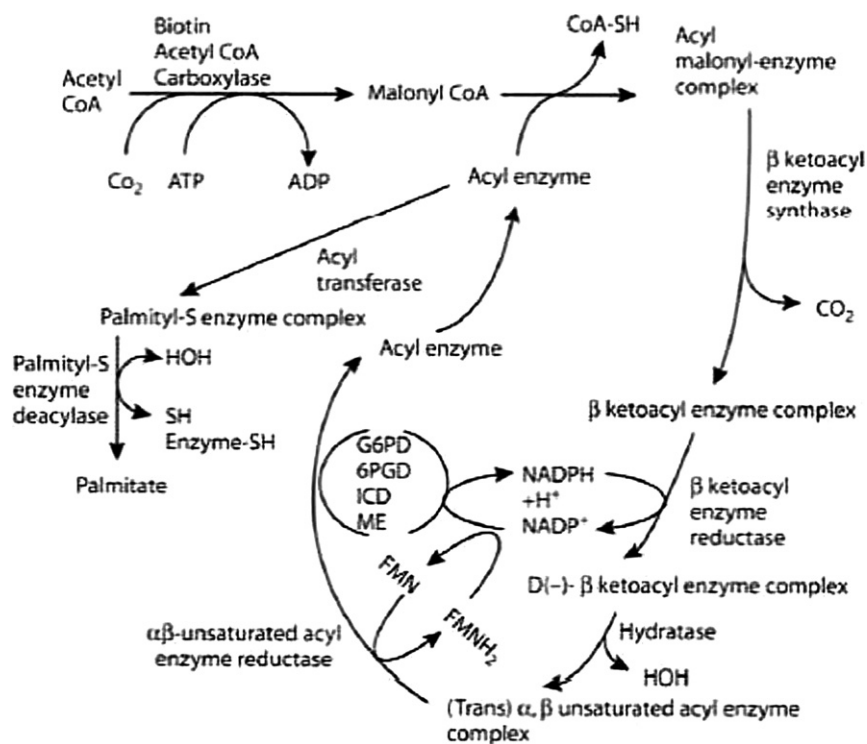
Figure 2.8.7 Lipogenesis. The synthesis of fatty acids from the two-carbon unit acetyl CoA.

in vitro is not a true measure of the activity of the pathway in question. Enzymes rarely work at saturation *in vivo*. The measurement of such activity *in vitro* is an optimized measurement. That is, all the needed substrates and cofactors are provided in optimal amounts. The best measurement of a metabolic pathway is through a dynamic measurement of the flux through the pathway. This is rarely done because of the difficulty in assessing back flow as well as disposal rates of the end products of the pathway. In some instances corrections for recycling must be applied and these are very difficult indeed to calculate and measure. An example of flux measurement reported by Smith et al. is shown in Table 2.8.6 [21]. The workers compared obese diabetic C57BL/6-*Lep^{ob}/Lep^{ob}* and normal (C57BL/6) mice. As expected, the obese hyperglycaemic mice were less glycolytic than the normal controls, as shown by the reduction in the fractional glucose use. Blood glucose levels were higher in the fed state in both genotypes and the mutant genotype had higher blood glucose levels regardless of feeding status. Note also that the mutant

genotype had higher glucose synthesis rates and glucose recycling was higher when the mice were in the fed state. Liver glycogen reflected the abnormal glucose metabolic flux: the mutant mice had far higher levels of glycogen in both fed and fasted state and this liver glycogen may reflect a defence of the animal against elevated blood glucose levels. By synthesizing and storing glucose as glycogen the blood glucose level can be reduced.

Similar shifts in fatty acid synthesis, deposition and lipolysis are observed in mice that have the obesity phenotype. The hyperphagia of these genotypes increases the intake of carbohydrate that must be metabolized. The carbohydrate can be oxidized, recycled as shown above or converted to fatty acids and stored as triglycerides. The increase in the amount of substrate that must be converted to fat induces an increase in the activities of those enzymes needed for such synthesis, as well as those proteins needed for transport and those needed to facilitate storage. Numerous reports exist that document the increase in *de novo* lipogenesis that occurs in obese strains of mice.
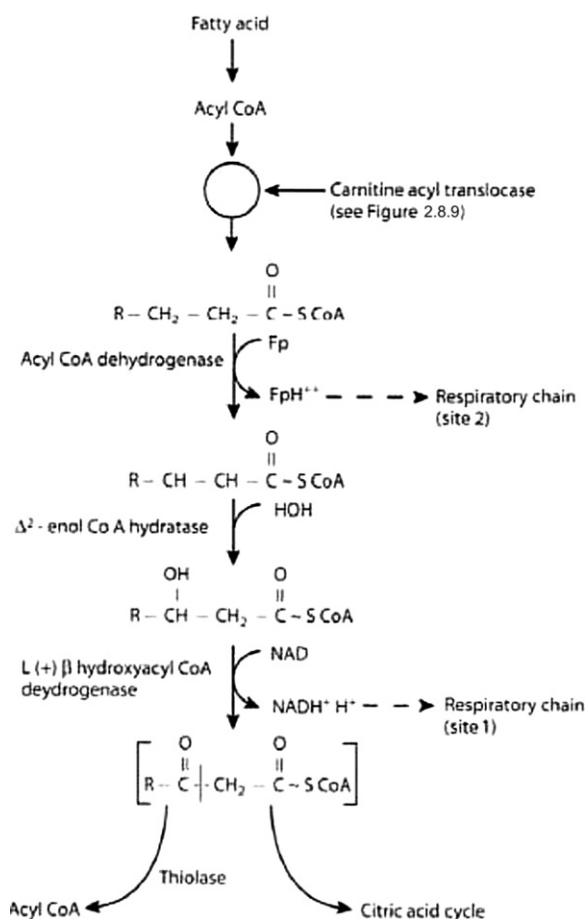
Figure 2.8.8 Fatty acid oxidation in the mitochondrial compartment. The key limitation of fatty acid oxidation rests with the entry of the fatty acid into the mitochondrial compartment.

Typically these strains have a fatty liver as well as enlarged adipose tissue storage depots.

In animals having mutations in genes relating to glucose use, the phenotype is that of diabetes [22]. In some the phenotype can also include obesity, but in others obesity is absent. The NOD mouse typifies the latter while the Lep^{ob}/Lep^{ob} and $Lepr^{dp}/Lepr^{dp}$ typify the former. The genetic defect thus determines the metabolic defect. In the obese Lep^{ob}/Lep^{ob} mouse, the mutant gene encodes leptin, the hormone produced by the adipocyte that signals satiety to the brain. In the $Lepr^{dp}/Lepr^{dp}$ mouse the defective gene encodes the leptin receptor. In both instances the satiety signal is aberrant and in both hyperphagia is part of the phenotype and can explain the metabolic patterns in these mice. The defect in the NOD mouse involves a defect in one or more components of the

immune system. These mice are not obese. Their pancreases have signs of autoimmune disease. The insulin production of these mice declines with age and diabetes is apparent. The metabolic patterns are those of insulin deficit. Glucose is not used appropriately and fatty acid mobilization occurs. This means an increase in peripheral lipolysis, fatty acid oxidation and, because of the insulin deficit, an increase in ketone production. When these mice develop these signs of abnormal intermediary metabolism they must receive exogenous insulin or they will die.

There are other genetically determined variants in metabolism in the mouse and these have been exploited by researchers interested in nutrition. Specific dietary components can influence the phenotypic expression of a specific genotype as illustrated by Surwit et al. [6]. These investigators used two strains of mice, C57BL/6J and A/J, fed either a stock diet or a high-fat–simple-carbohydrate diet. The latter diet induced obesity but only the C57BL/6J mice became glycaemic. Actually, the composition of the diet can determine when a particular phenotype will be observed. Leiter et al. [23] fed diets differing in carbohydrate content to db/db mice [23]. They found that a 60% simple-sugar diet elicited the diabetic state more rapidly than did an 8%- or 24%-sugar diet. Further, when these mice were fed a starch diet, the phenotypic expression of the diabetes genotype was delayed and lifespan was extended.

Diurnal variation in metabolism occurs in normal mice just as it occurs in other species. The synchronizer of these rhythms is not fully known, but the lighting schedule (hours of dark and light) influences the feeding pattern. In turn, the feeding pattern influences metabolic flux. When glycolysis is high, gluconeogenesis is low; when lipogenesis is high, lipolysis is low; when glycogen synthesis is high, glycogenolysis is low. These highs and lows follow a daily pattern that appears to be cued by light [1, 2, 24–26]. In contrast, genetically obese mice seem to lose this diurnal rhythm and eat fairly constantly throughout the day–night cycle. As a result, they also lose the above-described rhythm in metabolism. Changes in food availability will also affect metabolic rhythm. If mice are forced to consume all of their food in a single 2h meal instead of
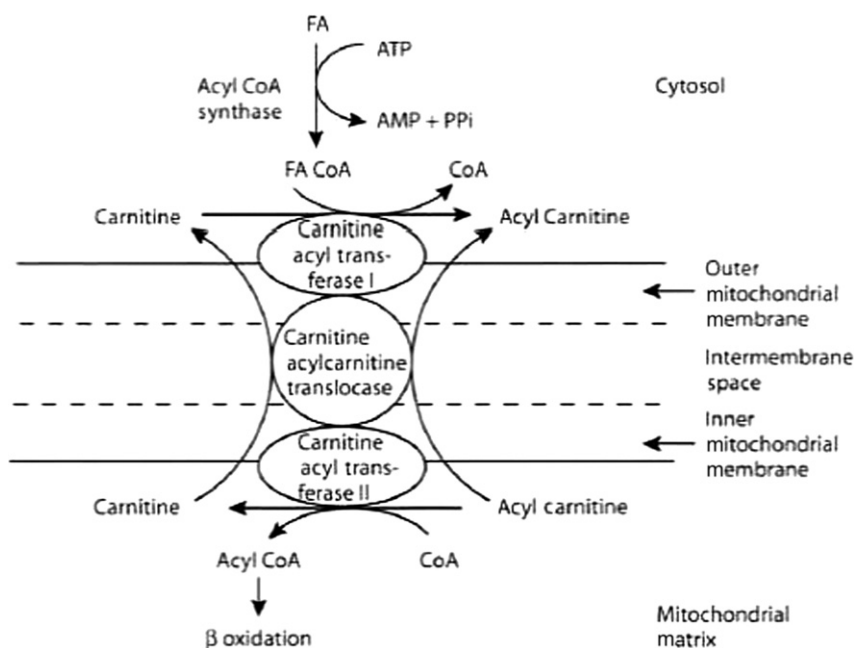
Figure 2.8.9 Scheme for the entry of fatty acids into the mitochondrial compartment.
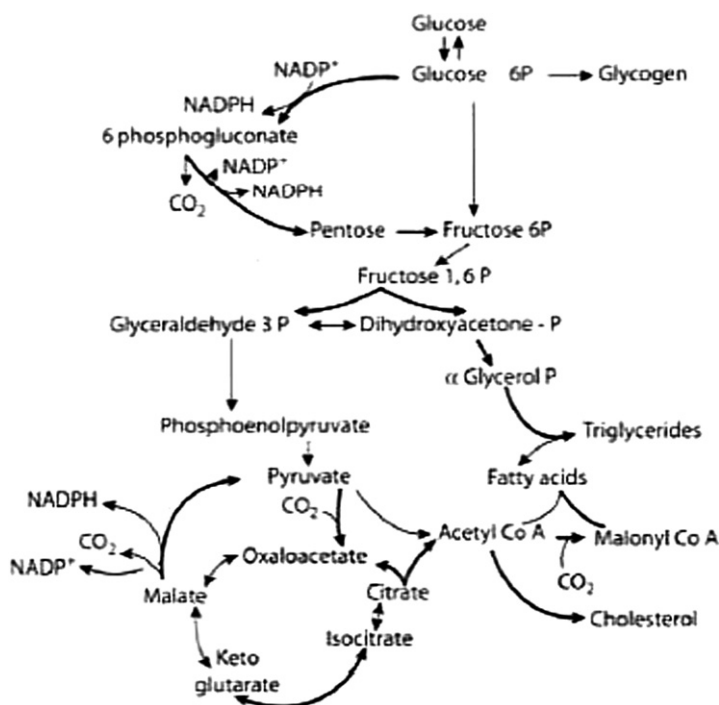
Figure 2.8.10 Intermediary metabolism. Heavy arrows show steps that are more active in lipogenic animals.

TABLE 2.8.5: Glucokinase activities in various mouse strains

Strain	Enzyme activity (nmoles substrate used/min per mg protein)
CBA/J	19.2 ± 1.25
SM/J	18.2 ± 0.53
C3H/HeJ	16.8 ± 0.63
C57Bl/6J	13.9 ± 0.55
AKR/J	13.1 ± 0.36
RF/J	7.41 ± 0.38
C58/J	7.33 ± 0.37
Transgenic FBP/m[a]	14.0 ± 2.0

[a]Mice expressing human hepatic glucokinase.

benefited the ob/ob mice as well. A third mouse strain (C57BL/6J) when food restricted had the reverse response: that is, their lifespans were reduced when food was restricted. No overt differences in metabolism were observed in these three strains yet differences in response were found.

Finally, it must be noted that the literature on metabolism is enormous. Mice have been studied extensively both as they respond to experimental manipulation of their diets and as they respond to genetic manipulation through the use of transgenic technology. Mice are excellent models for humans because of the similarity in metabolic patterns and the similarity of digestive systems. Two outstanding differences do exist, however: the mouse lacks the gallbladder found in humans, and the mouse

TABLE 2.8.6: Glucose flux in fed and fasted lean and obese mice

Measurement	Lean, fed	Lean, fasted	Obese, fed	Obese, fasted
Blood glucose (μmol/ml)	10.4 ± 0.5	6.0 ± 0.3	15.4 ± 0.9	9.9 ± 0.7
Frac. glucose use (%/min)	3.22 ± 0.60	3.52 ± 0.14	2.83 ± 0.17	3.18 ± 0.20
Glucose synthesis (μmol/min)	5.6 ± 0.6	4.4 ± 0.2	8.7 ± 0.8	6.4 ± 0.8
Cori cycle (%) (glucose recycling)	16.8 ± 0.8	24.1 ± 1.6	23.9 ± 2.4	25.4 ± 2.1
Liver glycogen (μmol/liver)	316 ± 28	34 ± 5	773 ± 33	356 ± 47

feeding *ad libitum*, the rhythm of anabolic processing of this food will be cued by the timing of food availability rather than by the lighting cycle [25].

Food restriction can not only affect metabolic rhythms but can also affect lifespan. In turn, lifespan can be genetically determined. Variation in the responses of different strains of mice to restricted feeding have been published [27]. Food restriction to two thirds that of *ad libitum*-fed mice extended the lifespan of several different strains of mice. Those that were normally long lived (B6CBAF-1 hybrids) had substantially longer lifespans than the shorter-lived ob/ob mice. Yet, food restriction

has a relatively large caecum compared to the human. These differences aside, the small size and short lifespan of the mouse makes it an ideal research tool for studies of metabolism and nutrient–gene interactions. What can be learned from the mouse can be applied to some degree to the human.

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CHAPTER 2.9

Haematology of the Mouse

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Introduction

This chapter is intended to provide useful and practical advice to those involved in assaying and interpreting haematological results from mice. The interpretation of haematological changes is similar whether the changes are due to an infectious disease, a toxin or a mutation. Identification of underlying causes is dependent on knowing which haematological tests should be used and how to interpret the test results.

A complete review of haematological malignancies of the mouse is beyond the scope of this chapter. However, the investigator who is studying murine haematology must be aware of the common haematologic malignancies of mice, and is referred to a review by Frith et al. [1]. Also available are review articles focusing on the haematology of certain genetically modified mice and mouse models of haematological diseases [2–4].

Much of the information in this chapter is not published, but has been gleaned from practical experience and from discussions with colleagues. When applicable, exact references have been specifically cited.

Terminology

Haematology is the study of the physiology and pathology of the cellular elements of blood. The three major cellular components of blood are red blood cells (erythrocytes), white blood cells (leukocytes) and platelets (thrombocytes). Basic haematological concepts are similar across most mammalian species, and can be found in several human and veterinary textbooks. The reader may consult any of the excellent haematology and clinical pathology reference books listed at the end of this chapter for guidance about general haematological principles.

Clinical terms (e.g. anaemia, leukopenia, lymphocytosis, pancytopenia) have specific medical connotations. These terms generally refer to conditions in which parameters fall outside of an appropriate reference range for a widely inclusive population (i.e. male humans, female dogs, dairy cows). In research, changes in haematological parameters for mice are usually compared to a control group or a very narrowly defined reference interval. Therefore, the use of these clinical terms is generally not appropriate in mouse research, and should not be used. Changes can be referred to as 'increases' or 'decreases' in the affected parameter. For example, instead of stating that an experimental treatment caused anaemia, one can state that mean haemoglobin concentration decreased by X g/dl and/or to X% of the control group mean. For more details about evaluation and interpretation of haematological changes in the context of experimental studies, the reader is referred to specific chapters of some haematology textbooks [5].

Blood collection and handling

Blood collection techniques

Several references describe methods for blood collection from mice [6-8]. For haematological testing, it is important to collect blood quickly with a minimum of tissue trauma. Thus, methods that collect blood directly from a vessel or plexus are preferred to those that may cause more tissue trauma. The following sites are most commonly used for blood collection for haematology in mice: orbital sinus, tail vein or artery, sublingual vein, heart, aorta and vena cava. Depending on the site used, blood collection must be a terminal procedure (heart, aorta, vena cava) or may be a survivable procedure (orbital sinus, tail, sublingual vein). Historically, blood collection from the retro-orbital plexus was used commonly as a survivable procedure. Today its use as a survivable procedure is discouraged because collection of blood from this site is associated with greater tissue injury, and thus may impact animal welfare.

Blood collected from a mouse should be immediately placed in a tube containing an anticoagulant. The preferred anticoagulant for routine haematological testing is EDTA (ethylenediamine tetra-acetic acid). If the blood is collected for preparation of smears only, blood without anticoagulant can be used, provided that smears are made immediately (seconds) after blood collection. Heparin should not be used as an anticoagulant because it tends to cause clumping of platelets, especially in mice, and negatively impacts the tinctorial quality of Romanowsky staining. When a capillary tube is used it is important to use plain rather than heparin-coated capillary tubes for blood collection.

Preparation of blood smears

Blood smear preparation is a skill that is easily learned and is covered in the standard haematology texts referenced at the end of this chapter. A blood smear can be prepared with a minimum of blood ($<50\,\mu$l), and thus can be a survivable procedure in mice. Blood smears should either be stained within a few hours of preparation or fixed, once thoroughly dry, with methanol.

Basics of haematological evaluation

Evaluation methods

The scope of haematological evaluation may vary greatly, depending on the needs of the investigator and the capabilities of the clinical pathology laboratory. The methods used will determine the type and number of parameters measured and the accuracy of the results. The haematological evaluation can include some or all of the following tests; these are listed below in the order of most simple and least expensive to those that are most complicated and require significant investment in instrumentation.

Blood smear evaluation

The blood smear is a landmark of any haematological evaluation and is examined microscopically. Regardless of the methods used to determine blood cell counts, blood smear evaluation remains the same. First, the density of white and red blood cells and platelets is estimated and compared to the counts obtained. A differential white blood cell count is performed. The blood smear is reviewed for morphologic changes in any of the cell populations (red blood cells, white blood cells and platelets). For this examination, only a microscope is required. The validity of results is highly dependent on the skill and experience level of the examiner. If automated differentials are conducted (see below), blood smears are still prepared, but are often examined only if deemed necessary after review of the automated results.

Spun haematocrit, haemocytometer cell counts and blood smear examination

A microcapillary tube is centrifuged to determine the spun haematocrit (also called *packed cell volume*). White and red blood cells and platelets are counted microscopically, using a haemacytometer. Haemoglobin concentration may be measured, and some or all red blood cell indices are calculated. The blood smear is examined microscopically, and a differential white blood cell count is performed. Absolute white blood cell differential counts are calculated. The blood smear is reviewed for morphologic changes. This method requires a minimum of equipment (microcapillary haematocrit centrifuge, hemacytometer, microscope, ± method of determining haemoglobin concentration). The results are moderately accurate.

Instrument cell counts and differential with blood smear review

Whole blood is analysed on a haematology cell counter using either impedance or optical technology. The cell counter determines red and white blood cell counts, platelet counts and haemoglobin concentration, and measures or calculates red blood cell indices. The blood smear is examined microscopically, and a differential white blood cell count is performed. Absolute white blood cell differential counts are

calculated. The blood smear may be reviewed for morphological changes. This method requires a dedicated haematology analyser and trained personnel. The results are very accurate if the instrument has been shown to be valid for the determination of mouse haematological tests, and if the operators have sufficient training to operate the instrument.

Instrument cell counts and optional blood smear examination

Whole blood is analysed on a flow cytometer-based haematology instrument. The instrument determines red and white blood cell counts, platelet counts and haemoglobin concentration, and measures or calculates red blood cell indices. The instrument also estimates the differential white blood cell count populations. The blood smear is examined microscopically to confirm the automated differential, and is also reviewed for morphological changes. This method requires a sophisticated haematology analyser and well-trained personnel. The results are very accurate if the instrument has been shown to be valid for the determination of mouse haematological tests, and if the operators have sufficient training to operate the instrument. Today the Siemens Advia 120 and the Sysmex XT-2000 IV are the most commonly used flow cytometry-based haematology analysers with software that can determine mouse white blood cell differential counts (Figure 2.9.1).

Almost all cell counters and flow cytometry-based analysers developed for haematology were originally designed to determine haematological parameters for humans. These instruments have specialized software to discriminate and count the different cellular constituents of blood. Some haematology analysers have been adapted for animal species by using species-specific software or settings. Analysers adapted for animal species vary markedly in their ability to accurately count and differentiate animal blood cells. This is especially true with blood from rodents. Therefore, for accurate results it is essential to use a laboratory with instrumentation validated for the analysis of mouse blood, and whose employees are skilled at mouse blood evaluation.

| Routine Operations | System Logs | Utilities | Special Procedures | Customize | Data Manager | Help |

Next Sample : HBSS   Mouse CBC / D / R
Standby   Auto Standby - Started

| Log On/Off | Startup | Manual Sample ID | Autosampler Reject Status |

Run Screen
Configuration Associate Hide Arrange Windows Print Display Off

Header - WB
SID:S11M-1123.2-7
Time & Date: 9/6/11 2:20:21 PM
Selectivity: CBC\DIFF\RETIC
Sample Type: PATIENT
Sex & Age: U
System:
Sequence: 263
Species: Mouse Playback
FOR LABORATORY USE ONLY
Hide Run Screen   Information

Routine Retic
%   #
Retic: 2.84 * 297.5 * x10⁹ cells/L
CHr: 16.0 * pg
CHm: 14.3   pg

RBC Volume   RBC HC   Platelet Vol
RBC Rate   HGB Trans   Retic CH

Perox   Baso
RBC V/HC   Retic Scatter Abs

Routine CBC
WBC: 11.00   x10³ cells/µL
RBC: H 10.47   x10⁶ cells/µL
HGB: H 15.4   g/dL
HCT: H 54.9   %
MCV: 52.4   fL
MCH: 14.7   pg
MCHC: L 28.0   g/dL
CHCM: L 25.4   g/dL
CH: L 13.3   pg
RDW: 13.4   %
HDW: L 1.60   g/dL
PLT: 1283   x10³ cells/µL
MPV: 7.4   fL

Routine WBC Differential
%   x10³ cells/µL
WBC: 11.00
Neut: 8.4   0.92
Lymph: 84.9   H 9.34
Mono: 2.5   0.27
Eos: 1.8   0.20
Baso: H 0.6   0.06
LUC: 1.9   0.21
LI: 2.29
MPXI: 9.2
WBCP: 10.10

PLT Scatter   Platelet
PLT: 1283 x10³ cells
MPV: 7.4   fL
PDW: H 60.4 %
PCT: 0.94 %
MPC: 20.8 g/dL
PCDW: 7.9   g/dL

Morphology Flags
MICRO:
MACRO:
HYPO:
HYPER:
ANISO:
HC VAR:
LEFT SHIFT:

Sample/System Flags

Figure 2.9.1 Example of analysis of mouse blood on a Siemens Advia 120 Automated Hematology Analyzer. The instrument uses a flow cytometry-based analyzer to measure individual white blood cell types (automated differential). Notice the scatterplots used to classify the various cell types.

Complete blood count

The report of results from a standard haematological evaluation is called a *complete blood count* (CBC). The CBC generally includes most or all of the parameters listed below. As discussed briefly above, these parameters are determined using one or all of the following methods: automated haematology analysers, haematology analysers plus microscopic examination, or by a combination of manual and automated methods. In most countries haematology results are expressed in SI units, as described by Laposata [9].

Red blood cell mass parameters

The functional red blood cell mass is measured by three parameters: red blood cell count (RBC), haematocrit (HCT) and haemoglobin concentration (HGB).

RED BLOOD CELL COUNT (RBC)

The RBC is the number of red blood cells in a given volume of whole blood. It is usually determined using an automated counter. In this method red blood cells are counted while they flow through an aperture in single file, using either impedance or optical technology. For manual haemocytometer counts, a commercially available diluent system (Unopette©, Becton-Dickinsen, Test 5850) is used to dilute blood prior to counting red blood cells. A qualitative estimation (density) of the RBC can be determined by microscopic evaluation of well-prepared blood smears. Mouse RBCs are higher than in most other species, because of the small size of their red blood cells. Counts range from approximately $7\text{–}11 \times 10^{12}$/L [10, 11]. Mouse red blood cells have smaller mean cell volumes than other species, but since their red cell counts are higher, mouse haematocrits are similar to those of other species.

The primary function of red blood cells is to carry oxygen from the lungs to tissues, and to carry carbon dioxide back to the lungs. In the mouse red blood cells are normally produced in bone marrow and in the spleen, even in adults. This is in contrast to other species in which splenic haematopoiesis does not occur in healthy adults. In the mouse the amount of extramedullary haematopoiesis in the spleen and liver
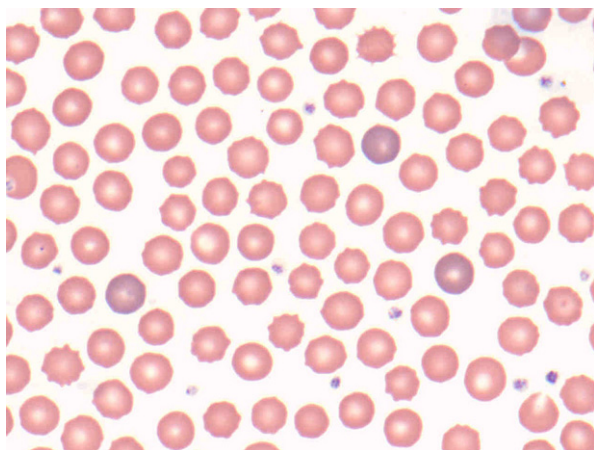
Figure 2.9.2 Peripheral blood erythrocytes from a Charles River CD-1 mouse (100× oil objective; Wright–Giemsa stain). Note the presence of polychromasia (light purple/blue staining cells), and variably sized cells (anisocytosis).

increases greatly when there is increased demand for red blood cells.

Compared to other mammalian species, the mouse red blood cell has a fairly short lifespan, estimated at 30–52 days [10, 12, 13]. The red blood cell lifespans of other common species are much longer (rats 45–50 days, dogs 110 days, humans 120 days). Because the lifespan of murine red cells is so short, there is a higher percentage of circulating immature red blood cells at any given time. Immature red blood cells are larger than mature erythrocytes and stain with a blue tint with Romanowsky-stained blood smears and are called *polychromatophils*. Therefore, *polychromasia* (purple/blue staining cells) and *anisocytosis* (variably sized red blood cells) occur to a greater extent in normal healthy mice compared to humans and many other animals. In addition, Howell-Jolly bodies (small, dark blue intracellular particles with Romanowsky-stained blood smears, which are remnants of nuclear DNA) are also more common in circulating red blood cells in mice (Figure 2.9.2).

HAEMOGLOBIN (HGB)

The haemoglobin concentration (HGB) is the measurement of total haemoglobin per volume of whole blood. It is determined spectrophotometrically after lysis of red blood cells. All forms of haemoglobin, whether functional or not, are included in the measurement of haemoglobin concentration. Mouse haemoglobin ranges from 130 to 180 g/L.

HAEMATOCRIT (HCT)

The HCT is a measurement of the volume of red blood cells as a percentage of whole blood. For automated procedures, the haematocrit is the product of the RBC and the mean cell volume (see below). For manual determinations, the haematocrit is measured after centrifugation of a microcapillary tube filled with whole blood. The percentage of blood composed of red blood cells is the haematocrit (sometimes called packed cell volume). Manual or 'spun' haematocrits tend to be a few percentage points higher than calculated haematocrits, because trapped plasma is included in the apparent red blood cell volume. Haematocrit is expressed as a number without units between 0.00 and 1.00. Haematocrit values for mice are generally between 0.40 and 0.50, but may range up to 0.60 depending on sampling site and fasting status.

Red blood cell indices and other red blood cell parameters

MEAN CELL VOLUME (MCV)

The MCV is the average size of red blood cells. When the RBC is determined by a haematology instrument, the MCV is measured. When cell counts are determined by a haemocytometer, MCV is calculated. For instrument-generated cell counts, red blood cell volume is measured during the cell count described above. A histogram is generated from the RBC and size (Figure 2.9.3) and the MCV is determined from this histogram. If cells are counted by a haemocytometer, the MCV is determined by dividing the haematocrit by the RBC. The MCV of mice is approximately 40–55 fl [10, 11, 14].

MEAN CELL HAEMOGLOBIN (MCH)

The MCH is the average amount of haemoglobin found in each individual red blood cell. It is determined by dividing the HBG by the RBC. In general, MCH is the least useful haematology parameter, because it is insensitive to change and provides little additional information than other red blood cell parameters. MCH for mice ranges from 13 to 17 pg. MCH is sometimes expressed in fmol.
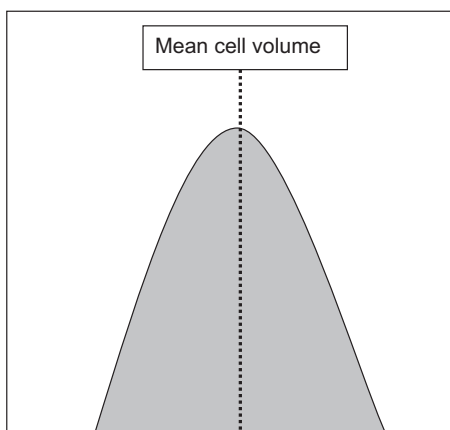
Figure 2.9.3 Red cell histogram. Red cell size is plotted on the x-axis, and measurement frequency is plotted on the y-axis. The mean value for red cell size is the mean cell volume (MCV); the frequency is the red cell count, and the spread of red cell histogram is reflected in the red cell distribution width (RDW).

MEAN CELL HAEMOGLOBIN CONCENTRATION (MCHC)

The MCHC is the HBG divided by the HCT, and thus is the average concentration of haemoglobin in all red blood cells. MCHC is much more relevant than the MCH, discussed above, because it is more sensitive to changes affecting red blood cells. Mouse MCHC generally ranges between 270 and 330 g/L but can vary between strains of mice.

RED BLOOD CELL DISTRIBUTION WIDTH (RDW)

The RDW is the coefficient of variation of the red blood cell volume, and is calculated from the red blood cell histogram described above and in Figure 2.9.3. It is therefore a quantitative indicator of the variation in red blood cell size (anisocytosis). Mouse RDW generally falls between 11 and 15%.

RETICULOCYTE COUNT (RETIC)

Reticulocytes are immature red blood cells containing residual RNA. Reticulocytes can be counted by instrumentation or by haemocytometer. Automated reticulocyte counts are performed similarly to automated RBCs. Before counting, red blood cells are stained with a dye that stains for nucleic acid (such as acridine orange) to differentiate reticulocytes from mature red blood cells. For manual reticulocyte counts, whole blood is mixed with a supravital dye such as new methylene blue and blood smears are prepared. The dye causes clumping and staining of residual nucleic acid present in immature cells. The stained cells (reticulocytes) are counted as a percentage of total red blood cells. The absolute reticulocyte count is determined by multiplying the total RBC by the percentage of reticulocytes. The number of circulating reticulocytes is higher in mice ($200\text{-}500 \times 10^9$/L) than in most other species, due to the short lifespan of the mouse red blood cell. Units for reticulocyte count vary among laboratories, but generally are reported in the same units as red blood cells or in the same units as platelets (cells $\times 10^9$/L).

Platelet parameters

PLATELET COUNT (PLT)

Platelets are essential for primary haemostasis and form a temporary haemostatic plug prior to activation of the clotting cascade. The PLT is the number of platelets in a given volume of whole blood. It is usually determined using an automated counter, using either impedance or optical technology. Platelets are counted as they flow through an aperture in single file, similar to red blood cells. Platelets can also be counted microscopically with a haemocytometer. A qualitative estimation of PLT can be determined by microscopic evaluation of well-prepared blood smears. Counting platelets in mouse blood is problematic because mouse platelets tend to form clumps (Figure 2.9.4). The presence of clumped platelets can interfere with the accuracy of both platelet and white blood cell counts, and
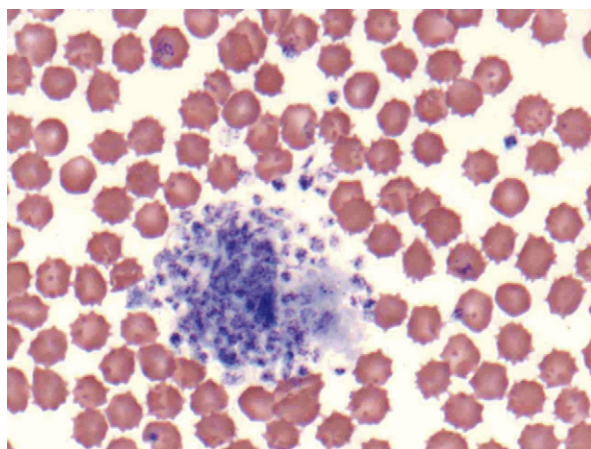
Figure 2.9.4 Platelet clump, peripheral blood smear (100× oil objective; Wright–Giemsa stain).

also invalidates PLT. Depending on the analyser, clumped platelets can either be counted as white blood cells or specifically as eosinophils. Therefore, regardless of the method used to count platelets, the smear must be evaluated for platelet clumps before the PLT value is accepted.

Mice have the highest circulating platelet count of any laboratory animal species (approximately 900–2000 $\times$ 10^9/L) [11, 14]. Mouse platelet half-life is approximately 4–5 days [11, 15, 16]. Platelets are variable in morphology, but range from 1 to 4 µm in diameter and 4 to 7 fl in volume. The primary growth factor controlling platelet production is thrombopoietin, but erythropoietin and iron status also affect platelet production. Under conditions of increased demand for platelets, platelet production is increased, and large platelets are sometimes observed on the peripheral blood smear.

MEAN PLATELET VOLUME (MPV)

The MPV is an estimation of the average size of platelets, and is analogous to the MCV of red blood cells. The MPV is only available on automated cell counters. As platelets are counted, their size is measured. A histogram is generated from the platelet counts and platelet sizes. The MPV is determined from this histogram. Units for MPV are femtolitres (fl).

White blood cell parameters

WHITE BLOOD CELL COUNT (WBC)

White blood cells participate in immune and inflammatory processes. The WBC may be determined quantitatively (automated analyser counts or manual haemocytometer counts) or qualitatively (determination of density with blood smear review). Before quantitative white blood cell determinations, red blood cells are lysed using a hypotonic solution. The resulting preparation contains only white blood cells and platelets. Automated counters measure white blood cells as they flow in single file past a detector. For manual haemocytometer counts, a commercially available diluent system (Unopette©, Becton-Dickinsen, Test 5855) is used to dilute blood and lyse red blood cells prior to counting white blood cells. A qualitative estimation of the WBC can be determined by microscopic evaluation of well-prepared blood

smears by a trained observer. The WBC of mice ranges from 2 to 10 $\times$ 10^9/L.

DIFFERENTIAL WHITE BLOOD CELL COUNT (DIFF)

The DIFF enumerates the various individual white blood cell types found in peripheral blood. The predominant circulating leukocytes in mice are lymphocytes, followed by neutrophils, monocytes, eosinophils and lastly basophils (Figures 2.9.5–2.9.7).

The DIFF can be performed on an automated instrument or by microscopy. Automated analysers with species-specific software are flow cytometers dedicated to differentiating peripheral white blood cells, and use nuclear and cytoplasmic characteristics of white blood cells for classification (see Figure 2.9.1). The DIFF can also be determined microscopically by examining and categorizing 100 white blood cells on a peripheral smear. The percentage of each cell type is multiplied by the total WBC to arrive at absolute differential counts for the various cell
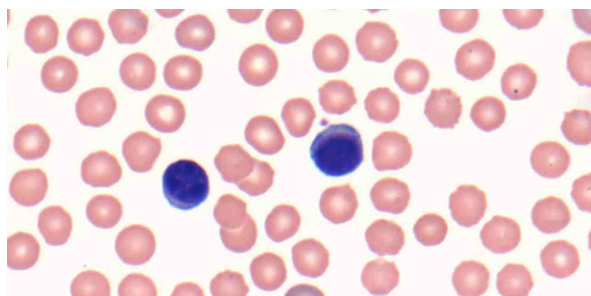
Figure 2.9.5 Lymphocytes, peripheral blood smear (100× oil objective; Wright–Giemsa stain). Note the high nuclear/cytoplasmic ratio, eccentric nucleus, smudged chromatin and blue cytoplasm.
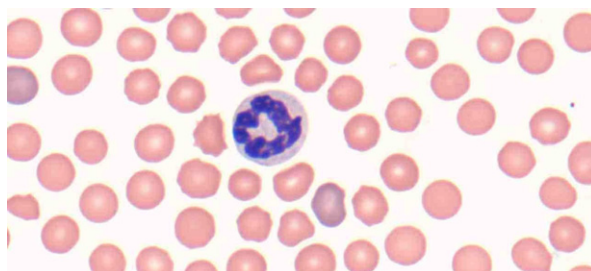
Figure 2.9.6 Neutrophil, peripheral blood smear (100× oil objective; Wright–Giemsa stain). The cytoplasm is clear or stains faintly. The nuclei of neutrophils show multilobulation and the chromatin is variably clear or condensed.
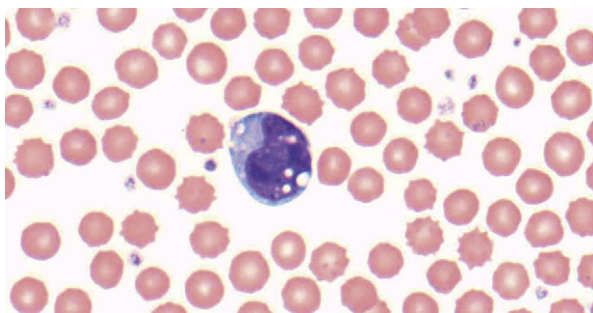
Figure 2.9.7 Monocyte, peripheral blood smear (100× oil objective; Wright–Giemsa stain). The monocyte is the largest peripheral blood cell of mice. Note the grey-blue vacuolated cytoplasm, lobulated nucleus and clear cytoplasmic vacuoles.

types. Interpretation of leukocyte changes should be based on absolute numbers (number of a given cell type per unit volume of blood) rather than relative numbers (percentage of a given cell type). Units for absolute DIFF are the same as for total WBC (cells $\times 10^9$/L).

The main categories of white blood cells counted during a differential count are lymphocytes, neutrophils, monocytes, eosinophils and basophils. Additional cell types may be observed; these might be subcategories of the five major cell types, or perhaps other cells not normally observed in peripheral blood. Interpretation of white blood cell changes is covered in a later section of this chapter.

1. *Lymphocytes* are responsible for immune surveillance, production of antibodies, production of cytokines and antigenic memory. Unlike other circulating leukocytes, lymphocytes are capable of division. Mouse lymphocytes are generally similar in appearance to those of other species. Both small and large lymphocytes can be observed on peripheral smears. Lymphocytes are approximately 7–12 μm in diameter, and have round to oval dark blue nuclei with pale blue scant cytoplasm on Romanowsky-stained blood smears. In the mouse, lymphocytes make up 70–80% of the differential count [11].

2. *Neutrophils* are circulating phagocytes and modulators of the immune response. In the mouse, neutrophils account for 20–30% of DIFF [11]. The morphologic appearance of the mouse neutrophil is similar in most respects to those of other species. Mouse neutrophils are

10–25 μm in diameter [10] and have pale granules. Mouse neutrophils sometimes exhibit circular doughnut-shaped nuclei; similar morphology is sometimes observed in rat neutrophils as well.

3. *Monocytes* and *eosinophils* are minor cell types. Generally there are more monocytes than eosinophils in peripheral blood. *Basophils* are very rarely observed in the peripheral blood of mice. Some authors have questioned the presence of basophils in mouse blood, however, microscopic and ultrastructural characteristics of murine basophils have been described in the literature [17, 18].

Morphological evaluation of the blood smear

The blood smear is evaluated microscopically for alterations in appearance (morphology) of white blood cells, red blood cells and platelets. At the same time, any other unusual findings are noted. Examples of appropriate comments would be observations of variation in size, shape, and coloration of cells; cellular organization (agglutination, rouleaux, platelet clumps); parasites and other relevant findings.

Bone marrow evaluation

Bone marrow smears are analysed to determine the underlying pathophysiology of peripheral blood changes. Both qualitative and quantitative evaluations of bone marrow cells can be useful in determining the cause of peripheral blood changes. Bone marrow smears can be qualitatively evaluated for relative proportions, maturity of precursor cells, storage pool and other changes. Quantitative evaluations of various cell types (differential) are generally not necessary, but may be done on occasion if extremely precise data is required. For a complete review and best-practice approach to bone marrow evaluation in laboratory animals, readers are referred to a best-practice publication [19]. This review article covers approaches for indications when to evaluate bone marrow in laboratory animals, with techniques available including histological, cytological or flow cytometric methods.
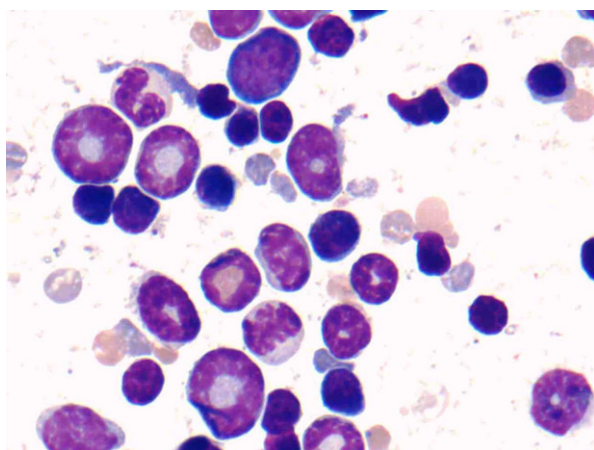
Figure 2.9.8 Bone marrow smear (100× oil objective; Wright–Giemsa stain). The majority of the cells in this field are late granulocytic precursors with doughnut-shaped nuclei, admixed with a few erythroid precursors.
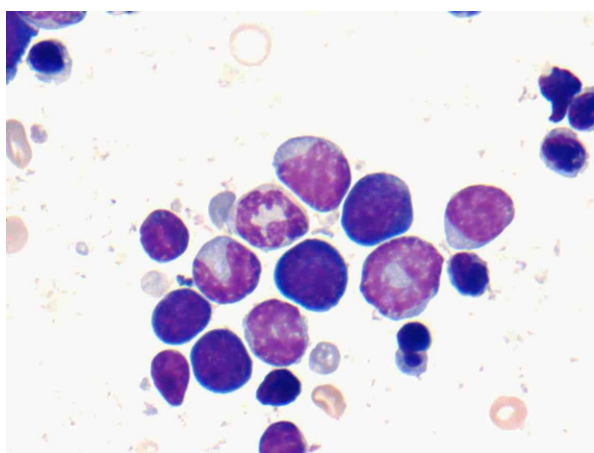
Figure 2.9.9 Bone marrow smear (100× oil objective; Wright–Giemsa stain). The majority of the cells in this field are erythroid precursors (dark nuclei, high nuclear/cytoplasmic ratio, blue cytoplasm), admixed with a few late-stage granulocytic precursors.

Variables affecting haematology results

Numerous factors can interfere with accurate analysis of haematology parameters. Potential interfering factors for analysis of mouse blood are listed in Table 2.9.1. Some of these interfering factors only occur with mouse samples, while others can occur with samples from other species.

The results of haematology tests are not only affected by pathologic processes, but are also affected by the status of the mouse at the time of blood collection, collection techniques and handling of blood prior to and during analysis. Therefore, it is essential to keep variables as consistent as possible so that results are comparable across experiments.

Haematological parameters vary with the status of the animal being tested. Fasting status, hydration status, time of collection, prior experimental manipulations, prior and concurrent anaesthesia, sex and a myriad of other factors will influence the results of haematology tests. In general, mice are not fasted prior to haematology sample collection, because fasted mice tend to drink less water and may become dehydrated.

Age has a very significant effect on haematological parameters. For example, the erythroid parameters for newborn mice are very different from those of mice aged 6 weeks or more. Newborn mice have much higher reticulocyte counts and lower red blood cell mass parameters. During the first few weeks of life, red blood cell

TABLE 2.9.1: Factors affecting results of haematology tests		
Animal physiology	**Collection process**	**Analysis**
Age	Study design	Sample quality (e.g. haemolysis, lipaemia, clots)
Sex	Movement of cages	Sample storage and handling
Strain	Prior handling or dosing	Instrumentation
Transport	Anaesthesia requirement	Order of analysis
Time of collection	Order of collection	Platelet clumps
Fasting/fed status	Site of collection	
Concurrent illness	Anticoagulant used	
Other experimental procedures	Anticoagulant/blood ratio	

mass actually decreases, but then rebounds steadily and is near adult levels at weaning.

The order of blood collection can affect haematology results. For this reason, it is important to collect blood from controls alternately with treated (experimentally manipulated) mice. Collection site can also play a role: in general, blood collected from central arteries or heart tends to have lower white and RBCs than blood collected from distal sites such as ocular plexus or tail [20]. Underfilling of tubes results in excess anticoagulant for the amount of blood, and can result in artefacts in haematological parameters. It is important to fill blood tubes to the volume recommended by the manufacturer.

Once collected it is important that all samples are stored appropriately (generally room temperature or refrigerated), and are analysed at approximately the same length of time after collection. A recent publication showed that mouse blood samples stored at 4 °C for 24 h or more had changes in some haematological parameters compared to those analysed within 1 h after collection [21].

Analytical variables that need to be avoided include operator error, bias due to order of analysis, instrument malfunction, outdated reagents or control material, dilution errors, calculation errors and calibration errors. For more information about general quality control and quality assurance in a veterinary clinical laboratory, the reader is referred to published guidelines from the American Society of Veterinary Clinical Pathology [22].

Pathophysiology and interpretation of results

Red blood cells

The most important effects on red blood cells are those that result in changes in red blood cell mass. These effects can be divided into those that increase red blood cell mass, and those that decrease red blood cell mass. Red blood cell mass changes are summarized in Table 2.9.2.

Increased red blood cell mass

Increases in red blood cell mass can be classified as either relative or absolute changes. Relative increases in red blood cell mass are the result of dehydration, which can occur very rapidly in mice. Dehydration may be associated with other signs of poor health, including inactivity, hunched appearance, decreased appetite and poor skin turgor.

Absolute increases in red blood cell mass result from increased red blood cell production, and may occur whenever there is increased production of growth factors or cytokines that stimulate erythropoiesis. The primary cytokine driving erythropoiesis is erythropoietin (EPO), but other cytokines, especially thrombopoietin, can also affect red blood cell production. EPO-like drugs may also have similar effects.

Active erythropoiesis, resulting in increased red blood cell mass, can be secondary and appropriate (occurring in response to a disorder with decreased oxygen delivery to tissues) or may be primary and inappropriate (occurring in the absence of any need for increased oxygen delivery). Causes of appropriately increased red blood cell mass include cardiovascular disorders, pulmonary disorders or abnormal oxygen-carrying capacity of haemoglobin. Causes of inappropriately increased red blood cell mass include conditions resulting in excess erythropoietin (autonomous production) or excess stimulation of the erythropoietin receptor (activating mutations). Polycythaemia, a myeloproliferative disease, also results in increased red blood cell mass.

In mice increased red blood cell production, regardless of the cause, is associated with extramedullary erythropoiesis in the spleen and liver, hypercellularity of bone marrow, and increased reticulocytes in the peripheral blood.

Decreased red blood cell mass

Decreases in circulating red blood cell mass are indicated by decreases in RBCs, haemoglobin concentration and haematocrit. Decreased red blood cell mass can be either relative (expansion of plasma volume) or absolute.

Relative decreases in red blood cell mass are rare, but may occur in pregnant dams and neonates, or as a result of other perturbations in

TABLE 2.9.2: Alterations in red blood cell mass parameters in mice	
Causes	**Characteristics**
Increased red cell mass	Increased haemoglobin, haematocrit, RBC
Relative increased red cell mass	
Dehydration	Recognized by clinical signs May see increased total protein or albumin
Absolute increased red cell mass	
Increased erythropoietin activity	Increased extramedullary erythropoiesis
Myeloproliferative disease	Increased reticulocytes
Decreased red cell mass	Decreased haemoglobin, haematocrit, RBC
Relative decrease in red cell mass	
Pregnant dams	Plasma volume expansion
Neonates	Rare occurrence
Absolute decreases in red cell mass	
Red cell loss (haemorrhage)	
Internal or external (overt, gastrointestinal, genitourinary)	Recognized by clinical signs
Red cell destruction	
Toxins, immune-mediated	↓ reticulocytes
Cell membrane alterations	↑ MCV
Biochemical alterations	↑ RDW
Vascular injury/turbulence	↓ MCHC
	↑ extramedullary erythropoiesis
	↑ splenic weights
Decreased red cell production	
Decreased erythropoietin	↓ reticulocytes
Renal disease	↓ MCV
Endocrine disease	↓ RDW
Chronic inflammatory disease	No change or ↑ MCHC
Bone marrow toxicity	
Abnormal maturation	
Abnormal haem or nucleic acid synthesis	
Haematopoietic neoplasia	

plasma volume homeostasis. Apparent decreases in red blood cell mass can be artefactual when blood cells are insufficiently resuspended in plasma due to poor mixing of the blood with anti-coagulant prior to analysis.

Absolute decreases in red blood cell mass are much more common than relative decreases, and result from loss (haemorrhage), increased destruction (haemolysis), or decreased production of red blood cells. Each of these causes can be recognized by particular changes in haematological parameters.

Haemorrhage

Loss of red blood cells is called haemorrhage, and can be either overt or occult. Overt haemorrhage can result from disorders of haemostasis,

ulcerated masses, surgical procedures or other trauma. Overt haemorrhage is a clinical diagnosis, and is best identified by observing the physical condition of the mouse. Occult haemorrhage is generally due to loss of red blood cells into the alimentary tract or urogenital tract, and can be detected by tests for occult haemorrhage such as Hemoccult© for faeces or dipstick tests for urine blood. In mice loss of red blood cells is generally accompanied by marked regeneration (increased reticulocytes), while very chronic loss of blood may result in decreased red blood cell production due to lack of iron, without a concurrent increase in reticulocyte count.

Increased destruction

Increased destruction of red blood cells, or haemolysis, results in decreased circulating half-life of red blood cells. In most cases increased destruction of red blood cells occurs by premature removal from circulation (extravascular haemolysis) by the reticuloendothelial system of the liver and spleen, rather than by rupture of red blood cells within the vasculature (intravascular haemolysis). Normally, red blood cell lifespan in the mouse is 30-52 days. With haemolysis, the lifespan of red blood cells can be decreased markedly. Destruction of red blood cells may be due to metabolic, immune-mediated or physical causes.

Haemolytic processes result in compensatory increased red blood cell production (regeneration). Regeneration is the result of increased erythropoietin in response to decreased oxygen tension. Erythropoietin recruits stem cells to differentiate into red blood cell precursors, and promotes survival of committed red blood cell precursors, resulting in increased circulating immature red blood cells in peripheral blood, and increased absolute reticulocyte count. The immature red blood cells (which include reticulocytes) are larger than mature red blood cells, and contain less haemoglobin. Therefore, in regenerative haemolytic anaemia, the altered parameters are associated with cell size (increased MCV, RDW, macrocytosis) and cell haemoglobin concentration (decreased MCHC).

In mice even minimal haemolysis results in compensatory hypercellularity of the bone marrow and splenic extramedullary erythropoiesis. This is in contrast to other species, in which extramedullary erythropoiesis only occurs under more severe haematological stress. In mice, splenic weights can be used as sensitive and objective measures of red blood cell regeneration. The presence of extramedullary erythropoiesis should be confirmed histologically.

Decreased production

Any process that has a deleterious effect on red blood cell precursors in the bone marrow can cause decreased red blood cell production. The effects can be grouped into effects on stem cells, growth factors, or synthesis of haemoglobin or nucleic acids.

Mice with decreased red blood cell mass due to decreased red blood cell production will have inappropriately low reticulocyte counts in peripheral blood, with respect to the change in red blood cell mass. The decrease in red blood cell mass due to decreased production is referred to as a non-regenerative or poorly regenerative process.

Reticulocytes, which are larger and have less haemoglobin than mature red blood cells, are normally present in a larger percentage in mice than in other species. Therefore, in mice decreased reticulocytes seen in decreased red blood cell mass due to decreased production may result in decreased polychromatophilic cells on the blood smear, decreased MCV and increased MCHC. The morphology of peripheral blood and/or bone marrow may help to elucidate the mechanism for decreased red blood cell production. When decreased red blood cell production is suspected, bone marrow histology and/or cytology should be evaluated to determine an underlying cause.

Anaemia of chronic disease

The most common cause of decreased red blood cell mass in humans and animals is anaemia of chronic disease [23-25]. This condition is usually secondary to hormonal or inflammatory conditions. It results from decreased red blood cell production and increased red blood cell destruction. Although the term 'chronic' implies that this effect only occurs after a long period, it can actually be observed within days to weeks. Because of shorter red blood cell half-lives in
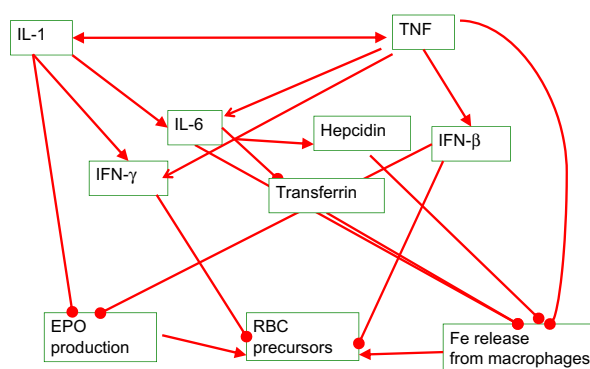
Figure 2.9.10 Pathophysiology of anaemia of chronic disease. *Adapted from Means and Krantz, 1992, Ganz, 2006, Nairz and Weiss, 2006.*

mice, processes affecting red cell mass have more rapid effects in mice than in other species. Figure 2.9.10 outlines the pathophysiology of anaemia of chronic disease.

Leukocytes

Interpretation of changes in total WBC should be made on the basis of changes in the absolute differential leukocyte counts. Leukocytes are summarized in Table 2.9.3.

Lymphocytes

In response to inflammation, mice usually have increased lymphocyte counts; this response is in contrast to that of most other laboratory species, but similar to that of rats. Increased circulating lymphocytes are also observed secondary to catecholamines (excitement), altered trafficking or lymphoid malignancy. Lymphocyte increases due to catecholamines are generally transitory (minutes to hours), whereas inflammatory-related increases are more persistent (days to weeks).

Decreased lymphocytes can be due to stress [26], altered trafficking or genetic manipulation resulting in immunomodulation. A decrease in lymphocytes due to stress occurs secondary to the effects of increased corticosteroids. This stress response in lymphocytes occurs within hours of the stressful event, and can persist for

TABLE 2.9.3: Most common peripheral blood leukocyte patterns in mice	
Short-term (acute) inflammation	Decreased neutrophils (may not be observed) during severe inflammation Immature neutrophils (less common in mice than larger animals)
Chronic inflammation	Mildly increased total leukocytes (young adults) Mildly to markedly increased total leukocytes (old mice) Increased neutrophils Increased lymphocytes Increased monocytes
Excitement (catecholamine/ adrenaline-induced changes)	Proportional increase in both lymphocytes and neutrophils
Stress (corticosterone-induced changes)	Increased neutrophils ± hypersegmentation of neutrophils Decreased lymphocytes Decreased eosinophils Decreased monocytes
Bone marrow toxicity	May involve only one cell type or may involve all three cell types If pancellular, cells are usually affected in the following order: leukocytes, platelets, then red blood cells Red cell effects: decreased reticulocytes, red cell distribution width, mean cell volume increased mean cell haemoglobin concentration
Neoplasia	Haematopoietic neoplasia: sometimes leukemic (neoplastic cells in circulation) Space-occupying lesions: sometimes leukopenic
Allergy or hypersensitivity	Increased eosinophils, possibly increased basophils

days to weeks. Often, decreased lymphocytes are incorrectly attributed to stress without correlative findings supporting the diagnosis. Generally, other effects such as weight loss, clinical signs or thymic atrophy are present when stress results in decreases in lymphocytes.

Total peripheral blood lymphocyte counts are only an estimate of immune system status. Unexplained effects on lymphocytes may be further explored using cytochemical or flow cytometric techniques. In addition, histopathology of other lymphoid tissues, such as lymph nodes and thymus, may be useful in the understanding of peripheral lymphocyte changes.

Neutrophils

Increases in circulating neutrophils can be due to excitement (demargination), increased neutrophil production or decreased neutrophil egress from circulation. Increased production is generally caused by increases in colony stimulating factors (G-CSF or GM-CSF), as a result of inflammation. Increased production can rarely be recognized by the presence of immature neutrophils in the peripheral blood. These neutrophils may have the morphological appearance of band neutrophils, or may show signs consistent with toxic change (increased granulation, basophilic cytoplasm and/or foamy cytoplasm), indicating accelerated neutrophil production. Decreased egress can be caused by lack of functional adhesion molecules (P-selectin or E-selectin deficiency) or absence of chemotactic factors to recruit neutrophils into tissues. Under conditions of decreased egress, increased nuclear segmentation may be observed in a subset of circulating neutrophils.

Decreased neutrophils can result from decreased bone marrow production, increased egress into tissue or destruction of neutrophils. Because the circulating lifespan of neutrophils is short (7-14 h) [27], decreased neutrophil count is often the first peripheral result of bone marrow toxicity, and usually occurs prior to platelet or red blood cell decreases. Increased egress can be due to peracute inflammation. In the initial stages of inflammation, many circulating neutrophils may be called to the site of inflammation by inflammatory cytokines. This may cause a temporary decrease in circulating neutrophils that is ameliorated when the bone marrow increases production of neutrophils to meet the peripheral demand. A decrease in neutrophils can also result from direct destruction by immunological or non-immunological mechanisms.

Monocytes, eosinophils and basophils

Increased circulating monocytes generally indicate an increased demand for tissue macrophages, and thus are an indicator of inflammation. A decrease in circulating monocytes is uncommon, but may indicate increased egress into tissues, or may be a result of increased corticosteroid activity [14].

Increased circulating eosinophils result from allergic or inflammatory conditions. Generally, increases in circulating eosinophils are accompanied by increased tissue eosinophils as well. Decreased eosinophils may occur in conjunction with decreased lymphocytes, as a result of increased circulating corticosteroids.

Increased basophil counts are very uncommon in mice. Because of the lack of basophils in most mouse samples, decreases in basophils do not generally occur.

Platelets

Effects on platelets may alter their number, size or function. The most common reason for an increase in platelets is a secondary response to accelerated production of red blood cells (increased erythropoietin). Other reasons for increased platelets include iron deficiency and increased thrombopoietin.

Decreased platelets can result from decreased production or increased destruction. Decreased production occurs as a result of injury to megakaryocytes, the bone marrow cells responsible for production of platelets. If decreased production is suspected, megakaryocyte number and morphology may help to elucidate the cause of decreased platelets. Decreased numbers or altered morphology of megakaryocytes supports the hypothesis that decreased platelet production has occurred. Often, processes that affect bone marrow production of one cell line can have ramifications in one of the other two cell lines produced in the bone marrow. Because platelets

and leukocytes have shorter half-lives than red blood cells, effects on bone marrow will often first manifest as peripheral blood abnormalities on these two cell lines.

Increased destruction/removal of platelets occurs if there is accelerated activation of platelets or immune recognition of platelets. If platelet half-life is shortened, megakaryocytes release large immature platelets containing rRNA. These 'reticulated platelets' can be measured using nucleic acid-binding dyes and flow cytometry. Alternatively, these large platelets can be detected by measuring MPV. Bone marrow generally shows increased numbers of megakaryocytes, with or without an increase in younger megakaryocytes.

Platelet function can also be studied in mice. Techniques such as aggregometry, bleeding time assays and flow cytometry [12, 28] can be used to elucidate functional changes.

Bone marrow evaluation

Murine haematopoiesis (production of granulocytes, monocytes, platelets and erythrocytes) primarily occurs in bone marrow at steady state, although a small amount of haematopoiesis also occurs in the spleens of healthy mice. Therefore, alterations in peripheral blood counts may be investigated by examination of the bone marrow. Mouse bone marrow includes stromal cells, macrophages, mast cells, megakaryocytes and megakaryocyte precursors; erythrocytes and erythroid precursors; granulocytes, monocytes and their precursors; and lymphocytes, as well as non-haematopoietic cells such as osteoblasts and osteoclasts.

Granulocytes (neutrophils, eosinophils and basophils) and red blood cells arise from precursor cells. The precursor cells of both granulocytes and red blood cells can be divided into proliferating cells (cells that are capable of undergoing division) and maturing cells (end-stage cells in the process of maturation). During a complete bone marrow differential cell count, these cells are counted, along with those cells mentioned above. For a more complete review of bone marrow smear collection techniques and evaluation, the readers are referred to Reagan et al. [19].

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CHAPTER 2.10

Studying Immunology in Mice

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Introduction

This chapter discusses four aspects of the use of mouse models for the analysis of the immune system: the importance of the health status of the mice used, the influence of the genetic background on the immune system, why mouse models are needed for the analysis of the function of the human immune system for the foreseeable future and how this could be achieved.

When the immune system is studied in mice several key parameters should be recorded and reported and important specimens should be collected in order to be able to verify findings retrospectively when needed. For each experiment, the age, sex, genetic background, health status and day/night cycle of the animals, and the time and date of the experiment, should be recorded at the end of the experiment and at least serum and a tissue sample of the liver or tail for later DNA preparation should be collected and stored for each mouse. Ideally, tissues of the central (thymus, bone marrow) and peripheral (spleen, lymph nodes) immune system should also be stored for histological analyses when needed. The serum will allow the determination of the concentration of immune system related proteins such as antibodies or cytokines; the DNA is a way to make sure that any genetic analysis prior to the experiment was correct and will also allow analysing the genetic background of the individual mouse when needed. Finally, the stored tissues can be used to verify major changes in the structure of the immune organs in case of mouse mutants or in case of strong immune responses.

Dependence of immune system status on microflora and other microorganisms

The mouse body typically harbours a symbiotic microflora, which can consist of protozoans, bacteria, fungi and viruses. Generally, the animal house unit of an organization will screen for most of the pathogens within this flora such as bacteria, viruses and parasites, a list of which is recommended by the Federation of Laboratory Animal Science Associations (FELASA) [1, 2]. The health status of animal facilities is not very well defined by terms like 'conventional facility' or 'specific pathogen free' (SPF). The definition of health status is much better defined when associated to the FELASA guidelines, but even then the strategies used to screen the colonies vary and not all infections altering the immune system are picked up all the time. In the 1980s, when T cell subsets became experimentally accessible, a big debate among immunologists started on the question what is the 'default' T cell differentiation pathway. Laboratories were divided into two groups, one group suggesting that the Th2 pathway is the default pathway, maybe nowadays linked to parasitic infections in the animal units, and the other group insisted that the Th1 pathway is the default pathway, nowadays most likely linked to helicobacter infections in the animal colony. In addition, the genetics of the mouse strain used is of importance, as discussed in the following section.

As the number of T cell subsets increases over time, more and more connections between the T cells in a given subset and the presence of certain bacterial strains in the gut have been discovered to shape the T cell subsets. A good example is a recent study by Ivanov et al. [3] in which the authors show that the number of Th17 cells in the gut is dependent on the presence of segmented filamentous bacteria. Another study on resident gut bacteria by Strauch et al. [4] showed that the bacterial antigens play an essential role in the generation of regulatory T cells in a healthy individual. Interestingly, the population and activity of the regulatory T cells (Tregs) correlate with the level of chronic infection, which in turn can affect the immune response to new infections. Colonizing the intestine of germ-free mice with the Charles River Altered Schaedler Flora (CRASF) was found to cause *De novo* generation of mucosal Tregs [5]. In an experimental model of filariasis, mice infected with the nematode *Brugia malayi* were found to preferentially expand the Tregs subset within the CD4+ T cell population. This supported the growing evidence that parasites initiate an immune response that skews the balance of the host immune system toward tolerance, preventing the immune system from eliminating the parasite [6].

What constitutes the ideal environment for immunological experiments on mice is still an open debate. Ideally, mice would be housed in a series of animal units with different microflora, from very clean and defined flora to conventional animal units. This would allow us to assess the immune system in mice by a comparison of phenotypes between these units. The most extreme measure is to construct an animal unit in which all mice are kept with a defined flora in isolators, and under certain circumstances in individually ventilated cages [7]. Mice kept in such an environment are 'very clean' in the sense that they lack exposure to immune pathogens and the basal activity of the immune system is very low. The number of IgA plasma cells, for example, is very low in such mice compared to mice housed in standard barrier sustained facilities in conventional cages. Even very sensitive mouse strains, like the interleukin (IL)-10 deficient mouse mutant [8], do not develop inflammation in the gut immune system and standard methods like inducing gut inflammation by dextran sulfate sodium (DSS) are governed by different rules compared to standard barrier sustained facilities. For example, in mice with the CRASF-defined flora, DSS-induced colitis is not dependent on the generation of interferon-gamma and there is no difference in the pathology in normal mice compared to *Ifngr2*-deficient mouse mutants [8a].

When mouse mutants are analysed for phenotypes in the immune system, a less defined

health status is more desirable and spontaneous phenotypes are easily visible. The IL-10 deficient mouse mutant, for example, will develop spontaneous chronic inflammation in the gut, which is most prominent when *Helicobacter hepaticus* is part of the gut flora. Ideally, larger facilities should be in a position to provide different animal units with different combinations of microorganisms. It has already been noted in the scientific community that some phenotypes, such as incidence of autoimmune diseases or inflammatory bowel disease (IBD), are dependent on a given animal unit and after transfer of mice into a different environment some phenotypes may be drastically reduced or disappear (e.g. due to different composition of diet). IL-10 deficient mice develop very severe IBD within a few weeks in a conventional animal unit, but when they are housed in an SPF unit the development of the disease takes a few months [8].

A series of papers is now appearing demonstrating the role of parasitic infections on other immune responses in the infected mice, demonstrating the power of immune regulation through parasites. The presence of certain infections, for example by virus or geohelminths, giving rise to tolerogenic response by the immune system can alter the expected immune response against known or experimental infections [6]. There is evidence of such 'pre-immunized' conditions in human populations also. Schoolchildren in certain parts of Indonesia who are readily exposed to geohelminthic infections such as hookworms or whipworms develop a general tolerance to infections, which effectively suppresses immune responses to BCG and *Plasmodium falciparum* infection [9]. In another human study of schistosomiasis it was found that the intensity of schistosome infection is inversely related to autoreactive antinuclear antibody (ANA) levels, which generally arise as a consequence of autoimmune disorders [10]. These findings for parasitic infections can also be translated to viral infections, as has been nicely demonstrated in the paper by Coutelier and colleagues in which the authors infected mice with viruses and found that the infection had an effect on Ig class switching and selectively stimulated the production of the IgG2a subclass [11].

As the environment is so important for the outcome of immunological experiments, it must be defined as precisely as possible, for example by characterization of the gut flora, and the documentation of other infections like viral infections and parasites. As long as these parameters are determined and this information is reported along with the immunological experiment, it can be used to judge the conclusions of the experiments performed.

Dependence of the immune system on the genetic background

Currently, many experiments are performed using a limited set of inbred mice, and small changes within a given inbred line can have enormous effects on the function of the immune system. In the area of gene targeting, two mouse strains are popular, the 129 mouse strains with many substrains and the C57BL/6 strain. The latter also has several substrains, the two major inbred lines being C57BL/6J and C57BL/6N. Most of the classic immunological experiments were performed in the C57BL/6J line, but in order to get stable efficient embryonic stem (ES) cells, the C57BL/6N line has now been selected for the new mouse mutants generated by high-throughput gene targeting. The community will switch to the C57BL/6N line in the future and the difference between the two lines is currently being investigated in a major scientific community effort. In the past ES cell lines were available from the 129 mouse strains. As immunologists prefer to use inbred lines, in order to perform cell transfer experiments for example, mouse mutants derived from the 129 line were being crossed onto the C57BL/6 background. While doing so Marina Botto's group found out that during backcrossing, some regions of the C57BL/6 lines are not compatible with the 129/Sv (129S6) genome and when certain regions are combined, offspring can develop a spontaneous autoimmune disease [12]. Some mutations introduced into the 129 genome by gene targeting were wrongly reported to be associated to autoimmunity. This one example demonstrates

a very important principle: that the phenotype of a defined mutation introduced into the mouse genome, for example by gene targeting, can be dependent on the genetic background. An example of the spontaneous autoimmune disease developing has been described in the analysis of the DNAse1 knockout mouse. This mouse mutant, generated in 129 ES cells, develops symptoms of systemic lupus erythematosus (SLE) in backcrosses to the C57BL/6 strain, which are lost when backcrossed for 10 generations [13].

Using the IL-10 deficient mouse mutant again as an example, we noticed early on in the analysis of the mutant that the phenotype is more severe in mice of the 129 background compared to mice backcrossed on to the C57BL/6 background. A simple experiment at the time showed us that more than one allele was involved in the differering severity of the disease. This difference was later more systematically used to map modifier genes of the phenotype in IL-10 deficient mouse mutants. The Mähler Group in Hannover, together with The Jackson Laboratory [14], used a quantitative trait loci (QTL) mapping method to identify loci modifying IBD in the IL-10 deficient mouse mutant. The Bleich group in Hannover then identified 16 genes that are expressed differentially between B6 and C3 IL-10 knockout mice, three of which (*Cd14*, *Gbp1* and *Pla2g2a*) are considered to be major candidate genes. Infection with *Helicobacter pylori*, an old associate of laboratory infections, has been studied in different mouse strains such as BALB/c, C57BL/6, C3H/HeN, C3H/HeJ, DBA/2J and FVB/N, and found to vary in its colonization of these mice [15].

Another example is the induction of diabetes in mice by using streptozotocin (STZ) to cause necrotic death of pancreatic cells. The STZ-induced diabetogenic mouse model is used to study autoimmunity. The dosage of STZ and the severity of diabetes varies depending on the genetic background of the mice [16]. Male FVB/NJ, BALB/cJ and A/J mice are resistant to STZ-induced diabetes, whereas male C57BL/6J mice are moderately susceptible and male NOD/ShiLtJ and CBA/J mice are highly susceptible. This guideline is used by The Jackson Laboratory in generating experimental diabetogenic mice.

These examples illustrate that it is important to think about the genetic background and gender of mice used in experiments, and this is the reason for keeping DNA of the individual mice used in experiments in order to analyse their genomic make-up later. We have to be aware that the immunological experiments are to some extent artificial, as most of the time we are working with inbred mice, which harbour many mutations in the homozygous condition when compared to wild mice, and as a consequence of inbreeding carry a very restricted MHC (major histocompatibility complex) haplotype. On the other hand, these defined experimental systems are both necessary and sufficient to answer fundamental questions in immunology. In the future new complex inbred mouse lines may overcome the limitations of the currently available inbred mouse lines.

The 'mouse trap', or why the mouse is needed in immunology

Is it enough to do human studies?

Generally, the study of basic immunology relies on various animal models and the study of human immunology is somewhat separate. Mark Davis, based at Stanford, has long been working on understanding the fundamentals of the immune system. His methods through the years—a combination of animal models, cell lines and genetic materials—have generated valuable contributions to immunology, particularly in the area of T cell receptor (TCR) repertoire development. Davis has stated boldly in a number of published articles and direct communications that the mouse model may not be as good as we think, or to put it in simple terms, that the mouse model is not a good model for human immunology. The reason why Davis objects to the use of mice is because he believes that, with the knowledge that we already have, there is now very little need to use mice to understand biology that can be applied to humans. Let us look into the points that he has raised to support his argument [17].

In recent decades immunology has established itself as an important branch of the life sciences. This has come about through the discovery of several key components of the immune system such as clonal selection, structure of antibodies, TCRs, the MHC, T helper cell subsets, cytokines, CD antigens (350 of them so far in humans), pathogen-associated molecular patterns (PAMPs) and pathogen recognition receptors (PRRs). Hayday and Peakman [18] are optimistic that because inflammation is central to the initiation of many infectious and also systemic diseases (e.g. atherosclerosis, type 2 diabetes), immunology will dominate biomedical research and clinical science in the near future.

In spite of so many developments in basic immunology, the truth is that very few of the basic discoveries have been successful in human translation or incorporated into standard clinical practice. The mouse has been so much more successful than all other animal models that most research using animals has switched to or been diverted into using mice, and this has influenced immunology research in such a way that there is an over-reliance on the mouse model, leading to the 'mouse trap' for clinical immunologists. The reasons why the mouse model is unsuccessful in clinical translation could be due to the possibility of inbred strains developing homozygous recessive defects, the artificiality of many protocols that are used to induce the 'human-like' disease (e.g. experimental autoimmune encephalomyelitis, EAE), and the phylogenetic and evolutionary distance between humans and mice. Importantly, the missing element in our research is that the human–mouse difference is not being studied systematically, which is bound to impede translation efforts.

It is not difficult to perceive why; although the mouse genome has so many conserved regions in common with the human genome, the mouse immune system still differs in structure and function from that of humans. Mice have evolved over 65–75 million years, living in very different conditions (environment and lifestyle) from humans, and this must have caused differences in the exposure to microbes and other immunogens, modulating the murine immune system differently from the human immune system. Mestas and Hughes [19] have reviewed the mouse–human differences in

innate and adaptive immunity and have discussed how we need to be aware of these differences when extrapolating results from mouse models to human trials. They have shown how, in spite of the differences, mouse models can be very helpful in designing therapies for human disease. The classical example is the EAE model, which is considered as the experimental model most closely resembling multiple sclerosis (MS). The treatment of MS with interferon-gamma, which in EAE has a protective role, has on the contrary been found to exacerbate the disease in humans. But in follow-up experiments the mouse model revealed that blocking VLA4–VCAM1 interaction helps in MS, and this has been successfully carried out in human trials. Mestas and Hughes clearly stated that, accepting all the differences between mouse and human and the failure in translation of mouse work, the mouse model is still not an invalid model system for human biology and will continue to provide insight into basic immunology for many more years to come. Hayday and Peakman [18] also admit that immunology was greatly empowered in a relatively short period by the vast knowledge gained from mouse models. They have emphasized that the contemporary drive to increase translational research that could possibly be less conclusive and cause inappropriate redirection of funding should not deny researchers the opportunity to choose the mouse as their pre-eminent model.

In an effort to overcome the human–mouse differences there is an increasing attempt to humanize the mouse model by introducing human cells into the transgenic immunocompromised mouse. These models are discussed later. Davis acknowledges the strength of such models but advises that they are not to be considered as equivalent to the human immune system.

The concern that arises from extensive mouse-based work and over-reliance on mouse models is that mouse research is probably not sufficiently dedicated to humans, but rather enjoys the status of 'blue sky' research. Hayday and Peakman [18] have highlighted this by providing statistics of mouse versus human publications in top immunology-focused journals: between 2007 and 2008 in a 6-month period only 15% of articles published were devoted solely to human immunology. Also, because of

the bias towards mouse model proposals by grant reviews or mouse model manuscripts by journals, there is tendency among current researchers who work on human immunology to include or totally switch to mouse models to further their career. This raises the question: is research now in a mousetrap?

All of these arguments are good and may possibly validate Davis' claim that the mouse model does not help us to understand sufficiently, or cannot be translated sufficiently to human immunology. But this is not a reason to switch off or reduce mouse work, because the laboratory mouse will still be the model of choice to study basic immunology. We can endorse this by simply pointing out that scientists like Davis and Hayday who are heading a movement to revolutionize human research and questioning the use of mouse models in understanding the human immune system, still use mice in many of their current research projects, as is evident from their recent publications [20, 21], and most likely will continue to do so. Mouse models can be considered as the starting point for investigating a certain basic principle, without having an aim to translate it into humans, just like other non-human models such as yeast, fruit fly or zebrafish, depending on the nature of the question in study: for example, studying the mechanism of heat shock proteins in the antigen presentation process or understanding the survival mechanism of the gut nematode against the host immune response. Let us remind ourselves that learning about life systems more frequently comes from non-human models, such as the genetically distant and simple yeast which was used by Paul Nurse to reveal secrets of the cell cycle which won him the Nobel Prize in 2001. Transgenic mouse models have been very effective in demonstrating principles of the immune response, and numerous transgenic models continue to be used today around the world. Tolerance is a crucial regulatory part of the immune response and the mechanism of how tolerance is induced at a certain stage of T cell development was not known for a long time. Rolf Zinkernagel and colleagues created a transgenic mouse in the late 1980s that expressed the CD8 TCR specific for a glycoprotein of the lymphocytic choriomeningitis virus (LCMV). Their model, which uniquely offered the way to study T cell tolerance to two independent antigens, showed that the same TCR can respond to different antigens causing a difference in the induced tolerance, and that tolerance is not induced at one specific stage of T cell development [22, 23]. This model and its derivatives are still used by different groups to understand T cell tolerance, pattern recognition and antigen presentation [24, 25].

In fact, mouse models have been so useful through the years in understanding fundamentals of the immune system that milestone research efforts have been rewarded with Nobel Prizes in the category of physiology or medicine. A quick review of these works will indicate the need for mouse models:

1. **1960**: Sir Frank Macfarlane Burnet and Peter Medawar on 'Raising Self-Awareness'. The team introduced and proved the concept of immunological tolerance by showing, through bovine and murine models, that self-recognition is not preprogrammed but rather learned throughout embryonic life. An animal could acquire tolerance and this offered the exciting possibility of enabling human tissue and organ transplantation.

2. **1980**: Baruj Benacerraf, Jean Dausset and George Snell on 'Seeking Signs of Compatibility'. The team, working individually on guinea-pigs, humans and mice, contributed to the discovery of the MHC genes, the self-identification system that is composed of a unique set of antigens on host cell surfaces that forms a kind of biological ID for distinguishing one individual from another.

3. **1984**: Niels Jerne, Georges Kohler and Cesar Milstein on 'Creating Supply on Demand'. The researchers showed that the body already has an (inherited) full repertoire of antibodies prior to encountering an immunogen and that one B cell will produce only one specific antibody. Their joint efforts invented the monoclonal antibody by creating the hybridoma cell and this has been promising in research, clinical diagnosis and clinical therapy.

4. **1987**: Susumu Tonegawa on 'Assembly Instructions for Antibodies'. By revealing how only a limited number of genes are rearranged in an individual during maturation to

become antibody-producing B cells to combat millions of different antigens, Tonegawa showed that genes are not fixed but can be rearranged within the lifetime of the individual.

5. **1996**: Peter Doherty and Rolf Zinkernagel on 'Double Checking Cells'. These researchers revealed the true nature of the self-recognition system, the MHC antigens. The MHC plays along with killer T cells and allows them to make the crucial life-or-death decision to prevent damage caused by viruses. By using virus-specific T cells they showed that lymphocytes need to recognize two different signals simultaneously on their target cell in order to destroy it—one from the invading virus that remains concealed, the other from the host cell. Interestingly, the infected host cell is distorted at the MHC level by the virus, which allows the T cell to recognize it as an altered self-cell.

These findings have significantly changed the immune concepts and, with the accelerating speed of technology and biological tools, the pressure to investigate deeper into molecular immunology to reveal greater mysteries remain a challenge. Perhaps there is even more need for mouse models now.

Davis gives the impression in his argument that we have discovered enough of basic immunology from mouse models and that we should largely shift to human clinical studies. We would like to differ from this view. We do not believe that we know all that we need to know, simply because we do not know what we need to know. The concepts of immunology have changed so many times in the last few decades with new information derived from mouse models that it would be unwise to consider we have reached the ultimate knowledge. It is logical to accept the proposal by Davis, supported by Hayday and Peakman, that now knowing so much of immunology (e.g. profile of 350 CD antigens, cytokines, receptors and antibody structure) the scientific community should formulate 'metrics' to clinically diagnose 'immune health'. But this will not be the rationale to reduce or end basic immunology research using mouse models because we may have many more truths to unfold. An example is the discovery of the Th17 pathway

during the past decade. The Th1 and Th2 pathways had been known to exist for quite some time and we believed that an immune response would take one of these two routes. This concept changed when certain inconsistencies came up in EAE experiments. EAE was based on the notion that there is a dysregulated Th1 response associated with organ-specific autoimmunity and that there is an interferon-gamma driven response. There was significant evidence for this too. When interferon-gamma- and interferon-gamma-R-deficient mice, and also mice deficient in other Th1-related factors (such as IL-12p35, IL-12R-beta-2 and IL-18), developed EAE (reviewed in [26]), the concept that an interferon-gamma-dependent Th1-driven condition causes organ-specific autoimmunity was challenged. The possibility that another subset of Th cells is involved became the hypothesis. IL-23 was discovered when the p19 chain was found to bind to the p40 unit of IL-12, forming a heterodimer [27]. This changed the view of how the inflammatory process worked around IL-12, because any approach that targeted the p40 subunit would involve both IL-12 and IL-23. Subsequently, IL-23p19 knockout mice were created and in these mice but not in the IL-12-deficient mice EAE was observed to be reduced, making a case for IL-23 association with the disease [28]. In a translational study there was an increased production of IL-23 by dendritic cells and increased IL-17 production by T cells of MS patients [29]. These experiments led to the further finding that IL-23 promotes T cells that produce IL-17, which initiate a newly known inflammatory Th17 pathway. It is now clear that at least in the EAE model, Th17 cells are not the answer, just like the Th1 cells in the past, and more work is required to identify the 'true' critical T cell subset.

One of the reasons as to why clinical trials as a follow-up of mouse model results do not work as expected is that there are wide variations in the disease stage (early or advanced) of the volunteering patients, genetic background, effect of drugs already administered, age, lifestyle (food, habitat, activity, etc.) and stress levels, and most often it is not possible to select a population that has the same attributes in every parameter. Failure to translate the results should not make the mouse model redundant, but urge us to take into account the mouse–human differences and

the differences in the study population. It may be true, as Davis claims, that mouse-human differences are not being studied systematically, something that requires the integration of zoological studies with molecular biology. But it may not be crucial for the mouse or similar model to be totally identical with humans if the study is only to provide a research direction. The translational studies will complement those findings; hence emphasis on translational aims in projects is the way to go.

To answer why so much of research has become mouse-biased, and—as Davis has mentioned—why the heavily invested mouse-based research structure encourages us to use mice as models, we need to remind ourselves why we keep using mostly mice or only mice, and do not use other animals as well or instead. The quick breeding, fast growth and multiple breeding features, and the lower maintenance cost of mouse colonies, are very good reasons. The current complexity in acquiring ethical permission to experiment readily with human tissue discourages researchers from considering human research, rather obliging them to turn to mouse models. This has been described as an obstacle to research by Hayday and Peakman [18]. So, although there is an abundance of human clinical samples around the world, the immunologist community gets access to only a very negligible fraction of that. Until changes to ethical screening and legislation are brought in to make research more practical and straightforward, researchers will continue to favour mouse models.

Discoveries in knockout mouse mutants and the relevance of such mutants to human diseases

A specialized category of mouse models is the knockout mouse, which is an exceptionally efficient tool for the study of cellular and genetic systems. An inventive way to study the function and pathway of a protein is by gene targeting in mouse models. To assess the biology of the organism in the absence of a particular protein, mice are created that have the relevant gene knocked out. This has allowed researchers to explore the need for the protein by analysing

the effect caused by the gap. The first knockout mouse was created in 1989 after Capecchi, Evans and Smithies, in a joint US-UK effort, led the way in gene targeting technology, which brought them the Nobel Prize in 2007. Since then many knockout mice have been generated and continue to be generated every year. A number of international efforts in knockout mice such as the International Gene Trap Consortium (IGTC), the Knockout Mouse Project (KOMP), the German Mouse Clinic (GMC), the European Mouse Disease Clinic (EUMODIC), the North American Conditional Mouse Mutagenesis project (NorCOMM) and several others now provide researchers ease of access to knockout-based research. Guan and colleagues [30] have reviewed and summarized the international knockout mouse resources. An important feature that encourages the use of mouse models as an aid in human studies is that the successfully sequenced mouse genome (Sanger Mouse Genome Project) can be used now to target specific mutations in genes by homologous recombination in mouse ES cells, which makes it possible to alter genes efficiently and precisely.

Immunology has advanced greatly in the last two decades with the aid of the knockout mouse. The discovery of fundamentals achieved by successful gene targeting in mice (reviewed in Mak and colleagues [31]) has changed our basic concepts of immunology, revolutionizing the drive in immunology research. A few examples will illustrate the impact of such findings. It used to be believed that the MHC class I genes are required for the development of T cells. This concept changed in 1990 when it was revealed independently by Smithies' and Jaenisch's groups that the requirement for the MHC class I is for the selection of MHC class I restricted T cells and their antigen recognition, but not for their development. This was studied in mice which had a deletion in the MHC class I expression caused by the disruption of the beta-2-microglobulin gene. The mice had normal development but there was a significant reduction of CD4− CD8+ cytotoxic T cells (CTLs). Another mouse that was deficient in CD8 TCR showed that the receptor was required for the development of CTLs but not for helper T cells [32]. The Rajewsky group has been a leader in B cell biology and created the B cell deficient mouse by disrupting the

immunoglobulin mu chain. The homozygous mutant animals showed an arrested development of B cells at the pre-B-cell stage, confirming the importance of the IgM expression on the membrane of developing cells [33]. In the following years successive mouse modelling using the gene targeting approach helped us to understand the roles of genes not only in the development of the immune system but also in autoimmunity, transplantation and infectious conditions. Knockout and transgenic mouse models have effectively helped studies of various neurological conditions (reviewed in [34]). To give only a few examples, we have learned that a non-functional p53 expression is protective, P-selectin has a role pathology, TNF is neuroprotective, thioredoxin reduces ischaemic brain injury, NF-kappaB promotes cell death, NGF protects against apoptotic cell death and Bcl-2 is neuroprotective. The research on stroke has advanced significantly in the past few decades and much of this is due to animal stroke models combined with knockout mice. The need to use animals to study stroke is largely due to the fact that stroke events cannot yet be mimicked satisfactorily *in vitro*. Mice are among the few animals that are used in stroke models (reviewed in [34]).

The knockout mouse model is successful and has been used to study human disorders (see examples in Table 2.10.1) but often it comes with its problems, one being the lethality of a gene mutation. The disruption of a gene in its locus in the ES cells can render it disrupted in all the cells throughout the whole body and in certain genes this would cause severe system malfunction in the phenotype, resulting in non-viability of the mouse. Because of such gene lethality phenotypes it became known that many genes of the immune system have a serious impact on embryonic development. This prompted the need for alternative means to overcome the problems of the total knockout method and to devise methods to disrupt genes in only targeted cells. In 1994 the Rajewsky group pioneered the creation of the cell-specific conditional knockout mouse [35], which then took gene targeting studies in mice to the next level. The Cre/Lox system in which the Cre recombinase targets the LoxP sites and splices the DNA was used by the group to delete genes only where the Cre and Lox were expressed. In Rajewsky's study this method

enabled the study of the impact of the mutation of DNA polymerase-beta only in T cells, avoiding embryonic lethality. As can be expected, the conditional knockout system has become increasingly popular over the years since then.

Understanding mechanisms quickly

It is true that in many cases the mouse model only partially represents the human system, making it difficult to translate findings straight into human conditions. This should not render the mouse model invalid. We have explained that the mouse model provides a basic understanding of a gene or protein and points the direction for human studies. What is important to comprehend is the speed at which we can understand mechanisms using the mouse model. The mouse has a short life and a short gestation period with multiple litters, making it very convenient for the researcher to track a particular biological pathway consecutively and consistently. Using other animal systems closer to humans or human tissue systems or cell lines *in vitro* to investigate the same problem would require much longer periods, but also the *in vitro* systems are far more condition controlled than the *in vivo* system available in the inbred mouse. The murine physiological environment offers a more complete biological environment than the *in vitro* human models. The *in vitro* system is very useful when we know exactly what factors we want to study the specific effect of on a given cellular system and when we wish to bypass the effect of other molecular factors; for example, assessing the effect of increased calcium sensing receptor (CaR) agonists and antagonists on the expression of CaR in (cultured) smooth muscle cells [71]. But when we wish to include the effects of all known and unknown variables of the total biological system we need to use the total organism, in which case mice are a good starting point since human volunteers will either not be approved for the particular study, or will possibly have an altered microenvironment due to a history of using drugs for treatment.

Reviewing the chronology of Nobel Prizes in physiology and medicine relating to immunological studies, we can see that the frequency

TABLE 2.10.1: Examples of mouse models that reflect human immune system-related diseases

Disorder in humans	Gene mutated	References in human studies	Corresponding mouse models
Systemic lupus erythematosus (SLE)	STAT4	Remmers et al. [36]	Singh et al. [37]
Autoimmune polyendocrinopathy syndrome type 1	Aire	Halonen et al. [38]	Liston [39]
Autoimmune lymphoproliferative syndrome	FAS	Holzelova et al. [40]	Rieux-Laucat et al. [41]
Bruton's agammaglobulinaemia	Bruton's tyrosine kinase	Hashimoto et al. [42]	Khan et al. [43]
Hyper-IgM syndrome	CD40 ligand	Agematsu et al. [44]; Atkinson et al. [45]	Kuraoka et al. [48]
	CD40	Lanzi et al. [46]	Kawabe et al. [49]
	AID	Revy et al. [47]	Muramatsu et al. [50]
Severe combined immune deficiency (SCID)			
Autosomal recessive	Janus kinase (JAK)-3	Macchi et al. [51]	Nosaka et al. [54]
	RAG-1, RAG-2	Asai et al. [52]; Villa et al. [53]	Mombaerts et al. [55]; Shinkai et al. [56]
X-linked	Interleukin-2-receptor-gamma chain	DiSanto et al. [57]	Ohbo et al. [58]
T cell immunodeficiency	ZAP-70	Picard et al. [59]; Arpaia et al. [60]	Sakaguchi et al. [61]
X-linked chronic granulomatous disease (CGD)	CYBB	Newburger et al. [62]; Jirapongsananuruk et al. [63]	Pollock et al. [64]
Wiskott—Aldrich syndrome (WAS)	WASP	Derry et al. [65]	Nguyen et al. [66]; Nikolov et al. [67]
Inflammatory bowel disease (IBD)	IL-10R	Glocker et al. [68]	Pils et al. [69]; Chaudhry et al. [70]

increased from 1980. Ground-breaking findings in immunology derived from mouse models were rewarded with the esteemed prize. We would like to correlate this advancement in immunology research with the improvement in genetics and mouse modelling and the speed that mouse work provides. What was achieved by the researchers in their prizewinning investigations in discovering the MHC and self-recognition, the biology of T cell development, antibody production, gene rearrangement, and tolerance, could perhaps have been achieved by non-mouse models, but certainly would have taken much longer if human studies or cell line models had been used. We would not have reached where we are in understanding immunology today had it not been for the speedy information retrieved from mice.

Using *in vitro* systems to replace animal experimentation

There are quite a number of examples of non-animal *in vitro* experimentation in immunology,

and increasingly these make the argument against the use of mouse experimentation stronger. The most extensive alternative methods to mouse modelling are found in the study of gut inflammation, but we are not sufficiently convinced to declare them substitutes for *in vivo* studies that may replace mouse work altogether. Here we discuss IBD as a classic experimentation scenario to illustrate important factors that are not sufficiently considered in methods aiming to replace animal experimentation.

It is well known that IBD is a serious problem globally that affects the whole life and livelihood of an individual. It is an immune-mediated chronic inflammatory condition of the gut mucosal lining that arises from dysregulation of the immune system. Animal models have been used to a great extent to mimic the IBD conditions by inducing chronic inflammation through various ways such as causing irritation by DSS, immunological challenge by bacterial peptidoglycan-polysaccharides, or adoptive transfer of T cells into transgenics that are predisposed to developing colitis (reviewed in [72]). The need to design studies that can better translate to clinical applications has prompted scientists to explore *in vitro* systems that arise largely from human cells or tissues. In this way intestinal epithelial cells have attracted much attention because evidence supports the critical role of cells in the inflammatory response.

Advances made using *in vitro* cell models for IBD research

A comparative view of *in vivo* and *in vitro* research publications listed in the IBD ZEBET database [73] shows the development of non-animal methods in IBD studies. In the 1990s much effort was dedicated to exploring methods that would allow researchers to use immune cells belonging to the gut environment *in vitro* to study inflammatory intestinal problems. Intestinal epithelial cells (IEC) harbour intestinal intraepithelial lymphocytes (iIEL). Christ and colleagues [74] cultured primary iIELs from the small and large intestine in regular RPMI medium supplemented with human serum. They compared the behaviour

of these cells in culture with that of peripheral blood lymphocytes and showed that these cells are structurally similar but may have functional differences because of the impact of the microenvironment. Bischoff and colleagues [75] attempted to find out whether human mast cells, which usually require the presence of 3T3 fibroblasts to culture, could be cultured without the help of any feeder cells. They isolated mast cells from border sections of excised intestinal tissue (free of tumour cells, as confirmed by histology) from patients who were diagnosed with intestinal cancer. They showed that these mast cells originating from human intestine can be maintained in culture without the need of feeder cells (in the presence of stem cell factor) and that the cells differentiate into being functionally capable of releasing mediators (such as histamine) more than freshly isolated mast cells.

Liu and colleagues [76] used lymphocytes from inflamed ileum or colon lamina propria of IBD patients and revealed the importance of IL-15 in the pathogenesis. The patient population was a mixture of those with or without drug treatment, but none had received any immunosuppressive medication. The control tissues came from non-IBD patients who were diagnosed with carcinoma and were found to be macroscopically normal, or non-inflamed lamina propria of IBD patients. The major finding in the study was that IL-15 could be an important therapeutic target in IBD as it is over-expressed in the inflamed mucosa and it affects the local T cells and macrophages in their activation, proliferation and (pro-inflammatory) cytokine production. However, it is hard to believe that in the microenvironment of the selected controls there would have been no effect of any factors that might have risen from the carcinoma or the IBD, or that the various drugs already administered to the test samples would not have altered the immune response. An absolute control in such human studies, as available in mouse model studies, would be almost impossible to find.

Mahida and colleagues [77] used freshly resected mucosal samples from human colon and terminal ileum to study the migratory path of cells out from the lamina propria into the lumen. Epithelial cells were removed without

the use of collagenase from mucosal strips and the denuded mucosal tissues, which are known to harbour lymphocytes and macrophages, were cultured and studied. The work revealed that there is a selective migration of T cells, macrophages and eosinophils, representing the lymphocytic population in the lamina propria. This gave implications on what kind of immune response may be expected in conditions such as IBD.

In a proteomics approach, Barceló-Batllori and colleagues [78] grew human adenocarcinoma cells, DLD-1, to study the unknown proteins that are induced by pro-inflammatory cytokines interferon-gamma, IL-6 and IL-1-beta. Proteomics is a powerful research method because it allows the assessment of the proteins that have been expressed in a given condition. The researchers also used epithelial cells *in vitro* obtained from various clinical samples including IBD, to compare the proteomics results.

The Caco-2 cell line has been used in some immunological studies of gastroenteric disease and is used widely by pharmaceutical companies as a human small-intestinal mucosa model to test drug products. This cell line has a composition of heterogeneous cells that originate from human epithelial colorectal adenocarcinoma cells. The usefulness of these cells is that they express many features that resemble the gut lining such as tight junctions, microvilli, enzymes and transporters. But the heterogeneity of the cells makes it difficult to compare results across laboratories around the world. Kucharzik and colleagues [79] used a combination of Caco-2 cells and IEC to study the effect of Th2 cytokines on the production of MCP-1. The study was aimed at improving therapeutics for IBD and found increased levels of MCP-1 in the supernatants of the cultured cells when stimulated with IL-1-beta or TNF-alpha, which could be reduced by IL-4, IL-10 and IL-13.

There are several other examples of the use of epithelial cells from colonic or ileal tissue for studying immune responses in gastrointestinal inflammation [80–84] and these have displayed the capability of human *in vitro* systems for being good working models. The key question now is, are these *in vitro* models sufficient or do we need mouse models to overcome limitations of the *in vitro* models? What would we not know now about IBD if we had stopped the use of mouse models for IBD and were just following the ZEBET guidelines?

Concept paper on replacement of animal studies

The Committee for Proprietary Medicinal Products (CPMP) held a meeting in London in February 1997 and adopted a position on the replacement of animal studies by *in vitro* studies [85]. The paper was prepared in consultation with the European Centre for the Validation of Alternative Methods (ECVAM) and was aimed at enforcing ethical considerations to limit the use of laboratory animals as much as possible. It addressed the feasibility of replacing animal studies by *in vitro* methods in the preclinical development of medicinal products. 'Reduction, refinement or replacement', also known as the '3Rs' rule, was proposed to guide investigators and organizations and research funding bodies to reduce animal work. The paper suggested assessment of toxicity by single dose administration, or repeated dose administration or evaluation of toxicity on reproductive functions as areas where *in vitro* methods can be considered to replace animal modelling. The recommendations made by the panel are quite justified and in most European countries, including the UK, the 3Rs have been followed in designing research protocols. However, these guidelines to replace animal work by *in vitro* techniques mostly apply to medicinal product research and/or where toxicity is a concern. Research in basic immunology is quite a different perspective and should continue to rely on animal models as well as corresponding *in vitro* systems.

Humanized mice

Probably the most important translational measure in experimental immunology in recent times has been the development of humanized mice, and it is conceivable that the use of this instrument in translational medicine will increase more in the near future. Mice engineered in a way so that they can functionally harbour and

integrate human haematolymphoid cells and tissues, so that they are humanized for the transplanted cells, are becoming popular. Importantly, these models try to counteract the flaws of small animal models such as the mouse-to-human phylogenetic and evolutionary species differences. Although there has been much debate about mouse modelling in immunology and incentives for replacement with alternative methods, mouse studies still remain indispensible in basic research and also in medicinal validation studies. So, the critical need to have mouse models and the urge to have them closer to human systems resulted in the generation of humanized mice, which are used to study the human system specifically.

Macchiarini and colleagues [86] have reviewed the successes and failures of humanized mice in immunological studies. Since the early studies with fetal sheep and genetically athymic nude mice in attempting to colonize human cells in experimental animals, the development of the humanized mouse has advanced significantly. The severe combined immunodeficiency ($Prkdc^{scid}$) mutant mouse, which lacks both T and B cells, has been a major tool in humanizing mice. Initially, engraftment of human cells in these mice had limited success because there was rejection caused by the host innate immune response. In successive years the cross of NOD mice with other mutants, e.g. $Prkdc^{scid}$, $Rag1^{null}$, $Rag2^{null}$, $B2m^{null}$, $Prf1^{null}$ and $IL2r\gamma^{null}$, has helped overcome these problems, resulting in longer-living mice that can retain the engraftment. There have been two main approaches in humanizing mice and these are described briefly here.

Approach 1: replacing mouse genes with human genes (gene targeting method)

This approach is largely targeted to producing human monoclonal antibodies for therapeutic purposes. The idea stemmed from the possibility of immunizing a mouse with human antigenic material and having the mouse producing human immunoglobulin (Ig). The Rajewsky Laboratory adopted a technique based on the cell-specific Cre/LoxP gene targeting system in

creating a humanized mouse strain to produce human–mouse chimeric antibodies [87]. Cre/LoxP is an efficient method of targeted mutagenesis in the mouse and the group used this to replace the extracellular part of mouse gene $C\gamma1$ with its human counterpart in the mouse ES cells. The $C\gamma1$ gene encodes the constant region of the heavy chain of IgG1 antibodies. As the constant region of the light chain was humanized as well, such mice produce chimeric humanized antibodies, the constant region being of human origin and the variable region from the mouse.

In an alternative approach Brüggemann and colleagues [88] have shown that DNA containing the large human immunoglobulin locus can be transferred into the mouse germ line and functions normally; such mice are able to produce human antibodies. The approach has been commercialized and, for example, the Kyowa Hakko Kirin, an innovative Japan-based antibody company, has created a mouse that can effectively produce fully human antibodies. This enables the antibodies to be captured in the form of hybridomas and produced in large quantities for treatment of diseases. This method is basically a commercialization of what Brüggemann and colleagues had achieved earlier.

Another aim of this approach is to develop a mouse model that will resolve the difficulties in TCR-related studies. The study of tolerance is an extremely important part of human immunology research, and a mouse model that expresses the human TCR repertoire would be an valuable tool for this. Human peptide antigens that differ between humans and mice could be studied in a mouse with a humanized T cell recognition system. The Blankenstein group developed a transgenic mouse humanized with the entire human TCRAlphaBeta gene loci in which functional CD8+ T cells were studied against several human tumour-associated antigens (TAAs) [89]. Yeast artificial chromosomes (YACs) containing the human TCRAlpha and TCRBeta were constructed and fused with mouse ES cells, and mice were generated from these ES cells. Crossing such mice with mice with deficiencies of murine TCR generation and rendering them transgenic for human HLA genes generated a mouse line

that is now expressing an authentic human TCR repertoire.

Approach 2: engraftment of immunodeficient mice with human immune cells or stem cells

Humanizing immunodeficient mice by engrafting such mice with human cells or tissues is now a very popular method and has been used in various experimental and clinical studies. The idea is to transplant a functional human immune system into a mouse body, which can then be used to evaluate immune response to infections and vaccines. This approach has been used for example in studying dengue fever, a mosquito-borne viral disease [90], which cannot be studied in normal mice. Humanized mice have also helped in studies of Epstein-Barr virus, diarrhoeal pathogens and the development of the influenza vaccine (reviewed by Macchiarini et al. [86]). The technology is already routine in the study of human immune system function. For example, the Harvard University Center for AIDS Research (CFAR) is dedicated to providing humanized mice to study human infectious agents and test potential vaccines. CFAR has established three mouse models of the human immune system—hNSG, BLT and GTL—and claims that the level of reconstitution with human immune cells in these mice is 40-90%. These mice have been used at CFAR to study HIV vaginal infection, immunization (DNA and protein based, use of adjuvants) and the human antibody gene repertoire. The engraftment technique was also used to create mice with a humanized T cell recognition system [89]. This model, which was basically a transgenic mouse with a diverse human TCR repertoire, has aided researchers to study tolerance in humans to self-antigens such as TAAs.

Conclusions

Studying the immune system in mice has given us very important insights into the building blocks of the immune system, allowed us to see the immune system in action and to understand immune regulation. Diseases of the immune system can be studied in the whole-body context and such studies simply cannot be replaced by *in vitro* systems. Limitations in translating findings in mice to human medicine can be overcome by systemic comparison of the mouse versus the human immune system and by the generation of humanized mice using various approaches. The study of the mouse immune system cannot be replaced by clinical immunology alone and will continue to be an important element in basic immunological research.

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The Behaviour of the House Mouse

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Introduction

The house mouse (*Mus musculus*) is the most successful and most widely distributed invasive mammal except for humans ([1], cited in [2]). For thousands of years, house mice have been commensal with and have been transported by humans throughout the world [3]. Archaeological and paleontological evidence shows that the association of humans and house mice took place immediately after the first postglacial temperature rebound during the Bølling/Allerød (12 700–10 700 BC). The subsequent house mouse expansion was initiated when new agricultural and husbandry practices were established, such as large-scale grain storage, during the Neolithic revolution (reviewed in [2]). Worldwide, house mice are found in a variety of habitats: in temperate and tropical zones, as well as in subarctic regions; on farmland or coral reef islands; in grain storage facilities, rice fields and in coal mines; in deserts and on tropical islands; from sea level to up to altitudes of several thousand metres; house mice have been even observed to reproduce successfully in frozen carcasses in cold stores in the port of London [4]. To allow such flexibility in habitat use and in distribution, flexibility in behaviour, especially in maternal and social strategies, is required.

Taxonomy and biogeography of the house mouse

Mice of the genus *Mus* evolved on the Indian subcontinent, from where they radiated in several directions [5]. All commensal house mice and laboratory mice belong to the species *Mus musculus*, which consists of the four well-described subspecies *Mus musculus domesticus*, *M. m. musculus*,

M. m. bactrianus and M. m. castaneus (for recent reviews on the taxonomy, systematics and bioge-ography of house mice see [6], [7] and Chapter 1.1). These subspecies have non-overlapping ranges under natural conditions, and are morpho-logically and molecularly distinct. Nevertheless, they can reproduce and produced fertile offspring in the laboratory [8].

The western house mouse (M. m. domesticus) followed a Mediterranean route out of its Near Eastern place of origin, and colonized western Europe, commensal with humans, 2000 years ago [2]. Nowadays it occurs in western Europe, North Africa and the Middle East to south-west Iran. The eastern house mouse (M. m. musculus), on the other hand, followed a route north of the Black Sea and along the Danube [9], and today is found all over northern Asia as well as in eastern, central and Scandinavian Europe. M. m. castaneus is found all over South East Asia, and M. m. bactrianus in Afghanistan and Pakistan (with some evidence that this group of 'central mice' might represent an ancestral population [7]).

Long-distance colonization of the rest of the world is a recent phenomenon. M. m. domesticus is the subspecies with the widest distribution, and expanded its range through passive trans-port with humans to the Americas, Australia, sub-Saharan Africa and Oceanian islands [2, 7]. The ecological success of house mice as an inva-sive species thus is linked to their commensal interaction with humans, which resulted in the species reaching and thriving in places outside its natural regions of origin.

The house mouse in research

The house mouse has been a model and a tool for medicine and biology for many centuries, and has contributed enormously to our knowledge of genetics and physiology of mammals (for a histor-ical overview and summary see [10]). The mouse was used for anatomical studies in the 17th century; it helped to document Mendelian segregation and linkage of genes in the early 20th century, and contributed to the neo-darwinian synthesis along with other classic organisms such as Drosophila; it

was the second mammal—after humans—for which the genome has been sequenced; it served and still serves as a model in evo-developmental studies, and is an important study species for biomedical research and pathology.

The origin and history of laboratory mice are described in detail in Chapter 1.1. Nowadays over 300 different inbred strains, and a variety of additional outbred strains, are known. Inherited variation is the basis of the differences between strains, but it has to be kept in mind that the commonly used strains only carry a small part of the variation found in wild mice. Behavioural analyses have been done for many inbred strains, with the discovery of genetic variability in behaviour within and between strains [11-13]. Differences have been documented for various traits, for example social behaviour [14], maternal behaviour [15-17], lactation perfor-mance [18, 19], activity and aggression [20], repro-ductive output and growth of pups after weaning [21], and length of an oestrous cycle or oestrous duration [22]; for a review of genetic differences described for naturally occurring populations see [23].

Genetically, all inbred strains analysed so far did not originate in only one house mouse subspe-cies, but are a combination of different subspecies. Nevertheless, M. m. domesticus is predominant [8]. Since most studies on free-living house mice, as well as on wild or laboratory mice kept in labora-tory animal facilities or in semi-natural enclosures, have been done on M. m. domesticus, the following review of house mouse behaviour is primarily based on observations on or experiments with this subspecies. Latham and Mason [23] recently compiled a review on the behavioural biology of free-living house mice, M. musculus.

Behavioural flexibility in the western house mouse

The western house mouse (M. m. domesticus) is considered a prime example of adaptability to

very diverse habitat and climatic conditions [3,24]. Behavioural flexibility is a remarkable feature of the subspecies and has been considered as a predisposition for its ecological success [25–27]. This is highlighted by the mouse's complex history of repeated successful colonization and the concomitant adaptations to new environments. Its association with human activities and dwellings, and the fact that humans may transport it over long distances, thus have a profound impact on the population dynamics and social structure of the species. As a result, house mice occur in patchily distributed small to large populations, and gene flow between populations varies in intensity both in space and in time. Most house mouse populations can thus be considered as genetically almost unique [24, 28].

In his classic review on the reproductive ecology of the house mouse, Bronson [3] emphasized ecological opportunism and colonizing abilities of the species. Nevertheless, he suggested classifying mouse populations according to whether they exist commensally with humans (commensal populations), or independently of human activity (feral populations). In Europe commensal populations occur in anthropogenic habitats, such as farm buildings and grain stores. Feral populations, on the other hand, are found in grasslands and cultivated areas, and are restricted to islands in Europe [29].

House mice have a high reproductive potential. Under favourable environmental conditions and in laboratory animal facilities, mice can sexually mature at 6–8 weeks, and females can give birth to a litter every month. Such an enormous reproductive output has been interpreted as an adaptation to a colonizing life strategy, which has to cope with variable environmental conditions and high mortality [30–32]. The average life expectancy of free-living house mice is only a few weeks (100–190 days), which is mainly due to high juvenile mortality [33–35]. Some individuals can nevertheless survive for more than two winters, and laboratory mice live up to 3 years or even longer.

Feeding and foraging behaviour

In terms of food, house mice are omnivorous. They feed predominantly on seeds, nuts, fruits and roots, but also eat meat and prey on living insects [4, 23]. Foraging takes place during regular patrolling of the territory [36]. Mice eat up to 20% of their body weight daily [37, 38], with lactating females more than doubling their caloric intake per day [19]. Typically, a mouse consumes about 200 small meals in a 24 h period, repeatedly visiting approximately 20–30 food sites [23]. Generally, mice are not reluctant to try new food. As pups they learn about food from their mother even before they are weaned. Subadults and adults assess by smell during grooming of group members what food the others have eaten, and can establish socially learned food preferences [39]. Such allo-grooming not only assists transfer of information about food, but also maintains social relationships. Self-grooming, on the other hand, is important for hygiene and insulation [23].

During foraging or exploration mice avoid unsheltered or exposed areas, and sites where there is a high risk of predation. They find carnivore faeces and related scents aversive, and avoid the urine and faeces of recently frightened conspecifics (reviewed in [23]). For females the local presence of a male, and status of his associated females, may also be important, as the breeding success of female mice is critically dependent on their ability to establish a nest site within a male territory [40, 41].

When mice explore a new area, they do so slowly and carefully, following physical structures such as barriers and walls. They frequently pause during excursions, and rear up or make long stretches forward, sniffing at new objects. Gradually they make longer excursions. In between such short-range explorative excursions, they often return to a familiar or safe area, following visual landmarks and the olfactory marks produced by the plantar glands on their feet [23, 36, 42].

Mice are very sensitive to movement and changes in light intensity, and also use visual cues to demarcate territorial boundaries [43], to navigate or to move to cover [44, 45]. They have little colour perception and are insensitive to red wavelengths (they lack a long-wavelength photo pigment). On the other hand, they are sensitive to ultraviolet wavelengths, which may be an adaptation to crepuscular activity [46], and may be used in navigation and in foraging

(many fruits, seeds and even larvae reflect in ultraviolet [23]). Hearing is well developed in mice and they are able to hear noises from 10 kHz to ultrasound over 100 kHz [47, 48].

Activity and territoriality

House mice are most active from dusk to dawn, and thus are considered as crepuscular or nocturnal. Still, commensal mice can also be active during the day in the absence of predation [36]. Little is known about how much time free-living mice are active. Laboratory mice spend less than 50% of the entire day active [23].

Commensal mice are territorial, and although the dominant male is usually the most aggressive, adults of both sexes contribute to territorial defence [23, 36, 49]. Territorial boundaries often overlap with physical structures in the environment. All group members mark territorial boundaries as well as conspicuous objects, or the surroundings of feeding and nesting sites, by urinary odour cues. In particular, dominant territorial males frequently deposit such scent marks at boundaries, since refreshing their own marks is a signal of competitive ability, providing information about territorial and sexual status [3, 50-53], and females prefer to mate with dominant males [54, 55]. Territorial males also try to over-mark their competitor's urine marks in neighbouring territories. Urine marks of all mice living in a territory can be deposited so frequently that they form 'pillars' several millimetres high [56, 57]. Once deposited, urine marks can last for up to 2 days, due to non-volatile major urinary proteins (MUP), which contain information about individuality, sex, dominance status and reproductive condition, and stimulate aggression among males and oestrous in females (for recent reviews on olfactory mediated information through MUPs see [53, 58-60]).

Besides such urinary cues, mice also deposit olfactory marks produced by plantar glands on the feet [23, 36], resulting in well-worn runways. Individuals travel their entire territory daily, covering and marking the same routes repeatedly. Commensal mice therefore have been described as creatures of habit [23]. Through these routines, mice acquire highly habitual responses (e.g. dashes to safety), which they can perform extremely rapidly and with minimal

sensory input. Predictable movement about the territory therefore is not only an essential part of territorial defence, but also allows the animal to build up a detailed, continually updated picture of its domain [36, 61].

Male intraspecific behaviour is characterized by aggressiveness and dominance, and functions to defend a territory and access to reproductively active females (still, intraspecific aggression varies between laboratory strains, between subspecies and also between different *Mus* species) [54, 62-65]. Unfamiliar intruders are generally aggressively driven out of the territory; if they are unable to escape, they are likely to face severe injury or even death [36, 61, 66].

Social structure

The social structure of the western house mouse varies with habitat, resource partitioning and density [3, 27, 67, 68]. Most typically, house mice live in small social groups that consist of a dominant male, one or several adult females with their litters and up to several subordinate mice of both sexes [3, 24, 49, 69-71]. The social system of house mice has been classified as polygynous, but there is increasing evidence that female house mice are actively polyandrous [35, 72-74]. The genetic mating system thus is better characterized as polygynandrous. Why females mate with several males during one oestrous cycle is not yet understood. It has been shown in experimental studies that polyandry increases offspring postnatal survival [75], facilitates inbreeding avoidance [76], selects for increased sperm numbers and motility [77] or may be understood as a female counterstrategy to mitigate the negative effects of a selfish genetic element, the t haplotype [35].

House mice are plural breeders, with several breeding females per group. Within groups adult females cooperate in some kinds of communal care, such as babysitting, social thermoregulation or defence of pups. The most striking example of cooperation, however, is non-offspring nursing, also prominent among laboratory mice (for a review see [78]). Such non-offspring nursing occurs when two, or sometimes more, females pool their litters in a communal nest and indiscriminately nurse their own young and non-offspring [79-82].

The causes and consequences of communal nursing will be discussed below.

Dispersal behaviour

In house mice, as in most other mammals, dispersal behaviour is one of the most important life-history traits [66, 83, 84]. A detailed study with wild house mice under semi-natural conditions [85, 86] revealed a dispersal pattern very much in agreement with knowledge from stable commensal populations [70, 84]. Nearly all male offspring disperse from the natal group unless an individual has successfully supplanted the current territorial male, which might happen to be the father of that male. The decision of when to disperse seems to be governed mainly by processes intrinsic to the dispersing animal, independent of sexual maturation. Young males often stay resident for some time after reaching sexual maturity, exhibiting no intrasexual aggression, and disperse late; others become aggressive early and disperse if not successful in gaining territory ownership [66, 86]. Females, on the other hand, stay in the natal group as long as there is a chance of starting to breed within that territory. Otherwise, at high local population densities, females disperse at about the same age as males, but without an observable increase in overt aggression [85, 86]. Since females can stay and successfully reproduce in their natal group, such groups often are extended family groups, comprising several related females.

Although male house mice are well known for their high aggressiveness, exceptions have been identified recently. In at least one population of wild house mice, adult males have been found to be reluctant to engage in aggressive interactions [87]. This confirms a finding of Benus and co-workers [88–90] who studied wild-stock-derived mice selected for long and short attack latency. These mice differ in a whole array of behavioural traits, including readiness for aggressive interactions for dominance status, anxiety in novel situations, and explorative activity. The authors argue that these two phenotypes reflect two dispersal strategies. One strategy would lead to socially competent males that are able to stay in the natal group for a longer period; the other one would produce males of a more active phenotype that is characterized by active exploration of opportunities for dominance and early dispersal. Hence, it is tempting to suppose that some ecological alterations have selected for more socially competent and less aggressive male mice under some circumstances, differing strongly from the aggressive and early-dispersing male phenotype encountered in most house mouse populations. Such selection might have taken place in house mice on the Isle of Man, where males are rather non-aggressive [87].

Laboratory mice are generally less aggressive and more socially tolerant towards conspecifics than wild house mice, presumably as the result of selection during domestication.

Flexibility in maternal reproductive strategies

As emphasized earlier, house mice have a very high reproductive potential. This is due to the fact that females give birth to 4–12 young after a gestation period of 19–21 days (average litter sizes differ for different inbred strains and populations [8]), have a postpartum oestrous 12–18 h after birth of a litter, and thus can produce the next litter after just 1 month. Implantation of the litter conceived during the postpartum oestrous is delayed by a few days [37] (reviewed in [91]).

Maternal care

Maternal behaviour in mammals is characterized by lactation. Via the milk, offspring are provided with nutrients, calories, vitamins, minerals, and passive immune protection (lymphocytes and antibodies), for growth and for metabolism [31, 92]. As a consequence, a long lactation period is beneficial for the pups. For the mother, on the other hand, the energetic costs of lactation may influence her survival and future reproduction. The metabolic demands of lactation are enormous, especially for small animals, and in house mice they are more than four times higher than

the energetic costs of gestation [93]. Daily energy output in milk per unit body weight is approximately 16 times higher than in an animal the size of a cow [94]. Such high and sustained milk production in small mammals is only possible because of a high metabolic rate that also relates to a decrease in lifespan [95, 96]. In addition, the longer the lactation period, or the more milk produced, the more delayed is the birth of the next litter [33, 34]. As a consequence, lactating females have to make a trade-off between current and future reproduction, and we expect flexible maternal strategies to compromise between offspring benefits and maternal costs during lactation under different environmental conditions. The potential for rather flexible maternal strategies is already illustrated by the observation that reproductive performance varies among different inbred strains [91, 97].

Maternal behaviour in house mice consists of nursing, licking and grooming pups (licking the anogenital region stimulates defecation in pups), nest-building behaviour, huddling over pups to keep them warm (under conditions of low temperature) and retrieving pups to the nest, either when females move the nest to another place, or when a nursing mother has left the nest, and some pups were dragged along because they were still attached to a teat [31]. It is interesting to note that male house mice, when kept in a monogamous pair with a female, show the same parental behaviours as females towards their offspring, except for nursing [31].

House mice as well as laboratory mice build nests in which they sleep or rest. Such nests are often relatively small and open, but are more closely built during periods of cold environmental conditions [91]. Pregnant and lactating females, on the other hand, build maternal nests (from approximately 4 days after mating onwards) that are two to three times the size of a sleeping nest, with one or two entrances and completely enclosed. Maternal nests are an important component of maternal behaviour. When given the choice between different bedding materials, including an option with no bedding material present, female laboratory mice never gave birth in cages without bedding [98]. Under natural or semi-natural conditions, access to a safe and protected nesting site seems to be a prerequisite for successful reproduction in females, because such

sites improve protection from disturbances by conspecifics [41, 99-101]. It is therefore highly recommended to provide mice with nest-building material in laboratory animal facilities.

Maternal aggression refers to aggressive behaviour of a lactating female when defending her litter [91]. Females become intensely aggressive towards other individuals during the final days of pregnancy and during lactation, and will vigorously protect their nest, biting intruders' heads and bellies [102, 103]. Such increased aggression may function to allow the female to defend her nest and pups against infanticidal conspecifics (both male and female house mice have been observed to kill pups when encountered in a foreign nest), or assess the dominance status of any intruding males [104-106].

Pup development and weaning

The development of house mouse pups can be summarized as follows [19, 31]. At birth (day of birth of a litter refers to day 1 of lactation), pups weigh 1.2-1.4 g, with individual pups from small litters being heavier that those from large litters. They are fully dependent on their mother for nutrition, thermoregulation and protection. By day 9 they are covered by dense fur (thin hair will have grown a few days earlier); they open their eyes at day 14; and they begin to actively leave the nest when 16 days old. Even during the first 2 weeks of life, mouse pups produce a variety of sounds, mostly ultrasounds, which function to elicit maternal care in a situation of distress, danger or hunger [107-109]. The rate of these ultrasound calls reaches a maximum at around day 8, and decreases after that until these calls disappear after 14-16 days [109-111].

Weaning of pups is a flexible maternal strategy in female house mice. Pups feed exclusively on milk until they are 16 days old; at 17 days they begin to eat solid food, but nevertheless are still nursed by their mother [31]. A drop in nursing activity to less than 1% defines weaning. Weaning age differs according to litter size, with small litters (litter size ≤6) being weaned at day 21, and larger litters at day 23. Furthermore, weaning is not completed before day 23 when

a female is simultaneously lactating and pregnant. After weaning nursing is replaced by resting with body contact to the mother, without nursing attempts by the pups. During days 17-22 pups regularly try to initiate nursing, and females often rest without body contact with the pups, for example in a separate corner of the cage, when they have finished nursing. If space is rather limited, pups often succeed in approaching the mother and initiate sucking. When the mice were kept in at least two small Macrolon cages (type 2) linked by tubes, however, females typically slept or rested alone in a cage other than the one with the nest, thereby avoiding such sucking attempts by their pups [31]. Resting alone and remaining far from the litter indicate the female's active role in avoiding the offspring's increasing demands during the period of weaning (days 17-22). Nevertheless, there is no maternal aggression towards the young during the weaning period. After weaning the relationship between mother and young appears free of conflict. Nursing is replaced by resting with body contact, and the offspring no longer try to suck [31].

Lactation performance

The main energy source in the milk of house mice is fat, which allows for rapid postnatal growth of a relatively large number of young. A detailed analysis of milk production over the whole lactation period in laboratory mice revealed that lipids provide on average more than 80% of the energy available for the suckling young [19]. Daily milk production, maternal body weight and food consumption increase after the first days of lactation to a maximum during days 9-16, and decrease again afterwards. Females do not store body fat during pregnancy to be used for milk production but have to increase their daily food consumption during lactation [93, 112]. As in other small rodents, a reversible enlargement of the digestive tract tissue may result in the same assimilation efficiency of lactating females as in non-reproducing individuals [113-116].

Females meet the energy demand of a growing litter not only by increasing the amount of milk produced, but also by improving the quality until day 16 of lactation [19]. During peak lactation both lipid concentrations and total solids reach maximal values. The concentration of calcium in the milk, which is essential for the bone synthesis of the young, also increases with age and growth of the litter until the onset of weaning. Iron, which is essential for synthesis of red blood cells, is maximal during the first week.

Although the energy needs for the pups' metabolism have only been measured through changes in pup body weight, metabolic use seems to increase steeply after day 16. Until then the sum of all energy losses of an individual young (growth, metabolism, urine, faeces) were approximately the same as the amount of energy absorbed via milk. After that, however, the energy demands of metabolism and growth were much higher than the energy supplied by the mother. This might suggest that young house mice have to increase their energy intake by consumption of solid food at the age of 17 days to allow for a positive energy budget—a necessity for growth [19]. This corresponds to the earliest age at which young mice were first seen to eat solid food [31].

With increasing litter size, females adjust the amount of milk produced according to the higher demands of many offspring, but regulation is imperfect [19]. Weaning weight of individual pups from large litters (litter size ≥8) is more than one gram lower than that of pups raised in small litters (litter size 5-6). Female house mice produce approximately 100 g milk of an energy equivalent of 1100-1200 kJ to rear a litter. With such an investment, a female can either wean a litter of six young with a body weight of 10.6 g each, or a litter of 7.3 young weighing 9.4 g each [19].

When analysed under different environmental and reproductive conditions, female house mice with litter sizes of 6-10 young adjust their maternal behaviour according to the developmental state of the litter and do not wean their pups below a minimal threshold weight of, on average, 9 g [31]. A weaning weight below this threshold, as found in young of very large litters (7.1 g per young in a litter of 12), would result in initial, but reversible, weight loss of pups. As a consequence of such low weaning weight, daughters will experience delayed maturity and delayed onset of reproduction [33]. A female rearing a very large litter therefore experiences

relatively low reproductive success in terms of granddaughters produced, compared to its relatively high energy expenditure in milk. A mother will gain higher reproductive success by dividing the energy available per lactation to the largest number of young she can raise to an average weaning weight of 9 g, instead of producing a smaller number of larger young or a larger number of smaller young [19].

In laboratory animal facilities weaning is typically imposed at 21 days, corresponding to the time when the next litter will be born if the female was mated postpartum [91]. However, weaning is only rarely finished at 21 days naturally and in laboratory mice, as previously illustrated. In addition, newborn pups do not suffer from delayed growth in the presence of an older litter [32]. The data presented on maternal strategies therefore recommend not weaning mouse pups before they are 23 days old.

Communal nursing of litters

As mentioned before, another maternal strategy in female house mice is communal nursing of pups. Non-offspring nursing is an integral part of the reproductive behaviour of house mice in multifemale groups, although females do not always communally nurse when given the opportunity to do so under free-living conditions (own observations). Communal or non-offspring nursing has been described for approximately 70 mammalian species (in 12 orders) and for reproducing and non-reproducing females; nevertheless, in only 10% of such species were non-offspring nursed as much as own young, as is the case in house mice [117-122].

When direct descendants of wild-caught house mice were kept under standardized conditions in the laboratory or in semi-natural enclosures, female lifetime reproductive success differed significantly as a function of the social environment. A female's lifetime reproductive success was defined as the number of offspring weaned within an experimental lifespan of 6 months, standardized as 120 days after first introduction of an unfamiliar, genetically unrelated adult male. Under natural conditions, such a life expectancy seems to be realistic for female house mice that survived at least until maturity [67, 34]. A female sharing a nest with a familiar

sister (i.e. females that grew up with each other and had never been separated) weaned significantly more offspring within an experimental lifespan than a female living monogamously and rearing litters alone. The lifetime reproductive success of a female living with a previously unfamiliar, unrelated partner lay somewhere in between [32, 80, 123]. Thus, communal rearing of young is beneficial due to improved individual lifetime reproductive success, as long as females choose a familiar sister. These laboratory data are supported by observations of free-living mice and of wild mice in semi-natural enclosures. First, the degree of relatedness among communally nesting females was higher than expected by chance, which has been interpreted as 'genetic assortment' among females [124]. Second, in natural populations and in semi-natural enclosures female house mice spatially associate and communally nest with kin [40, 41, 124-126]. Third, under semi-natural conditions females preferred for communal nursing a same-sex partner that had a similar major histocompatibility locus (MHC); since similarity in the MHC correlates with genetic relatedness, such assortative social partner choice has been interpreted as kin preference [127].

During mate choice house mice of both sexes also use MHC genetic cues; here, however, to avoid mating with unfamiliar related conspecifics and to mate preferentially with unrelated individuals (for recent reviews see [128, 129]). Proteins produced by the MHC are excreted through the urine and used as olfactory recognition cues. The recognition mechanism is probably phenotype matching, meaning that an individual with an odour similar to one's own is treated as kin. Such a mechanism may potentially also allow females to discriminate between a related and an unrelated potential social partner.

The effect of improved lifetime reproductive success was due to improved probability to reproduce and to improved offspring survival of females sharing a nest with a sister [130]. Familiarity during juvenile development and not genetic relatedness per se proved to be of paramount importance for this effect, despite the existence of a genetic mechanism to identify relatedness [131]. This may suggest that a physiological mechanism is involved which requires some period of adaptation to or some synchronization

with a partner. On the other hand, for communal nursing to be most productive it may be important that females have information about each other's behaviour, which is best guaranteed among familiar partners. Nevertheless, due to the basic family structure of house mouse breeding groups, there is a high probability that a female that chooses a familiar partner for communal care of young will direct her cooperation and care of non-offspring towards kin.

Despite such cooperation, females nevertheless compete over the opportunity to reproduce. Within breeding groups females establish either egalitarian reproductive relationships (both females wean young within their experimental lifespan) or despotic ones (only one female reproduces successfully). Lifetime reproductive success in egalitarian relationships is similar for both females, irrespective of the degree of relatedness to or familiarity with the female partner. However, egalitarian reproduction is significantly more common among familiar sisters than among unfamiliar and unrelated females, with sisters very rarely establishing despotic relationships. Future research has yet to show whether the presence of an adult, non-reproducing daughter improves a mother's reproductive success, as no helping effect has been documented for house mice so far.

Competition among females further increases with increasing group size. Individual lifetime reproductive success in groups of three females (either three sisters or three unfamiliar, unrelated females) is significantly lower than that of monogamous females. These data suggest that for successful cooperation it might be important for females to be able to establish stable pairs [78].

Nevertheless, irrespective of relatedness, communal care in pairs with egalitarian reproduction involves direct and mutualistic fitness benefits for both partners. The phenotypically altruistic behaviour of non-offspring nursing in house mice proves to be genetically 'selfish' by maximizing a female's lifetime reproductive success. Nevertheless, the mutualistic benefits are influenced by relatedness and juvenile familiarity among the females, because of improved probability for egalitarian reproduction among sisters that have grown up together.

During communal nursing, female house mice do not discriminate between own young and non-offspring, because of constraints in recognition abilities [80, 130]. As a consequence, cooperative females may run the risk of being exploited by a female partner with a larger litter size [78]. Because nursing is indiscriminate, lactating females adjust milk production not according to their own litter size but to the size of the communal nest. Energetic investment is then shared equally among the members of a communal nest (König and Neuhäusser-Wespy, in preparation). Such equalized investment therefore might be a prerequisite for cooperation, and suggests the importance of social partner choice for a female's reproductive success.

Recent research has shown that female house mice display non-random preferences for group members, and that social partner choice yields significant fitness benefits. Females were experimentally allowed to establish non-random associations to another female when kept in groups of six in seminatural enclosures. Afterwards, females were kept with either a previously preferred female partner or with a partner of random association, and mated with an adult, genetically unrelated male over an experimental lifespan of 6 months. Females kept in pairs with a preferred partner had a significantly higher probability of giving birth and establishing an egalitarian, cooperative relationship, resulting in higher reproductive success than females in non-preferred pairs [132].

Within groups, social relationships among females thus appear to be structured by cooperation and by the existence and resolution of conflicts. A highly flexible social and especially maternal behavioural repertoire nevertheless allows female house mice to reproduce under variable environmental conditions and adjust their parental investment even to changing situations. Such flexibility can be considered as an important component to understand the species' ecological success as an invasive species that is able to live in a large variety of habitats, and its almost worldwide distribution.

The Nobel Prize in Medicine in 2011 was awarded to Bruce A. Beutler, Jules A. Hoffmann and Ralph M. Steinman. The Nobel Prize was awarded to Bruce A. Beutler, for the positional cloning of the TLR4 gene in mice, together with Jules A. Hoffmann, who described the function of the TLR receptor in fruit flies.

Ralph M. Steinman received the Nobel Prize for the discovery of dendritic cells, first reported in mice [133, 134].

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Biological Rhythms of the Mouse

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Introduction

Most terrestrial organisms live in environments that are characterized by periodically changing conditions, the most obvious being the light–dark changes of day and night. For plants that exploit the energy of light for photosynthetic reactions it is very obvious that their entire biochemistry must be adapted to the daily illumination changes. But animals also have to divide their activities within a temporal framework, simply because some activities—such as sleeping and feeding—are mutually exclusive. Here again, day and night provide a suitable temporal framework for short-term adjustments. Hence animals show 24 h rhythms not only in behaviours such as sleep and wakefulness or feeding and locomotor activity but also in physiological functions such as cardiovascular or renal activity, metabolism and the endocrine system. In fact there is probably no cellular, physiological or behavioural function of an organism which does not possess a repetitive, oscillatory nature (for reviews see [1-5]). These periodic fluctuations are by no means trivial and the ranges of many parameters can amount up to several hundred per cent. Thus, in many if not most cases, it would certainly amount to scientific malpractice not to consider and take into account 24 h rhythms in an animal experiment using mice (or any other laboratory animal). And yet it is still common practice to perform experiments during the daytime (i.e. our usual working hours), regardless of the fact that this is the resting and sleeping time of most of our laboratory animals, which are nocturnal (rats, mice, hamsters); i.e. they are active at night when we and our animal care staff are usually asleep. There are also some practical considerations and consequences, which will be dealt with at the end of this chapter.

During the past 50 years chronobiology has grown exponentially into a truly multidisciplinary scientific field that cannot be covered in this short chapter. Two comprehensive textbooks

are available to which the reader is referred to for an overview of this rapidly expanding discipline [6, 7]. In this chapter we first consider the different time scales in which rhythmic processes in biological systems occur and analyse the formal properties of the rhythms. The focus will be mainly on the 24 h rhythms and what is known today about the anatomical structures and components from where these rhythms originate and where and how their molecular 'clockworks' are generated. There are now many mouse models available that lack one or several of the known clock genes or clock-controlled genes, and these are invaluable in unravelling the physiological importance of biological rhythms. Nevertheless, they can also show us the limits of our knowledge.

The biological clock

Types of rhythms

All true biological rhythms are endogenous rhythms that persist even under constant environmental conditions and are self-sustained, i.e. they do not require any exogenous stimulation. The scope of biological rhythms ranges from milliseconds to several years (Table 2.12.1) and there has never been any doubt about the

endogenous nature and the importance of the short-term rhythms, such as neuronal discharge or heartbeat. However, there are four biological rhythms that have evolved as an adaptation to, and in response to, geophysical cycles. For a long time there were questions as to their endogenous nature or their physiological relevance. These are the tidal rhythms, diurnal (24 h) rhythms, lunar rhythms, and annual rhythms. Because the tidal rhythms and the lunar rhythms concern primarily marine animals, or animals that live in the intertidal zone, these rhythms are only mentioned here for completeness and will not be dealt with any further. The annual rhythms can also be true endogenous rhythms or at least have an endogenous component. This can be very important for seasonally migrating birds, seasonally breeding animals and/or hibernating animals. But since the mouse belongs to neither of these categories, annual rhythms are of only marginal importance for mice. The daily rhythms, however, have a strong impact on all behavioural and physiological parameters, and therefore are of greatest relevance for the laboratory animal scientist. The daily light/dark (LD) cycle is the most obvious and most important external cue (chronobiologists use the term 'zeitgeber') to which the daily rhythm can synchronize or entrain. Laboratory animals are usually housed under an artificial daily light and dark period. We define 'zeitgeber time' (ZT) relative to this experimental LD cycle, and in an LD cycle of 12 h of light and 12 h of darkness (LD 12:12) the time of lights on is denoted by ZT0, and the time of lights off by ZT12. In the absence of a zeitgeber, these rhythms reveal their own endogenous period length (denoted by the Greek letter τ). Hence, under conditions of constant darkness (DD) or constant light (LL) the rhythm will continue, albeit with a τ that is significantly different from 24 h. Such a rhythm is called a 'free-running circadian rhythm' (from Latin '*circa*' = about and '*dies*' = day). In addition, some ultradian rhythms (i.e. shorter than circadian; e.g. GnRH pulses of 2–3 h) and infradian rhythms (longer than circadian; e.g. oestrous cycle) can be recognized and are of special interest. There are several claims that weekly or 'circaseptan' rhythms exist, but the experimental evidence is far from compelling and

TABLE 2.12.1: The main biological rhythms	
Biological rhythm	**Period**
Neural rhythms	0.001–1 s
Cardiac rhythm	0.05–3 s[a]
Respiratory rhythm	0.1–10 s[a]
Biochemical oscillations	30 s–20 min
Hormonal rhythms	10 min–3–5 h
Tidal rhythms	12.4 h
Circadian rhythms	~24 h
Ovarian cycle	1 day–1 year[a]
Lunar rhythm	29.5 days
Annual rhythm	1 year
Population rhythms	1–20 years

[a]Species dependent.
Source: modified from Goldbeter, A. (2008). Biological rhythms: clocks for all times. Curr. Biol. **18**, 751–753.

there is therefore no generally accepted scientific support for these claims.

Formal properties of endogenous clocks

A nocturnal animal species such as the house mouse is primarily active during the night-time and most of its exploratory activity, feeding and drinking is done during the hours of darkness. This behaviour has most likely evolved as an avoidance response to predation pressure by birds of prey which are primarily active during daytime. Running wheels can easily monitor locomotor activity of mice; passive infrared detectors, photodiodes or implantable transponders, and thus long-term recordings that do not disturb the animals, are possible. If such a recording is done under a regular LD cycle (most often in mice LD 12:12) the rhythm is entrained and shows a stable phase relationship (Ψ) with the zeitgeber

(Figure 2.12.1). Such activity recordings are usually depicted by plotting the data of consecutive days underneath each other. More complex and longer recordings are often presented as double plots where two consecutive days (48 h) are plotted side-by-side in one line and the second day is repeated in the next line underneath the first day, followed by the data of the third day next to it, and so on. This allows for good visual comparison of individual activity patterns of mice and also reveals characteristic differences between strains (see Figure 2.12.2 and also Figure 2.12.5). Under free-running conditions the activity period deviates from exactly 24 h and consequently drifts to the left if τ is less than 24 h, and to the right if it is greater than 24 h. Each cycle of the free-running circadian rhythm can be divided into 24 h of circadian time (CT), with CT0 being defined as onset of the subjective day and CT12 as onset of the subjective night.

Since the free-running τ is close to, but not exactly, 24 h, the internal clock will dissociate from the natural 24 h day unless a specific
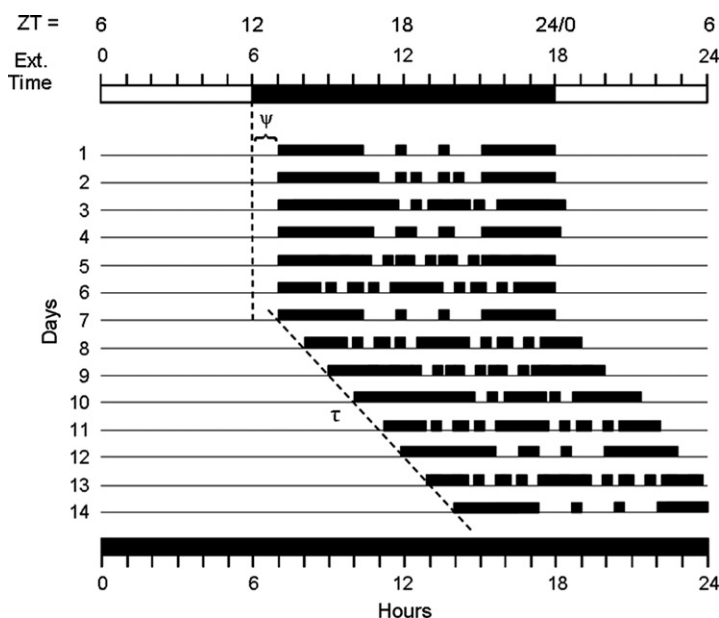
Figure 2.12.1 Schematic presentation of an activity rhythm in a so-called actogram. Each successive 24-h segment (here 14 days) is plotted below the preceding one. Horizontal black bars on each line (days) indicate bouts of activity (e.g. continuous wheel-running). For the first 7 days the mouse was housed under a L:D cycle of 12:12 as indicated by the white/black/white bar on top. The mouse was kept under an inverted light schedule, i.e. the dark time began at 6:00 h external time and ended at 18:00 h external time. This corresponds to Zeitgeber time (ZT) 12 for onset of darkness and ZT 0 for onset of light. Ψ indicates the phase angle of entrainment. From day 7 onwards the mouse was kept in constant darkness (DD) as indicated by the black bar at the bottom. The mouse subsequently shows a free-running rhythm with a period length $\tau = 25$ h, i.e. the onset of activity drifted to the right by 1 h each day ($= 7$ h in 7 days).
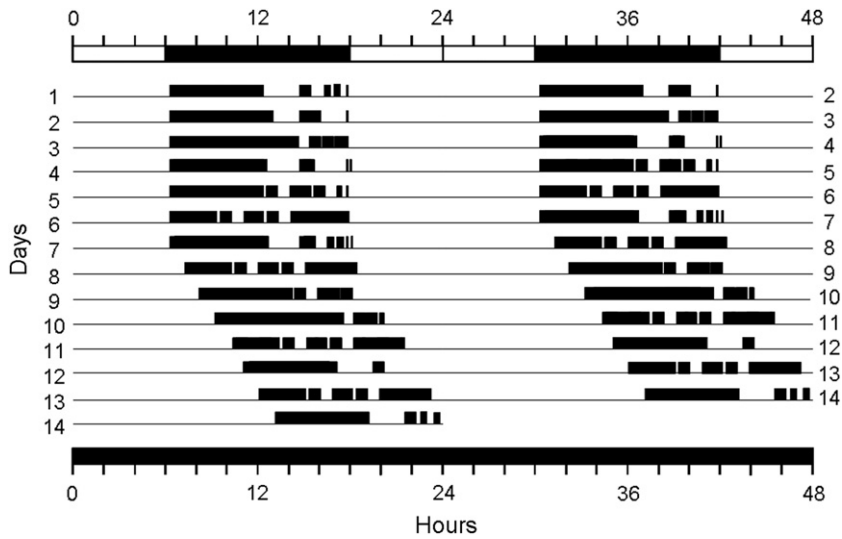
Figure 2.12.2 Similar data as in Figure 2.12.1 but double-plotted. Data for two consecutive days (48 h) are plotted in one line. Thus day 2 is plotted following day 1 (24–48 h) and re-plotted underneath day 1, followed by day 3, which is re-plotted underneath day 2, and so on. Again the white and black horizontal bars on top indicate the light and dark phases of the LD cycle. Starting from day 7 onwards the mouse was kept in constant darkness (DD). Double plots (or even triple plots) are used to facilitate inspection of activity records that drift over many days.
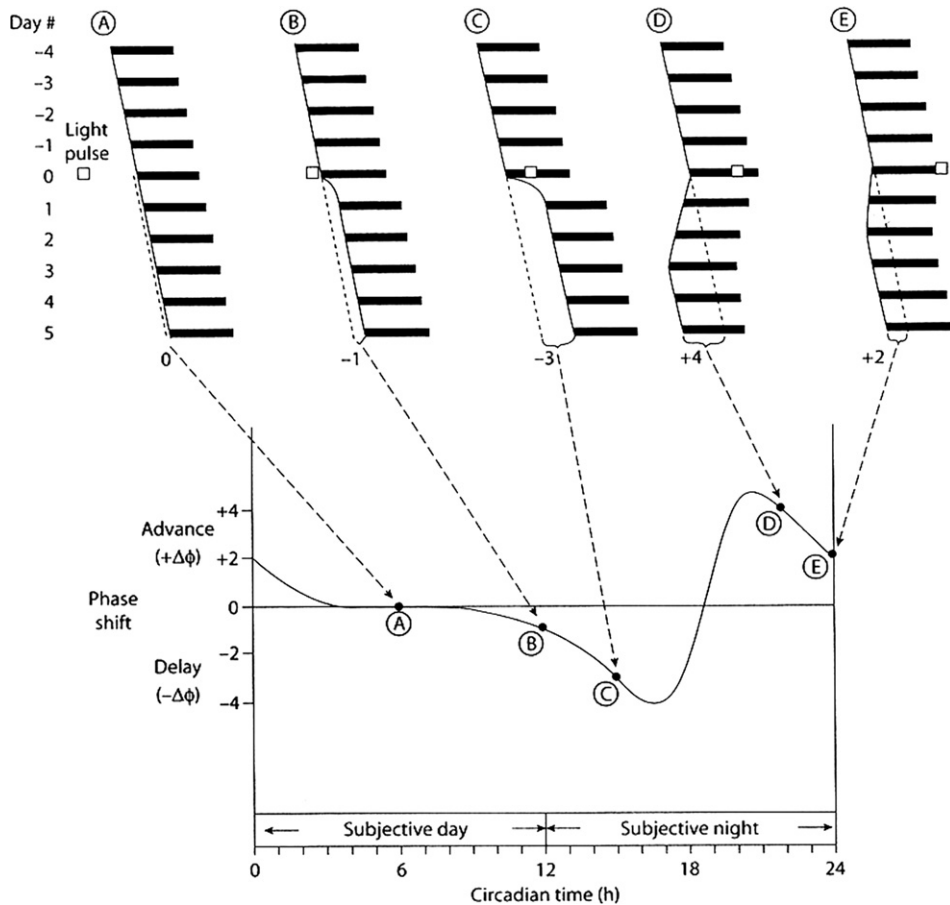
Figure 2.12.3 Schematic representation of the principle of constructing a phase response curve (PRC). Light pulses of identical duration and intensity are presented at different circadian times and the ensuing phase shifts are plotted as a function of circadian time. *From reference 13.*
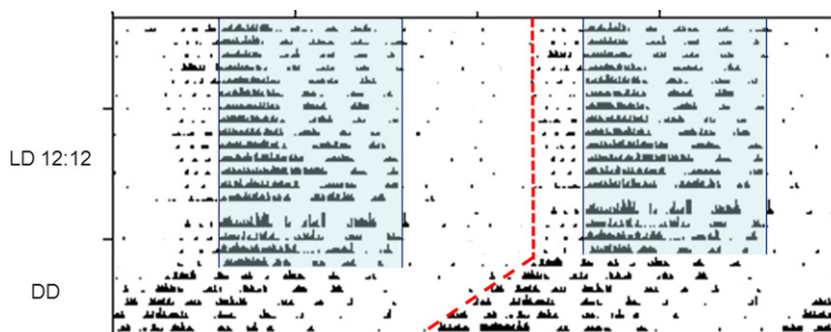
Figure 2.12.4 Masking by light–dark cycles in a *Per2*-KO mouse. During the LD 12:12 lighting the mouse showed its main activity during the darkness (blue shading). The 'true' onset of activity, however, is masked by the light phase as became evident when the mouse was released into constant darkness (DD). The mouse free-ran from a point about 3 h ahead of the preceding 'apparent' activity onset. Thus the 'true' onset of activity corresponds to the broken line (only shown on the left side to allow unbiased inspection of the plotted data).
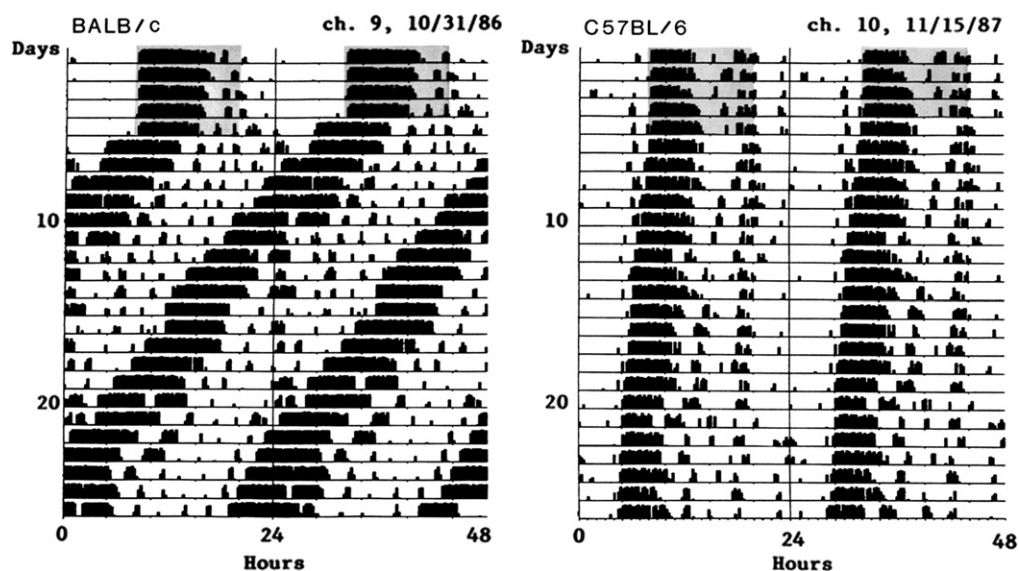
Figure 2.12.5 Double-plotted actograms of wheel-running activity of two inbred mouse strains to show the variability of the free-running period (τ). The final 5 days of entrainment to an LD 12:12 cycle are shown (scotophase shaded) before the mice were placed in constant darkness. In these cases τ was 22.88 h in the BALB/c mouse and 23.88 h in the C57BL/6 mouse. For group means see Table 2.12.2. *From reference 20, with permission.*

mechanism keeps it synchronized on a day-to-day basis. The adjustment of the free-running τ to an external zeitgeber cycle (*T*) is called *entrainment* and it leads to a specific phase relationship between τ and *T* with a stable phase angle (Ψ). In the case of the daily LD cycle, $T = 24$ h. In order to entrain its free-running circadian rhythm to either the artificial LD cycle in the laboratory or the natural day/night changes, the animal must reset its biological clock each day by either phase advancing or phase delaying the rhythm [5, 8-10]. The magnitude and the direction of the entraining shift are dependent on the time when the zeitgeber signal occurs. A light pulse during the early subjective night will cause a delay shift, while a light pulse towards the end of the subjective night will cause a phase advance. During the subjective day a light pulse will have relatively little or no effect. When the light pulses are given in a systematic manner throughout the circadian cycle and the ensuing phase shifts are plotted as a function of the phase of the circadian system at the time of the pulse, a *phase response curve* (PRC) is obtained

(Figure 2.12.3). The shape of the PRC is qualitatively similar in both nocturnal and diurnal animal species, as well as in humans, but the amplitude of the delay and advance shifts can vary [11-14]. A PRC is useful for estimating the sensitivity of an animal's circadian system to the phase-shifting effects of light. It also allows estimating the phase of the circadian cycle at which a light pulse must be given to keep the endogenous rhythm entrained with the LD cycle. If, for example, the mouse has a τ of 25.0 h, then its clock must be reset each day by a phase delay of −1 h in order to remain entrained with the natural day. Assuming that Figure 2.12.3 shows the PRC of this mouse, then in order to achieve such a phase delay the light pulse must be presented at CT12, i.e. at the onset of the subjective night.

Besides these phase-shifting effects, light during the (subjective) night also has acute effects on the locomotor activity of nocturnal rodents such as the mouse: the activity is acutely suppressed as long as the lights are on. This phenomenon is called 'masking' or, more precisely, 'negative masking' because the activity is decreased after an increase in illumination [15]. Figure 2.12.4 shows an example of masking in a *Per2* knockout mouse.

It is primarily the genetic background of the mouse that determines the circadian phenotype. Many inbred strains of mice show characteristic circadian features and a number of alterations in several parameters of circadian behaviour. This can affect the length of the circadian period τ as well as the pattern, the precision, and the robustness of the free-running rhythm [16-22]; for a review see [23]. Typical actograms of the inbred strains BALB/cByJ and C57BL/6J are shown in Figure 2.12.5. In these strains complete PRCs to light pulses were obtained and revealed distinct differences in their phase-shifting response to light pulses [20]. Examples of the variability of the endogenous τ in several inbred strains of mice are given in Table 2.12.2. In addition other factors such as old age, certain diseases or the photic history ('after effects') can influence the pattern of the rhythm and τ [24-27]. It is worth mentioning that while most rhythms deteriorate with age, e.g. the precision and the robustness of the activity rhythm decreases and the rhythm becomes fractionated, the rhythm of body

TABLE 2.12.2: Average length of the free-running period (τ) of wheel-running activity in 12 different inbred strains of mice living in the absence of any external Zeitgeber (continuous dark (DD) conditions)

Strain	n	Mean τ_DD SEM
129/J[a]	6	23.93 ± 0.07
RF/J[a]	6	23.92 ± 0.06
C57BL/6J	10	23.77 ± 0.02
SWR/J[a]	3	23.70 ± 0.02
SEC/1ReJ	8	23.59 ± 0.04
AKR/J[a]	5	23.52 ± 0.04
DBA/2J	3	23.46 ± 0.05
C57BL/10J	5	23.43 ± 0.01
C57L/J	4	23.42 ± 0.13
A/J[a]	10	23.37 ± 0.07
B10.D2(58N)/Sn	4	23.34 ± 0.15
BALB/cByJ[a]	8	22.94 ± 0.06

[a]Albino strains.
Source: *Data from reference 20.*

temperature in mice is maintained almost unaltered until death [28]. Between individuals—even of an inbred mouse strain—period length τ may vary conspicuously, but within an individual animal the rhythm can be very precise, i.e. the day-to-day variability can amount to only a few minutes per day [24, 29]; for a current view see [30].

Besides the dominant zeitgeber, the LD cycle, other periodically recurring signals can act as zeitgebers and are thus able to entrain circadian rhythms. Among these other zeitgebers are social cues, as in mother–pup interactions [31, 32], or daily temperature cycles, as demonstrated by Aschoff and Tokura [33] in the squirrel monkey. In addition, a periodic schedule of restricted feeding can be used as a zeitgeber [34-36]. Although restricted feeding as a zeitgeber appears to be weaker than light, it can nevertheless set the phase of many gastrointestinal, endocrine and metabolic functions (see below). During restricted feeding a 'food-anticipatory activity' is typically expressed, i.e. the mice show a bout of activity just prior to meal time (Figure 2.12.6). Together with the locomotor activity they also alter the rhythm of body temperature and corticosteroid secretion in correlation with the food availability rhythm [35, 37].
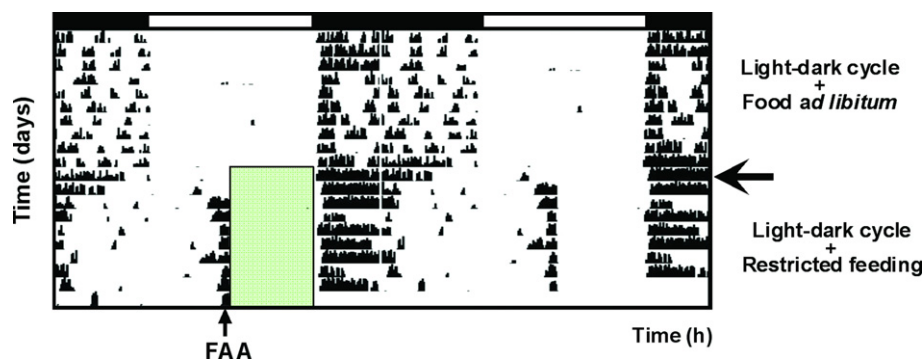
Figure 2.12.6 Wheel-running activity of a B6CBA mouse challenged with temporal restricted feeding under a light-dark cycle (symbolized by black and white bars on top of the double-plotted actogram). A bout of food-anticipatory activity (FAA) was expressed prior to the time of food restriction (green area). From reference 34, with permission.

Anatomical structures and components of the biological clock

The biological clock, the master generator of 24 h rhythms in mammals, is located in the suprachiasmatic nuclei (SCN) of the hypothalamus. These bilateral clusters of roughly 10 000 neurons each lie in the ventral hypothalamus, just dorsal to the optic chiasm and on both sides of the third ventricle (Figure 2.12.7) [38–41]. A lesion of the SCN will destroy the oscillator, and as a consequence, the animal will become arrhythmic [42]. However, a rhythm of locomotor activity can be restored by transplantation of foetal SCN tissue into the third ventricle [43, 44]. Similarly, in genetically arrhythmic mice circadian rhythmicity can be induced by SCN grafts from wild-type mice. Because light is the most important zeitgeber for circadian rhythms, the retina is essential in mammals for perception of the daily LD cycle [45]. A mammal that is blind due to enucleation or cutting of the optic nerves will still express its endogenous circadian rhythms but cannot entrain to LD cycles any more [42]. For a long time it was thought that the rods and cones of the retina were not only responsible for vision, but were also the light sensors for the circadian system. This was later refuted by showing that mice without rods or cones can still entrain to LD cycles with sensitivity indistinguishable from wild-type mice [46–48]. This led to the discovery of a novel photopigment, melanopsin, which is located in a subpopulation of specialized retinal ganglion cells [49–52]. This entirely independent light-detection system in the mammalian eye does not serve the image-forming visual system, but is designed to detect the level of illumination, the *irradiance*. Consequently it also sends signals to the circuit that controls light-activated pupil contraction [53, 54]. The unmyelinated axons from these melanopsin-positive cells form the monosynaptic retinohypothalamic tract (RHT) that connects the retina with the 'master clock', the SCN [55]. The SCN are composed of two anatomically and functionally distinct subdivisions, the 'core' and the 'shell' [56, 57]. The core is

Figure 2.12.7 Immunoreactive gastrin-releasing peptide (GRP) in the core region of the hypothalamic suprachiasmatic nuclei, the mammalian 'master clock'. 3 V, third ventricle; OC, optic chiasm.

the ventrolateral subdivision, whose cells are characterized by vasoactive intestinal polypeptide (VIP) and gastrin-releasing peptide (GRP) immunoreactivity. The shell is the dorsomedial subdivision delineated by arginine vasopressin (AVP)-containing neurons and receiving inputs from limbic, hypothalamic and brainstem nuclei [55, 56, 58, but see 59]. Only the neurons of the SCN core receive the direct light information via the RHT [40, 55, 56]. The excitatory amino acid glutamate is the primary neurotransmitter at the synapses of the RHT [60, 61]. It is released together with the neuromodulators substance P (SP) and pituitary adenylyl cyclase activating peptide (PACAP) whereby NMDA receptors are activated, causing an influx of Ca^{2+}, activation of MAP-kinases, and consequently the phosphorylation of cAMP-response-element-binding protein (CREB) [62, 63]. Activated CREB binds to the $Ca^{2+}/cAMP$ response element (CRE) in the promoter region of genes (e.g. *cfos*, *Per1* and *Per2*, see below) which subsequently leads to their transcription. The acutely light-activated neurons of the SCN core communicate with the SCN shell neurons and the periphery through the release of gamma-aminobutyric acid (GABA) and several neuropeptides such as NPY, GRP and SP [40, 59, 60]. Each of the neurons of the SCN shell contains the complete molecular machinery for producing

its own endogenous rhythm (see below). How the 20 000 separate cellular clocks are synchronized and can produce a homogenous output is still a mystery [40, 64-66].

There is a multitude of neuronal connections from the SCN to different brain areas that play a functional role in the generation of overt rhythms [4, 55, 67]. However, implantation of encapsulated SCN tissue that cannot make neuronal connections but can only release diffusible substances was able to restore rhythmic activity [68]. This shows that both neuronal [55, 57, 69, 70] and humoral signals [68, 71-73] function as output signals from the SCN to other regions of the brain and the periphery. It also shows that, for rhythmic expression of locomotor activity, a humoral signal from the SCN is sufficient.

So far the only anatomically and functionally well-characterized output pathway from the SCN is the connection to the pineal gland, controlling the production of melatonin (Figure 2.12.8). Axons from the SCN shell neurons connect to the paraventricular nucleus of the hypothalamus where sympathetic fibres originate and travel down to the spinal cord in the intermediolateral cell column. At level T1–T4 of the spinal cord preganglionic sympathetic fibres connect to the superior cervical ganglion. The superior cervical ganglia finally
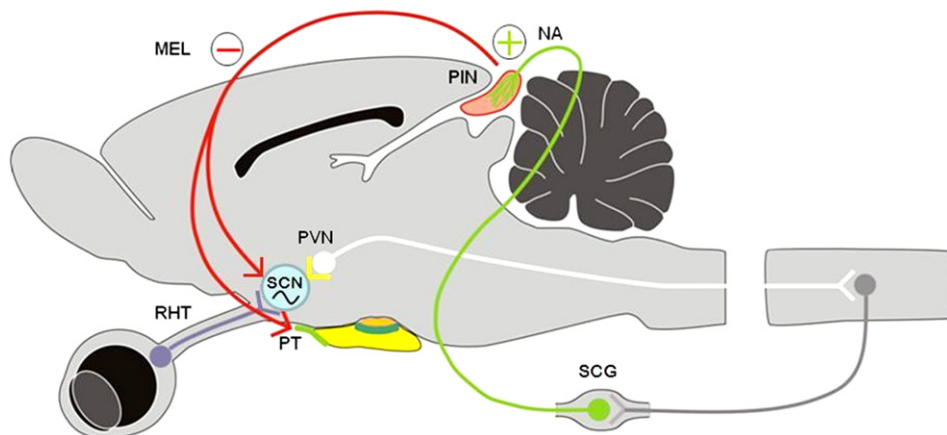
Figure 2.12.8 Schematic presentation of the photoneuroendocrine system. Light falling on the retina (RET) is perceived by specialized retinal ganglion cells and the information is transferred by the retino-hypothalamic tract (RHT) to the suprachiasmatic nuclei (SCN), the master clock. SCN output activates sympathetic nerves originating in the paraventricular nucleus (PVN). These send axons through the intermediolateral cell column to the thoracic spinal cord from where preganglionic sympathetic nerve fibres travel to the superior cervical ganglia (SCG). From the SCG postganglionic nerve fibres re-enter the brain and innervate the pineal gland as nervi conarii. Melatonin produced during the night feeds back on the SCN and also binds to receptors in the pars tuberalis of the pituitary gland. Modified from reference 168, with permission.

send postganglionic sympathetic fibres back into the brain as the nervi conarii which innervate the mammalian pineal gland. Noradrenalin is released only during darkness of the night at the synapses of these nerve endings, binds to β-adrenergic receptors, and activates the synthesis of melatonin. Melatonin is considered the 'chemical expression of darkness' [74] because light acutely inhibits melatonin synthesis and the duration of the nightly melatonin peak correlates with the duration of the dark phase [75, 76]. Melatonin released from the pineal gland at night is thought to be distributed throughout the body and binds to those brain areas and peripheral organs that are equipped with melatonin receptors, thereby informing the body of the time of day, or rather, time of night [74]. Melatonin is also thought to feed back to the neurons of the SCN where melatonin receptors are located [77-80]. This feedback probably can fine-tune the circadian output signal from the SCN [81]. However, most of the commonly used inbred mouse strains are unable to synthesize melatonin in the pineal gland due to malfunctions (mutations) in either arylalkylamine-N-acetyltransferase (AANAT) or hydroxyindole-O-methyltransferase (HIOMT), the two enzymes involved in melatonin synthesis from serotonin [82-84]. Only five inbred strains are known to produce a high-amplitude melatonin rhythm (Table 2.12.4). It should be pointed out that in C57BL/6 a small but significant short peak in pineal melatonin has been reported, despite the mutation in the AANAT [84-86]. Unlike other rodents and humans, mice do not excrete metabolized melatonin in the urine as 6-sulphatoxymelatonin. Instead, it is excreted as 6-glucoroylmelatonin [84].

The cellular clockwork

As mentioned above, even dissociated SCN neurons can display persistent circadian rhythmicity, showing that the clockwork is not the result of an orchestrated neuronal network but is generated at the level of the cell [64]. The cellular clockwork consists of a set of core clock genes forming transcriptional–translational feedback loops. The core clock genes are defined as genes whose protein products are necessary components for the generation and regulation of circadian rhythms within individual cells [2]. Several clock genes have been found and were characterized through naturally occurring, chemically induced or targeted (knockout) mutations. Because it turned out that in metazoans, several clock genes are evolutionarily conserved, comparative genomic approaches were successfully applied as well. Table 2.12.3 shows the presently known canonical clock genes and Figure 2.12.9 illustrates a model of the molecular clockwork within a cell. The first mammalian clock gene was found by screening the progeny of mice treated with the mutagen N-ethyl-N-nitrosourea (ENU) for altered circadian behaviour [87]. It turned out to be a semi-dominant mutation of a single gene and heterozygotes for this mutation had a significantly longer τ as compared to the B6 wild-type mice (24.8 h versus 23.6 h, respectively), whereas homozygotes had a very long τ of 27.3 h for the first days in DD, but thereafter the rhythm gradually vanished. Hence, the gene was called *Clock* (for circadian locomotor output cycles kaput). The proteins for which *Clock* and another core clock gene, *Bmal1* (also called *Mop3*) encode are members of the basic helix-loop-helix (bHLH)-PAS (*Period-Arnt-Singleminded*) transcription factor family [88-90]. Surprisingly, *Clock* mRNA and CLOCK protein are constitutively expressed, whereas *Bmal1* mRNA is rhythmic and peaks around CT15-18, i.e. in the middle of the subjective night [91, 92]. When BMAL1 is present in sufficient quantity in the cytoplasm it forms heterodimers with CLOCK [93]. This complex relocates into the nucleus, binds to the E-box in the promoter region of the period (*Per1, Per2*) and the cryptochrome (*Cry1, Cry2*) genes and drives their transcription [1]. This eventually leads to the accumulation of PER and CRY proteins in the cytoplasm during the day and the formation of PER:CRY heterodimers. Following phosphorylation by casein kinases this complex translocates back into the nucleus and interferes with the CLOCK:BMAL1 heterodimer at the E-box, thereby inhibiting the transcription of their own genes, namely the *period* and *cryptochrome* genes. Since the genes for retinoic acid-related orphan nuclear receptors *Rev-erbα* and *Rorα* also

TABLE 2.12.3: Mammalian circadian clock genes

Gene	Chromosome #	Classification	Function	Mutation phenotype[a]	Peak expression[b] (RNA/protein)
Clock	5	bHLH-PAS	Transcription factor	4.0 h longer period, arrythmicity in DD	Constitutive
Bmal1/Mop3	7	bHLH-PAS	Transcription factor	Arrhythmicity in DD	15−18/0−8
Per1	11	PAS domain	PER/CRY interaction CLOCK:BMAL1 inhibitor	0−1.1 h shorter period	4−6/10−14
Per2	1	PAS domain	PER/CRY interaction CLOCK:BMAL1 inhibitor	1.5 h shorter period; arrhythmicity in DD rhythmic in LL, 2 h longer period	6−12/10−14
Per3	4	PAS domain	PER/CRY interaction	0−0.5 h shorter period	4−9/10
Cry1	10	Flavoprotein	PER/CRY interaction CLOCK:BMAL1 inhibitor	1 h shorter period	8−12/12−18
Cry2	2	Flavoprotein	PER/CRY interaction CLOCK:BMAL1 inhibitor	1 h longer period	8−16/12−16
Rev-erbα	11	Orphan nuclear receptor	Inhibitor of Bmal1; links neg. + pos. feedback loops	0.5 h shorter period	2−6/ND
Rorα	?	Orphan nuclear receptor	Activator of Bmal1; links neg. + pos. feedback loops	Arrhythmic/various	6−10/?
CK1ε (Csnk1 ε)	15	Casein kinase	Phosphorylation of PERs, CRYs, and BMAL1	4 h shorter period (tau mutant hamster)	Constitutive
Npas2	1	bHLH-PAS	Transcription factor; Bmal1 paralogue	0.2 h shorter period	Not expressed in SCN

[a]Only the circadian phenotype is listed; there may be other phenotypes in other systems (see Table 2.12.4).
[b]Average circadian time (CT) at peak transcript level.
Source: *modified from references 2 and 161.*

TABLE 2.12.4: Melatonin-proficient and melatonin-deficient inbred mouse strains		
Origin	**Melatonin-proficient**	**Melatonin-deficient**
A Europe/USA	CBA/Ms, C3H/He	AKR/J, BALB/c, C57BL/6, C57BL/10, HTG, DBA/z, HTH, HT, RFM, 129/Sv, DDN, CF#1
B Japan		KR, KR/c, NC, OZB, OZC, OZD, OZH, OZK, CRN
C Crosses of A and B		BS, CS, DCR/c, IMV, Pony, WN, IIITes, IXBL, NZB/BLNJ, IS/CamEi, CAST/Ei
D Japan, wild	Mol-A, Mol-Nis, MOM	
E Crosses of A, B and D		AWB
F Europe /USA , wild	SK/CamEi, SF/CamEi, PERU-Atteck/CamEi	SK/Nga

Source: *Compiled from references [82] and [83].*
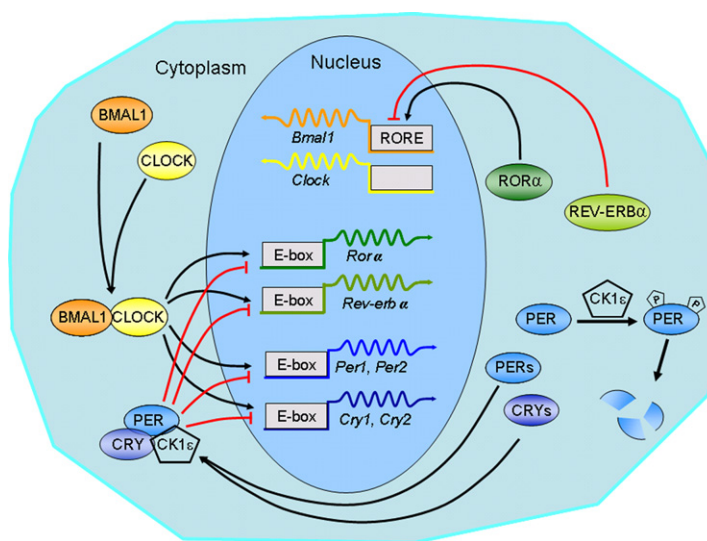
Figure 2.12.9 Simplified model of the molecular clockwork forming transcriptional–translational feedback loops. See text for further explanation.

have E-boxes, they are also targets of the CLOCK: BMAL1 heterodimer. REV-ERBα and RORα subsequently compete to bind to retinoic acid-related orphan receptor response elements (ROREs) located in the Bmal1 promoter. Thereby REV-ERBα protein provides an inhibitory break on *Bmal1* transcription, whereas RORα activates transcription of *Bmal1*. The cycle of the entire network of transcriptional-translational feedback loops takes about 24 h to complete. Casein kinase(s), especially casein kinase 1α (CK1ε) and casein kinase 1δ (CK1δ), play a critical role in determining the period length of the cycle. On the one hand, phosphorylation of the PER proteins leads to faster degradation [94]. On the other hand, however, CK1ε supports the translocation of the PER:CRY heterodimer into the nucleus. Hence, a mutation in the *ck1ε / ck1δ* genes leads to an altered circadian period as shown for the *tau* mutation in Syrian hamsters [95] or for the familial advanced sleep-phase syndrome (FASPS) in humans [96, 97]. The roles of other candidate clock genes, e.g. *Timeless, Dec1, Dec2* and *E4bp4* have not yet been clearly defined (for a review, see [2]) and it remains open how many other clock genes or clock-related genes will be found in the future [98]. In comprehensive screens of circadian-related quantitative trait loci (QTL) up to 14 loci were identified that affected circadian traits. Only one known core clock gene, *ck1ε*, mapped to one of these 14 QTLs within 10 cM. This genetic analysis has revealed previously undetected

complexity in the circadian system and points to the presence of many as yet undiscovered genes that contribute to the expression of circadian behaviour in mice. Although severe disruption of circadian rhythms may be caused by mutations in core clock genes, it is possible that the broad variety of circadian behaviour observed in mammalian species is the result of polymorphisms in multiple, interacting loci [99]. It must be emphasized that the molecular clockwork described here is a simplified version of the current model that is agreed upon by most chronobiologists. However, this model is by no means finalized and is still evolving [100]. There are probably still components of the clockwork to discover. There are probably redundancies (e.g. for CLOCK, see below), and quite likely post-transcriptional modifications will be important for fine-tuning the clock.

Peripheral clocks: slaves to the master clock?

The first indication for the existence of an autonomous oscillator outside the SCN came from work showing that rhythmic food-anticipatory activity (FAA, see Figure 2.12.6) can be observed in response to scheduled restricted feeding, even when the rats are arrhythmic due to an SCN lesion [101]. Although during the past 20 years several candidate areas in the limbic system [102], the hypothalamus [103, 104], and even in the stomach [105] have been proposed, the anatomical substrate for this food-entrainable oscillator is still unknown. Recent work has even shown that FAA is completely independent of the circadian clock because mutant mice lacking known circadian clock functions exhibit normal FAA [106]. The first anatomically localized autonomic oscillator outside the SCN was found in the retina [107]. Retina in tissue culture was shown to synthesize and release melatonin in a rhythmic fashion, entrained by light and oscillating in DD for several cycles. The function of this retinal melatonin is still unknown, but it has been speculated that it acts primarily within the retina itself, by changing the sensitivity of the retina and by timing the circadian rhythm of disc-shedding [108].

Following the identification and cloning of clock genes, many (25 +) other brain areas were found that contained the whole molecular clockwork and showed circadian rhythms *in vitro* and *in vivo* [109, 110]. The particular functions of all these clocks are presently unknown; some of these oscillators dampen out within several days when disconnected from SCN input; others, however, such as the mitral cells in the olfactory bulb, continue to cycle for some time. (In the latter, the SCN apparently entrains, but does not sustain the endogenous rhythmicity (see [111] for a review and for a comprehensive list of brain sites, see [112]).

A modification of the clock mechanism was detected in forebrain nuclei, where a paralogue of CLOCK, neuronal PAS domain protein 2 (NPAS2) functionally replaces CLOCK [113, 114]. It was recently shown that CLOCK-deficient mice continue to exhibit a robust behavioural and molecular rhythm, which is very surprising considering the central role of CLOCK in the model and given the massive phenotype of CLOCK mutant mice [115]. One attempt to rescue the model is the conjecture that NPAS2 could replace CLOCK in the SCN of CLOCK-deficient mice. Alternatively, there could be another partner for BMAL1 with a bHLH-PAS domain that can form transcriptionally active heterodimers and thereby substitute for the missing CLOCK [116].

Shortly after the discovery of the first clock genes in mammals, it became evident that circadian clocks were 'ticking,' not only in the neural tissue of SCN and the retina, but also in most, if not all, peripheral tissues [117]. This was most elegantly shown by creating an $mPer2^{Luciferase}$ knockin mouse in which the *Luc* gene is fused in-frame to the 3′ end of the endogenous *mPer2* gene. The resulting PER2::LUCIFERASE fusion protein is then used as a real-time reporter of circadian dynamics within cells and organs [118]. The number of reports concerning the clock function in peripheral tissues and organs has been exponentially increasing over the past years and it goes far beyond the scope of this short chapter to review even the most prominent ones. A number of excellent reviews are

available, to which the interested reader is referred [4, 119-121]. It will suffice to give one example of the oscillations of 14 canonical clock genes in heart, lung, liver, stomach, spleen, kidney and testis [122] and to summarize the present understanding. From Figure 2.12.10, it is evident that there are tissue-specific expression patterns for the different clock genes. For example, while *Clock* mRNA is constitutively expressed in the SCN, it cycles in most peripheral tissues, with the exception of the testis. The circadian peak times and the amplitude of
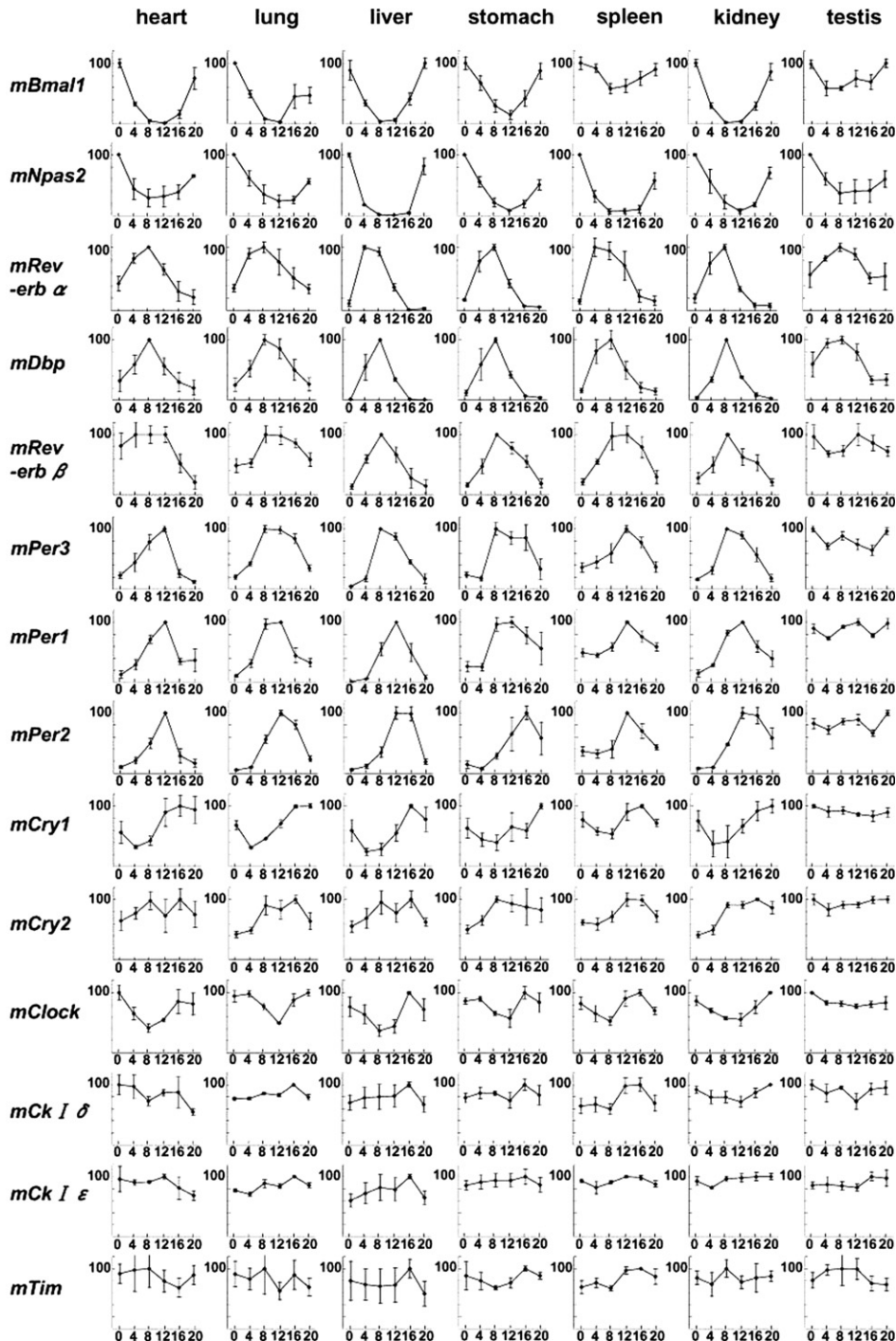
Figure 2.12.10 Circadian profiles of canonical clock genes and clock-controlled genes in peripheral organs of the mouse. *From reference 122.*

the rhythms vary across tissues; e.g. *Per2* peaks at CT12 in the heart but at CT16 in the liver. Remarkably, the testis appears to be the only tissue studied to date that does not exhibit rhythmic expression of the clock genes, but rather constitutively high expression [123]. It is generally agreed that the SCN is the master clock and that the SCN pacemaker—although being self-sustained—entrains the peripheral clocks. The SCN master clock coordinates the peripheral clocks either directly via neuronal or endocrine outputs, or indirectly by timing the sleep-wake cycle, locomotor activity, feeding, etc. [4, 124]. Daily resetting cues from the SCN master clock are necessary for long-term phase coherence of the peripheral 'slave' clocks. In addition, circulating glucocorticoids and the circadian cycle in body temperature can possibly serve as entraining signals for the peripheral clocks [117, 125]. Circadian gene profiling experiments have shown that a significant fraction of the transcriptome (3–10%) is controlled by the circadian clock [126–128]. In the liver, close to 1000 circadian transcripts were found. The majority of the pathways regulated by the clock in the liver belong to basic metabolic pathways, such as glycolysis, fatty acid metabolism, cholesterol biosynthesis, and xenobiotic and intermediate metabolism [129]. Rhythmically expressed liver genes encode key

enzymes involved in energy metabolism, the redox state of the cell, food processing and detoxification of xenobiotics [130]. Dibner and colleagues [4] hypothesize that the three main purposes of peripheral clocks are: (i) anticipation of metabolic pathways to optimize food processing; (ii) limitation of metabolic processes with adverse side effects to times when they are needed, and (iii) sequestration of chemically incompatible reactions to different time windows.

Phenotypic effects of mutations in circadian clock genes

Mice with either natural mutations or targeted mutations in clock genes have been very useful in unravelling the molecular cellular clockwork and have also helped to further elucidate how the biological clock affects organ functions. The circadian phenotype of mutations in canonical clock genes is listed in Table 2.12.3 and the phenotypes caused by these mutations downstream of the cellular clock in physiological functions are listed in Table 2.12.5. Some of these

TABLE 2.12.5: Circadian gene defects and their physiological phenotypes	
Disrupted gene	**Phenotype/physiological effects**
Bmal1	infertility, progressive arthropathy, abnormal gluconeogenesis, abnormal lipogenesis, altered sleep pattern
Clock$^{\Delta 19/\Delta 19}$	metabolic syndrome, abnormal gluconeogenesis, abnormal behavioural sensitization to psychostimulant, altered sleep pattern
Clock$^{-/-}$	no phenotype when deleted in SCN
Per1	abnormal apoptosis/cancer development, abnormal behavioural sensitization to psychostimulant, accelerated ageing, altered corticosteroid secretion
Per2	abnormal behavioural sensitization to psychostimulant, accelerated ageing, altered corticosteroid secretion
Per3	associated with delayed sleep-phase syndrome (human)
Cry1, Cry2	altered sleep pattern
Rorα	cerebellar ataxia, abnormal bone metabolism
Npas2	altered sleep pattern, impaired memory
CK1ϵ/ CK1δ	familial advanced sleep-phase syndrome
Source: Compiled from references.	

are further explained below in connection with the organs and the physiological systems involved.

Reproductive system

The reproductive system is profoundly influenced by circadian rhythms (for a review see [131]). It is therefore surprising that the testes of mice appear to be rather unaffected by the circadian clock and, although clock genes are present in testes, most of these are constitutively expressed and are thought to be primarily important during development [132, 133]. Male knockout mice targeted at clock genes are generally fertile with the exception of the *Bmal1* gene. *Bmal1* knockout mice—both males and females— turned out to be infertile [134]. In the female mice, circadian rhythms play a critical role at different levels and in different functions of the reproductive system. During a regular LD cycle, a surge of luteinizing hormone (LH) occurs on the day of proestrus at the end of the light phase and triggers ovulation about 6-9 h later in the middle of the dark period (oestrus). This and subsequent events (copulation, fertilization, embryo implantation and development) have to be timed in a precise manner in order to allow optimal reproductive outcome. SCN lesions abolish the LH surge and block ovulation, showing that the timing of ovulatory processes is clearly determined by the SCN [135]. In conjunction with the oestrus cycle, locomotor activity of the female mice changes systematically. Thus, on the day of proestrus, the onset of activity occurs earlier than on other days of the oestrus cycle. *Clock* mutant mice show subtle but significant effects in reproductive performance. Oestrus cycles are reported to be prolonged and irregular, litter size is slightly reduced and maintenance of pregnancies is compromised [136, 137]. The onset of puberty, however, appears to be normal. The latter is in contrast to findings in *Per* mutant mice, where onset of puberty is significantly advanced. Female *Per* mutant mice show a normal fertility while they are young, but already at 9 months of age, fertility and fecundity are drastically reduced. This points to an accelerated ageing process in these *Per* mutant mice, rather than a reproductive failure per se [138]. Female *Bmal1* knockout mice are infertile, as mentioned above. Although *Bmal1* knockout females ovulate, despite irregular oestrus cycles, mate and have fertilized ova, they show delayed implantation and early embryo loss, and as a consequence no full-term pregnancies occur [123]. The present view is that a multi-oscillatory action of hypothalamic, hypophyseal and ovarian clocks needs to be coordinated by the SCN in order to ensure successful reproduction [139].

Gastrointestinal tract and metabolism

Energy metabolism is of course intimately linked with the daily feeding-fasting cycle, which is, in turn, connected to the rest-activity cycle. Hence, there is growing evidence for interplay between energy metabolism and the circadian clock. Intestinal glucose transporters are expressed even before the animal starts to eat. This improves the efficiency of absorption by predicting recurring events rather than merely reacting to them [140]. Daily feeding-fasting cycles are dominant zeitgebers for several peripheral organs, especially those involved in food absorption and processing. In fact many rhythmically expressed liver genes are involved in metabolic pathways and are entrained by feeding [141]. Depending on their role in metabolism, they show stable phase relationships and thus allow the temporal separation of biochemically incompatible processes. For example, glycogen synthase and glycogen phosphorylase are regulated in anti-phase: during the absorptive phase glycogen synthase expression is upregulated, whereas during the postabsorptive phase glycogen phosphorylase expression is upregulated [142]. Restricted feeding of nocturnal rodents during daytime leads to a complete inversion of the gene expression rhythm in liver and pancreas [141, 143], without affecting the SCN. The consequence is an uncoupling of the peripheral liver clock from the SCN master clock. The molecular mechanism by which food can entrain the liver clock is still unknown. However it has been postulated that the intracellular redox state, i.e. the $[NAD(P)H]/[NAD(P)^+]$ ratio might be involved. It has been shown that the reduced cofactors NADH and NADPH stimulate the binding of the BMAL1-CLOCK or BMAL1-NPAS2 heterodimers to DNA, whereas the oxidized cofactors

NAD^+ and $NADP^+$ inhibit the binding [144]. Alternatively, postprandial temperature elevations or other food metabolites could act as entraining factors (reviewed in [145]).

The glucose level in blood is regulated by insulin and glucagon released from the endocrine pancreas. In healthy, i.e. non-diabetic individuals, insulin secretion follows a circadian rhythm that is generated within the pancreatic islets. This rhythm may be induced by melatonin which binds to melatonin receptors MT_1 and MT_2 at the islets' β-cells and can also cause a phase shift in insulin secretion [146]. Analysis of circadian gene expression of melatonin receptor knockout mice detected both phase shifts and amplitude changes. Regulation of insulin secretion and glucose homeostasis were monitored in melatonin receptor MT_1 or MT_2 single knockout mice or MT_1/MT_2 double knockout mice. Insulin secretion from isolated islets of the knockout mice was found to be increased relative to the wild type [147]. This points to a connection between circadian rhythms of insulin in pancreatic islets, its regulation by melatonin, and type 2 diabetes.

Clock mutant mice were reported to become obese due to hyperphagia, and eventually to develop a metabolic syndrome with the known symptoms of hyperleptinaemia, hyperlipidaemia, hepatic steatosis, hyperglycaemia, and hypoinsulinaemia [148]. The interrelationship between a dysfunction of the circadian clock and the development of metabolic abnormalities was also demonstrated in obese, diabetic ob/ob mice, in which the peripheral clocks were found to be impaired, but not the SCN clock. In addition, it was shown that in young ob/ob mice, the peripheral clocks are damaged before the onset of metabolic abnormalities. This suggests that the impairment of the peripheral clocks is not a consequence of the metabolic dysfunction but, rather, the other way round [149]; for a review see [150].

Cardiovascular system and adrenal function

Like other organ systems, the cardiovascular system is highly organized in time. Pronounced circadian variations can be measured in blood pressure, heart rate, peripheral resistance and pressure [151]; for a review see [152]. In addition, the release and activity of vasodilating hormones displays circadian rhythms. Surgical ablation of the SCN abolishes circadian variation in blood pressure [153]. In mice in which core clock genes are either deleted ($Bmal1^{-/-}$) or mutated ($Clock^{\Delta19/\delta19}$), the rhythms of blood pressure and heart rate are disrupted [154], vascular adaptation to changing blood flow is lost and they show a predisposition to thrombus [155]. It appears that the failure of vascular relaxation is due to a malfunction of the endothelial nitric oxide synthase, the enzyme that is necessary for endothelium-dependent nitric oxide.

Different forms of hypertension exhibit different circadian patterns. In primary hypertension there is usually a drop in blood pressure during the resting phase (in mice during the day, in humans during the night); these are called 'dippers'. In secondary hypertension due to, e.g. renal disease, Cushing's disease or diabetes mellitus, on the other hand, the rhythm in blood pressure is abolished ('non-dippers') or even reversed ('risers'). The lack of the decrease in blood pressure during resting time in non-dippers and risers is thought to lead to end-organ damage in cardiac, cerebral, vascular and renal tissues. Hence, these distinct forms of hypertension require different schedules for antihypertensive drug dosing [152].

Of special interest in the context of the cardiovascular system are the catecholamines that directly influence heart rate, stroke volume and also blood pressure via peripheral vasoconstriction. The adrenal glands are the body's main source of the circulating catecholamines and activation of the sympathetic nervous system, via the splanchnic nerves, triggers production and release of catecholamines from the adrenal medulla. Since the sympathetic tone is also controlled by the SCN, the catecholamines exhibit a clear circadian rhythm. Recently, a $Per1^{Luciferase}$ transgenic mouse was used to monitor *mPer1* expression *in vivo* in the adrenal glands [156]. The authors show that, following an acute light exposure after several days in DD, *mPer1* expression was strongly induced, and denervation of the adrenal gland abolishes the photic induction of *mPer1*. But more importantly, additionally the production of corticosterone was induced by this light exposure without a concomitant rise in adrenocorticotropic hormone (ACTH)

plasma levels. Thus, a new, more direct pathway for corticosterone production and release must be considered besides the 'classical' hypothalamic–pituitary–adrenal axis [157]. An adrenal clock was subsequently localized in the outer cortex and a gating mechanism for the production of corticosterones by ACTH was demonstrated [158]. It is therefore not surprising that mice with mutant or deleted clock genes show a disruption to their corticosteroid secretion [159, 160].

Altogether, it becomes obvious that mutations in one or several clock genes lead to multiple phenotypic changes and to specific alterations and/or problems in organ functions. It needs to be determined, however, whether these changes are the result of failure in clock mechanisms, or whether these clock genes have other, additional functions not related to the clock [161].

Practical recommendations from the viewpoint of chronobiology

Since experimental designs depend on the scientific question and are generally very different from one another, there cannot be recommendations valid for every experiment. For most experiments that are designed to evaluate anatomical differences (growth, fat accumulation, organ size, presence or absence of tumours, etc.) there is no need to consider time of day for sampling or dissection. However, there are several experimental approaches that require careful planning of the sampling times or the time for testing behaviour or locomotive skills. Usually this also requires careful planning of the animal husbandry and how to raise and keep the animals before and during the actual experimental phase.

Animal husbandry

Many animal facilities keep a standard LD cycle of 12:12 with lights on at 0600 h and off at 1800 h because this is the preferred working time for animal technicians and ancillary staff. For normal maintenance and cleaning the cages, as well as breeding, raising and checking the mice, this is quite adequate. However, for all experiments that require handling, testing and sampling the mice during their activity phase, this is very impractical for the scientific staff because they would always have to be on night shift. A compromise is to turn off the lights at 1200 h and on again at 2400 h, which gives the caretakers time for cleaning and maintenance in the morning and the scientific staff time to do their work in the afternoon. Such a schedule needs special structural provisions in the animal house, such as light-traps at the entrance to the animal rooms, so that no light enters the room when the door is opened. Even a single brief light pulse during the dark phase will acutely inhibit activity and lead to a drop in body temperature. Depending on the time during the scotophase when this light pulse occurs, the effect can last for up to 10 subsequent nights, as has been shown in hamsters, but it is probable that this will occur in mice as well [162].

Another compromise is to keep the main stock in the regular 0600 h to 1800 h schedule, and during the experiment put the animals into a reversed or phase-shifted LD cycle suitable for the experimental procedure and the sampling hours. This requires some additional time before the actual experiment, to make sure that the animals have adapted to, and are entrained to, the new LD cycle. I recommend allowing at least 2 days of adaptation for every hour shifted, i.e. 8 days for a 4 h shift, 24 days for a complete reversal of the LD cycle (= 12 h shift). I also recommend having all animal rooms equipped with constant dim red light similar to a photographic dark room (<1.5 lux or 2 mW/cm^2; $\lambda_{max} = 770$ nm). This allows one to enter the room during the dark phase and, after several minutes of dark adaptation, to be able to handle the animals and to perform manipulations that do not require bright light (e.g. injections). The use of a red head lamp or flash light must be discouraged because, when contrasted with complete darkness, even a short pulse of red light will have an arousing effect and cause a phase shift [163]. A continuous dim red light during the 'dark phase' contrasted to the bright daylight illumination, however, will be interpreted by the

animal as 'night' and they remain entrained to the LD cycle (actually a bright white light : dim red light cycle).

Following the twice yearly phase shifts of daylight saving time (or summer time) and back to winter time, as is the practice in many animal facilities, is not recommended. There is no real justification for doing so and it can be very detrimental, especially when the shift occurs during the course of an experiment.

Time for collection of samples and for testing

Since most hormones or metabolites show strong nycthemeral cycles it is important to choose the time of sampling judiciously. Especially when taking only a one time-point sample it is important to sample all animals in a group at approximately the same time of day and also to choose an equivalent time-point for the control group, otherwise the values might be meaningless.

Imagine being awakened in the middle of the night and asked to perform a difficult task. Would your performance be optimal? It should be logical to perform all behavioural tests and tests for locomotor skills during the activity phase of the animals. Also, a night-active animal such as the house mouse will be disturbed when the lights are turned on, look for a hiding place and stop being active. Therefore, the test should be done under suitable lighting conditions. Of course it is always the particular experiment and the scientific question behind it that determines the time of sampling or testing. For example, if you want to measure the resting metabolic rate of a mouse, it is advisable to do so during the daytime hours while the mouse is in its resting phase.

Dr Paul Pévet, who is head of the Strasbourg Department of Neurosciences, has established the first animal facility in the world that is especially designed for, and provides all necessary infrastructure, to perform experiments to chronobiological standards. This facility, called the 'Chronobiotron' is located at the University of Strasbourg, Institute for Cellular and Integrative Neurosciences, Neurobiology of Rhythms unit. This institute may be consulted for expert advice on adequate room and lighting conditions for

chronobiological studies [164]. Another source of advice and practical suggestions for performing chronobiological experiments in mice is given in [165].

Conclusion

Homeostasis has been—and still is—a fundamental principle in physiology. Yet, the field of chronobiology has demonstrated and documented during the past 50 years (at least) that there is an endogenous circadian variation in practically every physiological parameter. In order to acknowledge this fact, Nicholas Mrosovsky [166] has introduced the term *rheostasis* to describe regulation with rhythmic steady states as opposed to the constant steady states of homeostatically regulated systems. As Serge Daan, in his history of chronobiological concepts [167] has pointed out:

> There is great evolutionary advantage of changing homeostatic setpoints over time, in particular when the environmental changes are predictable, as is the case with cosmic cycles, such as the day or the year. By changing physiological setpoints over the daily cycle, animals and humans actively prepare for such changes instead of passively responding.

Laboratory animal scientists should be well aware of this concept and of its practical consequences.

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Diversity of Spontaneous Neoplasms in Commonly Used Inbred Strains of Laboratory Mice

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Introduction

Humans have maintained and domesticated many species over the centuries for food and labour. Non-human species were also maintained as companion animals or, in the case of rodents, often out of curiosity. Mice with spontaneous mutations that resulted in dramatic physical changes from normal, what we now call phenotypic deviants or mutant mice, were particularly prized. A notable example is the 'rhinoceros' mouse that lost all hair with age and developed prominent wrinkling [1], variations of which became the well-known hairless and rhino mice commonly used today in biomedical research. An extension of these observations was the understanding that these animals, particularly rodents, were useful as tools, or more specifically as biological models, for understanding similarities with human biology and especially disease. The Jackson Laboratory was founded in 1929 with the goal of using the laboratory mouse as a biomedical tool to unlock the secrets of mammary cancer. This led to the discovery that a filterable agent (the Bittner Agent), later determined to be a retrovirus (the mouse mammary tumour virus, MMTV), was the major cause of mammary cancer in some strains of mice [2]. More recently, we showed that mice can be infected with a papillomavirus that can cause cancer [3, 4] indicating that these discoveries continue.

It was once thought to be impossible to inbreed animals. In spite of this dogma, Little and Tyzzer in the early 1900s initiated the process of developing a large variety of inbred strains in many laboratories around the world [5]. As these strains became large colonies, a variety of diseases appeared in some colonies but not in others. As husbandry conditions improved through the 20th century, thereby eliminating most serious infectious diseases, background levels of cancer became more evident, particularly in ageing studies. Spontaneous cancers were important to understand because these lesions had to be differentiated from those caused by the experimental design of various studies [6]. The advent of genetic engineering further emphasized the need to understand background diseases in strains used to differentiate lesions induced by genetic manipulation from those that arise naturally [7-10]. As mouse research expanded exponentially in recent years [11], a demand for information on spontaneous background diseases that occur in each strain was needed so that experiments could be interpreted correctly. More importantly, with new genetic approaches now being available, such as genome-wide scans, these background diseases can be investigated as complex genetic traits rather than simply incidental findings [12].

Inbred mice are essentially identical except for sex because their genome is homogenous and stable. As we come to recognize that certain diseases only arise in some strains and not others, especially complex diseases such as cancer, these mice become irreplaceable as models to dissect the genetic bases of disease and their mechanisms. For example, rhabdomyosarcomas are rare malignant neoplasms of striated muscle that occur in a limited number of closely related strains, especially BALB/cJ, BALB/cByJ and A/J strains [13, 14]. It is now possible, with modern gene mapping tools, to dissect the complex polygenic nature of this neoplasm because genetic diversity between individuals is a known quantity with such a model. Similar studies are currently in progress with many types of cancer. For example, this approach has been applied to the juvenile ovarian granulosa cell tumour model using recombinant inbred approaches [15] and, more recently, to pulmonary adenomas [12] using aged inbred and wild-derived strains.

This chapter reviews tumour frequency records from the Aging Center at The Jackson Laboratory in which 28 of the most important and commonly used inbred strains of laboratory mice were aged and carefully evaluated for physiological changes as they aged, as well as the histopathological types and varieties of lesions they developed, including cancer. Tumour frequency, diagnoses, and representative photomicrographs for this study have recently been made available through public databases, together with data from many other strains [16]. The chapter also focuses on the variety of available search mechanisms and data formats available from two important public databases, the Mouse Tumor Biology database (MTB, http://tumor.informatics.jax.org/mtbwi/index.do) [11, 17, 18] and Pathbase

(http://www.pathbase.net/) [19-22], both of which store much of this information. Annotated colour images of specific mouse neoplasms or non-neoplastic diseases with extensive bibliographies are available from these databases and others that are provided in the list of websites in Table 3.1.2 later in this chapter.

The Mouse Tumor Biology Database and Pathbase

The MTB was first made available to the public in 1998 [11, 18, 23]. The MTB provides a centralized electronic resource that collects and integrates the many different types of data obtained from mouse cancer models in an easily searchable database. Data include incidence and latency of mouse tumours, pathology reports and representative images, as well as strain and somatic genetics. All data are attributed to the original reference, a contributor citation, or the website source. Controlled vocabularies and standardized nomenclature allow for integrated searches of data from different sources. Searches of MTB are accomplished using web-based query forms. Query forms use terms specific for data types in MTB, such as tumour class, mouse strain, genetics, images, reference and mouse homologues of human genes and associated data. Combined searches using terms from the strain, genetics, pathology image and tumour search forms simultaneously are also available using the advanced search form. The MTB includes data curated from the scientific literature, data submissions from cancer researchers and data downloaded from public databases. The MTB is part of the Mouse Genome Informatics (MGI) [24] Resource and can be accessed from the MGI website (http://www.informatics.jax.org/). The MTB also maintains a list of almost 300 antibodies available and how to use them for immunohistochemistry on mouse tissues with links to images of positive control results in both HTML and Microsoft Excel format [25].

Pathbase (http://www.pathbase.net/) was developed in 1999 by a group of European and North American pathologists as a community response to data identification and dissemination problems and was initially funded by the European Commission. It contains photomicrographs of representative images annotated to a set of defined controlled vocabularies and ontologies, which provide a public resource for the sharing of images of normal and abnormal tissues from mutant and background strains of laboratory mice [19-21, 26]. The images are annotated with details of strain, genotype, anatomical location and diagnosis, with key annotations derived from controlled vocabularies or Open Biomedical Ontologies (OBO)-compliant bio-ontologies [27] (e.g. mouse pathology ontology (MPATH), Mouse Anatomy Ontology (MA), e-Mouse Atlas Project (EMAP) developmental anatomy ontology, cell ontology (CA) and gene ontology (GO); see below). Nomenclature for mouse strains and mutant gene symbols are included, when provided, and follow the International Mouse Genetic Nomenclature Committee formats [28, 29]. This allows for comparison between studies addressing modifier genes or strain-specific diseases that might confuse interpretation.

The MA was developed to standardize anatomical terms [30]. It has a formal ontological structure built on the kind of framework contained in an anatomy reference book [31], but is open and under constant development and refinement. This is a dynamic process and, as such, MA is updated regularly as users provide input to curators. A textbook on comparative microscopic anatomy of the mouse and human is now available [32] and similar ones on embryology are also now available [33]. These resources provide further extension of comparative anatomical nomenclature. Pathology nomenclature for the mouse has been captured in the form of an ontology called the Mouse Pathology Ontology (MPATH). This was built using the expertise of the Mouse Pathology Ontology Consortium, a group of 20 veterinary and medical pathologists and biologists who meet regularly to review and update the ontology. This is one of the topics of an annual meeting known as *The Pathology of Mouse Models of Human Diseases* [34] and is often associated with similar European meetings [35]. These meetings provide for a regular review by a large panel of pathologists to curate and update the ontology, as well as to make it relevant to practising pathologists.

Pathbase has established links with the Aging Center at the Jackson Laboratory [36], the Mouse Phenome Database (MPD) [37] and the MTB [11, 18] to curate and host representative images from ongoing studies of age-related lesions and normal tissue variation from 31 inbred strains of mice. Quantitative data for other systems-based parameters are loaded into the MPD and the two datasets are integrated between MPD and Pathbase. Similar integration was recently established with the European Radiobiology Archives (ERA) database [38] and with the Northwestern University Janus radiobiology database (http://janus. northwestern.edu/janus2/) [39]; 50 000 individual mouse records were coded to MPATH to link the two datasets.

Strains

The Mouse Phenome Project defined the inbred strains of greatest importance in modern biomedical research (http://phenome.jax.org/ db/q?rtn=strains/search&reqpanel=MPD) [37, 40]. These represent not only inbred strains used for a variety of basic research projects but also those that are currently used for transgenesis (primarily C57BL/6 and FVB/N) or targeted mutagenesis (primarily various substrains of 129 and C57BL/6) experiments. The Jackson Laboratory maintains most of these strains in a repository (http://jaxmice.jax.org/) but many are very small colonies, so spontaneous neoplasia frequency data are limited. Due to space limitations, we limited the data presented here to tumour data from the 20 month time point of the Aging Center's inbred mouse strain ageing study (Table 3.1.1). These include nine of Castle's mouse-related strains (129S1/SvlmJ, A/J, AKR/J, C3H/HeJ, CBA/J, DBA/2J, LP/J, NZO/HlLtJ and NZW/LacJ), one of Castle's mouse-related substrain (BALB/cByJ), five C57 mouse-related strains (C57BL/10J, C57BL/6J, C57BLKS/J, C57BR/cdJ and C57L/J), five Swiss mouse-related strains (FVB/NJ, NON/LtJ, SJL/J, SM/J and SWR/J) and eight other strains (BTBR T+ tf/J, BUB/BnJ, KK/HIJ, MRL/MpJ, P/J, PL/J, PWD/PHJ, RIIIS/J and WSB/EiJ) to provide more genetic diversity (http://www.informatics. jax.org/external/festing/search_form.cgi).

Additional information on spontaneous cancers that occur in these and other inbred strains (C58/J, CE/J, MOLF/Ei, NOD/LtJ, NZB/ B1NJ, RF/J, STX/LeJ, SWL/J), substrains (129/ SvJ, A/HeJ, BUB/BnJ, C3H/HeOuJ, C3HeB/FeJ, C57BL/10J, C57BL/10SnJ, C57BR/cdJ, CBA/CaJ, I/LnJ, NZW/LacJ, RBF/DnJ, SEA/GnJ) and mutant strains are available on the MTB (http://tumor.informatics.jax.org/mtbwi/tumor FrequencyGrid.do). This database also provides a comprehensive selection of annotated photomicrographs of slides stained with haematoxylin and eosin, special stains or immunohistochemistry, as well as some electron micrographs with links to extensive references.

Other large-scale ageing studies using mice

Historically, much work was done on ageing lesions in strains of mice that were commonly used in biomedical research at the time. This provided background data to aid in interpreting other experimental procedures but also provided basic insight into the ageing process itself and how inbred strains differed from each other. This historical work focused on a few inbred and hybrid strains, such as BALB/cStCrlfC3H/Nctr and B6C3F1 ((C57BL/ 6N X C3H/HeN)F1) used by the National Center for Toxicologic Research, diet restriction studies (C57BL/6NNIA, DBA/2NNia, B6D2F1 (C57BL/ 6NNia X DBA/2NNia) or B6C3F1 (C57BL/ 6NNia X C3H/NNia) and a variety of other strains [41-44]. Husbandry, pathogen status and genetic quality control have all improved since much of these earlier data sets were generated, such that some of these data are no longer relevant.

Tumour incidence in inbred strains

Table 3.1.1 shows consolidated results of inbred mouse tumour frequencies from mice that were

TABLE 3.1.1: Tumour incidence in inbred strains from the 20 month Jackson Aging Study Cohort. Table shows tumour incidence sorted by inbred strain (male, female) vs tumour diagnosis and organ. Table is colour coded, with light grey indicating 1–24%, grey 25–69% and black 70–100%.

Organ	Diagnosis	129S1/SvImJ F	129S1/SvImJ M	A/J F	A/J M	AKR/J F	AKR/J M	BALB/cByJ F	BALB/cByJ M	BTBR T+ tf/J F	BTBR T+ tf/J M	BUB/BnJ F	BUB/BnJ M	C3H/HeJ F	C3H/HeJ M	C57BL/10J F	C57BL/10J M	C57BL/6J F	C57BL/6J M
Total	Cases	12	15	18	13	4	7	16	16	15	13	9	2	8	15	13	21	23	14
Adrenal gland	Adrenocortical adenoma							▒											
Vertebral body	Osteosarcoma																		
Caecum	Leiomyosarcoma																		
Duodenum	Polyp																		
Ear	Nerve sheath tumour																		
Eye lid	Mast cell tumour																		
Fat	White fat lipoma			▒															
Hematopoietic system	Lymphosarcoma					▓	█												
	Plasma cell tumour																		
	Histiocytic sarcoma																		
	Lymph node mast cell tumour																		
Ileum	Polyp							▒											
Kidney	Adenoma				▒														
Liver	Haemangiosarcoma																		
	Hepatoma									▒									
	Plasma cell tumour																		
Lung	Pulmonary adenoma	▒		▒	▓			▒		▓		▒							
	Myoepithelioma metastasis				▒														
Nail bed	Squamous cell carcinoma (locally invasive)									▒									

(Continued)

TABLE 3.1.1 CONT'D: Tumour incidence in inbred strains from the 20 month Jackson Aging Study Cohort. Table shows tumour incidence sorted by inbred strain (male, female) vs tumour diagnosis and organ. Table is colour coded, with light grey indicating 1–24%, grey 25–69% and black 70–100%

Organ	Diagnosis
Mammary gland	Myoepithelioma
Ovary	Luteal tumour
Uterus	Haemangioma
Peritoneal cavity	Lipoma
Testis	Leydig cell tumour
Skeletal muscle	Haemangiosarcoma
	Rhabdomyosarcoma
Skin	Basal cell carcinoma
Vibrissae	Squamous cell carcinoma
Forestomach	Papilloma
Glandular stomach	Adenoma

Organ	Diagnosis	C57BLKS/J F	C57BLKS/J M	C57BR/cdJ F	C57BR/cdJ M	C57L/J F	C57L/J M	CBA/J F	CBA/J M	DBA/2J F	DBA/2J M	FVB/NJ F	FVB/NJ M	KK/HlJ F	KK/HlJ M	LP/J F	LP/J M	MRL/MpJ F	MRL/MpJ M	NON/LtJ F	NON/LtJ M
Total	Cases	14	17	11	22	26	22	17	22	8	6	20	10	2	5	17	17	3	15	13	12
Adrenal gland	Adrenocortical adenoma																				
Vertebral body	Osteosarcoma																				
Caecum	Leiomyosarcoma																				
Duodenum	Polyp																				
Ear	Nerve sheath tumour																				
Eyelid	Mast cell tumour																				
Fat	White fat lipoma																				
Haematopoeitic system	Lymphosarcoma																				
	Plasma cell tumour																				

Organ	Neoplasm
	Histiocytic sarcoma
	Lymph node mast cell tumour
Ileum	Polyp
Kidney	Adenoma
Liver	Haemangiosarcoma
	Hepatoma
	Plasma cell tumour
Lung	Pulmonary adenoma
	Myoepithelioma metastasis
Nail bed	Squamous cell carcinoma (locally invasive)
Mammary gland	Myoepithelioma
Ovary	Luteal tumour
Uterus	Haemangioma
Peritoneal cavity	Lipoma
Testis	Leydig cell tumour
Skeletal muscle	Haemangiosarcoma
	rhabdomyosarcoma
Skin	Basal cell carcinoma
Vibrissae	Squamous cell carcinoma
Forestomach	Papilloma
Glandular stomach	Adenoma

TABLE 3.1.1: Tumour incidence in inbred strains from the 20 month Jackson Aging Study Cohort. Table shows tumour incidence sorted by inbred strain (male, female) vs tumour diagnosis and organ. Table is colour coded, with light grey indicating 1–24%, grey 25–69% and black 70–100%—cont'd

Organ	Diagnosis	NZO/H1LtJ F	NZO/H1LtJ M	NZW/LacJ F	NZW/LacJ M	P/J F	P/J M	PL/J F	PL/J M	PWD/PHJ F	PWD/PHJ M	RIIIS/J F	RIIIS/J M	SJL/J F	SJL/J M	SM/J F	SM/J M	SWR/J F	SWR/J M	WSB/EiJ F	WSB/EiJ M
Total	Cases	6	6	11	11	7	6	6	8	14	11	14	14	6	0	2	13	6	8	2	11
Adrenal gland	Adrenocortical adenoma																				
Vertebral body	Osteosarcoma													▓							
Caecum	Leiomyosarcoma																				
Duodenum	Polyp																				
Ear	Nerve sheath tumour																				
Eyelid	Mast cell tumour																				
Fat	White fat lipoma																				
Haematopoeitic system	Lymphosarcoma													▓							
	Plasma cell tumour													■							
	Histiocytic sarcoma													▓							
	Lymph node mast cell tumour																				
Ileum	Polyp																				
Kidney	Adenoma																				
Liver	Haemangiosarcoma																				
	Hepatoma																				
	Plasma cell tumour													▓							

Site	Neoplasm
Lung	Bronchoalveolar adenoma
	Myoepithelioma metastasis
Nail bed	Squamous cell carcinoma (locally invasive)
Mammary gland	Myoepithelioma
Ovary	Luteal tumour
Uterus	Haemangioma
Peritoneal cavity	Lipoma
Testis	Leydig cell tumour
Skeletal muscle	Haemangiosarcoma
	Rhabdomyosarcoma
Skin	Basal cell carcinoma
Vibrissae	Squamous cell carcinoma
Forestomach	Papilloma
Glandular stomach	Adenoma

TABLE 3.1.2: Mouse tumour databases accessible on the Internet	
Host	**URL**
The Mouse Tumor Biology database	http://tumor.informatics.jax.org/
eMICE: electronic Models Information, Communication, and Education	http://emice.nci.nih.gov/
Pathbase	http://www.pathbase.net/
Mouse Phenome Database (MPD)	http://phenome.jax.org/
Biology of the Mammary Gland Web Site	http://mammary.nih.gov/
Festing's Listing of Inbred Strains of Mice	http://www.informatics.jax.org/external/festing/search_form.cgi
JAX Mice Web Site	http://jaxmice.jax.org/
Mammary Cancer in Humans and Mice: A Tutorial for Comparative Pathology: The CD-ROM Web site3	http://ccm.ucdavis.edu/bcancercd/introduction.html

aged until they were 20 months old and subjected to complete necropsies by the Aging Center at The Jackson Laboratory [45]. Selected lesions were photographed at various magnifications and these images are available at both the Pathbase and MTB websites. We are currently performing whole-slide imaging that will allow users to scan the complete slide on their own computers in the form of a 'virtual slide'. This data set, while utilizing fewer mice, is more comprehensive than the dataset we published in the first edition of this book [46], which focused on histological diagnoses of tumours observed during gross examination of routine disease surveillance cases in which complete necropsies were rarely done. Figure 3.1.1 provides similar information for comparison from the MTB Tumor Frequency Grid, which is based on published data from many studies carried out by scientists worldwide.

The Aging Center study provides a detailed view of which anatomical systems have the highest tumour frequency among various inbred strains [16]. This study includes information on sex and tumour type as well. Across strains the systems that have the highest incidence of neoplasia are the female reproductive system, the haematopoietic system, liver and lung. Tumour frequency varies greatly between strains, however. The 129S1/SvlmJ and BTBR T+ tf/J mice have multiple different types of lesions and NOD.B10Sn-H2b/J shows no detectable types of cancer. The study also details strain-specific sex differences in tumour

frequency. Hepatomas were not detected in female CBA/J mice but were detected at high levels in the males. The previously mentioned BTBR T+ tf/J mice had multiple lesions detected but the majority of tumours were diagnosed in females. Only adrenocortical adenomas and pulmonary adenomas were detected in males, neither of which was detected in females.

The MTB Tumor Frequency Grid (Table 3.1.2) shows a slightly different view of information on neoplasms that spontaneously affect inbred strains. Unlike the Aging Center work, where all mice were subjected to complete necropsies, data in the MTB Tumor Frequency Grid is built from published data generated worldwide using a wide variety of protocols. The grid graphically displays the intersection of inbred strain or substrain with anatomical structure. The MTB grid does not differentiate between different types of neoplasms or gender. The highest rates of tumour frequency in the greatest number of strains are in the mammary gland, leukocytes, liver and lung. These results are similar, but not identical, to those of the Aging Center study. Strain results are also similar, with NOD mice showing almost no tumours and 129S1/SvlmJ having many different tumour types detected.

The Aging Center strain disease study and the MTB tumour grid present useful summary tables of tumour frequency as related to strain and tumour type and serve as good starting points for analysis. Much more detailed information is required for proper formulation of a course of
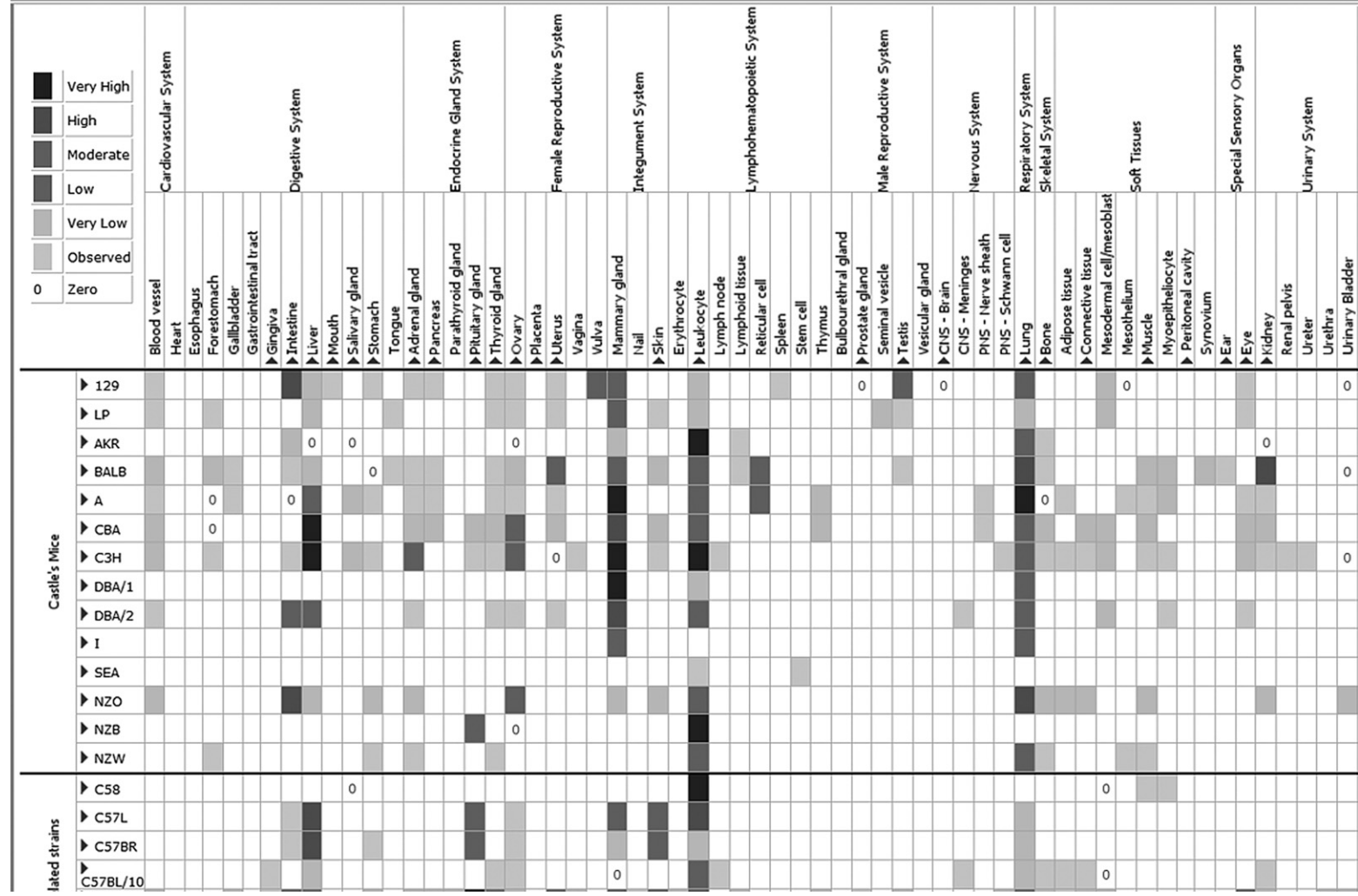
Figure 3.1.1 MTB tumour frequency grid.

scientific inquiry. Public databases, such as Pathbase and MTB, provide access to large amounts of previously published research and the analysis tools to examine these data. As an example, we will follow a hypothetical line of investigation for a scientist interested in lung tumours, specifically pulmonary adenomas.

An examination of the Aging Center data shows that the strains with the highest frequency of pulmonary adenomas are 129S1/SvlmJ, A/J, BALB/cByJ, LP/J, NZO/HlLtJ and RIIIS/J. The MTB Tumor Frequency Grid confirms the high incidence levels of lung tumours in 129, A, BALB and NZO strains, but only low levels in LP, and no recorded level for RIIIS.

Detailed tumour information and pathological images are available from both MTB and Pathbase. Tumour information in MTB can be accessed using multiple search engines. Tumour-specific information can be most directly obtained from the advanced search form (http://tumor.informatics.jax.org/mtbwi/

advancedSearch.do). A simple search can be accomplished by selecting 'adenoma' from the Tumor Classification pull-down menu, 'lung' from the Organ/Tissue of Origin pull-down menu and 'inbred' from the Strain Type pull-down menu. This search yields 829 tumour frequencies, 14 with photomicrographs (48 images), from numerous inbred strains including A/J, BTBR/J, C57L/J, WSB/EiJ, C57BR/cdJ, LP/J, A/J, A/HeJ, C3H/HeJ, DBA, CAST/EiJ, C57BL/6, BALB/C, 129S1/SvlmJ, SWR, BALB/CByJ, BALB/cJ, SWR/J, CBA/J, RIIIS/J, C57BL/6J, KK/HIJ , NON/ShiLtJ, P/J, SM/J, PWD/PhJ and FVB/NJ mice. These results include strain, gender, treatment, reproductive status, cohort size and frequency, age of onset and detection, any additional relevant information included in notes and pathology information/images. An example of a KK/HIJ lung adenoma image record is shown in Figure 3.1.2. The MTB also includes data on mutant and transgenic mice which are not detailed in these results.

Figure 3.1.2 MTB pathology image record of a pulmonary adenoma from 201 day old KK/HIJ mouse.

Figure 3.1.3 Pathbase data record of a pulmonary adenoma from 201 day old KK/HIJ mouse.

Pathbase also provides a detailed search engine (http://eulep.pdn.cam.ac.uk/Search_Pathbase/index.php) to identify specific records of interest. Search terms can be entered for organism, gender, MA, MPATH, gene and many other terms. A search of Pathbase for adenoma in the 'MPATH/Pathology' field and lung in the 'MA/Anatomical Site' field yields 22 records, which include papillary, Clara cell and pulmonary adenomas. Of these 69 records, 18 records from 5 mice were bronchioloalveolar (pulmonary) adenomas from BALB/cJ, KK/HIJ, RIIIS/J, C57BL/6J and FVB/NJ mice. An example of a pulmonary adenoma Pathbase record is shown in Figure 3.1.3. This is the same image illustrated in Figure 3.1.2 from MTB. The Pathbase records include details on gender, strain, genotype, text description of mouse details, genetic manipulation, images, the MA/Anatomical Site and MPATH/Pathology designations. Many more images from the Aging Center at The Jackson Laboratory are available and will be posted.

Mouse cancer websites

Many databases that provide access to mouse tumour data have been developed over the past few years (Table 3.1.2) [47]. At the present time, their contents reflect the interests of their curators and thus their scope remains limited. However, they provide room for more in-depth illustration than any textbook or regular paper in a scientific journal. Researchers are encouraged to contribute to these databases to increase the impact of their work.

Some of these databases are already linked to literature databases (such as PubMed and The Jackson Laboratory's MGI Database). Some also provide information about immunohistochemical methods with links to antibody manufacturers (MTB, for example). These databases have started to host some of the supplementary illustrations that cannot be published in regular scientific journals and this should increase over time. Also, as tumour gene databases develop, links to them will be established from the literature databases.

Conclusions

Most reported tumour rates in mice are based on 2 year ageing experiments. The Aging Center study provides tumour frequencies for mice during their 20 months of life primarily because many of the inbred strains do not normally survive long enough to reach 2 years of age [36, 48]. Data for additional strains and substrains are available on the websites of the MTB and Pathbase. Knowledge of tumour rates in young mice is crucial to the interpretation of most studies because many of them involve mice in their first months of life. Tumour frequency differs greatly among strains, possibly as a result of strain-specific genetic differences.

Scientists involved with mouse research need to be aware of these differences before initiating their research. In addition to the strain, numerous factors affect the rate of cancer in mice. These factors include gender, stress, temperature, bedding, caging density, altitude, diet, laboratory practices and time period [49]. Some of these factors may evolve with time as the diet and the husbandry practices evolve to reflect the advances in regulations. For example, *Helicobacter hepaticus* is now recognized as a pathogen and is eliminated in most research and vendor colonies [50, 51]. The same is true for *Klebsiella oxytoca*, especially in strains that have mutations in the Toll-like receptor 4 (*Tlr4*) gene [52]. Another example is elimination of exogenous mouse mammary tumour virus, and therefore the high frequency of mammary cancer, from C3H substrains that are distributed by commercial vendors [53]. Hence, spontaneous tumour rates are expected to evolve with time. Genetic drift may also alter cancer rates. The Jackson Laboratory and other large rodent repositories play a key role in preventing the genetic drift of inbred strains of mice. The ability to identify and analyse strain-specific tumour rates and other strain-associated tumour data from among the huge and increasing number of publications is of significant importance. Public databases provide important tools facilitating access to these data and will only get more essential as the volume of data increases.

Acknowledgements

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Viral Infections of Laboratory Mice

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Introduction

In interpreting the microbiological status of laboratory animals, it must be understood that infection and disease are not synonymous. *Infection* refers to the invasion and multiplication of microorganisms in body tissues and may occur with or without apparent disease. *Disease* refers to interruption or deviation from normal structure and function of any tissue, organ or system. Many of the infections with which we are concerned may not cause discernable disease in many strains of mice. However, they may cause inapparent or subclinical changes that can interfere with research. Such interference often remains undetected, and

therefore modified results may be obtained and published.

The types of interference of an agent with experimental results may be diverse. There is no doubt that research complications due to overt infectious disease are significant and that animals with clinical signs of disease should not be used for scientific experiments. But clinically inapparent infections may also have severe effects on animal experiments. There are numerous examples of influences of microorganisms on host physiology and hence of the interference of inapparent infections with the results of animal experiments. Many microorganisms have the potential to induce activation or suppression of the immune system, or both at the same time but on different parts of the

immune system, regardless of the level of pathogenicity. All infections, apparent or inapparent, are likely to increase interindividual variability and hence result in increased numbers of animals necessary to obtain reliable results. Microorganisms, in particular viruses, present in an animal may contaminate biological materials such as sera, cells or tumours [1, 2]. This may interfere with *in vitro* experiments conducted with such materials and may also lead to contamination of animals [3]. Mouse antibody production (MAP) testing or polymerase chain reaction (PCR) testing of biologics to be inoculated into mice is an important component of a disease prevention programme. Finally, latent infections may be activated by environmental factors, by experimental procedures, or by the combination and interaction between various microorganisms. For all these reasons, prevention of infection, not merely prevention of clinical disease, is essential.

Unfortunately, research complications due to infectious agents are usually considered artefacts and published only exceptionally. Information on influences of microorganisms on experiments is scattered in diverse scientific journals, and many articles are difficult to find. To address this problem, several meetings have been held on viral complications on research. The knowledge available is summarized in conference proceedings [4, 5] and has later repeatedly been reviewed [6-8].

Viral infections of mice have been studied in detail, and comprehensive information on their pathogenic potential, their impact on research, and the influence of host factors such as age, genotype, and immune status on the response to infection is available. The nomenclature and taxonomy of viruses is described based on recent nomenclature rules by the International Union of Microbiological Societies [9] and the Universal Virus Database of the International Committee on the Taxonomy of Viruses (http://www.ictvdb.org). Retroviruses are not covered in this chapter because they are not included in routine health surveillance programmes and cannot be eradicated with the methods presently available. This is because most of them are incorporated in the mouse genome as proviruses and thus are transmitted via the germline.

The ability to accurately determine whether or not laboratory animals or animal populations have been infected with a virus depends on the specificity and sensitivity of the detection methods used. Most viral infections in immunocompetent mice are acute or short term, and lesions are often subtle or subclinical. The absence of clinical disease and pathological changes has therefore only limited diagnostic value. However, clinical signs, altered behaviour or lesions may be the first indicator of an infection and often provide clues for further investigations.

Serology is the primary means of testing mouse colonies for exposure to viruses, largely because serological tests are sensitive and specific, are relatively inexpensive and allow screening for a multitude of agents with one serum sample. They are also employed to monitor biological materials for viral contamination using the MAP test. Serological tests detect specific antibodies, usually immunoglobulin G (IgG), produced by the host against the virus and do not actually test for the presence of the virus. An animal may have been infected, mounted an effective antibody response and cleared the virus, but remains seropositive for weeks or months or for ever, even though it is no longer infected or shedding the agent. Active infection can only be detected by using direct detection methods such as virus isolation, electron microscopy or PCR. Meanwhile, PCR assays have been established for the detection of almost every agent of interest. They are highly sensitive and, depending on the demands, they can be designed to broadly detect all members of a genus or only one species. However, good timing and selection of the appropriate specimen is critical for establishing the diagnosis. In practice, combinations of diagnostic tests are often necessary, including the use of sentinel animals or immunosuppression to get clear aetiological results or to avoid consequences from false-positive results.

Reports on the prevalence of viral infections in laboratory mice throughout the world have been published frequently. In general, the microbiological quality of laboratory mice has constantly improved during the last decades, and several agents (e.g. herpesviruses and polyomaviruses) have been essentially eliminated from contemporary colonies due to advances in diagnostic methodologies and modern husbandry and rederivation practices [10-15]. They may,

however, reappear, since most have been retained or are still being used experimentally. Furthermore, the general trend towards better microbiological quality is challenged by the increasing reliance of biomedical research on genetically modified and immunodeficient mice, whose responses to infection and disease can be unpredictable. Increasing numbers of scientists are creating genetically modified mice, with minimal or no awareness of infectious disease issues. As a consequence, these animals are more frequently infected than 'standard' strains of mice coming from commercial breeders, and available information on their health status is often insufficient. Frequently they are exchanged between laboratories, which amplifies the risk of introducing infections from a range of animal facilities. Breeding cessation strategies that have been reported to eliminate viruses from immunocompetent mouse colonies may prove to be costly and ineffective in genetically modified colonies of uncertain or incompetent immune status. It must also be expected that new agents will be detected, although only occasionally. Infections therefore remain a threat to biomedical research, and users of laboratory mice must be cognizant of infectious agents and the complications they can cause.

DNA viruses

Herpesviruses

Two members of the family Herpesviridae can cause natural infections in mice (*Mus musculus*). Mouse cytomegalovirus 1 (MCMV-1) or murid herpesvirus 1 (MuHV-1) belongs to the subfamily Betaherpesvirinae, genus *Muromegalovirus*. Murid herpesvirus 3 (MuHV-3) or mouse thymic virus (MTV) has not yet been assigned to a genus within the family Herpesviridae. Both are enveloped, double-stranded DNA viruses that are highly host-specific and relatively unstable to environmental conditions such as heat and acidic pH. Both agents are antigenically distinct and do not cross-react in serological tests, but their epidemiology is similar [16].

MCMV-1 is very uncommon in European and American colonies of laboratory mice and is found at a very low rate [11] or reported as not found [14, 15]. Seropositivity has, however, been reported from Asian countries [17, 18]. Testing for MTV is not frequently reported, and no sample tested positive in recent studies [11]. The data available suggest that the prevalence of both viruses in contemporary colonies and thus their importance for laboratory mice is negligible. However, both MCMV-1 and MTV are frequently found in wild mice, which may be coinfected with both viruses [8, 19-21].

Mouse cytomegalovirus 1 (MCMV-1) or murid herpesvirus 1 (MuHV-1)

Natural infection with MCMV-1 causes subclinical salivary gland infection in mice. Like other cytomegaloviruses, MCMV-1 is strictly host-specific. It persists in the salivary glands (particularly in the submaxillary glands) and also in other organs [22-24]. The virus can be cultured in mouse fibroblast lines like 3T3 cells, but primary mouse embryo fibroblasts are more sensitive to infection and produce higher virus titres. However, passage in cell culture results in its attenuation. To maintain virulence, the virus is best propagated by salivary gland passages of sublethal virus doses in weanling mice of a susceptible strain (e.g. BALB/c) [25].

Most information concerning the pathogenesis of MCMV-1 infection is based on experimental infection studies. These results are very difficult to summarize because the outcome of experimental infection in laboratory mice depends on various factors such as mouse strain and age, virus strain and passage history [26], virus dose and route of inoculation [24]. In general, newborn mice are most susceptible to clinical disease and to lethal infection and develop higher levels of resistance with increasing age. Infection of neonates leads to abnormal brain development [27, 28]. Virus replication is observed in newborn mice in many tissues and appears in the salivary glands towards the end of the first week of infection when virus concentrations in liver and spleen have already declined. Resistance develops rapidly after weaning between days 21 and 28 of age. Experimental infection of adult mice results in mortality only in susceptible strains and only if high doses are administered. Not even intravenous or intraperitoneal injections of adult mice usually produce

signs of illness in resistant strains [29]. Mice of the H-2^b (e.g. C57BL/6) and H-2^d (e.g. BALB/c) haplotype are more sensitive to experimental infection than are mice of the H-2^k haplotype (e.g. C3H), which are approximately 10-fold more resistant to mortality than those of the b or d haplotype [24].

Subclinical or latent infections can be activated by immunosuppression (e.g. with cyclophosphamide or cortisone) or critical illness such as sepsis [30]. Reactivation of MCMV-1 also occurs after implantation of latently infected salivary glands into $Prkdc^{scid}$ mice [31]. Immunodeficient mice lacking functional T cells or natural killer (NK) cells, such as $Foxn1^{nu}$ and $Lyst^{bg}$ mice are more susceptible than are immunocompetent animals. Experimental infection in $Prkdc^{scid}$ mice causes severe disease or is lethal, with necrosis in spleen, liver and other organs, and multinucleate syncythia with inclusion bodies in the liver [32]. Similarly to AIDS patients infected with human cytomegalovirus, athymic $Foxn1^{nu}$ mice experimentally infected with MCMV-1 also develop adrenal necrosis [33]. The virus also replicates in the lungs, leading to pneumonitis, whereas replication and disease are not seen in heterozygous ($Foxn1^{nu/+}$) littermates [34]. The pathogenesis of MCMV-1 infection in immunocompetent and in immunocompromised mice, as well as the role of the immune system, have been reviewed by Krmpotic et al. [35].

The most prominent histological finding of cytomegaloviruses is enlarged cells (cytomegaly) of salivary gland epithelium with eosinophilic nuclear and cytoplasmic inclusion bodies. The inclusion bodies contain viral material and are found also in other organs such as liver, spleen, ovary and pancreas [24]. Depending on inoculation route, dose, strain, and age of mice, experimental infections may result in inflammation or cytomegaly with inclusion bodies in a variety of tissues, pneumonitis, myocarditis, meningoencephalitis or splenic necrosis in susceptible strains [8, 24, 36].

Virus is transmitted by the oronasal route, by direct contact and is excreted in saliva, tears and urine for several months. The virus is ubiquitous in wild house mice worldwide. They serve as a natural reservoir for infection and can even be infected with different virus strains [37]. The virus is most frequently transmitted horizontally through mouse-to-mouse contact but does not easily spread between cages. Sexual transmission and transmission with tissues or organs is also possible. The virus does not cross the placenta in immunocompetent mice, although infection of pregnant females results in fetal death or resorption and wasting of borne pups. However, fetal infection is possible by direct injection of MCMV-1 into the placenta [38] and also occurs by transplacental transmission in mice with severe immunodeficiency [39]. Vertical transmission is also possible by milk during lactation [40].

It is generally assumed that MCMV-1 has a very low prevalence in contemporary colonies of laboratory mice. The risk of introduction into facilities housing laboratory mice is very low if wild mice are strictly excluded. Monitoring is necessary if populations of laboratory mice may have been contaminated by contact with wild mice. As for other viruses, different serological tests, including multiplex fluorescent immunoassay (MFIA) [41], are used for health surveillance of rodent colonies. As the virus persists, direct demonstration of MCMV-1 in infected mice is possible by PCR [42-44] or by virus isolation using mouse embryo fibroblasts (3T3 cells).

Although MCMV-1 does not play a significant role as a natural pathogen of laboratory mice, it is frequently used as a model for human cytomegalovirus infection [45]. These aspects have been discussed in detail by Shellam et al. [24]. MCMV-1 has also been used as a vaccine vector aiming at a disseminating mouse control agent by inducing immunocontraception in mice [46]. The virus is known to influence immune reactions in infected mice [47, 48] and may therefore have an impact on immunological research [6, 8].

Mouse thymic virus (MTV) or murid herpesvirus 3 (MuHV-3)

MTV was detected during studies in which samples from mice were passaged in newborn mice. Unlike other herpesviruses, MTV is difficult to culture *in vitro* and is usually propagated by intraperitoneal infection of newborn mice. The thymus is removed 7-10 days later, and thymus suspensions serve as virus material for further studies. The prevalence of MTV is believed to be low in laboratory mice, and for

this reason, and also due to the difficulties in virus production for serological assays, it is not included in many standard diagnostic or surveillance testing protocols. Limited data are available indicating that it is common in wild mice [8, 49]. Further, MTV obviously represents a significant source of contamination of MCMV-1 (and vice versa) if virus is prepared from salivary glands, since both viruses cause chronic or persistent salivary gland infections and can coinfect the same host.

All mouse strains are susceptible to infection, but natural or experimental infection of adult mice is subclinical. Gross lesions appear only in the thymus and only if experimental infection occurs at an age of less than about 5 days. Infection results in nuclear inclusions in thymocytes and their almost complete destruction within 2 weeks. Virus is present in the thymus but may also be found in the blood and in salivary glands of surviving animals. Salivary glands are the only site yielding positive virus isolations if animals are infected as adults. The virus persists here and is shed via saliva for months. MTV also establishes a persistent infection in athymic $Foxn1^{nu}$ mice, but virus shedding is reduced compared to euthymic mice, with virus recovery possible only in a lower percentage of mice [50].

Pathological changes caused by MTV occur in the thymus, and reduced thymus mass due to necrosis in suckling mice is the most characteristic gross lesion [36]. Lymphoid necrosis may also occur in lymph nodes and spleen [51], with necrosis and recovery similar to that in the thymus. In mice infected during the first 3 days after birth, necrosis of thymus becomes evident within 3–5 days, and the animals' size and weight are markedly reduced at day 12–14. Intranuclear inclusions may be present in thymocytes between days 10–14 after infection. The thymus and the affected peripheral tissues regenerate within 8 weeks after infection. Regardless of the age of mice at infection, a persistent infection is established in the salivary glands, and infected animals shed virus for life.

Several alterations of immune responses are associated with neonatal MTV infection. There is transient immunosuppression, attributable to lytic infection of T lymphocytes, but activity (e.g. response of spleen cells to T-cell mitogens) returns to normal as the histological repair

progresses [51]. Selective depletion of CD4+ T cells by MTV results in autoimmune disease [52, 53]. Information about additional influences on the immune system is given in textbooks [6, 8].

In experimentally infected newborn mice, oral and intraperitoneal infections similarly result in thymus necrosis, seroconversion and virus shedding, suggesting that the oral–nasal route is likely to be involved in natural transmission [54]. The virus spreads to cagemates after long periods of contact. It is transmitted between mice kept in close contact, and transmissibility from cage to cage seems to be low. MTV is not transmitted to fetuses by the transplacental route, and intravenous infection of pregnant mice does not lead to congenital damage, impairment in size or development, or abortion [55].

MTV and MCMV-1 do not cross-react serologically [16]. Serological monitoring of mouse populations for antibodies to MTV is possible by indirect immunofluorescent assay (IFA) testing, which is commercially available; enzyme-linked immunosorbent assays (ELISA) tests have also been established [41, 56]. ELISA and complement fixation yield similar results [57]. Serological tests based on recombinant proteins and direct detection of virus by PCR are currently not possible because the genome of the virus has not yet been sequenced. It must be noted that the immune response depends on the age at infection. Antibody responses are not detectable in mice infected as newborns, whereas adult mice develop high titres that are detectable by serological testing. If neonatal infection is suspected, homogenates of salivary glands or other materials can be inoculated into pathogen-free newborn mice followed by gross and histological examination of thymus, lymph nodes and spleens for lymphoid necrosis [49]. Alternatives to the *in vivo* infectivity assay for detecting MTV in infected tissues include a competition ELISA [58] and MAP testing, although this is slightly less sensitive than infectivity assays [59].

There is very little experience of eradication methods for MTV because of its low prevalence in contemporary mouse colonies. Methods that eliminate other herpesviruses will likely eliminate MTV. Procurement of animals of known negative MTV status is an appropriate strategy to prevent infection. Strict separation of laboratory mice from wild rodents is essential to avoid

introduction of the virus into laboratory animal facilities.

Other murid herpesviruses

Murid herpesvirus 2 (MuHV-2) or rat cytomegalovirus infects rats and is also a member of the genus *Muromegalovirus*. Murid herpesvirus 4 (MuHV-4) is a member of the genus *Rhadinovirus* in the subfamily Gammaherpesvirinae and is also known as mouse herpesvirus strain 68 (MHV-68). Other murid herpesviruses are not yet assigned to a subfamily within the family Herpesviridae. Among these is murid herpesvirus 3 (mouse thymic herpesvirus), but also murid herpesvirus 5 (field mouse herpesvirus) which infects voles (*Microtus pennsylvanicus*), murid herpesvirus 6 (sand rat nuclear inclusion agent), and murid herpesvirus 7 [60]. Furthermore, a gammaherpesvirus of house mice (*Mus musculus*) has been described recently which is clearly distinct from MHV-68 [61].

Experimental infection of laboratory mice with MHV-68 is a frequently used model system for the study of human gammaherpesvirus pathogenesis, e.g. of Kaposi's sarcoma-associated herpesvirus or Epstein–Barr virus (EBV) [62, 63] which are members of the same subfamily. They are also important models to study viral latency and immune mechanisms controlling latency [64–66]. *Mus musculus* is not the natural host for this virus; it was first isolated in Slovakia from bank voles (*Myodes glareolus*). Additional closely related strains (MHV-60, MHV-72) exist from the same host species, and similar strains (MHV-76, MHV-78) were isolated from wood mice (*Apodemus flavicollis* and *Apodemus sylvaticus*). *Apodemus* sp. seem to be the major hosts for MHV-68 in Great Britain [67]. Different virus strains exhibit different genetic and biological properties and also differ in their pathogenicity, e.g. for *Prkdc^scid* mice [68].

Infections in laboratory mice take the same course as in their natural hosts [69]. There are, however, some differences as, e.g. higher virus levels are reached in the lungs of BALB/c mice, and wood mice develop higher titres of neutralizing antibodies [70]. House mice develop an acute infection in the lungs after intranasal infection. A latent infection develops within 2 weeks and the virus persists lifelong in epithelial cells in the lungs and also spreads to the spleen and other organs (e.g. bone marrow, peritoneal cells) where it persists in different cells of the immune system. It behaves like a natural pathogen in inbred strains of mice and persists without causing disease.

Mousepox (ectromelia) virus

The mousepox (ectromelia) virus (ECTV) is a member of the genus *Orthopoxvirus* belonging to the family Poxviridae. It is antigenically and morphologically very similar to vaccinia virus and other orthopoxviruses. Poxviruses are the largest and most complex of all viruses, with a diameter of 200 nm and a length of 250–300 nm. Mousepox (ectromelia) virus contains one molecule of double-stranded DNA with a total genome length of nearly 210 000 nucleotides [71]. It is the causative agent of mousepox, a generalized disease in mice. Experimental transmission to young rats (up to 30 days of age) is possible [72]. Unlike various other orthopoxviruses, ectromelia virus does not infect humans [73].

The virus is resistant to desiccation, dry heat and many disinfectants. It is not consistently inactivated in serum heated for 30 min at 56 °C [3, 74] and remains active for months when maintained at 4 °C in fetal bovine serum [75]. Effective disinfectants include vapour-phase formaldehyde, sodium hypochlorite and iodophores [8, 76].

Historically, ECTV has been an extremely important natural pathogen of laboratory mice. The virus was widespread in mouse colonies worldwide and can still be found in several countries. Between 1950 and 1980 almost 40 individual ectromelia outbreaks were reported in the USA. The last major epizootic in the USA occurred in 1979–80 and has been described in great detail [77]. Severe outbreaks were also described in various European countries [78–80]. A more recent outbreak in the USA, which resulted in the eradication of almost 5000 mice in one institution, was described by Dick et al. [81]. Another recent and well-documented case of mousepox was published by Lipman et al. [3]. Few additional but unpublished cases of ectromelia have been observed since then; the latest report of an outbreak was published in 2009 [74]. In general, positive serological reactions are occasionally

reported from routine health surveillance studies [17] but the virus is extremely rare in European and American colonies of laboratory mice [13-15].

Natural infections manifest differently, depending on many factors. Mousepox may occur as a rapidly spreading outbreak with acute disease and deaths, or may be inconspicuous with slow spreading and mild clinical signs and may therefore be very difficult to diagnose [81]. The mortality rate can be very low in populations in which the virus has been present for long periods. The infection usually takes one of three clinical courses: acute asymptomatic infection, acute lethal infection (systemic form) or subacute to chronic infection (cutaneous form) [8, 81-83]. The systemic or visceral form is characterized clinically by facial oedema, conjunctivitis, multisystemic necrosis and usually high mortality. This form is less contagious than the cutaneous form because the animals die before there is virus shedding. The cutaneous form is characterized by typical dermal lesions and variable mortality. The outcome of infection depends on many factors including strain and dose of virus; route of viral entry; strain, age, and sex of mouse; husbandry methods and duration of infection in the colony. While all mouse strains seem to be susceptible to infection with ECTV, clinical signs and mortality are strain-dependent [84-86]. Acute lethal (systemic) infection occurs in highly susceptible inbred strains such as DBA/1, DBA/2, BALB/c, A and C3H/HeJ. Immunodeficient mice may also be very susceptible [87]. Outbreaks among susceptible mice can be explosive, with variable morbidity and high mortality (>80%). Clinical disease may not be evident in resistant strains such as C57BL/6 and AKR, and the virus can be endemic in a population for long periods before being recognized. Furthermore, females seem to be more resistant to disease than males, at least in certain strains of mice [84, 85]. Killer cells are necessary to control mousepox infections [88]. Mice that are resistant to mousepox may lose their resistance with increasing age, most likely due to the decreased number and activity of NK cells [89].

The mechanisms determining resistance versus susceptibility are not fully understood but appear to reflect the action of multiple genes. The genetic loci considered to be important include $H2D^b$ (termed $Rmp3$, resistance to mousepox), on chromosome 17 [90]; the $C5$ genes ($Rmp2$, on chromosome 2); $Rmp1$, localized to a region on chromosome 6 encoding the NK cell receptor NKR-P1 alloantigens [91]; the *nitric oxide synthase 2* locus on chromosome 11 [92]; and the *signal transducer and activator of transcription 6* locus on chromosome 10 [93]. Mousepox infections are controlled for several days during the initial course of infection by the complement system until the adaptive immune system can react. Loss of the complement system results in lethal infection [94]. Clearance of the virus by the immune system is dependent upon the effector functions of CD8+ T cells while NK cells, CD4+ T cells and macrophages are necessary for the generation of an optimal response [95, 96]. T- and B-cell interactions and antibodies play a central role during recovery from a secondary infection [97].

Mousepox (ectromelia) virus usually enters the host through the skin with local replication and extension to regional lymph nodes [8, 82, 86]. It escapes into the blood (primary viraemia) and infects splenic and hepatic macrophages, resulting in necrosis of these organs and a massive secondary viraemia. This sequence takes approximately 1 week. Many animals die at the end of this stage without premonitory signs of illness; others develop varying clinical signs including ruffled fur, hunched posture, swelling of the face or extremities, conjunctivitis and skin lesions (papules, erosions or encrustations mainly on ears, feet and tail; Figure 3.2.1). Necrotic amputation of limbs and tails can sometimes be seen in mice that survive the acute phase, hence the

Figure 3.2.1 The rash of mousepox in a hairless (hr) mutant mouse. *From Deerberg* et al. *[78]; with permission from Schlütersche Verlagsgesellschaft.*

Figure 3.2.2 Dry gangrene of the left hind foot of a mouse infected with ECTV.

original name of the disease: 'ectromelia' means absent or short limbs (Figure 3.2.2).

Common gross lesions of acute mousepox include enlarged lymph nodes, Peyer's patches, spleen and liver; multifocal to semiconfluent white foci of necrosis in the spleen and liver; and haemorrhage into the small intestinal lumen [36, 81, 86, 87]. In animals that survive, necrosis and scarring of the spleen can produce a mosaic pattern of white and red-brown areas that is a striking gross finding.

The most consistent histological lesions of acute mousepox are necroses of the spleen (Figure 3.2.3), lymph nodes, Peyer's patches, thymus and liver [3, 36, 81, 86, 87]. Occasionally, necrosis may also be observed in other organs such as ovaries, uterus, vagina, intestine and lungs. The primary skin lesion, which occurs

about a week after exposure at the site of inoculation (frequently on the head), is a localized swelling that enlarges from inflammatory oedema. Necrosis of dermal epithelium provokes a surface scab and heals as a deep, hairless scar. Secondary skin lesions (rash) develop 2–3 days later as the result of viraemia (Figure 3.2.1). They are often multiple and widespread and can be associated with conjunctivitis. The skin lesions also can ulcerate and scab before scarring. Mucosal and dermal epithelial cells may have characteristic intracytoplasmic eosinophilic (Cowdry type A) inclusion bodies (Figure 3.2.4). Basophilic (Cowdry type B) inclusions may be found in the cytoplasm of all infected cells, especially in hepatocytes.

Natural transmission of ECTV mainly occurs by direct contact and fomites [8, 82, 98, 99]. The primary route of infection is through skin abrasions. Faecal–oral and aerosol routes may also be involved [98]. In addition, the common practice of cannibalism by mice may contribute to the oral route of infection [99]. Intrauterine transmission is possible at least under experimental conditions [100]. Virus particles are shed from infected mice (mainly via scabs and/or faeces) for about 3–4 weeks, even though the virus can persist for months in the spleen of an occasional mouse [8, 99]. Cage-to-cage transmission of ECTV and transmission between rooms or units is usually low and largely depends on husbandry practices (e.g. mixing mice from

Figure 3.2.3 Section of the spleen of a mouse infected with ectromelia virus. There is marked parenchymal necrosis with extensive cellular debris and only few lymphoid cells left (H&E stain, magnification 200×). *Courtesy of Professor A. D. Gruber.*
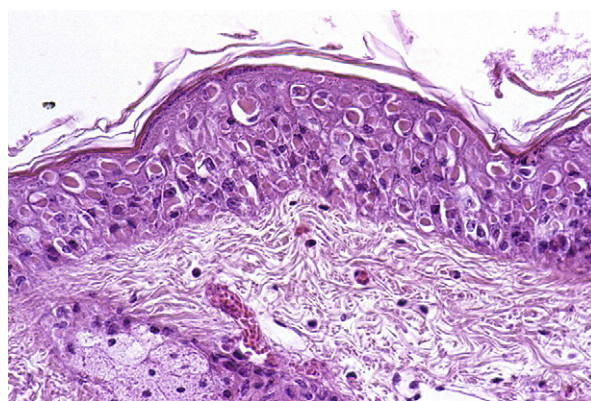
Figure 3.2.4 Section of the skin of a mouse infected with ECTV. Cutaneous hyperplasia with epithelial cell degeneration and numerous large intra-epithelial cytoplasmic viral inclusion bodies (Cowdry type A) are seen (H&E stain, magnification 400×). *Courtesy of Professor A. D. Gruber.*

different cages). Importantly, the virus may not be transmitted effectively to sentinel mice exposed to dirty bedding [3].

Various tests have been applied for the diagnosis of ectromelia. Previous epidemics were difficult to deal with because of limited published data and information on the biology of the virus and the lack of specific and sensitive assays [101]. In the 1950s, diagnosis relied on clinical signs, histopathology and animal passages of tissues from moribund and dead animals. Culture of the virus on the chorioallantoic membrane of embryonated eggs was also used. Serology is currently the primary means of routine health surveillance for testing mouse colonies for exposure to ECTV. The methods of choice are MFIA, ELISA and IFA; they are more sensitive and specific than the previously used haemagglutination inhibition (HI) assay [41, 102, 103]. Serological tests based on virus particles detect antibodies to orthopoxviruses and do not distinguish between ECTV and vaccinia virus or other orthopoxviruses, respectively. Vaccinia virus is commonly used as an antigen for serological testing to avoid the risk of infection for mice. Thus, false-positive serological reactions may be found after experimental administration of replication-competent vaccinia virus. It has been shown that even cage contact sentinels may develop antibodies, and vaccinia virus leading to seroconversion may even be transmitted by dirty bedding [104]. Confirmation of positive serological results is important before action is taken because vaccinia virus is increasingly prevalent in animal facilities as a research tool (e.g. for vaccination or gene therapy). As observed in different outbreaks, serological testing is of little value in the initial stages of the disease. For example, in the outbreak described by Dick et al. [81] depopulation was nearly completed before serological confirmation was possible. For this reason, negative serological results should be confirmed by direct detection methods (PCR, immunohistochemistry, virus isolation) or by histopathology, especially when clinical signs suggestive of mousepox are observed. PCR assays to detect different genes of poxviruses in infected tissues have been used [3, 81, 105]. Other PCR tests which were developed to detect smallpox virus have also been shown to detect ectromelia virus and can be used as well [106, 107].

The key to prevention and control of mousepox is early detection of infected mice and contaminated biological materials. All institutions that must introduce mice from other than commercial barrier facilities should have a health surveillance programme and test incoming mice. Perhaps even more important than living animals are samples from mice (tumours, sera, tissues). The virus replicates in lymphoma and hybridoma cell lines [108], and such cells or material derived from them may therefore be a vehicle for inadvertent transfer between laboratories. The last three published outbreaks of ectromelia were introduced into the facilities by mouse serum [3, 74, 81]. Lipman et al. [3] found that the contaminated serum originated from a pooled lot of 43 L that had been imported from China, but in both other cases, serum was obtained from animals in the USA. Because mouse serum is commonly sold to the end user in small aliquots (a few millilitres), it has to be expected that aliquots of the contaminated lot may still be stored in freezers. These published cases of ectromelia outbreaks provide excellent examples of why testing should be performed on all biological materials to be inoculated into mice. In the case of ectromelia virus it was shown that PCR is more sensitive, and MAP testing failed to detect contamination [74].

Eradication of mousepox has usually been accomplished by elimination of the affected colonies, disinfection of rooms and equipment, and disposal of all infected tissues and sera. While culling of entire mouse colonies is the safest method for eradication of mousepox, it is not a satisfactory method because of the uniqueness of numerous lines of genetically modified animals housed in many facilities. Several studies indicate that mousepox is not highly contagious [75, 84, 99] and that it may be self-limiting when adequate husbandry methods are applied. Therefore, strict quarantine procedures along with cessation of breeding (to permit resolution of infection) and frequent monitoring, with removal of clinically sick and seropositive animals, are a potential alternative. The period from the last births until the first matings after cessation of breeding should be at least 6 weeks [99]. Sequential testing of immunocompetent contact sentinels for seroconversion should be employed with this option.

In the past, immunization with live vaccinia virus was used to suppress clinical expression of mousepox. Vaccination may substantially reduce the mortality rate, but it does not prevent virus transmission or eradicate the agent from a population [109, 110]. After vaccination, typical pocks develop at the vaccination site, and infectious vaccinia virus is detectable in spleen, liver, lungs and thymus [111]. Vaccination also causes seroconversion so that serological tests are not applicable for health surveillance in vaccinated populations. It is therefore more prudent to control mousepox by quarantine and serological surveillance than by relying on vaccination.

Mortality and clinical disease are the major factors by which ECTV interferes with research. Severe disruption of research can also occur when drastic measures are taken to control the infection. The loss of time, animals and financial resources can be substantial.

Experimental mousepox infections are frequently used as a model to study various aspects of smallpox infections of humans [112-114]. Mousepox shares many aspects of virus biology and pathology, and models the course of human smallpox. Experimental mousepox infections are used to study vaccination procedures [115, 116] or anti-poxvirus therapies [117].

Murine adenoviruses

Murine adenoviruses (MAdV) are non-enveloped, double-stranded DNA viruses of the family Adenoviridae. Two distinct strains have been isolated from mice. The FL strain (MAdV-1) was first isolated in the USA as a contaminant of a Friend leukaemia [118] and has been classified as a member of the genus Mastadenovirus. The K87 strain (MAdV-2) was isolated in Japan from the faeces of a healthy mouse [119] and has not yet been assigned to a genus. Both strains are considered to represent different species [120-122]. They are host species specific and are not infectious for infant rats [123]. MAdV-1 can be cultured in vitro in mouse fibroblasts (e.g. 3T6 or L929 cells), MAdV-2 is usually cultured in vitro in a mouse rectum carcinoma cell line (CMT-93). In laboratory mice, seropositivity to adenoviruses was reported to be very low [11, 14, 15, 17, 18, 124] or negative [12, 13]. Antibodies to MAdV are also found in wild mice [21, 125] and in rats [21, 126].

Neither virus is known to cause clinical disease in naturally infected, immunocompetent mice. However, MAdV-1 can cause a fatal systemic disease in suckling mice after experimental inoculation [118, 127, 128]. Disease is characterized by scruffiness, lethargy, stunted growth and often death within 10 days. Experimental infection of adult mice with MAdV-1 is most often subclinical and persistent but can cause fatal haemorrhagic encephalomyelitis with neurological symptoms, including tremors, seizures, ataxia and paralysis in susceptible C57BL/6 and DBA/2J mice [129]. BALB/c mice are relatively resistant to this condition. Athymic $Foxn1^{nu}$ mice experimentally infected with MAdV-1 develop a lethal wasting disease [130]. Similarly, $Prkdc^{scid}$ mice succumb to experimental infection with MAdV-1 [131].

Gross lesions in response to natural MAdV infections are not detectable. Occasional lesions observed after experimental infection with MAdV-1 include small surface haemorrhages in the brain and spinal cord of C57BL/6 and DBA/2J mice [129], duodenal haemorrhage in $Foxn1^{nu}$ mice [130] and pale yellow livers in $Prkdc^{scid}$ mice [131].

Histologically, experimental MAdV-1 infection of suckling mice is characterized by multifocal necrosis and large basophilic intranuclear inclusion bodies in liver, adrenal gland, heart, kidney, salivary glands, spleen, brain, pancreas and brown fat [8, 36, 127, 132]. In experimentally induced haemorrhagic encephalomyelitis, multifocal petechial haemorrhages occur throughout the brain and spinal cord, predominantly in the white matter, and are attributed to infection and damage to the vascular epithelium of the central nervous system (CNS) [129]. Histopathological manifestations in MAdV-1-infected $Prkdc^{scid}$ mice are marked by microvesicular fatty degeneration of hepatocytes [131]. In contrast to MAdV-1, the tissue tropism of MAdV-2 is limited to the intestinal epithelium. Naturally or experimentally infected mice develop intranuclear inclusions in enterocytes, especially in the ileum and caecum [8, 36, 133].

Transmission of MAdV primarily occurs by ingestion. MAdV-1 is excreted in the urine and may be shed for up to 2 years [134]. MAdV-2 infects the intestinal tract and is shed in faeces for only a few weeks in immunocompetent

mice [135]; immunodeficient mice may shed the virus for longer periods [136].

Murine adenovirus infections are routinely diagnosed by serological tests. However, there is a one-sided cross-reactivity of MAdV-1 with MAdV-2 [137]. Serum from mice experimentally infected with MAdV-1 yielded positive reactions in serological tests with both viruses, while serum from mice infected with MAdV-2 reacted only with the homologous antigen [138]. Smith et al. [126] reported that sera might react with MAdV-1 or MAdV-2 or both antigens. Occasional reports of mice with lesions suggestive of adenovirus infections and negative serology (with MAdV-1) indicate that the infection may not be detected if only one virus is used as an antigen [139]. It is therefore usual to test sera for antibodies to both MAdV-1 and MAdV-2. The commonly used methods are IFA, ELISA and MFIA.

The low prevalence in colonies of laboratory mice indicates that MAdV can easily be eliminated (e.g. by hysterectomy derivation or embryo transfer) and that barrier maintenance has been very effective in preventing infection.

The low pathogenicity and the low prevalence in contemporary mouse populations are the main reasons why adenoviruses are considered to be of little importance, which is also indicated by the fact that recent publications about murine adenoviruses are very rare. However, the viruses might easily be spread by the exchange of genetically modified mice and therefore re-emerge. Only a few influences on research attributable to MAdV have been published. For example, it has been shown that MAdV-1 significantly aggravates the clinical course of scrapie disease in mice [140]. Natural infections with MAdV could also interfere with studies using adenovirus as a gene vector.

Other murine adenoviruses

A novel murine adenovirus classified as a Mastadenovirus has recently been isolated from a striped field mouse (*Apodemus agrarius*) [141]. It was cultured in Vero E6 cells and named MAdV type 3 (MAdV-3). It revealed the highest similarity to MAdV-1 but it represents a separate serotype. However, there is some cross-reactivity between MAdV-3 and both other mouse viruses [142]. In addition to serological and antigenic differences it also shows a unique organotropism and infects predominantly the heart tissue of C57BL/6N mice after experimental infection. Experimentally infected mice show no clinical signs. The virus is not easily transmitted from experimentally infected mice to contact sentinels [142].

Polyomaviruses

Polyomaviridae are enveloped, double-stranded DNA viruses. Two different agents of this family exclusively infect mice (*Mus musculus*), and both belong to the genus *Polyomavirus*. Murine pneumotropic virus (MPtV) was formerly known as 'newborn mouse pneumonitis virus' or 'K virus' (named after L. Kilham who first described the virus). The second is murine polyomavirus (MPyV). Both are related, but antigenically distinct, from each other [143], and also virus-like particles from the major capsid protein (VP1) do not cross-react [144]. They are enzootic in many populations of wild mice but are very uncommon in laboratory mice. Even older reports indicate that both have been eradicated from the vast majority of contemporary mouse colonies, and their importance is negligible [8]. Seropositivity to these viruses was not reported in a recent survey conducted in the USA [13], and other publications also indicate that these viruses do not presently play a significant role in laboratory mice [11, 14, 15]. Because of their low prevalence, neither virus is included in the list of agents for which testing is recommended on a regular basis by FELASA [145].

Although polyomavirus genes, especially those of SV40, are widely used in gene constructs for insertional mutagenesis, very few reports have been published on spontaneous or experimental disease due to MPyV or MPtV in the last 10–15 years. The reader is therefore referred to previous review articles for details [8, 146].

Murine pneumotropic virus (MPtV)

Natural infections with MPtV are subclinical. The prevalence of infection is usually low in an infected population. The virus may persist in infected animals for months and perhaps for life depending on the age at infection and is reactivated under conditions of immunosuppression.
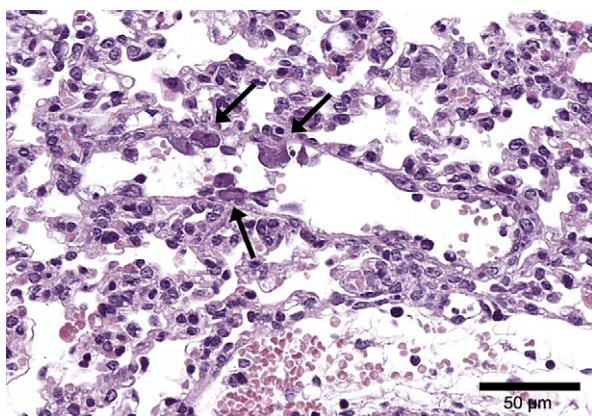
Figure 3.2.5 Section of the lung of a mouse infected with MPtV (K virus). Mild lymphohistiocytic interstitial pneumonia and large amphophilic to basophilic intranuclear inclusion bodies are visible (H&E stain). *Courtesy of Professor A. D. Gruber.*

Virus replicates primarily in endothelial cells, but renal tubular epithelial cells are the major site of viral persistence [147, 148].

Clinical signs are observed only after infection of infant mice less than 6–8 days of age. Infected pups suddenly develop respiratory symptoms after an incubation period of approximately 1 week, and many die within a few hours of onset of symptoms with an interstitial pneumonia caused by productive infection of and damage to pulmonary endothelium (Figure 3.2.5). Endothelial cells in other organs are also involved
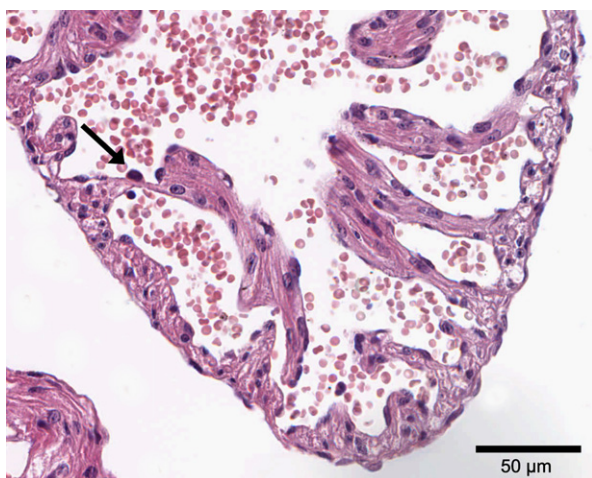
Figure 3.2.6 Section of the right auricle of a mouse infected with MPtV (K virus). Endothelial cell containing large amphophilic to basophilic intranuclear inclusion bodies (H&E stain). *Courtesy of Professor A. D. Gruber.*

in virus replication [148, 149] (Figure 3.2.6). In older suckling mice, MPtV produces a more protracted infection, and the virus or viral antigen can be detected for as long as 4 months. In adult animals, the virus produces a transient asymptomatic infection. Even in immunodeficient *Foxn1^{nu}* mice, experimental infection of adults is clinically asymptomatic, although virus is detectable for a period of several months [150].

In vitro cultivation of MPtV is difficult. No susceptible permanent cell line is known to support growth. It can be cultured in primary mouse embryonic cells, but viral titres are not sufficient for use in serological assays [151]. For this reason, the HI test using homogenates of livers and lungs of infected newborn mice is still frequently used, but IFA and ELISA tests are also available [152]. Furthermore, a PCR test for demonstration of MPtV in biological samples has also been published [153].

Murine polyomavirus (MPyV)

MPyV was first detected as a contaminant of murine leukaemia virus (MuLV) when sarcomas developed in mice after experimental inoculation of contaminated samples. It has later been shown to be a frequent contaminant of transplantable tumours [1]. Natural infection of mice is subclinical, and gross lesions including tumours are usually not found. Tumour formation occurs when mice are experimentally infected at a young age or when inoculated with high virus doses. Development of tumours may be preceded by multifocal necrosis and mortality during the viraemic stage [36]. Parotid, salivary gland and mammary tumours are common, and sarcomas or carcinomas of kidney, subcutis, adrenal glands, bone, cartilage, teeth, blood vessels and thyroid also occur. Virus strains vary with regard to the tumour types or lesions that they induce, and mouse strains vary in their susceptibility to different tumour types. Those of C57BL and C57BR/cd lineage are considered to be the most resistant strains; athymic *Foxn1^{nu}* mice are considered to be most susceptible; C3H mice are particularly susceptible to adrenal tumours and A mice tend to develop bone tumours. Immunosuppression or inoculation into immunodeficient strains (e.g. *Foxn1^{nu}*) also supports the growth of tumours. On the other hand, experimental infection of

adult immunocompetent mice does not result in tumour formation because the immune response suppresses tumour growth, and newborn immunocompetent mice develop runting only if inoculated with high virus doses [154].

After experimental intranasal infection, MPyV initially infects the respiratory tract followed by a systemic phase in which liver, spleen, kidney and colon become infected [155]. The virus is shed in faeces and in all body fluids, and transmission occurs rapidly by direct contact between animals, but also between cages in a room. Further, intrauterine transmission has been documented after experimental infection [156]. MPyV persists in all organs in $Prkdc^{scid}$ mice while viral DNA is detectable in immunocompetent mice after experimental infection for only a limited period of about 4 weeks [157]. However, virus may persist and can be reactivated by prolonged immunosuppression [158] or during pregnancy, at least in young mice [159]. It has been shown that interferon-gamma is an important factor of the host defence against tumour formation and MPyV infection [160]. Biological materials of mouse origin are likely to be the most common source of contamination of laboratory mice, emphasizing the importance of MAP or PCR screening of biological materials to be inoculated into mice.

The most frequently used tests for health surveillance of mouse colonies are ELISA, MFIA and IFA; in addition, the HI test is still used. Latent infections can be detected by intracerebral inoculation of neonate mice or by MAP testing, but direct demonstration of virus in biological samples is also possible by PCR testing [153].

While MPyV infections are of low importance for laboratory animal medicine, the virus is used in models of persistent virus infection [161, 162]. Virus-like particles from both murine polyomaviruses have been used as a vector for gene therapy or vaccines [163, 164].

Parvoviruses

Parvoviruses are non-enveloped small viruses (approximately 20 nm in diameter) with a single-stranded DNA genome of approximately 5000 nucleotides. Murine parvoviruses are members of the family Parvoviridae, genus *Parvovirus*.

They are remarkably resistant to environmental conditions like heat, desiccation, acidic and basic pH-values. Up to date, two distinct species that infect laboratory mice are officially listed: the minute virus of mice (MVM), previously named mice minute virus (MMV), and the mouse parvovirus (MPV). Non-structural proteins (NS-1 and NS-2) are highly conserved among both viruses whereas the capsid proteins (VP-1, VP-2, VP-3) are more divergent and determine the serogroup [165]. Both viruses require mitotically active cells for replication. Severe clinical signs are therefore not found in mature animals because of the lack of a sufficient number of susceptible cells in tissues. General aspects of rodent parvovirus infections and their potential effects on research results have been reviewed [6, 8, 166–170].

Mouse parvovirus (MPV)

Already in the mid-1980s mouse colonies were identified that gave positive reactions for MVM by IFA but not by HI tests. It was subsequently shown that these colonies were infected with a novel parvovirus, initially referred to as 'mouse orphan parvovirus'. The first isolate of MPV was detected as a contaminant of cultivated T-cell clones interfering with *in vitro* immune responses [171] and was named 'mouse parvovirus'. It does not replicate well in currently available cell cultures, and sufficient quantities of virus for serological tests are difficult to generate. Hitherto, only very few isolates of MPV have been cultured and subsequently characterized on a molecular basis [165, 172]. On the basis of epidemiological analyses, further parvoviruses were recently identified in mice, sequenced, and tentatively named serially MPV-2 and MPV-3 [173], MPV-4 (GenBank FJ440683) and MPV-5 (GenBank FJ441297). In addition, several variants are published for MPV-1 [172, 174, 175].

At present, MPV is among the most common viruses found in colonies of laboratory mice. The prevalence of sera positive for parvoviruses ranged from 1% to nearly 10% in Western Europe and North America, with the majority of sera being positive for MPV in studies differentiating between the two parvovirus species [12, 14, 15, 176]. These prevalence data are based on testing at commercial laboratories and do not reflect that, despite highly specific and sensitive test methods,

enzootic parvovirus infections are difficult to detect due to virus-associated characteristics [169, 170]. A recent survey conducted in the USA showed that during a 24–36 month period mouse parvoviruses were detected at almost all facilities that responded to a questionnaire, with MPV being more often diagnosed than MVM [13].

Clinical disease and gross or histological lesions have not been reported for mice naturally or experimentally infected with MPV. Infections are subclinical even in newborn and immuno-compromised animals [177, 178]. In contrast to many other viruses infecting mice, viral replication and excretion is not terminated by the onset of host immunity. Tissue necrosis has not been observed at any stage of infection in infected infant or adult mice [177, 178]. Humoral immunity to MPV does not protect against MVM infections, and vice versa [179].

Serological surveys have indicated that MPV naturally infects only mice, with the exception that MPV-3 shows genetic similarity to hamster parvovirus, suggesting that a cross-species transmission has occurred, where the mouse probably served as the natural host [173, 180]. Differences in mouse strain susceptibility to clinical MPV infection do not exist. However, seroconversion seems to be strain-dependent. After experimental infection with MPV-1b, seroconversion occurred in all C3H/HeN mice, fewer BALB/c, DBA/2 and ICR mice, and seroconversion could not be detected in C57BL/6 mice [181]. Upon MPV-1f inoculation, antibody response was absent in BALB/cArc mice [182]. Diagnosis of MPV infection by PCR testing of small intestine and mesenteric lymph nodes also depended on the mouse strain. MPV DNA was detected in all mouse strains evaluated except DBA/2 even though seroconversion was detected in these mice.

After oral infection, the intestine is the primary site of viral entry and replication. The virus spreads to the mesenteric lymph nodes and other lymphoid tissues, where it persists for more than 2 months [178], and seems to be excreted via the intestinal and the urinary tract. After experimental inoculation of weanling mice, MPV is transmitted to cagemates by direct contact for 2–6 weeks [177], and transmission by dirty bedding is also possible. These results implicate a role for urinary, faecal, and perhaps

respiratory excretion of virus. Another study showed that naturally infected mice might not transmit the virus under similar experimental conditions [183].

Serology is a useful tool to identify MPV infections in immunocompetent hosts, but reaching a diagnosis based on serological assays may be difficult and requires a good knowledge of the available techniques. Neither the virion ELISA nor HI is a practical screening test for MPV because they require large quantities of purified MPV, which is difficult to obtain. Diagnosis of MPV infections has long been made on the basis of an MVM HI-negative result coupled with an MVM IFA-positive result. IFA provides the opportunity to detect both serogroup-specific VP proteins as well as NS proteins that are conserved among mouse parvoviruses. A generic rodent parvovirus ELISA using a recombinant NS-1 protein as antigen has been developed [184], but MPV IFA and MPV HI assays are more sensitive techniques than the NS-1 ELISA and the MVM IFA [181]. In contrast, ELISA tests that use recombinant VP-2 provide sensitive and serogroup-specific assays for the diagnosis of MPV infections in mice [176, 185], although considerable cross-reactivity with heterologous capsid antigens exists [173]. Nevertheless, when using the ELISA technique, one needs to consider that MPV-2 may not consistently be detected by MPV-1 VP-2 ELISA [168, 173], especially when antibody titres are low (own observations). Therefore, ELISAs using MPV-2 VP-2 and MPV-3 VP-2 antigens are also used for diagnostics. As parvovirus diagnostics using recombinant assays should be based on a combination of antigens, bead-based mulitplex assays are a convenient extension of traditional ELISA, allowing the use of multiple antigens simultaneously.

In immunodeficient mice that do not generate a humoral immune response, PCR assays can be used to detect MPV [186, 187] and other parvoviruses. MPV has been shown to persist for at least 9 weeks in the mesenteric lymph nodes [178]. This tissue is considered the best suited for PCR analysis, but spleen and small intestine can also be used with good success [181]. For antemortem detection, shedding of parvoviruses can also be detected by PCR of faecal samples [188]. The virus persists sufficiently long in mesenteric lymph nodes so that PCR

assays may also be used as a primary screening tool for laboratories that do not have access to specific MPV antigen-based serological assays. The PCR is further a good confirmatory method for serological assays and has also been described for the detection of parvoviruses in cell lines and tumours [189]. In addition, the MAP test has been reported as a sensitive tool to detect MPV [183].

Given the high environmental stability of the virus and the potential fomite transmission, together with the long virus persistence in infected animals, spontaneous disappearance from a mouse population (e.g. by cessation of breeding) is unlikely. Eradication of infection is possible by elimination of infected animals and subsequent replacement with uninfected mice, and the agent can be eliminated from breeding populations by embryo transfer or by hysterectomy. It should be noted that recent studies suggest a risk of virus transmission by embryo transfer, though successful sanitation of immunodeficient mice was achieved despite antibody response in recipients and progeny after embryo transfer [190, 191].

Although there are few published reports of confounding effects of MPV on research, it is lymphocytotropic and may perturb immune responses *in vitro* and *in vivo*. Infections with MPV have been shown to influence rejection of skin and tumour grafts [192].

Minute virus of mice (MVM)

MVM is the type species of the genus *Parvovirus*. The virus was intermediately named mice minute virus (MMV). It was originally isolated by Crawford [193] from a stock of mouse adenovirus, and this prototype isolate was later designated MVMp. Its allotropic variant was detected as a contaminant of a transplantable mouse lymphoma [194] and designated MVMi because it exhibits immunosuppressive properties *in vitro*. Both variants have distinct cell tropisms *in vivo* and *in vitro*. MVMp infects fibroblast cell lines and does not cause clinical disease [195, 196]. MVMi grows lytically in T cells and inhibits various functions mediated by these cells *in vitro*. Both strains are apathogenic for adult mice, but the immunosuppressive variant is more pathogenic for neonatal mice than is MVMp. A third strain, the Cutter strain MVMc, was isolated

from BHK-21 cells [172]. In contrast to these three strains detected as cell culture contaminants, an isolate was obtained from naturally infected mice with a B-cell maturational defect maintained at the University of Missouri and therefore denominated MVMm [173].

Serological surveys show that the mouse is the primary natural host [19, 125, 197], but the virus is also infective for rats, hamsters [168, 198], and *Mastomys* [199] during fetal development or after parenteral inoculation.

Natural infections are usually asymptomatic in adults and infants, and the most common sign of infection is seroconversion. Kilham and Margolis [200] observed mild growth retardation a few days after experimental infection of neonatal mice with MVMp. Studies of transplacental infection yielded no pathological findings in mice [201]. The immunosuppressive variant, but not the prototype strain, is able to produce a runting syndrome after experimental infection of newborn mice [195]. Depending on the host genotype, experimental infections of fetal and neonatal mice with MVMi produce various clinical presentations and lesions. Infection in C57BL/6 mice is asymptomatic, but the virus causes lethal infections with intestinal haemorrhage in DBA/2 mice. Infection of strains such as BALB/c, CBA, C3H/He and SJL is also lethal and mice have renal papillary haemorrhage [196]. The MVMi also infects haematopoietic stem cells and mediates an acute myelosuppression [202, 203]. Because of its dependence on mitotically active tissues, the fetus is at particular risk for damage by parvoviruses. MVM and other parvoviruses may have severe teratogenic effects and cause fetal and neonatal abnormalities by destroying rapidly dividing cell populations, often resulting in fetal death. Adult *Prkdc^scid* mice develop an acute leukopenia 1 month after experimental infection with MVMi and die within 3 months. The virus persists lifelong in the bone marrow of these mice [204]. During a natural concurrent outbreak of MVMm and MPV, a runting syndrome with lymphohistiocytic renal inflammation and inclusion bodies in cells resembling splenic haematopoietic progenitor cells was reported in B-cell (*Ighm*)-deficient mice [205].

MMV is shed in faeces and urine. In faecal samples, MVM was detected for up to 4–6 weeks

by PCR [206, 207], although shorter periods (9-12 days) have been observed [208]. Notably, shedding re-occurred after immunosuppression by irradiation [207]. Contaminated food and bedding are important factors in viral transmission because the virus is very resistant to environmental conditions. Direct contact is also important and the virus does not easily spread between cages.

Routine health surveillance is usually conducted by serological methods. Unlike MPV, MVM can easily be cultured in cell lines so that antigen production for HI and ELISA (using whole purified virions) is easy. HI is a highly specific diagnostic test whereas IFA always exhibits some degree of cross-reactivity with MPV and other closely related parvoviruses. ELISA is probably the most frequently used test, but depending on the purity of the antigen preparation, cross-reactions with MPV may occur due to contamination with non-structural proteins that are common to both viruses. This problem can be avoided by the use of recombinant VP-2 antigen [176]. By using serological methods, one needs to consider that the mouse strain has a considerable effect on seroconversion so that an antibody response might not be detectable despite infection; while C57BL/6J mice showed good antibody response, seroconversion was observed only in some BALB/c, AKR/N, DBA/2J, FVB/N and C3H/HeN, but not in NMRI and ICR mice upon contact exposure to MVMi-inoculated mice [206]. Viral detection is also possible by PCR in biological materials, organs (intestine, mesenteric lymph node, kidney, spleen) and faeces from infected animals [187, 189, 206, 207, 209]. Although MVM was not thought to cause persistent infection in immunocompetent mice, recent data show that it can be detected in spleens for up to 16 weeks after exposure in some mouse strains [207]. Therefore, PCR may be considered as a confirmatory method for serology.

The virus can be eliminated from infected breeding populations by caesarean derivation or by embryo transfer. However, certain precautions such as careful washing and accompanying testing need to be minded, as MVM has been detected in reproductive organs and gametes and this virus firmly attaches to the zona pellucida or might even cross it [210, 211]. In experimental

colonies, elimination of infected animals and subsequent replacement with uninfected mice is practical if careful environmental sanitation is conducted by appropriate disinfection procedures. It is important that reintroduction is avoided by exclusion of wild mice and by strict separation from other infected populations and potentially contaminated materials in the same facility. Admission of biological materials must be restricted to samples that have been tested and found to be free from viral contamination.

Both allotropic variants of MVM have been used as models for molecular virology, and their small size and simple structure have facilitated examination of their molecular biology and expedited understanding of cell tropism, viral genetics and structure. The significance for laboratory mouse populations was considered low or uncertain because natural infections are inapparent. However, various effects on mouse-based research have been published [6, 7, 166, 167, 170]. Because of their predilection for replicating in mitotically active cells, they are frequently associated with tumour cells and have a marked oncosuppressive effect [212]. Special attention is also necessary for immunological research and other studies involving rapidly dividing cells (embryology, teratology). In addition, MVM is a common contaminant of transplantable tumours, murine leukaemias and other cell lines [1, 2, 213].

RNA viruses

Lactate dehydrogenase-elevating virus

Lactate dehydrogenase-elevating virus (LDV) is a single-stranded RNA virus of the genus *Arterivirus* belonging to the family Arteriviridae. The genome organization and replication of LDV and other arteriviruses, their cell biology and other molecular aspects have been reviewed by Snijder and Meulenberg [214]. LDV has repeatedly been detected in wild mice (*Mus musculus*), which are considered to be a virus reservoir [215, 216]. After infection of mice, virus titres of 10^{10}–10^{11} particles per ml serum are found within 12-14 h after infection. The virus titre drops to 10^5 particles per ml within 2-3 weeks and remains

constant at this level for life. It persists in infected mice for the whole lifetime although it stimulates various immune mechanisms [216–219]. The virus can be stored in undiluted mouse plasma at −70 °C without loss of infectivity, but it is not stable at room temperature and is very sensitive to environmental conditions. Only mice and primary mouse cells are susceptible to infection with LDV. It replicates in a subpopulation of macrophages in almost all tissues and persists in lymph nodes, spleen, liver, and testes tissues [220]. As suitable cell systems have not been available for virus production, routine serology has not been easily possible so that testing for LDV was not included in serological health monitoring programs. The prevalence of LDV in contemporary colonies of laboratory rodents is likely to be very low but detailed information about its prevalence comparable to most other agents is not available.

LDV was first detected during a study of methods that could be used in the early diagnosis of tumours [221]. It produces a persistent infection with continuous virus production and a lifelong viraemia despite LDV-specific immune reactions of the host [217]. LDV has been found in numerous biological materials that are serially passaged in mice such as transplantable tumours including human tumours or matrigel prepared from such materials [1, 2, 222, 223], monoclonal antibodies or ascitic fluids [224], or infectious agents (e.g. haemoprotozoans, K virus, *Clostridium piliforme*). These materials are contaminated after serial passage in an infected and viraemic mouse. Contamination with LDV leads to the infection of each sequential host and to transmission of the virus by the next passage and remains associated with the specimen. It is therefore the most frequently detected contaminant in biological materials [1, 2].

Infection with LDV is usually asymptomatic, and there are no gross lesions in immunocompetent as well as in immunodeficient mice. The only exception is poliomyelitis with flaccid paralysis of hindlimbs developing in C58 and AKR mice when they are immunosuppressed either naturally with ageing or experimentally. It has been shown that only mice harbouring cells in the CNS that express a specific endogenous MuLV are susceptible to poliomyelitis [225].

The characteristic feature of LDV infection is the increased activity of lactate dehydrogenase (LDH) and other plasma enzymes [8, 226], which is due to the continuous destruction of permissive macrophages that are responsible for the clearance of LDH from the circulation. As a consequence, the activity of plasma LDH begins to rise by only 24 h after infection and peaks 3-4 days after infection at 5-10-fold normal levels, or can even be up to 20-fold in SJL/J mice. The enzyme activity declines during the next 2 weeks but remains elevated throughout life.

Antigen–antibody complexes produced during infection circulate in the blood and are deposited in the glomeruli [226]. In contrast to other persistent virus infections (e.g. lymphocytic choriomeningitis virus), these complexes do not lead to immune complex disease and produce only a very mild glomerulopathy. The only gross finding associated with LDV infection is mild splenomegaly. Microscopically, necrosis of lymphoid tissues is visible during the first days of infection. In mouse strains that are susceptible to poliomyelitis, LDV induces lesions in the grey matter of the spinal cord and the brainstem.

LDV is not easily transmitted between mice, even in animals housed in the same cage. Fighting and cannibalism increase transmission between cagemates, most likely via blood and saliva. Infected females transmit the virus to their fetuses if they have been infected few days prior to birth and before IgG anti-LDV antibodies are produced, but developmental and immunological factors (e.g. gestational age, timing of maternal infection with LDV, placental barrier) are important in the regulation of transplacental LDV infection [227, 228]. Maternal immunity protects fetuses from intrauterine infection. Immunodeficient $Prkdc^{scid}$ mice also transmit virus to their offspring during chronic infection [229]. An important means of transmission is provided by experimental procedures such as mouse-to-mouse passage of contaminated biological materials or the use of the same needle for sequential inoculation of multiple mice.

In principle, serological methods such as IFA may be used for detecting LDV infection [230] but they are not of practical importance. Circulating virus–antibody complexes interfere with serological tests, and sufficient quantities of virus for serological tests are difficult to generate because LDV replicates only in specific subpopulations of primary cultures of murine

macrophages and monocytes for one cell cycle [226]. However, it is meanwhile possible to use recombinant viral proteins of LDV as antigens [231] in ELISA and MFIA tests so that routine testing by serology is possible. In the past, diagnosis of LDV infection has primarily been based on increased LDH activity in serum or plasma of mice. LDV activity in serum or plasma can be measured directly, or samples (e.g. plasma, cell or organ homogenates) are inoculated into pathogen-free mice and the increase in LDH activity within 3-4 days is measured. An 8-10-fold increase is indicative of LDV infection. Detection of infectivity of a plasma sample by the induction of increased LDH activity in the recipient animal is the most reliable means of identifying an infected animal. However, it is important to use clear non-haemolysed samples because haemolysis will (falsely) elevate activities of multiple serum or plasma enzymes, including LDH. This assay was usually included in a 'MAP test', but antibody detection similar to other viruses was not involved for reasons mentioned earlier. Persistent infection makes LDV an ideal candidate for PCR detection in plasma or in organ homogenates [232, 233]. However, reports exist that PCR may produce false-negative results and should be used cautiously [234]. Just as important as detecting LDV in animals is its detection in biological materials. This may be done by assay for increased LDH activity after inoculation of suspect material into pathogen-free mice [1, 2] or by PCR [232, 233, 235-237].

LDV spreads slowly in a population because direct contact is necessary. Therefore, LDV-negative breeding populations can easily be established by selecting animals with normal plasma LDH activity. Embryo transfer and hysterectomy derivation are also efficient. The presence of LDV in experimental populations may be indicative of contaminated biological materials. In such cases, it is essential that the virus is also eliminated from these samples. This is easily achieved by maintenance of cells by *in vitro* culture instead of by animal-to-animal passages [238]. Due to the extreme host specificity of the virus, contaminated tumour samples can also be sanitized by passages in nude rats [223] or other animal species. Another method to remove LDV from contaminated cells, which is based on cell sorting, has recently been described [239].

LDV is a potential confounder of any research using biological materials that are passaged in mice. Once present in an animal, the virus persists lifelong. The most obvious signs are increased levels of plasma LDH and several other enzymes. LDV may also exhibit numerous effects on the immune system (thymus involution, depression of cellular immunity, enhanced or diminished humoral responses, NK cell activation, development of autoimmunity, and suppression of development of diabetes in NOD mice); [218, 219, 224, 240-244] and enhance or suppress tumour growth [6, 7, 226]. Interaction with other viruses has also been described [245].

Lymphocytic choriomeningitis virus (LCMV)

Lymphocytic choriomeningitis virus (LCMV) is an enveloped, segmented single-stranded RNA virus of the genus *Arenavirus* family, Arenaviridae. It can easily be propagated in several commonly used cell lines like BHK-21 cells. However, cells are not lysed and a cytopathic effect (CPE) is not visible. The virus name refers to the condition that results from experimental intracerebral inoculation of the virus into adult mice and is not considered to be a feature of natural infections. Mice (*Mus musculus*) serve as the natural virus reservoir [246], but Syrian hamsters are also important hosts [247]. Additional species such as rabbits, guinea-pigs, squirrels, monkeys and humans are susceptible to natural or experimental infection [248]. Natural infection of callitrichid primates (marmosets and tamarins) leads to a progressive hepatic disease that is known as 'callitrichid hepatitis' [249, 250]. Antibodies to LCMV have been found in wild mice in Europe [251, 252], Africa [253], Asia [254], Australia [125] and America [255]. Thus, it is the only arenavirus with worldwide distribution. Infection with LCMV is rarely found in laboratory mice [248]. Seropositivity to LCMV in laboratory mice was reported to be low during the last decade [11, 15, 17, 124] or negative [12-14]. In addition to laboratory mice and other vertebrate hosts, the virus has frequently been found in transplantable tumours and tissue culture cell lines from mice and hamsters [2, 256].

Despite the low prevalence in laboratory mice, seropositivity to this zoonotic agent should raise serious concern for human health. LCMV is frequently transmitted to humans from wild mice and is also endemic to a varying degree in the human population [257-261] due to contact with wild mice. It has also been transmitted to humans by infected laboratory mice [262] and by pet and laboratory Syrian hamsters [263-266]. In addition, contaminated biological materials are important sources of infections for humans, and several outbreaks of LCM among laboratory personnel have been traced to transplantable tumours [267, 268]. Transmission of LCMV to humans also occurred repeatedly by organ transplantation and was most likely transmitted to organ donors by close contact with infected pets [266, 269]. LCMV can cause mild-to-serious or fatal disease in humans [262, 270, 271]. Congenital infection in humans may result in hydrocephalus, or fetal or neonatal death [272].

In mice, clinical signs of LCMV infection vary with strain and age of mouse, strain and dose of virus, and route of inoculation [8, 248, 251]. Two forms of natural LCMV infection are generally recognized: a persistent tolerant and an (acute) non-tolerant form. The persistent form results from infection of mice that are immunotolerant. This is the case if mice are infected in utero or during the first days after birth. This form is characterized by lifelong viraemia and viral shedding. Mice may show growth retardation, especially during the first 3-4 weeks, but they appear otherwise normal. Infectious virus is bound to specific antibodies and complement, and these complexes accumulate in the renal glomeruli, the choroid plexus, and sometimes also in synovial membranes and blood vessel walls. At 7-10 months of age, immune complex nephritis develops with ruffled fur, hunched posture, ascites and occasional deaths. This immunopathologic phenomenon is called 'late onset disease' or 'chronic immune complex disease'. The incidence of this type of disease varies between mouse strains. Gross lesions include enlarged spleen and lymph nodes due to lymphoid hyperplasia. Kidneys affected with glomerulonephritis may be enlarged with a granular surface texture or may be shrunken in later stages of the disease process. Microscopically, there is generalized lymphoid hyperplasia and immune complex deposition in glomeruli and vessel walls, resulting in glomerulonephritis and plasmacytic, lymphocytic perivascular cuffs in all visceral organs [36].

The non-tolerant acute form occurs when infection is acquired after the development of immunocompetence (in mice older than 1 week). These animals become viraemic but do not shed virus and may die within a few days or weeks. Natural infections of adults are usually asymptomatic. Surviving mice are seropositive and in most cases clear the virus to below detection levels of conventional methods. However, virus may persist at low levels in tissues (particularly spleen, lung and kidney) of mice for at least 12 weeks after infection as determined by sensitive assays such as nested reverse transcriptase (RT)-PCR or immunohistochemistry [273]. Such non-lethal infection leads to protection against otherwise lethal intracerebral challenge. Protection from lethal challenge is also achieved by maternally derived anti-LCMV antibodies through nursing or by the administration of anti-LDV monoclonal IgG2a antibodies [274].

In experimentally infected mice, the route of inoculation (subcutaneous, intraperitoneal, intravenous, intracerebral) also influences the type and degree of disease [248]. Intracerebral inoculation of adult immunocompetent mice typically results in tremors, convulsions and death due to meningoencephalitis and hepatitis. Neurological signs usually appear on day 6 after inoculation, and animals die within 1-3 days after the onset of symptoms, or recover within several days. The classic histological picture is of dense perivascular accumulations of lymphocytes and plasma cells in meninges and choroid plexus. While infection following subcutaneous inoculation usually remains inapparent, reaction of mice to intraperitoneal or intravenous inoculation depends on the virus strain and on the mouse strain. Infection by these routes primarily causes multifocal hepatic necrosis and necrosis of lymphoid cells. Athymic $Foxn1^{nu}$ mice and other immunodeficient mice do not develop disease but become persistently viraemic and shed virus.

As a general rule, all pathological alterations following LCMV infection are immune-mediated; and mice can be protected from LCMV-induced disease by immunosuppression [275]. LCMV disease is a prototype for virus-induced

T-lymphocyte-mediated immune injury and for immune complex disease. For detailed information on the pathogenesis, clinical and pathological features of LCMV infection, the reader is referred to review articles [248, 276, 277].

In nature, carrier mice with persistent infection serve as the principal source of virus. Intra-uterine transmission is very efficient, and with few exceptions all pups born from carrier mice are infected. Furthermore, persistently infected mice and hamsters can shed large numbers of infectious virions primarily in urine, but also in saliva and milk. The virus can replicate in the gastric mucosa after intragastric infection [278, 279]. Gastric inoculation elicits antibody responses of comparable magnitudes as intravenous inoculation and leads to active infection with LCMV, indicating that oral infection is possible, e.g. by ingestion of contaminated food or by cannibalism. A self-limiting infection frequently results from infection of adult mice. The virus does not spread rapidly after introduction in populations of adult mice, and the infectious chain usually ends. However, if the virus infects a pregnant dam or a newborn mouse, a lifelong infection results, and soon a whole breeding colony of mice may become infected if the mice live in close proximity (which is the case under laboratory conditions). The virus is not easily transmitted to dirty-bedding sentinels, and it is important that colony animals or animals having had direct contact with a population are tested to exclude LCMV infection [280].

LCMV is most commonly diagnosed by serological methods such as MFIA, IFA and ELISA [281]. All strains show a broad cross-reactivity and are serologically uniform. However, subclinical persistent infections may be difficult to detect because they may be associated with minimal or undetectable levels of circulating antibody. It is important that bleeding of mice is done carefully because of a potential risk due to viraemic animals. Historically, direct viral detection was performed by inoculating body fluids or tissue homogenates into the brain of LCMV-free mice or by subcutaneous injection into mice and subsequent serological testing (MAP test). More recently, PCR assays have been developed for the direct detection of viral RNA in clinical samples or animals [282-284]. Both MAP test and PCR can also be used to detect contamination of biological materials [235, 237]. Specifically for exclusion of contamination by LCMV, it was requested by different authorities that virus is inoculated intracerebrally at a lethal dose 3–4 weeks after administration of the material to be tested. In case of contamination by LCMV and subsequent seroconversion, animals survive the challenge infection.

Vertical transmission of LCMV by transuterine infection is efficient so that this virus cannot reliably be eliminated by caesarean rederivation [280]. Caesarean derivation may be effective if dams acquired infection after the development of immunocompetence (non-tolerant acute infection) and subsequently eliminated the virus, but such a strategy is difficult to justify in light of LCMV's zoonotic potential. In breeding colonies of great value, virus elimination might be possible soon after introduction into the colony by selecting non-viraemic breeders. This procedure is expensive and time consuming and requires special safety precautions.

Fortunately, infections of laboratory mice with LCMV are very uncommon. However, once LCMV has been detected in animals, or in biological materials, immediate destruction of all contaminated animals and materials is advisable to avoid risk of human infection. $Foxn1^{nu}$ and $Prkdc^{scid}$ mice may pose a special risk because infections are silent and chronic [268]. Cages and equipment should be autoclaved, and animal rooms should be fumigated with disinfectants such as formaldehyde, vaporized paraformaldehyde, hydrogen peroxide or other effective disinfectants. Prevention of introduction into an animal facility requires that wild mice cannot get access to the facility. Similarly important is screening of biological materials originating from mice and hamsters because these can be contaminated by LCMV. Finally, it has been shown that the virus can also be introduced into a population by mice with an undetected infection [280].

Appropriate precautions are necessary for experiments involving LCMV, or LCMV-infected animals or materials. Biological safety level (BSL) 2 will be considered to be sufficient in most cases. BSL 3 practices may be considered when working with infected animals owing to the increased risk of virus transmission by bite wounds, scratching or aerosol formation from the bedding. Animal

Biosafety Level (ABSL) 3 practices and facilities are generally recommended for work with infected hamsters. Appropriate precautions have been defined for different BSLs or ABSLs by CDC [285].

LCMV is frequently utilized as a model organism to study virus-host interactions, immunological tolerance, virus-induced immune complex disease, and a number of immunological mechanisms *in vivo* and *in vitro* [286-288]. Accidental transmission may have a severe impact on various kinds of experiments [6, 7, 248, 251] and also affect infection with other agents [289].

Mammalian orthoreovirus serotype 3 (MRV-3)

Mammalian orthoreoviruses (MRV) are non-enveloped, segmented double-stranded RNA viruses of the family Reoviridae, genus *Orthoreovirus*. They have a wide host range and are ubiquitous throughout the world. The designation *reo* stands for respiratory enteric orphan and reflects the original isolation of these viruses from human respiratory and intestinal tract without apparent disease. The term 'orphan' virus refers to a virus in search of a disease. Mammalian orthoreovirus can be grouped into three serotypes, numbered 1-3. Mammalian orthoreovirus-3 (synonyms: hepatoencephalomyelitis virus; ECHO 10 virus) infection remains prevalent in contemporary mouse colonies and has been reported in wild mice [20, 125, 290]. A study in France reported antibodies to MRV-3 in 9% of mouse colonies examined [10]. In more recent studies in North America and western Europe, such antibodies were detected in 0.01-0.2% of mice monitored [11, 14, 15]. Schoondermark-van de Ven et al. [12] found antibodies to MRV-3 in 0.6% of mouse samplings from western European institutions; and in a survey conducted by Carty [13], about 6% of responding institutions in the USA reported MRV-3 infection in their mouse colonies. In addition, contamination of mouse origin tumours and cell lines by MRV-3 has been reported many times [2, 8, 290]. Experimentally, MRV-3 infection of infant mice has been used to model human hepatobiliary disease, pancreatitis, diabetes mellitus and lymphoma [8, 291].

The literature on MRV-3 infections in mice is dominated by studies on experimentally infected animals. The virus can cause severe pantropic infection in infant mice [290-292]. After parenteral inoculation, virus can be recovered from the liver, brain, heart, pancreas, spleen, lymph nodes and blood vessels. Following oral inoculation, reoviruses gain entry by infecting specialized epithelial cells (M cells) that overlie Peyer's patches. The virus then becomes accessible to leukocytes and spreads to other organs by way of the lymphatic system and the bloodstream. Neural spread to the CNS has also been well documented [293, 294]. The mechanisms of viral pathogenesis and their interactions with the host cell as well as the host's immune response are reviewed in detail by Tyler et al. [295], Schiff et al. [296] and Ward et al. [291].

Natural infection by MRV-3 in a mouse colony is usually subclinical, although diarrhoea or steatorrhoea and oily hair effect in suckling mice may be noted [8, 36, 290-292]. The latter term has been used to describe the matted, unkempt appearance of the hair coat that results from steatorrhoea due to pancreatitis, maldigestion and biliary atresia. In addition, runting (attributed to immune-mediated destruction of cells in the pituitary gland that produce growth hormone), transient alopecia, jaundice (due to excessive bilirubin in the blood, which is attributed to the liver pathology, especially biliary atresia) and neurological signs such as incoordination, tremors or paralysis may develop. When present in natural infections, clinical signs and lesions are similar to but milder than in experimental neonatal infections. Early descriptions of naturally occurring disease may have been complicated by concurrent infections such as MHV (murine hepatitis virus) or murine rotavirus A (MuRV-A)/epizootic diarrhoea of infant mice (EDIM) virus that contributed to the severity of the lesions especially in liver, pancreas, CNS and intestine. The outcome of MRV-3 infection depends on age and immunological status of mouse, dose of virus and route of inoculation. Adult immunocompetent mice typically show no clinical signs and have no discernible lesions even in experimental infections. Mucosal and maternally conferred immunity are considered to be important in protection from or resolution of disease [297, 298]. Experimental infection of

adult *Prkdc^{scid}* mice is lethal [299]. Depending on the route of inoculation, experimental infection of adult *Foxn1^{nu}* mice is subclinical or results in liver disease [299, 300].

Histological findings reported to occur after experimental MRV-3 infection of neonatal mice include inflammation and necrosis in liver, pancreas, heart, adrenal, brain, and spinal cord; lymphoid depletion in thymus, spleen, and lymph nodes; and hepatic fibrosis with biliary atresia [36, 290–292, 298].

Transmission of reoviruses probably involves the aerosol as well as the faecal–oral route [8, 291]. Fomites may play an important role as passive vectors because reoviruses resist environmental conditions moderately well.

Serological screening with MFIA, ELISA or IFA is in widespread use for detection of antibodies to MRV-3 in diagnostic and health surveillance programmes. Both ELISA and IFA detect cross-reacting antibodies to heterologous MRV serotypes that can infect mice [301], although a recent report indicates that some IFA-positive MRV infections in mice may not be detected by commonly used ELISAs [302]. The HI test does not detect such cross-reacting antibodies but is prone to give false-positive results due to non-specific inhibitors of haemagglutination [301, 303]. RT-PCR methods for the detection of MRV-3 RNA [304, 305] or MRV RNA [302, 306] are also available. Reports on contamination of mouse origin tumours and cell lines by MRV-3 and its interference with transplantable tumour studies [307, 308] emphasize the importance of screening of biological materials to be inoculated into mice by MAP test or PCR. Natural seroconversion to MRV-3 without clinical disease is also observed in laboratory rats, hamsters and guinea-pigs [8, 290].

Caesarean derivation and barrier maintenance have proven effective in the control and prevention of MRV-3 infection [8, 291].

The virus may interfere with research involving transplantable tumours and cell lines of mouse origin. It has the potential to alter intestinal studies and multiple immune response functions in mice. In enzootically infected colonies, protection of neonates by maternal antibody could complicate or prevent experimental infections with reoviruses. It could further complicate experiments that require evaluation of liver, pancreas, CNS, heart, lymphoid organs and other tissues affected by the virus.

Murine hepatitis virus (MHV)

The term murine hepatitis virus (MHV; commonly referred to as 'mouse hepatitis virus') designates a large group of antigenically and genetically related, single-stranded RNA viruses belonging to the family Coronaviridae, genus *Coronavirus*. They are surrounded by an envelope with a corona of surface projections (spikes). MHV is antigenically related to rat coronaviruses and other coronaviruses of pigs, cattle and humans. Numerous different strains or isolates of MHV have been described. They can be distinguished by neutralization tests that detect strain-specific spike (S) antigens, by use of monoclonal antibodies, or by sequencing [309]. The best-studied strains are the prototype strains MHV-1, MHV-2, MHV-3, JHM (MHV-4), A59, and S, of which MHV-3 is regarded as the most virulent. Like other coronaviruses MHV mutates rapidly, and strains readily form recombinants, so that new (sub)strains are constantly evolving. Strains vary in their virulence, organotropism and cell tropism [310]. Based on their primary organotropism, MHV strains can be grouped into two biotypes: respiratory (or polytropic) and enterotropic. However, intermediate forms (enterotropic strains with tropism to other organs) also exist. Murine hepatitis virus is relatively resistant to repeated freezing and thawing, heating (56 °C for 30 min) and acid pH but is sensitive to drying and disinfectants, especially those with detergent activity [8]. Given the environmental conditions present in mouse rooms, MHV might remain infective for several days, at low humidity (20% relative humidity) or low temperatures (4 °C) even for weeks on surfaces [311].

Mus musculus is the natural host of MHV. It can be found in wild and laboratory mice throughout the world and is one of the most common viral pathogens in contemporary mouse colonies. While polytropic strains have historically been considered more common, this situation is thought to have reversed. Monitoring results for research institutions across North America and Europe indicate that the prevalence of MHV has decreased in the past, though it seems to have remained quite stable since the

1990s [11, 12]. Recently 1.57% of North American laboratory mouse serum samples tested positive [15]. In Europe, prevalence rates ranged from 3.25% to 12% [12, 14, 15]. A retrospective study in France covering the period from 1988 to 1997 reported antibodies to MHV in 67% of mouse colonies examined [10], and a survey performed in 2006 revealed that almost half of North American research institutions detected MHV in their mouse populations [13]. Suckling rats inoculated experimentally with MHV had transient virus replication in the nasal mucosa and seroconversion but no clinical disease [312]. Similarly, deer mice seroconverted but showed no clinical disease after experimental infection [313]. MHV is also a common contaminant of transplantable tumours [1, 2] and cell lines [314, 315].

The pathogenesis and outcome of MHV infections depend on interactions between numerous factors related to the virus (e.g. virulence and organotropism) and the host (e.g. age, genotype, immune status, and microbiological status) [8, 36, 309, 310, 316, 317]. MHV strains appear to possess a primary tropism for the upper respiratory or enteric mucosa. Those strains with respiratory tropism initiate infection in the nasal mucosa and then may disseminate via blood and lymphatics to a variety of other organs because of their polytropic nature. Respiratory (polytropic) strains include MHV-1, MHV-2, MHV-3, A59, S and JHM. Infection of mice with virulent polytropic MHV strains, infection of mice less than 2 weeks of age, infection of genetically susceptible strains of mice or infection of immunocompromised mice favour virus dissemination. Virus then secondarily replicates in vascular endothelium and parenchymal tissues, causing disease of the brain, liver, lymphoid organs, bone marrow and other sites. Infection of the brain by viraemic dissemination occurs primarily in immunocompromised or neonatal mice. Additionally, infection of adult mouse brain can occur by extension of virus along olfactory neural pathways, even in the absence of dissemination to other organs. In contrast, enterotropic MHV strains (e.g. LIVIM, MHV-D, MHV-Y) tend to selectively infect intestinal mucosal epithelium, with no or minimal dissemination to other organs such as mesenteric lymph nodes or liver.

All ages and strains are susceptible to active infection, but disease is largely age related.

Infection of neonatal mice results in severe necrotizing enterocolitis with high mortality within 48 h. Mortality and lesion severity diminish rapidly with advancing age at infection. Adult mice develop minimal lesions although replication of equal or higher titres of virus occurs compared with neonates. The age-dependent decrease in severity of enterotropic MHV disease is probably related to the higher mucosal epithelium turnover in older mice, allowing more rapid replacement of damaged mucosa. Another factor that is of considerable importance to the outcome of MHV infections is host genotype. For example, BALB/c mice are highly susceptible to enterotropic MHV disease while SJL mice, at the other end of the spectrum, are highly resistant [318]. Unlike in polytropic MHV infection where resistance is correlated with reduced virus replication in target cells [319], enterotropic MHV grows to comparable titres in SJL and BALB/c mice at all ages [318]. Therefore, the resistance of the SJL mouse to disease caused by enterotropic MHV seems to be mediated through an entirely different mechanism than resistance to polytropic MHV. Furthermore, mouse genotypes that are susceptible to disease caused by one MHV strain may be resistant to disease caused by another strain [316]. It is therefore not possible to strictly categorize mouse strains as susceptible or resistant. The genetic factors determining susceptibility versus resistance in MHV infections are as yet poorly understood. Both polytropic and enterotropic MHV infections are self-limiting in immunocompetent mice. Immune-mediated clearance of virus usually begins about a week after infection, and most mice eliminate the virus within 3–4 weeks [316, 318, 320]. Humoral and cellular immunity appear to participate in host defences to infection, and functional T cells are an absolute requirement [321-324]. Therefore, immunodeficient mice such as $Foxn1^{nu}$ and $Prkdc^{scid}$ mice cannot clear the virus [317, 325]. Similarly, some genetically modified strains of mice may have deficits in antiviral responses or other alterations that allow the development of persistent MHV infection [326]. Recovered immune mice are resistant to reinfection with the same MHV strain but remain susceptible to repeated infections with different strains of MHV [327-329]. Similarly, maternal immunity protects suckling mice

against homologous MHV strains but not necessarily against other strains [329, 330]. However, maternal immunity, even to homologous strains, depends on the presence of maternally acquired antibody in the lumen of the intestine [330]. Therefore, the susceptibility of young mice to infection significantly increases at weaning.

Most MHV infections are subclinical and follow one of two epidemiological patterns in immunocompetent mice [8, 310]. Enzootic (subclinical) infection, commonly seen in breeding colonies, occurs when a population has been in contact with the virus for a longer period (e.g. several weeks). Adults are immune (due to prior infection), sucklings are passively protected, and infection is perpetuated in weanlings. Epizootic (clinical) infection occurs when the virus is introduced into a naive population (housed in open cages). The infection rapidly spreads through the entire colony. Clinical signs depend upon the virus and mouse strains and are most evident in infant mice. Typically, they include diarrhoea, poor growth, lassitude, and death. In infections due to virulent enterotropic strains, mortality can reach 100% in infant mice. Some strains may also cause neurological signs such as flaccid paralysis of hindlimbs, convulsions and circling. Adult infections are again usually asymptomatic. As the infection becomes established in the colony, the epizootic pattern is replaced by the enzootic pattern. In immunodeficient (e.g. $Foxn1^{nu}$ and $Prkdc^{scid}$) mice, infection with virulent polytropic MHV strains is often rapidly fatal while less virulent strains cause chronic wasting disease [317]. In contrast, adult immunodeficient mice can tolerate chronic infection by enterotropic MHV, with slow emaciation and diarrhoea, or minimal clinical disease [316, 325]. Subclinical MHV infections can be activated by a variety of experimental procedures (e.g. thymectomy, whole body irradiation, treatment with chemotherapeutic agents, halothane anaesthesia) or by coinfections with other pathogens (e.g. *Eperythrozoon coccoides*, K virus; reviewed in [8, 309]).

In most natural infections, gross lesions are not present or are transient and not observed. Gross findings in neonates with clinical signs include dehydration, emaciation, and in contrast to EDIM, an empty stomach [309, 331, 332]. The intestine is distended and filled with watery to mucoid yellowish, sometimes gaseous contents. Haemorrhage or rupture of the intestine can occur. Depending on the virus strain, necrotic foci on the liver [36, 309, 332] and thymus involution [331, 333] may also be seen in susceptible mice. Liver involvement may be accompanied by jaundice and haemorrhagic peritoneal exudate. Splenomegaly may occur as a result of compensatory haematopoiesis [334].

Histopathological changes in susceptible mice infected with polytropic MHV strains include acute necrosis with syncytia in liver, spleen, lymph nodes, gut-associated lymphoid tissue, and bone marrow [8, 36, 309, 316] (Figure 3.2.7). Recently, pulmonary inflammation has been observed in susceptible mouse strains (C3H/HeJ and A/J) after intranasal inoculation with polytropic MHV-1 [335, 336]. Neonatally infected mice can have vascular-oriented necrotizing (meningo)encephalitis with demyelination in the brainstem and periependymal areas. Lesions in peritoneum, bone marrow, thymus and other tissues can be variably present. Mice can develop nasoencephalitis due to extension of infection from the nasal mucosa along olfactory pathways to the brain, with meningoencephalitis and demyelination, the latter of which is thought to be largely T-cell mediated [324]. This pattern of infection regularly occurs after intranasal inoculation of many MHV strains but is a relatively rare event after natural exposure. Syncytium arising from endothelium, parenchyma or leukocytes is a hallmark of infection in many tissues including intestine, lung, liver, lymph nodes, spleen, thymus, brain and bone marrow. Lesions are transient and seldom fully developed in adult immunocompetent mice, but they are manifest in immunocompromised mice. Highly unusual presentations can occur in mice with specific gene defects. For example, granulomatous peritonitis and pleuritis were found in interferon-gamma-deficient mice infected with MHV [337].

Histopathological changes caused by enterotropic strains of MHV are mainly confined to the intestinal tract and associated lymphoid tissues [8, 36, 309, 316]. The most common sites are terminal ileum, caecum and proximal colon. The severity of disease is primarily age-dependent, with neonatal mice being most severely affected. These mice show segmentally
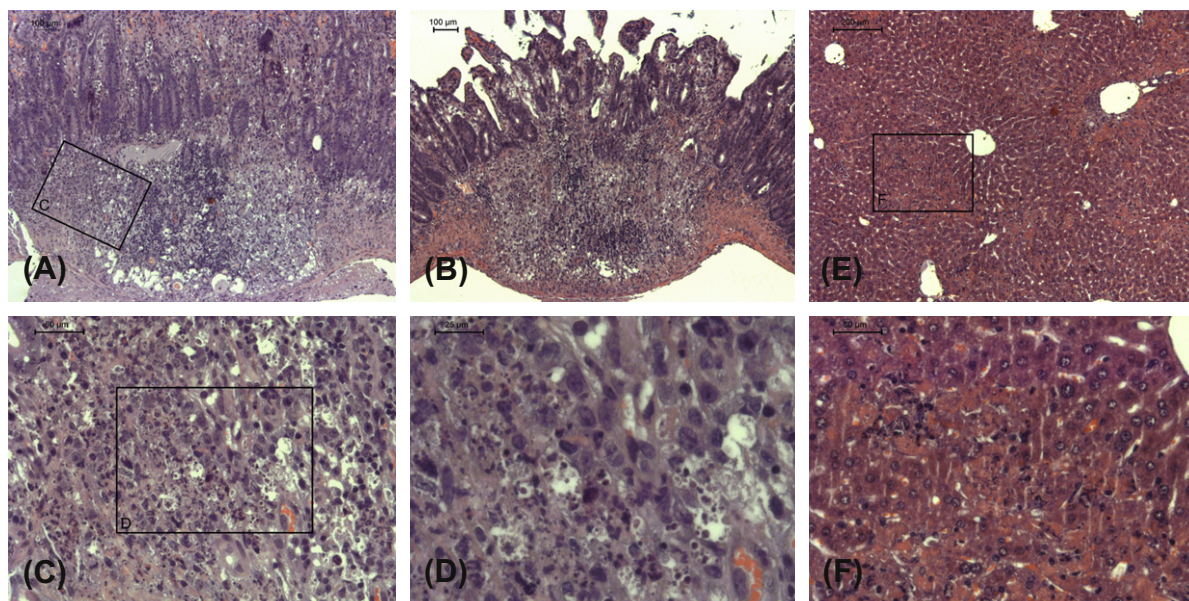
Figure 3.2.7 Mouse infected with a polytropic necrosis in intestine (A-D) and liver (E, F).

distributed areas of villus attenuation, enterocytic syncytia (balloon cells) and mucosal necrosis accompanied by leukocytic infiltration. Intracytoplasmic inclusions are present in enterocytes. Erosions, ulceration, and haemorrhage may be seen in more severe cases. Lesions can be fully developed within 24-48 h, but are usually more severe at 3-5 days after infection. Surviving mice may develop compensatory mucosal hyperplasia. Mesenteric lymph nodes usually contain lymphocytic syncytia, and mesenteric vessels may contain endothelial syncytia. Pathological changes in older mice are generally much more subtle and may only consist of transient syncytia. An occasional exception seems to occur in immunodeficient animals such as *Foxn1[nu]* mice, which can develop chronic hyperplastic typhlocolitis of varying severity [325], but other agents such as *Helicobacter spp.* may have been involved. In general, enterotropic MHV strains do not disseminate, but hepatitis and encephalitis can occur with some virus strains in certain mouse genotypes. In T-cell deficient mice, multisystemic lethal infection was observed after experimental infection with the enterotropic strain MHV-Y [338].

MHV is highly contagious. It is shed in faeces and nasopharyngeal secretions and appears to be transmitted via direct contact, aerosol and fomites [8,309]. Vertical (*in utero*) transmission has been demonstrated in experimental

infections [339] but does not seem to be of practical importance under natural conditions. MHV was transmitted by ovarian transplantation after reproductive organs became infected [340]. However, risk of MHV transmission by sperm or oocytes (IVF) or by embryo transfer seems to be low, though thorough washing of gametes and embryos is required [211, 340-342].

Diagnosis during the acute stage of infection can be made by histological demonstration of characteristic lesions with syncytia in target tissues, but clinical signs and lesions can be highly variable and may not be prominent. Suckling, genetically susceptible or immunocompromised mice are the best candidates for evaluation. Active infection can be confirmed by immunohistochemistry [343] or by virus isolation. Virus recovery from infected tissues is difficult but can be accomplished using primary macrophage cultures or a number of established cell lines such as NCTC 1469 or DBT [301]. These cells, however, may not be successful substrates for some enterotropic MHV strains. Virus in suspect tissue can also be confirmed by bioassays such as MAP testing or infant or *Foxn1[nu]* mouse inoculation [301, 344]. Amplification by passage in these mice increases the likelihood of detection of lesions and antigen, or virus recovery. Other direct diagnostic methods that have been successfully utilized to detect MHV in faeces or tissue of infected mice include

monoclonal antibody solution hybridization assay [345] and a number of RT-PCR assays [346–349]. Because of the transient nature of MHV infection in immunocompetent mice, serology is the most appropriate diagnostic tool for routine monitoring. Multiplex fluorescent immunoassay, ELISA and IFA are well established and sensitive, and all known MHV strains cross-react in these tests [301, 350, 351]. The magnitude of antibody response depends on MHV strain and mouse genotype [319, 352]. DBA/2 mice are poor antibody responders whereas C57BL/6 mice produce a high antibody titre and are therefore good sentinels. Antibody titres remain high over a period of at least 6 months [327, 329]. Infected mice may not develop detectable antibodies for up to 14 days after initial exposure [350]. In such cases, a direct diagnostic method, as discussed above, may be useful. Another drawback of serology is that mice weaned from immune dams can have maternal antibodies until they are 10 weeks of age [353]. This may impact serological monitoring because the possibility must be considered that low positive results are due to maternally derived passive immunity. Because the virus can be transmitted by transplantable tumours and other biological materials from mice, including hybridomas [354] and embryonic stem cells [355, 356] these materials should also be routinely screened for MHV contamination. Mouse inoculation bioassay, MAP test and RT-PCR can be used for this purpose. Therefore, surveillance programmes should combine careful evaluation of clinically ill animals, testing of biological materials and routine health monitoring. Soiled-bedding sentinel mice, which are frequently used for routine monitoring, are likely well suited for detecting enterotropic strains of MHV, but might not indicate the presence of less contagious respiratory strains of MHV [309] equally well. The mouse strain used as sentinel should be considered as a critical factor. Furthermore, duration of MHV shedding and stability of the virus, which seems to be lower in static microisolator cages than in IVC cages, might interfere with detection. The amount of bedding transferred seems not to be as critical as for, e.g. parvoviruses, at least for enterotropic strains [357]. Use of contact and exhaust air sentinels and testing of exhaust filters by PCR was also shown to be effective at detecting MHV [358].

The best means of MHV control is to prevent its entry into a facility. This can be accomplished by purchasing mice from virus-free sources and maintenance under effective barrier conditions monitored by a well-designed quality assurance programme. Control of wild mouse populations, proper husbandry and sanitation, and strict monitoring of biological materials that may harbour virus are also important measures to prevent infection. If infection occurs, the most effective elimination strategy is to cull the affected colony and obtain clean replacement stock. However, this is not always a feasible option when working with valuable mice (e.g. genetically modified lines, breeding stocks). Caesarean derivation or embryo transfer can be used to produce virus-free offspring, and foster-nursing has also been reported to be effective [359]. Quarantine of an affected colony with no breeding and no introduction of new animals for approximately 2 months has been effective in immunocompetent mice [360]. The infection is likely to be terminated because MHV requires a constant supply of susceptible animals. This method works best when working with small numbers of mice. Large populations favour the development of new MHV strains that may result in repeated infections with slightly different strains [361]. It may be practical to select a few future breeders from the infected population and quarantine them for approximately 3 weeks [317]. This can be achieved in isolators, or in individually ventilated cages if proper handling is guaranteed. After this interval, breeding can resume. The 3-week interval should permit recovery from active infection, and the additional 3-week gestation period effectively extends the total quarantine to 6 weeks. It is advisable to select seropositive breeders because the possibility of active infection is lower in such animals. The breeding cessation strategy may not be successful if immunodeficient mice are used because they are susceptible to chronic infection and viral excretion [325]. Genetically engineered mice of unclear, unknown or deficient immune status pose a special challenge because they may develop unusual manifestations of infection or may be unable to clear virus. Rederivation is likely to be the most cost-effective strategy in

such situations. Along with the measures described, proper sanitation and disinfection of caging and animal quarters, as well as stringent personal sanitation, are essential to eliminate infection. Careful testing with sentinel mice should be applied to evaluate the effectiveness of rederivation. If transplantable tumours are contaminated with MHV, virus elimination can be achieved by passage of tumours in athymic *Foxn1^{rnu}* rats [362].

MHV is one of the most important viral pathogens of laboratory mice and has been intensively studied from a number of research perspectives (e.g. as a model organism for studying coronavirus molecular biology or the pathogenesis of viral-induced demyelinating disease). Numerous reports document the effects of natural and experimental infections with MHV on host physiology and research, especially in the fields of immunology and tumour biology (reviewed in [6–8, 310, 316, 317]).

Murine norovirus (MNV)

Noroviruses are non-enveloped, single-stranded RNA viruses with high environmental resistance and belong to the family Caliciviridae, genus *Norovirus*. They were first identified after an outbreak of acute gastroenteritis at a school in Norwalk (Ohio, USA) in 1968 and cause about 90% of non-bacterial epidemic gastroenteritis in humans. Noroviruses found in animals include bovine, porcine and murine noroviruses. Noroviruses are not known to cross species. Murine norovirus (MNV) is endemic in many research mouse colonies and currently the most commonly detected viral agent in laboratory mice [14, 15, 363]. In the hitherto largest survey [15], about 32% of mouse serum samples examined had antibodies against MNV.

The first norovirus to infect mice was described in 2003 [364]. Experimental inoculation studies with this murine norovirus (MNV-1) show that duration of infection and disease manifestation vary depending on the mouse strain [363–365]. In immunocompetent strains, MNV infection is variable in length (e.g. ≥7–14 days in 129S6 mice, ≥5 weeks in Hsd:ICR mice) and does not induce clinical signs. Infection is associated with mild histopathological alterations in the small intestine (increase in inflammatory cells)

and spleen (red pulp hypertrophy and white pulp activation) of 129S6 mice. In certain immunodeficient strains, however, infection can cause lethal systemic disease (encephalitis, vasculitis, meningitis, hepatitis and pneumonia in interferon-alpha-beta-gamma-receptor-deficient and *Stat1^{tm1}* mice) or persist without symptoms (≥90 days in *Rag1^{−/−}* and *Rag2^{−/−}* mice). These findings indicate that components of the innate immune system are critical for resistance to MNV-1 induced disease. Consistent with this hypothesis, it was demonstrated that MNV-1 replicates in macrophages and dendritic cells [366]. Meanwhile, many additional strains of MNV with diverse biological properties were isolated [367, 368]. An analysis of 26 MNV isolates revealed 15 distinct MNV strains that comprise a single genogroup and serotype [368]. Experimental inoculation studies show that several MNV strains are able to persist in various tissues (small intestine, caecum, mesenteric lymph node, spleen) of immunocompetent (C57BL/6J, Hsd:ICR, Jcl:ICR) and immunodeficient (CB17-*Prkdc^{scid}*) mice with viral shedding in faeces for the duration of at least 35–60 days [367–369]. Murine norovirus is transmitted via the faecal-oral route and is efficiently transferred to sentinel mice by soiled bedding [370, 371].

MNV infection can be detected directly by RT-PCR on faecal pellets or tissue specimens (see above) and indirectly by serology (MFIA, ELISA, IFA) [363, 367, 369]. Detection is facilitated by high stability of MNV RNA in faeces (at least 2 weeks at room temperature) [371] and by broad serological cross-reactivity among different strains of MNV [367, 368].

Embryo transfer [370] and hysterectomy [369] are most likely effective means of eliminating MNV from mouse colonies. Since 1- to 3-day-old pups are resistant to infection, elimination of MNV may also be achieved by transferring neonates from infected dams to uninfected foster dams ('cross-fostering') [372]. This transfer should ideally be performed within 24 h after birth.

MNV is used as a surrogate to evaluate resistance of human noroviruses to disinfectants. The impact of MNV on animal experiments remains to be evaluated. Recent studies show that MNV is immunmodulatory and may alter disease phenotypes in mouse models of

inflammatory bowel disease [373–375] and other experimental mouse models [376, 377].

Murine pneumonia virus (PVM)

Murine pneumonia virus, commonly referred to as 'pneumonia virus of mice' (PVM), is an enveloped, single-stranded RNA virus of the family Paramyxoviridae, genus *Pneumovirus*. It is closely related to human respiratory syncytial virus (HRSV). The virus name is officially abbreviated as 'MPV' according to the International Union of Microbiological Societies [9]; however, the former designation 'PVM' will be used in this chapter to avoid confusion with the official abbreviation of mouse parvovirus (MPV). PVM infection remains prevalent in contemporary colonies of mice and rats throughout the world. A serological survey in France demonstrated antibodies to PVM in 16% of mouse colonies examined [10]. In more recent studies in North America and western Europe, the prevalence of PVM-specific antibodies in mice ranged between 0% and 0.1% [11, 14, 15]. Schoondermark-van de Ven et al. [12] found antibodies to PVM in 0.2% of mouse samplings from western European institutions. Antibodies to PVM have also been detected in hamsters, gerbils, cotton rats, guinea-pigs and rabbits [8, 378, 379]. Experimentally, PVM infection of mice is used as a model for HRSV infection and has therefore been extensively studied (reviewed by Rosenberg and Domachowske [380]).

In immunocompetent mice, natural infection with PVM is transient and usually not associated with clinical disease or pathological findings [8, 379, 381]. However, natural disease and persistent infection may occur in immunodeficient mice [382–384]. In particular, athymic *Foxn1^{nu}* mice seem to be susceptible to PVM infection, which can result in dyspnoea, cyanosis, emaciation and death due to pneumonia [383, 384]. Similar clinical signs have been reported for experimentally infected immunocompetent mice [385].

Necropsy findings in naturally infected *Foxn1^{nu}* mice include cachexia and diffuse pulmonary oedema or lobar consolidation [384]. Pulmonary consolidation (dark red or grey in color) has

also been found after experimental infection of immunocompetent mice [381].

Histologically, natural infection of *Foxn1^{nu}* mice with PVM presents as interstitial pneumonia [383, 384]. Experimental intranasal inoculation of immunocompetent mice can result in rhinitis, erosive bronchiolitis and interstitial pneumonia with prominent early pulmonary eosinophilia and neutrophilia [381, 386]. Hydrocephalus may result from intracerebral inoculation of neonatal mice [387]. Susceptibility to infection is influenced by age and strain of mouse, dose of virus, and a variety of local and systemic stressors [8, 379, 386]. In terms of the extent of the alveolar inflammatory response, 129/Sv and DBA/2 mice are susceptible to PVM infection, while BALB/c and C57BL/6 mice are relatively resistant [386]. In terms of the control of viral replication, mice of strains 129/Sv, DBA/2, BALB/c and C57BL/6 are susceptible to PVM infection, while SJL mice are relatively resistant.

PVM is labile in the environment and rapidly inactivated at room temperature [8, 379]. The virus is tropic for the respiratory epithelium [382, 385], and transmission is exclusively horizontal via the respiratory tract, mainly by direct contact and aerosol [8, 379]. Therefore, transmissibility in mouse colonies is low, and infections tend to be focal enzootics.

Serology (MFIA, ELISA, IFA or HI) is the primary means of testing mouse colonies for exposure to PVM. Immunohistochemistry has been applied to detect viral antigen in lung sections [382, 384]; however, proper sampling (see Chapter 4.4, 'Health Management and Monitoring') is critical for establishing the diagnosis due to the focal nature of the infection. An RT-PCR assay to detect viral RNA in respiratory tract tissues has also been reported [388]. However, the use of direct methods requires good timing because the virus is present for only up to about 10 days in immunocompetent mice [381].

Embryo transfer or caesarean derivation followed by barrier maintenance can be used to rear mice that are free of PVM. Because active infection is present in the individual immunocompetent mouse for only a short period, strict isolation of a few (preferably seropositive) mice with the temporary cessation of breeding might also be successful in eliminating the virus [8, 378].

PVM could interfere with studies involving the respiratory tract or immunological measurements in mice. In addition, PVM can have devastating effects on research using immunodeficient mice because they are particularly prone to develop fatal disease [383, 384] or become more susceptible to the deleterious effects of other agents such as *P. murina* [389].

Murine rotavirus A or epizootic diarrhoea of infant mice virus (MuRV-A/EDIM)

MuRV-A/EDIM (commonly referred to as 'mouse rotavirus' or 'epizootic diarrhoea of infant mice virus') is a non-enveloped, segmented double-stranded RNA virus of the family Reoviridae, genus *Rotavirus*. It is antigenically classified as a group A rotavirus, similar to rotaviruses of many other species that cause neonatal and infantile gastroenteritis [291]. MuRV-A/EDIM infection remains prevalent in contemporary mouse colonies and appears to occur worldwide. Large commercial laboratories found 0.6% to 9% of mouse sera from North American and European facilities to be positive for antibodies against MuRV-A/EDIM [11, 12, 14, 15], and up to 30% of mouse colonies in the USA were identified as affected in a survey performed in 2006 [13]. Experimentally, MuRV-A/EDIM infection in mice is used as a model for human rotavirus infection, especially in investigations on the mechanisms of rotavirus immunity and in the development of vaccination strategies [390].

Clinical symptoms following MuRV-A/EDIM infection range from inapparent or mild to severe, sometimes fatal, diarrhoea. 'Epizootic diarrhoea of infant mice' describes the clinical syndrome associated with natural or experimental infection by MuRV-A/EDIM during the first 2 weeks of life [8, 36, 291, 391, 392]. Diarrhoea usually begins around 48 h after infection and persists for about 1 week. Affected suckling mice have soft, yellow faeces that wet and stain the perianal region (Figure 3.2.8). In severe instances, the mice may be stunted, have dry scaly skin, or are virtually covered with faecal material. Morbidity is very high but mortality is usually low.

Gross lesions in affected mice are confined to the intestinal tract. The caecum and colon may be distended with gas and watery to paste-like contents that are frequently bright yellow. The stomach of diarrhoeic mice is almost always filled with milk, and this feature has been reported to be a reliable means to differentiate diarrhoea caused by rotavirus from the diarrhoea caused by MHV infection.

Histopathological changes may be subtle even in animals with significant diarrhoea (Figure 3.2.8). They are most prominent at the apices of villi, where rotaviruses infect and replicate within epithelial cells; the large intestinal surface mucosa may also be affected. Though inflammation is minimal, the lamina propria may be oedematous, lymphatics may be dilated and mild leukocytic infiltration in the large intestinal mucosa and submucosa has been observed in a recent outbreak of disease [36, 393]. Hydropic change of villous epithelial cells is the hallmark finding of acute disease. The villi become
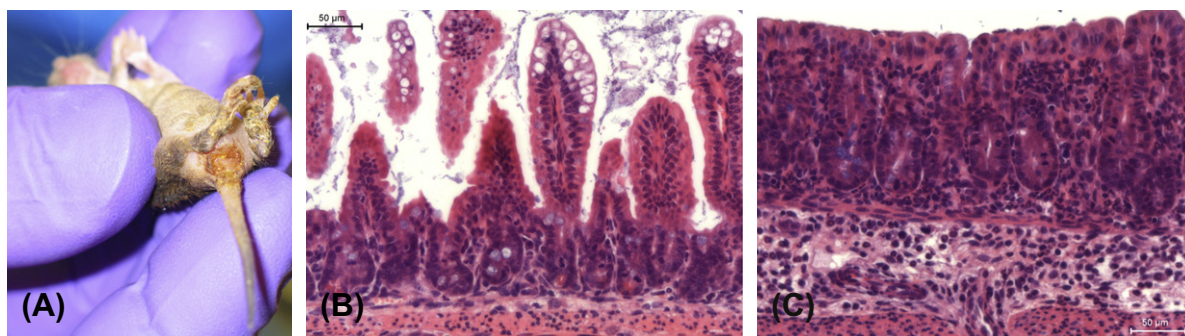
Figure 3.2.8 Clinical and histological presentation of EDIM in an affected suckling during an outbreak of disease. Watery to oily and yellow faeces and inflamed perianal region that appears wet and stained (A). Vacuolation and cytoplasmatic swelling of villar epithelial cells in the small intestine (B) and mixed infiltration of leucocytes in mucosa and submucosa (C) of the colon. *From Held* et al. *[393], used with permission from RSM Press.*

shortened, and the cells that initially replace the damaged cells are less differentiated, typically cuboidal instead of columnar, and lack a full complement of enzymes for digestion and absorption, resulting in diarrhoea due to maldigestion and malabsorption. Undigested milk in the small intestine promotes bacterial growth and exerts an osmotic effect, exacerbating damage to the villi. Intestinal fluid and electrolyte secretion is further enhanced by activation of the enteric nervous system [394] and through the effects of a viral enterotoxin called NSP4 (for non-structural protein 4) [395]. It is hypothesized that NSP4 is released from virus-infected cells and then triggers a signal transduction pathway that alters epithelial cell permeability and chloride secretion.

Susceptibility to EDIM depends on the age of the host and peaks between 4 and 14 days of age [8, 36, 291, 391, 392]. Mice older than about 2 weeks can still be infected with MuRV-A/EDIM, but small numbers of enterocytes become infected, there is little replication of virus and diarrhoea does not occur. The exact reason for this age-related resistance to disease is unknown. Pups suckling from immune dams are protected against EDIM during their period of disease susceptibility [396]. In general, the infection is self-limiting and resolves within days. Successful viral control and clearance is promoted by an intact immune response [396–399], and some immunodeficient mice (e.g. *Prkdc^{scid}* and *Rag2^{tm1Fwa}* mice) may shed virus for extended periods or become persistently infected [400, 401]. Protection against MuRV-A/EDIM reinfection is primarily mediated by antibodies [396, 397].

Murine rotavirus-A/EDIM is highly contagious and transmitted by the faecal-oral route [8, 291, 391]. Dissemination of the virus occurs through direct contact or contaminated fomites and aerosols and is facilitated by the general property of rotaviruses that they remain infectious outside the body, show resistance to inactivation (e.g. low pH, non-ionic detergents, hydrophobic organic liquids, proteolytic enzymes), and are shed in high quantities ($>10^{11}$ particles/g faeces) [291]. MuRV-A/EDIM is stable at $-70\,°C$ but otherwise tends to be susceptible to extreme environmental conditions, detergents and disinfectants containing phenols, chlorine or ethanol [291].

MFIA, ELISA and IFA are in widespread use for detection of serum antibodies to MuRV-A/EDIM in diagnostic and health surveillance programmes; other assay systems such as those using latex agglutination are also used [402]. As MuRV-A/EDIM shares the VP6 protein determined group A antigen, for example, with human, simian or bovine rotavirus strains, commercially available ELISA assays utilizing polyclonal or monoclonal antibodies have been used to detect rotavirus antigen in mice; however, great care must be taken in interpreting the results because some feeds have been reported to cause false-positive reactions with certain ELISA kits [403]. Electron microscopy of faeces of diarrhoeic pups should reveal typical wheel-shaped rotavirus particles, 60–80 nm in diameter. RT-PCR also can be used to detect rotavirus RNA in faecal samples [404]. Good timing is critical for establishing the diagnosis from faeces because virus is shed for only a few days in immunocompetent mice.

Embryo transfer or caesarean derivation followed by barrier maintenance is recommended for rederivation of breeding stocks [8]. In immunocompetent mice in which infection is effectively cleared, a breeding suspension strategy for 8-10 weeks combined with excellent sanitation, filter tops and conscientious serological testing of offspring and sentinel mice has also been reported to be effective, and prolongation of breeding cessation up to 12 weeks resolved infection even in immunocompromised mice [393].

MuRV-A/EDIM has the potential to interfere with any research using suckling mice. It may have a significant impact on studies where the intestinal tract of neonatal or infant mice is the target organ. The infection also poses a problem for infectious disease and immune response studies, particularly those involving enteropathogens in infant mice [405]. A disease-induced stress-related thymic necrosis may occur and alter immunology experiments [36]. In addition, runting could be interpreted erroneously as the effect of genetic manipulation or other experimental manipulation.

Sendai virus (SeV)

Sendai virus (SeV) is an enveloped, single-stranded RNA virus of the family Paramyxoviridae, genus *Respirovirus*. It is antigenically related to

human parainfluenza virus 1. The virus was named after Sendai, Japan, where it was first isolated from mice. Historically, infections were relatively common in mouse and rat colonies worldwide. In addition, there is evidence that hamsters, guinea-pigs and rabbits are susceptible to infection with SeV [8, 301, 406, 407]; however, some apparently seropositive guinea-pigs may in fact be seropositive to other parainfluenza viruses instead of SeV. A study in France reported antibodies to SeV in 17% of mouse colonies examined [10]. A low rate of seropositive mice (0.2%) was found in a survey in North America [11]. Schoondermark-van de Ven et al. [12] also found antibodies to SeV in 0.2% of mouse samplings from western European institutions. In more recent surveys in North America and western Europe, SeV infection was not detected [13-15], indicating that SeV, like most viruses, has meanwhile been eliminated from the majority of mouse colonies. SeV can contaminate biological materials [1].

SeV is pneumotropic and can cause significant respiratory disease in mice. The pneumotropism is partially a consequence of the action of respiratory serine proteases such as tryptase Clara, which activate viral infectivity by specific cleavage of the viral fusion glycoprotein [408]. In addition, the apical budding behaviour of SeV may hinder the spread of virus into subepithelial tissues and subsequently to distant organs via the blood.

Two epidemiologic patterns of SeV infection have been recognized, an enzootic (subclinical) and epizootic (clinically apparent) type [8, 379, 409]. Enzootic infections commonly occur in breeding or open colonies, where the constant supply of susceptible animals perpetuates the infection. In breeding colonies, mice are infected shortly after weaning as maternal antibody levels wane. Normally, the infection is subclinical, with virus persisting for approximately 2 weeks, accompanied by seroconversion that persists for a year or longer. Epizootic infections occur upon first introduction of the virus to a colony and either die out (self-cure) after 2-7 months or become enzootic depending on colony conditions. The epizootic form is generally acute, and morbidity is very high, resulting in nearly all susceptible animals becoming infected within a short time. Clinical signs vary and include rough hair coat, hunched posture, chattering, respiratory distress, prolonged gestation, death of neonates and sucklings and runting in young mice. Breeding colonies may return to normal productivity within 2 months and thereafter maintain the enzootic pattern of infection. Factors such as strain susceptibility, age, husbandry, transport and copathogens are important in precipitating overt disease. DBA and 129 strains of mice are very susceptible to SeV pneumonia, whereas SJL/J and C57BL/6/J and several outbred stocks are relatively resistant. Resistance to SeV infection is under multigenic control with epistatic involvement [410]. There is no evidence for persistent infection in immunocompetent mice, but persistent or prolonged infection may occur in immunodeficient mice and can result in wasting and death due to progressive pneumonia [411, 412]. Clearance of a primary SeV infection is mediated by CD8+ and CD4+ T-cell mechanisms [413, 414].

Heavier than normal, consolidated, plum-colored or grey lungs are a characteristic gross finding in severe SeV pneumonia [8, 36, 379, 409]. Lymphadenopathy and splenomegaly reflect the vigorous immune response to infection.

Histologically, three phases of disease can be recognized in susceptible immunocompetent mice: acute, reparative and resolution phases [36, 409]. Lesions of the acute phase, which lasts 8-12 days, are primarily attributed to the cell-mediated immune response that destroys infected respiratory epithelial cells and include necrotizing rhinitis, tracheitis, bronch(iol)itis and alveolitis. Epithelial syncytiae and cytoplasmic inclusion bodies in infected cells may be seen early in this phase. Alveoli contain sloughed necrotic epithelium, fibrin, neutrophils and mononuclear cells. Atelectasis, bronchiectasis and emphysema may occur as a result of damage and obstruction of airways. The reparative phase, which may overlap the acute phase but continues through about the third week after infection, is indicated by regeneration of airway lining epithelium. Adenomatous hyperplasia and squamous metaplasia (with multilayered flat epithelial cells instead of normal columnar cells) in the terminal bronchioles and alveoli are considered to be a hallmark of SeV pneumonia. Mixed inflammatory cell infiltrates in this phase tend to be primarily interstitial, rather than alveolar, as they are in the acute phase. The resolution phase may be complete by the fourth week after

infection and lesions may be difficult to subsequently identify. Residual, persistent lesions that may occur include organizing alveolitis and bronchiolitis fibrosa obliterans. Alveoli and bronchioles are replaced by collagen and fibroblasts, foamy macrophages and lymphoid infiltrates, often with foci of emphysema, cholesterol crystals and other debris, which represent attempts to organize and wall off residual necrotic debris and fibrin. Lesions are more severe and variable when additional pathogens such as *Mycoplasma pulmonis* are present [8]. Otitis media has also been reported in natural infections with SeV although some of these studies have been complicated by the presence of other pathogens [415]. SeV has been detected in the inner ear after experimental intracerebral inoculation of neonatal mice [416].

SeV is extremely contagious. Infectious virus is shed during the first 2 weeks of infection and appears to be transmitted by direct contact, contaminated fomites and respiratory aerosol [8, 379].

Serology (MFIA, ELISA, IFA, or HI) is the approach of choice for routine monitoring because serum antibodies to SeV are detectable soon after infection and persist at high levels for many months, although active infection lasts only 1-2 weeks in immunocompetent mice. The short period of active infection limits the utility of direct methods such as immunohistochemistry [382] and RT-PCR [388, 417]. Although SeV is considered to be highly contagious, studies have shown that dirty bedding sentinel systems do not reliably detect the infection and that outbred stocks may not seroconvert consistently [418, 419]. MAP testing and RT-PCR can be used to detect SeV in contaminated biological materials.

SeV infection in mouse colonies has proved to be one of the most difficult virus infections to control because the virus is highly infectious and easily disseminated. Depopulation of infected colonies is probably the most appropriate means of eliminating the virus in most situations. Embryo transfer, or caesarean derivation, followed by barrier maintenance, can also be used to eliminate the virus [8, 379]. A less effective alternative is to place the infected animals under strict quarantine, remove all young and pregnant mice, suspend all breeding and prevent addition of other susceptible animals for

approximately 2 months until the infection is extinguished, and then breeding and other normal activities are resumed. Vaccines against the virus have been developed [8, 379, 409], but these probably do not represent a practical means to achieve or maintain the seronegative status of colonies that is in demand today.

SeV has the potential to interfere with a wide variety of research involving mice. Reported effects include interference with early embryonic development and fetal growth; alterations of macrophage, NK-cell, and T- and B-cell function; altered responses to transplantable tumours and respiratory carcinogens; altered isograft rejection; and delayed wound healing (reviewed in [6–8]). Pulmonary changes during SeV infection can compromise interpretation of experimentally induced lesions and may lead to opportunistic infections by other agents. They could also affect the response to anaesthetics. In addition, natural SeV infection would interfere with studies using SeV as a gene vector.

Theiler's murine encephalomyelitis virus (TMEV)

Theiler's murine encephalomyelitis virus (TMEV), or murine poliovirus, is a member of the genus *Cardiovirus* in the family Picornaviridae. Members of this genus are non-enveloped viruses with single-stranded RNA. The virus is rapidly destroyed at temperatures above 50 °C. It is considered to be a primary pathogen of the CNS of mice and can cause clinical disease resembling that due to poliomyelitis virus infections in humans. Antibodies to TMEV have been identified in mouse colonies and feral populations worldwide, and *Mus musculus* is considered to be the natural host of TMEV [420]. The best-known and most frequently mentioned TMEV strain is GDVII, which is virulent for mice. Infant or young hamsters and laboratory rats are also susceptible to intracerebral infection. The original isolate is designated TO (Theiler's original) and represents a group of TMEV strains with low virulence for mice. Many additional virus strains have been isolated and studied, and they all fall in the broad grouping of TO and GDVII. A similar virus strain has also been isolated

from rats, but in contrast to mouse isolates, this virus is not pathogenic for rats and mice after intracerebral inoculation [421]. Recently, another rat isolate has been characterized and shown to be most closely related to, but quite distinct from, other TMEV viruses [422]. Antibodies to TMEV (strain GDVII) have been detected in guinea-pigs and are considered to indicate infection with another closely related cardiovirus [423].

Seropositivity to TMEV was reported in approximately 48% of French mouse colonies in a retrospective study [10]. In more recent studies, the prevalence of TMEV infections was found to be lower. Schoondermark-van de Ven et al. [12] detected antibodies to TMEV in 2.2% of mouse samplings from western European institutions. In a survey conducted by Carty [13], about 9% of responding institutions in the USA reported TMEV infection in their mouse colonies. Further surveys in North America and western Europe revealed antibodies in 0.09–0.26% of mice monitored [11, 14, 15].

TMEV is primarily an enteric pathogen, and virus strains are enterotropic. In natural infections, virus can be detected in intestinal mucosa and faecal matter, and in some cases it is also found in the mesenteric lymph nodes. However, histological lesions in the intestine are not discerned. Virus may be shed via intestinal contents for up to 22 weeks, sometimes intermittently [424], and transmission under natural conditions is via the faecal–oral route, by direct contact between mice, as well as by indirect contact (e.g. dirty bedding). The host immune response limits virus spread, but it does not immediately terminate virus replication in the intestines. Virus is cleared from extraneural tissues, but persists in the CNS for at least a year.

Clinical disease due to natural TMEV infection is rare, with a rate of only 1 in 1000–10 000 infected immunocompetent animals [36]. In immunodeficient mice, especially in weanlings, clinical signs may be more common and mortality may be higher [425]. This group of viruses usually causes asymptomatic infections of the intestinal tract. They may spread to the CNS as a rare event where they cause different neurological disease manifestations. The most typical clinical sign of TMEV infection is flaccid paralysis of hindlimbs. The animals appear otherwise healthy, and there is no mortality.

Experimental infection in mice provides models of poliomyelitis-like infection and virus-induced demyelinating disease including multiple sclerosis [426]. After experimental infection, TMEV causes a biphasic disease in susceptible strains of mice. The acute phase is characterized by early infection of neurons in the grey matter. Encephalomyelitis may develop during this phase and may be fatal, but most animals survive and enter the second phase of the disease at 1–3 months after the acute phase. This phase is characterized by viral persistence in the spinal cord white matter, mainly in macrophages, and leads to white matter demyelination. Persistence and demyelination occur only in genetically susceptible mouse strains, while resistant strains clear the infection after early grey matter encephalomyelitis through a cytotoxic T lymphocyte response.

The severity and nature of disease depend on virus strain, route of inoculation, host genotype and age [8, 36, 427]. In general, virus isolates with low virulence produce persistent CNS infection in mice whereas virulent strains are unable to cause persistent infection. Intracerebral inoculation results in the most severe infections, but the intranasal route is also effective. Experimental intracerebral infections with virulent FA and GDVII strains of TMEV are more likely to cause acute encephalomyelitis and death in weanling mice 4–5 days after inoculation ('early disease'). Death may be preceded by neurological manifestations of encephalitis such as hyperexcitability, convulsions, tremors, circling, rolling and weakness. Animals may develop typical flaccid paralysis of hindlimbs, and locomotion is possible only by use of the forelimbs. Interestingly, the tail is not paralyzed. Experimental infections with low-virulence virus strains (e.g. TO, DA, WW) are more likely to cause persistent infection with development of mild encephalomyelitis followed by a chronic demyelinating disease after a few months ('late disease'). These virus strains infect neurons in the grey matter of the brain and spinal cord during the acute phase of viral growth, followed by virus persistence in macrophages and glial cells in the spinal cord white matter. SJL, SWR and DBA/2 strains are most susceptible to this chronic

demyelinating disease. CBA and C3H/He are less susceptible strains, and strains A, C57BL/6, C57BL/10 and DBA/1 are relatively resistant [428]. Differences in humoral immune responses play a role in resistance to TMEV infection [429], but genetic factors are also important. Several genetic loci implicated in susceptibility to virus persistence, demyelination, or clinical disease have been identified, including the H-2D region of the major histocompatibility complex [430]. Furthermore, the age at infection influences the severity of clinical disease. In infant mice, intracerebral infection with low-virulence virus strains (e.g. TO) is often lethal. Young mice develop paralysis after an incubation period of 1–4 weeks while adult mice often show no clinical signs of infection.

The only gross lesions are secondary to the posterior paralysis and may include urine scald or dermatitis due to incontinence of urine and trauma to paralyzed limbs, or wasting or atrophy of the hindlimbs in long-term survivors.

TMEV infects neurons and glial cells, and histological changes in the CNS include non-suppurative meningitis, perivasculitis and polio-myelitis with neuronolysis, neuronophagia and microgliosis in the brainstem and ventral horns of the spinal cord [36]. Demyelination in immuno-competent mice is considered to be immune-mediated. Susceptible strains develop a specific delayed-type hypersensitivity response which is the basis for inflammation and demyelination. This reaction is mediated by T cells that release cytokines leading to recruitment of monocytes and macrophages as a consequence of infection of macrophages and other CNS-resident cells [431–433]. Protection from chronic demyelinating disease is possible by vaccination with live virus given previously by subcutaneous or intraperito-neal inoculation [434, 435]. Early immunosuppres-sion at the time of infection, e.g. by treatment with cyclophosphamide or antithymocyte serum, inhibits or diminishes demyelination. Immuno-suppression in mice chronically infected with TMEV leads to remyelination of oligodendrocytes [436]. Further details related to the pathogenesis of TMEV infections and the role of immune mechanisms have been reviewed by Yamada et al. [437], Kim et al. [432] and Lipton et al. [433].

Experimental infection of $Foxn1^{nu}$ mice results in acute encephalitis and demyelination. Demyelination associated with minimal inflam-mation and neurological signs, including the typical hindlimb paresis, develop 2 weeks after inoculation, and most animals die within 4 weeks. In $Foxn1^{nu}$ mice, demyelination is caused by a direct lytic effect of the virus on oligodendrocytes [438]. Demyelination and lethality are reduced after administration of neutralizing antibodies [439]. Histopathological changes in $Prkdc^{scid}$ mice are very similar to those in $Foxn1^{nu}$ mice [440].

Young mice born in infected populations usually acquire infection shortly after weaning and are almost all infected by 30 days of age. Intrauterine transmission to fetuses is possible during the early gestation period, but a placental barrier develops during gestation and later prevents intrauterine infection [441].

All TMEV isolates are closely related antigen-ically and form a single serogroup, as determined by complement fixation and HI [427]. Hemelt et al. [421] demonstrated cross-reactions among four strains used in experimental infections, but differences were evident in homologous and heterologous titres. The viral strain most commonly used as antigen for serological testing is GDVII. This strain agglutinates human type O erythrocytes at 4 °C, and HI has been the stan-dard test for routine screening of mouse popula-tions. Meanwhile, HI has been replaced by MFIA, ELISA or IFA, all of which are more sensitive and specific. Virus isolation is possible from brains or spinal cords of mice with clinical disease or from the intestinal contents of asymptomatic mice. PCR techniques are also available to test for virus-specific nucleotide sequences in biological samples [442].

Mice that have been shown to be free from TMEV by serological testing can be selected for breeding populations. If the virus is introduced into a mouse population, depopulation of infected colonies may be the most appropriate means to eliminate TMEV. Embryo transfer or caesarean derivation is the method of choice for eliminating virus from valuable breeding populations. Foster-nursing has been reported to be effective in generating virus-free offspring [359], although transplacental transmission has been demonstrated with experimental infection early in gestation.

Lesions of demyelination in CNS of mice with clinically inapparent chronic infection may

interfere with investigations that require evaluation of the CNS [443]. Conceivably, such lesions could also affect neuromuscular responses or coordination, and affect neurological and behavioural evaluations.

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Bacterial Infections of Laboratory Mice

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Introduction

Although this chapter focuses on the role of a few bacteria in causing disease, often in the context of genotype and a multitude of environmental factors, it is useful initially to briefly consider the bacterial milieu in which mice live. Of principal concern is the mouse microbiome, defined as being all of the bacterial species found in, or on, the mouse, and the interactions of those bacteria amongst themselves and with their environment [1]. The human microbiome is estimated to consist of ten times as many cells as the human host. Although similar data are not available for the mouse, since mice have a relatively larger caecum and colon than humans, one suspects that murine cells are also similarly outnumbered by bacteria. In addition to their microbiome, mice may also encounter any of a wide range of bacterial species in the environment, most of which are probably uncultivable by current techniques [2], necessitating a metagenomic approach to full characterization of the microbial milieu.

The normal flora of laboratory mice is not well known. Different vendors may associate mice with differing flora. Over time, depending on housing and husbandry, the flora will also become more complex, i.e. more species of bacteria will colonize the mice. Different colonies of the same strain, but from different vendors, or raised with different housing or biosecurity systems, may have divergent flora [3]. One important point which is often not appreciated is that

most bacterial monitoring and reporting in rodents only includes aerobic bacteria and is usually limited to the (likely) minority of bacterial species which may be cultured. In addition to the complex, variable and largely uncharacterized flora of the intestines [3], a complex flora also colonizes the skin [4] and adnexa and the mucous membranes of the conjunctiva, vagina and oral cavity.

As a result of the complexity of the mouse microbiome, brief mention of the nebulous distinctions between autochthonous flora, commensal bacteria, opportunistic infections and primary pathogens is indicated. *Autochthonous bacteria* are the indigenous bacteria that have coevolved with the host species, and have a generally mutualistic relationship, where both the bacteria and the host prosper. The autochthonous flora of mice has been reported to have a role in the normal development of the gastrointestinal tract (reviewed by [5]), innate and acquired immunity [6], endocrine responses [7] and nervous system [8, 9]. *Lactobacillus rhamnosus* strain GG has been shown to potentiate intestinal epithelial repair in a C57BL/6J mouse model [10]. It is likely that more aspects of the inter-relationship between mice and autochthonous flora will be uncovered in the future. *Commensal bacteria* are those which live on the surface or mucous membranes and do not affect the host. However, although it is unlikely that the vast majority of murine microbiota cause any harm in most mice, it is difficult to demonstrate that no harm could ever result, especially with severely immunodeficient or irradiated mice. In these, even normally commensal bacteria may be associated with disease if introduced into the body by trauma such as surgery. Thus, the definition of commensal versus pathogen must be considered in the context of genotype, immune status and experimental manipulation. Pathogens are often divided into *primary pathogens* (which will result in disease in a significant percentage of a given genotype without strong extenuating circumstances) and *opportunistic bacteria* capable of causing disease only in special situations. Opportunistic pathogen, often expressed as 'opportunistic agent' or even simply 'opportunist' is a useful working definition, but one which blends into both commensal flora and primary pathogens. Whether or not a specific bacterium poses a risk is usually situation-specific, depending largely on genotype and experimental manipulation.

The prevalence of bacterial pathogens in laboratory mice has changed over time. Previously prevalent pathogens such as *Salmonella*, *Mycoplasma pulmonis* and *Corynebacterium kutscheri* are no longer common [11, 12], probably as a result of improvements in housing and better control over husbandry materials such as feed, bedding and water. Improved supply of pathogen-free mice from vendors and better pest control may also have contributed to the decline in prevalence of many primary pathogens. Some of these historical pathogens, which are currently rare in laboratory mice in Europe and North America, are listed in Table 3.3.1. One should note that many of them may still be common in pet mice, and that no information on their prevalence in laboratory mice is available from some regions of the world.

TABLE 3.3.1: Bacterial diseases of primarily historic significance		
Pathogen or disease	**Major signs or lesions**	**References**
CAR bacillus	Bronchiectasis, bronchopneumonia	[98, 99]
Citrobacter rodentium	Colitis in weanlings	[44, 100, 101]
Corynebacterium kutscheri	Disseminated abscesses	[44, 102]
Mycoplasma pulmonis	Chronic suppurative inflammation of lung (with bronchiectasis), middle ear, oviduct, uterus	[103, 104]
Salmonella	Septicemia, typhlocolitis, lymphadenopathy	[105]
Streptobacillus moniliformis	Disseminated abscesses, arthritis	[44, 105]

Gram-positive Bacteria

Clostridia

Clostridium piliforme

BIOLOGY

Clostridium piliforme is an obligate intracellular bacterium that may stain as Gram-negative and have variable morphology in tissue section. Most often, it appears as a long, slender (i.e. hair-like or piliform) bacillus, approximately 8–10 μm long and 0.5 μm wide, in the cytoplasm of infected cells, although shorter, thicker, cigar-shaped forms may also occasionally be seen. Considered non-cultivable on artificial media, *Cl. piliforme* may be cultured on intestinal cell lines, on primary chick or mouse liver cells or in embryonated chicken eggs.

EPIDEMIOLOGY AND PREVALENCE

Cl. piliforme is the causative agent of Tyzzer's disease. It is spread by spores that are shed in the faeces. There is evidence for at least partial host specificity among *Cl. piliforme* strains isolated from different host species [13]. A high molecular weight exotoxin has been associated with pathogenicity *in vitro* and *in vivo* [14]. Not all strains produce this toxin, which may account for variation in pathogenicity among strains, and suggests that perhaps not all strains of *Cl. piliforme* are pathogenic. The cytopathogenesis of *Cl. piliforme* infection has been studied in Caco-2 cells, a human colon carcinoma line [15]. After ingestion of the spores, the bacterium is phagocytosed by intestinal epithelial cells. If the bacterium has been killed by formalin exposure, it is not taken up, suggesting a role for the organism in inducing phagocytosis. This also suggests that ingestion of killed spores, such as potentially could be present in autoclaved feed, might not result in an antibody response. Inside the cell, the vegetative form escapes the phagosome and begins replication in the cytoplasm. Eventually, the cell is killed and the bacteria are either deposited back into the lumen, or sometimes find their way deeper into the intestinal wall, where they may infect smooth muscle cells or gain access to the portal circulation. From the portal vein, bacteria may infect the liver and/or heart. Most mice will clear the infection and cease shedding after approximately 2 weeks. Infection is commonly asymptomatic, with disease occurring primarily in weanlings and mice with immune deficits, or perhaps in highly crowded situations, or where there is concurrent infection with other pathogens.

Serological surveys have either not reported the prevalence of *Cl. piliforme* antibodies [11] or have found that seroconversion is sporadic. Disease is, however, rare in current laboratory facilities.

DETECTION AND IDENTIFICATION

Serological surveillance for *Cl. piliforme* typically employs a whole-cell antigen preparation which includes a complex mixture of bacterial proteins. When positive serological results are obtained, it can be difficult to distinguish a false-positive result (the mouse had not been exposed to *Cl. piliforme*) from a true positive result. Although some hint may be gleaned from score of the positive titre (a high titre is more likely to indicate a true positive), a single positive serology result for *Cl. piliforme* must be followed by additional testing, most often screening of additional serum samples.

The two other laboratory methods for detection of *Cl. piliforme* are polymerase chain reaction (PCR) and screening for lesions. PCR can be used on fresh tissues (if the lesions are due to *Cl. piliforme*, then PCR will be positive), on sections cut from paraffin blocks after lesions are observed on histopathological evaluation, on faeces, or on environmental samples. If screening faeces from an asymptomatic group of mice by PCR for *Cl. piliforme*, the faeces should be collected from mice within 2 weeks of the presumed time of infection, which is suspected to be at weaning. Mice at 4–6 weeks of age from a suspect breeding colony are a good age group to screen. Older mice are likely to have cleared the infection and very young mice may be uninfected while protected by maternal antibodies. PCR on faecal samples for *Cl. piliforme* can also be complicated by the occasional presence in faeces of substances that can inhibit PCR reactions, and by the degree of protection afforded by the thick wall of the spore, which can make it difficult to extract the DNA.

CLINICAL SIGNS AND PATHOLOGY

Most infected mice are asymptomatic. If disease occurs, it is usually observed in recently weaned mice or mice with genetic or induced immune deficits. Although weanling rats with Tyzzer's disease have been reported to have a distended abdomen due to megaloileitis [16], this has not been reported in mice. Mice with Tyzzer's disease may have diarrhoea and perianal staining, or may seem thin and unkempt for a short period. Sudden deaths without premonitory signs may also occur. At necropsy, the ileum, caecum and colon may be slightly enlarged and reddened by hyperaemia or mild haemorrhage. Tyzzer's disease is not generally considered to produce markedly ulcerative enteritis. If the infection has disseminated past the intestinal tract, white or tan foci may be scattered throughout the liver, but can vary greatly in number and size (Figures 3.3.1, 3.3.2). Pale streaks or patches of myocardial necrosis (not to be mistaken for epicardial mineralization) may also be visible in the heart. Giemsa-stained impression smears of liver lesions will very often show the characteristic jumble of piliform organisms if the lesions are due to *Cl. piliforme* (Figure 3.3.3). Evaluation of such tissue smears is recommended as a useful method for a quick

Figure 3.3.2 Tyzzer's disease, due to *Cl. piliforme*. Haematoxylin and eosin, 20× objective. Almost the entire upper right area of the field has coagulative necrosis (white N), with a band of leukocytes separating the necrotic area from the more normal tissue on the left. Slightly bluish hepatocytes along the periphery of the necrosis (white arrow) are usually the best to examine for characteristic intracellular bacilli.

definitive diagnosis. Histologically, lesions in the liver and heart are foci of necrosis with a variable degree of leukocytic infiltration, depending on the duration of the lesion and the strain of mouse. Special stains (Warthin-Starry, Giemsa) will show long, thin bacilli within cells of the mucosa of the ileum, caecum or colon, or in nearby smooth

Figure 3.3.1 Tyzzer's disease, due to *Cl. piliforme*. This outbred ICR mouse has a single small focus of hepatic necrosis in the liver (white arrow). The ileum is hyperaemic (asterisk).
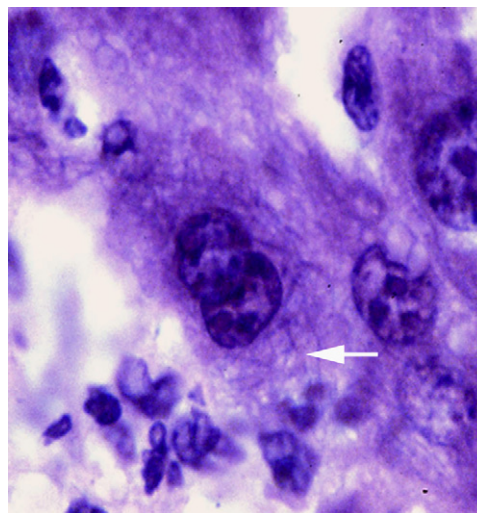
Figure 3.3.3 Tyzzer's disease, due to *Cl. piliforme*. Haematoxylin and eosin, 100× objective. Intracytoplasmic *Cl. piliforme* are usually more visible with a Warthin–Starry silver stain or a Giemsa stain, but may occasionally be seen with a standard H&E. Some of the numerous bacilli are indicated with a white arrow. Note the jumbled arrangement, which is characteristic of this bacterium.

muscle cells, or in hepatocytes or cardiac myofibres. The gold standard for confirmation of *Cl. piliforme* infection has traditionally been observation of the characteristic intracellular bacilli in lesions, although additional support from PCR should be sought when available.

MANAGEMENT AND CONTROL

Detection of antibodies to *Cl. piliforme* in mature mice not destined for immunocompromise may not always require elimination of the mice. Subclinical infection, which would occur in approximately the first 2 weeks after infection, can alter cytokine profiles and haemodynamic parameters [17] as well as tumour necrosis factor alpha and interferon gamma for at least several weeks [18]. The major concerns with the presence of *Cl. piliforme* are the risk for overt disease in young mice, especially in breeding colonies, and the difficulty in eliminating the spores from the environment. If an informed management decision is made that *Cl. piliforme* needs to be eliminated from mice in a research facility, then the probability of success will be enhanced by elimination of all mice, through cleaning (removal of all dirt) of all surfaces and equipment, and either autoclaving of all materials or disinfection with a high-level disinfectant capable of killing the clostridial spores [19, 20].

Other clostridia

Cl. perfringens and *Cl. difficile* have also been implicated as pathogens of mice. Both are anaerobic bacilli which can colonize the digestive tract of animals and which may be grown on artificial media. Both may be present in gut contents of asymptomatic animals, as well as those with diarrhoea, and may be seen in smears of gut contents or in histological sections. Therefore, mere observation of the bacteria is insufficient for a definitive diagnosis of clostridial disease, as perimortem intestinal congestion and postmortem autolysis may be mistaken for haemorrhage and necrosis, and these clostridia are among the bacteria that proliferate postmortem. Definitive diagnosis of disease due to *Cl. perfringens* or *Cl. difficile* depends on demonstration of their signature exotoxins, which, unfortunately, can be labile, together with morphological evidence of antemortem tissue injury [21].

Cl. perfringens has only rarely been reported as a cause of disease in mice, but it has been reported to cause sudden death in young mice (24–52 days of age) and in females of breeding age [22]. It has primarily been implicated in a poorly characterized syndrome of sudden death in lactating females, especially primiparous females in the second week of lactation with large litters [22–25]. Signs of hunched posture, distended abdomen and soft faeces have been reported, as have impacted faeces, or even death without observed clinical signs. At necropsy, distended and gas-filled large and small intestines are reported; intestines may also contain liquid contents. Petechial haemorrhages, and mucosal ulceration and pseudomembranes have also been reported and can help with preliminary differentiation from Tyzzer's disease. Histologically, the most consistent finding is mucosal necrosis, together with the (non-specific) presence of high numbers of bacilli in the intestinal lumen. Toxins identified in these cases include type D, and one that was simply identified as non-A, due to the unavailability of other antisera [26]. However, others have considered the inciting cause of enteropathy, ileus and death in lactating mice to be nutritional and that the clostridia are secondary, near-terminal exacerbating agents. Nutritional considerations mentioned include exhaustion of glucose or calcium and high-carbohydrate diets [22].

Cl. difficile is also found in mice, but has not been reported as a cause of naturally occurring disease, although experimental infection of germ-free mice, or of mice after antibiotic treatment, results in diarrhoea, typhlitis and pseudomembrane formation resembling *Cl. difficile* typhlocolitis in hamsters, humans and other species [21].

Corynebacteria
Corynebacterium bovis

BIOLOGY

Corynebacteria are Gram-positive, club-shaped bacilli, members of the Actinobacteria. Many corynebacteria are common in the environment [27] and as residents of the skin of humans and other mammals [28, 29]. *Co. bovis* has long been controversial for its potential role as a cause of bovine

mastitis, but has also been isolated from apparently normal bovine udders, human skin and mice. It has been demonstrated to cause generalized hyperkeratotic skin disease in some strains of mice [30–33]. The cell wall of *Co. bovis* contains mycolic acid [34]. The bacterium is resistant to desiccation, which makes it easy to transmit by fomites and complicates environmental decontamination.

EPIDEMIOLOGY AND PREVALENCE

Co. bovis has been found from the skin of both immunodeficient and immunocompetent mice, including nude and hairless mice, as well as those with a normal pelage. It is not clear, however, whether immunocompetent haired mice can be long-term carriers of the bacterium, or are merely briefly colonized after exposure [35]. The disease is relatively common in research facilities using nude mice, and has also been reported in mice from Europe, Asia and North America [11]. *Co. bovis* can be easily transferred by fomites such as cages, cage lids and gloved hands [36].

DETECTION AND IDENTIFICATION

Co. bovis grows well on blood agar, although growth may be enhanced by the addition of serum to the media [30, 33]. Punctate, white, pearlescent colonies are produced after 48 h at 37 °C, but these may be easy to miss when screening culture plates if there is abundant growth of other bacteria producing larger colonies. Suspect *Co. bovis* isolates are usually identified by biochemical profile, although the similarity of its profile to *Co. mastitidis* may cause some confusion. As a result, PCR may be used to confirm the identity of colonies [37]. The possibility of variable pathogenicity of various strains of *Co. bovis* in mice has not been addressed, although virulence varies among *Co. bovis* in cattle [38]. As mouse skin is also often colonized with many other corynebacteria which have not been demonstrated to be pathogenic in mice, caution in attributing significance to the initial detection of corynebacteria is advised.

Co. bovis is most often identified from skin swabs, but can also be detected by buccal swabs [30]. More recently, PCR has found it in high levels in faeces of infected mice (K. S. Henderson, personal communication, 2011), although whether this reflects colonization of the digestive tract or contamination of faecal pellets after expulsion is unknown. PCR of room surface swabs in infected facilities has also found *Co. bovis* in change hoods, the exterior surface of cages and even doorknobs, demonstrating a high potential for environmental dissemination of this pathogen. *Co. bovis* also has the potential to contaminate tumour lines passaged from mouse to mouse as fragments (brie), if there is contamination from the epidermis during collection and dicing of the tumours.

CLINICAL SIGNS AND PATHOLOGY

Susceptible mice include athymic nude mice, scid mice and immunocompetent SKH-1 hairless mice [30, 39]. Additional immunodeficient strains, such as RAG2 mice, are also likely to be susceptible. Clinical signs in nude mice appear approximately 7–10 days after exposure; this can be useful information in investigating the source of an outbreak. Signs may appear in only a minority of infected mice, although the typical histological features will often be found in more mice than show clinical signs. Signs consist of yellow-white flakes of keratin adherent to the epidermis. This hyperkeratosis may cover large areas of the body, leading to the informal appellation 'scaly skin disease' (Figure 3.3.4). Severe cases have led some observers to note that the mouse appeared as if it had been dipped in cornmeal.

Figure 3.3.4 Hyperkeratosis due to *Co. bovis* infection. The hyperkeratosis may affect only portions of the skin, as in the athymic nude mouse on the right, or may affect nearly the entire surface of the body with prominent yellow-white flakes of keratin. These mice appear slightly dehydrated, which is common with this disease. The mouse on the left also has conjunctivitis, a common condition in nude mice, which is not attributed to *Co. bovis* infection.

Affected mice may be dehydrated, and appear to have thickened skin. Areas of the skin which are undergoing active hair follicle cycles often seem to be preferentially affected, but this has not been experimentally confirmed and the skin in these areas is always thicker due to an increase in hypodermal fat thickness concomitant with elongation of the growing follicles. Reviews of the hair follicle cycles of mice are available elsewhere [40]. Clinical signs disappear after 7–10 days, although the mice remain infected, and the histological changes persist. How the bacteria induce hyperkeratosis and why the hyperkeratosis disappears are unknown. *Co. bovis* infection in newborn nude mice has been associated with high mortality, although lesions have not been described. Clinical signs of *Co. bovis* infection in scid mice include a scruffy appearance, with alopecia [32] (Figure 3.3.5). In addition to these clinical signs, infected mice have been reported to have slower growth of implanted tumours and to have increased mortality from chemotherapy [36].
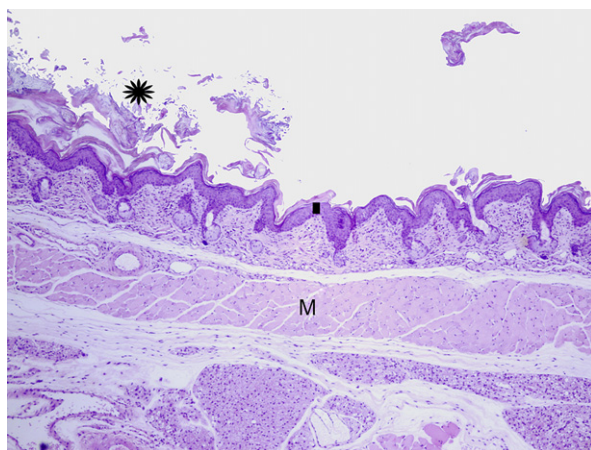
Figure 3.3.5 *Co. bovis* infection in an athymic nude mouse. Haematoxylin and eosin, 10× objective. The skin surface is at the top of this photomicrograph and subcuticular muscle (M) near the middle. Accumulations of keratin (Star) may be present, as on the left, but are not always seen. Acanthosis, thickening of the stratum spinosum, is a more consistent feature and may be diffuse. The epidermis in this field (black bar) is several times thicker than normal. Although sometimes thought to occur primarily in areas of active follicular growth (anagen), this area has resting (telogen) follicles, which do not extend below the dermis. Hypodermal fat between the dermis and the muscle is very thin, typical of this phase of the follicular growth cycle.

Microscopically, the most characteristic and persistent change is acanthosis [30]. Acanthosis will be present in areas where hyperkeratosis is not visible grossly, after hyperkeratosis has disappeared and in infected mice in which no hyperkeratosis was observed. Although acanthosis will occur locally as part of a regenerative response to a wide variety of epidermal insults, such as bite wounds, diffuse acanthosis, without ulceration, is considered the most characteristic change of *Co. bovis* infection in nude mice. Additional changes include mild non-suppurative dermatitis and often orthokeratotic hyperkeratosis, visible as clumps of material on the surface. With a Gram stain, Gram-positive corynebacteria in typical irregular branching arrays are usually visible in the clumps of keratin or in the necks of hair follicles. As noted above, however, caution should be exercised in attributing too much significance to the observation of corynebacteria on the skin, as many non-pathogenic species also colonize that niche. The histologic changes in *Prkdc^{scid}* mice also include acanthosis and hyperkeratosis [39].

MANAGEMENT AND CONTROL

Because of the high potential for wide dissemination in an infected facility and the high resistance to desiccation, *Co. bovis* can be extremely difficult to eradicate from an infected facility [41], even though it is susceptible to a variety of disinfectants. All infected mice, which are probably all mice exposed, should be eliminated. Infection is persistent and antibiotic treatment does not seem effective [33]. Any items that may have been contaminated by direct or indirect contact with mouse dander should be considered contaminated and should be replaced where practical. All remaining surfaces should be thoroughly cleaned and disinfected before the introduction of any new mice, which should only be obtained from *Co. bovis*-free sources.

Staphylococci

Staphylococci are Gram-positive aerobic non-motile cocci in the phylum Firmicutes. The genus includes at least 40 species, and are a minor component of soil flora, as well as a major component of the flora of skin and respiratory tract of mammals, including humans and birds [28].

Staphylococcus aureus

BIOLOGY

S. aureus is a commensal of mammalian skin and nasal cavities; it can be isolated from the nares of approximately 30% of humans [42], but can also be found by culture or molecular techniques in the digestive tract and in faeces. It grows well on artificial media including blood agar and produces gold or yellow-pigmented colonies. The pigmentation is due to a carotenoid pigment, staphyloxanthin, which acts as an antioxidant to help the organism evade oxygen-mediated killing. Host defence against S. aureus primarily rests with phagocytosis and killing of bacteria by neutrophils and macrophages, and involves both innate and acquired immunity [43]. These professional phagocytes are attracted to areas of tissue invasion by molecules secreted by S. aureus, via direct recruitment or via chemokines generated by monocytes, T cells and endothelial cells. Significant chemoattractant molecules from S. aureus include lipoteichoic acid, capsular polysaccharide, enterotoxins A and B, and toxic shock syndrome toxin-1. Mice with deficient phagocyte number or function are at particular risk. Whereas staphylococci are less often a primary cause of disease in immunocompetent mice, they are among the most common isolates from cutaneous furunculosis or ulcerative dermatitis and are almost always isolated from suppurative adenitis of the preputial gland [44]. Disease most often results when S. aureus is introduced into tissues by injuries such as biting or surgery.

EPIDEMIOLOGY AND PREVALENCE

The rate of carriage of S. aureus by mice is difficult to determine, and may be underestimated by routine cultures, in which a single sample is collected from one site; however, estimates based on more extensive sampling of each animal, or using molecular techniques, are not available. In addition to these possible reasons for underestimation of S. aureus prevalence, there is anecdotal evidence that transfer of soiled bedding to sentinels may not always effectively transmit S. aureus.

With these warnings in mind, 6–11% of mice from sources other than directly from a major vendor have been found to be positive for S. aureus at a large rodent diagnostic laboratory [11], indicating that the organism is common in mice.

DETECTION AND IDENTIFICATION

S. aureus is most often detected by culture on sheep blood agar of nasal samples, either swabs of nasopharyngeal lavage or suspect lesions. It forms yellow-gold colonies with beta-haemolysis, and the non-motile cocci are often in grape-like (staphylo-) clusters on smears. The bacteria are catalase-positive, which helps distinguish them from enterococci and streptococci, and are coagulase-positive, which helps distinguish them from other staphylococci, although a few strains of S. aureus may be coagulase-negative [45]. PCR can be a useful adjunct for definitive identification, and can also be applied to screening for specific virulence or antibiotic resistance genes. PCR can also be applied to screening swabs or other samples, as well as for identification of suspect bacteria observed in histological lesions.

Antibiotic resistance of S. aureus has not been reported in natural infections of laboratory mice, nor has any zoonotic potential been documented. Review of isolates at a large rodent diagnostic laboratory (R. Fister, personal communication, 2011) suggests that strains of S. aureus detected in laboratory mice are susceptible to a broad range of antibiotics, and that methicillin-resistant S. aureus (MRSA) has not been isolated.

CLINICAL SIGNS AND PATHOLOGY

Most mice carrying S. aureus will be asymptomatic; disease is seen only in a minority of colonized mice, regardless of immune status [44]. The remainder of this section deals only with that minority. One of the most common lesions associated with S. aureus in immunocompetent mice is suppurative inflammation of the preputial glands (Figure 3.3.6), usually called preputial gland abscesses even though the inflammation may be contained within the lumen of the distended gland and thus does not meet the strict definition of an abscess. C57BL/6 mice are highly susceptible to preputial adenitis, and S. aureus is almost always isolated from the lesions. Mice free of S. aureus do not develop preputial adenitis. A similar lesion is also occasionally found in other stocks and strains, as well as in clitoral glands. S. aureus is also a common secondary invader of skin lesions, and its elaboration of
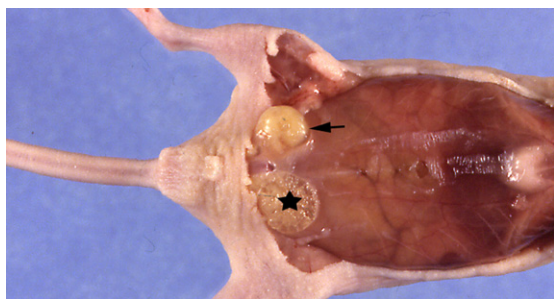
Figure 3.3.6 Athymic nude mouse with an inflamed preputial gland. One preputial gland (black star) is normal. The other gland (black arrow) is distended with suppurative inflammation. Suppurative preputial adenitis may lead to fistulation and drainage. *S. aureus* is the most frequently isolated bacterium from these lesions.

toxins can greatly exacerbate skin wounds [44]. It is also a common finding in contaminated surgical sites, including catheter tracts, where its ability to adhere to fibrinogen and fibrin facilitates its colonization of blood clots (Figure 3.3.7). *S. aureus* has also been associated with osteomyelitis in mice [46].

In immunodeficient mice, especially athymic nude mice, *S. aureus* can cause large subcutaneous abscesses, sometimes surrounding foreign material [44]. The lack of cilia (eyelashes) in nude mice allows foreign matter to accumulate in the conjunctival sac, which can provide a nidus for infection with *S. aureus* or other bacteria, such as *Pasteurella pneumotropica*.

Histologically, *S. aureus* is usually easily recognized in the centre of lesions or on ulcerated
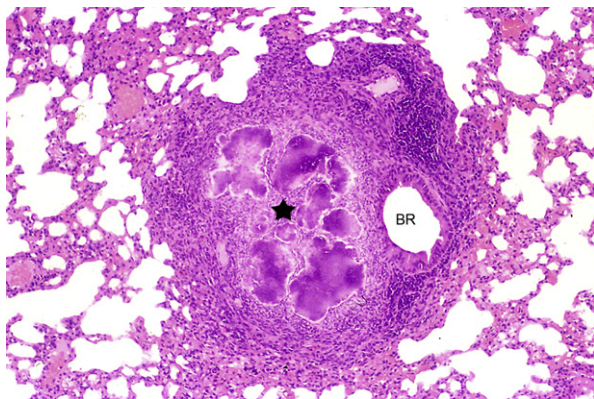
Figure 3.3.7 *S. aureus* embolic vasculitis in a mouse lung. Haematoxylin and eosin, 10× objective. Colonies of *S. aureus* (black star) surrounded by a dense transmural inflammatory infiltrated distended artery adjacent to a bronchiole (BR). These lesions are a common sequela to chronic jugular catheterization.
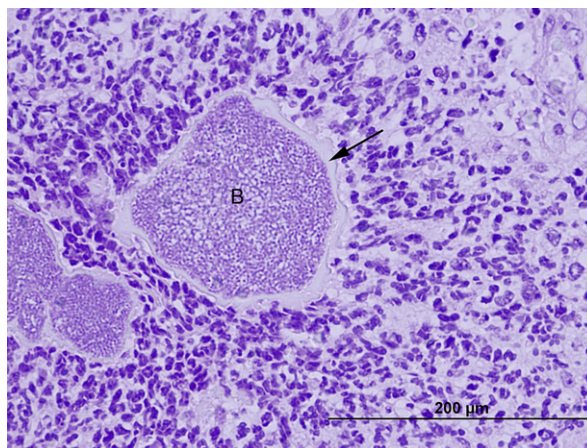
Figure 3.3.8 *S. aureus* infection. Haematoxylin and eosin, 10× objective. *S. aureus* are often easily found in lesions as colonies of large coccoid bacteria (B) surrounded by Splendore–Hoeppli material (black arrow). External to this is a mass of neutrophils, many of which are degenerating. A few macrophages are present at the upper right.

surfaces as prominent colonies of large Gram-positive cocci, often surrounded by amorphous eosinophilic hyaline material known as Splendore-Hoeppli phenomenon (Figure 3.3.8).

MANAGEMENT AND CONTROL

As *S. aureus* rarely causes disease in immunocompetent mice, no control is usually attempted. Mice with profound genetic or acquired immune deficits should be raised from birth free of any contact with humans or infected mice, or fomites that may be contaminated by them. Elimination of *S. aureus* from a line of mice can be reliably achieved only by rederivation. Antibiotic treatment is discouraged as it is unlikely to clear infection from all mice and unlikely to achieve bactericidal concentrations on surfaces where the bacteria dwell.

Other staphylococci

Staphylococcus xylosus, a coagulase-negative *Staphylococcus* sp., has been occasionally reported as a cause of disease in mice with immune deficits and as a cause of ulcerative lesions of the tail in SJL mice. Nude mice have been reported to suffer fatal dermatitis from *S. xylosus*. This single report from 1993 [47] isolated *S. xylosus* from 14/23 affected mice with full-thickness coagulative necrosis of the skin, and experimentally reproduced it in 6/24 mice. Experimental inoculation

included implantation of a suture for 24 h or inclusion of 1.25% agar at the inoculation site. No lesions were noted in tissues other than the skin. No reports to corroborate these findings have followed. Mice with deficiencies in oxygen-mediated killing by phagocytes have also been reported to have lesions due to *S. xylosus* [48] similar to those of chronic granulomatous disease of humans, in which patients have deficient oxygen-mediated killing. Finally, SJL mice have been reported to have ulcerative dermatitis of the tails, from which *S. xylosus* is consistently isolated. Implantation of a suture soaked in *S. xylosus* culture into the skin of the tail reproduced the lesions [49].

Streptococci

Biology

Streptococci are members of the Lactobacillales order of the Firmicutes. Whereas staphylococci divide along multiple planes to produce clusters, streptococci divide along a single plane to produce chains. Most are oxidase-negative and catalase-negative, and primary differentiation is usually based on patterns of haemolysis when grown on blood agar: alpha-haemolysis is greenish, beta-haemolysis produces a clear zone, and gamma-'haemolysis' is the term applied for no observable haemolysis. Beta-haemolytic streptococci are usually subdivided into Lancefield groups A, B, C and G based on serotyping of the capsular polysaccharides.

Epidemiology and prevalence

Streptococci are common commensal bacteria of the skin, upper respiratory tract and digestive tract of mammals. These bacteria are transmitted to laboratory mice by infected mice, humans or other species, or by contaminated fomites. *Strep. pneumoniae*, an alpha-haemolytic streptococcus, appears to be very rare in contemporary laboratory mice [50], and although mice are highly susceptible to experimental infection, no reports of naturally occurring disease due to *Strep. pneumoniae* could be found, so it will not be discussed further.

Beta-haemolytic streptococci have a low prevalence, overall, in laboratory mice [50], but can have a moderate to high prevalence in infected breeding colonies. They are occasionally reported as causes of disease in mice.

Clinical signs and pathology

Most mice carrying beta-haemolytic streptococci will be asymptomatic; disease is seen only in a minority of immunocompetent or immunodeficient mice, and then almost exclusively with group B streptococcus, also known as *Strep. agalactiae*. The remainder of this section will deal only with those situations. A non-haemolytic group B streptococcus was reported as a cause of suppurative meningoencephalitis and rhinitis in recently weaned athymic nude mice, and was also isolated from brain, blood and nasal cultures of heterozygous breeding females in the colony [51]. Group B streptococcus has also been reported to cause septicaemia, with suppurative inflammation in the heart, uterus, liver and lung of DBA/2 mice and F1 hybrids produced from that colony, but could not be found in NOD, C3H or C57BL/6 mice housed within the same microbiological barrier room, suggesting the possibility of mouse strain susceptibility differences [52].

Management and control

As streptococci rarely cause disease in immunocompetent mice, no control is usually attempted. Mice that must be free of streptococci should be maintained from birth in housing adequate to prevent colonization with human-borne bacteria. Elimination of streptococci from a line of mice can be reliably achieved only by rederivation. Antibiotic treatment is discouraged as it is unlikely to clear infection from all mice and unlikely to achieve bactericidal concentrations on surfaces where the bacteria dwell.

Gram-negative bacteria

Pasteurellaceae

The Pasteurellaceae are a large and diverse group of Gram-negative obligate parasites, primarily commensal organisms of the respiratory tract of birds and mammals. The family includes at least 13 genera, of which *Pasteurella*, *Actinobacillus*, and *Haemophilus* are the best-known to laboratory animal professionals. Many of the

species are poorly characterized and their ecology in laboratory mice is not well understood. *Pasteurella pneumotropica* is the only member of the Pasteurellaceae which is of current and well-established significance in mice. A comprehensive discussion of the Pasteurellaceae in mice is available in the excellent review by Nicklas [53].

Pasteurella pneumotropica

BIOLOGY

P. pneumotropica is a Gram-negative, non-spore-forming coccobacillus or bacillus. The species as most often defined includes two biotypes, Jawetz and Heyl, and this definition will be used in this section. Readers should note, however, that molecular characterization shows that the Heyl biotype is sufficiently distinct to represent another species and genus, and that the Jawetz biotype may encompass several different species. It grows well on blood agar to produce non-haemolytic, grey-white, smooth colonies, although sometimes colonies of the Heyl biotype may be yellow [53]. *P. pneumotropica* is most commonly isolated from mucosal surfaces such as vagina, conjunctiva and nasopharynx, although molecular techniques also allow its detection with high sensitivity in faecal samples.

EPIDEMIOLOGY AND PREVALENCE

P. pneumotropica is commonly detected in routine cultures of mice, with one report finding it in almost 13% of mice received from non-vendor clients at a large rodent diagnostic laboratory [11]. As *P. pneumotropica* is an obligate parasite, i.e. it does not survive or proliferate for long periods of time in the environment, and because it is not commonly carried by humans, it is transmitted primarily by contact with infected mice. It has been reported to survive for only 2 h on mouse fur and as little as 30 min on a laboratory coat, and is only transferred with difficulty to sentinel mice exposed solely by soiled bedding [54].

DETECTION AND IDENTIFICATION

P. pneumotropica is detected in routine screening or in lesions by microbiological culture, by PCR of swabs or faeces, or by PCR on deparaffinized histological sections of suspect lesions. Suspicious colonies on blood agar may be identified by biochemical profile or by PCR. PCR should use primers for both Jawetz and Heyl biotypes. Biochemically [53], *P. pneumotropica* of mice produces positive reactions for alkaline phosphatase and beta-galactosidase, is ribose and trehalose positive and urease positive, does not ferment mannitol, dulcitol, cellobiose, salicin or esculin and does not produce gas from glucose. It has a positive reaction for alpha-glucosidase, and has negative reactions for beta-glucosidase and alpha-fucosidase. Reactions to inositol and lactose are variable. Most strains also produce ornithine decarboxylase, glucuronidase, and beta-xylosidase.

CLINICAL SIGNS AND PATHOLOGY

Most mice carrying *P. pneumotropica* will be asymptomatic; disease is seen only in a minority of immunodeficient mice (Figure 3.3.9) and very rarely in immunocompetent mice. The most common lesion in nude mice infected with *P. pneumotropica* is necrotizing and suppurative infection of the Harderian gland [55] (Figure 3.3.10). Other immunodeficient strains or lines of genetically modified mice may also be affected [56]. It has also been associated with suppurative infection of other tissues, including the middle ear [55], subcutaneous tissue, mammary gland and uterus. Historically, *P. pneumotropica* was considered to be a cause of pneumonia in mice, although very little has been published in the last 30 years to indicate it as

Figure 3.3.9 Athymic nude mouse with *P. pneumotropica* infection. The right eye is exophthalmic due to a large retroorbital abscess. The abscess is visible posterior to the eye. In nude mice used as contact sentinels for quarantine of genetically engineered mice, these abscesses are almost always due to infection with *P. pneumotropica*.
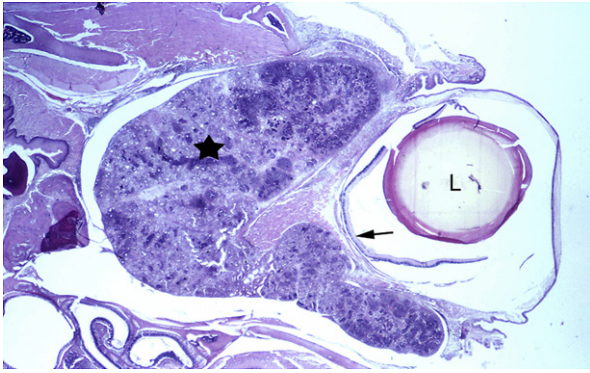
Figure 3.3.10 Retroorbital suppurative Harderian adenitis due to *P. pneumotropica* infection in an athymic nude mouse. Haematoxylin and eosin, 2× objective. The Harderian gland (black star) is distended, diffusely necrotic and infiltrated with neutrophils (leukocyte type not discernible at this magnification). The eye is slightly exophthalmic. The atrophic retina (black arrow) is most likely due to the *rd1* mutation (L, lens).

a primary cause of pneumonia in immunocompetent or immunodeficient mice. For example, the most recent papers describe pneumonia due to coinfection of B cell deficient mice with *Pneumocystis murina* (a primary pathogen of immunodeficient mice) and *P. pneumotropica* [57].

MANAGEMENT AND CONTROL

Mice infected with *P. pneumotropica* are also often infected with other agents such as helicobacter and murine norovirus, so discovery of *P. pneumotropica* does not often trigger rederivation efforts. In addition, *P. pneumotropica* rarely causes overt disease in immunocompetent mice. However, at least two factors might support a decision to eliminate it. First, because it persists only in infected rodents, not in the environment or in humans, *P. pneumotropica* is more easily successfully eliminated from a vivarium and thereafter excluded than many other agents. Second, because it can be found growing to high numbers in the uterus, its presence is more difficult to tolerate in breeding cores, especially those generating immunodeficient mice or genetically modified mice of questionable immune status.

Enterobacteriaceae

The Enterobacteriaceae are a large family of Gram-negative, non-spore-forming bacilli found in soil and water, as well as in plants and in animals, both vertebrates and invertebrates. Among notable genera are *Escherichia*, *Salmonella*, *Enterobacter*, *Klebsiella*, *Citrobacter*, *Shigella* and *Yersinia*. *Klebsiella* are considered coliform bacteria (fermenting lactose to produce acid and gas at 37 °C), but are not faecal coliforms, i.e. those bacteria which can grow at 44 °C and are not inhibited by the presence of bile salts.

Klebsiella

Although *Klebsiella* spp. are considered ubiquitous in nature, clinical concern is primarily directed toward *K. pneumoniae* and *K. oxytoca*.

No reports of disease due to *K. pneumoniae* in mice could be found since 1976 [58]. In that report, *K. pneumoniae* capsule type 6 (there are at least 77 different capsular types) was isolated in pure culture from Swiss and athymic nude mice dying of septicaemia, with abscesses in cervical lymph nodes, kidney, liver and lung. Disease was reproduced by inoculation of a cultured isolate intraperitoneally in peptone broth. No surveillance for concomitant viral infection was mentioned.

K. oxytoca has been reported as the cause of disease in one outbreak in immunocompetent mice, and in several reports in diabetic mice and mice with immune deficits. In immunocompetent mice, it was associated with suppurative inflammation of the reproductive tract of ageing B6C3F1 mice, from which it was isolated [59]. Experimental inoculation resulted in only a few cases of mild disease, suggesting that environmental factors or concomitant infections were important cofactors. For example, the mice were also positive for *Mycoplasma arthritidis*. The authors noted that alterations in room husbandry were followed by a disappearance of new cases, without changes in microbiological status, i.e. the disease disappeared although the colony was still positive for *K. oxytoca*. This suggests that *K. oxytoca* was not the sole cause of the lesions. More recently, *K. oxytoca* has been isolated from abdominal abscesses in diabetic mice and from mice with immune deficits such as oxygen-mediated killing [60, 61] In addition, C3H/HeJ mice, a strain with defective TLR4 which renders the strain hyporesponsive to Gram-negative bacteria, are

reported to develop a high incidence of otitis media by 12 months of age when chronically infected with *K. oxytoca* [62].

Because *Klebsiella* spp. can be carried by humans and can persist in the environment, long-term successful exclusion from animal facilities will require strict biosecurity measures adequate to prevent contact with humans, infected animals, or fomites. Elimination is likely to only be possible by rederivation.

Bordetella

Bordetella hinzii

Bordetella are Gram-negative non-spore-forming coccobacilli. *Bordetella hinzii* has recently been identified as a cause of tracheobronchitis in mice [63].

BIOLOGY

B. hinzii is closely related to *B. avium*, which was previously known as *Alcaligenes faecalis*. Diagnostic reports of *B. avium* should be carefully considered to insure that *B. avium* was properly differentiated from *B. hinzii*.

EPIDEMIOLOGY AND PREVALENCE

The prevalence of this emerging agent is not known, although it is occasionally identified in several laboratories in Japan and North America, primarily in genetically modified mice [64].

DETECTION AND IDENTIFICATION

B. hinzii grows well on blood agar, and is distinguished from *B. avium* by the production of alkali from malonate [63].

CLINICAL SIGNS AND PATHOLOGY

Infection with Bordetella is most likely persistent. The morbidity is unknown. Some infected mice may develop dyspnoea. At necropsy, there may be mild bronchopneumonia. Microscopically, bronchiectasis and bronchopneumonia resembles that produced by infection with CAR bacillus. Silver stains such as Warthin–Starry show numerous short bacilli nestled amongst the cilia, as seen in CAR bacillus infection (Figure 3.3.11). However, as a coccobacillus, *B. hinzii* is shorter than the surrounding cilia and is much shorter than CAR bacillus (Table 3.3.1), which is longer than the cilia. Diagnosis of *B. hinzii*
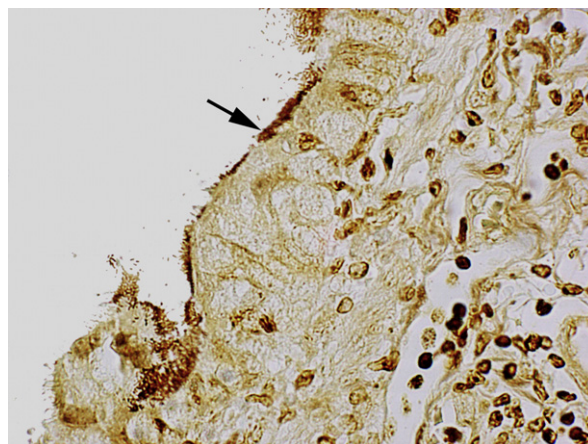
Figure 3.3.11 *B. hinzii* **bronchiolitis in a mouse. Warthin–Starry silver stain, 40× objective.** Numerous short dark bacilli (black arrow) cover the luminal surface of the bronchiolar epithelium. A few cilia, which stain very faintly, are present at the lower left. In contrast to *B. hinzii*, CAR bacillus would be longer than the cilia.

infection should be confirmed by culture or, if available, PCR.

MANAGEMENT AND CONTROL

Eradication protocols have not been reported for *B. hinzii*. However, rederivation of infected lines of mice should be successful. The organism is not expected to have a high degree of environmental persistence, and would not require extraordinary disinfection procedures, as might be required for *Co. bovis*. The potential for persistence in water, as has been reported for *B. bronchiseptica* or for colonization of other species, is unknown.

Other bordetella species

B. avium and *B. bronchiseptica* have also been reported in mice. Based on the recent report of Hayashimoto et al. [63] on *B. hinzii*, it is possible that reports of *B. avium* represent misidentification, but it is also possible that both species, *B. avium* and *B. hinzii*, may infect mice. Reports of *B. bronchiseptica* in mice are several decades old [65]. Although mice are used as experimental hosts for *B. bronchiseptica*, it has not been found in recent large multicentre and multiyear surveys [11], and it is unknown if it occurs in contemporary laboratory mouse populations, or if natural infection of mice with *B. bronchiseptica* is pathogenic.

Helicobacter

Helicobacters are Gram-negative, obligate parasites of homeothermic digestive tracts. In general, they are divided into those that primarily inhabit the stomach and those that inhabit the intestines and sometimes the hepatobiliary tract [66]. The latter are referred to as enterohepatic helicobacter. All currently known helicobacters of significance in laboratory mice are in the enterohepatic group and are transmitted by the faecal-oral route [66]. Mice may be experimentally infected with *H. pylori* but are not natural hosts for this human pathogen or other gastric helicobacters. For additional information, readers are referred to excellent in-depth reviews of helicobacter infections of laboratory rodents [67–69].

Helicobacter hepaticus

BIOLOGY

H. hepaticus is a Gram-negative spiral bacterium with bipolar single-sheathed flagella. Like most other helicobacters, it is considered highly sensitive to desiccation. *H. hepaticus* colonizes the caecal and colonic crypts in the large intestine, but also bile canaliculi in the liver of many strains of mice [69].

EPIDEMIOLOGY AND PREVALENCE

Infection is transmitted by the faecal-oral route. Newborn mice of infected dams are not infected until after the first few days of life [70, 71], but infection persists thereafter. *H. hepaticus* is the most prevalent helicobacter of mice, with a prevalence greater than all other helicobacter species combined [11]. It is especially common in lines of genetically modified rodents [68]. Most, or all, major vendors are free of it, as self-reported on their websites.

DETECTION AND IDENTIFICATION

H. hepaticus may be detected by PCR, microbiological culture, serology or histopathology. PCR is most often used for routine surveillance, usually on pooled faecal samples [69, 72]. Although the standard methods of sentinel exposure using soiled bedding are often successful [73], the high sensitivity of *H. hepaticus* to desiccation may also lead to some failures of soiled-bedding sentinel systems to detect *H. hepaticus* and other

helicobacter species in the mice being monitored. PCR can employ 'generic' primers capable of detecting any helicobacter species, or may use specific primers to identify species. Microbiological culture is sometimes conducted, although different species of helicobacter require varying culture methods; thus, culture is less practical as a screening tool. Serology has also been attempted, but is not commercially available, perhaps due to two major drawbacks [74]. First, mice may not seroconvert for several months after infection, so the diagnostic sensitivity may be less than PCR on faeces, and secondly, the specificity is variable. Histopathology with staining techniques such as Warthin–Starry or Steiner silver stains ([75]; see below) can identify characteristic organisms in bile canaliculi in suspicious lesions, but the sensitivity is significantly less than that of PCR [69]. Although finding characteristic histological lesions in the caecum and colon is also diagnostically helpful, PCR is still necessary to confirm the diagnosis. Observation of spiral bacteria in crypts in the caecum or colon is non-specific, as some of the autochthonous microbiota are spirochetes [76, 77].

CLINICAL SIGNS AND PATHOLOGY

Although all strains of mice may be infected, susceptibility to helicobacter-induced inflammatory or neoplastic disease is strain-related. Inflammatory lesions of the caecum and colon may be observed in A/JCr and some other immunocompetent mice [44]. In A/JCr mice, females experience worse intestinal inflammation [78], although male A/JCr mice develop a higher incidence of helicobacter-induced liver neoplasia. Lesions are especially prominent in mice with profound immunodeficiency, such as athymic nude or scid [79], or in mice with impaired regulation of inflammation, such as IL-10-deficient mice with a variety of genetic backgrounds [67]. The most common clinical sign is rectal prolapse (Figure 3.3.12); helicobacter infection, usually *H. hepaticus*, is the most common cause of rectal prolapse in contemporary laboratory mice. Grossly, the caecum and especially the colon often appear segmentally thickened. Mesenteric lymph nodes may be enlarged. Microscopically, the mucosa is much thicker than normal, due to epithelial hyperplasia with immature and mitotically active cells being present from the base to

Figure 3.3.12 Prolapsed rectum due to *H. hepaticus* infection in an athymic nude mouse. Helicobacter infection, usually *H. hepaticus*, is currently the most common cause of prolapsed rectum in mice.

the neck of the crypts. The lamina propria is thickened with a marked mononuclear cell infiltration, with a lymphoid component that may be pronounced near the rectum. With chronic hepatic involvement, the liver may appear roughened or irregular. Microscopically [80, 81], hepatic changes due to *H. hepaticus* infection are characterized by mixed mononuclear infiltrates and hepatocellular necrosis (Figure 3.3.13). As lesions become more chronic, biliary hyperplasia is accompanied by hyperplasia and hypertrophy
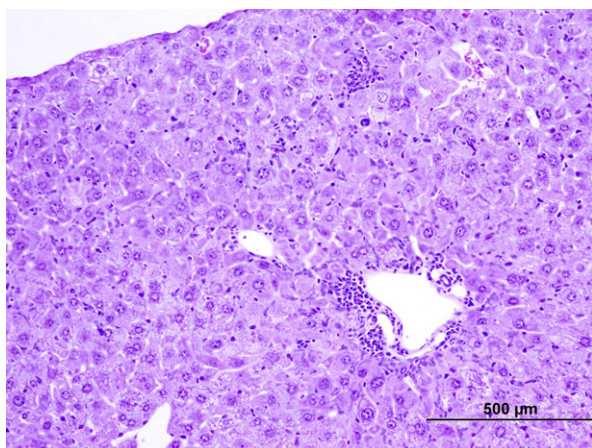
Figure 3.3.13 *H. hepaticus* hepatitis in a mouse. Haematoxylin and eosin, 10× objective. This is a mild chronic diffuse hepatitis. The surface of the liver, upper left, is irregular and hepatic cords are slightly distorted. A mixed mononuclear cell infiltration is scattered in the sinusoids and concentrated in periportal areas.

of oval cells, Ito cells and Kupffer cells. Over time, hepatocytomegaly, fibrosis and lipofuscinosis of Kupffer cells become prominent. Special stains such as a Steiner or Warthin–Starry silver stain will demonstrate spiral organisms in bile canaliculi (Figure 3.3.14). In immunodeficient mice, necrosis may be a significant component, such that Tyzzer's disease may be a differential diagnosis at necropsy [82].

MANAGEMENT AND CONTROL

Management and control of all helicobacter species is similar. Because *H. hepaticus* is only found in infected rodents and does not survive well in the environment, careful husbandry practices can maintain helicobacter-free mice safely in the same facility, room or even rack (assuming filter-top or individually ventilated caging) as infected mice. In addition, the high environmental sensitivity of the bacteria means that eradication of helicobacter from a vivarium need not be accompanied by environmental disinfection. Helicobacter can be eliminated from infected mice either by antibiotic treatment or by rederivation. Antibiotics can be administered in medicated feed [83], or by gavage. In general, antibiotic treatment carries a possibility of a single animal not being completely cleared of helicobacter; such an animal could then serve as a nidus for reinfection of the group. The larger the group of mice treated, the greater the chance of a failure. However, treatment followed by subsequent repeat testing to confirm
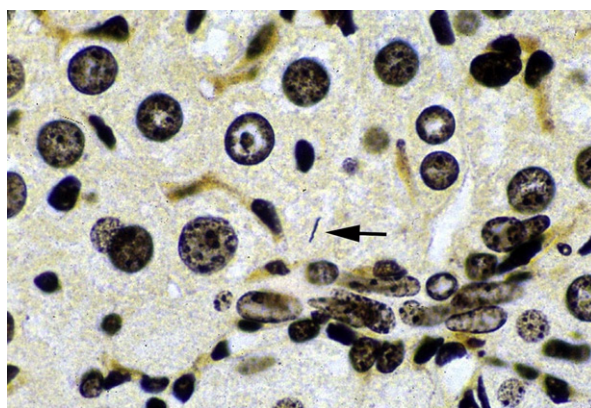
Figure 3.3.14 *H. hepaticus* hepatitis in a mouse. Warthin–Starry silver stain, 100× objective. A spiral bacterium (black arrow) characteristic of *H. hepaticus* is present in a bile canaliculus.

eradication may be the best option in some circumstances.

Rederivation of an infected line of mice may be done by one of several means. Embryo transfer and caesarian section have the advantage of eliminating not only helicobacter, but also all other infectious agents except those that are vertically transmitted [69]. Particular attention should be paid to the health status of the vasectomized males and recipient females, and offspring should be quarantined and tested before release into the general population. The other option for rederivation is by transfer of pups less than 1 week of age (day 1 is usually recommended) to clean foster dams [70, 71]. This method has been reported as highly successful for elimination of helicobacter, as well as murine norovirus and other agents to which the newborn pup is temporarily not susceptible [84].

Helicobacter bilis

BIOLOGY

H. bilis is a curved fusiform bacterium with periplasmic fibres and 3-14 bipolar-sheathed flagella [85]. Environmental susceptibility to desiccation is thought to be similar to that for *H. hepaticus*.

EPIDEMIOLOGY AND PREVALENCE

Like *H. hepaticus*, *H. bilis* is transmitted by the faecal–oral route. However, unlike *H. hepaticus*, *H. bilis* has been detected in multiple host species, including dogs, rats, gerbils and humans. In humans, *H. bilis* in the biliary tract and gallbladder has been associated with cholecystitis [86] and, loosely, with neoplasia [87].

DETECTION AND IDENTIFICATION

H. bilis is detected primarily by PCR on pooled faecal samples for surveillance [72], and on deparaffinized sections for elucidation of a putative role of *H. bilis* in histopathological lesions. It may also be cultured with similar conditions to *H. hepaticus*.

CLINICAL SIGNS AND PATHOLOGY

In mice with immune deficits, *H. bilis* causes rectal prolapse, proliferative typhlocolitis and chronic hepatitis [88]. Chronic hepatitis may also occur in ageing outbred Swiss-Webster mice, with a predilection for females [89]. Liver lesions were characterized by minimal to moderate non-suppurative portal and periportal inflammation. Bacteria consistent with the morphology of *H. bilis* were observed in silver-stained sections.

MANAGEMENT AND CONTROL

H. bilis is managed similarly to *H. hepaticus*.

Other helicobacter species

Other helicobacters detected in mice include *H. ganmani*, *H. rodentium*, *H. typhlonius*, *H. muridarum*, *H. mastomyrinus*, *H. 'muricola'*, and *H. 'rappini'*. Biologically, all are similar, although they may be differentiated by morphology and biochemical cultural characteristics [69]. In addition, *H. ganmani* is an anaerobe, whereas the others are micro-aerophilic.

H. rodentium has been reported to exacerbate intestinal disease in conjunction with *H. bilis* in *Prkdc^{scid}* mice [90] and in conjunction with *H. typhlonius* in IL10$-/-$ mice on a C57BL/6J background [91]. IL-10-deficient mice on a C57BL/6J background have been reported to have decreased pregnancy rates and pup survival when infected with *H. rodentium*, *H. typhlonius*, or both species. The greatest effect was seen for dual-infected mice, the least effect with monoinfection by *H. rodentium*, and an intermediate impact with *H. typhlonius* monoinfection [92]. *H. typhlonius* has also been associated with typhlocolitis in scid mice [93] and IL-10-deficient mice [94]. The other species of helicobacter have not been associated with clinical disease in laboratory mice.

Mycoplasma

Mycoplasma arginini

Although cell lines are frequently infected with mycoplasma [95, 96], these rarely have any ability to infect mice, and are of consequence primarily because of their effects on the cell cultures. For example, no reports of *M. arthritidis*, *M. neurolyticum* or *M. collis* infecting cell lines, and only a single abstract reporting *M. pulmonis* could be found [95]. However, *M. arginini*, which is occasionally found as a contaminant of cell lines, has recently been identified as a cause of pyogranulomatous arthritis in immunodeficient

mice following inoculation with infected cell lines [97].

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CHAPTER 3.4

Parasitic Infections of Laboratory Mice

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Introduction

A parasite is an organism that has evolved to live in or on other species. Parasites generally cause some harm, or at least give no benefit, to the host. Parasites have evolved in parallel with the species they parasitize, and so attempt to elude host defences while remaining in their niche [1]. Parasites may influence host behaviour [2, 3], alter host immune status [4-7] and affect host growth [8, 9]. Although mice can host a large number of parasites, few parasites are found in modern laboratory mouse facilities. Nevertheless, sporadic outbreaks of relatively benign parasites, such as pinworms, regularly occur. These parasites may be rare in modern laboratory animal facilities, but incursion by wild or feral rodents greatly increases the risk of parasitic (and other) infection. Arthropod pests in the animal facility can also pose a hazard to resident mice, because they may act as vectors or intermediate hosts for parasites. A pest control programme that addresses rodent and arthropod pests should be in place to manage this risk.

Both the detection of parasitic infections and their diagnosis can be challenging [10-14]. Care should be taken not to confuse pseudoparasites with true parasites. Plant or other material in faeces may appear to be parasites or their eggs. Free-living arthropods may also be confused with true parasites. External parasites may be present in low numbers, or may spend only part of their time on the host. Relying solely on one diagnostic method for detection of parasites may result in failure to diagnose parasites not easily detected by that method.

Wild-caught or feral mice may have a much greater parasite load than immunocompetent laboratory mice kept under controlled conditions. A greater variety of parasites may also be present in these animals. It is beyond the scope of this chapter to address every possible parasite of mice, so we will focus on parasites that are currently seen in laboratory mouse facilities. For those working with wild mice, *Flynn's Parasites of Laboratory Animals* [15] may prove a useful resource.

When ascribing negative effects to parasitic infections of mice, the older literature cited both here and elsewhere must be evaluated with a keen eye towards the overall health of the animals. In many cases, older literature does not always specify the microbiological status of the animals in question and infections, e.g., with *Helicobacter*, may lead to clinical signs that were previously described as being due to parasite infection [16–18]. The continued elimination of viral and bacterial cofactors may affect clinical signs purportedly produced by parasitic infections. Alternatively, more sophisticated research methods in use in the future may reveal previously unknown effects of parasites on mice.

Internal parasites of laboratory mice

Internal parasites of laboratory mice can be divided into two broad categories: protozoa and helminths. Helminth parasites can be further divided into nematode and cestode parasites. Cestode parasites are more commonly known as tapeworms and are rarely found in modern laboratory mice. Of helminth parasites, the pinworms are the most commonly seen. At least one other nematode parasite of mice, *Heligmosomoides polygyrus*, may be encountered in the laboratory setting, as it is regularly used to study parasitic immunology and assess the efficacy of anthelmintic compounds.

Non-pathogenic protozoa

Protozoan parasites may be found in the intestines or the tissues. Those present in tissues, such as *Klossiella*, *Hepatozoon*, *Babesia*, *Toxoplasma*,

and *Plasmodium*, are almost never found in modern laboratory mouse facilities, but may infect wild mice. If these parasites are being used to model human parasitic infections, precautions to prevent transmission to naive populations should be in place. Non-pathogenic enteric protozoan species, however, are by far the most common enteric parasites seen in modern laboratory mice. These include *Entamoeba muris*, *Chilomastix bettencourti* and various species of trichomonads.

Entamoeba muris

Entamoeba muris is a non-flagellate enteric protozoan parasite of mice (Figure 3.4.1). The spherical cysts and trophozoites of this parasite may be found in the lumen of the caecum, and occasionally the colon, of mice [19]. Cysts may be smaller than trophozoites (9–20 µm vs 25 µm), and cysts of *Entamoeba* have a characteristic appearance due to the presence of eight nuclei. *E. muris* has a direct life cycle and is transmitted through ingestion of faeces. Diagnosis is typically made through light microscopic examination of a direct wet mount of caecal mucosal scrapings. Cysts may be found in faeces by flotation or faecal concentration techniques.
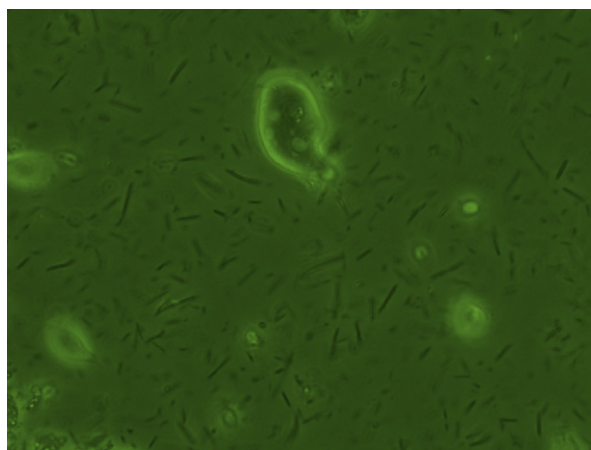
Figure 3.4.1 Phase contrast photomicrograph of *Entamoeba muris* (400×). Multinucleated trophozoites measure 25–30 µm in diameter. *Entamoeba* trophozoites may be seen to form pseudopodia although they may not be motile. If *Entamoeba* trophozoites are suspected, infection should always be confirmed by verifying the presence of cysts. *E. muris* cysts are round, measure 9–20 µm in diameter and contain eight nuclei.

Chilomastix bettencourti

Chilomastix bettencourti is a flagellate enteric protozoan parasite of mice (Figure 3.4.2). It occurs in the lumen of the caecum and ascending colon, where it can be found in two forms, a cyst and a trophozoite [20]. Cysts are smaller than trophozoites, which measure approximately 15 µm × 7 µm. Trophozoites are pear-shaped, with flagella and a nucleus located at the anterior end. Cysts are ovoid, non-flagellate, and have a visible nucleus. The life cycle is direct and cysts are shed in faeces. Infection occurs when cysts are ingested. *C. bettencourti* is typically diagnosed via light microscopic examination of a direct wet mount of caecal mucosal scrapings. Cysts may be found in faeces by flotation or faecal concentration techniques.

Trichomonads

At least four species of trichomonads are known to infect laboratory mice. They are generally not speciated, however, and just identified to the genus level based on their characteristic morphology. The most commonly found in laboratory mice is *Tritrichomonas muris* (Figure 3.4.3). *T. muris* has an anterior nucleus, and is flagellated, with three anterior flagella and one recurrent flagellum with a free posterior portion. This

Figure 3.4.3 Phase contrast photomicrograph of *Tritrichomonas muris* (400×). *T. muris* is the most common species of trichomonad found in mice. It measures 16–26 µm long by 10–14 µm wide. It has three anterior flagella, two of which are easily visible in this photomicrograph and a posterior flagellum. Trichomonads have an undulating membrane, easily seen under the microscope, that helps differentiate them from other protozoans.

recurrent flagellum forms an undulating membrane, which gives trichomonads their characteristic ruffled appearance microscopically. Trophozoites are approximately 21 µm × 12 µm. A true cyst is not formed and typical measurements of the pseudocysts formed by *T. muris* are unknown, but are probably smaller than the trophozoites. In pseudocysts, the flagella are internalized. The life cycle is direct and pseudocysts are shed in faeces. Infection occurs when pseudocysts are ingested. *T. muris* is typically diagnosed via light microscopic examination of a direct wet mount of caecal mucosal scrapings. As well as the undulating membrane, *T. muris* has a characteristic rolling motility when found in wet mounts.

Management and control

The three protozoa described above are non-pathogenic and are considered to have little or no effect on most research. If a colony formerly negative for these organisms becomes positive, it is a marker of a possible problem with biosecurity. Treatment for these parasites is not recommended. Rederivation of animals via embryo transfer into clean recipient females will eradicate infections.

Figure 3.4.2 Phase contrast photomicrograph of *Chilomastix bettencourti* (400×). Trophozoites measure 8.3–20.9 µm long by 6.6–8.4 µm wide. They are pyriform and possess three anterior flagella, one of which is faintly visible in this photomicrograph. The cysts are usually lemon-shaped and contain one nucleus and the organelles of the trophozoite.

Pathogenic protozoa

Few enteric protozoa of mice are described as pathogenic, and of those few, almost none are found in modern laboratory mouse facilities. The three species of protozoan parasites that are described as pathogenic in laboratory mice are *Giardia muris*, *Spironucleus muris* and *Cryptosporidium muris*. *S. muris* remains relatively common in modern laboratory mice, but *G. muris* and *C. muris* are rarely seen.

Giardia muris

G. muris is a pear-shaped parasite whose trophozoite is easily distinguished from others by its two prominent anterior nuclei and paired posterior flagella (Figure 3.4.4). The trophozoite is 7-13 × 5-10 μm and the ellipsoid cyst is 15 × 17 μm. *G. muris* trophozoites have a characteristic 'falling leaf' motility if seen in a wet mount of intestinal mucosa. These parasites are found in the lumen of the small intestine, in close association with the brush border. Clinical signs include weight loss and decreased growth in susceptible mice [21].
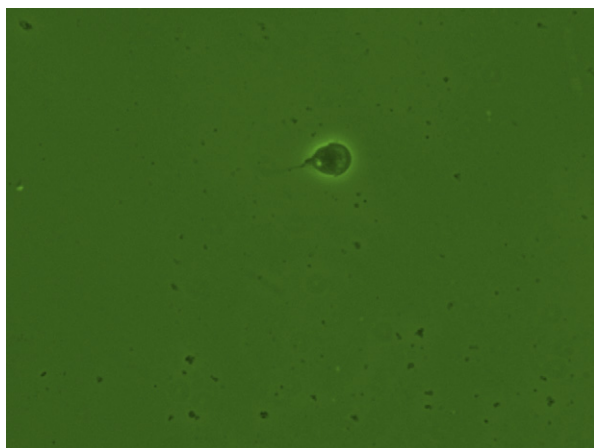
Figure 3.4.4 Phase contrast photomicrograph of *Giardia muris* (400×). *G. muris* measures 7–13 μm long and 5–10 μm wide. Trophozoites have a broadly rounded anterior end and eight flagella emerging at different locations. Two slender axostyles emerge from the posterior end. The typical 'owl eyes' appearance of *Giardia* spp. is due to a pair of darkly-staining median bodies. Movement is characterized by a cupping motion, rotating side to side as the cup flexes. *Giardia* is considered a pathogenic protozoan, although most infections do not result in clinical signs.

G. muris may be diagnosed through direct examination of the mucosa of the small intestines. Cysts can be found in the faeces and faecal PCR is also available to detect organisms in faeces.

Spironucleus muris

S. muris is a parasite found in the mucus layer of the small intestine and the crypts of Lieberkuhn. It is a small, tapered ellipsoid, measuring 10-15 × 3-4 μm, and has a rapid, zigzag motility when seen on wet mount. *S. muris* is multiflagellated, with both anterior and posterior flagella, and has two anterior nuclei. The presence of this parasite, since it is in such close association with the host, can cause an alteration of immunoreactivity. In immunodeficient mice, clinical disease, consisting of chronic enteritis and wasting, may be seen [22]. *S. muris* may be diagnosed by wet mount of intestinal mucosal scrapings, or by examination of faeces for cysts. Cysts may be detected by PCR of faeces, or by faecal centrifugation and concentration techniques.

Cryptosporidium muris

C. muris is a small, round to ellipsoid protozoan. Its oocysts typically measure 5 × 7 μm and the meronts are approximately the same size. The parasite is described as intracellular, but extracytoplasmic and is located in the cells of the gastric mucosa. Chronic infection of immunodeficient mice is possible, resulting in sticky faeces and weight loss, although immunocompetent mice show no clinical signs [23]. *C. muris* may be seen on faecal flotation, or in wet mount of gastric mucosal scrapings.

Management and control

If a colony formerly negative for these organisms becomes positive, it is a marker of a problem with biosecurity. Treatment for these parasites is not recommended. Rederivation of animals via embryo transfer into clean recipient females will eradicate infections.

Oxyurids (pinworms)

After protozoa, the next most commonly seen internal parasites of laboratory mice are the

oxyurids, or pinworms. Pinworms reside in the caecum and colon and are rarely associated with clinical signs, although their presence may interfere with research through modulation of the immune system [24, 25]. Mice are primarily infected with two species of pinworms, *Syphacia obvelata* and *Aspiculuris tetraptera* and mice can be infected with both species at the same time. [26] The rat pinworm, *Syphacia muris*, may be seen in mice, but it will not be discussed in depth. *S. muris*, *S. obvelata*, and *A. tetraptera* are easily differentiated by both egg and worm morphology. A guide to the basic characteristics of all three species is presented in Table 3.4.1.

Syphacia obvelata

S. obvelata is the most common helminth parasite of laboratory mice as reported by surveys and diagnostic laboratories [27-29]. The morphology of adult *S. obvelata* differs from *A. tetraptera* in many aspects, including the lips, the cervical alae, the presence of mamelons on the male and the presence of spicules. *S. obvelata* has three prominent, fleshy lips surrounding the mouth and a round oesophageal bulb, as well as small cervical alae. These features are readily visible with light microscopy. *S. obvelata* males are smaller than females, with males 1–1.5 mm and females 3.5-6 mm long. Male *S. obvelata* have a prominent spicule and two or three rounded mamelons, the organs used to grasp females during copulation. Female *S. obvelata* are markedly larger than males and eggs are visible within the body of mature females. The vulvar pore is located in the anterior of the body. The eggs average 134 × 36 μm, and are banana-shaped (flattened on one side) (Figure 3.4.5).

The life cycle of *S. obvelata* is short and direct, with eggs hatching 5-20 h after release from the female, and a prepatent period of 11-15 days. Infection is generally through ingestion of embryonated eggs, although retroinfection by larvae from the perineal skin is theoretically possible [30]. The larvae migrate to the caecum, and most females are fertilized by 6 days after hatching. *S. obvelata* lives in the caecum, where it feeds on free-living caecal bacteria. The gravid

TABLE 3.4.1: Physical characteristics and life cycles of *Syphacia obvelata*, *Syphacia muris*, and *Aspiculuris tetraptera*

	S. obvelata	S. muris	A. tetraptera
PHYSICAL CHARACTERISTICS			
Length			
Female	3.5–6 mm	3–4 mm	3–4 mm
Male	1–1.5 mm	2–3 mm	2–3 mm
Cervical alae	Subtle	Moderate	Prominent
Female tail	Long and pointed	Long and pointed (longer than S. obvelata)	Conical
Vulvar location	Anterior body	Anterior body	Middle of body
Male mamelons	Present	Present	Absent
Spicule	Present	Present	Absent
Ova	Average 134 × 36 μm, flattened on one side	Average 78 × 31 μm, slightly flattened on one side	Average 86 × 37 μm, ovoid and symmetrical
LIFE CYCLE			
Location in host	Caecum and colon	Caecum and colon	Colon
Prepatent period	11–15 days	7–8 days	21–25 days
Location of ova	Perianal skin	Perianal skin	Faeces
Time to infectivity of ova	5–20 h	5–20 h	5–8 days

Figure 3.4.5a The anterior of *Syphacia obvelata*. Note the round oesophageal bulb and the fleshy lips at the mouth.
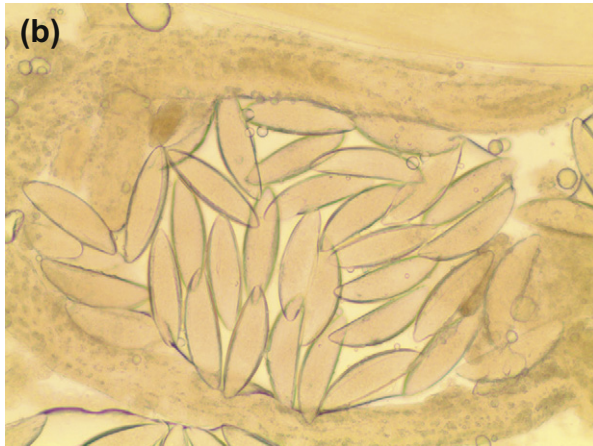
Figure 3.4.5b *Syphacia obvelata* eggs. This photomicrograph shows *eggs* contained within a gravid female. Eggs present on the perineal hair are generally present in smaller numbers.

females travel to the perineum, where they lay an average of 350 eggs and then die [31].

Aspiculuris tetraptera

A. tetraptera differs from *S. obvelata* in morphology and size of both males and females. As distinguishing features, *A. tetraptera* has an oval esophageal bulb, smaller lips and broader cervical alae. Female *A. tetraptera* are 3-4 mm long and approximately 250 μm wide, while males are 2-4 mm long and approximately 160 μm wide. *A. tetraptera* males are larger than *S. obvelata* males, and both mamelons and

spicules are absent in *A. tetraptera*. The vulvar pore of the female *A. tetraptera* is located further posteriorly than in *S. obvelata*. The oval-shaped eggs average 86 × 37 μm and the embryo within the egg is in the morula stage at the time of release (Figure 3.4.6).

The life cycle of *A. tetraptera* is short and direct, but longer than that of *S. obvelata*. The prepatent period is 21-25 days. Adults live in the lumen of the proximal colon and females travel from the proximal to distal colon to lay their eggs, which are incorporated into the faeces. Each female releases approximately 17 eggs per day and then returns to the proximal colon. Egg release continues for 21-24 days after the first release, so the total lifespan of a female is 45-50 days [32]. Infection is via ingestion of embryonated eggs, but eggs of *A. tetraptera* are not infective until 5-8 days after release from the female. The larvae hatch in the caecum, then migrate to the colon, where they mature within the crypts of Lieberkühn.

Diagnosis is usually made by examination of colony or sentinel animals. The gold standard is considered to be examination at necropsy of the caecal and colonic contents for the presence of adult worms. Other diagnostic methods include fur plucks of the perineal area and perineal tape tests [14]. The two methods should be combined because *A. tetraptera* eggs are only rarely found on a perineal tape test and faecal
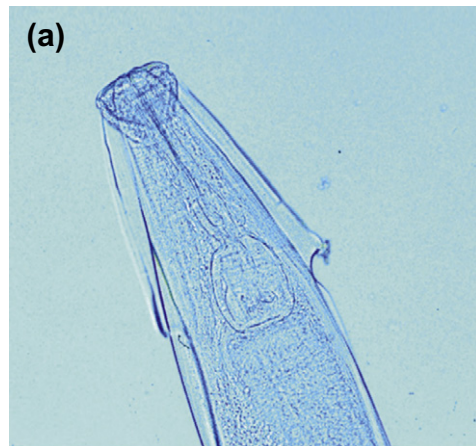
Figure 3.4.6a The anterior of *Aspiculuris tetraptera*. The oesophageal bulb is oval, not round, the lips are smaller, and the worm has prominent cervical alae. These characteristics help distinguish *A. tetraptera* from *S. obvelata*.
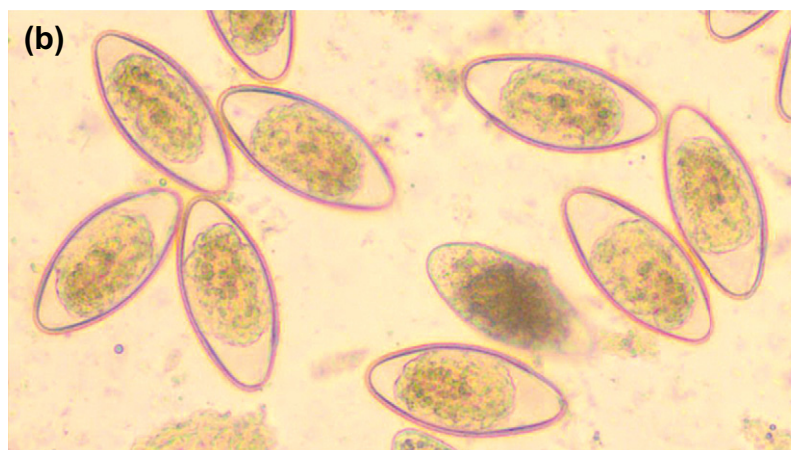
Figure 3.4.6b Eggs of *A. tetraptera* from a faecal flotation. The ellipsoid eggs of *A. tetraptera* contain an embryo that does not fill the egg.

flotation is not often successful in diagnosing *S. obvelata*, since the eggs of this parasite are firmly adherent to the perineal hairs. Faecal flotation should be a routine part of parasitology screening for mouse colonies because this will detect other helminth parasites as well. PCR of faeces is a promising method for diagnosing pinworm infections of both species. The PCR targets the 16s ribosomal DNA sequence unique to *A. tetraptera* or *S. obvelata* since this is present in every cell of the animal from egg to adult [33].

Clinical signs and pathology

Clinical signs associated with pinworm infections are rarely seen, even with heavy infections. Older literature does mention heavy infections causing decreased growth rate, catarrhal enteritis, hepatic granulomas and perianal irritation, but these animals may have been coinfected with other agents that could contribute to such clinical signs (e.g. *Helicobacter*) [17, 18, 34, 35]. Pinworms have the potential to interfere with research through their modification of the host immune system. Young animals have the highest worm burdens and these burdens decline with age due to activation of the host immune system. [34, 36–38] Host-parasite interaction is complex, involving both B and T cell mediated effects. Pinworm infections have been shown to induce lymphoma [39], ablate self-tolerance [40], induce Th2 responses [25] and increase the host humoral immune response [41].

Management and control

As with all enteric agents, rederivation via embryo transfer into clean dams and relocation of those dams to a clean environment will eradicate both *S. obvelata* and *A. tetraptera*. This may be a drastic response, however, if pinworms are the only infectious problem in the facility. Many facilities have been successful in eliminating infections from colonies through a combination of anthelmintic treatment of animals and stringent environmental decontamination. The most successful anthelmintic appears to be fenbendazole, since it acts on all stages of the worm's life cycle (egg, larva and adult) [42, 43]. A typical treatment regimen is to give feed compounded with 150 ppm fenbendazole for four alternating weeks, combined with a thorough environmental decontamination.

Although pinworms may affect research, treating pinworms may also influence research results. Fenbendazole may occasionally act as a tumour promoter if certain initiators are given concurrently [44]. Recent studies also suggest that fenbendazole may affect the immune system of mice, but that the effects will probably resolve after 4–6 weeks on normal diet [45]. Ivermectin is also used to treat animals for pinworms. Selamectin does not appear to be as efficacious for pinworms in mice [46, 47]. Mice with a compromised blood-brain barrier should not be treated with ivermectin [48] and care should be taken with young mice and transgenic mice [49, 50]. The eggs of pinworms appear to survive well in the environment,

although few controlled studies have been performed. Eggs are definitely susceptible to autoclaving and strong oxidizing disinfectants [51, 52].

Nematode parasites

Heligmosomoides polygyrus is another helminth parasite of mice. This trichostrongyloid nematode parasite was once common in research facilities, but is not found in modern facilities unless it is being used as a model of host–parasite interaction or to test anthelmintic efficacy. The life cycle is direct, and the larvae are infective 4-5 days after the eggs hatch in the environment. Larvae travel through the tissues to the small intestines, where they mature into adults that penetrate tissues and feed on tissue components. Adults of both sexes are tightly coiled red worms but females are larger than males, at 9-12 mm and 4-6 mm in length, respectively. The prepatent period is 9-12 days. Diagnosis may be made by faecal flotation or visualization of the parasites in the intestines at necropsy. Treatment of *Heligmosomoides* is usually with ivermectin, and effects on the host are generally related to the immune system [53-56].

Cestode parasites

Cestode parasites of laboratory mice are vanishingly rare in modern animal facilities. These parasites may also be used to study parasite–host interactions, and if so, care should be taken to avoid infecting naïve colonies. Mice are the definitive hosts for three cestodes: *Hymenolepis diminuta*, *Rodentolepis microstoma* and *Rodentolepis nana*. General characteristics of these parasites may be found in Table 3.4.2. Cestode parasites share a general form, with a scolex, or head, attached to the host by suckers, and a strobila, or body, attached to the scolex. The scolex contains a rostellum, which may be armed with hooks. The strobila is composed of segments called proglottids that contain eggs. These proglottids break off the strobila and are passed in the faeces (Figure 3.4.7).

Hymenolepis diminuta

Although commonly known as the rat tapeworm, *H. diminuta* can infect mice. If an infection is established, this cestode will be found in the small intestines. It attaches to the wall of the small intestine with a scolex with four suckers, but an unarmed rostellum, and feeds on host interstitial fluids. Adult worms are 3-4 mm wide and 20-60 mm long. *H. diminuta* eggs measure approximately 70×68 μm, and contain three pairs of small hooks. The eggs of *H. diminuta* are more spherical than those of *R. nana* or *R. microstoma*, and do not contain polar filaments.

H. diminuta requires an intermediate host for its life cycle. Cysticercoid larvae develop

TABLE 3.4.2: Physical characteristics and life cycles of *Hymenolepis diminuta*, *Rodentolepis microstoma*, and *Rodentolepis nana*

	H. diminuta	R. microstoma	R. nana
PHYSICAL CHARACTERISTICS			
Length	20–60 mm	8–50 mm (up to 120 mm)	25–40 mm
Width	4 mm	0.5 mm -4 mm	0.25–0.5 mm
Armed rostellum	No	Yes	Yes
Egg diameter	~70 μm	~85 μm	~45 μm
LIFE CYCLE			
Requires intermediate host	Yes	Yes	No
Location in host	Small intestine	Bile duct	Small intestine
Prepatent period	19–20 days	16–17 days	14–16 days
Zoonotic	Yes	No	Yes
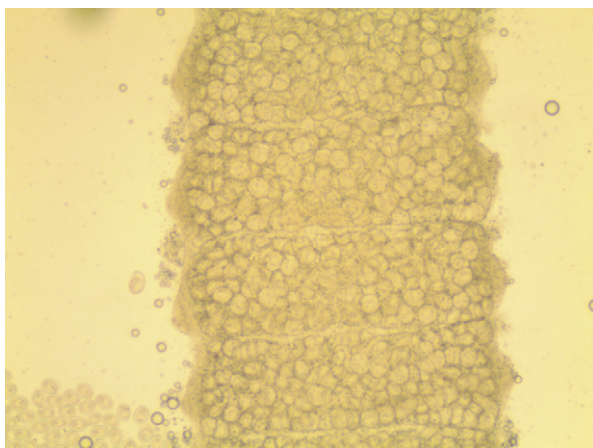
Figure 3.4.7 Proglottids of *H. diminuta*. Proglottids are shed in the faeces and are infectious when ingested. The adult worm measures 20–60 mm in length and 3–4 mm in width. The eggs are almost spherical, measure 62–88 μm by 52–81 μm, and contain an embryo which possesses three pairs of small hooks and lacks polar filaments.

in an insect, usually a flour beetle, moth or flea, after consumption of eggs. The mouse must consume the intermediate host to become infected and pass infective proglottids in the faeces. After ingestion of the intermediate host, adult worms are found in 19-21 days. Human infection with *H. diminuta* is possible, but must occur through ingestion of the arthropod intermediate host.

Rodentolepis (Hymenolepis) microstoma

Adult *R. microstoma* is a tapeworm found in the bile ducts of mice. It measures 2 mm in width, but varies widely in length from 80 mm to 350 mm. The scolex has 4 suckers and the rostellum is armed with approximately 25 hooklets. The ovoid eggs average 72×60 μm and contain both polar filaments and three pairs of small hooks. The eggs are very similar in appearance to those of *R. nana*, but larger.

 R. microstoma also has an indirect life cycle, but it can have a direct life cycle in immunodeficient mice [57]. The intermediate hosts are the same as *H. diminuta*—moths, fleas and the flour beetle. After ingestion of the intermediate host, the pre-patent period is 14 days, during which the worms migrate from the proximal small intestine to the bile duct. Adult worms attain their adult size by 25 days after infection. Human infection

with *R. microstoma* has been described in the literature [58].

Rodentolepis (Hymenolepis) nana

R. nana is also known as the dwarf tapeworm of mice. Adult worms are less than 1 mm wide and 25-40 mm long, making them the smallest of the commonly described mouse cestodes. The scolex of this worm has four suckers and a rostellum armed with approximately 25 small hooks. Eggs of *R. nana* are oval, measuring approximately 50×35 μm. The eggs contain an embryo with three pairs of hooks and polar filaments. *R. nana* attaches to the wall of the small intestine where it feeds on interstitial fluids. *R. nana* is considered a potential zoonotic infection, although current literature indicates that there may be human-specific isolates of *R. nana* [59].

 Unlike both *H. diminuta*, *R. microstoma* and, in fact, all other cestodes, *R. nana* has both a direct and indirect life cycle in immunocompetent animals. In the indirect life cycle, mice must ingest an intermediate host containing cysticercoid larvae, such as the flour beetle, to become infected. Grain beetles and some fleas may also serve as intermediate hosts. However, a direct life cycle, in which mice ingest faeces containing *R. nana* eggs and become infected, is also possible. In this life cycle, the eggs are ingested by a mouse, become cysticercoid larvae within the mucosa of the intestinal villi, then re-enter the intestinal lumen of the same mouse. The indirect life cycle is variable in length due to the time it takes for the cysticercoid larvae to develop within an insect. The direct life cycle takes 14-16 days, 4-5 days as larvae, then another 10-11 days to develop to full maturity within the intestine.

 Diagnosis of cestode infection is usually through discovery of eggs on faecal flotation, or adult worms in their typical location. These parasites are common in wild mice and mice from pet shops. PCR assays are described in the literature as well.

Clinical signs and pathology

Clinical signs associated with cestode infection may be seen with heavy infections. These can include catarrhal enteritis, growth retardation,

weight loss and possibly intestinal blockage [35]. Light infections may not produce clinical signs. Effects on research are related to modulation of the immune system produced by parasitism [60, 61]. In infections with *R. microstoma*, biliary inflammation and mucosal erosion are seen [62]. Experimental infections with *R. microstoma* may be associated with more severe changes such as intestinal mastocytosis and hepatitis [63].

Taenia taeniaeformis (cat tapeworm)

The mouse is the intermediate host of the cat tapeworm, *Taenia taeniaeformis*. Strobilocerci of this parasite may be found in the muscle and liver of infected mice approximately 30 days after ingestion of eggs. The strobilocerci are 4–10 mm in diameter, and contain a scolex and segmented strobila. In older literature, strobilocerci may be given their own scientific name, *Cysticercus fasciolaris* [64]. There are generally no clinical signs associated with this parasite, and it can only be found at necropsy. Finding this parasite in mice means that the mice have ingested cat faeces. A thorough inquiry as to how this might have occurred is of paramount importance.

Management and control

Treatment of cestodes is possible, but not recommended. An oral dose of praziquantel at a dose of 140–210 ppm in feed for 7 days is reported to be effective for *R. nana* infections [65]. Treatment regimens for *H. diminuta* and *R. microstoma* are probably similar, but little information is available in the literature. The eggs of the parasites may persist in the environment, however, and if intermediate hosts are not excluded from the animal areas, reinfection may occur. As with all enteric agents, rederivation via embryo transfer into clean dams and relocation of those dams to a clean environment will eradicate all three cestodes.

External parasites of laboratory mice

External parasites of laboratory mice include ticks, lice, fleas and mites. The only parasites that remain prevalent in modern facilities are mites [27–29]; the rest are only of concern if working with wild-caught mice. A facility's pest control programme should consider the local population level of wild mice and the potential for incursion of external parasites harboured by wild mice. If wild-caught mice are brought into a facility for study, quarantine should include appropriate treatment for external parasites. Again, if a facility works with wild-caught mice, *Flynn's Parasites of Laboratory Animals* [15] may be of help in identifying uncommon parasites.

Mites

Many species of mites may infest mice; the following addresses only species that are likely to be seen or that have zoonotic significance. Notably, mice must have hair for a fur mite infestation to occur; nude or hairless mice are not susceptible to mites. Modern laboratory mice are most commonly affected by three species of mites: *Myobia musculi*, *Myocoptes musculinus*, and *Radfordia affinis*. *Radfordia ensifera*, a mite of rats, may also be found on mice, but this is not common. Other uncommon species that may be found on mice are *Demodex musculi* and *Ornithonyssus bacoti*. Mites are typically distinguished by the morphology of the adults, as the eggs appear similar (Figure 3.4.8). A summary of physical
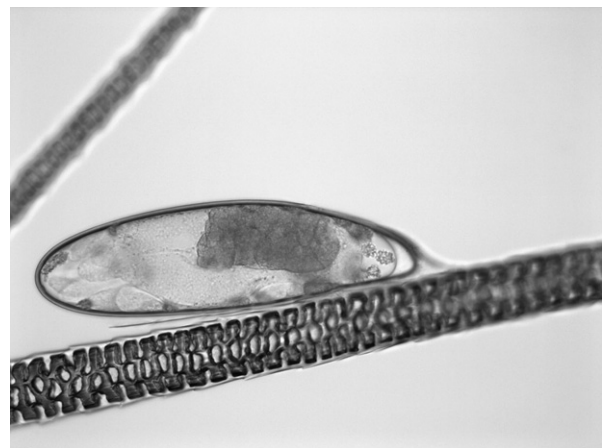
Figure 3.4.8 An egg of *Myocoptes musculinus*, shown tightly adherent to the hair shaft. Differentiating mites by eggs alone is difficult; always seek out adults or nymphs to properly speciate the mites seen.

	Myobia musculi	Myocoptes musculinus	R. affinis	R. ensifera
TABLE 3.4.3: Physical characteristics and life cycles of *Myobia musculi*, *Myocoptes musculinus*, *Radfordia affinis* and *Radfordia ensifera*				
PHYSICAL CHARACTERISTICS				
Length				
Female	400–500 μm	300–325 μm	400–500 μm	400–500 μm
Male	280–320 μm	190–200 μm	280–320 μm	280–320 μm
Width	160–180 μm	130–135 μm	180–200 μm	180–200 μm
Distinguishing feature	Single empodial claw on 2nd leg	Heavily chitinized 3rd and 4th legs	Paired empodial claws on 2nd leg; unequal length	Paired empodial claws on 2nd leg; equal length
Resembles	*Radfordia*	—	*Myobia*	*Myobia*
Feeds on	Cellular interstitial fluid	Skin debris	Cellular interstitial fluid	Cellular interstitial fluid
Egg location	Proximal hair shaft	Distal hair shaft	Proximal hair shaft	Proximal hair shaft
Life cycle	23 days	14 days	?	?
Zoonotic	no	no	no	no

characteristics of common mites is found in Table 3.4.3.

All mites share a typical life cycle that differs from that of insects. The mites described below progress from an egg into one or two larval stages, and from there into two or three nymphal stages. The larval stage(s) has six legs, but nymphs have eight legs and often appear to be miniature adults. Adults are sexually mature and once a female is fertilized, she will lay a variable number of eggs during her lifespan. Unfortunately, detailed information on the life cycle is not available for every species of mouse fur mite, as it is on mite species of economic or human health importance.

Myobia musculi

Like other species of mites, *Myobia musculi* has eight legs (Figure 3.4.9). The first two legs are highly adapted to grasp hair, so the mite appears to have six legs and a pair of 'pincers'. The body of this mite has characteristic bulges between the legs, and each of the walking legs has a single empodial claw. The female is larger than the male (400-500 μm long compared to 285-320 μm). Females may be seen to contain eggs. *Myobia* feeds on cellular interstitial fluid, so is immunologically sensitizing.
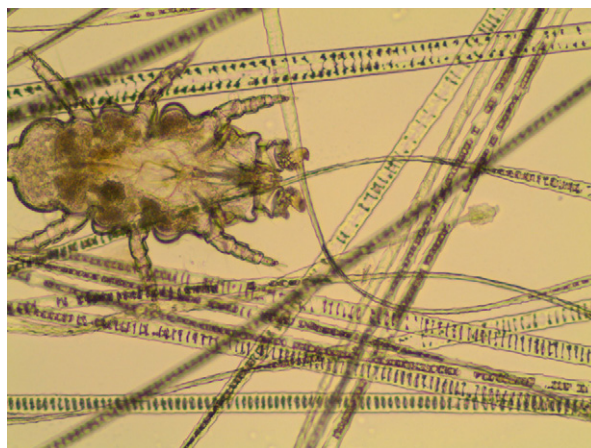
Figure 3.4.9 *Myobia musculi* in a fur sample mounted on tape. Note the first legs, which appear almost like pincers at the rostral end of the mite. These legs are adapted to grasp hair. Also note the bulges of the body between the other legs; this is typical of both *Myobia* and *Radfordia*.

Transmission of *Myobia* definitely occurs through direct contact with infested mice. It is theoretically possible that animals could become infested through contact with shed fur containing eggs, or through mobile adults in the environment. Adult mites are known to leave dead mice as the carcass cools, but this mite is generally described as spending its entire life cycle on the fur of

Figure 3.4.10 *Myocoptes musculinus* **in a fur sample mounted on tape.** The third and fourth legs are heavily chitinized. In very heavy infestations, these legs can give an orange-yellow cast to the fur of white animals. In contrast to *Myobia* and *Radfordia*, these mites have a smooth, elongated body.

mice. The life cycle of *Myobia* is complete in approximately 23 days. The survival time of eggs in the environment is unknown. When laid on the animal, eggs hatch in 7–8 days. Eggs of *Myobia* are described as being attached to the proximal part of the hair shaft, when compared to those of *Myocoptes* [66]. *M. musculi* infests neonates at 7–8 days of age.

Myocoptes musculinus

Myocoptes musculinus is another commonly seen fur mite of mice. Of the eight pairs of legs, the 3rd and 4th pairs are modified for hair clasping and heavily chitinized (Figure 3.4.10). In very heavy infestations, the appearance of the legs can make the fur of albino animals appear brownish at sites of mite concentration. Female *Myocoptes* measure approximately $380 \times 130 \, \mu m$, while the smaller males measure $175 \times 135 \, \mu m$. *Myocoptes* is described as more mobile than *Myobia* and will tend to spread out over the body. Rather than plasma, *Myocoptes* feeds on skin debris, so it is less immunologically sensitizing than *Myobia*. In mixed infestations, *Myocoptes* is described as outcompeting *Myobia*.

The life cycle of *Myocoptes* is complete in 14 days, but the lifespan of individual mites is unknown. Eggs of *Myocoptes* are described as being attached more distally on the hair shaft than those of *Myobia* [66]. The eggs hatch 5 days after being laid on the fur of the host. *Myocoptes* can infest

neonates as early as 4–5 days of age. As with *Myobia*, transmission of *Myocoptes* primarily occurs through direct contact with infested mice. It is theoretically possible that animals could become infested through contact with shed fur containing eggs, or through mobile adults in the environment. Adult mites are known to leave dead mice as the carcass cools, but this mite is generally described as spending its entire life cycle on the fur of mice. The length of time eggs remain viable in the environment is unknown.

Radfordia affinis

Radfordia affinis is a mouse fur mite very similar to *Myobia musculi* but distinguished from it by having two tarsal claws of uneven length on the second pair of legs (*Myobia* has a single empodial claw and *R. ensifera* has paired claws of equal length). Little is known about either *Radfordia* species and their biology, life cycle and effects on research are assumed to be similar to that of *Myobia*.

Detection and identification

Fur mite diagnosis is often performed antemortem, since treatments other than rederivation have the potential to be effective. Antemortem diagnostic methods include PCR, fur pluck and fur scrape techniques [14]. PCR, while exquisitely sensitive in detecting the presence of mites, is unlikely to be helpful in determining the success of treatment since parts of the mite may be present in the pelage for several months [12]. The same caveat should apply to any examination of skin scrapings or fur. Postmortem diagnosis is also effective and can be accomplished through direct examination of mice for mites, or tests involving waiting for mites to leave a cooling carcass [14]. When performing any test involving the pelage, be sure to examine the mouse thoroughly. Although the literature lists 'typical' locations for mites, mites do not read the literature and can be found anywhere on the animal. All of these methods can be performed to detect mites directly on colony animals or indirectly, via the use of sentinels. Recent work has brought into question the ability of dirty bedding sentinels to accurately detect fur mite infestations, but the sensitivity of sentinels is likely related to both mite ecological factors and the level of infestation in a colony [11, 12, 67].

Clinical signs and pathology

As noted above, animals need hair to be infested with mites, so hairless and nude animals will be resistant. The animals' natural grooming patterns are important to keep mite populations down and animals with impaired grooming abilities will have higher mite populations [68, 69]. In older literature, heavy infestations are described as causing a decreased lifespan, as well as depressed reproduction and decreased body weight [70]. If animals have wounds secondary to pruritis caused by mite infestations, secondary infection is certainly possible [71]. *Myobia* in particular, with its more intimate association with the animal, will effect a change in the IgE response, which can affect studies involving allergic response or response to parasites [72, 73].

Management and control

Treatment of *Myobia*, *Myocoptes*, and *Radfordia* is accomplished in a similar fashion. Generally, this is through the administration of ivermectin. Ivermectin has been administered to mite-infested colonies of animals in the feed [74], in the water [75] and as a topical treatment [76]. Older literature discusses treating bedding with various agents, including organophosphates, pyrethrins and permethrins as an adjunct method of managing mite infestations [77-79]. In some cases, adding insecticide-treated nesting material has also been found to be a useful adjunct to other treatments [80, 81]. As with other parasitic agents discussed in this chapter, embryo transfer, hysterectomy rederivation, or fostering neonates onto clean dams will also eradicate these parasites.

Less common mites

Demodex musculi is a hair follicle mite of mice. This small, cigar-shaped mite has been primarily found in mice with immunodeficiencies [82]. Little is known about the life cycle or treatment of this mite. Even in severely immunodeficient mice, there are no clinical signs associated with *D. musculi* [82]. If treatment is desired, treatments effective against canine demodicosis, such as topical amitraz or oral ivermectin, should be effective. Diagnosis is via deep skin scrapings of the dorsum.

Ornithonyssus bacoti is also known as the tropical rat mite. This mite is an obligate intermittent, but not fastidious, blood feeder and is rarely found on animals unless feeding. These are relatively large mites, measuring 750 μm to 1 mm. Female mites are yellow-white to tan, but reddish-black if they have had a recent blood meal. Their life cycle is complete in 13 days, and the life span of females is approximately 70 days, during which she will lay about 100 eggs. *O. bacoti* are zoonotic and an infestation in a colony is often discovered when caretakers and research staff develop dermatitis after being in contact with animals or working in the animal facility [83, 84]. The mites are usually found in proximity to animals, such as on bedding or caging, rather than on animals. Treatment should be focused on the environment, rather than the animals. Organophosphates and pyrethrins have been used successfully to treat infestations. Since *O. bacoti* infests a wide variety of hosts, infestation of laboratory colonies is usually related to incursions of wild rodents.

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Housing and Maintenance

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Introduction

Proper housing takes into account the physical and social environment of the animals. Well-organized colony management and correctly followed animal care regulations are indispensable prerequisites for animal experiments of high quality and reproducibility. In this chapter appropriate conditions for breeding, maintenance and experimentation are described with respect to various hygienic levels. In animal experimentation the established (national) guidelines for the care and use of laboratory animals in line with the local animal welfare regulations have to be followed. For details, see [1, 2] and also Chapters 4.2 and 6.1.

General aspects

The husbandry must provide a standardized macro- and micro-environment as a basis of reliable and reproducible research results, but should also take into account the welfare of the animals and avoid, minimize and alleviate distress. Several recommendations have been published which serve as guidelines for proper (e.g. [2-8]), but not necessarily comfortable ([9], Chapter 4.2) housing of mice. These guidelines refer to requirements on ventilation, temperature, humidity, lighting, noise levels, health status (see Chapter 4.4; 10), feeding, water supply, animal enclosures, handling and experimentation (see Chapter 5.1) including anaesthesia, analgesia and euthanasia (see Chapter 5.4). The environmental requirements of mice are summarized in Table 4.1.1, the space requirements according to ILAR [2] and the European standards [11] are listed in Table 4.1.2.

So far temperature, relative humidity, pollutants and ventilation standards are based rather on room conditions than in-cage conditions, and this can have serious affects on the well-being of the mice, depending on the primary enclosure system in use. Cage ventilation rates and their

TABLE 4.1.1: Environmental requirements of mice

Temperature	20–24 °C
Relative humidity	55 ± 10%
Ventilation (air change/h) in room (open cages)	~15
Ventilation (air change/h) in cage (IVC)	30–80
Photoperiod (light/dark[a])	12/12, 14/10
Light intensity	60[b]–400[c] lux
Noise	~50[d] to <85 dB
Water intake[a]	5–8 ml/day[e]
Food intake[a]	4–8 g/day[e]

See also references [7, 18, 19, 134, 135, 136].
[a] Working light during dark phase <630 nm [18, 19].
[b] At cage level.
[c] Center of room.
[d] Empty room.
[e] According to Harkness and Wagner [136]: 15 ml/100 g BW/day, or 15 g/100 g BW/day.

ambient temperature by altered activity, metabolic and respiratory rate, as well as behavioural thermoregulation, by moving towards a zone where the habitat is less stressful. Because of these effects it is advisable to keep the temperature constant without wide fluctuation [15]. While room temperatures in the range of 20-26 °C are generally considered acceptable [2], the optimal temperature for mice varies. Mice prefer higher temperatures as they age, with warmer temperatures during the light period (28-29 °C) and cooler temperatures during the dark period (23-24 °C) [16]. Temperatures above 30 °C can lead to embryonic mortality and teratogenesis [17].

Another environmental variable, the photoperiod, has profound regulatory effects on circadian rhythms and should therefore be properly controlled using qualified installations (timers). In general, light-dark cycles of 12 h each are used. However, it might be more appropriate to provide 14 h of light and 10 h of darkness, as breeding productivity might increase and, in view of daylight saving, time a fixed unaltered scheme throughout will permit longer access to the animals without interfering with their

effect on intracage ammonia and carbon dioxide concentration [7, 12] are discussed later, as well as air quality.

Mice can adapt to a range of temperatures [13, 14] and will compensate for changes in the

TABLE 4.1.2: Space requirements for mice

	Body weight (g)		Minimum floor area (cm²)		Minimum cage height (cm)		Floor area per animal (cm²)	
	EU[a]	ILAR[b]	EU[a]	ILAR[b]	EU[a]	ILAR[b]	EU[a]	ILAR[b]
In stock and during procedure		<10				12.7		39.24
		<15				12.7		51.6
		<20	330				60	
	<25	<25	330		12	12.7	70	77.4
	<30		330		12		80	
		>25				12.7		96.75
	>30		330		12		100	
Breeding			330[c]	[d]	12	12.7		

[a] European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes, ETS 123, Appendix A [1, 11].
[b] ILAR Guide for the Care and Use of Laboratory Animals [2].
[c] For a monogamous pair or a trio; for each additional female plus litter 180 cm² should be added.
[d] Mice are typically bred in female to male ratios of 1:1 or 2:1. If bred permanently monogamous (1:1), the female and any litter of pups may be kept together with the male until the pups are weaned. In case of a trio (two females and one male) kept in a microisolator cage, it is recommended that one of the two females be removed to a separate cage once observed to be pregnant. This facilitates compliance with housing standards and furthermore permits unobscured pedigree documentation. It is, however, permissible to keep the trio and the pups of one or two litters together providing that when the eldest litter turns 14 days of age there are no more than 12 pups in total in the cage [56]. Higher female ratios (3:1, 4:1) are not recommended unless pregnant females are removed once observed to be pregnant. Weaning of pups is recommended by ILAR [2] at 18–21 days of age especially when intensive mating is done where the male is kept with the female(s) permitting breeding on the postpartum oestrus. Mouse pups should be weaned by 28 days of age at the latest, or if a new litter is borne by the still nursing female.

circadian rhythm (see Chapter 2.12). Further, since mice as nocturnal animals cannot discern red light ($\geq$630 nm wavelength) from darkness [18, 19], emergency lights as well as door-viewing windows should be appropriately tinted. Inside an animal room the intensity should not exceed 350–400 lux in the centre [2] and 60 lux at cage level [14, 18]; special attention should therefore be given to cages on the upper shelves.

Mice are sensitive to certain sounds, and high noise levels can be stressors to the animals. The noise level in an empty room should be 50 dB or less, but in a room stacked with mice it may be as much as 85 dB, especially during the animals' activity phase (i.e. dark phase). Noise exposure, especially in the high-frequency range, has broad systemic effects on mice. These may have an impact not only on the auditory system, but also on various other physiological parameters, like the neuroendo-crine and cardiovascular systems, seizure suscep-tibility, etc. In particular, fire alarms require special attention; these should emit sounds at less than 1000 Hz [20].

Mice are commonly housed in closed cages consisting of different types of plastic material such as polycarbonate (Makrolon, Lexan), poly-sulfone or polyetherimide. The latter materials are more expensive, but also much more resistant to chemicals, heat and mechanical stressing and can therefore be sterilized by autoclaving without the risk of being destroyed by the heat. Cages with bottom grids do not offer an appro-priate environment for the animals. They may exceptionally be necessary for a restricted time period and should no longer be used for long-term housing.

Except for specific reasons, mice should have permanent access to uncontaminated food and water and receive both *ad libitum*. Depending on the local situation, untreated tap water may be given. Since water quality can vary depending on locality, this needs to be tested for pH, hard-ness and microbial or chemical contamination, in order to decide whether its quality is accept-able, or requires a specific treatment (in partic-ular, type of purification). Nevertheless, in general it is common to treat water by auto-claving, filtration, chlorination or acidification in order to avoid introduction or transmission of agents.

Depending on the production process, food is heated during the pelleting or extrusion process and therefore contains only small numbers of microorganisms; only spore formers may be found at higher numbers. Subsequent contami-nation by wild rodents is possible, however. Also, due to the overall presence of wild mice there is a high risk that wild rodents carrying pathogens may accidentally be included in food. In case of infections by heat-resistant organisms, such as parvoviruses, these can be a source of contamination. To avoid the risk of introduction by food it is reasonable and increasingly common to autoclave food at suitably high temperatures. Detailed information on the nutrition of labora-tory mice is given in Chapter 4.3.

Laboratory mice are in contact with bedding during their whole lifetime, and this is therefore an important part of animal care and enrich-ment. Bedding must be added to a cage to provide an environment that absorbs humidity and keeps cages dry. This helps to avoid bacterial growth and the production of ammonia and other noxious or toxic compounds. Mice also use bedding to deposit odour signatures. However, ingredients of bedding may have an impact on physiological functions and thus unwanted side effects [21, 22]. Most commonly, wood products are used as bedding material. They are usually produced from soft wood such as aspen, but coniferous wood is also used. These products are available in various sizes and shapes ranging from small chips to shavings. During the produc-tion process they are dried to inactivate harmful microorganisms, or heat-treated, which also helps to eliminate volatile compounds and resins that are found in higher concentrations especially in coniferous wood. These compounds must be reduced to a minimum because they may influ-ence liver metabolism. Due to the low microbial contamination rate this treatment may be suffi-cient to eliminate infectious risks, so that auto-claving or irradiation is not always necessary. Wood products are highly absorbent and emit moisture very easily. Especially in cages, moisture is quickly removed, thus making the cage envi-ronment very dry. It is important that the wood used is not contaminated by pesticides or heavy metals and that the dust content is minimal to avoid health risks for personnel exposed to dust. Wood products are produced without use

of additives and are therefore environmentally friendly. Shavings are frequently added as additional nesting materials.

Corncob products are frequently used as bedding in the USA, but less commonly so in Europe. Untreated, the absorptive capacity is exceptional, but much lower when autoclaved [23]. This means that with less absorbed urine, evaporation is higher in an IVC-system with its higher ventilation due to drying, and therefore the amount of NH3 is reduced. As the microbial contamination rate of the raw product is high, corncob bedding needs to be autoclaved or irradiated prior to use. When corncobs are stored during the colder months moulds can grow which may produce mycotoxins. These cannot be removed by autoclaving or any other treatment. Corncob bedding may be ingested by animals in appreciable amounts and must be avoided in studies where fasting is required. Another disadvantage is the higher bulk weight, which makes it more difficult for caretakers to handle.

Paper products are soft and even more absorbent that wood products, but do not release moisture easily. They are manufactured from wood chips, which are mechanically and chemically treated to produce paper. They are available as natural pulp in a brown colour or bleached to a grey or white colour. Paper products contain chemicals used for the pulping or bleaching process but have lower contents of biological contamination [24]. Mixtures of different types of bedding are also available.

Other products, e.g. from rice ('paddy husk') are occasionally used but are less important.

Disposal of soiled bedding is an important issue and leads to significant costs. Used bedding is usually considered microbiologically contaminated and may also contain chemicals used in experimental studies, or their metabolites. Incineration is the safest method; it eliminates all risks and also has the advantage of greatly reducing the volume of treated materials. The usually low content of plastic material in waste from animal housing and the high percentage of bedding material (e.g. wood shavings) resulting in a high energy yield make incineration the method of choice. Other options are sending soiled bedding to landfill or using it for mulching or composting. National and local regulations must, however, be followed.

All personnel in charge of the facility and handling the animals, as well as those conducting experiments, need to be educated and well trained in the care of laboratory animals and their handling e.g. according to FELASA [25–28] recommendations. It is important to develop compliance with a conscientious culture of care and a deliberate and consistent attitude towards the humane treatment and use of the animals. The well-being of the animals is of utmost importance and is a legal requirement in most countries. All (standard) procedures have to be strictly defined such as (i) general hygienic procedures for personnel; (ii) daily monitoring of animals for adequate environmental conditions; their general health, e.g. as required by European regulations [1, 11]; (iii) provision of sufficient feed (defined diet) and potable water (with regular checking for blocked or leaking sipper tubes, or proper functioning of valves in case of automatic watering); (iv) housing in adequate cages with sufficient bedding and nesting material; (v) regular cleaning and sterilization of cages, water bottles, sipper tubes, racks and other equipment; and (vi) sanitation programmes.

Hygienic characteristics of laboratory mice

Earlier descriptions of housing systems for small rodents have not lost their principal validity (see e.g. [29-33]) although many refinements have been introduced meanwhile [7, 34, 35]. The scientific demands, international standards and the risk of contamination within the building and/or different hygienic areas should be established according to the animal house premises.

In principle, these could be either *germfree* or *axenic,* designating a status in which no microorganisms are present except those integrated in the genome; or *gnotobiotic* or *gnotoxenic* where the animals are colonized with a fully defined flora which may induce some resistance to ubiquitous microorganisms. This could be on the basis of a prestimulation of the innate immune response and/or on interference between bacterial strains

including yeast in the resident flora of the intestinal tract. This quality can be maintained only when all maintenance and experimental procedures are conducted under sterile conditions and when animals are housed in isolators. This makes gnotobiotic animals extremely expensive and difficult to care for, so that they play only a minor role in experimental studies.

Specified pathogen free (SPF) describes by definition the respective animals as being free from the pathogens specified individually and for which the colony is regularly monitored [10]. It is important to mention that there is no uniform definition of the term 'SPF'. The term is often misused, as it is frequently considered to stand for microbiologically 'clean' animals without specifying the agents that are not found in the population nor describing test methods, sample sizes, frequency of monitoring, age of animals tested, sentinel programmes, etc. Suggestions for defining the health status as well as methods of monitoring can be found in Chapters 4.4 and 4.6.

Quarantine originally used to cover the potential latency period of infections by opportunistic or pathogenic agents, but in the present context it describes a status in which, in particular, newly introduced animals from other institutions have to be imported, maintained and put through repeated and thorough hygienic examination (screening for viral, bacterial, endo- and ectoparasitic infections) before the animals may be transferred into the respective animal quarters or before the new strain undergoes a rederivation process. As a consequence of genetic manipulation, the exchange of breeding stocks between institutions has rapidly increased. Because of the presumably different hygienic constitutions, the single stocks should be preserved in their own microbiological status until rederivation can be performed. This can be achieved by the use of individually ventilated cages (IVCs) in *positive* pressure within a separate barrier unit in *negative* pressure.

Infectious refers to a status of animals either being infected naturally or within experimental research. They are a risk for all other animals within the facility and/or possibly for humans. Animals with undefined microbiological status should generally be treated like infectious animals [36]. *Natural* infections require rederivation, in particular when rarely available stocks

are concerned. IVCs or isolators in negative pressure should be used for containment until rederivation is completed. In *experimental* infections the pathogenicity of the microorganisms and the immune status of the animals determines the housing either in biocontainment IVCs or in (static) microisolators. Further biosafety measures may be necessary depending on the agent.

'*Conventional*' animals are housed under conditions that do not necessarily aim at appropriate protection from contamination, or the term may describe animals that are not carefully health monitored, so that the health status is not known. Like 'SPF', this definition is insufficient and does not reflect the microbiological quality.

Housing systems

In the last decade, IVC systems have come into increased use. This set-up not only affects facility construction, but also animal care management and health monitoring. While about $20\,m^2$ is considered to be the optimal size for animal rooms [36], we consider this to hold true primarily for a set-up with a conventional open cage system. Room size and layout need to be adapted to the specific needs, and room sizes with IVC systems installed may range between 45–70 m^2 [37]. IVCs provide for the maximum number of cages, i.e. mouse-holding capacity, for the available floor space.

Compared to conventional open caging, the air change of a single cage is several-fold higher in IVCs, allowing some extension of the cage-changing interval, which may at least in part compensate for the higher labour intensity. If correctly designed, the use of IVCs considerably decreases aeroallergens and odour levels in the animal rooms.

Barrier units

Physical barriers, together with an appropriate management system and operating procedures, aim to prevent the introduction of unwanted agents. However, animals housed behind barriers are not necessarily free of pathogens. Also, no statement on residual microorganisms is given,

implying the probability of extensive differences from one unit to another ([38–41], Chapters 4.4 and 4.6). Therefore, when transferring animals from one unit into another, it must be borne in mind that additional microorganisms may be introduced which can have an effect on the microbiological equilibrium. This is particularly to be expected if immunocompromised animals are being maintained [42]. Barrier units are used for breeding, usually with restricted access for personnel (including researchers), or for long-term experiments, and also for every other purpose. Animals are protected by strict hygienic barriers with a supply of filtered air. Food, cages, bedding and any other material should be treated to prevent agent introduction, while all personnel, including researchers, have to pass a water or air shower as entry lock [31, 33, 43]. A conventional open caging system or IVC system may be used within the unit. After microbial decontamination, for example with formaldehyde or hydrogen peroxide [44], animals with a known microbiological status or even gnotobiotic animals can be introduced, e.g. via a chemical entry lock. If standardized diets are introduced by steam sterilization the diet has to be enriched ('fortified') with increased concentrations of heat-labile vitamins to ensure that sufficient amounts remain after the heat treatment (see also Chapter 4.3). As an alternative, gamma-irradiated standardized diet (25 kGy) packed in waterproof evacuated bags can be transferred into the barrier unit after sufficient decontamination of external surfaces.

The drinking water can be sterilized either by heat, ultrafiltration and/or fluorescent (UV) light and then acidified (using hydrochloric acid or acetic acid) to a pH of ideally 2.8–3.0, or chlorinated at pH 5 using stabilized hypochlorite to 6–8 ppm free chlorine [45] in order to minimize microbial growth. Bedding material should be dust-free (<1% dust), should not contain toxic substances (e.g. pine and cedar softwood that may induce hepatic monooxygenases) and should be steam sterilized (see Table 4.1.3) to eliminate contaminating pathogens (such as bacteria or yeast found in corncob bedding).

The personnel entering the barrier bring about a higher risk in terms of microbial contamination. Therefore, only well-trained and highly motivated staff [25–28], who have had no contact to external rodents of a lower hygienic quality for several days and are free of infections, should be allowed to enter the holding unit [33].

Animals of the same or closely related species pose the highest risk of agent introduction for an animal population. It is therefore of crucial importance that introduction of animals follows strict rules. It has become common for populations of a high (economic and scientific) value that animals are allowed to be introduced only by embryo transfer or hysterectomy (e.g. in breeding populations), or in the case of long-term experiments, for example, that supplementary animal introduction is not permitted at all or only under strictly defined terms and conditions.

The *microbiological status* of the animals should be monitored on a regular basis (see Chapter 4.4). All sick animals must be removed from the unit (unless the disease status is part of the animal's genetic constitution or the experiment) and submitted to health monitoring (microbiological examination, necropsy). In addition, colony animals or sentinels should be checked at predetermined intervals. Regular disinfection of floors, walls and racks should be a routine operational procedure in order to minimize the possibility of infections (alternating between disinfectants used). If properly managed, such units may be maintained free of unwanted microorganisms for many years. In case of an infection within the unit it is unlikely that this can be restricted to a few cages when using conventional caging; this may be circumvented only by the use of IVCs if handled properly.

IVC systems

The individual ventilation of the cages with HEPA-filtered air allows long-term bio-containment on the cage level when the handling of the interior of the cage follows aseptic rules. IVC systems are used to breed and maintain animals in order to reduce the risk of miscellaneous microbial contamination and to improve environmental conditions, which may be of special interest when maintaining immunocompromised animals. IVC systems are particularly useful when a barrier-sustained unit is not available and in experimental units, where easy access to the animals by the scientists is indispensable.

TABLE 4.1.3: Suggested autoclaving temperatures and times

Material	Pre-vacuum (cycles × min)	Sterilization		Vacuum for drying
		°C	min	
Cage (empty), hoods,	1 × 5	121[a]	10	10 min
Bottles (empty), lids in stacks		134	5	
Bedding in bags[b]	3 × 10	121	30	30 min
		134	20	
Bedding in cages	3 × 10	121	15	15 min
		134	10	
Food[c]	1 × 5	121	15	5—10 min
		134	5	5—10 min
Water[d]	1 × 5	121	20	~10 h (pressure kept at 3.0 bar)
		134	10	
Supply cylinder		121	30	
Cages with bedding	3 × 5			20 min
Bottles (empty), lids, food		134	15	
IVCs fitted with cages	3 × 5	121	10	

The autoclaving protocols may have to be adapted because of enormous differences between products.
[a] 121 °C for polycarbonate (Makrolon) material; 134 °C may be used for polysulfonate material.
[b] Bedding in perforated plastic bags (15 kg); one layer on shelf.
[c] In perforated paper bags (15 kg); one layer on shelf; 15 min warming before pre-vacuum.
[d] Water in bottles (cover not tightly fixed) with measurement of reference temperature in one vessel.

In addition, IVCs with positive pressure are also ideal for a preliminary or timed containment of animals derived from different sources to maintain their respective hygienic status, if the area is barrier protected. For quarantine purposes without an additional barrier system and for infectious experiments these racks should be used with negative pressure. In case of high biocontainment requirements (ABSL3) the use of tightly sealed cages ensuring consistent negative pressure with HEPA-filtered exhaust (BCU/ISO) is required.

A problem of IVCs is health monitoring, requiring *sensu strictu* testing of each individual cage. Direct health testing of colony animals is possible, but large numbers of animals from different cages should be tested, which is expensive. Health monitoring in IVC cages is typically performed indirectly via sentinels which are exposed to pooled soiled bedding from colony cages, sometimes also to food or drinking bottles from other cages. Detection of infection depends on transmission of infection to sentinels, which means that these must be exposed to an infectious dose of the agent. There is a high risk of diluting the infectious dose when pooling bedding from many cages, so that the number of agents transmitted may be too low to get seroconversion. Also, agents with a low prevalence rate (only few cages may be infected) may not be detected. Further, not all agents are easily transmitted to sentinels. Sentinels may acquire resistance with increasing age, so that rotavirus or parvoviruses (and other agents) remain undetected. In general, transmission of respiratory agents (e.g. Sendai virus, CAR bacillus, mycoplasma, Pasteurellaceae) and mites is poor [46, 47]. Also, certain strains show reduced susceptibility to specific agents (MVM, MPV, *Streptobacillus moniliformis*) so that, depending on the strain used as sentinel, certain agents may not be found [48]. Other agents (e.g. pneumocystis, *Corynebacterium bovis*) are detectable only when immunodeficient animals are used as sentinels. It must also be considered that seroconversion may be late (>10 weeks exposure time) in case

of many bacterial pathogens (Pasteurellaceae, *Streptobacillus moniliformis*).

The microbiological status between cages may differ significantly. This includes not only pathogen status, but also the natural 'background' flora, leading to a significant cage-to-cage variation in the animals' physiological reactions. Especially after use of antibiotics, isolation of animals on the cage level prohibits exchange of microorganisms and re-establishment of a stable gut flora.

While wet bedding and increased ammonia concentrations are of major concern in cages that are not actively ventilated, these are not a problem in ventilated cages because of the higher air exchange rate. The cage change intervals can therefore be extended, and change intervals for bedding of once a week or even less are frequently common and acceptable. This also reduces stress to the animals and improves animal welfare by allowing them to stay longer in their familiar environment. However, nude mice may feel uncomfortable in IVC cages, especially when the air velocity is high. Appropriate nesting material should be provided for them, and the air velocity should be turned as low as possible, while maintaining sufficient air changes.

Ventilation of the cages can be performed by blowers in the animal room. In this case the supply of air is drawn from the room; the exhaust air duct should be loosely interconnected to the room's exhaust. This system permits individual adjustment for each rack. On the other hand, if a facility is to be newly constructed, separate channels for air supply/exhaust for the room and the IVCs might be installed. This avoids blower units within the room and may allow decreasing room ventilation and biofilter capacity, but requires a narrow temperature control and a back-up air-conditioning system. The latter system permits the total stocking rate per room to be increased, because it is not limited to the lower ventilation capacity of the room. The stocking rate must, however, be in accordance with national animal welfare regulations regarding maximum stocking density per cage [1, 2].

For most IVC-rack types the intracage pressure can be adjusted to be either positive or negative. This makes the units versatile in their use under different hygienic conditions. Ventilation with positive pressure in the cages counteracts the leakiness of the system. Negative cage pressure can prevent the escape of microorganisms and aeroallergens from the IVCs. The different aspects of ventilated cage systems have been described in overviews by Lipman [37, 49] and special topics presented by others [50–61]. Various products are commercially available in which the air is delivered either directly into the cage or distributed by a wide filter mesh in the hood to reduce the intracage air velocity. In many systems the exhaust air passes through a filter in the cover to retain dust from the exhaust pipes. What is decisive for the animals' well-being is apparently not the frequency of ventilation, but the air velocity that may be disturbing to the animals. Not for this reason alone, but rather as a measure to increase well-being, suitable bedding material always needs to be provided, permitting the mice to use this material as an enrichment for building proper nests [62, 63] and providing different temperature zones within a cage (Figure 4.1.1).

A critical event in IVCs is a failure of the air supply [64] by an electrical defect, blower breakdown or disconnection of tubes. Depending on the system in use, this may lead to a critically high carbon dioxide exposure within a short period of time [65]. It is therefore recommended to install an airflow controller in the supply air duct (positive pressure) or exhaust duct (negative pressure) respectively, which is connected to the alarm system of the animal facility [66].

Handling of IVCs

The sterile handling procedure for IVCs is labour intensive. This can be partially compensated for by extending the cage change interval. Due to the higher intracage ventilation, carbon dioxide and ammonia concentrations are lower and bedding material stays dry for longer. The increase in the change interval reduces the stress for the animals [67] and increases breeding efficacy [68]. Thus, when changing cages the transfer of part of the nest, or the whole nest in case there is a new litter (>3 days; in case of younger litters cage changing should be postponed), will be less of a disturbance to the mice and they will adjust more quickly to the new environment [63].

Figure 4.1.1 Nest built with a combination of shredded paper strips and tissue. C3H/HeNZtm mouse in the centre of a nest made of shredded paper strips. The nesting material has been gathered up to form a complete dome, with a small hole on top of the dome. During cage change part of the nest should always be transferred to the clean cage in order to minimize stress on the mice due to the new environment [63].

The proper management of IVCs is critical and quite often underestimated. The IVC area may be distinguished into three different hygienic levels that have to be considered: (i) The sterility level of the autoclaved material: *cage* with bedding, lid and hood (sterilized as a loosely closed unit, or separately in a container or foil), *diet* (autoclaved or gamma-irradiated at 25 kGy) and *water* bottles, transferred sterile into the laminar flow changing station (e.g. within a filter hood covered cage); (ii) the hygienic level of the animal room (i.e. the direct environment of the animal enclosure, termed 'room-clean'); and (iii) the hygienic status of the animals. To maintain the health status of the animals within the individual cages it is advisable to run the regular caretaking routines by two animal technicians (a 'clean' and a 'room-clean' person; Table 4.1.4). In certain IVC systems with water bottles outside the cage there is a high risk of contaminating the mice when bottles are not changed along with regular cage changing in the laminar flow cage changing station.

Power loss or other technical failures, such as clogged filters, could lead to increased levels of carbon dioxide, ammonia and humidity and, finally, shortage of oxygen, depending on the biocontainment system in use. Therefore, it is paramount to have a proper back-up air supply system (or an emergency power supply with battery back-up) as well as alarm systems installed, following accepted standards, best practices and methods.

Filter-top cages, static microisolators

Static microisolators are non-ventilated, partially perforated boxes with a tight filter medium covering the perforation and a cover that has to be sealed [69]. Although these filters are coarse (unless made of HEPA filter material), the lack of pressure difference prevents particles penetrating filters. These cages provide a microenvironment that is protected from adventitious contamination from outside. It is affected by the type of filter, bedding material, number of animals within the cage, their strain and sex and their activity. Microisolators are still in use (e.g. Han-Gnotocage, see Figure 4.1.2) and mostly recommended for the transport of animals within vivaria [70]. They can also be used for short-term housing of germfree or gnotobiotic

TABLE 4.1.4: Handling of IVC racks: two-person procedure

Step 1	Laminar flow cage changing station or laminar flow hood is running (30 min in advance)
Step 2	A fast-acting sterilization compound (e.g. Clidox) is freshly diluted for gloves, bench worktop and (in separate beakers) for the forceps to handle the mice (alternately used) and pincers to handle the lid
Step 3	The animal technicians handling the cages and animals should at least wear gloves, but it is recommended to also wear a surgical gown, cap and mask
	Room-clean person: The cage to be changed is placed into the changing station, the cage tag is removed and hood and lid partially displaced backwards. An autoclaved cage with bedding, lid and hood is placed into the bench and partially opened without contacting the inner side of the cage
	Clean person: Sterilized diet is transferred into the clean cage from the external sterilized, irradiated food-bag. Sterile water bottle is inserted. Mice are transferred in the sterile cage with the disinfected forceps and the lid is closed with the pincers
Step 4	*Room-clean person:* Hood is replaced, cage tag remounted and both cages removed
Step 5	Worktop and gloves are disinfected after each step with the sterilizing solution

Note: If animals are infected, the contaminated cages have to be autoclaved with the filter tops/lids in place.

Figure 4.1.2 Microisolator, Han-Gnotocage.

foster mothers (in a laminar flow cabinet). The disadvantage of these microisolators, however, is their impeded intracage ventilation allowing the accumulation of heat, humidity, carbon dioxide and ammonia.

The ammonia inside the cage is produced by the action of bacteria found in the animals' faeces and urine [71], and is known to be influenced by the number of animals per cage, the number of days of housing, the frequency of bedding change, temperature, humidity and ventilation [72].

In contrast to IVCs, where heat, humidity and toxic gases are optimally removed with about 60 air-changes/h (30–100/h), and to open cages with wire-bar lids in which 6–17 air-changes/h [33, 73] result in tolerable ammonia concentrations, only 0.6 air-changes/h will be achieved in static micro-isolators with filter tops. The concentration of ammonia and carbon dioxide can differ between cages, and furthermore may have a negative effect on the experimental results and thus become intolerable. However, static filter-top cages prevent the release of allergens and are therefore well suited for transport of mice within an institution. With increasing animal density and humidity in the bedding material, static microisolators can become unsuitable for the health and comfort of the mice.

Positive pressure isolators

Positive pressure isolators are generally used for housing animals when contamination by environmental microorganisms is not tolerable. When properly handled, they also reliably prohibit contact between humans and animals and therefore prevent the transmission of human agents to animals. Before embryos or sperm were routinely frozen (see Chapter 4.7), it was common to breed a nucleus of each strain in an isolator and to protect it from loss by infections.

Ventilated isolators in the positive pressure version are indispensable for breeding and maintaining germ-free and gnotobiotic animals. They consist of a closed construction with a germ-tight air inlet filter, a liquid air outlet trap or a germ-tight outlet filter, long-arm gloves and a chemically sterilizable lock for the supply via an autoclave cylinder (see e.g. [33, 74]). Freshly mixed buffered peracetic acid, diluted to 2.0%,

is commonly used to sterilize the interior of the isolator and the entry lock. For details on how to generally operate isolators the reader is referred to Chapter 4.6. As a consequence of sterilization for 30 min at 134 °C the diet may become less palatable [75] and masticable than a surrogate gamma-irradiated diet (50 kGy) supplied in evacuated double-lined bags.

Gnotobiotic animals are normally derived from germ-free animals by oral application of a gnotobiotic flora, consisting mainly of anaerobic, well-defined microorganisms [76]. The reproductivity in this hygienic status may be restored and the resistance to outside environment may be improved in comparison to germ-free animals [77, 78]. Therefore, this status can be recommended for immunocompromised strains—at least for their breeding stocks—and for foster mothers used for embryo transfer. A gnotobiotic status can only be preserved in isolators, a fact that may restrict the expansion of colonies. This limitation may be circumvented by the use of individually ventilated cage systems, although contamination with other microorganisms cannot be totally excluded.

Negative pressure isolators

The equipment of most commercially available isolators allows their alternative use with negative pressure. In this version isolators protect the environment (other mouse colonies or humans) from infections that are present in the isolator by means of a HEPA filter in the exhaust valve. Their use is obligatory for experiments with high-risk pathogens. It is furthermore recommended for new strains from other sources that are infected with agents imposing a high infectious risk to the existing colonies. All materials of isolators or quarantine have to be autoclaved when leaving the infectious area.

Filter cabinets

Small numbers of animals sometimes need to be housed close to the experimenters. This can be done in filter cabinets, which are available with positive or negative pressure. Access to animals is easy, through doors. Like isolators or IVCs, these systems provide protection of personnel

from exposure to allergens, dust and other contaminants from animals ('biocontainment') as well as protection of animals from the environment ('bioexclusion'). They offer the possibility of housing animals under a defined day–night cycle, where temperature, humidity and air changes can also be adjusted according to needs.

Housing conditions for specific purposes

Quarantine

Quarantine describes a temporary or permanent isolation of animals after introduction from outside, during which their microbiological status can be defined by appropriate testing, or continued. Quarantine is in most cases not considered necessary when animals originate from a few named and well-known breeding units, primarily from commercial breeders. It is highly recommended or even necessary for animals with unknown status, especially for those from research colonies (e.g. genetically modified animals). As a consequence of their unique genetic modification, the exchange of breeding stocks between institutions has rapidly increased. These animals are more likely to be infected than animals from commercial vendors [79]. Because of the presumably different hygienic constitutions, single stocks should be preserved in their own microbiological status until rederivation can be performed. This can be achieved by the use of positive-pressure IVCs within a separate barrier unit in negative pressure. In general one should introduce new strains from quarantine into the main facility via embryo transfer, unless the animals have been purchased from a vendor who maintains the colonies under high or maximum barrier conditions and demonstrates their good hygienic quality by reliable health reports.

Quarantine precautions should also be established when cellular material or any other biologicals with unknown microbiological status have to be inoculated into experimental animals. If contaminated, this biological material could be

of risk for the animal facility [36, 80] and infect the whole unit with unwanted viruses, such as ectromelia virus [32, 81-83], mice minute virus [84] or any other virus. Bacterial pathogens have been found as contaminants in biological materials. Animals that have been severely immunosuppressed by gamma-irradiation or by drugs should also be submitted to a quarantine-type holding area and not returned to their previous quarters. However, specific containers (sealed to avoid contamination of animals, with sufficient air volume to maintain oxygen supply for a certain period of time) have been developed for irradiation, which may allow reintroduction of these mice into a barrier unit after the process of whole-body irradiation.

Genetically engineered strains

The technology now available to genetically engineer mice has brought about an exponential increase of new strains/stocks specifically designed for different aspects of research—see the activities by several initiatives, KOMP, NorCOMM, EUCOMM, MMRRC, IMSR, IGTC, IKMC, and RIKEN that aim to generate mouse embryonic stem (ES) cells containing a constitutive or conditional null mutation in every gene in the mouse genome. The KOMP Phenotyping Program provides new tools and methods and a comprehensive mutant mouse database and library, and conducts broad, standardized and high-throughput analysis of mutant mouse models. In general, donor mice of oocytes or early embryonic stages are bought from commercial breeders. If these mice are maintained separately and manipulation of the oocytes/ embryos (laminar flow, medium, washing, handling) is designed to interrupt a potential transmission of microorganisms, the risk of transmission of an infection should be low. The status of the foster mothers is by far the most decisive factor for the hygienic status of the genetically manipulated progeny. Their handling is of special importance when creating immunodeficient strains.

Because of the enormous number of genetically modified strains, their phenotypic discrimination is practically impossible. In addition to the risk of mixing identities in the course of cage changing, false genotyping or false identification

of the animals may be a source of errors. Therefore, regular screening of homozygous or still segregating strains/stocks is indispensable. In the process of backcrossing a transgene or targeted mutation on to a defined background it is advisable to select and analyse markers sufficiently differentiating between the background strain and the donor stock of the genetic modification. Genetic monitoring using markers similar to a marker-assisted selection protocol (speed congenics production; see Chapter 4.5; [85, 86]) is recommended.

Immunocompromised mice and preventive care

Immunodeficient animals, at least those with severe immunodeficiencies such as homozygous *Foxn1^{nu}* nude mice, *Prkdc^{scid}*, *Rag1^{tm1}*, *Rag2^{tm1}* and mice with multiple deficiencies like scid-beige, or in combination with IL-2 receptor gamma-chain knockout (e.g. NSG, NRG$^{@}$) should be strictly isolated from other animals, especially when immunodeficient mice need to be housed for weeks or months or when infected animals are housed in the same facility. Humans may also play a significant role as carriers of opportunistic agents such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and many others. Immunodeficient animals usually survive several weeks or a few months when specific and pathogenic agents are not present in the population. However, not only agents with known pathogenicity but also numerous opportunistic agents, even with unexpected pathogenicity, e.g. *Burkholderia gladioli* [87], may reduce the life expectancy. Absence of such agents is essential for breeding colonies of immunodeficient mice. Isolators provide the safest housing system and are recommended for breeding. However, various IVC systems exist in which long-term holding of immunodeficient mice is possible.

Treating immunocompromised breeders with immunocompetent cells (from syngeneic donors such as an immunocompetent genetic background or F1 hybrids of the strain to be reconstituted and the immunocompetent strain) can assist in the propagation of highly immunocompromised strains [88, 89]. For the reconstitution of nude mice, *Foxn1^{nu}*, thymus homogenates can be injected intraperitoneally to reconstitute their T-cell defect. As a preventive measure for other mutants or lines (e.g. *Prkdc^{scid}*, *Rag1^{tm1}*, *Rag2^{tm1}*, etc.) an intraperitoneal injection of syngeneic or F1 spleen cells (1-2 $\times$ 10^7), or bone marrow cells (2-5 $\times$ 10^6) intravenously into juvenile mice, has been shown to be very efficient (H. Mossmann, personal communication, 2003). This treatment improves the constitution of the individual mice and thus predestines them as breeders (and only breeders).

Infected animals and infection experiments

Housing infected animals requires precautions to prevent transmission of microorganisms between animal populations and, in the case of zoonotic agents, to humans. The zoonotic risk arising from naturally infected rodents is low because most rodent pathogens do not infect humans [90]. Only few rarely found agents like LCMV, hantaviruses, or *Streptobacillus moniliformis* have the potential to cause severe infections in humans and might be prevalent in colonies of laboratory rodents. Severe disease outbreaks in humans associated with infected colonies of laboratory rodents have been reported [91, 92], and safety programmes are therefore necessary to prevent laboratory-associated infections and infections transmitted by laboratory animals.

Experimental infections are more likely to pose a risk for humans. A broad spectrum of infectious agents can be introduced accidentally with patient specimens, and many laboratory animals are used for specific infectious experiments. In general, health precautions are very similar for clinical or research laboratories and for animal facilities. In many cases, however, an increased risk may arise from infected animals due to bite wound infections or when pathogens are transmissible by dust or by aerosols.

$^{@}$NSG = NOD.Cg-Prkdcscid Il2rgtm1Wjl; NRG = NOD.

In case of infection experiments, microorganisms must be classified into safety levels. Four safety levels have been established [93] which consist of combinations of laboratory practices and techniques, safety equipment and recommendations for operation of laboratory facilities. The classification of an organism or parts of it (DNA, toxin) is based on various factors such as the host spectrum, virulence for healthy humans and animals, minimal infectious dose, mode of transmission, epidemiological situation (prevalence in a given population), availability of antibiotics, vaccines or other treatments and tenacity. The recommended levels represent those conditions under which the organism can ordinarily be safely handled. Sometimes, more stringent practices may be necessary. For example, hantaviruses are typical BSL-3 pathogens. Many researchers consider hantaviruses ABSL-4 agents when inoculated into laboratory animals, especially into rats, since there is clear evidence of aerosol transmission or transmission by bites from infected animals.

Biosafety level 1 (BSL-1) applies to the use of characterized microorganisms not known to cause disease in healthy human adults. BSL-1 organisms are, for example, bacteria that do not multiply in warm-blooded organisms, saprophytes and bacteria that have been used for the production of foodstuffs (e.g. lactobacillus). BSL-2 is used for work involving agents that represent a moderate hazard for personnel and the environment, for farm or wild-living animals or for plants. Most vertebrate viruses and a broad spectrum of bacteria (e.g. *Escherichia coli, Staphylococcus aureus, Clostridium tetani, Vibrio cholerae*) are classified as BSL-2 organisms. Biosafety level 3 is used when it is necessary to work with indigenous or exotic agents that may cause serious or potentially lethal disease as a result of autoinoculation or ingestion or with a potential for aerosol transmission. Among viruses, yellow fever virus, HIV or herpes B are classified as BSL-3 pathogens. Only few bacterial species (e.g. *Mycobacterium tuberculosis, Yersinia pestis, Pseudomonas mallei, Coxiella burnetii*), fungi or parasites pose a serious risk for laboratory workers or animals or a moderate risk for the population and may therefore be classified as BSL-3.

BSL-4 practices are applicable for work with highly contagious and pathogenic or exotic organisms that may cause lethal infections, for which there is no available vaccine or therapy and which may be transmitted by the aerosol route. Examples are Lassa fever virus, Marburg virus, Ebola virus, smallpox, rinderpest, and foot and mouth disease. At present no bacterial pathogens, fungi or parasites are classified as BSL-4.

Biosafety criteria for housing vertebrates have been defined in the USA by the Centers for Disease Control and Prevention, National Institutes of Health (CDC/HIH) [94]. In general, biosafety levels recommended for working with infectious materials *in vitro* and *in vivo* are comparable although there are some differences, because activities of the animals themselves can introduce new hazards by producing dust or aerosols, or they may traumatize humans by biting and scratching. Therefore, CDC/NIH [94] established standards for activities involving infected animals which are designed 'animal biosafety levels' (ABSL) 1–4. These combinations describe animal facilities and practices applicable to work on animals infected with agents assigned to the corresponding level (BSL 1–4). Similar regulations also exist in other countries.

Reduction of the risk of disease transmission can be achieved by very general procedures, which are common practice in most well-run animal facilities housing animals behind physical barriers. The first point must be adherence to safety procedures and proper behaviour, such as the use of personal protective equipment. Prohibition of eating, drinking, smoking, handling of contact lenses and the application of cosmetics in the laboratory are other basic rules, as is the separation of food storage refrigerators from laboratory refrigerators. The most likely route of infection is direct contact with contaminated animals or materials. Microorganisms do not usually penetrate intact skin. The risk of infection can therefore be reduced by repeated hand decontamination and by decontamination of surfaces or contaminated instruments.

Handling of infected animals should be allowed only for experienced and skilled personnel, to prevent bite wounds. Working in

safety cabinets helps to avoid inhalation of infectious aerosols and airborne particles, which are easily generated in cages when animals scratch or play. Filter-top, IVC, or sealed negative-pressure biocontainment unit (BCU/ISO) cages are often used in animal facilities for transport within the facility to avoid exposure of humans to allergens. Such cages also help to reduce the risk of spreading microorganisms during transportation.

In most animal facilities containment equipment (filter-top, IVC-, or BCU/ISO cages, or isolators) is used if infected animals might be a hazard for humans or other animals. Experiments with infectious agents will usually be conducted in separate areas, which fulfil all safety requirements such as ventilation (negative pressure in laboratories to prevent air flow into non-laboratory areas), or better, in isolators, which represent the most stringent containment system. For safety reasons, containment is generally necessary if animals are artificially infected with pathogenic microorganisms. In the case of low-pathogenicity organisms, microisolator cages might be sufficient. Individually ventilated cages operating under negative pressure are better suited than static microisolators to prevent spreading of microorganisms if they are properly handled. The highest level of safety can be achieved by using a negative-pressure isolator. The risk of infection during handling is reduced if all work with open cages is conducted in changing cabinets or in laminar flow benches.

An important part of safety programmes in laboratories, and especially in laboratory animal facilities, is waste management. Unlike radioactive or chemical waste, infectious waste cannot be identified objectively. In case animals are not experimentally infected, the judgement of whether waste is infectious or not depends on the person in charge. There is, however, no doubt if animals have been infected experimentally. In such cases the presence of a pathogen allows evaluation of the risk.

Infectious waste from animal houses (bedding material, animal carcasses) can be submitted to chemical or thermal disinfection, but incineration and steam sterilization are the most common treatment methods.

Refinement of housing and environmental enrichment

Animal welfare regulations (see also Chapters 4.2 and 6.1) require that the environment of the animals maintained should meet their physiological and behavioural needs and that the housing conditions must facilitate the performance of natural behavioural patterns and allow for adequate social contacts [5]. Current debate on enrichment of the animals' environment and space requirements has not come to a general conclusion. There is no doubt that all cages should be equipped with proper nesting material (e.g. shredded paper strips, nestlets, paper towels) [63, 95]. The published opinion is rather contradictory with respect to the effects on research data obtained under 'standard' vs. structurally and/or spatially enriched housing systems. While several groups report on distinct effects on behaviour, such as increased locomotion, exploration, learning ability or reduced anxiety ([96–102]; Chapter 4.2), other studies indicate that an enrichment of the housing cage may lead to an increase in aggressive behaviour [103–106], or to an increase in the coefficient of variation of several parameters depending on the strains analysed [107–110]. The latter result may suggest that the estimates of the appropriate number of animals to be used have to be increased because of the greater variation of various parameters from one experiment to another and between different environments. Marashi et al. [111] report on an increase in aggressive behaviour in male mice and elevated levels of stress hormones in mice housed in an environment that was structurally enriched (plastic inset and wooden scaffolding) and also spatially enriched (richly structured by a complete, passable enriched cage, extra plains, plastic stairs, wooden footpaths, hemp ropes and a climbing tree). Interestingly, the conclusion is that stress is a most important prerequisite to achieve good welfare. This controversy with the previously agreed notion that animals should be housed with the goal of maximizing species-specific

behaviour and minimizing stress-induced behaviours needs further analysis.

Therapeutic treatment

The administration of therapeutics can influence the outcome of animal experiments in various ways or might be associated with toxic effects [112, 113]. For example, some knockout mice (targeted disruption of the multidrug resistance gene) and CD-1 mice have been shown to be very sensitive to ivermectin, with resulting mortality. The use of drugs should not become a routine procedure, nor is it a way of substituting for improved hygienic standards. Antibiotic treatment guided by microbial resistance testing may be a necessity in certain natural and induced mutants (e.g. *Ncf1*) due to their defective granulocyte bactericidal activity [114] unless maintained under germ-free or strict gnotobiotic conditions. The treatment of parasitic invasions in particular is dependent on the accompanying hygienic procedures, e.g. chemical and/or physical disinfection of the animal rooms, cages, lids, bottles, etc. In general, therapeutic drugs such as antibiotics must be given in a sufficiently high dose for an adequate period (for drug dosages see Hawk et al. [115]). It is important to mention that infectious agents can only exceptionally be eliminated by the use of antibiotics, although many reports exist of successful agent eradication by drugs. Successful eradication with drugs is possible for parasites such as pinworms [116] and mites [117]. However, various side effects have been described even for fenbendazol, which is most commonly used [118–123].

Permanent antibiotic treatment of infections may induce bacterial resistance to antibiotics especially when used on a large scale. This will not only affect the animal colony [124] by a shift in the gut flora [125] and other derangements in physiological functions [126]; it may also lead to a contamination of the environment with multiresistant bacteria that could be a hazard to humans due to overgrowth of unwanted possibly facultative pathogenic bacteria hosted within the colony at a low level, previously not disclosed. All bedding material of treated animals should therefore be adequately steam-sterilized before disposal.

Prophylactic use of antibiotics is not uncommon when immunodeficient animals are housed for longer periods to prevent clinical disease by environmental or opportunistic agents. It even happens that breeding populations are treated permanently (over years). This may interfere with the outcome of many experiments and does not really solve problems permanently. Agents that might be pathogenic for a certain population should rather be eliminated by rederivation procedures, and animals should be housed under conditions that prevent reintroduction. Agents that are clinically significant for a certain population are best detected by comprehensive health monitoring of sick animals.

Antibiotic treatment is very common after sublethal irradiation of mice and helps to reduce the death rate. Opportunistic bacteria can cross the intestine–blood barrier and cause a sepsis due to the immunosuppression and the destruction of the intestinal mucosa by irradiation. Many different antibiotics are given prophylactically with good success, commonly without previous sensitivity testing. Elimination of opportunistic agents causing clinical problems is, however, a better approach for conducting experiments that are undisturbed by the use of drugs. It is likely that animals that are free from opportunistic agents will survive without administration of antibiotics. The major risk during the first week is due to fluid loss into the gut, since the rapidly dividing cells are damaged or killed. The mice just need good nursing care for a week or two until their gut mucosa recovers.

Identification systems

Regulations published by the European Union [1] and by ILAR [2] stress the importance of proper animal identification in sound research and humane animal care (Table 4.1.5). The identification of single mice has become an indispensable tool, even more so as a consequence of genetic manipulation, where genotyping results have to comply with the respective individual. Normally,

TABLE 4.1.5: Principles of proper colony management	
1	During regular handling only one cage at a time should be managed. This will prevent accidental exchange of animals from different cages
2	Animals that have escaped or dropped to the floor must never be returned to the suspected cage. Animals caught outside the cage should be isolated, if identification is possible, otherwise killed
3	Cages and hoods should be in sufficient condition that no animal can escape or enter another cage—a problem more often encountered in mouse than in rat breeding units
4	For ease of identification and in order to prevent an inadvertent mix-up, cage tags should have a strain-specific colour code and a strain-specific number (code)
5	Cage tags should always be filled out properly, including the strain name, strain number, identification numbering of the animals in the cage, parentage, date of birth, generation, and in case of experimental use the name and licence number of the scientist
6	If a cage tag is lost, one should not redefine the cage except in the case of definite proof of identity through marked animals within the cage
7	If at weaning the number of animals is larger than that recorded at birth, the whole litter should be discarded or submitted to genetic monitoring
8	Any change in phenotype and/or increase in productivity should immediately be reported to the colony supervisor. The latter change should always be considered suspect for a possible genetic contamination
9	Regular training programmes on basic Mendelian genetics, systems of mating and the reproductive physiology of the animals maintained should make animal technicians and care givers conscious of the consequences any mistake will impose on the colonies. Further training should stress the importance of a search for deviants as potentially new models for biomedical research

correct labelling of the cages gives the first hint to strain, sex, age and individual numbering. The *Guide for the Care and Use of Laboratory Animals* as well as a number of other sources, lists many acceptable identification methods for most common laboratory animal species. Several methods are in use:

1. *Metal ear tags* are an easy method of permanently marking laboratory animals. The procedure is simple, using an ear punch and a specific applicator, and does not require anaesthesia. The tags are available with a numbering system. This implies a consecutive numbering of all mice, irrespective of the strain or a numbering within a strain. Ear tags with different colours per strain within a unit may be of help. Unfortunately, mice sometimes lose their tags, requiring remarking. Metal tags may be made of different alloys. Some contain nickel and therefore frequently induce nickel allergies with severe side effects.

According to the Swiss regulations on animal experimentation [127] the application of ear tags is not permitted.

2. *Tattooing* of footpads, toes, tail, etc. is an acceptable alternative, which may be especially helpful in marking newborn mice.

3. *Ear notches and punches*: Exterior markings of the pinna can be done on mice after 2 weeks of age without anaesthesia. Ear punch identification may be obliterated after several weeks because of wound healing or by fighting between individuals. A system of holes and notches permits a numbering from 1 to 9, or 10 to 90 using both ears (see Figure 4.1.3), allowing for an individual numbering system for up to 100 mice.

4. *Toe-clipping*: This method involves removal of the first phalanges of one toe of up to all four limbs, corresponding to a predetermined numbering code [128]. The different digits removed code the identifier. Because toe-clipping is a potentially painful procedure that can also alter the gait or weight-bearing ability
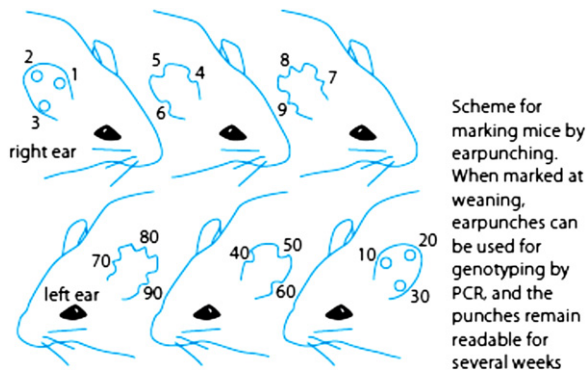
Figure 4.1.3 Standard pattern for ear notch/punch numbering in mice.

Scheme for marking mice by earpunching. When marked at weaning, earpunches can be used for genotyping by PCR, and the punches remain readable for several weeks

of a rodent's rear limbs the ILAR *Guide for the Care and Use of Laboratory Animals* [2] and Swiss regulations [127] limit its use only to justified instances, and may require anaesthesia and analgesia [129, 130]. In other countries this method is under debate (e.g. Norway) or not permitted.

5. *Transponder*: The subcutaneous implantation of a transponder is without doubt the most reliable, but most expensive, method for identification. This system is practically unlimited in the number of individuals that can be differentiated and is convenient when animals have to be identified very often, e.g. if monitoring of body weight or other parameters like body temperature have to be recorded together with the identification number [131]. Some transponders appear large compared to the size of a mouse and need to be implanted with thick needles, but smaller transponders have been developed in recent years and should preferably be used for mice. However, if the mice are to be scanned in MMR or μCT scanners (see Chapter 5.6) electromagnetic transponders should not be used for marking as they can seriously distort or interfere with the imaging.

Computer-assisted management of animal facilities

The production and breeding of genetically manipulated mouse strains in most institutions has increased exponentially in recent years. In addition, the need to document animal breeding and experimentation for governmental and scientific purposes is also increasing. Vivarium management software will facilitate the efficient and complex storage and retrieval of all related information, such as mouse strains, breeding status and availability, services required/provided, registered users and ethically reviewed projects (e.g. by the Institutional Animal Care and Use Committee, IACUC). Such a relational database should not only be multiuser compatible and platform-independent within the computer network of the institution but should be designed in such a manner as to assist the facility administrator, researchers, animal technicians and the animal welfare officers to maintain an overview of the work, as well as to support communication with each other and to reduce the workload [132].

Typical functions of such a database include:

1. *Mouse strains* with information on all (sub) strains and sublines maintained using proper international nomenclature as well as internal laboratory denominations, a coding system, strain owner, responsible investigator, genotype, phenotype, breeding information, pedigree, breeding/holding unit, etc.
2. *Animal ordering system* allowing users to submit jobs to the breeding facilities, such as ordering of animals for experimentation or shipment, ordering of tissues for genotyping, cryopreservation, immunization, embryo transfer, isolator breeding, etc. The animal technicians in the breeding facilities will then process these duties and the status of each duty should be traceable.
3. *Animal welfare*: In all projects approved by IACUC or AWB and governmental granting agencies, animals should be readily accessible to those responsible for oversight, as well as the type of experimental procedures permitted, the number of animals allowed and used, registered personnel, etc.
4. *User list*: All users of animals could be stored, along with strain access information. Personal licences as well as expertise required by welfare regulations should also be added.
5. *Colony analysis*: In order to properly monitor colonies, it is not only necessary to register the litters and offspring of all breeding for each

strain. The information stored should also provide a full animal and cage inventory, sex, phenotype, genotype, genealogy, dates and results of hygienic and genetic monitoring, etc.

6. *Inventory*: It is important to be able to track the number of mice or cages present in the breeding facility, and obtain information on weaning, breeding and transgenic and knockout mouse colonies. This will assist the management in allocating breeding and holding space, and eases cost calculations.

7. *Invoices*: If required, it should be possible to generate invoices for animals, cages or special animal care, or for specific diagnostics or sample collection, as well as for other services rendered.

8. *Online information*: Data sheets or comments could be integrated, which describe the use of the database and aspects of animal work, some of which may be specific to the institution.

9. *Language preference*: The employees in many research institutes may not have a common mother language. For this reason, it could be useful if the database can accommodate a preferred language for each user.

Such a database should help to maintain an overview of experimentation with animals. It should stimulate efficient communication between the different user groups and save work.

1. *Researchers* should be able to see which strains and ethically reviewed projects they are responsible for, including the current number of animals used and the status of all jobs they have submitted. They may also be allowed to run a detailed analysis of genotype frequencies, pedigrees, reproductive data, etc.

2. *Animal technicians* could check which animals have been ordered for a specific date and where (holding unit/laboratory) these should be delivered/shipped.

3. *Animal welfare officers* should be able to verify how many animals have been ordered for which purpose, the duration of the treatment, etc. They could use this information to easily create a report providing a summary of the animals used in any experiment. This report may be submitted directly to the government authorities, if required.

4. *Breeding administrators* can identify which animals belong to whom and how many mice

have been used. They can thus ensure that sufficient numbers per sex and per strain will be available within the institution. If desired, invoices for the services provided could also be created.

Several databases have been developed for colony management, animal breeding, experimentation, budgeting and many other purposes. Some are available free of charge, whereas others can be very complex and are very expensive. Before purchasing or developing a database, it is important that an institution clearly defines its needs and potential applications in detail. These must be considered in advance and before decisions are made [133].

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Mouse Enrichment

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Introduction

It is tempting to consider the laboratory mouse simply as the common house mouse, *Mus musculus*, and therefore a homogeneous animal for which enrichment can easily be provided. The laboratory mouse is a ubiquitously used research subject whose genetics, anatomy, physiology, immunology and behaviour have been studied in detail for generations. Thus, it might seem that providing a housing environment that is species-appropriate would be a simple matter. However, it would be a serious mistake to approach mouse enrichment as a one-size-fits-all husbandry procedure. The laboratory mouse is still considered behaviourally similar to wild mice in many ways [1], though it differs somewhat from the wild-type ancestor in its behaviour, with running behaviour, open-field freezing behaviour and a generally higher level of activity more evident in wild-type mice than in laboratory-bred animals [2]. Over decades of purposeful breeding, a variety of characteristics (e.g. ease of handling) have been either deliberately or inadvertently introduced into the behaviour profile of the laboratory mouse. Today, the increasing trend in the use of transgenic mice has only amplified the diversity of traits being bred for, and thus the potential exists for both extensive and subtle differences in mouse behaviour and physiology as well as their response to their environment.

This behavioural breadth of the species may help to account for the fact that the literature is replete with contradictory findings and diverse conclusions about the potential benefits and unexpected consequences from providing enrichment to laboratory mice. Although such contradictions may be frustrating for laboratory managers who wish to provide meaningful and practical enrichment to the mice in their colonies, they clearly signal both inadequate objective information regarding the behaviour of the

mice we use in research and the need for additional basic studies to better characterize the animal model as its genome is modified. Due to the unpredictability of the effect of many enrichment techniques, the reality is that mouse enrichment programmes are complex, must be thoroughly researched, and implemented on the basis of input from investigators, veterinarians and husbandry personnel.

For these reasons, this chapter cannot provide a roadmap to designing a single perfect enrichment programme for all laboratory mice. Rather, the more general landscape of common approaches to providing enrichment to mice, issues of cage space, effects of enrichment on both the animal and the research, assessing the value of enrichment and pragmatic approaches to providing enrichment will be described. The goal, then, is to provide the reader with tools to critically evaluate the types and relative merits of enrichment available and to consider ways to enhance the welfare of the mice without compromising their research purpose.

Implementing environmental enrichment

Conceptual issues

The goal of enrichment

Environmental enrichment has been variously defined, but generally includes the goal of improving the welfare of the animal through the thoughtful inclusion of meaningful features to the cage environment. More than 10 years ago, enrichment was described as:

> any modification in the environment of the captive animals that seeks to enhance its physical and psychological well-being by providing stimuli meeting the animals' species-specific needs [3].

More recently, the aim of enrichment has been described as:

> a method to enhance animal well-being by providing animals with sensory and motor stimulation, through structures and resources that facilitate the expression of species-typical behaviours and promote psychological well-being through physical exercise, manipulative activities and cognitive challenges according to species-specific characteristics [4].

In some cases, the objective of enrichment is to increase the expression of certain behaviours while, in other cases, reduction of specific behaviours is intended. For example, reduction in the expression of stereotypic behaviours may be a goal, which is achieved by providing resources such as a shelter [5]. In all instances, the provision of enrichment should not negatively impact either the health and safety of the animal, or its utility for research.

There are several general characteristics of non-social enrichments that are desired and which may drive the selection process among enrichment choices. Primary among these is that ideally there should be demonstrable value derived from the enrichment technique in enhancing the welfare of the animal. Optimally, this evidence should be contained in the peer-reviewed literature and it should be possible to extrapolate the results of the published data to the specific context of the institution considering implementing that type of enrichment. It is worthy to note that enrichment is typically intended to improve animal welfare over some established baseline. Often, the wild counterpart of the laboratory animal is held up as the standard for comparison. However, as noted above, this comparison may be inadequate because of the potential for changes to have occurred in the biology of the laboratory mouse following generations of targeted breeding. The question thus becomes what standard should be used as the benchmark for mouse well-being. If one considers that a C57BL/6 or p53 knockout mouse should serve as its own control for characterizing the welfare of the entire strain, then it becomes a monumental task to accomplish this for each strain and transgenic line created. Clearly, the logistics, time investment and cost of such an approach are prohibitive. Relying on past experience alone and retrospectively assessing how the welfare of various mouse strains and lines is affected by the housing environment entails the potential for welfare to have been unintentionally compromised before the error is detected, if it is detected at all.

One reasonable approach is to base welfare assessments on a composite of types of mice that evidence similar behaviours, responses to experimental challenges, or fragility. In this manner, groupings of strains or lines of mice would be made and common approaches to assessing welfare could be applied. Needless to say, the success of such a strategy would be dependent on the accuracy of the groupings and, of necessity, would rely on the availability of information to make these judgements. In some circumstances, scoring systems for behavioural phenotyping facilitate the description of behaviours of transgenic and knockout mice. These systems typically involve analysis of a battery of responses to stimuli and resting activities [6], as well as physical characteristics (e.g. bald patches in the hair coat). This tool may aid in the grouping of mice by determining when an animal differs from its prototype, which could be an indicator of altered welfare. In the absence of an obvious metric for assessing the welfare of the diverse range of mice used in research, there may be an inclination to rely on the wild-type mouse or on an inappropriate laboratory strain or line as the basis for comparison. And, although progress has been made in identifying pain and distress in mice—such as the mouse grimace scale [7], changes in activity [8] and changes in behaviours such as flinching, writhing, rear leg lift and press [9]—strain differences continue to plague us, making some of these strategies broadly utilitarian [9]. Also, the value of systems based primarily on behaviour change for animals in a prolonged state of compromised welfare (e.g. chronic pain) has not been determined [10].

Enrichment and refinement

The 'Three Rs' of Russell and Burch [11]—Replacement, Reduction and Refinement—have become mainstream principles for the care and use of laboratory animals. In their discussion of the Three Rs, the authors define refinement as a strategy whose 'object is simply to reduce to an absolute minimum the amount of distress imposed on those animals that are still used.' As the publication of their thesis regarding the Three Rs predated notions of providing environmental enrichment to laboratory animals, it is not surprising that, as noted by Buchanan-Smith and colleagues, the concept of refinement has changed substantially since it was first proposed [12]. Over time, as the tenets of the Three Rs have gained in international stature and have been referenced in several pivotal guidance documents, the general consensus that environmental enrichment is a prominent method of refinement has also gained momentum. For example, both enrichment and the Three Rs are referenced in the *Guide* [4], an internationally accepted standard for the treatment of research animals, and more recently both concepts have been adopted into the International Guiding Principles for Biomedical Research Involving Animals of the Council of International Organizations of Medical Science (CIOMS) which is used by the international scientific community to guide the responsible use of vertebrate animals in scientific and educational activities [13]. The 2012 version of the *Principles* is explicit in stating that the 'tenets of the Three Rs—Replacement, Reduction and Refinement—should be incorporated in the design and conduct of scientific and/or educational activities that involve animals,' and that 'Measures should be taken to ensure that the animals' environment and management are appropriate for the species and contribute to the animals' well-being…' In addition, the World Organisation for Animal Health (OIE) cites the Three Rs in its *Terrestrial Animal Code* and encourages the use of environmental enrichment as a:

> means of increasing the complexity (e.g. with toys, cage furniture, foraging opportunities, social housing, etc.) in a captive animal's environment to foster the expression of non-injurious species-typical behaviours and reduce the expression of maladaptive behaviours, as well as provide cognitive stimulation [14].

These sources reflect the widely held notion that refinement strategies often entail attention to the animal's housing environment and concomitant enrichment techniques.

This view is evident in the United Kingdom's National Centre for the Replacement, Refinement and Reduction of Animals in Research (http://www.nc3rs.org.uk/) whose mission is to use the Three Rs to support science, innovation and animal welfare in the biosciences. The NC3Rs supported a special initiative to fund

research projects that would develop refinements in rodent husbandry, care and procedures, and the organization's website includes recommendations for housing elements (http://www.nc3rs.org.uk/category.asp?catID=39), many of which are complexities aimed at enhancing mouse welfare, such as something to gnaw on, tubes, nesting material, nest boxes and bedding. In addition, the Canadian Council on Animal Care (CCAC) notes in its policy statement on 'Ethics of animal investigation' [15] that investigator adherence to the Three Rs is required, and the CCAC website includes a Three Rs microsite (http://threers.ccac.ca/en/alternatives/intro.html) as a resource for the scientific community. It is here that the CCAC defines refinement as the 'modification of husbandry or experimental procedures to minimize pain and distress, and to enhance the welfare of an animal used in science from the time it is born until its death,' thus clearly linking enrichment with refinement. Buchanan-Smith et al. [12] underscore the importance of the relationship between enrichment and refinement in their recommendation that a proactive approach to refinement should be taken which includes enhancing animal well-being through environmental enrichment techniques.

Caging characteristics

The characteristics of the caging provided to mice can significantly impact the welfare of the animals. Caging, properly designed and constructed, can support species-appropriate behaviours, and more specifically a variety of meaningful enrichment strategies. Consideration should be given to the material from which the cage is manufactured; the floor space, cage height, and thus cage volume; the quality of the cage space; and the accessibility of key resources.

Cage size

Perhaps the most contentious topic regarding housing is the amount of cage space that should be provided for mice. A very cursory scan of the literature pertaining to this subject reveals quite different recommendations as to what is considered adequate. A key factor contributing to this debate is the fact that different strains and lines of mice respond quite differently to environmental conditions. The mouse is perhaps the most genetically manipulated laboratory animal that we study. As a result, mice can evince both subtle and obvious differences in behaviour, reinforcing the notion that environmental conditions that may be optimal for one type of mouse may be inadequate for another type. Further confounding resolution of this question is the fact that different parameters are measured across studies, or different methods are used to achieve the differences in floor space afforded the subject animals. Depending on the study, animal responses to cage size might be assessed based on weight gain/body weight [16-22], food and water consumption [17-19], immunological parameters [16-18], behaviour [21, 23], emotionality of the animals [24], impact on first- or second-generation pups derived from litters of dams raised with different available cage space [25], and mortality [16-19]. Cage environmental conditions such as ammonia, carbon dioxide, temperature and relative humidity [18, 20, 26] have also been evaluated as a function of animal density and the impact of increasing animal density on the health of the cage occupants. Two primary methods of modifying the cage space available to an individual animal are typically used. In some studies (e.g. [16, 17]) the number of animals in the cage space remains the same while the cage size is reduced. In other studies (e.g. [18, 19]), cage density is increased while the actual cage size remains static. A criticism of the latter approach is the potential confounding effect on the data by social interactions and pressures that occur with crowding of animals, but such studies also use commercially available cage equipment rather than customized caging of incrementally different sizes, thereby perhaps facilitating extrapolation of findings into typical animal housing units.

Several recommendations have been made regarding what constitutes adequate cage space. These range from a performance-based approach, such as providing sufficient space for exercise and normal social behaviour and the inclusion of enrichment items [27], to varying the amount of cage space based on the strain, number of animals in the cage, age of the animals, reproductive status, familiarity of the animals with each other and the work being

done with them [28]. While it has been postulated that the quality of the cage space is more important than simply a larger floor area [29], other work has shown that animals evaluated using a consumer demand paradigm will work to gain access to additional cage space that is empty except for bedding [30]. Still others have suggested that the amount of cage space is important, in and of itself, as it can become the limiting factor for the provision of enrichment [28]. Of interest is the finding in female C57BL-derived mice that the motivation to access additional cage space did not depend on the amount of additional space accessed [30]. In this study, a single mouse would move away from its social group, food, water and nesting material to access additional space (a bedded cage of varying dimensions). The test mouse had been acquainted with the additional cage space to mitigate possible novelty effects. Despite increasing demands (number of presses on a switch) to access the additional space, mice demonstrated strong motivation to do so. The author suggests that this sustained response could represent a drive for exploration, territorial monitoring or escape from cagemates. However, it may be argued that the additional space offered, which prompted the expression of exploratory behaviour, does not equate to enhanced welfare.

Jennings et al. [28] propose that in the context of a lack of concurrence regarding what constitutes 'optimum' cage space, the dynamic resulting from the space, design and construction of the cage, the animals, and the enrichment should serve as a guide for establishing space requirements. However, the element missing from this recommendation is that the scientific use of the animal should also be considered, as the cage environment should not only foster animal welfare but also facilitate high quality science. While there may not be absolute concurrence regarding an optimum cage size, the scientific literature provides many examples of studies demonstrating that some strains can be housed at higher densities with no adverse effect (e.g., [16-19, 23, 31, 32]), with the strains evaluated including C57BL/6 lines, BALB/c lines, NOD/LtJ, FVB/NJ and MF1. Several of these studies also show, however, that while mice appear to be able to be housed at densities greater than the cage space recommendations in the *Guide*

[4], there is a limit to the increase [33, 34]. Smith and Corrow [35] postulate that the reason for the reduced aggression observed in mice housed at higher densities (e.g. [23]) is because there is less defensible territory with less floor space. This hypothesis is supported by findings in wild mice, which are strongly territorial when population density is low or moderate [36].

Informative summaries of the measured effects of different cage sizes on various physiological and behavioural parameters for different strains of mice are available in the literature [18, 20]. The significant point derived from these summaries is that cage size effects simply cannot be extrapolated from one strain of mouse to another, as the response may be quite different (reduced, unaffected or increased). Thus, the dilemma faced by colony managers housing mouse strains, or lines for which there is no objective, peer-reviewed data regarding the animals' response to different cage sizes, is what standard to use in determining the most appropriate cage size and housing density. For two common strains of mice, C57BL/6J and BALB/cJ, Nicolson et al. [20] suggest that the most reliable metrics for determining appropriate cage size are body weight gain, adrenal gland size and percentage adrenal cortex (the corticosterone-producing portion of the gland), faecal corticosterone metabolites, in-cage telemetry of activity and heart rate, behaviours such as barbering, whisker-picking and fighting, and formal tests of anxiety. Clearly, experience with the animals will provide practical information upon which to base cage size determinations, albeit in a *post hoc* manner. However, a potential flaw with this scheme is that until the *post hoc* analysis has been done, animals may not be housed in an appropriately sized cage (e.g. more available cage space with resulting negative consequences such as increased aggression [23] or lower body weight [22]; alternatively, less available cage space with negative consequences such as increased serum corticosterone [16, 35] or gastritis [33].

Structural enrichment

Nests

Nesting behaviour appears to be an activity that is well preserved from wild-type progenitor

mice [1]. The provision of nesting material to caged mice has received widespread support because there appears to be a strong motivation for mice to build nests (even among non-breeding mice). It can enhance pup survivability, it is a behaviour that is commonly performed by numerous strains of mice and it offers the opportunity for mice to better thermoregulate in their environment [37, 38]. Numerous studies have assessed the relative merits of different kinds of nesting material, including commercially available Nestlets, paper strips, tissue or paper towel, cotton string, wood wool and wood shavings. The value of the nesting material to the mouse has also been critically evaluated, using the complexity or architecture of the nest as a metric for the quality of the nesting material provided [39, 40] or the mouse's willingness to work to access nesting material [41]. Of significant welfare benefit is the finding that some kinds of nesting material (e.g. corn husks) reduce aggressive behaviour in a line of BALB/c mice, as indicated by observed decreased wounding of the animals [42], possibly due to the availability of areas to escape aggressive animals. Clearly the type of nesting material impacts this welfare benefit, as aggression was decreased in 7 week old male BALB/c type mice provided with tissue torn into strips [43], though intracage fighting was not reduced by providing wood wool as the nesting material to BALB/c and C57BL/6J mice [44] and actually increased fighting in NIH/S male mice [45]. Yet, there is evidence that some strains of mice, such as BALB/c and CD-1 mice, show reduced signs of stress, including lower urine corticosterone levels and heavier thymuses, if they are provided with nesting material and if the nest is transferred during cage cleaning procedures [46, 47]. Although there are contradictions in the literature regarding optimal nesting material (e.g. paper strips [40] versus tissue or paper towel [48]), an important consideration is the planned use of the mice. As Pasalic and colleagues [49] determined, tissue nesting material can be a confounding variable for studies of allergic asthma in BALB/c mice, resulting in increased total cell number, eosinophil number and IL-13 concentration in bronchoalveolar lavage fluid as compared with non-enriched control animals.

Also, warnings have been given with regard to some types of nest-building material that can entangle the limbs of pups [28].

Nest boxes/shelters

As a prey species, wild-type mice will attempt to flee and hide from predators, and it has been postulated that laboratory mice retain this fear response behaviour. For example, laboratory mice may exhibit aggression to handlers if startled or fearful, and thus the provision of shelters has been suggested as a means of reducing the mouse's fear response [50]. The inclusion of shelters or nest boxes has been evaluated as a single enrichment and in combination with other enrichments (e.g. nesting material, running wheels). As has been demonstrated by investigations into other forms of enrichment, varying results have been obtained on the merits of providing shelters, depending on the strain of mouse, whether nesting material was also present, the number of openings in the nest box and the material from which the nest box or shelter was constructed (e.g. metal, plastic, wood, paper product). In fact, the material of which the nest box is constructed has been proposed as a significant factor in preferences expressed by mice [51].

In some cases, the shelter provided is a tube (perforated along the sides of the tube or non-perforated), while in others it is designed to function more specifically for nesting. Baumans et al. [29] suggest that partitioning the cage space with structures like shelters allows mice to use separate areas for feeding, resting and urination/defaecation and that shelters aid mice in controlling their environment, such as exposure to illumination. The relative value of the shelter may vary with its location in the cage or if other enrichments are also available. A tube-shaped shelter within the cage may be located either directly on the cage floor or suspended from the cage wall, though animals appear to use the tube shelter more frequently if it is located on the cage floor. Even the location of the nest box or shelter on the cage floor will be adjusted by mice in individually ventilated cages, with animals moving the shelter to a position under the food hopper [52]. Sherwin [53] determined that individually housed male TO mice would not use a tube for sleeping if sawdust was provided as bedding in the cage;

rather, these mice used the tube for refuge and as a latrine.

Recently, it has been shown that the number of days of survival of Tabby jimpy (*Ta-jp*) mice (*TaPlp1^{jp}/$^+$*) resulting from the breeding of heterozygous females (*TaPlpN$^{jp/++}$*) with white-bellied agouti males (B6CBACa-W^{w-J}/A) was increased in those animals provided with a nest box constructed of paper boxes [54]. These animals also had a higher weaning rate, had a statistically significant higher weaning weight and developed few abnormal jumping behaviours. Because the nest box was fabricated from a paper product, the dams could create additional holes in the shelter and use the shredded paper as a component in their nest-building activity, thereby adding value to this type of shelter. Male BALB/c mice also had increased longevity if they had access to a shelter [55]. Mice living in cages containing a nest box, nesting material, chew blocks and a running wheel consumed less feed than mice housed in standard cages that were allowed to self-administer an anxiolytic, and spent less time performing bar-related behaviours and bar-circling stereotypies [56]. Another dimension to the complex picture of shelter use is provided by a third report regarding C57BL/6J mouse use of a polyvinyl chloride (PVC) nest box. Animals were also provided with a wooden chew block, a cardboard tube and nesting material [57]. The authors determined that in this arrangement, use of the shelter increased between 4 and 8 weeks of age, but the animals used the shelter as a nesting/sleeping site only at 4 weeks of age, and at an older age generally preferred to use the nesting material for sleeping. Of note is the finding that, for some strains of mice, inclusion of a nest box or shelter has been implicated in increased levels of aggression in animals [43], though this is not always the case [55, 58].

Wheel-running

Although wheel-running has been described as an artefact of captivity [59], there is general agreement that it is a highly robust behaviour among mice that are afforded the opportunity to engage in the activity. Reports of mice running 2-5 km over a 24 h period (mostly during the dark cycle) emphasize the motivation of mice to use this type of apparatus [60, 61]. The question of whether wheel-running actually benefits the welfare of the mouse, and thus can be considered an enrichment strategy, has been debated. Wheel-running has variously been described as substitute activity for other kinds of behaviours [60] and an incentive-induced motivated behaviour [61]. Sherwin [59] offers a detailed and well-balanced review of the sometimes conflicting data regarding causal factors and impacts.

Wheel-running does not reduce the expression of stereotypic motor behaviour in deer mice [62] and it can alter the time budget for other activities [61]. Specifically, female C57BL/6 mice provided with a wheel for running had altered hourly patterns of movement and reduced time in a shelter, though they had overall higher levels of activity. Much of the change in motor activity was reflected in less time moving on the cage floor. The authors concluded that wheel-running should not be considered simply an extended opportunity for locomotion because it actually reorganizes daily behaviour, with strain differences apparent between DBA/2 and C57BL/6 mice [63]. Female C57BL/6 mice housed with a wheel also exhibited angiogenesis, enhanced motor coordination and some behavioural changes such as improved water maze performance [64], which led the authors to caution use of a running wheel as a standard enrichment component due to the need to control for its effect for some types of studies. The potential for wheel-running to effect experimental data is of wide concern. Recently, Zajac and colleagues [65] documented that wheel-running significantly increased total brain-derived neurotrophic factor (BDNF). Disruption of BDNF gene expression is critical to the development of symptoms in Huntington's disease (HD). Wheel-running in HD mice results in a delay in the onset of motor deficits across several tests [66]. In addition to physical changes associated with mice using a running wheel, behavioural effects have also been observed. For example, intermittent individual housing of female BALB/c mice typically affects open-field behaviour, but these differences can be mitigated by access to a running wheel [67]. Wheel-running may also be a stereotypy [68]. Perhaps the most significant behavioural effect associated with the running wheel is the evidence of increased

aggression associated with its presence in the cage, as well as data suggesting that the wheel may be disrupting social organization in the cage [69].

Cage or shelter colour

Contradictions in optimal characteristics of the housing environment are as fundamental as the colour of the cage itself. Rodents are considered to be dichromats, able to perceive colours in the green-yellow region of the spectrum and in the ultraviolet range, but with red appearing dark [70]. Sherwin and Glen [71] evaluated the choice of cage colour for female CBA mice by preference test. The authors housed the mice in a home cage painted either black, red, green or white. Five weeks later, the mouse was allowed to choose the colour of its home cage among these same colour types. All mice showed a statistically significant preference for the white cages, with red being the least preferred colour. However, such findings must be approached with caution as the authors did not specify the substrain of the mouse, and CBA/J carries the $Pde6b^{rd1}$ mutation, which can cause blindness by the age of weaning [72].

This finding led to an assessment of a commercially available mouse shelter constructed of red transparent thermoplastic material and a shelter made of a paper-based product [73]. When given a choice of a cage with the red thermoplastic shelter or the paper shelter, female mice of BALB/c, C3H and C57BL/6J lines chose the shelter fabricated from the paper product significantly more often. The authors considered the red colour of the thermoplastic shelter, the shelter material (i.e. the thermoplastic could not be modified by chewing on it, whereas the paper product could be modified by the mice), and the fact that the paper product was sufficiently lightweight to move around the cage, as possible factors driving the preference toward the paper-based shelter. However, a subsequent study of male mice of BALB/c, C57BL/6, CBA and NMRI lines provided with only a red thermoplastic cage, plus bedding and nesting material, showed differences among the strains regarding the time spent in the shelter and number of entries into the shelter, though the shelter was routinely used [74]. Thus, there appears to be some variability in the literature regarding mouse response to shelters; however, these preliminary data suggest that mice will rank different types of shelter if offered a choice, but that if no choice is offered, the shelter will certainly be used by the animals to varying degrees.

Effects of enrichment

Effects on the animals

One of the challenges associated with cataloguing the effects of environmental enrichment on mice is that reports of effects from studies using 'enrichment' may be confounded by the fact that the items provided in the cage to increase structural complexity were not objects that actually enhanced the welfare of the animals. Clearly, semantics plays a role in this problem, as any addition to the cage environment seems to be automatically labelled as an enrichment, whether the actual definition of enrichment is achieved or not. As described above, many preconceived notions about the benefits of certain cage structures must be discarded as evidence mounts regarding their value as true enrichments. Further complicating the picture is the variability among strains of mice in terms of responses to enrichment items or structural additions to the cage environment. For example, Hutchinson et al. [1] describe the differences in the number of litters produced by female mice living in enriched or standard housing. In these studies, the enriched cages included a ladder and jar with nesting material, while the standard cage had bedding. BALB/c and Swiss-Webster females produced significantly fewer litters (p <0.001) and had fewer pups per litter when housed in the enriched cage as compared to the standard cage. However, CB17-$Prkdc^{scid}$, B6D2F2 and ICR mice did not show any difference in number of litters or pups per litter when housed in standard or enriched cages. The authors also detail striking gender differences for BALB/c and Swiss-Webster mice. Specifically, females of these two strains (but not males) demonstrated

significantly lower levels of thymocytes when living in an enriched cage as compared to the standard cage.

An understanding of the effects of providing an enriched, or stimulating, environment to rodents has its roots in studies done with rats and assessing effects of handling and maze training on brain chemistry and anatomy [75]. Since then, the body of information regarding the influence of cage complexities on the mouse has grown considerably and new findings continue to be published. These findings can generally be categorized into effects on the behaviour or biology of the animals, often described in the context of changes in a specific animal model.

Behaviour

The standard cage provides limited scope for the expression of species-appropriate behaviours in the laboratory mouse. Signs of this deficiency include abnormal behaviours such as stereotypies (e.g. bar-mouthing, jumping, circling [76]) and compulsive behaviours such as barbering [77], elevated stress hormone levels, fearful and anxiety-like behaviour [78] and impaired thermo-regulation [79]. It is therefore not unexpected that the addition of complexities to the cage environment can evoke a change in behaviour. General activity level, which in some studies is dissected into the more specific behaviours of exploration and locomotion, as well as sleep, stress or anxiety-related behaviours (sometimes referred to as emotionality), social behaviours, appetitive behaviour and grooming are among the parameters evaluated when one or more objects is introduced to the cage environment. Results vary among strains, gender and type of object(s) introduced. But, the data converge in demonstrating reduced stereotypies [80], increased exploratory behaviour [81], at least initially; increases in aggression between animals, with many, though not all, types of enrichment [43, 82]; alterations in the open-field test behaviours [83]; and general 'use' of the variety of enrichments.

Neurological effects

Although initial studies regarding the influence of enrichment on the central nervous system were conducted with rats, there are similarly numerous reports of neurological effects of enrichment on mice and some excellent reviews have been published on this subject [84, 85]. As has been observed in rats, exposure of adult CBA/B6 hybrid mice to enrichment induces structural changes in the brain [86, 87]. For example, mice placed in a large box containing toys, wooden blocks, a running wheel and shelters for 3 h/day had altered mRNA levels of genes associated with structural changes in the brain. The authors identified an upregulation of dynactin, a cytoskeletal protein involved in retrograde axonal transport, which is thought to have a role in neuronal growth and synaptogenesis. The protein cortactin, which is involved in synaptic formation and plasticity, was also upregulated in the enriched animals. The authors also determined that the protein known as defender against cell death 1 (DAD1) was upregulated after exposure to enrichment. They concluded that environmental enrichment influences the expression of several genes linked to neuronal structure, synaptic signalling and plasticity which have a role in learning, memory and age-related memory deficits. Mitigating effects of enrichment on age-related memory deficits has also been demonstrated by Frick and colleagues [87] using C57BL/6 mice. In this research, the effect of enrichment on the spatial memory of 'middle-aged' (7 months) male and female mice was evaluated in a Morris water maze. Enrichment, consisting of a running wheel and various shaped toys, was provided in the home cage for 25–29 days. Data indicated that enrichment provided to middle-aged mice reduced age-related spatial reference memory deficits (both spatial task acquisition and retention) as compared to socially housed control animals.

Much of the research into the effect of enrichment on neural structure has focused on the hippocampus due to evidence that enrichment enhances hippocampal function, including long-term potentiation, neurogenesis, dendritic spine growth and neurotrophin mRNA expression [88], which in the hippocampus are implicated in learning and memory functions. Indeed, the hippocampus appears to be one of the more susceptible areas of the brain to the influence of enrichment [89]. Diabetic mice

show structural alterations in the hippocampus, including reduced neurogenesis in the dentate gyrus, decreased dendritic complexity and reduced vascularization of the dentate gyrus. However, an exposure of 10 days' duration to 16 week old C57BL/6 mice that had streptozotocin-induced diabetes resulted in neural cell proliferation, differentiation and retention, vascularization of the dentate gyrus and enhanced dendritic complexity of hippocampal neurons [90]. The mechanism for this activity was not definitively determined, although an increase in BDNF or a modulating effect of enrichment on the hypothalamic–pituitary–adrenal axis were postulated as possible pathways. A relevant point is that Zhu et al. [89] observed increased levels of BDNF in C57BL/6 male and female mice that lived in an enriched cage environment for 4 months, thus lending further support to the hypothesis that neurotropin levels may be modulated by external stimuli. Increased levels of BDNF have also been detected in the retina of enriched mice, with a concomitant increase in retinal ganglion cell (RGC) dendrites, even preventing the typical suppression of dendritic branching caused by dark rearing which suggests that visual stimulation alone is not the modulating factor for RGC dendritic enhancement [91].

The effect of enrichment (including a running wheel) on mice used to study several neurological disorders has recently been reviewed [84, 85]. The modulating effect of enrichment has been consistently demonstrated in several mouse models of neurological disease. For example, results in a delayed onset and progression of the motor deficits in mice afflicted with HD [92–94]; a reduction of amyloid deposition in transgenic mice used to study Alzheimer's disease; increased resistance to MPTP, used to induce Parkinson's disease and enhanced recovery of motor function [95]; extended lifespan and enhanced Rotor-Rod performance in mice with amyotrophic lateral sclerosis [96, 97]; increased dendritic branching and spine density in mice with fragile X syndrome [98]; delayed and reduced neurological deficits, including motor impairment and anxiety behaviours, in mice lacking *Mecp2* and used as a model for Rett syndrome [99]; reduced central nervous system cell infiltration by Piry virus (used to induce

encephalitis) and more rapid viral clearance, with reduced microgliosis in infected mice [100]; and enhanced spatial memory acquisition in female Ts65Dn mice (a model for Down's syndrome), but, of note, reduced spatial memory acquisition in the trisomic male mice [101].

Effects on research
Beneficial effects or confounding variable?

The provision of environmental enrichment may result in unintended consequences for both animals and research results [102]. However, studies specifically examining the effects of environmental enrichment on the variation of experimental results have demonstrated mixed results. Van de Weerd and colleagues [103] evaluated both behavioural and physiological parameters in mice used for potency testing for tetanus vaccine and stress-induced hyperthermia. Although they observed some differences in body weight and open-field testing responses between control and experimental animals, there was no increase in variability among the two groups, and for some measures there was less variability for the experimental animals. Similarly, Augustsson et al. [104] determined that the greatest effect on variation for C57BL/6 and BALB/c mice used in a light/dark test using diazepam as an anxiolytic agent was the strain of mouse rather than the presence of enrichment.

Clearly, the welfare of laboratory mice may be seriously altered by housing them in inappropriately designed and insufficiently complex cages. Attenuating these adverse effects through adequate environmental enrichment is likely to improve not only the animals' well-being, but also the scientific validity of a wide range of experiments conducted with them. As Baumans [50] has suggested, with the provision of enrichment, the mouse can exhibit more species-appropriate behaviour, and thus may be able to better cope with unexpected changes to its environment and respond more uniformly to different challenges. Abnormal behaviour, stress, fear and anxiety, and impaired thermoregulation are potentially confounding variables that may adversely affect the outcome of animal experiments and consequently increase variation in the data. Therefore, it is a reasonable conclusion

that the most appropriate enrichment is in the best interest of both the animals and the research.

Nevertheless, environmental enrichment is still far from being a standard operational procedure in most mouse facilities, though one study of 22 animal facilities indicated that 73% provided structural enrichment to their mice, with 20 of the facilities using nesting material [1]. One reason for the reluctance to provide enrichment is the concern that environmental enrichment itself could be a confounding variable that adversely affects the scientific validity of animal experiments. In particular, it has been argued that environmental enrichment might disrupt environmental standardization in ways that are detrimental to the precision and reproducibility of results from animal experiments. If true, this would mean that environmental enrichment may create a conflict between the welfare of the animals and the validity of the research, and that the benefits of enrichment in terms of better animal welfare need to be gauged against its costs in terms of poorer scientific validity.

Environmental standardization

Standardization in animal experimentation and its implications for the precision, reproducibility and validity of animal experiments has typically been the backdrop for the conduct of research. According to textbooks on laboratory animal science, standardization refers to 'the defining of the properties of any given animal (or animal population) and its environment' [105]. Like genetic standardization, environmental standardization essentially serves two distinct goals. First, by defining environmental conditions and exposing all animals used in an experiment to the same defined conditions, environmental standardization is aimed at reducing within-experiment variation, that is, variation due to individual differences resulting from individual animals being exposed to different environmental conditions. Although the 'defining of the properties' does not necessarily implicate identical environmental conditions for all the animals of an experiment, environmental standardization is generally equated with such environmental *homogenization*. Thus, environmental standardization renders animals within experiments more homogeneous. The intention behind

such uniformity is to minimize within-experiment variation in order to maximize test sensitivity. Higher test sensitivity means that a given difference between the means of two populations is statistically more significant, or that the same level of statistical significance is achieved with fewer animals. Therefore, environmental standardization has the effect, both economically and ethically, of reducing the number of animals needed per experiment. However, this benefit is achieved with an increasingly narrow spectrum of phenotypes. Therefore, the external validity of experimental results obtained from such a homogeneous population may be limited to a narrow range of environmental conditions [106]. This has important implications for the reproducibility of the results, as discussed below.

Second, by using the same defined environmental conditions across different experiments, environmental standardization is aimed at reducing between-experiment variation, that is, variation in the outcome of an experiment resulting from experimental populations being exposed to different environmental conditions. The intention behind such *harmonization* of environmental conditions across experiments is to minimize between-experiment variation in order to maximize the reproducibility of results. Reproducibility is a cornerstone of all scientific research and is particularly crucial in animal research where the lives of the animals are highly valuable. For example, in the USA animal care and use regulations require scientists not to 'unnecessarily duplicate previous experiments' [107]. This requirement critically depends on the results of animal experiments being reproducible both within and between laboratories, and harmonization of environmental conditions across experiments and laboratories aims to guarantee such reproducibility.

Concerns that environmental enrichment might disrupt environmental standardization relate to both of these aspects. Thus, it has been argued that enrichment might increase either within-experiment variation or between-experiment variation or both (for example, see [108]).

Enrichment and within-experiment variation

Concerns that enrichment would increase within-experiment variation rest on the hypothesis that

a more complex environment produces a greater diversity of phenotypes among the animals of a study population. On the one hand, a more complex environment might create more opportunities for individuation, for example, by providing different niches within an environment so that the different animals within a cage are exposed to different environmental conditions. On the other hand, an unenriched environment might increase individual differences, as indicated by the occurrence of abnormal behaviours such as stereotypies, resulting in variable and individual coping responses. Whether phenotypic diversity is a function of environmental complexity, and whether this relationship is positive or negative, are empirical questions that have never been systematically addressed. However, several studies have examined the effects of various enrichment protocols on within-experiment variation in physiological and behavioural measures [96, 97, 109]. None of them identified evidence that enrichment would affect within-experiment variation consistently in one or the other direction.

Present evidence therefore suggests that the welfare of mice can be improved by provision of suitable environmental enrichment without increasing within-experiment variation, provided the enrichment is adequate for the animals and does not itself constitute a stressor, in which case variation in experimental results may indeed be enhanced.

Enrichment and between-experiment variation

The hypothesis that enrichment might increase within-experiment variation is also used to argue that enrichment might compromise the reproducibility of experimental results. However, reproducibility is not determined by variation within experiments but by variation between experiments, and a recent multilaboratory study showed that even extensive enrichment had no adverse effect on between-experiment variation, demonstrating that a more complex environment does not compromise reproducibility [109, 110].

Others are concerned that more complex housing conditions would inevitably lead to greater differences in the environmental conditions between laboratories, because different institutions would choose different enrichment items, use different products, arrange them differently within cages and differ in how often they replace them. However, besides the possibility to standardize all of these aspects, it is unlikely that they represent a significant problem, given the variation in environmental conditions that exists anyway between different laboratories. Thus, there are many environmental factors that simply cannot be standardized (including staff, room architecture, noise, smells, air exchange rates, illumination levels and vibrations). It is therefore unavoidable that different laboratories standardize to different local environmental conditions, and enrichment is one more factor that varies across laboratories. Given that environmental variation among laboratories (and even between experiments within the same laboratory) is a matter of fact, results will only be reproducible if they generalize to at least the range of conditions covered by these inherent laboratory differences. Therefore, reproducibility primarily depends on the external validity of the results, which is partly determined by the treatment and the measured response (some treatment effects are fairly robust against variation of conditions), and partly by the experimental design.

Enrichment and scientific validity

Validity in the context of animal experiments refers to whether an experiment or a measured result is scientifically meaningful. There is a range of different concepts of validity, including internal vs external validity, convergent vs discriminant validity, and face vs construct vs predictive validity. The latter forms of validity all depend on experimental design, types of measurements and measured outcomes, and are independent of whether or not the animals' cages are enriched. Validity in the more general sense, however, may well be affected by environmental enrichment. Thus, scientific validity in this general sense encompasses the rationale of the study and all aspects of the methodology, and thereby determines whether the results obtained meet all of the requirements of good scientific practice. Any aspect that violates scientific principles or

principles of Good Laboratory Practice will compromise the integrity of the results.

Assessing the value of enrichment

For rodent enrichment to be considered a meaningful addition to standard animal care practices, a harm–benefit analysis should be undertaken. This analysis should be inclusive of effects on the animal and on the research. Clearly, some types of research will be less impacted by the often subtle impact on the animal, while others will be exquisitely sensitive to small changes in the animal's physiology. In many instances, the inclusion of enrichment in a mouse's cage has led to intriguing discoveries regarding the effect of environmental complexity on a particular animal model of human disease, leading to new theories of pathogenesis as well as potential treatment adjuncts.

Several fundamental questions bracket the implementation of mouse enrichment. The most basic of these is whether the animal 'uses' the enrichment. Animal use of the enrichment can be described as moving the object around, making contact with it (e.g. climbing, resting or running on it), entering it (e.g. a shelter), or changing its configuration (e.g. nesting material). Next, it should be determined how the animal is using the enrichment. For example, if the animal is defending the enrichment to the point of increasing its aggressive behaviour towards other animals, an alternative form of enrichment, or possibly other approaches to improving the animals' welfare, and their value as a research subject, should be considered. Finally, and importantly, is the need for a sound understanding of the potential ramifications of the enrichment technique on the animal's biology and whether this may have consequences for the intended research use of the animal. The scientific literature is rich in studies that have evaluated this topic, although much remains to be done, which can give direction to this determination.

As already described, enrichment offered with the best of intentions can have a negative impact on the animal. The mouse's response appears to depend on the type of enrichment; strain, sex and age of mouse; individual or social housing; and whether the enrichment is provided in the home cage or the animal is moved to a separate enrichment space. These responses can include stress, fear and anxiety; aggression; injury due to the enrichment itself and potentially the introduction of contaminants into the mouse environment. Yet, the evidence is clear that a sterile cage environment results in mice with deficits in brain development and exhibiting abnormal behaviour [79], which is certainly a welfare concern, but also presents doubts regarding the validity of the animal as a research subject. Because not providing an adequate housing environment for mice has both welfare and scientific implications, and because providing mice with inappropriate enrichment also has both welfare and scientific repercussions, the approach to optimizing mouse cage environments should be a measured one. In addition to establishing a team of professionals at the institution to assess the specific circumstances and determine the proper strategy for housing the mice in question, consideration should be given to implementing enrichment in an incremental manner, if possible, so that the impact on the animals and the research can be reviewed and any necessary adjustments to the programme made accordingly. While preference testing has been conducted to evaluate the relative importance of enrichments to mice (e.g. [72, 111]), it should be recognized that this method of testing has limitations and does not address the suitability of the enrichment for the specific mouse strain, sex or age.

Keeping mouse enrichment practical

Ease of husbandry

For an enrichment technique to be widely accepted within an institution, it should not perceptibly impede the work of the animal care staff. Husbandry procedures may be facilitated if consideration is given to the sanitizability of the enrichment item(s), or alternatively if the

item is sufficiently inexpensive that it can be disposed of when degraded or soiled. It is very helpful to have husbandry staff participate in the selection, planning and implementation of the mouse enrichment programme because of their detailed knowledge of, and experience with, the cage equipment and routine feeding and cleaning procedures. Concerns regarding enrichment in mouse cages tend to cluster into five general themes: (i) the enrichment precludes adequate visualization of the animals for daily observation of health status, (ii) the enrichment takes up so much space that the animals are crowded, (iii) the enrichment item is difficult to sanitize or complicates routine cage change operations, (iv) the enrichment impedes removing the animals from the cage, and (v) less frequently reported, though a significant potential problem, the enrichment can accidentally bump up against the automatic watering device or water bottle, resulting in flooding of the cage.

The impact of husbandry procedures and conditions on mice can be significant. To mitigate some of the effects of the routine cage change procedures and the potential social disruption that may ensue due to the sudden absence of familiar odours, the transfer of nesting material during cage changing has been recommended as one method to reduce aggression in male BALB/c mice [46], though this has not been replicated in SJL/J mice [35] and aggression in mice has been linked to caging with a high ventilation rate alone [112]. It is also suggested that nests and shelters can be manipulated by the mice to modulate the ventilation and illumination levels experienced by the animals [52] and so, while they may impede daily observation of the mice unless they are moved by the care staff, these items foster an ability in the animals to better control environmental conditions. The approach of 'super-enrichment' described by Hutchinson et al. [1] entails providing a variety of enrichments either simultaneously or through a rotation schedule. If provided simultaneously, there is the possibility that the cage becomes too crowded for the animals to move around freely or for husbandry and research staff to easily access the animals, and items may 'wick' water into the cage from the watering system. Therefore, although there is evidence that super-enrichment has positive effects on neural plasticity, care

should be exercised to not excessively crowd the cage with enrichment devices.

Although much of the evidence pertaining to the effect of enrichment on efficient husbandry is anecdotal and experiential, Moons and colleagues [113] objectively evaluated the time necessary to catch mice from a cage containing two PVC tubes as compared to mice housed in cages without the tubes. The study animals were male, 10 week old FVB and NMRI mice. They determined that the PVC tubes decreased the time to catch the NMRI mice and had no significant effect on catching the FVB mice. In addition, the presence of the tubes did not make the animals harder to handle, as assessed by there being no difference in resistance to being held for a sham injection procedure between the control and enriched mice. In addition, a recent study has shown that picking mice up out of their cage using a tube, rather than manually by the tail, resulted in a low anxiety, voluntary approach of the mice to the experimenter, and greater acceptance of physical restraint [114].

Budgetary and other considerations

Animal *per diem* costs are a constant concern to researchers and facility managers alike. Thus, the type and design of caging system used should support and, optimally, enhance the animal's health and welfare so that the most refined animal model is available for the planned scientific enquiry. To that end, the enrichment selected should meet the criteria of fostering animal well-being and enhancing the value of the animal for the experiment, and it should be economical. It should be recognized that there may be an initial investment in different types of enrichment, with the selection of the types of enrichment based on information from the scientific literature, to assess their utility in the context of the individual institutional animal care and use programme. Following this selection process, the enrichments should be included in the budget in the same manner as other recurring expenses, such as bedding.

A mechanism should be established to ensure adequate communication between the personnel who are implementing the mouse enrichment

programme and the research team whose animals are impacted by those actions. This communication should occur before enrichments are put in the cages of animals that have just been obtained for a study, and should continue regularly to allow for feedback regarding the enrichment programme and to update research teams about any planned changes in the programme. Husbandry personnel should be trained to be sensitive to mouse behaviour and able to interpret changes relative to the enrichment programme. Staff should be alert both to positive effects resulting from the programme (e.g. animals that are easier to handle) as well as possible concerns. These latter outcomes should be reported and addressed in a timely manner.

Conclusions

While there is general consensus that an unenriched cage environment is not recommended because of the detrimental effects on the animals, and the concomitant potential for the quality of the research data to be negatively impacted, it is equally clear that a 'cookie-cutter' approach to mouse enrichment is naive and possibly harmful both to the animal and to the research. While mouse enrichment is a highly complex topic, and one that certainly merits further investigation, studies on the effects of enrichment have yielded surprising results that offer intriguing glimpses into biological mechanisms and suggest fascinating new paths of research.

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Nutrition of the Laboratory Mouse

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Introduction

Laboratory mice and rats have always made up a large percentage of the total number of animals used for biomedical research purposes. This percentage is usually around 80-90% of the total number of animals used. For this reason, these species have been well-characterized in many ways. The use of the laboratory mouse (*Mus musculus*) has increased even more dramatically over the last decades, due to the possibility of studying gene function *in vivo* by the use of genetic modification techniques which have resulted in many newly established mouse strains.

The nutrition of the laboratory mouse (and rat) has also been well-studied and well-defined in comparison with that of other species (for an overview see [1]). This chapter concentrates on important aspects of feeding laboratory mice. One needs to be aware of how nutrition and feeding as an environmental factor can interact with experimental results and animal welfare when nutrition is not the main focus of study. Moreover, when the mouse is used as an animal model for human nutritional conditions, specific experimental conditions need to be taken care of.

This is important in order to obtain reliable experimental results and optimal welfare of the animals simultaneously.

Nutritional requirements

Energy

Under *ad libitum* feeding conditions animals usually eat an amount of food that is determined by the their energy requirements [2]. In Table 4.3.1 the energy need is given according to the stage of life the mouse (or other animal species) is in. During lactation an animal has a much higher energy need than during the maintenance phase where the animal has stopped growing and is not pregnant or lactating. 'Metabolic kilos' ($kg^{0.75}$) are used in order to be able to compare species of different sizes. One cannot compare species on the basis of mass in kilograms, as depending on the size of the animal, the metabolic rate per kilogram of body weight is different. Using metabolic kilos compensates for these differences, so that a reliable comparison between species can be made.

That part of the energy in the diet capable of transformation by the body is called *metabolizable energy* [3]. Usually the metabolizable energy content (MEC) of a diet can be obtained from the feed manufacturers. In case it is not provided, it can also be estimated. For this purpose one uses the levels of energy-producing substances in the diets, i.e. fat, carbohydrates and protein. Fats have an MEC of about 37 kJ/g, and protein and carbohydrates about 17 kJ/g. As different types of carbohydrates have different MECs, metabolic studies are required in case one needs to know the exact MEC for specific studies. The contribution made by fibre to dietary energy content is usually negligible [2].

The need for energy depends not only on the stage of life of the mice, but also on other factors such as the environmental temperature and the amount of activity. As laboratory conditions are usually standardized to a certain room temperature and cage size, which prevents unusually high activity, these general formulae for energy requirements according to the stage of life can be used reliably in the current laboratory setting. A mouse showing stereotyped behaviour, such as circling, will have a higher energy need than the cagemate that does not exhibit this behaviour. This will increase variation in results. In order to prevent stereotyped behaviour, environmental conditions must meet the animals' essential needs [4].

Food intake according to energy need will only hold when the rest of the diet lives up to the minimal needs. In case there is a shortage of an essential nutrient, the animal may become (sub)clinically affected, which may lead to a reduced food intake. A reduced food intake may also occur in the case of a test substance with a bad taste being added to the diet. This can interfere with the reliability of the experimental results. On the other hand, where diets have a very good taste, mice are expected to ingest more than their energy need, leading to obesity.

Nutrient requirements

The National Research Council (NRC) provides scientific documentation on nutritional requirements for mice and other species [5]. There is a lot of data published on these requirements, and the NRC establishes committees to review these as new scientific data become available. On the basis of this review process, these committees then publish guidelines on the estimated nutrient requirements [5, 6]. The estimated nutrient requirements for the mouse are shown in Table 4.3.2 [4]. The nutrient amounts per kg diet as well as the nutrient amounts per 100 kJ of diet are shown. As mice eat according to energy need under *ad libitum* conditions, it is

TABLE 4.3.1: Food intake under *ad libitum* conditions is based on the energy requirement, which is related to the stage of life	
Stage of life	Energy need (MJ/$kg^{0.75}$)
Growth	1.20
Maintenance	0.45
Pregnancy	0.60
Lactation	1.30
Source: *Beynen and Coates [2]*.	

Nutrient	Amount	
	Per kg diet	Per 100 kJ
Metabolizable energy	16 500 kJ	
Lipid	50.0 g	0.30 g
Linoleic acid	6.8 g	0.04 g
Protein (growth)	180.0 g	1.09 g
AMINO ACIDS		
Arginine	3.0 g	18.18 mg
Histidine	2.0 g	12.12 mg
Isoleucine	4.0 g	24.24 mg
Leucine	7.0 g	42.42 mg
Valine	5.0 g	30.30 mg
Threonine	4.0 g	24.24 mg
Lysine	4.0 g	24.24 mg
Methionine	5.0 g	30.30 mg
Phenylalanine	7.6 g	46.06 mg
Tryptophan	1.0 g	6.06 mg
MINERALS		
Calcium	5.0 g	0.03 g
Chloride	0.5 g	3.03 mg
Magnesium	0.5 g	3.03 mg
Phosphorus	3.0 g	18.18 mg
Potassium	2.0 g	12.12 mg
Sodium	0.5 g	3.03 mg
Copper	6.0 mg	36.36 µg
Iron	35.0 mg	0.21 mg
Manganese	10.0 mg	60.61 µg
Zinc	10.0 mg	60.61 µg
Iodine	150.0 µg	0.91 µg
Molybdenum	150.0 µg	0.91 µg
Selenium	150.0 µg	0.91 µg
VITAMINS		
A (retinol)	0.72 mg	4.36 µg
D (cholecalciferol)	0.03 mg	0.15 µg
E (R,R,R-α-tocopherol)	22.0 mg	0.13 mg
K (phylloquinone)	1.0 mg	6.06 µg
Biotin (D-biotin)	0.2 mg	1.21 µg
Choline (bitartrate)	2.0 g	12.12 mg
Folic acid	0.5 mg	3.03 µg
Niacin (nicotinic acid)	15.0 mg	90.91 µg
Panthothenate	16.0 mg	96.97 µg
Riboflavin	7.0 mg	42.42 µg
Thiamin	5.0 mg	30.30 µg
B_6 (pyridoxine-HCl)	8.0 mg	48.48 µg
B_{12}	10.0 µg	0.06 µg

TABLE 4.3.2: Estimated nutrient requirements of mice

Source: *Council of Europe [4]*.

advantageous to present the diets in amounts per kJ. This makes it easier to judge whether experimental diet compositions live up to all the essential nutrient needs under *ad libitum* conditions. The NRC guidelines [5] recommend nutrient allowances that are greater than the minimum requirements, as they are often based on the criterion for obtaining maximum growth [2]. This is not necessarily the best situation for obtaining optimal (long-term) health. However, as these recommendations are the best scientifically documented requirements of essential nutrients available at the moment, it is advisable to use them until new scientific proof becomes available.

The recommendations do not take into account that there can be differences in minimum requirements between different strains [2]. Interactions between the nutritional requirements and genetic background of mouse strains can occur. Dystrophic cardiac calcification is a postmortem finding in various strains, which coincides with calcifications in the tongue, lungs and diaphragms [7]. The inbred strains DBA/2Ola and C3H/Ola are susceptible to the development of soft tissue calcifications, whereas C57BL/6Ola and BALB/cOla are resistant towards the disorder (see Table 4.3.3; [7]). The region on chromosome 7 containing the gene *Hrc* (coding for the histidine-rich calcium-binding protein in the sarcoplasmatic reticulum) is likely to be associated with soft tissue calcifications in DBA/2 mice [8]. Nutritional measurements can be taken to prevent excessive calcifications. The diet of susceptible mice, especially at a young age, should contain adequate amounts of magnesium and fluoride [9], whereas excessive phosphorus and vitamin D intake should be avoided [10].

Genetic modification may also alter nutrient requirements. In order to make sure of satisfying the requirements of a particular genetically modified strain, it may be necessary to add special nutrients to the diets.

The recommendations do not necessarily hold for germ-free mice either. Vitamins K and B$_{12}$, for example, are synthesized by the gut flora of conventional mice and will be sufficiently ingested as a result of *coprophagy*. Grit floors do not prevent coprophagy, as the mice can still eat the faeces directly from the anus. For germ-free and specified pathogen free (SPF) mice it is advisable to include higher vitamin B and K levels in the diets, as the microflora of SPF animals may not contain all vitamin-synthesizing organisms [2].

Toxic levels

Toxic levels of nutrients have been defined for a range of nutrients for laboratory rodents [5], and specifically for minerals [11] and vitamins [12]. These tend to focus on extreme excesses that are unlikely to occur in carefully formulated and appropriately used diets, but even modest excesses of some nutrients, for example of protein [1], calcium and phosphorus [13], may be deleterious, though not toxic. In Europe, deliberate experimental use of toxic levels of nutrients would be controlled by both institutional

TABLE 4.3.3: Cardiac calcification in four mouse strains				
Strain/parameter	DBA/2	BALB/c	C3H	C57BL/6
Heart histology				
Incidence[a]	7/7	0/7	7/7	0/7
Score[b]	0.3−2.6	0.0−0.0	0.1−2.8	0.0−0.0
Mineral content (µmol/g dry wt)				
Calcium[c]	43.6 ± 16.7	17.2 ± 1.8	42.9 ± 33.6	17.1 ± 2.2

[a]The number of mice that were scored positive in histological sections for the presence of cardiac calcification out of a group of seven mice.
[b]Range of average scores per mouse.
[c]Mean ± standard deviation.
Source: *Van den Broek* et al. *[7]*.

and national bodies tasked with the regulation of animal welfare.

Toxic effects of nutrients occur rarely and are usually due to lack of knowledge or accident. The following are examples:

- Hyperhomocysteinaemia, an independent risk factor for atherothrombosis, was induced in apoE-deficient mice by adding extra methionine to the diets [14]. As shown in Figure 4.3.1, both the 'low' and 'high' methionine groups did not live until the scheduled end of the study. The total dietary methionine levels fed to the mice in these groups were 2.2% and 4.4%, respectively, levels that with hindsight were obviously toxic for mice. The minimum recommended dietary methionine level for mice during growth is only 0.3% [5]. As a dietary methionine level of 2.3% decreased body weight in Wistar rats [15], higher dietary concentrations must be chosen with care, in order to avoid the risk of toxic effects. Decreasing the excess dietary methionine level to 1.4% allowed the mice to survive until the end of the study without obvious clinical or toxicity problems (Figure 4.3.2; [14]). By carefully screening the literature

before starting animal experiments, the most optimal high dietary levels can be calculated and chosen in accordance with the purpose of the study without causing toxicity.

- A change in the optical isomer of choline bitartrate led to the development of kidney and bladder stones in rodents, followed by renal failure. Synthetic DL-tartaric acid was substituted for the previously used natural L-tartaric acid isomer without informing the diet manufacturers. DL-Tartaric acid has been shown to induce renal damage in rats [16, 17].

Contaminants

Contaminants can be defined as undesirable substances (usually extraneous in origin) which, when present at a sufficiently high concentration in the food, may affect the animal and therefore the outcome of the experiments [18]. Possible contaminants include industrial chemicals (e.g. PCBs), pesticides (e.g. DDT), mycotoxins (e.g. aflatoxin), heavy metals, nitrosamines, nitrates and nitrites. The list also typically includes microbiological parameters, some of which may be deleterious to the animal (e.g. salmonella) while others,
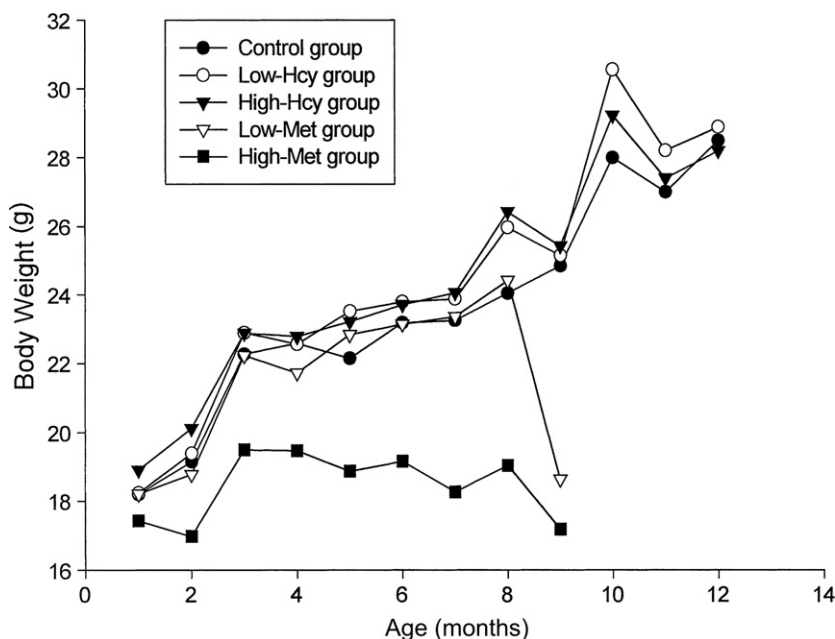
Figure 4.3.1 Body weight development in apoE-deficient mice fed five different experimental diets [10]. A 'high' (4.4%, High-Met group) and 'low' (2.2%, Low-Met group) dietary methionine level fed to apoE-deficient mice caused weight loss and death before the scheduled end of the study. Reproduced with permission from *Zhou, J.* et al. *2001 [14]*.
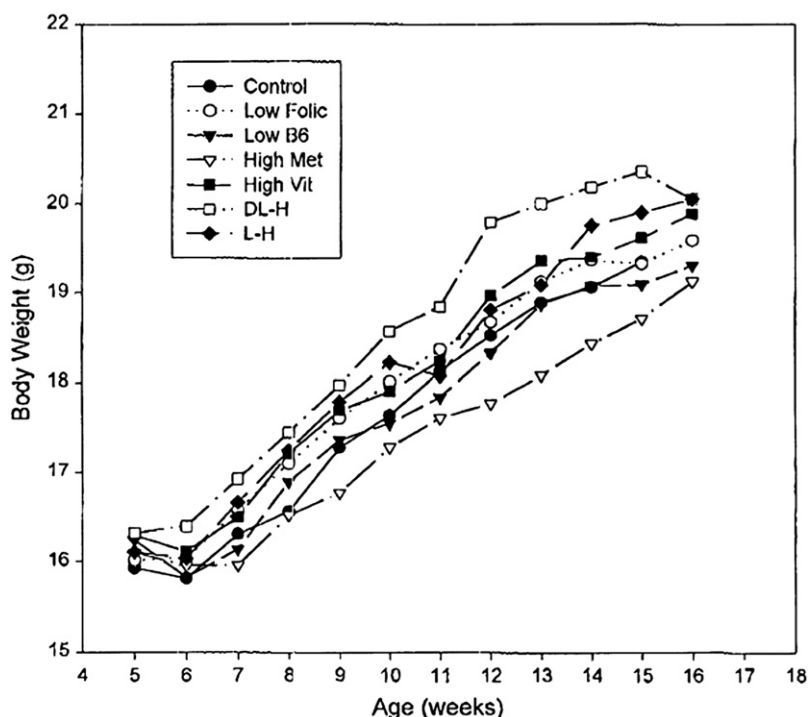
Figure 4.3.2 The body weight development af apoE-deficient mice on seven different purified diets. The 'high' methionine level of 1.4% did not appear to cause adverse effects during the course of the study [39].

such as total viable count and moulds, are indicators of hygiene standards in the feed mill, although at very high levels they could be deleterious to the animal.

Both the US Environmental Protection Agency (EPA) and FDA (Food and Drug Administration) were central in introducing monitoring of contaminant levels in laboratory animal diet, but it is important to distinguish between their guidelines for *in vivo* toxicology and similar studies carried out in accordance with Good Laboratory Practice (GLP), and for studies carried out for their own requirements or purposes. Those for the latter were really contractual specifications to be met by diet suppliers.

EPA and FDA both stipulated that for *in vivo* toxicology studies carried out to GLP the feed should be analysed periodically to ensure that contaminants known to be capable of interfering with the study and reasonably expected to be present in such feed were not present at levels above those specified by the tester in the protocol [19, 20]. These requirements have been reaffirmed in updates to the original publications. More recently, the OECD [21] has stated that, for regulatory carcinogenicity studies, the content of dietary contaminants, such as pesticide residues, persistent organic pollutants, phytooestrogens, heavy metals and mycotoxins, that might influence the outcome of the test, should be as low as possible. Furthermore the diet should be analysed for such contaminants at least at the beginning of the study and when there is a change in the batch used. The introduction of phytooestrogens to the list is novel and is discussed below. None of these agencies defined either specific contaminants or limits to their levels in diet but assumed these would be determined by those conducting the studies in light of the substances being tested and the potential for interference from possible contaminants in the diet. In further guidelines, FDA cautioned against assuming that analysis of a blanket range of analytes would always be appropriate [22]; nevertheless this does happen in practice.

Despite the general nature of contaminant guidelines from the EPA and FDA, they have provided specific statements on contaminants and the maximum allowable concentrations (MAC). However, these were in the form of specifications for diet to be used as part of the testing

programme to support the EPA Toxic Substances Control Act [23] and for in-house studies by government agencies such as the National Institute of Environmental Health Sciences and National Centre for Toxicological Research [24–26]. These generally became *de facto* standards for US diet manufacturers and their customers.

In Europe, these guidelines have been extended by BARQA [18] and GV-Solas [27] to include guidelines on appropriate ranges for the nutrient levels claimed by manufacturers. The list of contaminants and MAC (see Table 4.3.4) show some commonality but also reflect

individual historical experiences. Individual diet manufacturers have also generated lists of contaminants, generally based on the lists above, but also reflecting their historical experience and that of their major customers.

In recent years, phytooestrogens in laboratory animal diet have been recognized as having potentially significant confounding effects on experimental studies. Although endogenous in nature, they may be regarded as contaminants [21]. These substances, mainly in the form of isoflavones and primarily associated with soybean meal, can vary considerably in concentration in

TABLE 4.3.4: Comparison of three different guidelines on maximum allowed dietary levels of contaminants for mice (and rats)

Contaminant	BARQA[a]	GV SOLAS[b]	EPA[c]
Fluorine mg/kg	40	150	
Nitrate mg/kg	100		
Nitrite mg/kg	5.0	15.0	
Nitrosamines mg/kg		NDEA[d] 0.01 NDMA[e] 0.01	0.01
Lead mg/kg	3.0	1.5[f]	1.5
Arsenic mg/kg	1.0	1.0	1
Cadmium mg/kg	0.5	0.4	0.15
Mercury mg/kg	0.1	0.1	0.1
Selenium mg/kg	0.5		0.1–0.5
Aflatoxins μg/kg	5.0	B1 10 B2 5 G1 5 G2 5	5[g]
PCB μg/kg	50	50	50
DDT (total) μg/kg	100	50[h]	100
Dieldrin μg/kg	20	10[i]	20
Endrin μg/kg		10	
Lindane μg/kg	100	100	20
Heptachlor μg/kg			20
HCB μg/kg		10[j]	
α,β,δ-HCH μg/kg		20	
α,γ-chlordan μg/kg		20	
α,β-endosulfan and endosulfate μg/kg		100	
Malathion μg/kg	500	1000	2500
Fenitrothion μg/kg		1000	

(Continued)

TABLE 4.3.4: Comparison of three different guidelines on maximum allowed dietary levels of contaminants for mice (and rats)—cont'd

Contaminant	BARQA[a]	GV SOLAS[b]	EPA[c]
Pirimiphos (-methyl) µg/kg		1000	
Chlorpyriphos (-methyl) µg/kg		1000	
Other phosphoric acid esters µg/kg		500	
Oestrogenic activity µg/kg			1
TVO per g[k]	20 000	Fibre <7% 100 000 Fibre >7% 500 000	
Mesophilic spores per g	20,000		
Salmonellae per g	none	None	
E. coli per g	none	10	
Fungal units per g	200	Fibre <7% 1000 Fibre >7% 5000	
Fusarium toxins mg/kg		Deoxynivalenol 0.50 Ochratoxin 0.10 Zearalenone 0.10	
A/B activity per g	None		None

[a]British Association of Research Quality Assurance, 1992 [18].
[b]German Association for Laboratory Animal Science, 2002 [27].
[c]Environmental Protection Agency, 1979 [19].
[d]Nitrosodiethylamine.
[e]Nitrosodimethylamine.
[f]With a dietary protein level of over 20% or with a crude fibre level of over 12%, values of up to 2.5 mg/kg feed are possible.
[g]Aflatoxin B1, B2, G1, G2.
[h]DDT + DDE + DDD.
[i]Dieldrin + aldrin.
[j]Heptachlor and heptachlor epoxy.
[k]Total viable organisms.

different batches of feed, and affect the animal through a variety of mechanisms. They can influence reproduction, the response to endocrine disruptor chemicals, tumour studies (e.g. mammary, prostate, lung, colorectal and bladder cancers), the cardiovascular system, immunology and inflammation, diabetes, bone metabolism and neurobiology studies (e.g. memory, cognition, anxiety and neurodegeneration) [1, 28].

Unfortunately most contaminant lists are outdated and include contaminants that are no longer found in diet, but exclude some of more current relevance. For example it is difficult to understand the continuing inclusion of oestrogenic activity in some parts of Europe (though not in the USA) which was primarily intended to detect contamination with highly potent diethylstilbestrol and which should not be, but has been, confused with the detection of phytooestrogens [29]. Likely contaminants in diet have been reviewed by Tobin et al. [1].

The nature and level of contaminants are influenced by the ingredients used in the diet: fish meal, a very good quality protein source and widely used in laboratory animal diets, is also potentially a source of nitrosamines and heavy metals. Much of the mercury in fish meal is in the form of organic methyl mercury, which is highly absorbed, in contrast to the inorganic mercury associated with other ingredients [30]. Soybean meal, and alfalfa are rich sources of oestrogenic isoflavones. Avoidance of certain ingredients can be used to minimize the level of contaminants that might be of concern to the user.

Most commercial manufacturers provide diets with batch analysis certificates that include analysis of key nutrients and contaminants. This provides buyers with the opportunity to judge whether this specific batch is suitable for the purpose of their particular study or to buy an alternative batch. After the completion of the study, the batch analysis certificate is also valuable for the interpretation of the results. Although small deviations in nutrients may be acceptable, if explained, it should be unusual for a manufacturer to release a batch that exceeds an MAC for an inorganic and organic contaminant since this is likely to require significant justification to regulatory authorities.

Types of diets

Natural-ingredient diets

The two most commonly used types of mouse diets in the laboratory are natural-ingredient and purified diets. A third category of diets, the chemically defined diets in which amino acids may be used instead of a protein source, or fatty acids instead of fat, are of minor interest, rarely used, and are not discussed in this chapter (but see Beynen and Coates [2] for information). The pelleted diets that are often standard in most laboratory animal facilities are usually produced from natural ingredients.

Natural-ingredient diets can also be divided into open- and closed-formula diets [31]. In open-formula diets, all dietary ingredients and their concentrations are reported and should not vary from batch to batch. In closed-formula diets, the dietary ingredients used are usually reported, but the concentration of each dietary ingredient is not stated by the manufacturing company.

Closed-formula diets can be manufactured to either a fixed or a variable formula (open-formula diets are always fixed). In a variable-formula diet, the objective is to try to maintain the nutrient levels as constant as possible, by adjusting the inclusion of ingredients to allow for any nutrient variability in different batches of ingredients. In practice it is impossible to achieve such consistency and the focus is usually on consistency of protein level. The concentration of dietary ingredients in the diet may vary from batch to batch or with availability of ingredients [31].

In a fixed-formula diet, the same proportions of raw material ingredients are used each time a batch is produced [18]. As natural ingredients can differ in nutrient levels, natural-ingredient diets are subject to variation, though with careful selection of ingredients and suppliers, and quality control of incoming materials, variation of nutrients can be minimized. It should be possible in a fixed-formula diet to achieve a coefficient of variation in a nutrient such as protein as low as 2–3%, which compares favourably with variable formula diets. For a fuller discussion see Tobin [1].

While most natural-ingredient diets for mice are pelleted, it is possible to obtain extruded (expanded) rodent diets. These offer several advantages particularly for autoclaving, for higher fat diets, and for weaker strains of mice [1, 32].

Manufacturers provide information on the diets in their catalogues and on their websites. The amount of information provided and the way the catalogue values for nutrient concentrations have been established is not standardized, though it is usually possible to contact the individual firms to find out any additional details required. There is considerable difference between manufacturers in the nutrient levels for diets with a similar purpose (i.e. breeding and maintenance diets). In some cases a maintenance diet from one manufacturer can have higher or similar nutrient levels to those in a breeding diet from another. It is important to compare suitability of diets based on nutrient levels rather than name.

Researchers often assume that the nutrient levels in the manufacturers' data sheets will be accurately reflected in a particular batch of diet. Manufacturers' data sheets should be treated as a guide to nutrient levels and usually reflect the values calculated from typical nutrient levels in the ingredients, either from the manufacturers' own ingredient data or published tables. Only by carrying out analytical measurements can the values be stated with certainty. Where high nutrient accuracy is required *a priori*, that is best achieved by the use of purified diets [33]. The deviation between actual and datasheet values can be considerable and is brought about mainly by variation in nutrients in ingredients, mixing inhomogeneity, sampling errors (typical

batch sizes can be as great as 20 tonnes), and analytical errors. Even legislation on nutrient declarations and specifications [27] can encourage deliberate under-reporting of average for crude protein and over-reporting of crude fibre to minimize the risk of deviating from specifications (the data are normally distributed but the specifications may not be).

Inevitably between-batch variation can increase variation within a study and certainly between studies: any increase in the standard deviations increases the number of animals required to find statistically significant findings [34]. This runs counter to the objectives of Russell and Burch's principle of the 3Rs (reduction, refinement and replacement) in which one of the goals is to limit the number of animals used. From the point of view of standardization, it is advisable to use purified diets in experimental studies instead of natural-ingredient diets wherever practicable. Where natural-ingredient diets are used, it is advisable to buy them with a batch-analysis certificate, so that one at least has information on the exact levels of selected nutrients and contaminants of the specific batch use; the user should at least measure the nutrients of concern for the experiment.

Purified diets

Purified or semi-purified diets (also called synthetic or semi-synthetic diets) are defined as being formulated with a combination of ingredients, each of which is largely a source of a nutrient type. This results in diets having a much more standardized composition than natural-ingredient diets, consequently leading to more reproducible results, both within and between laboratories. Purified diets also allow precise and accurate changes in individual nutrients in a way not achievable with natural-ingredient diets. One common mistake in many studies is to use a natural-ingredient diet as the control and a purified diet as the 'experimental' diet. This may mean that a considerable part of the observed difference is because of the control, rather than the variable being investigated [35].

Improvements in accuracy and precision achieved with purified diets contribute to a more responsible use of laboratory animals, as the number of animals needed for reaching statistically significant results can be reduced, and experimental results between studies and laboratories can be compared more directly. Table 4.3.5 illustrates the analysed values versus the targeted levels in purified diets [14].

The American Institute of Nutrition has developed general-purpose purified diets, initially AIN-76A (the original AIN-76 was modified because it resulted in vitamin K deficiency) and, in 1993 further improvements were made, resulting in AIN-93G for growth and AIN-93M for maintenance [36–38]. It is possible that the diets can be further improved by further supplementation with vitamin B_{12} and selenium [14, 39]. Table 4.3.6 gives a typical example of a diet designed according to AIN-93 guidelines, with these modifications. The AIN-76A and AIN-93 diets are often the basis for further modifications for specific experimental purposes.

TABLE 4.3.5: Critical nutrients in five purified diets in concentrations as aimed for (in brackets) and according to actual analysis

Type of diet	Vitamin B_6 (mg/kg)	Vitamin B_{12} (μg/kg)	Folate (mg/kg)	Methionine (g/kg)
Control	8.9 (8)	6.4 (10)	0.53 (0.5)	4.3 (5)
Low folate	7.2 (8)	10 (10)	0.02[a]	4.5 (5)
Low vitamin B_6	3.7 (4)	5.3 (10)	0.86 (0.5)	4.1 (5)
Low methionine	6.8 (8)	6.4 (10)	0.8 (0.5)	13.7 (15)
High vitamins	18 (24)	31 (30)	2.3 (1.5)	4.4 (5)

[a]As low as possible.
Source: *Zhou [14]*.

TABLE 4.3.6: Composition of a purified mouse diet, according to AIN-93 guidelines

Ingredient	Amount (g)
Casein	14.0
Corn flour	71.5
Solkafloc (cellulose)	5.0
Corn oil	4.0
Choline bitartrate	0.2
L-Cystine	0.2
Vitamin and mineral mix	5.0
Methionine	0.1
Total	100.0

Source: *Reeves* et al. [38]; *Zhou* [14]; *Zhou* et al. [39].

Most of the major diet manufacturers have a technical group that can design or recommend purified diets for customers, and some companies manufacture almost entirely purified diets. In Harlan, about 900–1000 new formulas are designed each year.

The largest use of purified diets is in the study of metabolic syndrome (diabetes, obesity). Purified diets are also used to obtain low concentrations of particular nutrients that typically cannot be reached when using natural-ingredient diets, due to the relatively high concentrations naturally present in the raw materials.

Particularly with high fat inclusion, it may be impossible to pellet purified diets, though this can be influenced by the type of fat and carbohydrate: the inclusion of dextrin can often improve pelletability. The example of the diet given in Table 4.3.6 is of such a composition. When pelleting is not possible, the use of special feeding devices becomes necessary [40].

The use of purified diets in toxicology studies has been promoted from time to time [41, 42], primarily because of their ability to minimize nutrient variability within and between studies, and avoid potentially confounding non-nutrients and contaminants associated with natural-ingredient diets. AIN-93M was developed for possible use in toxicology studies. Unfortunately there are some practical difficulties in its widespread use in toxicology, including significantly increased cost, availability of sufficient New Zealand white casein, difficulty of manufacture of the large amounts needed for such studies and limited experience of their use. Furthermore, studies in the 1980s of comparisons between natural-ingredient diets and AIN-76A were disappointing [43, 44] but this may have been due in part to the high levels of sucrose and protein in the diet [45]. Later studies carried out with AIN-93M, which corrected these issues, have provided some encouragement but mortality on purified diets seems to exceed that on natural-ingredient diets, even with diet restriction [46-48].

Storage conditions

In order to ensure that the nutrient contents of the natural-ingredient diets remain within the specifications until the recommended expiry date, diets should be stored in a cool (about 18-21 °C) and dry place (less than 65% relative humidity) and free from pests. In order to avoid cross-contamination, the storage area must be dedicated to non-medicated diets. A full discussion on diet storage is given in Tobin et al. [1].

Storage of purified diets, particularly high-fat ones, poses particular problems. Fullerton et al. [49] reported that vitamin losses and rancidity could be substantially decreased by reducing storage temperature from ambient to 4 °C, and in the case of AIN-76A (which would be typical of many purified diets), expiration could be extended to 6 months. Shelf life can be further prolonged by keeping the diets in a freezer at −20 °C instead of 4 °C. Storage of AIN-93 at −20 °C for 3 months did not lead to fat oxidation as measured by the peroxide value, in contrast to storage at +5 °C (unpublished observations). When storing diet at −20 °C, addition of antioxidants is not considered necessary. This is important where antioxidants may interfere with the purpose of studies, e.g. atherosclerosis induction, and are best avoided. Working with highly unsaturated oils like fish oil needs particular care since they are very easily oxidized, altering the nature of the fatty acids and possibly decreasing vitamin E levels. When adding fish oil to diets, the best procedure is to keep the oil stored at −80 °C, and then mix the oil freshly through the purified diet each day, just before feeding [50].

Pellet hardness

Although mice are less sensitive than rats to diet hardness [51], it is still an important factor in their growth and reproduction, particularly for weaker animals such as many inbred, genetically modified and mutant strains.

Even mice that seem unaffected by diet hardness may be affected in periods where increased food intake is required. Koopman and colleagues [52, 53] showed that even robust female outbred Swiss mice were restricted in their ability to raise litters when fed a hard diet because they were unable to increase their food intake adequately to meet the increased energy demands of lactation. Subsequently, although the pups had a lower weaning weight, the offspring were able to grow at almost a normal rate despite the hardness of the diet.

There are several reviews on the measurement of pellet hardness, and the influence of process conditions and raw materials on diet hardness [1, 54-56]. The hardness measurement is influenced not only by the true hardness of the diet but also by the method of measurement and the size of the pellet, making it difficult to compare measurements carried out in different laboratories. In addition there is often considerable variation between individual pellets from a single batch of diet [53, 57], and a reliable mean value needs to be obtained from at least 10 pellets, and possibly up to 30.

Autoclaving/irradiation

Diets that are to be used behind microbiological barriers are usually steam autoclaved or gamma irradiated [58]. The most common autoclaving conditions are 121-124 °C for 15-30 minutes or 134-137 °C for 3 minutes. Autoclaving has little effect on the overall protein level, but damages individual amino acids, particularly methionine, cystine and lysine, and causes reactions between amino acids, and between amino acids and carbohydrates that reduce the availability and biological quality of the protein [59, 60]. There is also a considerable loss of some vitamins, particularly

thiamine (B_1), vitamin B_{12}, vitamin K_3, vitamin B_6 and pantothenate. Losses of vitamin A, D_3 and folic acid are modest while riboflavin, biotin, niacin and vitamin E are little affected.

Measurements of crude protein, crude fat and crude fibre, ash [61, 62], and fatty acid patterns do not appear to be measurably influenced by autoclaving. Manufacturers provide special diets with increased levels of vitamins (and sometimes amino acids such as methionine and lysine) in order to compensate for autoclaving losses.

Autoclaving also has adverse effects on the physical nature of pelleted diets, often resulting in diet clumping and hardness [1, 57]. These effects can be largely eliminated by the use of extruded diets.

For irradiation it is advisable to use 21 kGy as a minimal dose (typically equating to an average of 25 kGy) for diets to be used in SPF units, and 40 kGy for germ-free animals (average 50 kGy). Lower doses can be used when an analysis of the microbial quality prior to irradiation indicates that this will be sufficient, for example, with high fat purified diets. Irradiation is less damaging to nutrients than a comparable level of steam sterilization, for example, protein and amino acids [61-63]. Vitamin losses are typically less than 20% [1].

The main concern with irradiation of diet is the potential for the free radicals that are produced to oxidize fats and produce peroxides [64, 65]. This seems not to be a problem with standard diets but may adversely affect the intake of irradiated high-fat diets [66].

Quality control

Both BARQA [18] and GV-Solas [27] give guidance on the quality control of laboratory animal diets. Users should follow the adage 'Trust but verify', ideally by auditing the manufacturer (by visit or questionnaire). The quality of the finished product depends on the quality of the incoming materials, care in the manufacturing process (including pest control and cleaning), the quality and training of staff, and rigorous QC/QA systems, including documented operating procedures. Finally, a series of physical and analytical tests should confirm that the product is what it

purports to be and that it meets specification before it is released for use. Any storage areas for finished product should be clean, well-organized, pest free and of the desired temperature and humidity: ideally the store should be temperature controlled. It should be possible to obtain evidence of all these factors by examining retained records. Ideally independent verification of the quality system should be available, for example ISO 9000 certification. For GLP studies, the diet is usually analysed for a wide range of nutrients and contaminants before release, though the analytes will vary between different countries. Such a diet is known by various terms—certified diet, extended analysis diet, GLP diet. Apart from the analysis, there should be no difference in the quality of diet for GLP and non-GLP studies.

Ad libitum feeding versus food restriction

Ad libitum feeding means that the diet is available at all times. Restricted feeding refers to restricting the amount of food while still ensuring nutritional adequacy [67]. This implies that only the amount of energy has been restricted. It is still common practice to feed laboratory mice (and rats) *ad libitum* (less than 1% of the total number of papers on rodents mention the use of food restriction; [68]), even though this is undesirable from the point of view of animal welfare, as well as the validity of the experimental results. Keenan et al. [69] have stated that *ad libitum* feeding is currently our worst standardized experimental factor in laboratory units. In some toxicology laboratories it has become common practice to restrict feed intake of rodents to 75-80% of *ad libitum* intake, as this gives better standardization and longer survival. This implies, however, that animals are housed individually, as there are no appropriate housing and feeding systems available for the restricted feeding of group-housed rodents. As rodents are social species, the resolution of the Council of Europe [4] requires that social species need to

be group-housed whenever possible. For that reason it is necessary that appropriate feeding systems and/or methods be developed that both guarantee restricted feeding as well as social housing conditions. Until then consideration should be given—whenever possible—to feeding the animals individually, and then providing social housing as soon as the feeding period is over. For rats the use of diet boards has secured working for food and, as a consequence, lowering of body weight, even though the diet is available 24 h/day, and additionally led to a longer survival time as compared to normal *ad libitum* conditions [70].

When comparing *ad libitum* feeding versus food restriction, it becomes clear that *ad libitum* feeding has a negative impact on the health of rodents. *Ad libitum* feeding will lead to more obesity, a shorter survival time, increased degenerative kidney and heart diseases, a shorter latency time and higher incidence of cancer as compared with restricted feeding [67]. Table 4.3.7 shows a comparison between *ad libitum* and food-restricted B6C3F1 mice that served as control groups in long-term toxicity studies [67]. Food restriction gave a reduced body weight as compared with *ad libitum* feeding, and also a decreased incidence of liver tumours in both sexes, decreased lung tumour incidence in males and decreased malignant lymphomas in females. Food restriction led to an improved survival time, especially in females. A recent meta-analysis revealed that mice in control groups in the National Toxicology Program showed an 11.8% increase in body weight per decade from 1982 to 2003 in females. In male mice there was a 10.5% increase of body weight per decade [71].

A large and elaborate study has been conducted on the effects of diet composition and food consumption in rats. Significant effects of diet reduction (20% reduction) were found on longevity, degenerative disease development and neoplasia in Wistar rats [72]. Similar effects can be expected for mice.

During the 1980s and 1990s the variation in results of long-term bioassays in rodents that were on *ad libitum* feeding schemes increased [69]. Therefore the feeding of a standardized restricted amount of food to each individual in this type of experiment is considered a necessity in order to obtain standardized results without

TABLE 4.3.7: Some results of control groups of B6C3F1 mice on *ad libitum* or food restriction schedules				
Tumour type and survival	Males		Females	
	AL	FR	AL	FR
Liver tumours (%)	54	22**	45	8**
Lung tumours (%)	28	17*	2	12
Malignant lymphoma (%)	9	8	16	4*
Haemangio(sarco)ma (%)	5	2	8	2
Survival (%)	80	88	65	94**

AL, *ad libitum*; FR, food restriction.
*(p < 0.05),
**(p < 0.01) significantly different from AL-fed controls.
Source: *Hart* et al. [67].

unnecessarily large variations. This will also increase the reliability of the interpretation of toxicity of test substances, as the amount of the test substance ingested through the diet becomes standardized for each individual. The standardization of food intake will also contribute to a lower variation in experimental results, and thus provide Reduction potential [71]. However, a recent study in rats into the Reduction potential of food restriction as compared to *ad libitum* feeding revealed that the resulting variation depended on the parameter measured [73]. For body weights the Reduction effect was clear and significant, but the type of feeding had no consistent effect on the variation of several standard blood parameters measured. Besides a better standardization, another advantage of food restriction is that animals become 'more robust', i.e. they are better in coping with stress factors [69]. That way they can be exposed to test substances in higher concentrations and/or for longer periods without compromising the animal's health, physiology or metabolic profile [67]. As food restriction to 75% of *ad libitum* intake improves longevity (as seen above), animals can be exposed to test compounds for a longer period, thereby improving the sensitivity of bioassays to detect compound-specific chronic toxicity and carcinogenicity [67].

The level of nutrients in natural-ingredient diets is usually far above the levels needed for fulfilling the nutrient requirements [5]. Manufacturers do this in order to guarantee that no deficiencies will occur, even after longer storage periods. Moreover, the NRC requirements support maximum growth. Therefore limiting food intake to 75% of *ad libitum* intake will still make sure that the diet lives up to the minimum levels of essential nutrients.

Pair feeding

Where a carcinogenic test substance is mixed through the diet and has a bad taste, this will reduce food intake, particularly when feeding is *ad libitum*. The control group will ingest a higher amount of food than the test group, thereby developing cancer at a higher frequency and at an earlier age. The outcome could thus be false-negative, i.e. that one does not judge the carcinogenic test substance to be carcinogenic. In order to avoid these problems, pair-feeding is necessary. How much food the test animals ingest is measured, and then the same amount of food is given to the control group/animals the next day. This assumes that the food intake of each individual is measured [2, 40].

Normal feeding behaviour versus food restriction

Rodents are nocturnally active animals and ingest most of their food during the dark period. Food restriction (60% of *ad libitum* intake) as compared with *ad libitum* feeding will affect physiological variables, circadian rhythms, activity and feeding behaviour [67]. Table 4.3.8 illustrates the effects of *ad libitum* feeding and food restriction during the dark or the light period, on various physiological and behavioural variables in 28 month old male B6C3F1 mice [67, 74]. Total food and caloric consumption was decreased in food-restricted groups, as expected. Water consumption remained nearly the same in the restricted group fed during the dark period as compared with the *ad libitum* fed B6C3F1 mice, so that total water consumption increased in relation to food intake (Table 4.3.8). However, when fed restrictedly during the light period, water

TABLE 4.3.8: Effects of diet and feeding time on physiological and behavioural variables in old male B6C3F1 mice (aged 28 months)

Measurement	AL group Mean ± SE	LF restricted group Mean ± SE	% of AL	DF restricted group Mean ± SE	% of AL	Significance levels		
						AL vs LF	AL vs DF	LF vs DF
Total food consumption (g)	5.21 ± 0.10	3.35 ± 0.02	64.3	3.41 ± 0.08	65.5	A***	B***	_****
Caloric consumption (kcal/g)	22.66 ± 0.43	14.57 ± 0.09	64.3	14.83 ± 0.35	65.5	A***	B***	_****
Total water consumption (g)	3.64 ± 0.38	5.15 ± 1.07	141.5	3.72 ± 0.07	102.2	A*	_****	C*
Number of feeding episodes	16.51 ± 0.69	2.80 ± 0.23	17.0	4.32 ± 0.37	25.6	A***	B***	C*
Number of drinking episodes	11.13 ± 1.28	9.03 ± 0.44	81.1	8.99 ± 0.67	80.8	_***	_***	_***
Average body temperature (°C)	36.78 ± 0.08	35.54 ± 0.15	96.6	35.11 ± 0.18	95.5	A***	B***	_***
Max−min body temperature (range; °C)	37.98−35.81 (2.18)	38.15−32.24 (5.91)	271.1	37.52−32.93 (4.59)	210.6	A***	B***	_***
Average activity (pulse/h)	10.54 ± 2.30	18.26 ± 1.71	173.2	26.50 ± 5.57	251.4	A*	B**	_***
Average O_2 consumption (g/LBM) (ml g^{-1} h^{-1})	3.34 ± 0.16	3.44 ± 0.16	103.0	3.19 ± 0.06	95.5	_***	_***	_***
Max−min respiratory quotient (range)	0.95−0.86 (0.09)	0.99−0.80 (0.19)	211.0	1.01−0.77 (0.24)	267.0	A***	B***	_***

AL, *ad libitum*; LF, restricted group fed during light period; DF, restricted group fed during dark period; LBM, lean body mass.
Results of Student's t-test analysis:
A= AL × LF restricted comparison (significant effect)
B= AL × DF restricted comparison (significant effect)
C= LF restricted × DF restricted comparison (significant effect; adapted from Duffy et al., 1990).
*p < 0.05,
**p < 0.01,
***p < 0.001,
****p > 0.05.
Source: *Duffy* et al. [74]; *Hart* et al. [67].

intake increased to a level of about 141% that of the *ad libitum* fed mice. This may be related to the fact that the mice do not have food available during their normal active period in which they ingest food, thereby possibly using drinking water as a substitute. The duration of food consumption was compressed by restriction, so that most food was consumed during the first few hours immediately after feeding commenced. Restricted mice ate fewer meals (feeding episodes) but spent more time feeding per meal and consumed more food per meal than the *ad libitum* fed mice. The number of drinking episodes was also decreased by food restriction. Average body temperature decreased, whereas the range in body temperature increased by dietary restriction. Spontaneous activity was increased by food restriction. Average oxygen consumption increased in the restricted group fed during the light period and decreased in the restricted group fed during the dark period. This may be explained by the fact that, in the first group, the mice were actively searching for food during the entire dark period, which is their normal feeding time. The daily variations in the respiratory coefficient (RQ) were increased by dietary restriction, indicating rapid substrate-dependent shifts in metabolic pathways from carbohydrate metabolism (immediately after feeding) to fatty acid metabolism (several hours before feeding). This may be related to an enhanced metabolic efficiency [67].

Because mice are nocturnally active animals, it is advisable that when animals are fed restrictedly, food is provided during the normal feeding time, i.e. the dark period, in order to fulfil the animals' basic needs. The relationship between circadian rhythms and nutrition is beyond the scope of this chapter, but further information can be found in Ritskes-Hoitinga and Strubbe [75].

Individual housing versus group housing

After the 1970s, in mouse studies performed in the National Toxicology Program, body weights showed a tendency to increase [67, 76, 77]. This may be related to the fact that the protocol changed from group housing to individual housing. Individually housed animals had higher body weights and higher tumour incidence than group-housed mice [77]. The variance in body weight in group-housed animals (two, four or eight per cage) was smaller than in individually housed mice [78]. According to the National Toxicology Program, female mice are now once again housed in groups, but male mice are still housed individually to avoid the problem of fighting among group-housed males [67, 77].

Different types of studies based on the route of administration (e.g. inhalation, feed, corn oil gavage, etc.) are registered in the National Toxicology Program database [67]. Each of these types of study treats control animals differently, resulting in different results, for example in average tumour incidence in mice (Table 4.3.9; [67]). In inhalation studies a clear relationship is seen between body weight biomarkers and tumour incidence. Mice are almost always singly housed in inhalation chambers, so that any dietary modulation of body weight and spontaneous tumourigenesis is not confounded by social interactions among the animals. Some feed and corn oil gavage studies also use single housing for males, in order to prevent fighting. When compared with similar studies that used group housing, group housing increased the percentage of animals surviving and decreased the incidence of lymphomas (Table 4.3.10; [67]). The clear relationship between body weight and survival and (liver) tumour incidences is found in singly housed animals only; in group housing this relationship is confounded [67].

Isocaloric exchange

When designing experiments in which the dietary fat content is intended to be altered in the test groups, it is important to consider the basic facts in order to reach properly controlled and standardized diet compositions. The MEC of fat (37 MJ/kg) is about twice as high as that of carbohydrates or protein (17 MJ/kg). So when the dietary fat content is increased, the dietary energy concentration will increase. In case of

TABLE 4.3.9: Average body weight at 12 months on test (BW12) and tumour incidences for different study types for B6C3F1 mice								
Type of test	i		c		g		f	
	M	F	M	F	M	F	M	F
BW12 (g)	40.1	34.6	44.0	36.2	43.1	38.0	40.9	38.1
Liver tumour incidence (%)	39.6	18.6	41.4	12.2	38.8	10.6	33.2	15.5
Lymphoma incidence (%)	8.7	21.1	16.7	39.1	15.6	43.6	10.0	37.2
Pituitary tumour incidence (%)	0.5	23.2	1.1	33.3	2.6	23.6	0.3	20.0

i, inhalation studies (n = 8); c, corn oil gavage studies (n = 21); g, water gavage studies (n = 5); f, feed studies (n = 17); M, males; F, females. Tumour incidences were total incidences after 24 months on test.
Source: Hart et al. [67].

TABLE 4.3.10: Average survival and lymphoma incidence in single and group-housed male B6C3F1 mice		
Type of housing	Single-housed	Group-housed
Survival (%)	65 ± 9	84 ± 10
Lymphoma incidence (%)	15 ± 5	7 ± 7

Single-housed combines results of 9 studies, group-housed 12 studies.
Source: Hart et al. [67].

ad libitum food intake, the animals ingest according to energy need, so the food intake in grams will decrease. When an exchange with carbohydrates, for example, is done on the basis of weight, test and control groups will ingest different amounts of all nutrients (Table 4.3.11; [2]). In order to reach full standardization, the exchange of fats and carbohydrates needs to be done on the basis of calories (isocaloric exchange; see Table 4.3.9). In this way, the intake in grams of all nutrients will be similar in control and test groups; only the intake in fats and carbohydrates will be different as this is inherent to the design of the diets.

When the intention is to examine the influence of certain dietary fatty acids, it is advisable to keep the total fat content similar and vary the types of fats used, in order to obtain variable concentrations of fatty acids only. An example of how to design two different dietary fat levels with varying fatty acid concentrations within each fat level is illustrated by Ritskes-Hoitinga et al. [79].

Mouse models in nutrition research

Animal models can contribute to the understanding of (parts of) human processes. Russell and Burch [80], in their famous book *The Principles of Humane Experimental Technique*, distinguished between two types of animal models: the High Fidelity and the Discrimination models. In the High Fidelity model all characteristics resemble those in humans, whereas in a Discrimination model only one characteristic is reproduced. Researchers need to be aware of which type of model they are using, in order to make reliable comparisons with the human situation [81]. A part of what is studied is independent of the interference of scientists, but another part is influenced by the choices scientists make [82]. The choices that are made regarding the model, the design and environmental conditions are of major importance for the outcome of the study [83]. Results of animal studies should never be extrapolated directly to the human situation, but need discussion and critical evaluation. Literature/systematic reviews of other animal studies, epidemiological studies and clinical trials must be part of the entire evaluation.

Influence of dietary linoleic acid on mammary cancer development

In a study by De Wille et al. [84] the influence of dietary linoleic acid concentration on mammary

TABLE 4.3.11: Isocaloric exchange

	Diet 1 Low-fat	Diet 2 High-fat	Diet 3 High-fat, adjusted	Diet 4 High-fat, adjusted
DIET INGREDIENT				
Protein (g)	20	20	20	20
Carbohydrate (g)	60	40	15	15
Fat (g)	10	30	30	30
Fibre (g)	4	4	4	4
Mineral mix (g)	4	4	4	4
Vitamin mix (g)	1	1	1	1
Test compound (g)	1	1	1	1
'Inert'compound (g)	—	—	—	25
TOTAL (g)	100	100	75	100
Energy value (kcal/g)	4.10	5.10	5.47	4.10
EXPECTED INTAKE				
Energy (kcal/day)	82	82	82	82
Food (g/day)	20	16	15	20
Protein (g/day)	4	3.2	4	4
Carbohydrate (g/day)	12	6.4	3	3
Fat (g/day)	2	4.8	6	6
Fibre (g)	0.8	0.64	0.8	0.8
Mineral mix (g/day)	0.8	0.64	0.8	0.8
Vitamin mix (g/day)	0.2	0.16	0.2	0.2
Test compound (g/day)	0.2	0.16	0.2	0.2
'Inert'compound (g/day)	—	—	—	5

Source: *Beynen and Coates [2].*

tumour development in transgenic mice (MMTV/v-Ha-*ras)* was studied. Three levels of dietary linoleic acid were given, 0%, 1.2% and 6.7%. There was a significant reduction of mammary tumour development on the 0% dietary level of linoleic acid, as compared with the other two dietary groups (Figure 4.3.3). As linoleic acid is an essential fatty acid necessary for the development of cell membranes [5], this cannot be considered a reliable control group. Where the diet contains no linoleic acid at all, general health is expected to be compromised. In the study by De Wille et al. [84] it was reported that there were initially 25 animals in the 0% level linoleic acid group; however, in the results section only data from 15 animals were presented. What happened to the missing 10 animals was not
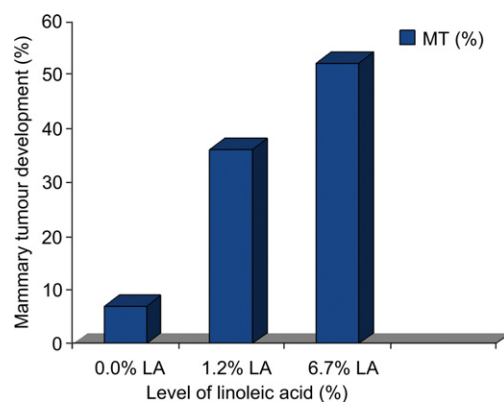
Figure 4.3.3 Dietary linoleic acid level (LA) and mammary tumour frequency (MT) [84].

described. It is essential to mention all details in scientific reports and publications, in order to fully understand the course of events and to be able to reproduce studies. The control group in the study by De Wille et al. [84] should have contained the minimum necessary level of linoleic acid in the diet (0.05%; 5), in order to obtain control animals in good health and reliable results. By leaving out dietary linoleic acid completely, one would not expect that cell membranes or tumours could develop.

In a literature review by Ritskes-Hoitinga et al. [83] the results of studies examining the influence of dietary linoleic acid in different rodent models were compared. The results were dependent on the type of animal model used. In Table 4.3.12 the results from the mouse models are given. When using dimethyl-benz(*a*)anthracene (DMBA) to induce mammary tumours, higher dietary linoleic acid concentrations were associated with increased mammary tumour incidences [85, 86]. In spontaneous [87] and the BALB/c-MMTV

TABLE 4.3.12: Mammary tumour incidence in different mouse models					
Reference	**Strain**	**Type of model**	**Dietary fat (%)**	**Dietary linoleic acid level (%)**	**Mammary tumour incidence (%)**
Fischer [85]	Sencar mouse	DMBA induction	15	0.8	23
			15	4.5	43
			15	8.4	50
Craig-Schmidt [86]	BALB/c mouse	DMBA induction	20	1.4	36
			20	1.5	45
			20	11.6	77
Brown [87]	C3H mouse	Spontaneous	5	0.5	13
			5	0.9	3
			5	1.1	2
			5	2.9	3
			5	3.0	7
			17	1.6	8
			17	3.2	15
			17	3.6	12
			17	9.7	8
			17	10.1	5
De Wille [47]	MMTV/v-Ha-*ras* mouse	MMTV	0	0.0	7
			2	1.2	36
			11	6.7	52
Ritskes-Hoitinga [45]	BALB/c-MTV	MMTV	7	0.9	40
			7	1.3	30
			7	2.6	44
			7	4.3	32
			16	0.9	52
			16	1.3	30
			16	2.6	52
			16	4.3	38

DMBA, dimethylbenz(*a*)anthracene; MMTV, mouse mammary tumour virus.
Source: *Ritskes-Hoitinga* et al. *[79]*.

mouse models [79], no clear association was detected between dietary linoleic acid concentrations and mammary tumour development. As mentioned above, the study by De Wille et al. [84] showed a higher mammary tumour incidence in the higher dietary linoleic acid groups. This may have been an artefactual finding, as the control group lacked linoleic acid completely. Depending on the model, the latency period differed [83]. It may be that the amount of linoleic acid needed for tumour development is dependent on the latency period: by using DMBA, rapid tumour development is induced, which may be associated with a high linoleic acid 'requirement' for tumour growth. This illustrates that the choice of animal models and set of experimental conditions, as well as the interpretation of results, needs careful consideration.

Atherosclerosis-inducing diets

Mice are historically resistant to the development of atherosclerosis [88]. On a normal chow diet (about 4.5% fat and 0.02% cholesterol) most of the plasma cholesterol is in the form of high density lipid (HDL), the anti-atherogenic fraction of cholesterol [88]. High fat diets will result in the development of atherosclerotic lesions over time in susceptible strains such as C57BL/6. After 7 weeks on the high fat diet, these mice develop fatty streaks and progress to more complicated lesions by 14 weeks. Other strains, such as the BALB/c and C3H strains never develop atherosclerotic lesions [88]. Special diets used to induce atherosclerosis are a 'Western-type' diet, which contains about 21% fat and 0.15% cholesterol, and an 'atherogenic' diet, which contains 15% fat, 1.25% cholesterol and 0.5% cholic acid [88]. The latter is also referred to as the 'Paigen's diet.' Historically, this diet was used to induce gallstones. It is known to be hepatotoxic and to induce a proinflammatory state [88]. When using the Paigen's diet, atherosclerotic plaques can be induced; however, hepatotoxicity and gallstones are induced simultaneously. Hepatotoxicity may interfere with the development of atherosclerotic lesions, as was seen in rabbits [50]. It is therefore considered necessary to at least evaluate the condition of the liver, and publish this, when atherosclerosis studies are performed. As gallstones are painful in humans, it may be expected to be the same for mice as well.

The use of transgenic mouse models instead of wild-type mice models may be a good alternative solution, as it then becomes possible to omit the cholate from the diet. On a normal chow diet, apoE-deficient mice develop plasma cholesterol levels that are at least 10 times as high as in wild-type mice and most of the cholesterol is in the highly atherogenic VLDL form [88]. apoE-deficient mice develop atherosclerotic lesions on normal chow and on a Western-type diet, but lesions develop more rapidly and at an earlier age on the Western-type diet. The atherosclerotic lesions in apoE-deficient mice have strikingly similar pathological characteristics and anatomical distributions to those of humans [88]. The low density lipid (LDL) receptor-deficient mouse does not develop atherosclerosis on a normal chow diet, but this can be induced by feeding a high-fat diet. Lesion characteristics are the same as in the apoE-deficient mouse, but lesion formation is more controllable by dietary changes [88]. Plasma cholesterol levels are lower than in the apoE-deficient mice and are thus more human-like.

If an atherosclerosis-inducing test diet is used next to a control diet, care must be taken that a proper isocaloric exchange is made between the control and test diet. With isocalorically exchanged diets, only the fat and carbohydrate intake (in grams) will be different between the two groups. The intake of all other nutrients will be similar, thereby allowing a more reliable interpretation of results due to optimal standardization.

Diabetes and obesity models

Diabetes mellitus, often simply referred to as diabetes, is a group of metabolic diseases characterized by high blood glucose levels, either because the body does not produce enough insulin in the beta cells of the islets of Langerhans in the pancreas, or because cells in all parts of the body, except the brain, do not respond in a normal way to the insulin that is produced. This normally means that the cell membrane becomes more permeable to glucose and muscle and liver cells increase conversion of glucose to glycogen. These high blood glucose levels produce the classical symptoms of polyuria, polydipsia and polyphagia.

There are three main types of diabetes: type 1 diabetes, type 2 diabetes and gestational diabetes. Type 1 diabetes results from the body's failure to produce insulin, and at present requires the person to inject insulin. It is therefore referred to as insulin-dependent diabetes mellitus (IDDM) or juvenile diabetes. Type 2 diabetes results from insulin resistance, a condition in which the cells fail to respond to insulin properly, sometimes combined with an absolute insulin deficiency and thus formerly referred to as non-insulin-dependent diabetes mellitus (NIDDM) or adult-onset diabetes. Gestational diabetes is seen in pregnant women, who have never had diabetes before, as a high blood glucose level during pregnancy; it may precede development of type 2 diabetes.

Other forms of diabetes mellitus include congenital diabetes, which is due to genetic defects of insulin secretion, cystic fibrosis-related diabetes, steroid diabetes induced by high doses of glucocorticoids, and several forms of monogenic diabetes.

Both type 1 and 2 diabetes are chronic diseases that usually cannot be cured and need lifelong treatment with insulin or insulin analogues, although type 2 diabetes at an early stage can be controlled by rigorous changes in lifestyle, e.g. increased exercise and weight loss. Pancreas transplants have been tried with limited success in type 1 diabetes and gastric bypass surgery has successfully cured type 2 diabetes, but require a complete change in lifestyle and might have still unknown long-term side effects. Gestational diabetes usually resolves after delivery.

An estimated 285 million people, about 6.4% of the world's adult population, were living with diabetes in 2010. The number is expected to grow to 438 million by 2030, about 7.8% of the adult population. Diabetes is one of the major causes of premature illness and death worldwide. Type 2 diabetes is responsible for 85–95% of all diabetes in high-income countries and may account for an even higher percentage in low- and middle-income countries. Non-communicable diseases, including diabetes, account for 60% of all deaths worldwide. Almost 80% of type 2 diabetes is preventable by changing diet, increasing physical activity and improving the living environment. Yet, without effective prevention and control programmes, the incidence of diabetes is likely to continue rising globally.

Insulin is vital for the survival of people with type 1 diabetes and often ultimately required by people with type 2 diabetes. Even though its indispensible nature is recognized by its inclusion in the WHO's Essential Medicines List, uninterrupted supplies of insulin are still not available in many parts of the developing world.

Diabetes without adequate treatment can cause many complications. Acute complications are due to either low blood glucose levels causing hypoglycaemia or high blood glucose levels causing hyperglycaemia or diabetes ketoacidosis. Chronic and severe late complications include high blood pressure and heart problems leading to heart attacks and heart failure, difficulty in vision and eye problems leading to blindness, kidney problems leading to kidney failure, nerve damage primarily leading to problems of the feet, but also problems like diarrhoea, constipation, nausea and vomiting.

Adequate research into the pathogenesis, prevention and treatment, and hopefully cure, of diabetes is important and hence the knowledge and use of appropriate diabetes and obesity animal models is extremely important for future research into diabetes [88a, 88b].

The non-obese diabetic (NOD) mouse

The non-obese diabetic (NOD) mouse is a well known and important model of type 1 diabetes and has become the model of choice. The NOD mouse was originally developed by Makino and colleagues [89, 90] in Japan during the selection of a cataract-prone strain derived from the outbred Jcl:ICR line of mice. During the selection of this cataract-prone strain, the NOD strain was established, through repetitive brother–sister mating, as a subline that spontaneously developed diabetes. The incidence of spontaneous diabetes is very gender dependent, as being 60–80% in females and only 20–30% in males [90, 91], but also dependent on various environmental factors. The highest incidence occurs when mice are housed in a relatively germ-free environment rather than more conventional 'dirty' housing environments [92], whereas housing under environmentally enriched conditions versus non-enriched condition did not show any difference in diabetes onset or incidence [93]. The diabetes onset typically occurs at 12–14 weeks of age in female mice and slightly later in male mice.

The histological changes due to the autoimmune process in the pancreas with infiltration of immune cells can be noted from the age of 3-4 weeks, when both male and female mice begin to demonstrate mononuclear infiltrates surrounding the islet and later invade the islet over the next few weeks, finally giving rise to severe insulitis by the age of 10 weeks [94]. Studies have shown that T cells are intimately involved in the pathogenesis of diabetes in NOD mice with CD4+ T cells being very much involved in the pathogenesis of disease and directly mediating islet cell destruction. However, the CD8+ T cells promote the disease as well, and it has been suggested that the CD8+ T cells might be causing a sufficient islet cell destruction to prime the more robust CD4+ T cell response [95]. However, the genetically linked autoimmunity in the NOD mice is a complex process that likely results from the summation of multiple defective tolerance mechanisms, and which shares many similarities to human type 1 diabetes. On the other hand, intrinsic differences in the biology of the respective species such as the pathophysiological changes associated with diabetes in the mouse may not be identical to those in humans. For example, some of the secondary pathological changes associated with the morbidity of chronic type 1 diabetes in humans, such as kidney lesions, neuropathies, and retinopathy, have not yet been reported in chronically diabetic NOD mice [96]. Still the NOD mouse has helped investigators in recent development of a number of potential therapies that may alter the development of the disease and its progression in patients with type 1 diabetes.

The obese diabetic mouse (Lep^{ob}/Lep^{ob} or ob/ob)

The obese diabetic mouse (ob/ob) is an excellent model for the study of obesity and diabetes as it has a longer lifespan and less severe clinical symptoms than the diabetic mouse (db/db). The obese spontaneous model was originally developed by the Jackson Laboratory in 1949 by a spontaneous mutation with only homozygotes being obese, reaching a maximum body weight of 60-70 g at an age of 7-8 months, and heterozygotes and wild types being lean, reaching their normal weight of 30-40 g at the age of 3-4 months. Infertility due to atrophic ovaries and uterus is

characteristic for the ob/ob females [97], whereas the ob/ob males will occasionally breed.

The obese diabetic (ob/ob) mice are characterized by marked obesity due to a greatly increased food intake 2-3 weeks after weaning. The hyperphagia is accompanied by an increased efficiency of energy utilization with the rate of lipogenesis in the liver and the adipose tissue being more than doubled and both intraperitoneal and subcutaneous deposit of fat being increased [98]. Further, a transient hyperglycaemia and markedly elevated plasma insulin concentration associated with an increase in number and size of the beta cells of the islets of Langerhans is seen [99]. The secretion of glucagon is also elevated and this hyperfunction of the alpha cells in the pancreas of the obese mice might be involved in the pathogenesis of the obese-hyperglycaemic syndrome [100].

The diabetic mouse (Lepr^{db}/Lepr^{db} or db/db)

The diabetic mouse (db/db) develops severe and potentially fatal diabetes due to a mutational inactivation of the leptin receptor gene [101, 102]. The mouse strain was initially developed by Hummel et al. in 1959 by discovering a (Lepr^{db}) mutation in mice with a C57BLKS background. The phenotype of the homozygous mice includes obesity, insulin resistance and diabetes, with secondary derangement of renal function and morphological changes. The pathogenesis of the renal failure may be due to renal sensitivity to insulin in the hyperinsulinaemic state with early signs of proteinuria before evidence of renal lesions, which include extracellular matrix expansion.

Mice homozygous for the diabetes spontaneous mutation (Lepr^{db}) are sterile and become obese at approximately 3-4 weeks of age. Elevations of plasma insulin begin at 10-14 days and elevations of blood sugar at 4-8 weeks. Homozygous mutant mice are polyphagic, polydipsic and polyuric. The severity of disease on this genetic background leads to an uncontrolled rise in blood sugar, severe depletion of the insulin-producing beta cells of the pancreatic islets, and death by 10 months of age. Exogenous insulin fails to control blood glucose levels and gluconeogenic enzyme activity increases. Peripheral neuropathy and myocardial disease are seen in C57BLKS-Lepr^{db} homozygotes as well as

delayed wound healing and increased metabolic efficiency. Mice heterozygous for the diabetes spontaneous mutation (*Lepr^db*) have a normal body weight, blood glucose and plasma insulin levels, but increased metabolic efficiency; they will survive longer fasting periods than controls.

Despite the severe and fatal development of diabetes, this model is widely used in diabetes and obesity research, but must be closely monitored with well-established humane end-points because of the severe changes in phenotype.

Fasting

Fasting is often used in pharmacokinetic and toxicological studies and in metabolic tests of glucose homeostasis.

For metabolic studies, overnight fast (14-18 h) or morning fast (5-6 h) procedures are typically used [103]. Since blood glucose varies with food intake, the mice are fasted in order to obtain a basal blood glucose value [104]. Mice are nocturnal animals and consume about two-thirds of their total food intake during the night [105-107]. Because mice are nocturnal and have a higher metabolic rate than humans [108], overnight fasting cannot be considered to cause similar levels of physiological distress in mice and in humans.

Several changes in physiological and biochemical processes are seen with fasting, as the mice readily enter a catabolic state, due to their metabolic rate (Table 4.3.13). Due to the numerous physiological changes caused by fasting, it can have confounding effects if the appropriate length of fasting is not considered, thereby influencing the scientific results.

The fasting of mice for 18 h causes a 14-17% weight loss [105, 134, 135]. Further, fasting can provoke mice to enter torpor, a state where the metabolic rate can be lowered to around 30% of the basal metabolic rate and the body temperature can decrease by up to 15 °C [108, 137]. The thermoneutral ambient temperature for mice is approximately 30 °C [138], hence housing mice at about 23 °C will increase the metabolic stress caused by fasting. Fasting causes significantly increased corticosterone levels [139-141], increasing gradually with increasing duration of the fasting period [140].

In general, fasting induces change from anabolic to catabolic metabolism. The glycogen in the liver provides an energy reserve that can be utilized immediately to maintain glucose homeostasis. The liver glycogen content is significantly lowered after 18 h of fasting compared to 5 h of fasting [105]. As the liver glycogen reserve is depleted, the breakdown of adipose tissue is initiated, providing triglycerides utilized for production of ketone bodies such as β-hydroxybutyrate. Plasma β-hydroxybutyrate is significantly increased in mice fasted for 14 h [142]. Hepatic steatosis can be seen after 16 h of fasting [143], but strain differences exist regarding hepatic triglyceride levels during fasting [143].

Haemoglobin A1c (HbA1c) is a long term measure of the daily blood glucose average. It has been shown in three strains of mice that the glucose level measured after 6 h of fasting correlates more closely with HbA1c than the glucose level measured after an overnight fast, although both values did correlate [104]. A morning fast of 5-6 h is preferred to an overnight fast in order to reduce physiological distress and thereby increase animal welfare and yet result in a valid blood glucose measurement [144, 145].

Different responses to fasting have been reported between male and female mice [140] and between different strains [146], but overall, fasting for more than 5-6 h will result in physiological changes that should be considered for confounding effects and negative animal welfare impact in all studies [147], and should be used only if considered imperative [78].

When reporting experiments that include fasting procedures, it is important to describe the duration of the fast and the time of initiation of the fast in relation to the light-dark cycle.

Welfare considerations and enrichment

The use of genetically modified mice has increased dramatically. When using these models, the possible health and welfare problems of the specific models must be evaluated in order

TABLE 4.3.13: Effects of fasting

References	Strain	Duration of fasting (h)	Parameters measured	Direction of effect
105, 109	C57BL/6J, CF-1	5–18, 28	Adipose tissue	↓
104, 110–113, 131, 139,	C57BL, FVB/N, C57BL/6J, SV129, 3 strains, A-ZIP/F, SV129/OlaHsd; C57BL/6J	48, 24, 48–72, 24, 6–14, 24, 9–24	Blood-β-hydroxybutyrate	↑
114, 130	C57BL/6J, NMRI	2–16, 24	Blood alanine transaminase	↔
114, 130	C57BL/6J, NMRI	2–16, 24	Blood aspartate transaminase	↔
130, 131, 139	C57BL, C57BL/6J, FVB/N	48, 2–16, 24	Blood corticosterone	↑
110–113	C57BL/6J, SV129, 3 strains, A-ZIP/F, SV129/OlaHsd; C57BL/6J	48–72, 24, 6–14, 24, 9–24	Blood free fatty acids	↑
111, 112	—	—	Blood glucagon	↑
110, 112, 113, 124, 130, 139	C57BL, C57BL/6J, C57BL/6J, A-ZIP/F, SV129/OlaHsd;C57BL/6J, C3H-S	48, 2–16, 48–72, 24, 9–24, 29	Blood glucose	↓
116, 130, 132–134	C57BL/6J, 2 strains, Dbh, Aston, WT	2–16, 24, 24, 24, 24	Blood leptin	↓
114	Albino	24–120	Blood phospholipids	↓
111, 115–118, 127, 131	3 strains, albino, C57BL/6J, SV129, WT, C57BL/6, C57BL/6J	0–72, 5–46, 0–72, 24, 24, 16–48, 24–48	Body temperature	↓
130, 131, 139	C57BL, C57BL/6J, FVB/N	48, 2–16, 24	Blood testosterone	↓
131	FVB/N	24	Blood thyroid stimulating hormone	↔
130, 131, 139	C57BL, C57BL/6J, FVB/N	48, 2–16, 24	Blood thyroxine	↓
130, 131	C57BL/6J, FVB/N	2–16, 24	Blood triiodothyronine	↓
119	Albino	24–120	Blood volume	↓
112, 114, 120, 121, 122, 135, 139	C57BL, C57BL/6J, NA, A-ZIP/F, CD1, FVB, NMRI, C57BL/6J	48, 5–18, 24–72, 24, 3–18, 72, 24, 24–48	Body weight	↓
123–125, 135	CD1, C3H-S, CD1, Bantin and Kingman White mice	18, 29, 3–18, 24	Gastric content	↓
126	DS	24	Gastric emptying rate constant	↑
127	Ddy	48	Ghrelin mRNA	↑
122	C57BL/6J	4–48	Heart rate	↓
110	C57BL/6J	48–72	Heart triglyceride	↔

130	C57BL/6J	2—16	Haematocrit	↓
130	C57BL/6J	2—16	Haemoglobin	↔
114	NMRI	24	Hepatic aminopyrine N-demethylase activity	↑
114	NMRI	24	Hepatic aniline hydroxylase activity	↑
128	Swiss white mice	12	Hepatic aryl hydrocarbon hydroxylase activity	↔
110	C57BL/6J	48—72	Hepatic carnitine	↑
114, 128	NMRI, Swiss white mice	24, 12	Hepatic cytochrome P-450	↓, ↔
110, 114, 121, 131	C57BL/6J, FVB/N, C57BL/6J, NMRI	5—18, 24, 48—72, 24	Hepatic glycogen	↓
128	Swiss white mice	12	Hepatic glucuronyl transferase activity	↑
114, 128	NMRI, Swiss white mice	24, 12	Hepatic microsomal protein	↔
114	NMRI	24	Hepatic NADPH-cytochrome c-reductase activity	↔
114	NMRI	24	Hepatic sorbitol dehydrogenase	↔
110, 114, 117, 142	C57BL/6J, C57BL/6, 3 strains, NMRI	48—72, 4—16, 6—14, 24	Hepatic triglyceride	↑
135	CD1	3—18	Intestinal transit time of charcoal	↓
110	C57BL/6J	48—72	Kidney triglyceride	↔
114	NMRI	24	Liver/body weight	↔
114	NMRI	24	Liver water content	↔
114, 120	NA, NMRI	24—72, 24	Liver weight	↓
120	NA	24—72	Liver weight/100 g body weight ratio	↓
122	C57BL/6J	24—48	Mean arterial pressure	↓
130	C57BL/6J	2—16	Mean cell haemoglobin concentration	↔
130	C57BL/6J	2—16	Mean cell volume	↕
130	C57BL/6J	2—16	Mean corpuscular haemoglobin	↔
111	SV129	24	Metabolic rate	↓
131	FVB/N	24	Muscle glycogen	↓
117	C57BL/6	4—16	Muscle-specific glucose uptake	↑
117	C57BL/6	4—16	Skeletal muscle triglyceride	↔

(*Continued*)

TABLE 4.3.13: Effects of fasting—cont'd

References	Strain	Duration of fasting (h)	Parameters measured	Direction of effect
139	C57BL	48	Neuropeptide Y mRNA	↑
139	C57BL	48	Plasma adrenocorticotropic hormone	↑
130	C57BL/6J	2–16	Plasma amylase	↑
113	SV129/OlaHsd;C57BL/6J	9–24	Plasma cholesterol	↑
113, 130, 139, 144	C57BL, C57BL/6J, C57BL/6J, SV129/OlaHsd;C57BL/6J	48, 3–24, 2–16, 9–24	Plasma insulin	↓
117	C57BL/6	4–16	Plasma ketone bodies	↑
110, 111	C57BL/6J, SV129	48–72, 24	Plasma lactate	↓
110, 113, 130	C57BL/6J, C57BL/6J, SV129/olaHsd;C57BL/6J	2–16, 48–72, 9–24	Plasma triglyceride	↕
130, 131	C57BL/6J, FVB/N	2–16, 24	Plasma urea	↔
130	C57BL/6J	2–16	Platelets	↑
130	C57BL/6J	2–16	Red blood cells	↑
139	C57BL	48	Serum luteinizing hormone	↔
129	C57BL/6J	48	Serum resistin	↓
131	FVB/N	24	Testis weight	↔
117	C57BL/6	4–16	Total hepatic glucose production	↔
102, 122, 130	MIL/ha/ICR, 7 strains, C57BL/6J	24–120, NA, 24–48	Water intake	↕
130	C57BL/6J	2–16	White blood cells	↕
117	C57BL/6	4–16	Whole body insulin sensitivity index	↑

The arrows indicate whether the parameters increase, decrease, do both or stay level during fasting: ↓, decrease; ↑, increase; ↕, decrease and increase; ↔, no change.
Modified from [147].

to prevent unnecessary suffering. Mouse models of muscular dystrophy died at a very young age, as these mice were physically unable to reach for the food in the feeding trough (unpublished observations). By providing food pellets at the bottom of the cages and a longer drinking nipple, the animals could survive until a much older age without any obvious clinical problems.

After surgery the mice are sometimes unable to stretch their bodies and/or heads towards the feeding trough and drinking nipple. In this case a small feeding device with feeding pellets mixed with water must be provided on the bottom of the cage, until the animals are completely recovered.

Currently, the enrichment of housing conditions receives a lot of attention. Much attention is given to environmental factors, e.g. space, cage furniture and nesting material. As animals spend a large proportion of their active time searching for food, modifying the way we feed our laboratory animals has a large potential for enrichment. This has not yet been investigated to a large extent, and it is the challenge of the coming years to investigate and use this potential. For rabbits it was found that when feeding a restricted amount of food at a time point approaching the 'normal' feeding hours, stereotyped behaviour was registered as significantly decreased as compared with *ad libitum* or feeding restrictedly at another time point [148]. This may imply that when a restricted amount of food is fed at the 'proper time', better welfare, better health and more standardized results can be obtained [75].

Mice are social species, so if single housing is a necessity for the experimental design, welfare considerations become necessary. According to the Resolution of the Council of Europe [4] social species must be housed socially, unless there are good reasons to do otherwise. In certain cases mice may be housed individually at the time of feeding, and housed socially outside the feeding period. Another possibility is to divide the cage with bars, so that the mice can be in close contact without being able to reach each other's food. Human contact may also compensate to a certain degree for a shortage in social contact with conspecifics. During procedures, e.g. weighing the animals, individually housed animals may be temporarily housed in a group. It goes without saying that this needs effective individual identification. The animals may regard the social contact provided during procedures as a positive reward, thereby making the handling and procedures easier.

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Health Management and Monitoring

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Introduction

In animal research validity and reproducibility of the data are critically influenced by the microbiological status of the experimental animals. Only animals of good microbiological quality will give any kind of guarantee of an experiment undisturbed by health hazards. The use of such animals reduces the number of animals needed and makes an important contribution to animal welfare.

Health monitoring aims to produce animals that meet preset requirements of microbiological quality and to aid in the maintenance of this quality during experiments. The microbiological quality of laboratory animals is a direct result of colony management practices. Health monitoring by itself does not influence the microbiological quality of animals but provides an after-the-fact assessment of the adequacy of those practices and is therefore an important management tool. It is a prerequisite for microbiological standardization of laboratory animals. Health monitoring is therefore an integrated part of any quality assurance system, e.g. Good Laboratory Practice (GLP), the accreditation programme of the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC), or the International Organization for Standardization (ISO).

Significance of infectious agents

Several groups of microorganisms (viruses, bacteria, fungi and parasites) are responsible for

> **BOX 4.4.1**
> **Possible consequences of infections in laboratory mice**
>
> - Outbreak of clinical disease, eventually with lethal outcome
> - Hazardous for personnel if the agent is zoonotic
> - Morphological changes in organs
> - Changed behaviour
> - Lower growth rate
> - Reduction of lifespan
> - Reduction of breeding efficacy
> - Increase in interindividual variation
>
> - Impact on physiological parameters (immunology, haematology, enzymology, clinical chemistry)
> - Interference with studies of infectious disease
> - Modulation of oncogenesis (induction of tumours, reduction of the incidence of tumours, enhancement or suppression of chemical or viral carcinogenesis, altered growth rate of transplantable tumours)
> - Contamination of samples and tissue specimens (transplantable tumours, cells, sera, monoclonal antibodies)

infections in mice. They may affect animals (or humans) in various ways (Box 4.4.1). Some are pathogenic and may induce clinical signs with variable morbidity or mortality (see Chapter 3.2 on viral infections, Chapter 3.3 on bacterial infections and Chapter 3.4 on parasitic infections). Concomitant morphological changes in organs can confound diagnostic pathology, particularly in toxicological studies or in the phenotypic evaluation of genetically altered mice. In mice, infectious pathology is most commonly found in the respiratory tract and the digestive system. While clinical signs or lesions due to various pathogens were frequently described 2-3 decades ago, they are now less common, most likely because mice are nowadays housed under optimal and standardized conditions (housing, climate, nutrition) and because fewer pathogens which could cause lesions are still prevalent. Most infectious diseases are multifactorial, i.e. an infectious agent alone or in insufficient quantities is usually not able to elicit the disease, and support by other factors is necessary. Silent infections are often activated by experimental procedures (immunosuppression, toxic substances, tumours), environmental influences (physical, social, nutritional stress) or emergence of a second or more infectious agent(s) (interaction of microorganisms). In addition, genetic factors are important determinants of the susceptibility of mice to disease. For example, infection with ectromelia virus results in high mortality in the CBA, C3H, DBA/2 and BALB/c strains, whereas other strains such as C57BL/6 and C57BL/10 are almost resistant to clinical disease. Similarly, various microorganisms (e.g. *Corynebacterium bovis*, *Staphylococcus aureus*,

Pneumocystis murina) that usually do not cause clinical signs in immunocompetent animals can be highly pathogenic for immunodeficient animals. The outcome of infection is also dependent on specific properties of the infectious agent. There are different strains of many viruses with different organotropism, e.g. enterotropic and polytropic strains of murine hepatitis virus (MHV). This influences the disease rate and the mortality as well as the type and severity of pathological changes.

It has to be emphasized that most infections in laboratory mice are subclinical. The absence of clinical manifestations has therefore only limited diagnostic value. However, modifications of research results due to natural infections often occur in the absence of clinical disease. Such modifications may be devastating for experiments because they often remain undetected. The types of interference of an agent with experimental results may be diverse. There are numerous examples of influences of microorganisms on the physiology of the laboratory animal and hence of the interference of inapparent infections with results of animal experiments. Infected mice may show altered behaviour, suppressed body weight or reduced life expectancy, which may, for example, influence the tumour rate. Many microorganisms have the potential to induce functional suppression or activation of the immune system, or both at the same time but on different parts of the immune system. Sometimes only T cells, or B cells of specific subpopulations are influenced. Therefore, most infections are detrimental for immunological research and infectious disease

TABLE 4.4.1: Examples of interference with research: MHV	
Immunology	Virus replication in macrophages, T cells, and B cells; dysfunctions of macrophages, T cells, and B cells
	Activation of natural killer cells and induction of interferon
	Immunosuppression and immunostimulation depending on the time of infection
	Reduced *in vitro* cytokine production by spleen cells
	Permanent decrease of skin graft rejection and T cell-dependent antibody responses after recovery from infection
Microbiology	Reduced susceptibility to viral infections (Sendai virus, murine pneumonia virus)
	Enhanced resistance to *Salmonella typhimurium* infection
	Confusion about the origin of Tettnang virus isolates
Physiology	Alteration of liver enzyme levels and protein synthesis
	Changes in peripheral blood cell counts
	Increased monocyte procoagulant activity
	Decrease of the incidence of diabetes in non-obese diabetic mice
Oncology	Abnormal tumour passage intervals and tumour invasion pattern
	Rejection of human xenografts in *Foxn^{1nu}* mice
	Contamination of transplantable tumours

studies. Microorganisms, in particular viruses, present in animals can also lead to contamination of biological materials such as transplantable tumours, cells and sera. This may interfere with experiments conducted with such materials. For all these reasons, monitoring the health status of laboratory animals is of crucial importance.

As an example of interference with research, a detailed list of the potential influences of MHV, a frequently occurring mouse pathogen, is given in Table 4.4.1. More information about the considerable effects on research due to infectious agents can be found in various review articles or textbooks [1-7].

Definitions of microbiological status

Laboratory animals can be arbitrarily classified according to a number of different microbiological qualities. Unfortunately, a universal reporting terminology for clear and consistent definition of pathogen status in mouse populations does not exist. The animal's microbiological quality has traditionally been described by the terms 'gnotobiotic', 'specified pathogen-free' and 'conventional', but only the term 'gnotobiotic' is clearly defined. Additional terms such as 'specific and opportunistic pathogen-free', 'barrier-reared', 'virus-antibody-free' (VAF), 'clean conventional', 'pathogen-free' or 'murine pathogen-free' (MPF), 'optimal hygienic conditions' (OHC) and 'health-monitored' are used; however, they describe concepts rather than the microbiological quality of animals.

Gnotobiotic animals harbour a microflora and fauna that is entirely known (from the Greek words *gnotos* = known and *biota* = flora and fauna). They may be germ-free (axenic) or associated with one or more defined microorganism(s). Endogenous retroviruses, e.g. murine leukaemia viruses that occur in all mice, are also present in axenic mice. Gnotobiotic animals are derived by hysterectomy or embryo transfer, then reared and maintained in an isolator using germ-free techniques.

A frequently used term to describe the microbiological quality is 'specified pathogen-free' (SPF). This term requires explicit definition every time it is used. It means that the absence of individually listed microorganisms has been demonstrated by regular monitoring of a sufficient number of animals of appropriate age and by accepted methods. The microbiota of SPF animals is not entirely known or identical among animals. The use of the term 'SPF' alone is insufficient and useless because it does not define which agents are present or absent in a population, but only which agents have not been detected. SPF animals originate from gnotobiotic animals and subsequently lose their gnotobiotic status by contact with environmental and human microorganisms. Such animals are bred and housed under barrier conditions that prevent the introduction of unwanted microorganisms. SPF animals are morphologically and physiologically normal, well-suited for modelling the situation of a human population. Some breeders use the term 'specific and opportunistic pathogen-free' (SOPF) in order to describe the microbiological quality of animals that are also free of certain major interfering opportunistic agents such as Klebsiella species (spp.), Proteus spp., Pseudomonas aeruginosa, or Staphylococcus aureus (e.g. when breeding immunodeficient mouse lines under strict barrier conditions). Proper usage of this term also requires a list of specified agents and thus differs very little from 'SPF'.

Conventional animals have an undefined or nominally defined microbiological status that includes common pathogens. They are generally housed without special precautions to prevent entry of infectious agents. However, this does not mean that they are necessarily infected with a number of pathogens. Furthermore, it is considered less prudent to monitor for the presence of unwanted agents in conventional animals than in their rederived, barrier-maintained counterparts.

Sources of infections

Laboratory mice can become infected with unwanted microorganisms by various routes and materials. Important sources of infections are: other laboratory animals, biological materials, personnel, vermin and equipment. The risk of inadvertently introducing microorganisms into experimental units is generally higher than for breeding units, as animals and various experimental materials need to be introduced into experimental facilities. In addition, a number of personnel may have access to animals due to the requirements of the experiments.

Apart from constructive measures, an appropriate management system is essential for the prevention of infections as well as for their detection and control. It is important for the management of an animal facility to understand how microorganisms might be introduced or spread under the specific conditions given. Management of all animal facilities in an institution is best centralized. This means that all information dealing with the purchase of animals, use of experimental materials and equipment, as well as the performance of animal experiments, flows through one office, thereby reducing the opportunity for failures of communication. A centralized management can best establish comprehensive monitoring programmes to evaluate important risk factors, such as animals and/or biological materials, before they are introduced into a facility. Contamination of animals can generally happen in two ways: (i) the introduction of microorganisms coming from outside and (ii) transmission of microorganisms within a colony. Both can be influenced by the management and the housing system.

Animals

The greatest risk of contamination arises from infected animals of the same or closely related species (e.g. rats). The importance of animals as sources of infections becomes obvious from recent surveys of the prevalence of infections among laboratory mice and rats in North America and western Europe [8-13]. In the largest survey [13] so far, the most prevalent infectious agents (i.e. murine norovirus (MNV), Helicobacter spp., Pasteurella pneumotropica, trichomonads) were detected in 9-32% of mice monitored. These data, however, are not generally valid as the spectrum of agents and the infection rate may be different in laboratory animals from other parts of the world [14-17]. A much higher infection rate

must also be considered when animals, primarily genetically modified mice originating from experimental colonies, are introduced into a facility. Such animals are frequently exchanged between institutions and are more frequently infected than animals from commercial breeders.

Most animal facilities are multipurpose and must therefore house a variety of species coming from various sources. The risk of introducing pathogens via animals from external sources is relatively low when animals are available from very few sources of well-known microbiological status and when these animals have been protected from contamination during shipment. In many cases, direct transfer of such animals (without quarantine) into an experimental unit is necessary; however, spot checks should be performed from time to time to redefine the status upon arrival. While it may be acceptable to introduce such animals into experimental units, they should never be transferred into a breeding unit, especially if many different strains are comaintained. In the latter case, new animals should only be introduced via embryo transfer or hysterectomy. Most breeders implement health monitoring programmes and supply their test results, indicating that many commercial breeding colonies are free of pathogens. However, procurement from breeders is not always possible; for example, in the case of genetically modified mice. These are usually produced and bred in experimental facilities, where less attention is paid to preventive hygienic measures as compared with breeding units, and available information on their health status is often insufficient. Frequently, they are exchanged between institutions, with a high risk of introducing pathogens from a range of animal facilities. As a consequence, they are much more frequently infected than mice coming from commercial breeders. In addition, only a few animals are usually sent and at short notice so that a proper evaluation of their health status upon arrival is not always possible. It must also be emphasized that a specific risk of transmitting microorganisms may arise from immunodeficient animals. Many virus infections (e.g. MHV, Sendai virus, murine pneumonia virus) are limited in immunocompetent mice and the virus may be eliminated completely.

Immunodeficient mice, however, may shed infectious virus for longer periods of time or may be infected persistently. As a general rule, all animals coming from sources of unknown microbiological status should be regarded as infected unless their status has been defined. This is especially important when animals are introduced from other experimental colonies. These animals must be separately housed from others in quarantine areas. If possible, quarantine facilities should be physically isolated from the rest of the facility. If an isolated area is not available, this can best be achieved in isolators or, if proper handling is guaranteed, in individually ventilated cages (IVCs) or in filter-top (microisolator) cages. Here, the animals should be maintained until health monitoring has been performed to define their status. If health testing shows the absence of unwanted microorganisms, they may be transferred to the experimental facility. In cases of hazardous infection, rederivation, further separation, or other forms of risk management of animals must be considered.

Biological materials

Biological materials represent a high risk of contamination if they originate or have been propagated in animals [18-28]. In particular, tumours, viruses, or parasites that are serially passaged in animals often pick up infectious agents, and therefore a high percentage of these are contaminated. The infectious agents may survive for years or decades when contaminated samples are stored frozen or freeze-dried.

In principle, biological materials can contain the same contaminants, notably intracellular microorganisms, that are present in live animals. Most contaminations are viral, but bacteria (e.g. *Mycoplasma pulmonis*, *Pasteurella pneumotropica*) and fungi (e.g. *Encephalitozoon cuniculi*) have also been found as contaminants. The problem of viral contamination in biological materials is obvious from studies by Collins and Parker [19] and Nicklas et al. [24]. They found 70% of mouse tumours propagated *in vivo* positive for murine viruses. The most frequent contaminant was lactate dehydrogenase-elevating virus (LDV) followed by mammalian orthoreovirus-3, lymphocytic choriomeningitis virus, minute virus of

mice (MVM), and MHV. Many organisms disappear under *in vitro* conditions, so that the contamination rate of tumour cells after *in vitro* passages is lower. Pathogenic microorganisms can also be transmitted by other contaminated biological materials such as monoclonal antibodies [22] and viruses [20]. The most recent outbreaks of ectromelia in the USA resulted from the use of contaminated serum samples [25, 26, 28]. In colonies of genetically engineered mice, embryonic stem (ES) cells, sperm and embryos should be considered as potential sources of infection [29]. ES cells especially are at increased risk of infection because they require growth factors that are usually supplemented by coculture with primary mouse cells. It has been shown by Okumura et al. [30] and Kyuwa [31] that ES cells are susceptible to persistent infection with MHV and may produce virus although they appear to be functionally intact.

Mycoplasma contamination of cell lines is also of concern [27, 32]. The most frequent contaminant mycoplasmas of cell cultures are the human *Mycoplasma orale*, *M. fermentans*, *M. hominis*, the bovine *M. arginini* and the porcine species *M. hyorhinis*, indicating that the sources for *Mycoplasma* contaminations in cell culture are mainly laboratory personnel, contaminated serum or reagents, and other contaminated cell cultures. Contamination of murine ES cells may affect various cell parameters, germline transmission and postnatal development of chimeric progeny [32]. Caution is further warranted as the administration of mycoplasma-contaminated cells to immunocompromised mice may result in clinical diseases [33]. In most cases, however, mycoplasmas of non-murine origin are likely to be eliminated by macrophages when administered to mice and do not therefore cause disease.

It is recommended that biological materials be considered as contaminated and that animal experiments be performed under conditions of strict containment (isolation), unless the biological materials have been tested and found free of contamination. Monitoring has traditionally been done by the mouse antibody production (MAP) test. This test is based on the serum antibody response to microorganisms which is stimulated in pathogen- and antibody-free animals if the material injected is contaminated.

Meanwhile, polymerase chain reaction (PCR) assays have been established to replace the MAP test as the preferred method for detecting viral contaminants in biological materials [34, 35].

Personnel

Humans are unlikely to be an appropriate host where mouse pathogens can reside and replicate. However, the importance of humans as mechanical vectors should not be underestimated, and several microorganisms of human origin (e.g. *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Streptococcus agalactiae*) can cause infectious disease or research complications in mice, particularly in immunodeficient mice. Transmission from humans to animals (or vice versa) certainly cannot be avoided in barrier-maintained colonies, even by wearing gloves and surgical masks and taking other precautions. It may only be avoided by establishing strict barriers as provided, for example, by isolator maintenance. Immunodeficient mice, at least mice used for breeding or long-term experiments, which are known to have an increased sensitivity to infection with microorganisms of human origin should therefore be housed in isolators, microisolators, or IVCs, respectively.

Little published information is available on the role of humans as mechanical vectors. There is no doubt that microorganisms can be transmitted by handling [36]. Microorganisms can even be transported from pets to laboratory animals by human vectors [37]. Such examples emphasize the need for proper hygienic measures and the importance of education and positive motivation of staff. It is an important task of the management of an animal facility to ensure that personnel coming into contact with animals have no contact with animals of a lower microbiological quality.

Vermin

Vermin is another potential source of infection. Flying insects do not present a serious problem because they can easily be removed from the incoming air by air filters or insect-electrocuting devices. Crawling insects such as cockroaches are

more difficult to control. The most serious problem arises from wild rodents, which are frequently carriers of infections [38-41]. Wild as well as escaped rodents are attracted by animal diets, bedding and waste. Usually, the design of a modern animal facility in combination with proper hygienic measures are able to control vermin and to reduce their importance to a minimum.

Materials and equipment

Husbandry supplies, research equipment and other materials that have been in contact with infected animals may be contaminated and may act as potential vectors. However, many of them (cages, food, water, bedding, etc.) can easily be decontaminated by disinfection, sterilization or other hygienic procedures so that the risk of contamination is relatively low. The effect of these measures should be monitored regularly. For example, autoclaving can be monitored by recording time, temperature and pressure or by examining whether test organisms were killed by the treatment or not.

The interested reader is directed to other publications [1, 4, 6, 23, 42, 43] for more detailed information about possible routes of infection and bioexclusion practices.

Health monitoring programme

Health monitoring of laboratory animals can be defined as the science of evaluating representative sample groups from given units against a specific listing of infectious agents to define the microbiological status of the source colony. Health monitoring procedures in laboratory animal populations differ from procedures used in human medicine. Especially in populations of laboratory rodents, a single animal usually has only limited value. Health monitoring of laboratory rodents aims at detecting health problems or defining the pathogen status in a population rather than in an individual. Therefore, systematic laboratory investigations (health monitoring programmes) are necessary to

determine the colony status and, most importantly, to prevent influences on experiments. Disease monitoring differs from routine health monitoring in that abnormalities are the subject of testing. This testing is not scheduled, and tests are directed towards identifying those pathogens most likely to cause the lesion or change in established patterns of reaction to experimental protocols.

While the need for health monitoring programmes is generally accepted, there is a great diversity of opinions about their design. Each institution may select its own list of pathogens, test procedures, sampling strategy, frequency of sampling and reporting terminology, and the terms used vary greatly in precision and meaning [4, 44, 45]. An individual programme is usually tailored to the conditions it is to serve. The type of programme will differ between institutions or between different units of the same facility in its dependence on (i) research objectives, (ii) physical conditions and the layout of the animal facility, (iii) husbandry methods, (iv) sources of animals, (v) staff quantity and qualification, (vi) diagnostic laboratory support and (vii) finances. Professional guidance is necessary to shape the monitoring to meet specific institutional needs. The Federation of European Laboratory Animal Science Associations (FELASA) recommends that each facility appoints a person with sufficient understanding of the principles of health monitoring, who is responsible for devising and maintaining the health monitoring programme [46]. It should also be noted that health monitoring is not confined to laboratory reporting. In any animal facility, there should be a culture of communication between animal care staff, facility managers, veterinarians and researchers so that observed abnormalities in breeding animals and experimental data can rapidly be evaluated and acted upon.

Detailed recommendations for the health monitoring of rodent and rabbit colonies in breeding and experimental units have been published by FELASA [46]. This paper sets common standards for which agents to test for, which methods and samples to use, how many samples to test, how frequently this should be done and how test results should be reported. The FELASA recommendations are periodically reviewed and amendments are published as

necessary (www.felasa.eu). Although these recommendations are widely applied in many parts of the world, a global harmonization of health monitoring procedures would be desirable [47]. Furthermore, FELASA has issued a set of guidelines for the accreditation of health monitoring programmes and laboratory animal diagnostic laboratories [48, 49]. These guidelines aim at promoting the standardization of health monitoring of laboratory animals by increasing the significance and reliability of health reports.

Selection of agents

A difficult problem which often leads to discussions between breeders and users of laboratory animals is the selection of relevant microorganisms for which animals should be monitored. Lists of infectious agents to be monitored in routine programmes have been published by various organizations [4, 46, 50, 51] and can be used for guidance. Regular monitoring for all the agents mentioned is neither realistic nor necessary. The most important microorganisms are those that are indigenous and pose a threat to research or to the health of animals and, in addition, those which can be eliminated. Therefore, oncogenic retroviruses are excluded as they integrate into the host genome, and thus cannot be eradicated by the presently available methods. It should be self-evident that agents which have zoonotic potential and could therefore affect the health of humans should also be included. Other microorganisms may be less important as they are unlikely to occur in good quality mice due to repeated rederivation procedures (e.g. murine polyoma virus, *Eperythrozoon coccoides*, *Leptospira* spp., *Yersinia pseudotuberculosis*, *Encephalitozoon cuniculi*, *Toxoplasma gondii*). Most cestodes (with the exception of *Rodentolepis nana*) are unlikely to be found because they require an intermediate host. On the other hand, too strict adherence to existing lists may also bear the risk that monitoring does not include important agents (e.g. those recently detected, or not of general relevance). Monitoring for additional agents is advised when they are associated with disease or when there is evidence that they affect physiological parameters or breeding performance. Especially in immunocompromised mice or in infectious experiments, monitoring for a comprehensive list of microorganisms is reasonable. Various microorganisms (e.g. *Corynebacterium bovis*, *Helicobacter hepaticus*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Pneumocystis murina*) that usually do not cause disease in immunocompetent animals may cause serious problems in immunodeficient animals. It is therefore necessary to monitor immunodeficient mice not only for strong or weak pathogens, but also for opportunistic agents or commensals. In other cases, microorganisms of low clinical importance may cause disease or have a severe impact on research if animals are concomitantly infected with several agents, e.g. Sendai virus and *Pasteurella pneumotropica* [52].

Each institution should define a list of those microorganisms that are not acceptable in the facility or only in parts of it. This list is easiest to establish for viruses. A large amount of information is available on their pathogenic potential and on their ability to compromise the object of research. It is generally accepted that mouse colonies should be free from viruses, even if they are usually not pathogenic. For some viruses (e.g. mouse thymic virus, murine polyoma virus), the only question is whether or not monitoring is necessary because they have been eradicated from the vast majority of mouse colonies many years or even decades ago. Very few new mouse viruses have been detected during the last few years, e.g. MNV [53], and it has to be expected that new viruses will be isolated, although only occasionally.

Less is known about the ability of most parasites to influence research results. They are regarded as an indicator of a low hygiene level and are therefore eradicated from mouse colonies. Some apathogenic flagellates such as trichomonads are occasionally detectable. At present, no evidence exists that they have any impact on the physiology of their host. They might, however, be an indicator of a leak in the barrier system or of direct or indirect contact with wild rodents.

The most complex problems exist for bacteria. In contrast to viruses, their importance for laboratory mice is usually estimated on the basis of their ability to induce pathological changes or clinical disease since very little is known about most bacterial species with regard

to their potential to cause other effects on their hosts and on experiments. Insufficient information exists on the taxonomy and proper identification for various rodent-specific bacterial species such as *Pasteurella pneumotropica* or other members of the Pasteurellaceae (e.g. *Actinobacillus muris*, *Haemophilus influenzaemurium*). Lack of detailed information on the characteristics of these organisms, together with the presently unclear taxonomic situation, often leads to misidentification, and the lack of knowledge about species-specificity impedes their elimination. FELASA has therefore decided to recommend that rodents should be monitored for all Pasteurellaceae [46]. There is, however, evidence that some growth factor-dependent Pasteurellaceae found in rodents are closely related to *Haemophilus parainfluenzae* and might therefore be transmitted by humans [54]. It is unclear if these bacteria can be successfully eradicated from barrier units because exposure of barrier-produced animals to humans represents a permanent risk for reinfection. The same is true for several members of the Enterobacteriaceae (*Escherichia coli*, *Klebsiella* spp., *Proteus* spp.), *Pseudomonas aeruginosa*, *Staphylococcus aureus*, β-haemolytic streptococci, and *Streptococcus pneumoniae* for which humans are the reservoir. Another problem arises from the fact that many bacteria are presently being reclassified, resulting in name changes. For example, the mouse-specific organism known as '*Citrobacter freundii* 4280' has been reclassified as *Citrobacter rodentium* [55]. Whole genera have been renamed, and additional bacterial species have been detected, e.g. *Helicobacter (H.) hepaticus* [56], *H. bilis* [57], and *H. typhlonius* [58]. Some of these fastidious organisms are not detected or not properly identified by each monitoring laboratory.

The above examples show that the whole spectrum of microorganisms as a concept is not a permanent list for all time; it rather represents a moving boundary in which old pathogens are eradicated and new ones are added. In practice, such lists of agents do not differ much between research facilities or commercial breeders in different parts of the world, at least for viruses and parasites. The most important viruses, bacteria and parasites for which mice should be monitored according to FELASA [46] are listed in Appendix 4.4.1.

Sampling

Proper sampling is necessary in order to detect an infection in a given population as early as possible. In general, animals are the most crucial point in a monitoring programme. Animals coming from outside have to be checked to assess or exclude the risk of introducing unwanted microorganisms, and animals already within the unit need to be monitored to define their status and to obtain information on the presence or absence of infectious agents in the colony. In order to know the actual health status of a colony, it would be necessary to examine all the animals within the colony. Because this procedure is usually not possible, only a fraction of the animals are selected for examination and the results are used to describe the entire colony. Therefore, if one animal is infected with a certain microorganism, the entire colony is considered infected with that particular organism, and if the infection is not found in any of the animals sampled, the entire colony is considered free of that organism. For this practice, it is essential to define the microbiological entity (unit), i.e. a definition of the group of animals for which a sample is predictive, which is a very complicated matter. One isolator, one IVC (if handled properly), or a simple barrier-protected animal room used for breeding may be defined as one microbiological entity each, as the idea of the system is to prevent entry of unwanted agents and also because there is close contact between the animals within the system. In experimental facilities, however, it is often difficult to define the microbiological entity because barrier measures are less stringent and as a consequence some microorganisms may spread from one room to another. Depending on the actual measures taken and on the professional judgement of the person responsible for health monitoring, the total facility may be considered as multiple units or a single unit.

It is obvious that a sufficient number of animals has to be monitored to obtain relevant information on a given population. Guidelines for determining sample sizes have been provided by Cannon and Roe [59] and others [60–63]. In essence, these guidelines show that, as the prevalence of infection decreases, the sample size required to detect infection with

a high level of confidence increases [61]. The prevalence that a certain infection reaches depends on many factors, e.g. housing conditions and contact among the animals, susceptibility of the animals and characteristics of the agent itself. Based on a recommendation by the Institute for Laboratory Animal Resources Committee on Long-Term Holding of Laboratory Rodents [60], it has become common practice in traditional barrier units with housing in open cages to monitor at least nine randomly sampled animals per (microbiological) unit. This theoretically results in a 95% chance of detecting an infection if at least 30% of a population (with 100 or more animals) is infected. The formula used by ILAR to determine the sample size for an estimated prevalence rate is given in Table 4.4.2. This formula is applicable only in populations of at least 100 animals, if the infection is randomly distributed in the unit, and if the animals are randomly sampled. However, because the distribution of an infection may be dependent on sex and age, attention should also be given to sampling animals of both sexes and of different ages. Younger animals often

have a greater parasite burden, whereas older animals (≥ 3 months) are more suitable for detecting viral infections. Similarly, there may be strain differences in susceptibility to infection and serological response to agents. Therefore, if more than one strain is present, as many as possible or even all strains should be screened and each strain should be monitored at least once a year, where possible. Alternatively, the use of sentinel animals (see below) may be considered. It is also important to consider that the sample size needed to detect a specific agent in a colony is influenced by the sensitivity of the diagnostic method applied. For example, PCR might require a smaller sample size to detect or exclude *Helicobacter* infections than (less sensitive and more interference-prone) bacterial culture [64]. Furthermore, indirect detection of a viral infection by serological methods is usually preferable to direct detection methods because antibodies persist for long periods of time and are easier to detect than the virus itself (e.g. by culture or PCR). Independent of animals that are scheduled for monitoring, all clinically sick or dead animals

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TABLE 4.4.2: Calculation of the number of animals to be monitored

Assumptions

1. Both sexes are infected at the same rate
2. Population size >100 animals
3. Random sampling
4. Random distribution of infection

Formula

$$\frac{\log(1 - \text{desired confidence level})}{\log(1 - \text{assumed prevalence rate})} = \text{no. of animals to be monitored}$$

Number of animals required to detect an infection

Assumed prevalence rate	Sample sizes at different confidence levels		
	95%	99%	99.9%
10	29	44	66
20	14	21	31
30	9	13	20
40	6	10	14
50	5	7	10

Example

Nine animals should be monitored to be 95% confident of finding at least one positive animal if the suspected prevalence rate of an infection is >30%.

should be examined; they are a valuable source of information about the hygienic status of the colony.

Sampling strategies and calculation of sample sizes have been discussed in more detail by Selwyn and Shek [62] and Clifford [63].

Statistical considerations are not useful to determine a reasonable sample size for health monitoring when small numbers of animals are housed in hygienic units, e.g. in isolators or in laminar flow cabinets. Small or undefined hygienic units are nowadays the common situation, as large numbers of mice are housed in IVC systems. Contact between cages is dependent on the maintenance procedures but is generally less than in open cages. Under these conditions, strategies for proper sampling need to be developed for breeding and experimental populations independent from statistical considerations. To obtain reliable information on the health status for animals housed under such conditions, much higher numbers of animals need to be tested. Most institutions rely on testing of sentinel animals (see below).

Sentinels

Random sampling of sufficient numbers of animals from a hygienic unit is the ideal strategy for health monitoring. This could comprise any animal that is representative for a population, e.g. retired breeders, surplus from breeding or from experiments or vasectomized males. Ideally, animals of different strains and ages and also of both sexes should be sampled.

Random sampling for health monitoring is not a serious problem in large breeding colonies, but it may be impossible in some experimental units and colonies of genetically modified or immunodeficient mice. It may even be inappropriate to carry out health monitoring in such colonies. For example, serological testing of immunovague or immunodeficient mice may yield false-negative results because these mice may not produce sufficient amounts of antibodies. Health monitoring may then be carried out on sentinel mice, which act as surveillance substitutes. However, the use of sentinels is not covered by the formulas published by ILAR (Table 4.4.2) and others.

Sentinels are animals obtained from a breeding colony of known pathogen status that are placed together with animals of the same (or another) species to aid in evaluation of their microbiological status. They are submitted to testing after a sufficient exposure period. Sentinels are frequently introduced from outside, but it is also correct to use the term for animals that are taken from the principal population. If sentinels are not bred within the colony that is being monitored, they must be obtained from a colony that is known to be free from all agents to be monitored and free from antibodies to these agents. This is especially important when sentinels are used for health surveillance of immunodeficient animals. Immunocompetent sentinels may be colonized with agents that are acceptable and even common in immunocompetent animals and therefore not declared in a health report but may cause clinical problems or even death in immunodeficient mice. Sentinel animals must be housed for a sufficiently long period in the population that is to be monitored to develop detectable antibody titres (for serology) or parasitic stages. It is common to house sentinels within a population for at least 4-6 weeks prior to testing but longer exposure periods of 10-12 weeks are better. Seroconversion can be very slow, e.g. in case of bacterial agents living on mucous membranes. Longer exposure times may also be necessary for sufficient sero-conversion against bacterial pathogens such as *Mycoplasma* spp., *Streptobacillus moniliformis*, and Pasteurellaceae.

The mouse strain may also be considered in some circumstances, because there are well-known differences in susceptibility to various microorganisms even among immunocompetent strains of mice. For example, C57BL/6 mice are low antibody responders to infection with mouse parvovirus (MPV) [65]. This strain, however, responds well to infection with MVM in contrast to mice of strains FVB/N, NMRI, ICR and C3H/HeN [66].

Sentinels must be immunocompetent for serological testing. Most commonly, outbred mice are used as sentinels because they are inexpensive. Under certain conditions, i.e. to detect specific agents, inbred or even immunodeficient sentinels may be used. Agents such as *Pneumocystis murina* or *Corynebacterium bovis* are detectable by

sentinel programmes only when immunodeficient sentinels are used.

Sentinels should be kept in a manner that maximizes their exposure to potential infections. Provided that the animals in the general population are in open cages, exposure of sentinels to possible infectious agents might be enhanced by putting them into open cages throughout the unit in locations where possible exposure to infectious agents is known or thought to be maximal (usually on the bottom shelves). Transmission of infectious agents should be further enhanced by exposing the sentinel animals to soiled bedding, water and feed taken from the cages of the experimental animals. It must be noted, however, that transfer of soiled bedding, the customary method for sentinel exposure, may be inefficient in transmitting certain agents. In particular, transmission of respiratory pathogens such as Sendai virus [67] or *Pasteurella pneumotropica* [68] is insufficient. Experience has shown in recent years that many additional agents including fur mites [69, 70], *Helicobacter* spp. and MNV are not easily transmitted to sentinels without direct contact between animals. However, the advantage of sentinels exposed to soiled bedding and/or aerosols is that few sentinels represent a whole colony.

Another option is the use of contact sentinels, which means that sentinel animals are directly exposed to experimental animals by placing them in the same cage. Agents are very likely to be transmitted to cage contact sentinels. Females are used for this purpose to avoid fighting. They are housed with colony mice for 2-3 weeks (or longer) and tested 8-10 weeks later. Transmission of agents can be increased after the period of direct exposure by transfer of soiled bedding, food and water bottles. However, use of contact sentinels is feasible only in small populations; high numbers of sentinels would be necessary in larger colonies. Use of contact sentinels (also in combination with the transfer of soiled bedding) is ideal when small numbers of animals have to be tested, e.g. one or two breeding pairs of genetically modified animals from external sources [71].

During the last decade, additional housing systems such as filter cabinets, microisolator cages and IVCs have emerged. They offer the advantage of separating small populations from each other and are frequently used for housing immunocompromised or infected mice. If handled properly, they very efficiently prevent transmission of infectious agents and must therefore be considered as self-contained microbiological entities. Health monitoring under such housing conditions is usually conducted by the use of very few sentinel animals. If colony animals are housed in microisolators or IVCs, sentinels are also kept in filter-top cages. When cages are changed in changing cabinets, sufficient amounts of soiled bedding from several cages are transferred into a separate cage which is used to house sentinels. Weekly changes of donor cages may then give insight into the microbiological status of the whole population. Other examples of methods that may be considered for monitoring are testing of exhaust filters or cage surfaces by using PCR [72, 73].

Numerous options and variants exist depending on local conditions. A strain or line bred in an IVC may be considered a hygienic unit, and sampling of colony animals from various cages may be a good option. Cohousing of animals from various cages for 4-6 weeks before testing may increase the likelihood of detecting an agent. It is, however, important that, independent of the selected approach, sufficient numbers of animals are submitted to testing.

Monitoring isolator-housed animals is usually also conducted by the use of sentinel animals. In many cases, only small numbers of animals are available for testing due to limited space, which may be acceptable if sentinels are properly handled. Sentinel animals are housed in one or several cages (depending on the isolator size) on soiled bedding taken from as many cages as possible. Commonly, a maximum of 3-5 animals per isolator is available for monitoring.

Frequency of monitoring

Health monitoring must be performed on a regular basis to detect unwanted microorganisms in good time. The frequency of monitoring mainly depends on the specific purpose of the population in question, the importance of a pathogen or other contaminant to the use of the population, the risk of introducing agents, and economic considerations. The FELASA

recommendations [46] state that monitoring for the most relevant infections should be conducted at least quarterly. Most commercial breeders of laboratory mice monitor more frequently (every 4–6 weeks). In most multipurpose experimental units where animals are regularly bought and introduced, more frequent monitoring is also preferable as this results in earlier detection of an infection. As a general rule, monitoring small numbers of animals from each unit at high frequency is more reliable in picking up infections than monitoring larger numbers taken at less frequent intervals (e.g. 3–5 animals every 4–6 weeks instead of 10 animals every 3 months) [74]. Under practical conditions, not every animal may be monitored for all microorganisms. Depending on the factors already mentioned, the frequency of testing may be different for different agents. Monitoring for frequently occurring organisms or for agents that have a serious impact on research should be performed more frequently (e.g. monthly), whereas testing for unusual organisms like mouse cytomegalovirus 1 can be done less frequently (e.g. annually). If germ-free or gnotobiotic mice are housed in isolators, monitoring for bacteria (environmental organisms) should be conducted more often than monitoring for viruses and parasites due to the higher risk of the former being introduced in the event of a barrier breakdown.

Test methods

Health monitoring of laboratory mice is accomplished using a variety of methods. Traditional methods used to detect infectious agents or disease processes include pathology, parasitological examinations, bacterial culture and serological tests. Molecular methods have become increasingly important over the last few years. The technique used to identify a specific microorganism depends on a number of factors, including the type of organism, the fastidiousness of the organism, the immunological status of the host, the ecology of the organism and the particular tests that have been developed to detect and identify the organism.

Testing of mice usually starts with routine necropsy and blood sampling for serology, followed by microscopic examination for parasites and sampling of organs for bacteriology, pathology and, in rare cases, virological examinations. All major organs should be inspected macroscopically to decide whether further investigation by histopathology is needed, though histopathological examination is seldom the method of choice for routine monitoring and is more efficient as a tool for disease diagnosis and validation of the impact of a certain microorganism.

Microscopic methods such as stereomicroscopy are commonly used for monitoring for ectoparasites. Endoparasites (enteric helminths, protozoans) may be diagnosed by direct microscopy after flotation of intestinal contents, on smears from intestinal contents and on adhesive tapes used for sampling around the anus.

Culture is the diagnostic method of choice for most bacteria and fungi because many species can be readily grown on artificial media. Samples for bacterial culture are typically taken from the respiratory tract (nasopharynx, trachea, lungs), the intestinal tract (caecal contents or faeces), and the urogenital tract (vagina or prepuce, kidney). In the case of pathological changes, additional organs (liver, spleen, mammary gland, lymph nodes, conjunctiva, skin, etc.) should be monitored. Culture of bacterial agents is generally accomplished by inoculating non-selective and selective media with the specimen and incubating the media in appropriate conditions to allow growth. Some bacteria such as *Corynebacterium bovis* grow very slowly so that culture media have to be incubated for sufficiently long periods. Morphologically distinct colonies are then evaluated by Gram stain and subjected to a panel of biochemical tests to determine the specific genus and species of each colony type. Commercial identification kits for human and veterinary pathogenic bacteria are sometimes not suitable to correctly identify bacteria from mice, e.g. Pasteurellaceae, *Citrobacter rodentium*, and *Corynebacterium bovis*. Some bacterial species are difficult to grow *in vitro* because they require special media, environmental conditions, or the presence of mammalian cells. For example, helicobacters require a microaerophilic atmosphere and *Clostridium piliforme* requires cultivation in certain cell cultures. For these agents, alternative diagnostic approaches such as PCR or serological

assays are often used. Molecular methods are also increasingly used to supplement traditional bacteriology. For example, PCR amplification of certain genes from bacterial cultures and subsequent sequencing of the gene(s) and comparison with sequence databases can be used to identify bacteria.

Serology is the most widely used methodology for monitoring infections in mice and relies on the detection of specific antibodies, usually immunoglobulin G (IgG), produced during an infection. It is applied especially for the detection of viral infections. Furthermore, a few bacteria such as *Clostridium piliforme*, *Corynebacterium kutscheri*, *Bordetella bronchiseptica*, Pasteurellaceae, and *Mycoplasma* spp. are screened by serological assays. Suitable and presently used methods include the multiplex fluorescent immunoassay (MFIA), the enzyme-linked immunosorbent assay (ELISA), the indirect immunofluorescence assay (IFA), and the haemagglutination inhibition (HI) test. Serological methods must be selected properly as they may differ in sensitivity and specificity, and unexpected serological results should always be confirmed by a second method, by a second laboratory, or by monitoring additional animals. In general, MFIA, ELISA and IFA are more sensitive than HI and so should be used as primary tests. The specificity of the tests is primarily determined by the antigen chosen and the methods used for antigen preparation. Immunofluorescence assay, for example, measures cross-reacting antibodies to various parvoviruses, whereas HI is specific for the virus (MVM, MPV). Although extremely useful, serological methods have certain limitations. Serology represents historical evidence of prior exposure to a microorganism and is not necessarily indicative of the continuing presence of the organism. Serology is also inadequate for detecting exposure to microorganisms in immunodeficient mice and in infant mice with an immature immune system. Serological methods for detection of antibodies in the IgG class will not detect exposure of an animal to a pathogen early in the course of infection. It takes some time (generally a number of days, sometimes much longer) after the initial contact with an antigen, before the amount of antibodies in the blood serum will exceed the detection limit of a test. Finally, some pathogenic microorganisms do not reliably induce an antibody response, particularly if cellular immunity is required to clear the organism (e.g. *Pneumocystis murina*). In the case of acute infection or where antibodies are not developed, a direct assay such as PCR may be the best option.

Molecular methods have become an integral part of health monitoring programmes, supplementing and replacing traditional techniques, and are meanwhile available for all bacterial agents, viruses, fungi and many parasites. They are aimed at detecting specific nucleic acid (DNA or RNA) sequences from infectious agents. The most common molecular methods used to detect infectious agents utilize PCR methodology. Such assays represent an attractive choice for the detection of microorganisms which cannot be reliably detected by traditional methods, and for monitoring biological materials. They are also used to evaluate the risk of agent transmission from animals, for example by testing excretions and other samples from animals coming from external sources. Major advantages of PCR are its high sensitivity, which allows detection of minute levels of infectious agents, and its high specificity, which allows differentiation of closely related organisms. Another advantage of PCR is that it is rapid. Disadvantages of PCR-based testing are the expense and the potential for false-positive or false-negative results. Diagnosis of infectious agents by PCR requires careful attention to the selection of animals and tissues for evaluation since the organism must be present at the time of testing and in the specimen evaluated. Improper selection of animals or specimens can result in false-negative results. False-negative reactions may also be the result of inadequate purification of the nucleic acids from the specimen resulting in samples containing substances that inhibit polymerase.

Monitoring for microorganisms is usually done by commercial laboratories, and is thus determined by their capabilities. In addition, most commercial breeders, as well as many research institutes and universities, have dedicated diagnostic laboratories. It is important that all investigations be performed in laboratories with sufficient expertise in microbiology or pathology of laboratory mice. Serological and molecular tests also require technical competence to ensure sufficient standardization of

tests (including controls) and accurate interpretation of results. Accreditation increases the trustworthiness of a laboratory. FELASA advocates accreditation of laboratory animal diagnostic laboratories according to DIN EN ISO/IEC 17025 (formerly ISO Guide 25), in which special emphasis is placed on competency of the staff, validation of test methods, and participation in interlaboratory testing [48, 75].

FELASA has recently established working procedures for an accreditation process of health monitoring programmes and for testing laboratories involved in health monitoring of laboratory animals [49]. An accreditation board evaluates programmes after voluntary application for accreditation, and official FELASA accreditation can be given to health monitoring schemes and/or to laboratories if they conform to the quality standards described in the FELASA recommendations. The FELASA accreditation process focuses on the scientific relevance of procedures implemented, competency of staff, interlaboratory/proficiency testing of laboratories, and appropriate procedures for managing animals submitted for health monitoring.

For more information about the diagnostic detection and identification of microorganisms in laboratory mice, the reader is referred to special publications [34, 46, 74, 76-78] and textbooks [79, 80].

Health report

A detailed health report should be required before the introduction of animals from outside sources, in order to better assess the hygienic risk from them. These reports are usually made available by the vendor for all purchasers of the animals, e.g. by publishing them on the internet. Similarly, health monitoring data should be made available to the researchers in experimental facilities. The data are part of the experimental work and should therefore be evaluated for their influence on the results of experiments, and included in scientific reports and publications as part of the animal specification.

A health report must contain sufficient data to provide reliable information on the quality of a population and thus differs from a test report issued by a diagnostic laboratory. Box 4.4.2 provides a checklist of the basic information that should be included in a health report. Usually, each animal facility or breeder has its own style of report sheets, which are sometimes difficult to read and to interpret. In order to easily compare monitoring reports from different breeders and users, FELASA has developed report forms for common species of laboratory rodents and rabbits [46]. The form recommended for reporting health monitoring data on mice is shown in Appendix 4.4.1.

APPENDIX 4.4.1: Health report form recommended by FELASA [46]						
Health monitoring in accordance with FELASA recommendations						
Location: **Species: Mouse** **Species and strains present within the unit:**			**Date of issue:** **Housing: (Barrier/non-barrier/IVC/isolator)** **Strain:**			
	Test frequency	**Lastest test date**	**Latest results**	**Testing laboratory**	**Test method**	**Historical results (≤18 months)**
Viruses						
Murine hepatitis virus	3 months					
Murine rotavirus (EDIM)	3 months					
Parvoviruses						
Mice minute virus	3 months					
Mouse parvovirus	3 months					
Murine pneumonia virus	3 months					
Sendai virus	3 months					
Theiler's murine encephalomyelitis virus	3 months					
Ectromelia virus	Annually					
Lymphocytic choriomeningitis virus	Annually					
Murine adenovirus type 1 (FL)	Annually					
Murine adenovirus type 2 (K87)	Annually					
Mouse cytomegalovirus 1	Annually					
Reovirus type 3	Annually					
Additional organisms tested:						
Bacteria, mycoplasma and fungi						
Citrobacter rodentium	3 months					
Clostridium piliforme (Tyzzer's disease)	3 months					
Corynebacterium kutscheri	3 months					
Mycoplasma spp.	3 months					
Pasteurellaceae	3 months					
Salmonella spp.	3 months					
Streptococci β-haemolytic (not group D)	3 months					
Streptococcus pneumoniae	3 months					
Helicobacter spp.	Annually					
Streptobacillus moniliformis	Annually					
Parasites						
Ectoparasites:	3 months					

APPENDIX 4.4.1: Health report form recommended by FELASA [46]—cont'd

Health monitoring in accordance with FELASA recommendations

Location:
Species: Mouse
Species and strains
present within the unit:

Date of issue:
Housing: (Barrier/non-barrier/IVC/isolator)
Strain:

	Test frequency	Lastest test date	Latest results	Testing laboratory	Test method	Historical results (≤18 months)
Species designation						
Endoparasites:	3 months					
Species designation						
Pathological lesions observed	3 months					

Data are expressed as number positive/number tested.
Positive findings in other species in the same unit:
Abbreviations used in this report:
ELISA, enzyme-linked immunosorbent assay; MICR, microscopy; IFA, immunofluorescence assay; CULT, culture; PATH, gross pathology; PCR, polymerase chain reaction; HIST, histopathology; NT, not tested

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CHAPTER 4.5

Genetic Monitoring of Inbred Strains of Mice

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Introduction

The wealth of knowledge that is available on laboratory rodents and the abundance of strains displaying different characteristics have made these animals, in particular mice and rats, an extremely valuable tool in biomedical research. It is widely accepted that the controlled management of variables is of great importance for the outcome of any animal experimentation. Hence the use of genetically defined laboratory animals plays an important factor. Inbred strains allow for much better standardization of the test conditions and improve the quality of results obtainable. Genetically defined inbred strains make it possible to reduce the analysis to keep the experiments at a minimum by selecting an appropriate genetic

model. Moreover, such a principle is in line with animal welfare legislation calling for reduction and refinement. Geneticists can provide not only homogeneous populations, but also, if desired, genetically heterogeneous populations with specific characters that can be synthesized repeatedly over many generations from the same inbred strains; each of the resynthesized populations will be essentially identical to the preceding ones.

Experimentation based on laboratory animals by and large depends on the genetic uniformity and constancy of the animals to be used. Therefore, inbred strains are preferred as they are considered to be homozygous after more than 20 generations of brother × sister inbreeding. This assumption depends on the strength of the mathematical theory of inbreeding.

A detailed knowledge of the various categories of inbred strains is required for a proper selection of a strain or the most suitable combination of strains including genetic modifications.

Inbred strains

An inbred strain is defined as a strain which is derived from at least 20 consecutive generations of brother × sister mating. All animals of an inbred colony will be homozygous at more than 99% of all genetic loci with exception of sex differences. The genetic background of an inbred strain should be well defined and it must be genetically constant for a long time period. The genetic constitution of an inbred strain represents a fixed composition of loci out of the genetic pool of its species. Therefore, phenotypic expression, including pathological processes, might be identical among all individuals of an inbred colony, but it has to be borne in mind that sex differences and non-genetic factors may have an effect on phenotypes. Further possible reasons for differences between individuals of an inbred colony are incomplete penetrance and variable expressivity of phenotypes.

The history of inbred strains of mice began in 1909, initiated by C. C. Little who investigated the pathology of cancer [1, 2]. Nowadays, inbred strains of mice and their derivatives are used in all fields of biomedical research and a multitude of different mouse models, mostly genetically

modified, is available for various types of diseases and disorders such as autoimmunity, metabolic diseases, cardiac disorders, neurological disorders and cancer. Genetic homogeneity is one of the preconditions for standardized experiments, meaning that the experimental group and the control group are genetically identical and also that animals in subsequent studies do not differ from those of previous studies. Thus a purposeful breeding scheme (see 'Maintenance of inbred strains of mice') including a genetic monitoring programme must be imposed on the colony to maintain genetic authenticity.

In spite of adequate breeding and monitoring programmes, animals of an inbred strain are not absolutely identical in all genetic loci due to a residual heterozygosity (<0.2%) and recent mutations, which remained undetected. These circumstances can and will lead to substrains, especially when colonies have branched off from a particular resource colony.

Substrains

Substrains are derivatives of an inbred strain and they are either known or suspected to be genetically different from their origin. Substrains arise by definition when branches (i) are separated after 20 generations but before 40 generations and when (ii) an inbred line is maintained separately from the ancestral strain for ≥20 generations (from a common ancestor 10 generations in the reference line and 10 in the new colony) and when (iii) differences are detected by genetic analysis. Genetic variances between substrains can be considerable and may lead to clearly phenotypic differences. One popular example dating from 1998 is the C3H/HeJ substrain. C3H/HeJ obtained from Heston (He) by The Jackson Laboratory (J) in 1947 differs from other C3H substrains in a mutation in $Tlr4^{lps-d}$ (Toll-like-receptor 4) on chromosome 4. This mutation renders the substrain more resistant to endotoxic lipopolysaccharide [3-5].

Worldwide, C57BL/6 is the most distributed inbred strain of mice. Thus a huge number of substrains exist with genetic and phenotypic differences [6-8]. In fact, the genealogy of C57BL/6 substrains is often under-reported and the degree of genetic diversity among these substrains is neglected by researchers. A huge

problem is the intensive usage of different C57BL/6 substrains as genetic controls and genetic background for the vast number of congenic strains carrying genetic modifications. Unfortunately, not all of them have been sufficiently backcrossed. These strains/stocks quite often harbour one or more genetically modified genes as well as a mixed genetic background of different (sub)strains. Despite appropriate breeding techniques applied by the vendors, accompanied by genetic monitoring programmes, quite often researchers derive their animals from various sources without paying attention to genetic uniformity of the strains in use; they also deliver their combined models to other laboratories. Genetic standardization is complicated further by the fact that embryonic stem cells used for genetic manipulation are derived from 129 different strains.

Factors that compromise genetic quality of inbred strains

In nature genetic diversity within a population and a species is important for its survival and for adapting to changes in the environment. Increase in genetic diversity can lead to speciation and plays an important role in evolution. The genetic diversity of a population is assessed by the genetic variation between individuals, which is determined by the number of polymorphic loci within the genetic pool of a population and the number of individuals heterozygous for these loci. The genetic variation between sexual reproductive individuals is driven by meiotic events, in particular, an independent assortment of chromosomes and crossing-over. Mutations are further important factors for increasing the genetic variation between individuals and they can help to adapt better to the current environment. On the other hand, mutations are a drawback for the population when they impede reproduction or cause health risks such as susceptibility to diseases or congenital disorders.

It contradicts all principles of genetic diversity in nature to develop an inbred strain and try to freeze a homozygote genome over generations for a long time period. In fact inbred strains are not protected from alterations of the genome even though they are maintained using adequate breeding programmes. Three sources compromise the genetic stability of an inbred strain: genetic contamination, residual heterozygosity and mutations.

Genetic contamination

Genetic contamination of an inbred strain results from an unintended outcross. The maintainance of inbred animals should therefore never be left to untrained personnel. This kind of genetic alteration can easily be discovered by suitable genetic monitoring programmes, if not by the coat colour of descendants. Genetic contaminations that remain uncovered result in mixed and undefined genetic backgrounds. Interestingly, some differences between existing sublines are due to genetic contaminations [9–12].

Residual heterozygosity

A certain percentage of gene loci remain heterozygous despite a strict brother × sister mating scheme. In 1965 Fisher has estimated the residual heterozygosity for certain inbred generations [13]. Based on the assumption of 20 chromosomes and an overall genome length of 2500 cM, he calculated it would take approximately 60 generations of either brother × sister matings, or younger parent × offspring matings, for a 98.9% probability that a genome is completely homozygous. Here the primary factor is not the number of chromosomes but the total length of the genome, and his calculations considered neither mutations nor selection. The overall length of the mouse genome is currently estimated as 3.4×10^9 bp, corresponding to 1453 cM [14, 15] and 99.8% homozygosity after F60 is generally accepted [11].

Heterozygosity can persist in spite of inbreeding, as a result of the reduced fertility and viability of homozygous genotypes. In 1953 Hayman and Mather [16] showed that even a moderate advantage of heterozygotes over the homozygotes can greatly retard or even arrest progress towards

complete homozygosity. However, this effect does not play a major role in laboratory mammals [17]. It is unlikely that more than just a few genes remain permanently heterozygous.

Spontaneous mutation

In addition to outcrossing and differential fixation of originally heterozygous loci, the third factor altering the genetic constitution of an inbred strain is mutation. A spontaneous mutation results from errors in DNA replication and repair. Mutations can occur in coding sequences as well as in non-coding sequences of the genome, but the frequency and spectrum of mutations in coding DNA differs from that in non-coding DNA. Some progress has been made in estimating the mutation rate and it is interesting how many mutations occur in germ cells and how many of them finally emerge in a population.

Individuals affected by a mutation leading to dominant alteration of a phenotype can be easily identified, while mutation rates in recessive phenotypes can only be estimated using indirect approaches. The mutation rate μ is the ratio of the number of germ cells affected by a mutation to the number of germ cells at risk for carrying that mutation [18]. New alleles that are lost from the population's gene pool by selection are continually substituted by new mutations. In the course of time both selection and mutation will reach an equilibrium. If the mutation rate per locus per gamete is μ then the probability of a mutation arising in any one generation is $2N\mu$, where N is the number of breeding individuals. The probability that a mutant gene of one of these gametes will become fixed equals its original frequency, $1/2N$, if there is no selective advantage. Thus the probability of a mutation from any single generation being incorporated in homozygous conditions equals μ regardless of the size of the colony. The probability of encountering at least one mutant when comparing individuals from two sublines that have been separated for a specified number of generations since subline dichotomy (n_1 and n_2, respectively) was given in 1977 by Bailey [19] as:

$$p = 1 - (1 - \mu)^{n1 + n2}$$

The average mutation rate has been generally accepted as 1×10^{-5} assuming 3×10^4 structural genes in the genome [20, 21]. This estimation is still applicable today because the latest genome analysis revealed 22 667 mouse protein coding genes plus 4948 pseudogenes and all in all 46 375 genes are predicted [15]. Thus any one gamete carries on average 0.3 mutations. For 10 generations past dichotomy, the chance that two sublines differ in at least one locus is thus $p = 0.9992$. However, one should keep in mind that the rates of spontaneous mutations differ markedly for the various traits such as cancer, transplantation antigens, microsatellites, conserved genes and certain chromosomal regions [22-24].

Mutations occurring in coding sequences are classified into synonymous (silent) and non-synonymous mutations. The so-called silent mutations do not affect the primary structure of a protein, but they can alter the secondary structure of the mRNA, which may have an effect on the stability of the mRNA and its migration from the nucleus into the cytosol.

Mutations in non-coding sequences can affect gene expression, and thus a phenotype, when they occur in promoter sequences or some other DNA sequences, e.g. enhancer, silencer and microRNA that regulate gene expression [25, 26]. Even when mutations are present in introns they can interfere with correct splicing of the transcribed pre-mRNA [27]. In addition to this basic classification, mutations are named in detail according to their different mechanisms and functions.

Maintenance of inbred strains

Colony structure

Breeding of inbred mice has to satisfy all demands for genetic authenticity even when a large number of animals is required. Thus breeding colonies are specifically structured in order to avoid intrastrain variability. The breeding of inbred strains is compartmentalized into three main parts, a foundation or nucleus colony, a pedigreed expansion colony and a multiplication colony. Nucleus colonies are sufficient when only small numbers of animals, about 10-15 mice per week, are needed. A subordinate pedigreed expansion colony must be built

up if there is a demand for up to 50 mice per week, and multiplication colonies have to be set up when more than 50 animals per week are needed. The actual size of each colony has to adjust according to requirements.

Nucleus colony

A nucleus colony (also called stem-line, foundation colony, and primary type colony) is a self-perpetuating entity and is the source colony for further subcultivation of the strain. Any genetic alteration within the nucleus colony that remains undetected is transferred to subsequent generations and subcolonies. The genetic background of a nucleus colony must be described and genetically monitored using different sets of markers (see the section 'Marker systems and strategies for genetic monitoring of inbred strains'), especially when different nucleus colonies are maintained in the same unit. Brother × sister mating is indispensable for nucleus colonies. In addition, a small colony size of about 10–30 breeding cages and constant generation intervals of about 6 months support the breeding of a sufficient number of laboratory animals. An important criterion of a nucleus colony is that all descendants can be traced back to a common ancestor.

Breeding systems

Two different colony systems can be recommended to maintain a nucleus colony, either a single-line system or a modified parallel-line system.

SINGLE-LINE SYSTEM

According to Festing [28], in a single-line system all breeders trace back to a common ancestor after six generations, meaning that a nucleus colony does not exceed seven generations. The reason for this procedure is that unfixed mutations may remain undetected when the nucleus is cut back to an ancestral pair within less than five generations. After the second generation the breeding line can split into two major sublines (Figure 4.5.1).

Single-line systems do not prevent genetic drift in nucleus colonies. The Jackson Laboratory has therefore established a so-called Genetic Stability Program (GSP) to minimize genetic alterations within an inbred strain and to prevent cumulative genetic drift for a long time period. The modification of this system compared to the traditional single-line system is the refreshment of the nucleus about every five generations using cryopreserved embryos frozen up to 25 years ago. Such a colony set-up was first mentioned in 1979 [29] and has been used by The Jackson Laboratory since 2003 [30].

MODIFIED PARALLEL-LINE SYSTEM

This system aims to minimize the likelihood that an undetected mutation is transferred to subsequent generations and colonies, and to have a back-up in case of an unintended outcross. In the modified parallel-line system three sublines are kept for four generations and the number of breeding pairs should not exceed 5-10. In general new generations are set up from third litters. When a nucleus has to be restored after the fourth generation, one breeder pair is selected from one of the three parallel lines as the new common ancestor. The other lines are pruned. Of this selected breeding pair, three new sublines are built up using the third-party offspring. As in the single-line system the choice of the subline depends not only on the genetic profile, but also on its reproductive performance. In practice this results on average in a cycle length of four generations within a 2 year interval (Figure 4.5.2).

Record-keeping systems

Documentation of the breeding of laboratory animals, especially genetically modified animals, is required by law in several countries. Independent of legal requirements, the documentation of breeding is an important tool to assure genetic quality and to reflect the history of an inbred strain. A record-keeping system should make it possible to trace the relationship of each animal in various generations back to one common ancestor and to summarize the data-yield systematically. In addition, documented breeding data permit an overview of the reproduction performance (colony index, CI) of an inbred strain. The data are documented on the breeding record card, on the cage label and, where required, in breeding books. The following demographic information must be documented: species, strain denomination; breeding pair number; generation;
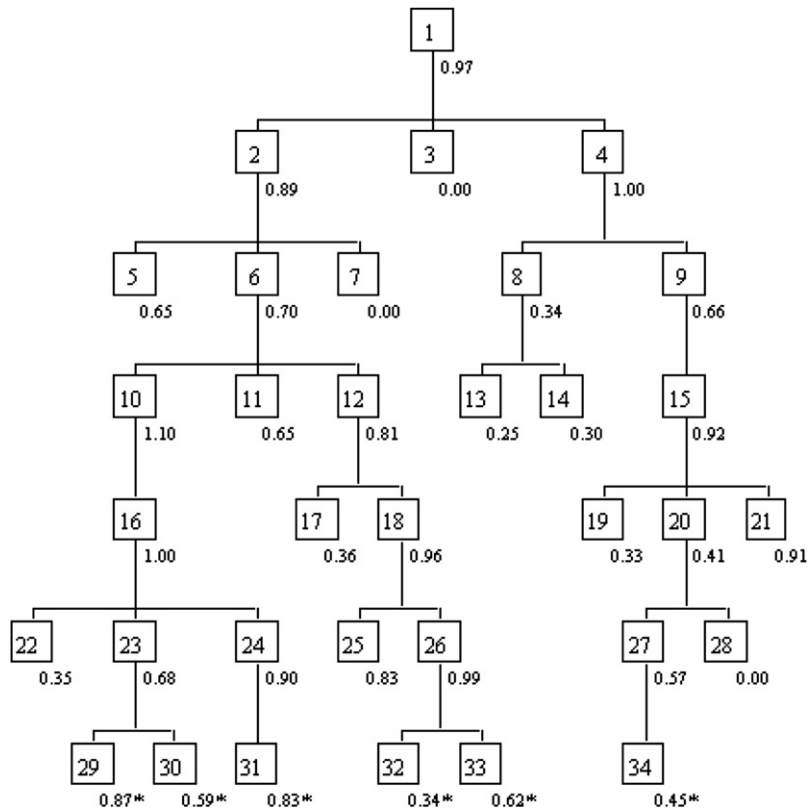
Figure 4.5.1 Nucleus colony maintained in a single-line system. Breeding pair numbers are given within the squares, and the mean productivity of each pair, expressed as young weaned/female/week is stated below each square (see colony index). All pairs trace back to a common ancestor. Two major sublines arise in generation 2 and have been maintained for a further five generations [28].
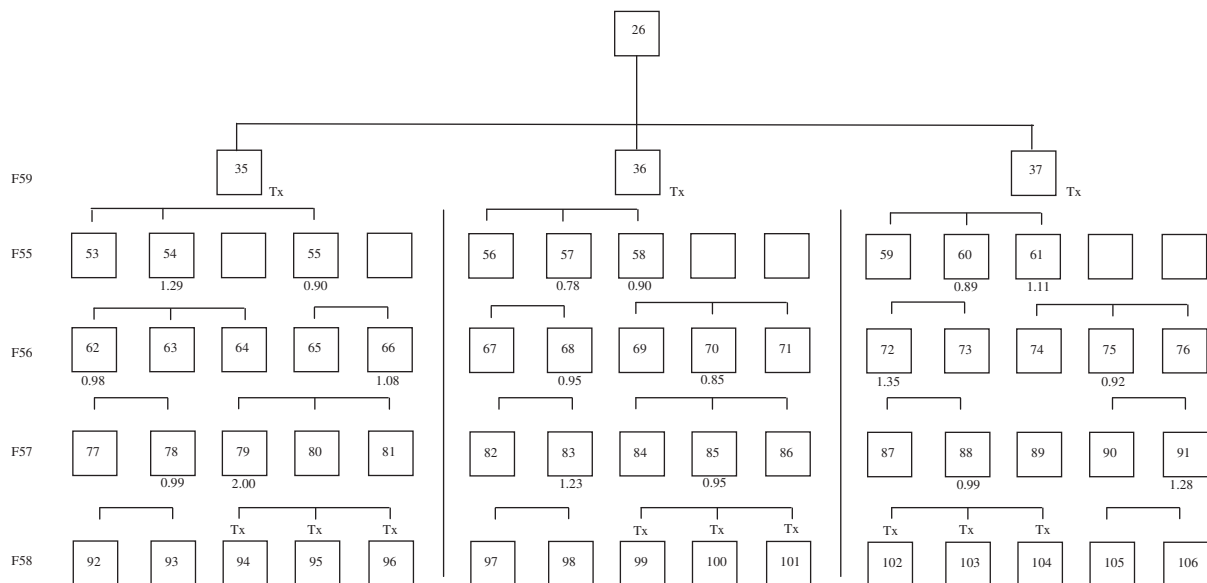
Figure 4.5.2 Modified parallel-line system. This system is based on the assumption that genetic contamination or fixation of deleterious mutants are rather unlikely to occur simultaneously in all three lines. Histocompatibility mutations can be detected by skin grafting of the prospective line parents (nos. 94, 95, 96, 99, 100, 101, 102, 103, 104) indicated by Tx. The mean productivity of each subline is given by the colony index (young weaned/female/week).

parental pair number; date of birth, mating, and death; date of birth of each litter; number born; number and sex weaned and disposition. Cage cards with a specific colour code provide an extra measure against confusion, when several strains are kept together in one breeding unit. Furthermore, a pedigree chart must be kept current with the set-up of new matings in nucleus colonies. In addition there should be some extra space on the chart for notes on genetic monitoring, reproductive performance, environmental changes (e.g. diet) and alterations in health status. Several developers provide colony management software and this has now been introduced into the record-keeping system in some institutions (see also Chapter 4.1).

Pedigreed expansion colony

This colony type is set up either to meet a limited weekly demand or to provide replacement pairs for large-scale production units. Pedigreed expansion colonies are maintained by brother × sister matings. They are directly related to the nucleus colonies but not self-regenerating. A first-grade pedigreed expansion colony is always built up from those offspring in the nucleus colony that are not required as future breeders or for experiments. The colony size should be specifically tailored for the multiplication colony requirement. This is a function of the colony output requested and the productivity (i.e. litter size, viability of offspring and litter interval) of the respective strain. Record-keeping for a pedigreed expansion colony is the same as for a nucleus colony.

Multiplication colony

The production of large numbers of inbred animals may run into practical difficulties. One serious disadvantage is reduced fertility due to inbreeding decline in many inbred strains. Thus breeding colonies are structured like a pyramid with the nucleus on top, pedigreed expansion colonies directly related to the nucleus, and up to four multiplication colonies at the base. A further difficulty is the genetic divergence likely to occur between sublines that have been separated from the common ancestor by 10-20

generations. Therefore, the aim must be to keep the number of propagating steps (generations) between the research animals and the common ancestral pair limited to less than 10. In a multiplication colony there is no particular need to continue brother × sister mating as the loss of homozygosity is negligible. Breeders may be mated at random, so long as it is guaranteed that the number of multiplication steps does not exceed four. Matings should be set up only between members of the same generation.

Abandoning inbreeding can promote the spread of mutations within a colony, primarily if they are recessive and of neutral type. It further acts against fixation (in homozygous form)-even more so if the altered gene exerts a deleterious effect. Thus, after strict brother × sister breeding is discontinued the degree of homozygosity will decline, with increasing spread from generation to generation. Furthermore, great care has to be taken to prevent genetic contamination. In those multiplication colonies whose offspring enter research directly, outcrosses may not become evident within two generations if mating is at random, and if the number of animals submitted to genetic monitoring is well above statistical significance.

In a multiplication colony, record-keeping can be reduced to the amount essential for efficient management. It may be advisable to maintain the last pedigreed expansion colony together with the multiplication colony as a source for constant supply without the risk of hygienic contamination due to frequent transfer between barrier units. As with pedigreed expansion colonies, in multiplication colonies the number of multiplication steps (≤4) and the size is determined by local needs, depending on the fluctuation in supply and demand.

Interaction with the genetic monitoring laboratory

Neither genetic colony management nor genetic monitoring by itself will guarantee that the animals provided for research are authentic. It is the mutual interaction between the two that is necessary to assure the genetic integrity of a given strain. If there is good communication between the management and monitoring groups,

a divergence in any colony will generally be found before it can interfere with an experiment.

Depending on the pedigree chart, the person in charge of the nucleus colonies has to be in close contact with the monitoring laboratory to arrange the procedures for genetic monitoring. On the other hand, the monitoring laboratory has to provide feedback on the different colonies. In addition, the monitoring laboratory should follow the set-up of new breeding cages on a copy of the pedigree chart provided at regular intervals by the colony management, and request the required samples in case of delay.

For routine screening of pedigreed expansion colonies and multiplication colonies the monitoring laboratory must take samples twice a year. The sample size depends on the stated colony size.

Principles and strategies of genetic monitoring

The demand for laboratory animals that are genetically defined and authenticated is increasingly pressing today. As biomedical research has become very sophisticated the live tools, i.e. the laboratory animals (inbred strains) used should also meet these standards. They must be specified not only by name according to nomenclature rules, but also by the laboratory code and holder symbol, as well as in genetic terms. Furthermore, the degree of conformity with published data that has been tested needs to be stated. However, genetic monitoring programmes cannot be restricted to laboratory evaluations only. The determination of a certain genetic status is inadequate and would merely result in a compilation of data without any significance to the respective colonies. The number of markers that can easily be tested covers just a very small proportion of the genome. The risk of genetic contamination depends on the set-up of the animal house, number of strains maintained, colony structure and size. Thus the sample size to be tested with a certain significance level has to be adjusted to each specific unit. It is a mutual interaction

between animal house and genetic monitoring laboratory that assures the production of high-quality laboratory animals. Sophisticated laboratory methods will not compensate for mistakes that may happen during regular handling of the animals. Colony management by qualified and well-trained animal technicians is the basic step towards proper propagation of authentic laboratory animals. A statement by Falconer [17] five decades ago concerning the quality of laboratory animals has still not lost any of its validity:

> The maintenance of inbred strains is a responsible matter which should not be left to untrained workers. The only guarantee that an inbred strain is what it purports to be is the reputation of those who have maintained it in the past.

Once fully inbred (>60 generations), the strains are rather stable in their genetic composition. However, in early generations differential fixation of alleles in residual heterozygous loci may result in differences within and between sublines. Mutations and inadvertent outcrossing (genetic contamination) are other factors that can alter the genetic constitution of an established inbred strain. While the first two factors result in minor differences that are rarely detected, but may be of scientific significance, the extent of the genetic alteration caused by genetic contamination is so serious that the strain can no longer be used for research.

By what means can scientists, central animal facilities and commercial breeders verify the integrity of the strains maintained, or guarantee that only authenticated animals have been used for research? The measures required (genetic monitoring) have to be adjusted to the specific requirements, and depend on the scientific purpose, physical maintenance conditions and laboratory equipment. They are also strongly influenced by economic factors. There is no fixed universal scheme for genetic monitoring, but there are clear facts to be considered in various situations. For example, there is a difference between a scientist using strains that have been developed in their own laboratory, and a scientist using several strains obtained from various sources, but in both situations the scientist has a pertinent interest in the strains being of the expected genotype.

Nevertheless, it is of the utmost importance to establish accurate colony management that fits the colony type (nucleus or expansion colony). Mistakes at the nucleus colony level cannot be corrected. Well-trained personnel and organizational measures are the first step in preventing mix-ups. A genetic quality programme should then consider criteria such as a set of specific markers (genetic profile) and possibly isohistogenicity (permanent take of skin grafts exchanged among members of an inbred strain).

Which measures are then to be recommended for genetic monitoring and appear to be essential?

1. A scientist who has developed a strain, or who knows the characteristics of a strain by long-standing experience, will most probably test the specific characteristics by the set-up of the experiment or in controls. In this case genetic monitoring may be limited to a regular determination of isohistogenicity of that strain. However, testing only (e.g.) for genetic modification and not for the contamination risk within the unit is not sufficient.

2. If several strains are comaintained, the scientist should either compare the strains with reference strains, or preferably restock the colonies on a regular basis by certified pedigreed breeders from a reference source known to authenticate the strains by genetic profiling.

3. Central animal facilities that breed and maintain a larger panel of different strains and supply various research groups have a greater responsibility for the authenticity of the animals distributed than any scientist who breeds the animals just for their own research. These institutions should establish a genetic monitoring system even if it is a regular routine to restock colonies from a reliable or frozen source. Depending on the genetic profile of the strains maintained, it is necessary to regularly determine markers characteristically differentiating between the strains in addition to the isohistogenicity check. If the institution cannot do these tests, reference or monitoring centres should be commissioned to type the respective colonies for their critical subsets.

4. For commercial breeders distributing large numbers of animals from different strains, the responsibility is much greater. If non-authentic animals are being distributed, the scientific damage to be expected increases with the number of customers. Commercial breeders should therefore indicate their genetic monitoring programme not only by methods/markers but also by sample size, frequency and date of sampling/typing, and typing results. Again, commercial breeders may also reduce their genetic monitoring efforts by regularly restocking their colonies from reference colonies or frozen sources known to be authenticated.

In principle, genetic monitoring programmes should first be structured to authenticate a strain. Subsequently samples of all strains comaintained in a functional unit will have to be typed for the expression of differentiating markers (critical subset). These tests do not obviate an inadvertent outcross, but the probability that non-authentic animals are submitted to research will be minimized.

For any authentication it is necessary to determine a genetic profile that can be compared with published data. In general this profile is composed of monogenetic markers (molecular markers, immunological markers, biochemical markers, morphological markers, behavioural profiles and, if necessary, any marker capable of identifying and distinguishing an inbred strain), and ideally represents a sample of the genome. Thorough genetic monitoring is laborious and thus expensive per tested animal, but calculated on the basis of colony output and the scientific and economic value of a biomedical test, the costs are acceptable.

Recommendations for assuring genetic quality of inbred strains

Heritable traits that can be utilized for genetic monitoring programs are described in the section 'Marker systems and strategies for genetic monitoring of inbred strains'. The large number of potentially useful markers raises questions concerning the objectives, costs and feasibility of a genetic quality assurance programme. With respect to costs it should be remembered that the total expenditure for laboratory animals

usually constitutes less than 10% of the budget of a research project. Furthermore, the costs of a genetic monitoring programme must be balanced against the possibility of compromising an entire study, either by using genetically contaminated animals or by making erroneous assumptions concerning the genome of a particular strain. If genetic quality assurance is integrated into the basic colony management programme, it should prove practicable to produce genetically defined inbred strains for biomedical research.

Objectives of genetic monitoring

The most fundamental consideration in designing a genetic monitoring system is to establish the objectives of the programme. These objectives—and their limitations—should be understood by both the producers and users of the mice. Most monitoring programmes are designed to authenticate the genetic profile for a particular strain and thereafter minimize or eliminate the possibility of undetected outcrosses in the production of the inbred mice. These programmes have only limited ability to detect spontaneous mutations and/or subline divergence and are best addressed by a programme of embryo cryopreservation.

In stating the objectives of the monitoring programme, the limitations of the system must be indicated. The same level of assurance, for example, cannot be provided when sampling a large production colony rather than monitoring a small nucleus colony. Monitoring programmes do not preclude such errors as mislabelling during shipment to the user. Although these considerations may seem obvious to many, they are often ignored. It is often assumed that a monitoring programme provides a higher level of assurance than is in fact the case.

Since any genetic monitoring programme has to consider colony structure and type (nucleus colony, pedigreed expansion colony, multiplication colony), it is recommended that specific documentation on the type of monitoring be furnished with the animals. This documentation should include evidence that the strain is authentic, e.g. a genetic profile, and affirmation

that a routine genetic monitoring programme based on accepted methodology has been carried out. The monitoring methods used and the method of sampling should be indicated. This latter statement should include the time when typing was last performed and the relationship of typed animals to the animals in question. The colony manager should be prepared to discuss in more detail questions such as the critical subset used and whether all colonies—pedigreed and expansion—have been sampled. An assessment of the risk of contamination is also warranted.

Authentication of a strain

The initial step in establishing a genetic quality assurance programme is to authenticate the genetic profiles of the strains being maintained. This process establishes that the animals are inbred and conform to published profiles for those strains. Heterozygosity and/or deviations from the expected profiles must be addressed and resolved. Potential sources of unexpected genetic variation in laboratory rodents are described in the section 'Factors that compromise genetic quality of inbred strains.' If unexpected variation is detected, a decision must be made as to the probable cause and whether to rename or possibly to discard the strain. Deviation at a single marker may reflect a fixed mutation while a variation at a number of loci suggests an earlier problem in the breeding of the animals.

Selection of markers

The single most important requirement in selecting a set of markers for use in a genetic monitoring programme is that they provide the necessary information. Beyond this obvious consideration, most laboratories will select markers and techniques with which they have experience and/or the requisite equipment. It should be emphasized, however, that a series of markers requiring a battery of tests is often needed to achieve the stated objectives. Neither molecular nor immunogenetic, serological, biochemical or morphological markers are sufficient by themselves to define an inbred strain. Only a combination of these markers allows for a unique characterization of an inbred strain.

Once this genetic profile is established, the markers that will be utilized in further routine monitoring must be determined. The selection criterion is based on the maintenance condition and on the strains maintained in close proximity.

The following factors should be considered when selecting markers for use in a monitoring programme:

1. Some markers, in particular, *single locus alleles,* provide specific information as to the nature of a particular problem. If there has been genetic contamination, for example, these markers might suggest the origin of the inappropriate animal. On the other hand, skin graft rejection would only indicate that the strain is no longer isohistogenic, but would not allow differentiation between mutations and contaminations.

2. *The strains to be monitored:* the selected markers should be efficient in distinguishing the monitored animals. Some markers are relatively invariant within strains. Testing for these markers would provide little useful information and might consume a great deal of time and effort. Similarly, markers that are only of interest with respect to one strain are usually less efficient than a battery of three or four markers which, in combination, will distinguish all or most strains.

3. *Genetic variability:* ideally, the markers should be highly polymorphic with codominant expression of alleles. Heterozygous phenotypes should be easy to distinguish from either homozygote.

4. *Economic criteria:* factors which influence the ability to set up routine testing on a large-scale basis include the age of the animal at the time of testing, the number of tissue samples that must be obtained from a given animal and the ability to store the tissue prior to testing. Furthermore, the speed with which the results have to be obtained should also be considered. Although many techniques can be completed within one day, this is not true for all tests; for instance, skin grafts must be observed for at least 120 days.

5. *The need to sacrifice the animal to be tested:* the ability to leave an animal in the colony for breeding purposes offers obvious advantages over systems that require removal and subsequent sacrifice.

6. *The ease of obtaining and storing reagents:* some assays usually utilize commercially available reagents. Other techniques may require that the reagents be developed or produced and standardized within the laboratory. These processes may require more time than the actual monitoring programme itself.

7. *Stability/reproducibility of results:* the detection methods for the chosen marker system have to produce stable and reproducible results. The methods should be well established at the monitoring laboratory and they should be adapted by experienced personnel.

From long-standing experience, it appears that testing for isohistogenicity is still an appropriate and worthwhile approach. Although skin grafting is a time-consuming procedure, which requires an observation period of at least 100 days, it should be implemented to assure genetic quality. No other method can replace this technique for detecting the products of all histocompatibility loci, which are thought to serve as markers on virtually every chromosome. Determination of isohistogenicity is usually restricted to the nucleus colony. The small number of animals that can be tested and the delay in obtaining the results often preclude the testing of animals from multiplication colonies.

For routine genetic monitoring, where it is the primary goal to preclude genetic contamination beyond dispute, a certain number of markers is selected depending on the colony set-up and the multiplication step. In the case of monotribal maintenance (one strain per unit) where genetic contamination is impossible, monitoring can be restricted to test isohistogenicity every four generations in the nucleus colony. As soon as more than one strain is maintained within one unit, samples have to be taken and submitted to the monitoring laboratory.

Critical subsets

To uncover a genetic contamination it is not necessary to test each animal for a number of markers that will uniquely identify each strain being maintained within a functional unit. Only the minimum number of markers differentiating between the strains in question needs to be determined. This will provide the colony

management with sufficient information as to whether contamination has taken place, and possibly about its source.

Monitoring by critical subset typing is only acceptable if the genetic profile of the strains is known or if the animals were originally obtained from a monitored and certified colony.

Sampling procedure and sample size

A final factor to consider in establishing a monitoring programme is the sampling procedures. The frequency of sampling, the sample size and the markers to be selected for monitoring have to be adjusted to the colony type and the risk of contamination. The underlying principle in determining sampling procedures is based on Mendel's second law of independent assortment. In practical terms, this law predicts that an F2 or later generation offspring of an unintended outcross might conform to the expected genetic profile due to chance. Similarly, the offspring of an F1 hybrid backcrossed to the original parental strain might also display an appropriate profile. This principle must be considered when designing sampling procedures for various colonies.

Nucleus colony

The nucleus colony of any inbred strain deserves the closest monitoring. Undetected outcrossing at this level would not only result in the contamination and possible loss of subsequent generations and multiplying colonies, but in the loss of the original inbred strain. It is therefore necessary to test every breeding pair established in the colony, if a single-line system is being employed. Three different sampling systems have been used to achieve this objective:

1. *Test both parental animals at the time of mating:* while effective, this system can be utilized only if monitoring does not require sacrificing the animals.
2. *Test both parental animals at the termination of their breeding life:* testing retired breeders permits the sacrifice of the animals, but necessitates being able to locate all offspring of that mating in subsequent generations should a problem be detected.

3. *Test one offspring from the first litter of every mated pair:* when established, this system ensures that every breeding cage has been tested shortly after first parity. This system requires that staff in the animal facility ensure that the required animals are sent to the monitoring laboratory. If desired, the retired breeding animals can be sent to the laboratory.

Animals taken for any of the above sampling protocols are tested only for their critical subset. The entire genetic profile of the strain should be verified at some periodic interval. Several laboratories verify the profiles of their strains every four generations or whenever new lines are established.

Based on these suppositions, the number of tests required to be performed by the monitoring laboratory can easily exceed a manageable size when many strains have to be authenticated and if the nucleus size exceeds 5–10 breeding cages. The required size and the set-up of nucleus colonies have been described in the section 'Maintenance of inbred strains.' Nevertheless, if the strain to be assessed is regarded as invaluable one of the above three sampling systems has to be used. If in the case of poly- or oligotribal maintenance a modified parallel-line system is employed, full-scale monitoring is required every four generations when new lines are set up. As it is unlikely that all three lines will become genetically contaminated at one time, a critical subset determination on samples of common line ancestry every second generation or on random samples from each line at specific intervals will provide enough reliability.

Expansion colonies

The sheer number of breeding animals in the expansion colonies precludes testing on a large scale as used in the nucleus colony. While there is no consensus on how to best approach this problem, most facilities test sample animals from their expansion colonies on a periodic basis. The purpose of testing is to preclude high levels of contamination in the colonies and to provide the management with empirical data on the frequency of any problems. It must be emphasized that this testing does not preclude the issuing of contaminated animals. Expansion colonies are dynamic structures, with a small percentage of the

breeders being replaced at short intervals. Testing such a colony in March, therefore, provides little information about the animals present in April.

A random sample should be taken from an expansion colony, recognizing that any contamination may not be random in physical distribution. Steps should be therefore followed to ensure that a random sample of a random distribution is taken. Sampling should always be marked on the records of the cages sampled. The number of animals taken is dependent on the level of monitoring desired. The frequency of sampling is usually twice yearly.

Although these procedures do not preclude undetected outcrosses, they will detect contaminations if there has been a massive introduction of a foreign genome. A low rate of outcrossing may never be detected, but this problem should at least be minimal if no further breeding stock is collected from this colony.

Marker systems and strategies for genetic monitoring of inbred strains

A genetic marker is defined as a heritable variation among the individuals of a population. These variations are either easily discernible, such as morphological alterations, or can be detected by adequate techniques when they occur at protein and DNA level. For any authentication of an inbred strain it is necessary to determine its genetic profile, which is composed of all strain characteristics. Modern molecular techniques make it possible to define a set of DNA markers for each inbred strain densely covering the whole genome. These opportunities must not lead to the false logic that all the other marker systems are not needed for genetic quality assurance. Modern molecular marker systems cannot cover the whole genome and sequencing of the complete genome is not a feasible option for genetic monitoring. In the following sections, applicable marker systems for genetic monitoring are introduced.

Phenotypic traits

Visible traits can be distinguished easily during regular handling of the animals, for instance coat colour and pelage variations, morphological alterations, deviant behavioural abnormalities and lethargy due to manifested diseases. These traits can be used as markers for genetic authenticity of an inbred strain without the need for special equipment. It has to be considered that some variations, for instance white spots, are not uncommon [31–34]. In this case variations must be observed to see whether they alter over time. For other visible traits, such as manifestation of a particular disease or reproductive performance, it must be taken into account that environmental factors can have an impact on expressivity and penetrance.

Pigmentation

The coat colour of strains is a method of detecting genetic contamination by direct inspection if animals with different colours were outcrossed. Over 50 gene loci are known to affect the coat colour of mice. The most eye-catching coat colour genes are listed and described in Table 4.5.1 [35, 36]. Some of these genes have considerably more than two alleles. Thus new spontaneous mutations in coat colour genes are not unusual and coat colour variability cannot be solely attributed to genetic contamination. On the other hand, spontaneously arising coat colour changes in litters, especially recessive variants, indicate segregating loci within the colony.

Reproductive performance

The reproductive performance of an inbred strain is, in most cases, poor compared with that of F1 hybrids or outbred colonies. The reproductive performance of an inbred strain is represented as a CI, which is calculated by dividing the total number of offspring weaned per week or month by the female inventory for that week or month. A litter size dramatically higher than that calculated by the strain-specific CI raises suspicion. It has to be considered that a CI is specific for a strain maintained under given conditions; the

TABLE 4.5.1: Genes affecting pigmentation in mice

Gene	Annotation	Old symbol	Old annotation	No. of alleles	Chromosome
$Mlph^{ln}$	Melanophilin	*ln*	Leaden	1	MMU1
a	non-agouti	*a*	Non-agouti	58	MMU2
$Trp1^b$	Tyrosinase-related protein	*b*	Brown	8	MMU4
Tyr^c	Tyrosinase	*c*	Albino	27	MMU7
$Myo5a^d$	Myosin 5a	*d*	Diluted	51	MMU9
$Oca2^p$	Oculocutaneus albinism2	*p*	Pink-eyed dilution	19	MMU7

The most eye-catching coat colour genes, number of variants [34, 35] and position on chromosomes.

CI of a strain can increase or decrease over time when conditions are changed, for instance light cycle, temperature or health status.

Behaviour

Deviant behaviour of animals within an inbred colony can indicate genetic alterations, for instance, circling behaviour, ataxia, star-gazing, epileptic seizures, disorientation, jumping and lethargy. It has to be proven by several means (breeding experiments, microbiological tests, pathological investigations) whether the deviant behaviour is due to a genetic alteration or an infection.

Morphological alterations

Any visible alteration, such as a change in pelage, paralysis or polydactyly, could possibly be attributed to a mutation.

Pathophysiological traits

Unexpected manifestations of complex diseases such as metabolic disorders, cardiovascular diseases, autoimmunity, and a frequent occurrence of tumour development are signs of genetic alterations within an inbred colony. Affected animals can become apparent through changes in weight, insidious lethargy, and specific symptoms, so that specific tests are required for a clear description of the cause. Further investigations are needed to clarify whether the phenotype can be attributed to infection. On the other hand, in populations of inbred strains expressing a pathophysiological trait, a decreasing incidence of a trait can also indicate a genetic alteration.

Isohistogenicity (skin grafting)

Skin grafting is the method of choice to determine whether individuals carry an identical set of transplantation antigens. Although only histocompatibility loci are tested, it is to a certain extent reasonable to put isohistogenity on a par with identity (isohistogenicity). Skin grafting is the method that makes it possible to test isohistogenicity independently of any laboratory equipment. Another advantage of this method is its sensitivity, because a large number of major and minor transplantation antigens (<100) are determined, located on virtually every chromosome. The disadvantage is that skin grafting is time consuming, because disparity at minor transplantation loci causes a graft rejection that may vary from an acute to a very weak rejection pattern even exceeding 200 days. This technique requires holding capacity for the grafted animals, and in the case of an outcross, it does not provide the information on the contaminating source. Furthermore, the method needs well-trained staff and local animal welfare legislation has to be observed.

Marker systems requiring laboratory techniques
Immunological markers

Immunological markers in general are detected by serological methods using polyclonal or monoclonal antibodies. They may be located on the cell

surface, such as cell membrane-associated alloantigen loci (erythrocyte alloantigens, differentiation alloantigens and histocompatibility loci) or be soluble molecules like immunoglobulins. These markers can be identified by several techniques such as haemagglutination, complement-dependent cytotoxity test, antibody binding assays (flow cytometry; enzyme-linked immunosorbent assay, ELISA) A large number of immunological markers are known and their respective antibodies can be easily obtained commercially.

Biochemical markers

Biochemical markers are isozymes and variants of other proteins. They can be distinguished by several electrophoresis techniques using different media (STAGE, CAE, PAGE and IEF) followed by corresponding histochemical staining of the protein. The use of biochemical markers has decreased, although a large number of genetic variants of enzymes and proteins are known in the mouse [37]. One reason for this is that quite a number of animals have to be sacrificed, because expression of markers is organ-specific.

DNA markers

Recently, DNA markers have superseded many of the markers formerly used, not only because they are polymorphic with at least two alleles, but also because they are densely spread over the whole genome. During the last three decades DNA markers such as restriction fragment length polymorphism (RFLP), amplified fragment length polymorphism (AFLP), random amplified of polymorphic DNA (RAPD), variable number of tandem repeats (VNTR) and single sequence conformation polymorphism (SSCP) have been established [38-42]. These markers can only be used to distinguish genetically different inbred strains in individual cases, because their polymorphic content is often limited. Also, the detection methods are time consuming, and in some cases the results are elusive and not reproducible.

In the early 1980s microsatellite markers were also described and, by the end of that decade, their high density throughout all mammalian genomes as well as their extremely polymorphic content had become apparent [14].

Furthermore, microsatellite markers can be easily detected by polymerase chain reaction (PCR) and gel electrophoresis. Features such as high density, high polymorphism, and easy and rapid detection in a large number of individuals have emphasized their suitability for routine genetic monitoring. Today, the majority of inbred strains of mice have been characterized using microsatellite markers; the genetic profiles are available and can be compared against each other in several databases [34].

Another milestone in genome research was the discovery of single nucleotide polymorphisms (SNPs) and the development of fast detection methods such as PCR-based techniques combined with special endonucleases and/or fluorescence-labelled allele-specific oligonucleotides [43, 44]. Although the polymorphism of these markers is restricted to only four variants (A, G, C and T) and generally only two alleles are discernible, these markers can be used for genetic monitoring because of their high density throughout the whole genome (every 500-1000 bp). The genetic profile of SNPs of a large number of inbred strains of mice is published in databases as for microsatellite markers [15, 34].

In designing a critical subset of markers (see the section 'Critical subsets') it is undoubtedly an advantage to use current databases that provide a genetic profile of an inbred strain and, further, to be able to compare the genetic profile of two or more inbred strains. Although the microsatellite and SNP profiles of many inbred strains have been published, the genetic profile of the vast majority of substrains remains almost unknown. Thus, the information content of databases is limited and any marker that is selected must always be verified before being used.

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Gnotobiology and Breeding Techniques

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Gnotobiology

Introduction and historical background: evolution of applications

Interaction between environmental microbism and animal health, either beneficial or deleterious, was noticed very early in laboratory animals. Pasteur and, a few years later in 1907, Nencki and Metchnikoff, investigated the survival of several species in the absence, or the presence, of various bacteria, as cited by Luckey [1] in 1963 and Pleasants [2] in 1973. In 1907, the Russian scientist Metchnikoff [3], at that time a professor at the Pasteur Institute in Paris and a future Nobel laureate, was the first to introduce the concept of the positive role played by certain bacteria and suggested that it would be possible to modify the gut flora and to replace 'harmful' microbes with 'useful' ones. He suggested that the ageing process results from the activity of putrefactive (i.e. proteolytic) microbes producing toxic substances in the large bowel.

The first germfree animals were produced after overcoming the problems of preparation of sterile diet, the design of the first isolator systems and the use of germicide products [1]. The breeding of germfree rats was reported in 1946 by Gustafsson, Trexler and Reynolds, using different types of isolators and sterilization systems. The use of aseptic hysterectomy or hysterotomy in the reproduction and maintenance of gnotobiotic rat and mouse colonies became a common practice in the late 1950s. The first research applications addressed the effects of microbial microflora on animal physiology, nutrition and metabolism [4-7]. Later

developments included extended experimental work in the fields of leukaemia, carcinogenesis, disease resistance, immunological response and cell line differentiation [2, 7-10]. Later on, when the breeding and experimental use of 'specific pathogen free' (SPF) rodents became the rule, the techniques developed in gnotobiology were used for rederivation of contaminated lines or colonies to gnotoxenic conditions before microflora transfer and maintenance under barrier conditions, i.e. under less demanding bioexclusion conditions, no longer aiming to preserve an axenic or oligoxenic definition but to prevent contamination by a well-defined list of species-specific pathogenic agents ('bioexclusion list'). Depending on the context of use or the author, these animals were also described as 'disease-free', 'heteroxenic' or 'caesarean-obtained and barrier sustained' (COBS) [11-17]. Their use in biomedical research grew quickly and outpaced that of germfree, gnotoxenic or even 'conventional' rodents. Last but not least, concurrently with the development of murine virology and serology, as well as molecular biology techniques, the SPF definitions were progressively extended to the absence of all undesirable organisms, i.e. not only zoonotic and pathogenic agents but also those interfering with research [18-20]. The extensive use of immunocompromised research models, beginning with the nude mouse models, extended first to other mutations affecting the immune response (immunocompromised models), then to various spontaneous or target mutations (fragilized disease models), paved the way to more comprehensive SPF definitions including opportunistic organisms [16, 21, 22]. Considering the development of research applications in mice, we may expect to find newly identified agents known or suspected to interfere with more or less specific research fields, generally or under special conditions [18].

The greatest colony contamination risk from undesirable agents is linked to the introduction of contaminated individuals or strains from the outside either directly (by introducing live individuals into the same animal resources or exposure to murine biological products) or indirectly (via human or animal carriers, and fomites). These potential contamination risks should be duly addressed in health management and monitoring programmes.

Health standards: definitions and categories

Gnotobiology

The original meaning of gnotobiology describes it as the 'study of gnotobiotic animals (i.e. with a fully defined and controlled microflora) in order to elucidate associated biological phenomena' [1]. Table 4.6.1 below includes the most common definitions used in gnotobiology, as well the related bioexclusion systems and derivation techniques.

Conventional

Conventional colonies of animals are, at best, defined as clinically healthy animals, free from apparent signs of disease or infection. This category includes mice with an uncontrolled, undefined or unknown associated microflora.

Even when a colony is under close veterinary supervision, to guarantee the absence of clinical signs of disease or lesions, one can never exclude the presence of a carrier state with potentially pathogenic, zoonotic or interfering agents. In consequence, the use of such non-controlled rodent models is not consistent with good scientific practices, quality assurance standards and ethical principles in animal experimentation [23], which are now integrated into best practice and the regulations governing the use of animals for scientific purposes [24].

Holoxenic

Holoxenic animals include those with an associated microflora that is generally similar to the microflora acquired by wild conspecific individuals after exposure to their natural environment. The term is often used in opposition to *heteroxenic*, which defines the microflora originally obtained by transferring a controlled microflora (gnotoxenic step) into germfree animals (obtained by aseptic hysterectomy), subsequently maintained in research facilities

	Gnotobiotic			Agnotobiotic	
	Axenic or germfree	Gnotoxenic microbio-logically defined	Heteroxenic (SOPF)	Heteroxenic (SPF)	Holoxenic or conventional
Definition	No detectable microorganism, 'sterility'	Implantation and maintenance of a strictly defined microflora, positive definition	SOPF: SPF exclusion list plus opportunistic agent list	SPF: SPF exclusion list includes zoonotic, pathogenic and interfering agents	No control, no definition or knowledge of microflora; contamination possible
Microflora	None	Strict control and maintenance of original gnotoxenic microflora		Progressive enrichment of gnotoxenic microflora with environmental and human-derived organisms	Uncontrolled and unknown microflora, as found in the wild
Type of bioexclusion system	Isolator only	Isolator only	Isolator or filter-top and laminar hood cage system in barrier room	Barrier room, open cages or microisolation cages with less strict procedures	None
Original derivation technique	Aseptic hysterectomy	Aseptic hysterectomy Embryo transfer	Aseptic hysterectomy Embryo transfer Progressive enrichment of gnotoxenic microflora with environmental organisms but control of aerobic opportunistic microflora	Aseptic hysterectomy Embryo transfer	None

SPF, specific pathogen free; SOPF, specific and opportunistic pathogen free.

TABLE 4.6.2: Sterile and non-sterile organs	
Normally sterile organs	**Normally septic organs and normal microflora**
Respiratory tract (below vocal cords)	Skin: $10-10^5/cm^2$, (*S. epidermidis, S. aureus*, fungi)
Sinuses at middle ear	Nasopharynx: $10^9/mL$ (anaerobic/aerobic: 1000/1, *S. pneumoniae, H. influenzae, S. aureus, N. meningitidis*, etc.)
Pleura and peritoneum	
Liver and biliar vesicle	Oesophagus/stomach: $10^2-10^3/mL$ (*H. pylori*)
Urinary tract (except terminal part of urethra)	Small intestine: $10^2-10^3/mL$ (lactobacilli)
Bones, joints	Bowel: $10^{10}-10^{12}/mL$ (anaerobic: *Eubacterium, Clostridium, Basteroïdes, Veilonella*, etc. Aerobic: *E. coli*, etc.)
Cephalorachidian fluid	Vagina: $10^8/mL$ (anaerobic and lactobacilli, faecal microflora)
Muscles, blood	Terminal urethra: $10^2/cm^2$ (*S. epidermidis*, lactobacilli, *E. coli*)
Source: *from [27].*	

where the original implantation microflora is gradually enriched by microbial species originating from this environment, including from human origin (Tables 4.6.1 and 4.6.2) [25]. Holoxenic animals can either be 'conventional' (when they do not benefit from an adequate health definition and monitoring programme; see above) or they can be defined and managed as an SPF colony (see later).

Gnotoxenic, axenic, polyxenic, oligoxenic

'Gnotoxenic' or 'gnotobiotic' (Table 4.6.2) refers to animals living in the absence of detectable microorganisms (axenic or germfree) or associated with a well-defined microflora in the digestive tract (which normally harbours the highest number of associated microorganisms), on the skin and in various cavities and organs (Table 4.6.1; [26–28]).

It is easy to understand the complexity of creating and managing a gnotoxenic colony. Technically, it requires one to start by creating an axenic (or germfree) colony, as described later. When the research purpose requires the use of this category of animals, they must be kept under strict bioexclusion conditions in order to maintain them in a sterile environment (see Figures 4.6.2 G–I below). For other applications, an artificially selected bacterial microflora is implanted on axenic individuals to create

a *polyxenic* or *oligoxenic* colony (depending on the number of associated microbial species). All operations and housing conditions require the use of germfree isolators, with a very strict management of bioexclusion conditions avoiding any source of microbial contamination: sterility of water, feed, various supplies and transfers, and high-efficiency air filtration [29, 30].

A classic technical limitation is the difficulty of controlling and detecting all categories of microorganisms. One example is the anaerobic microflora: testing for the absence of these very oxygen-sensitive agents requires specific and strictly anaerobic culture conditions both for isolation and for identification, when culture is even possible. With recent molecular biology techniques such as l6S ribosomal RNA-based polymerase chain reaction (PCR), temporal temperature gradient gel electrophoresis (TTGE) profiles, DNA microarrays and gene chips it is possible to assess the diversity and the composition of the dominant intestinal microflora and its stability over time, and to detect composition shifts related to environmental or dietary changes, to treatments, or to different life stages [31–33]. Since viruses are now included in the definition (originally gnotobiology exclusively addressed bacterial and parasitic agents), it is likely that most of those affecting rodents are still to be discovered. Some viruses are almost impossible to exclude, e.g. vertically or epigenetically transmitted microorganisms [34–36].

Agnotoxenic, heteroxenic

More commonly, and depending on the breeder's rederivation procedures, mice are kept under axenic conditions only for a limited period of time before being associated with a defined microflora as illustrated in Box 4.6.1 [11, 12, 16, 37], but are not kept in strict bioexclusion. As a consequence, the originally implanted microflora is enriched by microorganisms originating from the environment.

For practical and economic reasons, a vast majority of rodents used in biomedical research are not strictly gnotoxenic but rather *agnotoxenic* (i.e. with no positive and comprehensive definition of their resident microflora, including all infectious, opportunistic or commensal agents) and *heteroxenic* (i.e. with a resident microflora acquired in a laboratory environment).

Specific/specified pathogen free (SPF)

Agnotoxenic and heteroxenic animals deriving from axenic individuals are usually defined as 'specific pathogen free' or 'specified pathogen free' (SPF). Originally, 'specific' refers to the absence of 'species-specific' pathogenic agents, whereas 'specified' implies a predefined and specified exclusion list (Table 4.6.3).

After transfer of an implantation microflora, gnotoxenic animals are transferred into isolators or barrier rooms (Figure 4.6.1) where they will be progressively exposed to new environmental or undefined microorganisms (Table 4.6.2), hence the 'heteroxenic' definition [25]. As shown in Table 4.6.3, such animals are defined as SPF when they benefit from a health management and monitoring programme based on an exclusion list, i.e. a negative definition detailing all organisms being excluded. The exclusion list is predetermined (according to research and animal-related requirements) to include all undesirable parasites (uni- or multicellular), bacteria and viruses [19, 20, 38-42] and, for very specific applications, ecotropic retroviruses or non-conventional transmissible agents (prions).

Each breeder or user should establish, validate and guarantee a more or less restrictive SPF definition matching specific expectations and research activities, with a clear reference to the methods used to monitor the breeding colony and guarantee compliance with the exclusion list [19]. An SPF exclusion list should at least include the primary species pathogens, the zoonotic species and the major interfering agents, i.e. a list adapted according to the type of research activities envisaged. Commercial breeders generally adopt comprehensive health definitions in order to meet the global expectations of the research community. As mentioned already, the wording

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BOX 4.6.1

E x a m p l e s   o f   c o l o n i z a t i o n   m i c r o f l o r a

Original Schaedler microflora [37]

1. *E. coli* var. *mutabilis* (aerobic growth)
2. *Streptococcus faecalis* (aerobic growth)
3. *Lactobacillus acidophilus* (aerobic growth, and some on aerobic plates)
4. *Lactobacillus salivarius* (aerobic growth, and some on aerobic plates)
5. Group N streptococcus (strictly anaerobic growth)
6. *Bacteroides distasonis* (strictly anaerobic growth)
7. Unidentified clostridium (strictly anaerobic growth)
8. Unidentified fusiform-shaped bacterium, strain ASF 356 (strictly anaerobic growth)

Altered Schaedler microflora (ASF) [31]

1. *Lactobacillus acidophilus*, (Schaedler LI), strain ASF 360
2. *Lactobacillus salivarius*, (Schaedler LIII), strain ASF 361
3. Spirochete, strain ASF 457
4. *Bacteroides distasonis* (Schaedler 19X), strain ASF 519
5. Fusiform EOS@ bacterium (*Firmicutes*, *Bacillus-Clostridium* group), strain ASF 356
6. *Eubacterium plexicaudatum* (*Firmicutes*, *Bacillus-Clostridium* group), strain ASF 492
7. Fusiform EOS bacterium (*Firmicutes*, *Bacillus-Clostridium* group), strain ASF 500
8. Fusiform EOS bacterium (*Firmicutes*, *Bacillus-Clostridium* group), strain ASF 502

TABLE 4.6.3: Examples of heteroxenic mouse definitions (SPF and SOPF)

A. Example of SPF list

Bacteria and fungi	Parasites	Viruses
Mycoplasma pulmonis	All ectoparasites	Reovirus 3
Bordetella bronchiseptica	All endoparasites	Mouse hepatitis virus (MHV)
Pasteurella multocida		Sendai virus
Pasteurella pneumotropica		Pneumonia virus of mouse (PVM)
Corynebacterium murium		Theiler virus (GD VII)
Streptococcus pneumoniae		Hantaan virus
Salmonella		Lymphocytic
		Choriomeningitis virus (LCMV)
Citrobacter freundii		Mouse norovirus (MVN)
β-haemolitic Streptococci		Ectromelia virus
Clostridium piliforme		Minute virus of mouse (MVM)
(Tyzzer's disease)		Mouse parvovirus 1 and 2 (MPV-1 and 2)
Streptobacillus moniliformis		Mouse adenovirus 1 and 2 (Mad-1 and 2)
Helicobacter hepaticus		Mouse rotavirus
CAR bacillus		Mouse thymic virus (MTV)
Dermatophytes		Mouse cytomegalovirus (MCMV)
		K virus, polyomavirus
		Lactic dehydrogenase virus (LDHV)

B. Example of SOPF: SPF definition plus the following microorganisms:

Staphylococcus aureus[a]

Proteus[a]

Pseudomonas aeruginosa[a]

Klebsiella pneumoniae/oxytoca[a]

Pneumocystis carinii

Helicobacter hepaticus/bilis

Corynebacterium bovis

Helicobacter

All protozoans

Any microorganism associated to lesions as a causative agent in immunodeficient lines

Experimentally based extension of the SPF virus list

C. Example of predefined 'policies' after contamination of SPF colonies

Breeding colonies	Experimental colonies
Immediate stop of shipments and initiation of recycle	Invalidation of all studies and immediate recycle
Shipment upon documented acceptance and planned recycle	Limited/selective study invalidation and planned recycle
Reporting only, no recycle	Reporting only, no recycle

Pathology: absence of lesion and clinical signs
[a] These frequent opportunistic agents are also used as indicators to detect a breach in isolator barrier or aseptic handling of cages and/or animals, which may result in other opportunistic contaminations.
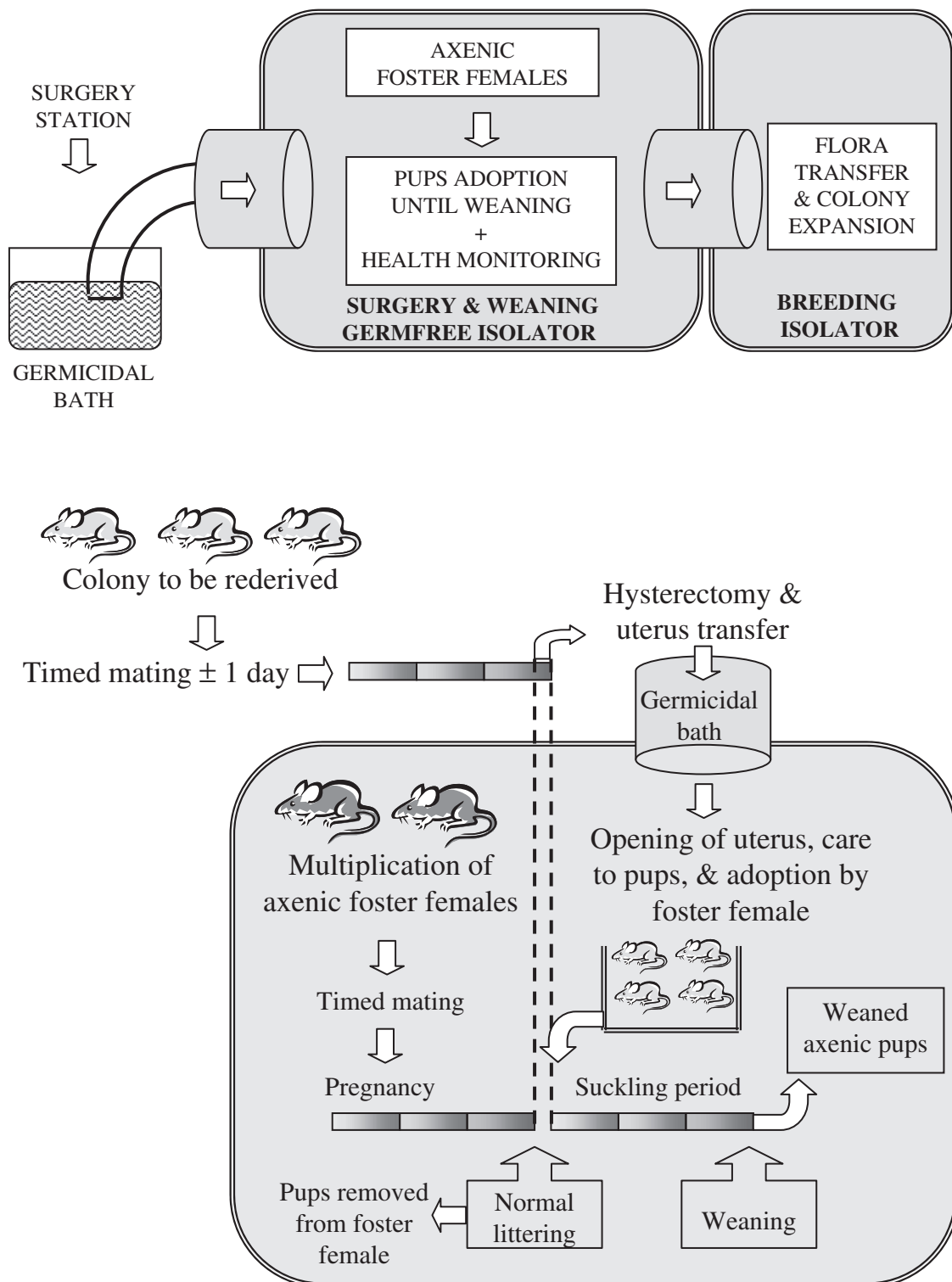
Figure 4.6.1 Aseptic hysterectomy.

'*specific* pathogen free' refers originally to the screening and exclusion of *species*-specific pathogens. Considering the progressive extension of the definition to include an increasing number of purely interfering agents [42-48], it is also common to refer to '*specified* pathogen free' colonies in order to stress the need to associate this wording with an accurately defined exclusion list.

At this stage, when defining the health standard and monitoring techniques, it is critical to keep in mind the difference between the *screening* list and the *exclusion* list. The first can be purely informative, i.e. a simple monitoring of the resident microflora in order to assess the global efficiency of the bioexclusion system. The second will generate, in the case of a positive result, a decision to invalidate experimental results obtained using such animals and for the breeder, to recycle the breeding colony. In both cases, in addition to the research impact, ethical, practical and economic consequences are generally severe [19, 49-51].

Some commercial breeders, mainly in the USA, have registered SPF 'trademarks' such as VAF (for 'virus antibody free', focusing on the absence of viruses which are mostly screened by serological methods) or MPF (for 'murine pathogen free'). Whatever the wording, it may be confusing when it is not associated with detailed information about:

1. The rederivation methods used to create the breeding colonies
2. The health management policy of isolator or barrier-maintained colonies
3. The screening list, with frequencies of testing, sampling and testing methods
4. The fully detailed exclusion list, with the recycle policy for each agent, e.g. 'immediate recycle' for pathogenic and major interfering agents, 'planned recycle' for minor interfering agents (i.e. with a very limited impact on experimental projects or research activities) or 'no action' for the purely commensal microorganisms (Table 4.6.3).

Extended SPF definitions

The creation of immunocompromised or fragilized mice generated the need for additional bioexclusion techniques (Figures 4.6.2 A–C, G–I, 4.6.3 and 4.6.4 A–D; Table 4.6.4; Boxes 4.6.2 and 4.6.3). These are necessary not only to exclude the specific pathogens but also an extended list of opportunistic agents (mainly human-borne and environmental bacteria), potentially able to cause infection, either locally (subcutaneous or preputial abscesses, conjunctivitis, etc.) or systemically (septicaemia and 'wasting syndrome', respiratory infections, reduced lifespan or breeding life). An *opportunistic* microorganism or pathogen is defined as a microorganism (bacterial, viral, fungal or protozoan) that is normally commensal in a healthy and non-immunocompromised host, but can cause a disease when the host's resistance is abnormally low: newborn or aged individuals, malnutrition, concomitant infection or disease, immunosuppression or chemotherapy treatment, genetic predisposition (including immunocompromised models), skin or mucous damage (e.g. wound, catheter, burn), antibiotic treatment, invasive medical procedures, pregnancy, immunodeficiency virus infection. Common opportunistic pathogens in humans are *Candida albicans*, *Staphylococcus aureus* and *Pseudomonas aeruginosa*.

In other cases an extended SPF standard is used to avoid experimental interferences with more sensitive or fragilized strains [21] or to investigate genetic-microbial interactions in genetically modified mice [52, 53]. In order to emphasize the extension of the exclusion list, i.e. of the classical SPF definitions, some breeders have described such colonies as 'isolator reared', in opposition to 'barrier-reared.' Obviously, it is much more accurate and relevant to rely on a definition based on the exclusion list rather than on the bioexclusion system, such as SOPF (specific and opportunistic pathogen free) or RF (restricted flora) (Table 4.6.3).

When monitoring these 'opportunistic-free' colonies, for economic and practical reasons as well as to avoid fastidious routine screenings of all human-borne and environmental bacteria, the selection and use of a set of indicator agents can be a very convenient alternative. Each indicator should ideally be representative of a specific contamination route or the most common causes of barrier breach (due to personnel, supplies, water, etc.). Table 4.6.3B illustrates an example of a SOPF definition including a set of marker opportunistic bacteria selected because of their frequency in the environment or on humans, their common detection in barrier-maintained mouse colonies, their easy detection and identification by routine bacteriological techniques
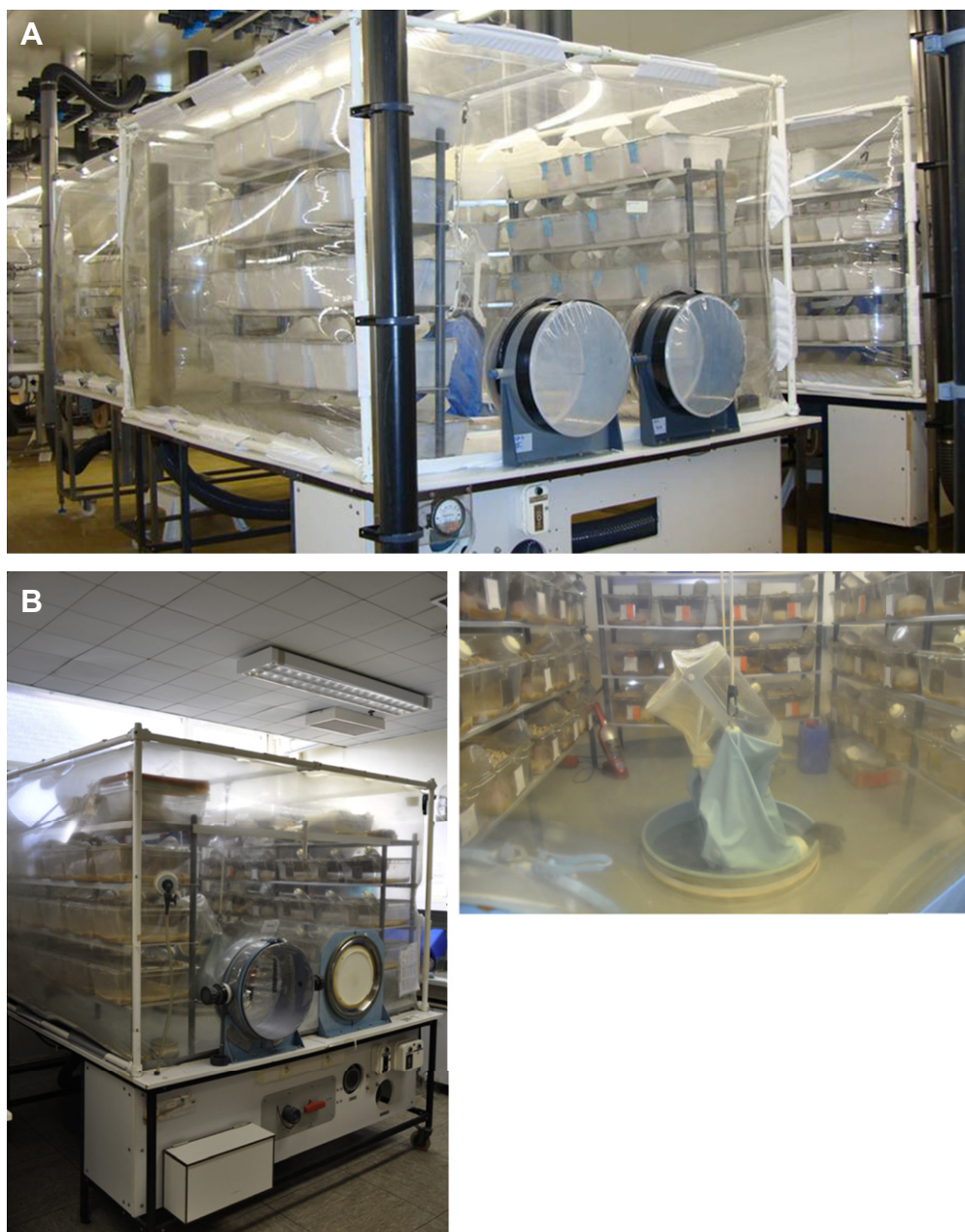
Figure 4.6.2 Isolator technology. A. Large, flexible-film, positive-pressure, half-suit breeding isolator equipped with two standard transfer ports (photo credit: Janvier). B. Left: Large, flexible-film, positive-pressure, half-suit breeding isolator equipped with one standard transfer port and one rapid-transfer door. Right: Detail of half-suit (photo credit: Institut Pasteur Lille).

(without having to sacrifice the animals) and their potential adverse effects on immunodeficient mice.

In addition to these indicators, other agents may be more directly involved in lesions observed in immunodeficient colonies or cause very specific interference with research. Of course, any opportunistic agent associated with a lesion as a causative agent in immunocompromised strains (alone or in synergy with other microorganisms) should be considered for inclusion in a SOPF exclusion list [21, 54, 55].

Microflora categories

The microflora associated with metazoan host organisms is normally very complex, highly

Figure 4.6.2—**Cont'd**. C. Left: Large, flexible-film, positive-pressure, half-suit breeding isolator. Right: Transfer isolator, ready to be connected through the transfer sleeve equipped with a rapid-transfer door. (photo credit: Institut Pasteur Lille). D. Transfer container equipped with rapid-transfer door and two filter openings allowing steam sterilization (photo credit: Institut Pasteur Lille). E. Rigid/negative pressure standard isolator, usable for biocontainment or quarantine (photo credit: Institut Pasteur Lille). F. Peracetic acid vaporizer for isolator sterilization (photo credit: Aeroflux France Merial R & D).

variable and incredibly rich: a normal body, and particularly the intestinal tract, contains up to 10 times more bacteria than endogenous cells [27]. The commensal microflora is both environment- and species-dependent. When defining a health standard (exclusion and screening lists) and a monitoring programme, it is essential to be aware of the various categories of microorganisms colonizing the metazoan host.

Figure 4.6.2—Cont'd. G. Rigid-body, positive-pressure standard fixed isolators, on two levels, for small breeding projects, with standard connection port (photo credit: Taconic). H. Rigid-body, positive-pressure standard movable isolators, for breeding projects, with rapid-transfer door (photo credit: Institut Pasteur Lille). I. Flexible-film, positive-pressure, standard movable isolators, on two levels, for small breeding projects, with standard connection port (photo credit: Janvier).

Figure 4.6.3 Full-barrier room. A. Barrier area fittings: rodent shipping chute with communication window. (C) Left: Breeding barrier side. Right: Shipping room side (photo credit: Janvier). B. Barrier area fittings. Left: hydrogen peroxide chamber. Right: Steam autoclave (photo credit: Sanofi R&D).

Commensal or resident microflora

This is defined as the normal, stable microflora found associated with a healthy host organism. The commensal microflora can include the following categories.

IMPLANTATION MICROFLORA

After evidencing the success of a rederivation, germfree individuals are generally exposed to a selected microflora defined as the *implantation microflora* [11, 37, 56]. After this implantation step, depending on the environmental conditions, the original commensal microflora will start to become enriched with various microorganisms originating from the environment and humans (*microflora drift*).

SYNERGISTIC/BARRIER MICROFLORA, PROBIOTICS

A *synergistic* microflora can be defined as one having a demonstrable benefit to the health of the host, i.e. by improving digestion, metabolism, immunity or by causing an antibiosis phenomenon, i.e. a biological interaction between the host commensal microflora organisms and other undesirable microorganisms, which are
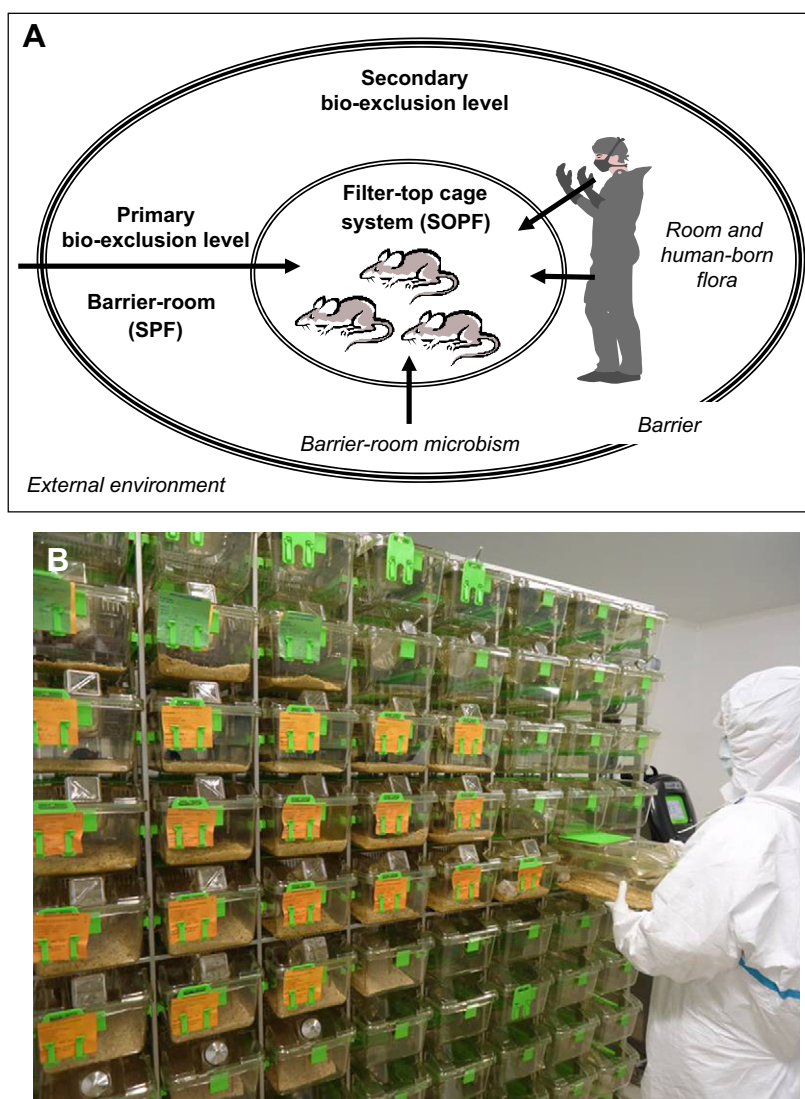
Figure 4.6.4 Filter-top cage systems used for SOPF mice. A. Two-level bioexclusion filter-top cage system. The barrier-room barrier excludes external contaminants to guarantee an SPF status environment. The second barrier (at cage level) complements the first and also excludes barrier-room opportunistic agents (including human-borne) to maintain SOPF status. B. Static/passive ventilation filter-top cages in a full barrier room and personnel equipment, allowing the exclusion of specific pathogens and opportunistic agents (including human-borne) to maintain SOPF status (photo credit: Janvier).

detrimental to the latter. As an example, intestinal or skin bacteria ecologically adapted to the host organism may prevent colonization by other less adapted microorganisms, including pathogens and opportunists, and contribute significantly to the stability of a healthy condition. Such a microflora 'barrier' effect is one of the reasons why breeders should pay particular attention to the implantation microflora when creating isolator security colonies.

At the time of this early intestinal microflora implantation, or at a later stage, it might also be useful to consider using *probiotic* microorganisms, defined as 'live microorganisms, which if administered in adequate amounts, confer a health benefit on the host [57-59]'. Most often, the 'probiotic' bacteria belong to the *Lactobacillus* or *Bifidobacterium* genus, e.g. *Lactobacillus acidophilus* and *Bifidobacterium bifidus* or, even if their benefit is more controversial, to the yeasts, e.g. *Saccharomyces boulardii.*
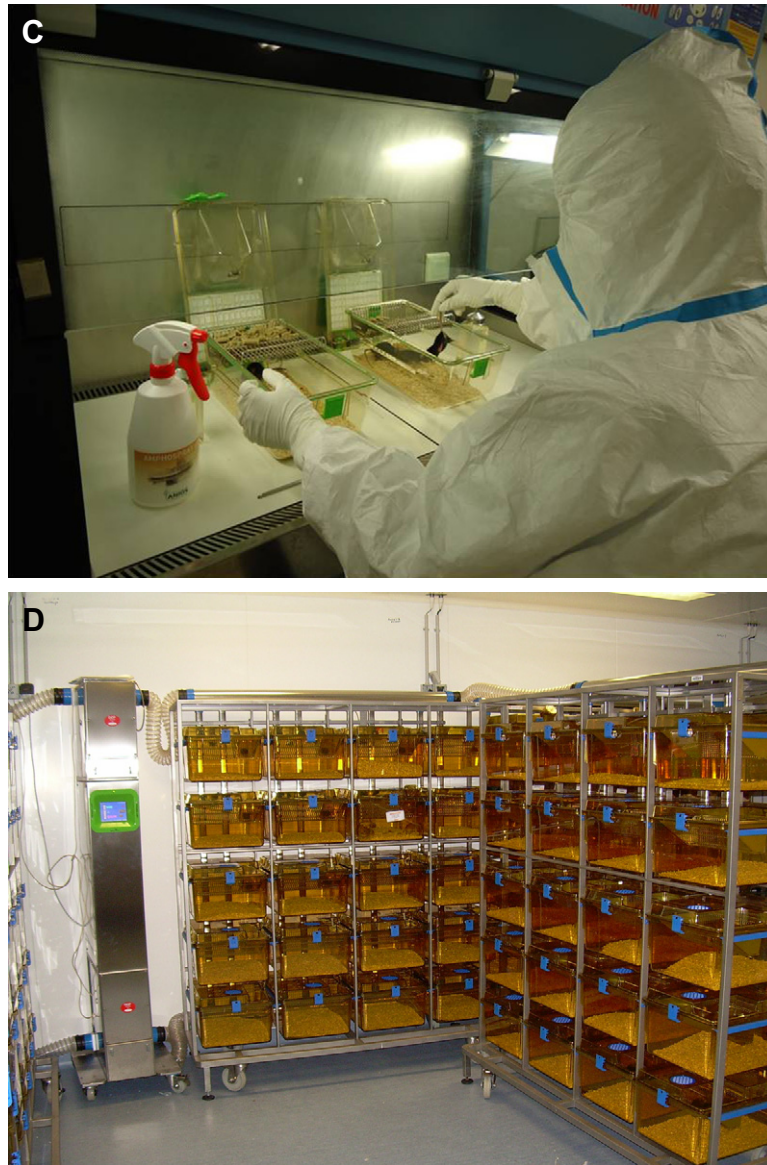
Figure 4.6.4—**Cont'd**. C. Aseptic cage opening and handling in laminar-flow working cabinet, in a full barrier-room environment to maintain SOPF status (photo credit; Janvier). D. Right: Ventilated filter-top cage racks in a full barrier room, allowing the exclusion of specific pathogens and opportunistic agents (including human-borne) to maintain SPF or SOPF status. Left: The in/out ventilation and air filtration unit (photo credit: Merial R&D).

Probiotics should not be confused with *prebiotics*, which are defined as 'non-digestible food ingredients' (e.g. oligo- and polysaccharides) that stimulate the growth and/or activity of bacteria in the digestive system and are beneficial to the health of the body', i.e. that are used as a substrate by microorganisms already in the host's intestine [60, 61]. When probiotics and prebiotics are mixed together, they form a *synbiotic*. Therapeutic or prophylactic use of probiotics and/or prebiotics may help to stimulate the production of IgA antibodies and macrophage activity phagocytosis, to decrease the number of inflammatory mediators and to decrease intestinal pH and thus the growth and activity of undesirable intestinal bacteria [3, 62]. However, it appears that the composition of the microflora of most individuals is quite

TABLE 4.6.4: Comparison of two bioexclusion systems usable for SOPF status

Characteristics	Isolator	Filter-top cage system (static or ventilated cages)
Capacity limitation	Isolator capacity	Room capacity
Handling procedures	Difficult	Easier (in laminar-flow cabinet)
Fixed and variable costs	Higher	Lower (static) or higher (ventilated)
Capital expenditure	Higher	Lower (static) or higher (ventilated)
Adaptability/versatility	Lower	Higher
Maintenance of SOPF status	Yes	Yes
Maintenance of germfree status	Yes	No
Biocontainment of contaminants	Yes	Less reliable, cage-type dependent
Health monitoring (sampling)	Easy	Complex

stable, especially in the large intestine. It is easier to modify the microflora of the small intestine, which harbours a much lower number of bacteria. Influencing an established small-intestine microflora may require 10^4-10^5 bacteria per dose and repeated doses, with no guarantee of lasting effect. A much higher dosage is required for the large intestine.

BOX 4.6.2
Barrier bioexclusion processes

Autoclave cycles

Theoretical conditions:

15 min at 121 °C
10 min at 126 °C
3 min at 134 °C

In practice, for bioexclusion:

20 min at 121 °C
10 min at 134 °C

Application in rodent units (examples of 'plateau' temperatures and durations)

Feed and bedding: 30 min at 107 °C
Stainless steel equipment: 10 min at 134 °C
Plastic cages: 10 min at 118 °C
Waterbottles (filled): 10 min at 115 °C (plateau)

Gamma radiation (applied to feed and bedding)

Type of radiation (gamma rays)
Vacuum packaging
Exposure time/dose adapted to type and density of irradiated product

Doses for:

'Germfree' rodents: 40–50 kGy (5 Mrad)
'SOPF' or 'SPF' rodents: 25 kGy (2.5 Mrad)
Heat-sensitive feed formula: 10 kGy (1 Mrad)

Other types of barrier processes

Chemical disinfection (peracetic acid, hydrogen peroxide, chlorine dioxide-based products):
Isolator or barrier spray port
Chemical lock or dunk tank
Air: prefiltration (opacimetric/gravimetric filter) and filtration (HEPA filter)
Water: autoclaved in bottles, chlorination, acidification, prefiltration, filtration at 0.1 or 0.2 μm, ultraviolet treatment and combinations of these processes
Gowning locks, with water and/or air shower

BOX 4.6.3
Example of bioexclusion principles in a filter-top cage system ('static' or 'ventilated' cages)

- Dedicated full-barrier unit
- Exclusive introduction and housing of SOPF rodents
- Introduction of animals after rederivation and adequate health monitoring results
- Exclusive use of filter-top cages (no open cages in the same unit)
- All 'open cage' operations in laminar-flow work-stations (cage changes, animal handling, etc.)

- Aseptic handling, with regular glove disinfection
- Adequately skilled and trained staff
- Autoclaved caging equipment (cage, lid/feeder, bottle, enrichment items)
- Feed: 2.5 Mrad irradiation or autoclaved
- Bedding: 2.5 Mrad irradiation or autoclaved
- Sterilized drinking water: 0.1 μm end-filter (daily disinfected) or autoclaved

MICROFLORA AND RESEARCH MODEL DEFINITION

As well as having a demonstrated benefit to the host health or general conditions, the intestinal microflora can also influence the host phenotype, especially with genetically engineered mouse models [28, 53]. Such a potential influence on the definition and sensitivity of the animal model or on the experimental outcome may have critical importance for the research applications. A first example is the quality and composition of the intestinal microflora, which is a key regulating factor of host immune response, both locally (by activating, modulating and regulating the intestinal immune system or mucosal homeostasis) [33] and systematically (by modulating different subclasses of IgG) [62-65]. Other examples can be found in various research fields, such as obesity [66], gut inflammatory diseases [67, 68], cancer [69] and regulation of energy metabolism [70].

OPPORTUNISTIC MICROFLORA

As defined earlier, some components of the commensal microflora not belonging to the SPF exclusion list can be defined as opportunistic agents that are able to cause either pathological conditions or experimental interference under special conditions [21, 71] such as immunodeficiency, stress, poor environmental conditions or fragilization (disease or ageing models). It is the responsibility of the breeder and of the investigator to decide on their relevance and add them to the exclusion list or the screening list [18]. Agents interfering with a specific research application or playing a synergistic role when associated with other viruses or other bacteria

should be addressed in a similar way. The concepts of both 'interfering' (see later) and 'opportunistic' agents are relative, varying with the research environment and sometimes with variable documented evidence in peer-reviewed literature [22, 53, 54, 72].

TRANSIT MICROFLORA

Unlike the stable commensal or resident microflora, some environmental or human-borne microorganisms can be detected over a short period of time before disappearing or reaching a non-detectable level. The current FELASA recommendation is to stop reporting these agents when they are not detected after 18 months and six successive quarterly screenings [19].

PATHOGENIC AGENTS

All viruses, bacteria, fungi, protozoans and metazoan endo- and ectoparasites known as major or minor pathogens should be listed as such in the SPF exclusion list (Table 4.6.3).

INTERFERING AGENTS

It is the rule to add to the primary SPF list all additional microorganisms known or suspected to cause experimental interference in the principal research fields (Table 4.6.3) [18]. Most murine viruses are purely interfering agents.

ZOONOTIC AGENTS

To avoid health risks to personnel, the zoonotic risk should be managed in a very specific way. The main concern, relying on biocontainment measures, is to prevent any possibility of accidental transmission of these animal-borne

human pathogens. Their absence should always be guaranteed by the strict implementation of biosecurity and health monitoring programmes. Any suspicion of contamination should be immediately reported and addressed. A classic example is the mouse lymphochorio-meningitis virus (LCMV), an arenavirus causing severe flu-like symptoms in humans. A relevant health monitoring programme based on serology is the only way to guarantee its absence, even if transmission to humans and clinical cases are rare, especially with barrier-bred laboratory mice.

HEALTHY CARRIERS

It is critical to bear in mind that mouse pathogenic, parasitic or zoonotic agents (such as LCMV) can also be found in healthy carriers, i.e. individuals not displaying any clinical sign or lesion, hence the importance of health monitoring.

ANTIBODY-FREE ANIMALS

Although it is not common with mouse models, some research or testing applications (e.g. with biologicals) may require the provision of 'antibody-free' animals in addition to the classical SPF requirements, i.e. animals free from predefined antibodies. When relevant, such a requirement may have to be added to the health specifications.

MICROFLORA AND RESEARCH MODEL DEFINITION

Because an animal model is a complex biological system, it is necessary to consider each of its components in order to define and obtain the required standard, then to make sure that each parameter is adequately monitored over the entire duration of the study.

These components are generally divided into three categories:

1. The *intrinsic animal definition*: species, sex, age, genetic characteristics
2. The *health definition* and *microflora*, which is environment-related and specifies the microbiological status of the animal, including the associated microflora, parasites, etc
3. The *living environment* (both micro- and macro-environment, i.e. at cage and room level), which includes parameters such as housing conditions (e.g. temperature, humidity, ventilation, lighting, noise level), the caging system (group or single housing, type of cage or

enclosure, floor and/or bedding quality, type of environmental enrichment), feeding and nutrition (composition, consistency, quality and quantity of feed, type of water supply), care, nursing and handling practices, etc.

Like the other components, the microflora and health definition strongly influence the relevance, quality and consistency of the research model definition, the quality of the study and the experimental outcome. Because of the interference potential of undesirable microorganisms and parasites (which are model- or research-dependent), the resident/commensal microflora may also be considered as a component of the research model definition (see the section 'Commensal or resident microflora') [53].

Because all research model components interact with each other and are relevant to the nature of the research, a comprehensive understanding and review of requirements is necessary before the specifications of the animal model are established.

Rederivation techniques

When a mouse strain is confirmed as 'contaminated', i.e. not complying with the SPF exclusion list, several techniques can be used to eliminate non-acceptable agents or, to use the common wording, to *rederive* the strain. The selection of a technique will depend on the one hand on the number and the nature of the agent(s) to eliminate and, on the other hand, on the expected use of the animals. The term 'rederivation' is generally used for the most efficient and sophisticated techniques such as aseptic hysterectomy or embryo transfer. Other techniques are also briefly described because they may help to rescue a breeding colony, when no other alternative is acceptable for time, budget or technical reasons. Of course, they do not provide the same level of reliability and efficiency as aseptic hysterectomy and embryo transfer, which rely on a very simple (but not absolute) principle: that the reproductive tract is sterile during pregnancy until the onset of delivery and opening of the cervix. This principle is valid for most, but not all parasitic and infectious agents, and depends on the risk of 'false' vertical transmission, due to a contamination during the rederivation process [73] or of

'real' vertical transmission (transfer before or after embryo nidation and placentation). These limitations will be addressed later.

Non-medical/non-surgical methods

QUARANTINE AND SPONTANEOUS 'ERADICATION'

Some purely opportunistic bacteria originating from another species (mainly humans) and having a poor ecological tropism for the mouse may spontaneously 'disappear' from the micro-flora, i.e. become at least undetectable by the usual techniques over a long period of time. However, this phenomenon is unreliable, has unpredictable results and is mainly reported in larger species. The colony has to be kept under strict biocontainment, to avoid the spreading of the agent and the contamination of neighbouring naive individuals, until clear evidence of negativity is obtained via repeated screenings over at least 6-12 months [19].

QUARANTINE AND ERADICATION AFTER SEROCONVERSION

This 'burnout' procedure is valid only in non-breeding colonies. The most common example in mice relates to the frequently isolated mouse hepatitis virus (MHV) strains. Being highly infective (the rate of infection approaches 100%), they spread rapidly and disappear when the entire colony has recovered from the infection and has seroconverted, if the colony is small, mature and immunocompetent, and when there is no introduction of naive mice and no breeding activity [74, 75]. Depending on the exposure level of mice to the virus and on various other factors, the whole process takes from a few weeks to several months. The only reliable indicator of success is the absence of infection when naive mice are introduced into the colony. With MHV, one success factor is also its low viability in the environment. In addition to stringent isolation of the colony, it is critical to eliminate any potential chronic carriers and excretors such as immunocompromised or naive individuals. All introduction of external animals should be prohibited during the burnout process, as well as any breeding, because naive individuals and immunologically immature pups are easily infected and keep spreading the virus.

QUARANTINE, SELECTION AND TIME

This process may be used for a variety of agents (one at a time), which do not immediately infect all individuals in a colony. It requires a sufficient number of mice in the contaminated colony and a highly sensitive screening method for live mice. It is based on individual separation in micro-isolator cages and procedures preventing inter-cage cross-contamination. Each cage is screened for the presence of the agent and followed by immediate culling of these animals if the result is positive. If enough animals are found negative after repeated screenings, there is a chance that it may be possible to restart a negative colony from their cages. One important drawback is the size reduction of outbred breeding colonies, potentially causing a prohibitive 'genetic bottleneck' effect (critical loss of genetic polymorphism). A variant for eradication of helicobacter has been described, using rapid neonatal transfer of pups to helicobacter-negative foster mothers on the first day of life [76].

Medical treatment

Most internal and external parasites can be eliminated using a well-designed and conducted global anti-parasitic treatment targeting all stages of parasitic development, e.g. ivermectin for *Syphacia muris* and *S. obvelata* or mites [77-80]. In addition to repeated dosage, this may also require parallel drastic hygienic measures to eliminate all eggs from the environment, because parasite eggs are highly resistant to the usual disinfectants. Again, the colony should be carefully isolated during this treatment until repeated screenings demonstrate the success of the process [81].

Aseptic hysterectomy

Technical details about aseptic hysterectomy are available in publications, e.g. [30]. A classical method can be summarized as follows (Figure 4.6.1). Future foster mothers originating from a germfree, gnotoxenic or SPF colony are time-mated in order to become pregnant in synchrony with contaminated 'donor' females. Matings are scheduled such that the aseptic hysterectomy can be carried out on 'donor' females a few hours before their normal littering time (i.e. cervix opening) and a

few hours after the foster females' littering. Hysterectomies are carried out after euthanasia of the donor females in order to introduce the clamped gravid uterus aseptically into a sterile isolator through a germicidal bath. Then, after uterus incision, the pups are removed, warmed up and substituted for the foster mother's litter. Isolator health screening is conducted at least once by weaning age before validating the success of the process. Repeated checks are highly recommended at intervals of a few weeks. Depending on the breeding objective and genetic issues (outbred vs inbred) the total number of rederived females may vary significantly. Using germfree foster mothers for this process is technically demanding, but has the major advantage of easily and quickly detecting bacteria contaminations due to a breach in the aseptic process.

Embryo transfer

Technical details about embryo transfer are available in specialized handbooks [82] and other publications [83-86] and in Chapter 4.7. In a few words, contaminated donor females are mated in synchrony with SPF or SOPF recipient females mated to vasectomized males to become pseudopregnant. Technical variants of this process are the use of superovulation or *in vitro* fertilization. Donor females are anaesthetized in order to collect two-cell embryos which are washed and morphologically selected before implantation into the oviduct of synchronized/pseudopregnant recipient females. After transfer into an isolator and weaning of the pups, the recipient females are screened for the presence of contaminants in order to assess the success of the rederivation, with specific focus on all undesirable agents identified before rederivation. One advantage of embryo transfer when compared with hysterectomy is the avoidance of postimplantation vertical transmission [87].

Aseptic hysterectomy or embryo transfer associated with other procedures

Eliminating a vertically transmitted agent such as LCMV or avoiding intrauterine contamination with agents like *Mycoplasma pulmonis* [73] depends on the nature of the contaminant and may require combining the use of classical rederivation techniques with separation of individuals

and selection by serological screening, embryo washing, antibiotic treatment, etc. To be reliable, several screenings should be conducted before validating the success. One difficulty is the vertical transmission described for agents including endogenous ecotropic retroviruses such as murine leukaemia virus (MuLV) [34, 36].

Genetic issues when rederiving an outbred colony

The number of pairs and origins used to obtain the pups composing the next generation, after rederivation, are key issues with outbred stocks. It is critical to avoid a genetic bottleneck [88]. Multiple rederivation series should be conducted in order to obtain progeny from the highest number of pairs (e.g. 80 pairs), each pair contributing equally to the next generation (see the section 'Genetic standards and breeding systems').

Bioexclusion, biocontainment and health monitoring

The maintenance of a defined health standard depends on the proper implementation of a bioexclusion programme, i.e. methods and resources used in a consistent and integrated programme aiming at avoiding contamination of the breeding colony, relying on all required resources (design of the facility, fittings, equipment, management, qualified personnel). Mice of an adequate health standard are maintained in bioexclusion conditions in order to exclude undesirable biological contaminants or sources of contamination due to the environment, fomites, humans or other animals. The strictest conditions are required to maintain a germfree environment, which can only be achieved in a positive-pressure isolator, with transfer procedures and supplies consistent with the expected sterility level (Figure 4.6.2).

Bioexclusion equipment and procedures used for microflora-associated colonies (isolators, a filter-top cage system or barrier rooms; Tables 4.6.4, Boxes 4.6.2 and 4.6.3, Figures 4.6.4 A-D) are less demanding than using a germfree isolator. A comprehensive health monitoring programme should be designed and duly implemented (see Chapter 4.4). Other

mice that are microbiologically undefined or contaminated should be kept under biocontainment conditions in order to avoid the dissemination of their undesirable or hazardous microflora components to cleaner animals, directly or through fomites or human contact. When the aim of biocontainment is to protect operators and/or the environment from a biological hazard, we speak of *biosafety* programmes and procedures. Various specific regulations and guidelines address such biocontainment situations for infectious agents and for genetically modified organisms (classification of pathogens, level and type of biocontainment, use of biosafety cabinets and waste management). In practice, animal resources and equipment should allow the parallel use of biocontainment (for reception, quarantine and health screening of contaminated non-microbiologically defined animals) and bioexclusion (for rederivation and subsequent housing of 'clean' animals).

Colony termination and recycling policy

Examples of situations illustrating when breeding colony recycling has to be considered include the following:

1. In case of a major microbial contamination (i.e. with an agent on the exclusion list)
2. Less urgently, in case of opportunistic contamination(s) causing a drift of the colony microflora associated with clinical signs or lesions
3. After a genetic contamination of an inbred colony
4. When different stocks and strains have to be reorganized and reallocated to different barrier rooms.

Breeding techniques

A prerequisite for managing a breeding activity is to have a good understanding of reproductive biology as well as genetics and strain/stock definitions. Housing, maintenance and nutrition are other critical factors in breeding management,

as well as access to health and genetic monitoring resources. Assisted reproduction techniques and cryopreservation may also be essential for some projects: they are addressed in Chapter 4.7.

Genetic standards and breeding systems
Definitions

An *outbred* stock is a population with a large pool of allelic forms [89, 90], defined by its allelic variants and their frequencies. Each individual from a population is like a random allelic sample and differs from other members of the population, even if originating from the same allelic pool. When adequately managed, animal breeds can be defined as closed populations. Such populations are generally characterized by common phenotypic traits. Individuals can only be defined as originating from a genetically well managed and identified population, sharing the population phenotypic characteristics. A careful genetic management system is necessary over time to minimize genetic drift and to avoid increasing inbreeding coefficient [89, 91-93].

An *inbred* strain, or line, is defined as a breeding group originating from a common ancestor pair and having an inbreeding coefficient over 98.6%, which corresponds to a minimum of 20 generations of brother × sister mating. Most inbred strains are mice and rats (some are also available for chickens and guinea-pigs).

The *inbreeding coefficient* of a strain is the percentage of alleles which are fixed as homozygous. Lines are managed so as to maintain or increase this coefficient, as well as to avoid genetic contamination and genetic drift into substrains. Inbred animals [94, 95] belonging to the same strain are homozygous and isogenic (i.e. possessing the same genotype). They are histocompatible and accept tissue grafts from each other without any rejection. This particularity is of value in developing models of cancer based on syngenic grafts, i.e. various types of tumours originating from inbred strains which are grafted on to histocompatible individuals.

The first filial generation (*F1 hybrids*) are offspring resulting from a cross-mating of distinctly different parental types. The term is

commonly used to refer to cross-mating between two species (e.g. mules, which are F1 hybrids between horse and donkey).

F1 hybrid rodents are produced by crossing individuals of two different inbred strains. In this case, each strain provides one of the two allelic forms of each locus. Examples are B6D2F1 (produced by mating a C56BL/6 female, abbreviated to B6, and a DBA/2 male, abbreviated to D2) or D2B6F1 (obtained by mating a D2 female with a B6 male). F1 hybrids produced by the same mating are isogenic and heterozygous at all loci. In consequence, they are genetically and phenotypically uniform, as inbred strains are, but as they are no longer homozygous, rather than being subject to 'inbreeding depression' they benefit from *heterosis*, also known as 'hybrid vigour'. They are generally robust animal models, highly valuable for many research applications. F1 hybrids can be generated for as long as the two parental strains exist. The F2 offspring produced by mating F1 mice are no longer isogenic, but are a random mixture of alleles from both parental strains.

Foundation colonies

The goal of foundation colonies is to serve as a genetic reference in order to guarantee long-term genetic stability (i.e. to minimize the genetic drift) over generations and intercolony homogeneity when several breeding colonies of the same stock or strain are managed in parallel (Figures 4.6.6 and 4.6.8).

For outbred stocks, the objective is to ensure that the population remains as constant as possible in all characteristics [96-100], i.e. to maintain the highest level of heterozygosity and to keep the population's original allelic forms and frequencies as stable as possible over generations [89, 90, 93, 101-103].

For inbred strains (Figure 4.6.8A, B), the primary purpose is to preserve strain isogenicity and a maximum inbreeding coefficient [94, 104]. This last goal is achieved by avoiding the three main causes of divergence in inbred strains: genetic contamination, mutation/fixation [105] and residual heterozygosity.

By definition, for either inbred strains or outbred stocks, the foundation or reference

colony is unique. When it cannot produce enough animals for experimental use, one or more production colonies are created. In consequence, a genetic management programme should address:

1. The genetic management and breeding system in the foundation colony and its related production colonies
2. Migrations and interactions between foundation and production colonies (Figure 4.6.6 and Figure 4.6.8).

Inbred and outbred management systems are totally different. Factors which may rapidly affect the genetic quality of a population are [94, 99, 101]:

1. The introduction of external breeders, accidentally or purposefully (inbred and outbred)
2. The population size and 'founder' effect (outbred)
3. The type of mating scheme (inbred and outbred)
4. Mutations and their fixation (inbred and outbred)
5. Conscious or unconscious selection (outbred).

Outbred stocks

The long-term goals of an outbred foundation colony and its genetic management system are:

1. To maintain maximum genetic variability/polymorphism
2. To guarantee production colonies genetically similar to the foundation colony and to each other, i.e. their genetic uniformity whatever their number and location
3. To avoid the introduction of new allelic forms
4. To maintain stable allelic frequencies from generation to generation
5. To minimize the increase of inbreeding from generation to generation.

General recommendations for the long-term maintenance of genetic polymorphism are listed in Box 4.6.4 [101], as well as an example of practical application.

When creating a new outbred colony one should guarantee the highest level of genetic polymorphism, using a high number of pairs from several independent origins having the least possible common ancestorship.

BOX 4.6.4
Genetic management of an outbred stock foundation colony (guidelines and example of practical implementation)

Long-term maintenance of genetic variability/polymorphism (based on [95], [101], [106])

- Closed colony, no introduction of new breeders (i.e. foreign allelic forms)
- Population size (number of breeding pairs, males and females) as large as possible
- Equal chance for each individual of participating to the next generation (random mating)
- No selection criteria (i.e. loss of allelic forms)
- High interval between generations (to slow down genetic drift)
- Mating scheme using subgroups to minimize risk of inbreeding
- Prevention of mutations is not under control

Example of application in rodent colonies: genetic management system of a foundation colony

- Closed colony, no introduction of new breeders
- Minimum 200 permanent breeding pairs
- Overlapping generations, constant mean age of colony
- Fixed reproduction life: 30 weeks
- Mating scheme: four-unit-Robertson's mating scheme
- Future breeders from fourth litter, maximum 3 future breeders per pair
- Minimum 84% of pairs contribute to the next generation
- No selection to increase productivity

BOX 4.6.5
Outbred foundation colony simulation of genetic drift/ inbreeding increase (simplified formulas)

A. Increase of homozygosity over generation in an outbred population

(Wright's formula in [101])

$$F_x = S\left[0.5^{n+n'-1}(1+F_a)\right]$$

where

F_x is the inbreeding coefficient of the individual x;
S is the symbol for sum if repeated inbreeding towards the same or several common ancestors occurs;
n is the number of generations between the individual x and the common ancestor from the father's side y, without adding x and y;
n' is the number of generations between the individual x and the common ancestor from the mother's side y, without adding x and y;
F_a is the inbreeding coefficient of a common inbred ancestor.

B. Increase of inbreeding per generation [96]

Mating scheme	Inbreeding increase per generation (%)
Random mating	I.I. = 1/8 N_{ma} + 1/8 N_{fe}
Maximum avoidance mating	I.I. = 1/16 N_{ma} + 1/16 N_{fe}

I.I., increase of inbreeding per generation; N_{ma}, number of males; N_{fe}, number of females.

C. Example of simplified simulation, showing the influence of the less represented sex (males) with a maximum avoidance mating system

2 males, 20 females = 3.53%
12 males, 24 females = 0.78%
40 males, 200 females = 0.19%
120 males, 120 females = 0.10%

BOX 4.6.6
Outbred foundation colony: simulation of genetic drift/inbreeding increase—long-term influence of the 'bottleneck'/founder effects due to a temporary reduction of the colony size (simplified formulas)

Mean population size: $n_e = g/\{1/n_1 + 1/n_2 + \ldots + 1/n_t\}$

where

n_e is the mean population size calculated over n generations

g is the number of generations

$n_1, n_2, \ldots, n_t$ is the actual population size from generations 1 to t

Examples of simplified simulation, showing the influence of a genetic bottleneck of 40 individuals (20 pairs) at N2, before colony re-expansion

$n_1 = 240 - n_2 = 40 - n_3 = 80 - n_4 = 160 - n_5 = 240 - n_e = 96$

$n_1 = 240 - n_2 = 40 - n_3 = 40 - n_4 = 80 - n_5 = 160 - n_e = 68$

$n_1 = 20 - n_2 = 100 - n_3 = 1000 - n_4 = 1000 - n_e = 64$

Source: *from [88].*

Before adopting a breeding system, one should evaluate its performance. Simplified formulas can be used for simulation, as illustrated in Boxes 4.6.5 and 4.6.6.

As a minimum goal, the inbreeding coefficient of an outbred stock should increase by far less than 1% per generation (Figure 4.6.5A, B). A monogamous system is highly recommended in foundation colonies, with one pedigreed and identified pair per cage, in order to allow genealogical records and selection of future breeding pairs. The ideal result is obtained by using computer-based mating systems based on maximal avoidance of inbreeding [100], i.e. selecting the 'less related' males and females to create new pairs. For practical reasons, simpler (non-computer-based) mating systems may be used (Figures 4.6.5A, B and 4.6.7A, B) [91, 101, 106, 107].

A long generation interval is another critical factor helping to minimize the drift in the long term. This can be achieved by keeping the breeding pairs as long as their performance is acceptable. A common practice is to use pups from the fifth litter to create the breeding pairs of next generation. Purposeful or unconscious selection should be carefully avoided at this step: each individual should have the same chance to contribute to the next generation. This last requirement does not prevent excluding a limited number of individuals displaying abnormal phenotypic characteristics when selecting future breeders. However, conscious or unconscious selection criteria may result in a tendency towards inbreeding and should be carefully avoided (selection for large litter size, fecundity, ability to bring a litter to term, behaviour).

As a next step, the foundation colony is used to create production colonies, guaranteeing long-term intercolony homogeneity. This goal can be achieved either by restarting production colonies on a regular basis with pairs from the foundation colony (as illustrated in Figure 4.6.6) and/or by the use of a migration system.

Inbred strains

Genetic contamination makes an inbred colony unusable. In consequence, genetic monitoring is essential to assure the integrity of the colony. If contamination is detected, the only option is to cull the colony and to restart it from scratch with pure pedigreed inbred breeders.

Substrains arise from the process of genetic drift, which results from the accumulation of mutations that become fixed at random in the colony. Major divergences lead to the development of substrains, which may progressively display different phenotypes and research-related characteristics.

A

	Number of males	Number of females	F$_I$ (%) *	MIA **
A	5	5	5.00	\
B	5	40	2.81	\
C	15	15	1.67	\
D	15	15	1.67	0.84
E	10	20	1.88	\
F	10	50	1.50	\
G	25	25	1.00	\
H	25	25	1.00	0.50
I	80	80	0.31	\
J	80	80	0.31	0.16
K	20	120	0.73	\
L	120	120	0.21	\
M	120	120	0.21	0.11

* F$_I$ = Increase of inbreeding per generation (%)
** Use of Miaximum Inbreeding Avoidance (MIA) scheme with equal sex numbers

B Inbreeding coefficient (%)
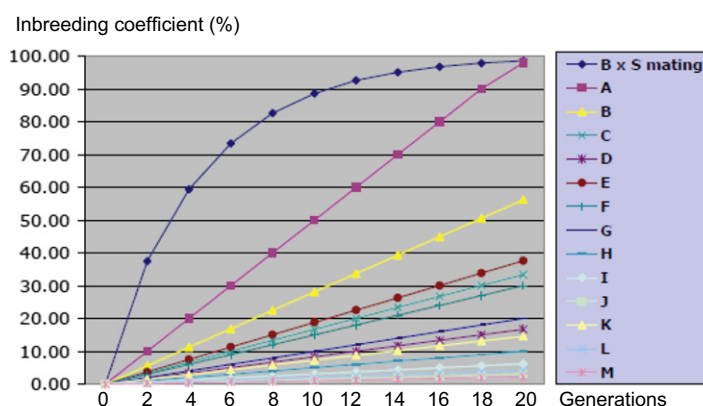
Figure 4.6.5 Evolution of the inbreeding coefficient in an outbred colony. A. Evolution per generation with different colony size and mating systems A–M. B. Evolution with different colony size and mating systems A–M. The brother × sister mating system (B × S) is shown as a comparison with inbreeding.

Genetic contamination should be prevented by strict maintenance of breeding records and cage identification, avoiding any source or situation of accidental mating (i.e. of genetic contamination), such as accidental mating after an escape from a cage. As an example of good practice, strains having the same coat colours

Figure 4.6.6 Starting or restarting outbred production colonies (at a defined frequency), with a large number of pairs from the foundation colony.

should not be kept in the same physical enclosure. Any escaped and unidentified mouse should be removed from breeding and euthanized. Any abnormal observation (phenotypic deviance) should be immediately reported as a potential sign of genetic contamination or mutation and closely investigated as such (e.g. sudden increase of litter size may result from heterosis, abnormal coat colour or behaviour). Animal technicians should be trained to ensure a close surveillance of breeding colonies and careful record-keeping. Unlike outbred populations, the size of an inbred foundation colony is not critical. In theory, just one pedigreed pair from a selected reference origin could be used. In practice, for security reasons and to speed up the colony expansion process, i.e. the later creation of production colonies, a larger number of cages and pairs is generally used. All are pedigreed and individually identified, hence the term 'foundation and pedigree identified pairs'.

To avoid a decrease of the inbreeding coefficient, a foundation colony should be managed by strict application of continuous inbreeding. The foundation colony is self-supporting and is used both to start new production colonies and to replace their breeders. Individuals from a foundation colony are used to propagate lineal descendants for a defined and limited number of generations (e.g. 10 or 15 generations) of brother × sister matings from the common ancestor in the foundation stock, plus a strictly limited number of generations (e.g. [2-3]) in production colonies [94, 104, 108]. When creating a production colony, pairs from the foundation colony are transferred to create a pedigreed nucleus (PN) group, using brother × sister matings. This is the first step in the creation of a production colony (Figure 4.6.8). The next step, using PN originating pairs, is to create a non-pedigreed expansion (NPE) group.

To ensure strict compliance to a genetic standard, it is necessary to keep introducing new pedigreed pairs on a regular basis (e.g. every 10-20 generations) from the foundation colony into the PN level of production colonies.

Ideally, a bank of cryopreserved embryos should be kept as security and as a back-up for the foundation colony. Such a bank can also be used to better control genetic drift and prevent cumulative change by thawing embryos on a regular basis (e.g. every five generations) in order to renew the foundation colony. Sufficient embryo numbers should be frozen to act as a reservoir in the long term.

Microbiological security of a colony

In addition to the availability of genetic security (i.e. the foundation colony), a health security colony should also be created in order to restart outbred or inbred breeding groups in case of microbiological contamination. Considering the limited space required to house a mouse foundation colony, it can easily be kept in a large isolator or in several smaller isolators, hence being also usable as a health security colony (Figure 4.6.2 A-C, G-I). If this is not done, recreating a foundation colony with an adequate

health standard will require rederivation of the contaminated colony through aseptic hysterectomy or embryo transfer, as described in the section on gnotobiology, and will require the availability of foster or embryo-recipient females originating from a colony with the adequate health standard.

Production colonies
Outbred stocks

The most common practice is to use harem matings (number of females per male adapted to the cage dimensions and to the stock reproductive capabilities), using a simplified mating system between subgroups of the production colony (Figure 4.6.7A). Around the 14th day of pregnancy, when it is visually detectable, pregnant females are removed from the mating cages to be kept in all-female groups. At birth, smaller groups are generally created. Except for specific requests, such as time-mated females or dated births, there is no urgent need to sex the pups at this stage, but rather to guarantee growth homogeneity. For this reason, it is recommended to give exactly the same number of suckling pups (born on the same day) to each female, e.g. 10 per female. At weaning age (18-21 days), pups are sexed, males and females are separated and stock groups are created for growth in cages identified with the birth date. If necessary, the caging density of these groups is adapted weekly to the caging standards. From these cages, experimental groups are made up either by weight or age bracket or both.

Inbred strains

As mentioned earlier, when starting a production colony, the first step is to create a PN group with brother × sister pairs directly originating from the foundation colony. The PN is then used to generate an NPE group where brother × sister matings or trios are used. The last production step is the random mated production (RMP) level, which is the only one where 'small harem' mating (1 male with 3-4 females) is generally adopted in order to maximize the productivity and generate large homogenous groups for research use (Figure 4.6.8 A, B).

A

3-block system, not recommended for Foundation Colonies and genetic management, usable only in Production Colonies

Generations 1, 3, 5, 7…   Generations 2, 4, 6, 8…

Generations 1, 3, 5, 7…   Generations 2, 4, 6, 8…

In all schemes: ——→ = *male transfers between groups*

B

MALE ORIGIN		FEMALE ORIGIN		NEW PAIR
1	X	2	→	1
3	X	4	→	2
2	X	1	→	3
4	X	3	→	4
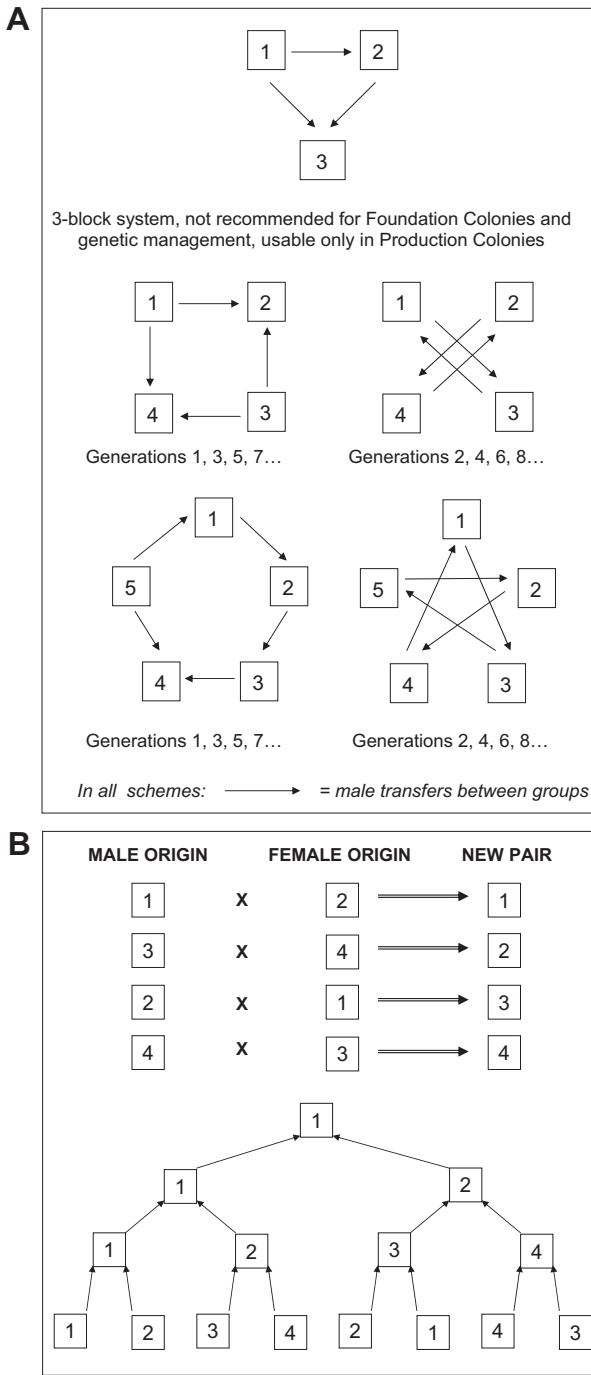
Figure 4.6.7 Avoidance of inbreeding in outbred colonies. A. Circular group mating systems in blocks of three, four or five, with male rotation (adapted from [91], [101], [107]). B. Robertson's four-block mating system, mainly used in foundation colonies.

At the NPE and RMP levels the mating system can be adapted to strain characteristics and zootechnical issues (using pairs, trios or harems)

A

Isolators   FC   Foundation Colony (pedigreed, B X S)

Production Colony   PN   Pedigreed Nucleus (B X S)

Barrier Room   NPE   Non-Pedigreed Expansion (B X S)

RMP   Random-Mating Production

Inbred strains available for research

B

FC

PN
NPE
RMP
Production Colony 1

PN
NPE
RMP
Production Colony 2

PN
NPE
RMP
Production Colony 3
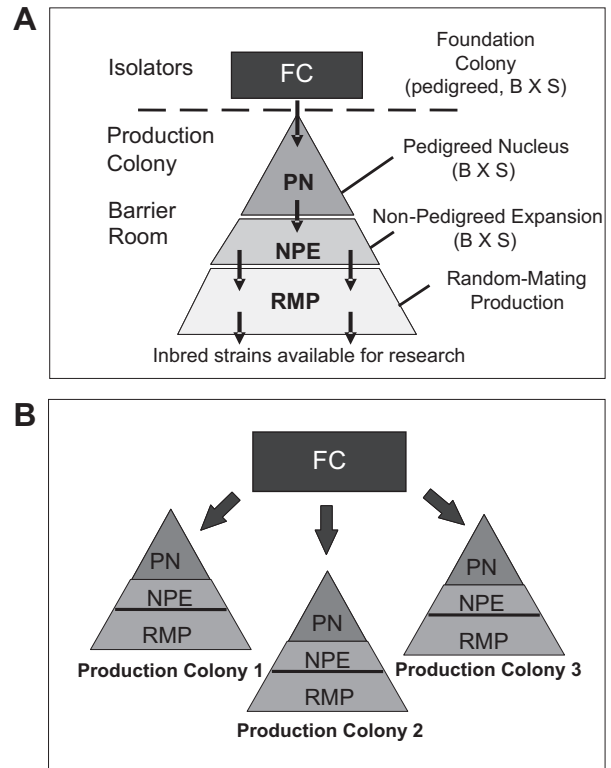
Figure 4.6.8 Organization of inbred breeding. A. From foundation colony to final production step. B. Inbred breeding with three independent production colonies. B × S, brother × sister matings; FC, foundation colony; NPE, non-pedigreed expansion; PN, pedigreed nucleus; RMP, random mating production.

in order to optimize productivity. Some sensitive strains may have to be bred in pairs, even at RMP level.

Note that the type of breeding system described above is generally adopted for large breeding operations. Depending on the context and the expected use of the animals, a simpler breeding system can be designed and implemented.

F1 hybrids

F1 hybrids are produced by mating two inbred strains together. This type of breeding, associated with inbred colonies, does not have any specific requirements except that, by definition, the colony is not self-perpetuating and requires the availability of the two parental strains in

the same room. In consequence, the risk of mismating and genetic contamination should be carefully addressed, especially when strains with similar coat colours are kept in the same breeding room. As a rule, when strains or stocks with similar coat colours cannot be separated, identification and strict procedures should be adopted, as well as genetic monitoring and surveillance.

Selection: oligogenic and polygenic traits

Polygenic traits (corresponding to quantitative trait loci, i.e. sets of genes that together control a phenotypic trait not completely determined by one gene acting alone) can be subject to selection, starting from a group of individuals expected to contain as many allelic forms as possible [109, 110]. For this reason, it is recommended to create a breeding colony with breeders from various and independent origins. After creating this new, highly allele polymorphic colony, each new generation will be created with males and females displaying the expected phenotype (e.g. arterial hypertension, body size or weight, growth, fatness, food intake, alcohol preference) until the phenotype stabilizes. The selection can be monodirectional or bidirectional. Such selection causes a severe increase of the inbreeding coefficient. To maintain an acceptable level of breeding performance, a productivity criterion (at least, the ability to keep breeding adequately to perpetuate the colony) has also to be included in the selection process. After reaching the expected phenotype, an inbred breeding system is generally used, while maintaining a simultaneous continuous phenotypic selection of the future breeders [111].

Mutations and transgenes

For practical or experimental reasons, such as managing a small colony size or controlling the influence of modifier genes and ensuring a stable phenotype, most mutations (spontaneous, induced chemically or by homologous recombination) are maintained on an inbred background. One critical issue, too often neglected, is to control the genetic background and guarantee isogenicity between individuals carrying the gene of interest and their control wild-type line [112]. This can be achieved (i) by selecting the optimal genetic background for the mutation, then creating a strain that is coisogenic to its control strain (i.e. generating transgenics or mutants with a defined genetic background such as C57BL/6 or FVB); (ii) by choosing a strain genetically identical to the ES cell line used for homologous recombination; or (iii) by generating congenic strains, using traditional backcross or marker-assisted speed congenic protocols as shown in Figure 4.6.9A, B [112-115]. Homozygous mating with an inbred genetic background requires reaching a minimum residual heterozygosity (i.e. an inbreeding coefficient of at least 99.9%) before establishing a close colony. With outbred backgrounds, for heterozygous or hemizygous production, it may be more convenient to use a mating scheme with regular infusions from the reference outbred colony. Depending on the type of mutation (recessive or dominant, autosomal or not) and in case of undesirable associated phenotype (expression of lethality or sterility alleles), one has to choose the most productive and adapted mating scheme. A critical step in the creation of a new mouse model is the selection of the genetic background to be used with any gene of interest, in order to avoid undesirable phenotypic or experimental interference, such as early blindness in behaviour studies or spontaneous tumour incidence with oncogene expression.

The identification of some genotypes requires the use of a validated genotyping method: Southern blot, PCR, single nucleotide polymorphism (SNP) markers, protein analyis (Table 4.6.5D). The maintenance of an outbred background requires the strict implementation of a relevant outbred management system guaranteeing the expected result. After creating a transgenic line by random gene integration, one should ensure that the individuals used to start a colony derive from a common founder selected beforehand to carry a unique, identical and stable integration site, with the same number of transgene copies.

A

Generation	Inbr. Coef.
N0	0
N1	0.5
N2	0.75
N3	0.875
N4	0.938
N5	0.969
N6	0.984
N7	0.992
N8	0.996
N9	0.998
N10	0.999

Backcross to inbred strain

B

N3	inbreeding coefficient ~ 0.985
N5	inbreeding coefficient ~ 0.999
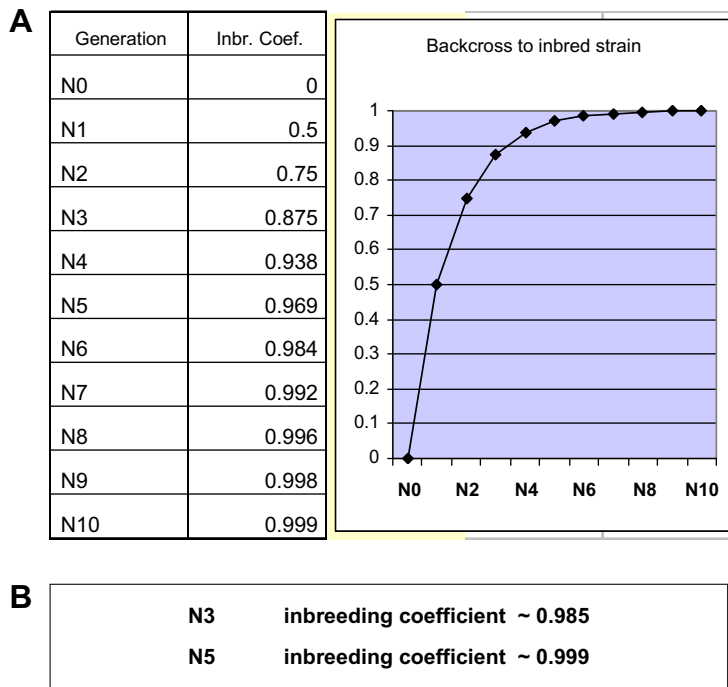
Figure 4.6.9 Evolution of the inbreeding coefficient. A. In a traditional/random-backcrossing protocol. B. In marker-assisted backcrossing protocols (speed congenics method).

Mating systems and breeding techniques

Reproduction data and mating systems

Breeding data

Some breeding and development data are detailed in Box 4.6.7.

Mating systems

Different systems can be used (Table 4.6.6) depending on several decision parameters related to the type of colony or the breeding step (foundation colony, expansion, full production). One should first consider the principal goal of the mating system. Pair mating is ideal in foundation colonies, where priority is given to pedigree follow-up and individual identification, to ensure minimum drift and maximum inbreeding avoidance in the long term, with limited concern for productivity. In production colonies, priority is given to productivity to benefit from the availability of large groups of homogenous animals for experimentation purposes (i.e. within a homogenous weight and age bracket), leading to the use of harem mating, at least for outbred stocks.

Timed pregnant females or timed births and use of 'plugged' females

After mating, a vaginal plug is formed by the secretions of the male vesicular and coagulating glands, filling the vagina in a variable way (often from cervix to vulva) and persisting for 8-24 h [116]. It is a convenient and easily visible indicator that mating has occurred. With most strains, 80-90% of 'plug positive' females are pregnant. Ovulation and mating usually occur overnight. The first day of gestation is considered to be the day after the vaginal plug is observed. For large production programmes, oestrus synchronization can be used prior to mating (see section on 'Practical applications of pheromone effects').

Superovulation

See Chapter 4.7.

TABLE 4.6.5: Examples of mating schemes for mutations and transgenes

A. Production of CBy. Cg- *Foxn1*^{nu}/*Foxn1*^{nu} (homozygous)

Autosomal and recessive mutation: *Foxn1*^{nu}

Male	Female	Progeny
nu/nu	*nu/*+	*nu/nu* (50%)
		nu/+ (50%)

After 10 generations a backcross is carried out: *nu/nu* males are mated with +/+ females originating from the BALB/cBy reference colony.

B. Production of C57BL/6−Tg/+ (hemizygous)

Example for a single insertion locus

Male	Female	Progeny
Tg/+	+/+	*Tg/*+ (50%)
		+/+ (50%)

Infusion with C57BL/6 females is carried out at each generation.

C. Production of BKS. Cg-m *Lepr*^{db}/*Lepr*^{db} (homozygous)

Autosomal and recessive mutation: *Lepr*^{db}

Example of double heterozygote repulsion mating with *m* mutation (misty coat colour marker)

Male	Female	Progeny
db +/+ *m*	*db* +/+ *m*	*db*+/*db* + (25%)
		db +/+ *m* (50%)
		+ *m*/+ *m* (25%)

db +/ *db* + (fat and black)
+ *m*/+ *m* (lean and dark grey)
db +/+ *m* (lean and black)

D. Production of BKS.V-*Lep*^{ob}*Lep*^{ob} (homozygous)

Autosomal and recessive mutation: *Lep*^{ob}

Use of PCR genotyping to genotype (lean) mice

Male	Female	Progeny
ob/+	*ob/*+	*ob/ob* (25%)
		ob/+ (50%)
		+/+ (25%)

ob/ob (fat and black)
ob/+ (lean and black) +/+ (lean and black) → PCR phenotyping

nu, nude; current nomenclature, *Foxn1*^{nu}
db, diabetic; current nomenclature, *Lepr*^{db}
ob, obese; current nomenclature, *Lep*^{ob}

Pseudopregnancy

Mating a female with a sterile or vasectomized male will cause a pseudopregnancy hormonally similar to pregnancy and lasting a few days, before the next oestrus cycle about 11 days later. Pseudopregnant females can be used as recipients for embryo transfer from synchronized donor females.

BOX 4.6.7
Some mouse breeding and development data

Ovulation: polyoestrus species, spontaneous, during oestrus, usually around midnight

Oestrus cycle: 4—5 days

Oestrus duration: ~12 h

Gestation period: 18—22 days (average 19—20)

Birth: most births occur at night or late afternoon

Litter size: 4—9 (with extremes from 2 to 12; varies greatly depending on the strain/stock, the age, the order of the litter, the environmental conditions)

Postnatal development:
  External ear opening: 3 days
  Coat: well developed at 2 weeks
  Vagina opening: 3—5 weeks
  First oestrus: 5—6 weeks

Postpartum oestrus: generally about 12—24 h after parturition, causing simultaneous gestation and lactation in permanent breeding groups (i.e. when the male is permanently present in the maternity cage)

TABLE 4.6.6: Different types of mating systems

Pros	Cons	Applications
MONOGAMOUS PERMANENT PAIRS (1 MALE + 1 FEMALE PER CAGE, MALE NOT REMOVED)		
Genealogical records	Large number of males	Mutations, transgene
Female zootechnical records	Space consuming	Project start
Postpartum oestrus	Labour consuming	Foundation colonies
		Inbred PES
	Male present at littering (can increase pup losses[a])	
		Backcross/progeny test
		Breeding to homozygocity
		Sensitive strains
PERMANENT TRIOS (1 MALE + 2 FEMALES PER CAGE, MALE NOT REMOVED)		
Genealogical records	Competition between litters	Same as monogamous
Productivity per m^2	Male present at littering	Intermediate production level
Postpartum oestrus		
Olfactive stimulation		Inbred NPE-RHP production colonies
PERMANENT HAREM MATING (FEMALES REMAIN WITH MALES FOR LITTERING AND SUCKLING, NOT RECOMMENDED)		
Productivity per m^2	Fights (strain-dependent)	Large-scale production
Postpartum oestrus	No genealogical records	Outbred production colony
Olfactive stimulation	Cage size	(1 male + up to 13 females)
Synchronization of littering	Male breeding capacity	
	Competition between litters	
BOXING-OUT HAREM MATING (1 MALE AND 4—13 FEMALES PER CAGE, FEMALES REMOVED WHEN PREGNANT)		
Productivity per m^2	No postpartum oestrus	Large-scale production
Olfactive stimulation	Use	Timed births (pregnant females removed to individual cages)
Synchronization of littering	Fights (strain-dependent)	
Lower pup mortality	Cage size	
Genealogical records (if 1 pregnant female per cage)	Male breeding capacity	Outbred production colony (1 male + up to 13 females)
Less competition between litters (homogenous birth dates)		Inbred production stock 2 (1 male + up to 4 females)

[a] Example: *B6.129P2-Apoe^{tm1Unc}* productivity index is ~5.0 without male and ~3.5 with male.
[b] If no limitation due to cage size.

Postpartum oestrus

A first postpartum oestrus cycle with an ovulation is generally observed about 124–248 h after parturition.

Practical applications of pheromone effects

Crowding females causes oestrus suppression, followed by oestrus synchronization when this inhibition is removed [117, 118]. This technique can be used before placing 1–3 females with 1 male. As the presence of a male in a female group not only overrides the negative female stimuli under crowded conditions but regulates and accelerates the oestrus, another technique is to use a double-compartment cage, separating the male from 2–3 mature females (minimum age 8 weeks) by a removable grid-type partition for 2–3 days. Then, the partition is removed, matings take place and plugs are checked. One should be aware that crowding can also cause increased pre- and postimplantational mortality or male fighting [119–121].

Record-keeping (cage cards and pedigree charts)

Depending on the type of breeding system and colony, a suitable record-keeping system has to be designed and duly used. At least in foundation colonies it relies mainly on the use of cage and individual identification, cage card records and pedigree charts. These records should make it possible to trace the ancestors, the progeny and the collateral relatives of any individual as well as the number of pups at birth and at weaning, and the parturition dates in order to monitor breeding performances. Breeding performance indicators are very useful to monitor the adequacy of breeding environment and nutrition.

Birth, sexing and weaning

At birth, pups are examined and may be sexed at this stage. In outbred stocks, to benefit from more homogenous growth, a common practice is to balance the size of litters at birth, giving the same number of pups to each female (e.g. [10]). Pups are sexed at the latest upon weaning, usually by 19–21 days, or a few days earlier when required.

Postweaning and growth; future breeders

After weaning, male and female growth groups are created, according to experimental and cage dimension specifications. An important indicator calculated at weaning age from data recorded on the cage card or by a computer system is the *productivity index*, the average number of pups weaned per female and per week, integrating the litter size at weaning (i.e. litter size at parturition minus preweaning mortality) and the parturition interval. This index varies according to the strain, the breeding system, nursing care and numerous environmental influences. When breeding conditions are defined and standardized (caging, density, feed), the breeder should determine and regularly update the growth curve as well as other relevant biological data.

Retirement of breeders

The breeding life is generally longer in foundation colonies, in order to increase the generation time and to minimize the long-term genetic drift. In production colonies, breeders are retired when the litter size starts decreasing significantly. With outbred stocks, this is usually after 7–8 litters in foundation and PES colonies or after 4–6 litters in production colonies. Breeders are retired early in case of abnormal observations (excessive weight, skin lesion, poor health condition). In inbred strains, females are usually mated at 7–8 weeks of age and retired after about 28–32 weeks. Retired individuals are very useful for diagnostic or health monitoring purposes.

Genetic monitoring and genotyping

This topic is addressed in Chapter 4.5. Genetic monitoring is mainly used to detect genetic contamination in inbred strains, using routine monitoring not only of DNA markers such as microsatellites/short tandem repeats or SNPs but also genetically determined and relevant phenotypic traits or markers. Genetic markers

should be spread throughout the genome and be as discriminant as possible between the different lines bred on site. Short marker scans aim to detect genetic contamination and extended scans (80–120 markers) are used in accelerated backcrossing or in gross strain comparison. It is critical to bear in mind that minor genetic drifts and substrain differences are generally not detectable by genetic monitoring, even with the extended scans used for accelerated backcrossing: the quality and rigour of the genetic management system is the only guarantee of the purity of a genetic standard. With transgenes or mutations, it is usually necessary to rely on routine genotyping to identify the gene of interest and its zygosity or to evaluate the number of transgene copies and their integration site(s) using standard or real-time PCR techniques, Southern blot, fluorescent *in situ* hybridization (FISH) or restriction enzymes. Identification and reporting of phenotypic deviants is critical, especially in foundation and expansion colonies.

Husbandry practices, caging and environmental conditions

Nutrition, husbandry practices, caging and environmental conditions are of course critical both for ethical and breeding performance reasons and may have to be adapted to the line sensitivity and its specific requirements [122].

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Cryopreservation of Preimplantation Embryos and Gametes, and Associated Methods

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Introduction

The purposes of freezing gametes and embryos are multifarious. One intention is to preserve the genetic pool of different strains. An embryo or gamete bank serves as an important back-up for vitally maintained strains in case of disease, reduced reproduction, genetic contamination or incidental environmental events. Storage in liquid nitrogen (LN_2) at $-196\,°C$ prevents genetic drift (mutations) [1, 2]. In addition, the exponentially growing number of genetically modified strains already exceeds the capacities of most animal houses. This trend continues, reflected in the fact that international programmes such as EUCOMM [3], NorCOMM [4] and KOMP [5] have been established for setting up libraries of mouse embryonic stem (ES) cell lines which carry conditional, as well as constitutive, knockout mutations for all known genes. The cryopreservation of strains that are not actually used for scientific procedures might be an alternative to maintaining live animals.

Today, numerous protocols for gamete and embryo cryopreservation exist. The greatest success is achieved by cryopreserving preimplantation embryos and spermatozoa. However, one has to keep in mind that genetic manipulations or mutations may have unexpected adverse effects on the development of embryos. This may result in the impossibility of recovering a strain from cryopreserved sperm or embryos.

In this chapter we describe the methods that we routinely and successfully use in our laboratory.

Cryopreservation of epididymal sperm

Cryopreservation of sperm has become important for mutagenesis programmes as well as for storing genetically modified strains. The cryopreservation of spermatozoa is fast and cheap, not requiring expensive equipment. Only the spermatozoa of a few males are needed to fertilize the oocytes of numerous females. However, one must take into account that *in vitro* fertilization (IVF) and embryo transfer to foster mothers have to be performed after thawing, and oocyte donors of an appropriate strain have to be available for this. If possible, oocyte donors should be coisogenic to the background strain of the sperm donors.

As only the haploid genome is preserved, all resulting animals are heterozygous for the modified gene or mutation after IVF, if the sperm donor is homozygous. If the sperm donor is heterozygous, only 50% of the offspring carry the gene of interest. In any case, backcross programmes are needed to produce animals with the desired genetic status. The additional time required for this must be considered.

Quite a number of protocols have been published and are in use. Interestingly, a medium composed of raffinose and skimmed milk seems to be optimal [6]. Nevertheless, the success of sperm freezing depends strongly on the genetic background of the mice. Until recently, the results for the widely used C57BL/6 strains

were quite poor. A recently published protocol [7] seems to have overcome this problem. This protocol, known as the 'JAX protocol' is routinely used at The Jackson Laboratory, Maine, USA. The freezing method described by Takeshima et al. [8] in combination with the raffinose–skim milk medium also gives good results for a variety of inbred strains (pellet method) [9]. Both protocols are described below.

Cryopreservation medium

Raffinose (18%) and skim milk (3%) are suspended in H_2O at 60 °C. After centrifuging at $10 \times 10^3\,g$ for 15 min the supernatant is filtered using a sterile, pyrogen-free, disposable filter holder (0.45 μm). The filtrate is used as cryopreservation medium (CPM). Osmolarity should be between 470 and 490 mOsm. Aliquots can be stored for up to 1 year at −20 °C.

Sampling of spermatozoa for cryopreservation

The best quality and quantity are achieved with adult males, which have not mated in the period 3 days prior to sperm preparation. Ideally, the males should be 8-12 weeks of age. For preparation the male is sacrificed by inhaling carbon dioxide, to prevent ejaculation. The abdominal cavity is opened and the epididymis (caput, cauda and vas) is dissected out. Both epididymi of one male are transferred to a 2 mL plastic reaction tube containing 0.5 mL of the appropriate CPM. The tissue is cut into pieces with fine scissors and gently mixed. After incubation at room temperature for 15 min the sperm swim up and tissue fragments sink to the bottom. One drop of the supernatant containing the sperm is checked under a stereomicroscope for morphological integrity and motility. The sperm count is determined by using a haemocytometer. Songsasen and Leibo [10] suggest using only preparations in which at least 60% of total spermatozoa are motile with vigorously progressive movement and with a concentration of 2-4 $\times 10^7$ total sperm/mL. Next, the supernatant containing the sperm is diluted to a concentration of approximately 1×10^7 sperm/mL with CPM.

Sperm freezing

JAX protocol

The JAX freezing procedure [7] begins with the preparation of the 'freezing chamber', a polystyrene box filled with LN$_2$. A 'raft' is placed on the liquid surface, which allows nitrogen vapour to pass through (Figure 4.7.1).

Prior to use, CPM is thawed in a 37 °C water bath. Alpha-monothioglycerol (MTG, FW = 108.16) is added to a final concentration of 477 µM (CPM + MTG). Sperm is prepared in CPM + MTG as described above. French straws, filled as shown in Figure 4.7.2, are used as cryocontainers. This can easily be done by attaching a 1 mL syringe to the side of the cotton plug. First a 1 cm air column is drawn into the straw, followed by a 5.5 cm CPM + MTG column. A second air column is followed by 10 µL sperm suspension in CPM + MTG. The filled straw is heat-sealed on both sides. Subsequently, the straw is immersed in the nitrogen gas by placing it on the 'raft' (−170 °C) for 10 min before transfer to LN$_2$ for storage.

Pellet method

In the pellet method of sperm freezing [9], CPM is first thawed in a 37 °C water bath before use. Sperm is prepared in CPM as described above. Holes (diameter 0.5 cm, depth 0.3 cm) are made in a block (15 cm × 15 cm × 5 cm) of dry ice (−70 °C) using a steel punch (Figure 4.7.3). The difference in temperature between the steel punch (approximately 20 °C) and the dry ice (−78.5 °C) makes this possible. Then, 50 µL of the sperm suspension are pipetted into each hole. After 5 min the sperm suspension forms pellets, which are directly transferred to LN$_2$ for an additional 5 min. Finally, the pellets are inserted into precooled 1 mL cryotubes with screw caps. The 'sperm pellets' of one male should be divided into two or three cryotubes. The cryotubes are stored in LN$_2$.

Cryopreservation of preimplantation mouse embryos

In principle, all preimplantation stages can be cryopreserved and revitalized. However, the

Figure 4.7.1 The 'freezing chamber', a polystyrene box filled with liquid nitrogen. A bracket that is normally used to hold plastic reaction tubes is used as a raft. Cryocontainers (here Minitüb) can be placed on the raft to be cooled in nitrogen vapour.
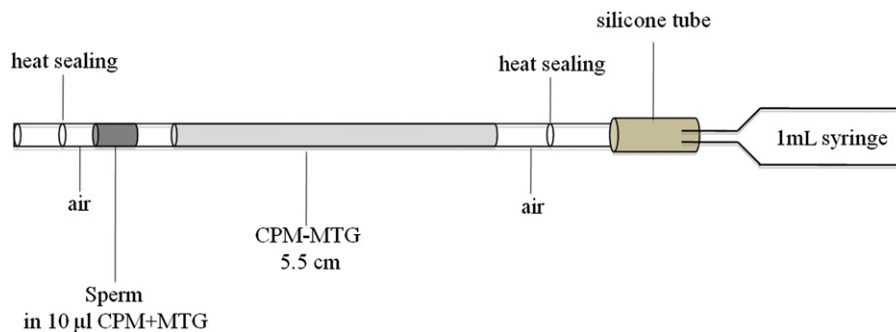
Figure 4.7.2 Filling a French straw for the cryopreservation of sperm.
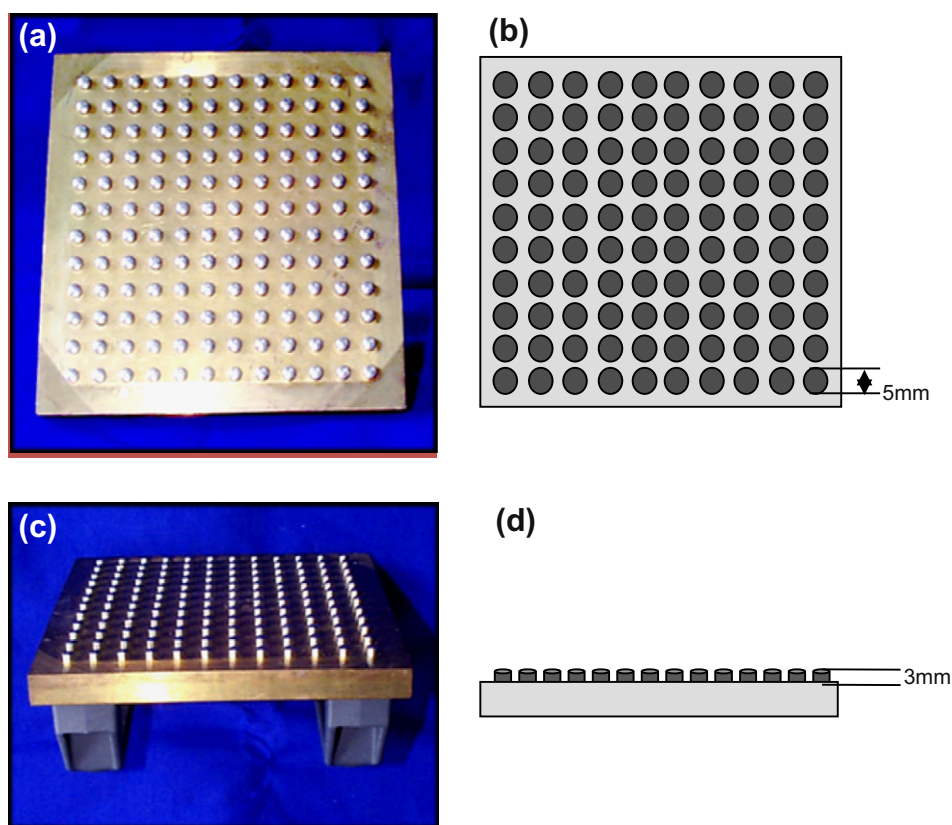
Figure 4.7.3 Steel punch for melting holes into a block of dry ice for the freezing of mouse sperm. (a, b) top view; (c, d) side view.

results for cryopreserving zygotes are not yet satisfactory [11]. Cryopreservation of two-cell to eight-cell embryos gives the best results after revitalization. Ultrarapid freezing (vitrification) [12] is a technique not requiring expensive technical equipment. Nonetheless, according to our experience, the revitalization rates of this method are not as satisfactory as those for a modified two-step method [13, 14]. The modified two-step method is described below. The genetic background and genetic manipulations may affect the embryonic survivability after freezing independent of the freezing method.

Media for embryo handling

For embryo collection and handling a medium with stable pH is needed. The phosphate-buffered medium (PB1, Table 4.7.1) described by Whittingham [15] is suitable, but the commercially available M2 medium can also be used.

TABLE 4.7.1: Phosphate-buffered medium: PB1	
Substance	**Final concentration (mM)**
H_2O	
NaCl	100.93
KCl	2.73
Na pyruvate	0.33
KH_2PO_4	1.42
$MgCl_2 \cdot 6H_2O$	0.48
$Na_2HPO_4 \cdot 12H_2O$	8.05
$CaCl_2 \cdot 2H_2O$	0.94
Phenol red	0.01 mg/mL
Penicillin G	1 mg/mL
Streptomycin	0.05 mg/mL
Glucose	1 mg/mL
BSA	3 mg/mL

Substances have to be mixed in the given order. PB1 can be stored for 3 weeks at 4 °C.
Source: ref. [15].

Collection of preimplantation embryos

Collection of one-cell embryos (zygotes)

Cryopreservation of zygotes is suggested [17] as a back-up for microinjection to produce transgenic animals. To reduce the number of female donors, superovulated animals should be used (see the section 'Superovulation'). Only females with a copulatory plug the morning after mating are used for embryo collection. They are killed at the appropriate stage of pregnancy (Table 4.7.2) under carbon dioxide anaesthesia by cervical dislocation. For the collection of zygotes the females are sacrificed 12-14 h after the second injection for superovulation (day 0.5 of pregnancy).

The animal is laid in a supine position and the abdomen disinfected. The skin is cut open by means of a small transverse middle incision and the skin is then pulled towards the head and tail, respectively. The cavity is opened through a transversal incision beneath the xiphoid cartilage from right to left. Both oviducts are removed, as well as a small part of the respective uterine horn. They are transferred to a Petri dish (35×10 mm) containing 2.0 mL PB1 at room temperature. By using a stereomicroscope the swollen ampulla in the upper part of the oviduct (Figure 4.7.4) can easily be located.

The swelling of the ampulla at this stage of development is a result of the cumulus cells surrounding the zygotes. The ampulla is pulled open using watchmaker's forceps and the cumulus–oocyte complexes pour into the medium. The oviducts are then removed from the Petri dish. The cumulus cells are removed by adding hyaluronidase to a final concentration

TABLE 4.7.2: Early development (data of a cross of C57BL/6 female and a CBA male)

Time (h)	Stage	Appearance	Location
0	Fertilization	Ova surrounded by cumulus cells	Ampulla
10	Pronuclei become visible	Male and female pronuclei are clearly visible Second polar body is visible Fewer cumulus cells surround the ova	Ampulla
20	State of first mitotic cleavage	Cumulus cells have disappeared Fusion of pronuclei	1–2 loop of oviduct
24	2-cell stage	Two blastomeres of equal size, surrounded by the zona pellucida	2 loop of oviduct
48	4-cell stage	Two blastomeres of equal size, surrounded by the zona pellucida	Lower half of the oviduct
52	8-cell stage (stage of compaction)	The contours of the blastomeres are no longer distinctly visible	Lower half of the oviduct
72	Morula	The blastomeres are not equal in size	Pars intramuralis tubae (connection from oviduct to uterus)
96	Blastocyst	The zona pellucida has disappeared (hatched blastocyst)	Uterus
120	Beginning of implantation	Distinct differences in the degree of development in the same litter	Blastocyst adheres to the uterine epithelium

Source: [16].
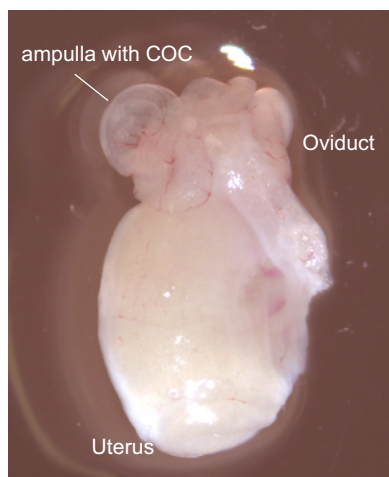
Figure 4.7.4 Oviduct of a day 0.5 pregnant mouse. The cumulus–oocyte complexes (COCs) are visible in the swollen ampulla.

of 10 000 IU/mL. The cumulus cells detach from the zygote within 2–3 min. After washing them in fresh PB1 they can be cryopreserved or used for micromanipulation.

Collection of two-cell embryos to morulae

Embryos from the two-cell to early morula stage are flushed out from the oviduct. The females are sacrificed at the appropriate time of embryonic development (Table 4.7.2). The abdominal cavity is opened as described above and the oviducts are excised. Subsequently, each oviduct is placed in a drop of PB1. Under the stereomicroscope a 33G blunt-ended needle, attached to a syringe filled with PB1, is inserted into the infundibular opening. The needle is held in position using watchmaker's forceps (Figure 4.7.5). The embryos are flushed out with 3–4 drops of PB1 into one well of a four-well Petri dish (35 × 10 mm). Morphologically intact embryos are then washed several times with fresh PB1; most of the known germs are removed from the zona in this way [18, 19]. The embryos can then be transferred to the freezing medium for cryopreservation (described below).

Collection of blastocysts

Depending on the strain, blastocysts can be collected on day 3.5–4.5 of pregnancy. Embryos at this stage have reached the oviduct–uterus junction and are flushed out from the uterine
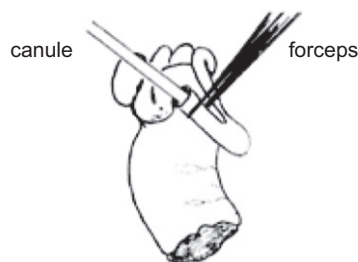
Figure 4.7.5 Flushing of preimplantation embryos from the oviduct. A blunt-ended canula is inserted into the infundibular opening. It is held in place with forceps.

horns. The abdominal cavity is opened as described above and both uterine horns are removed with the corpus uteri. The uterus is transferred to a 35 × 10 mm Petri dish containing 2 mL PB1. Each horn is flushed retrograde with 5 drops of PB1 by inserting a blunt-ended 33G needle through the cervix. The blastocysts are washed several times in fresh PB1. They are now ready for further use.

Modified two-step freezing method for cryopreservation of preimplantation embryos

The modified two-step method can be used for freezing up to the blastocyst stage. Cooling to $-32\,°C$ is done in a programmable automatic cooling bath (an example is shown in Figure 4.7.6) with ethanol as coolant. We use 250 μL plastic straws as cryocontainers.

For the embryo-freezing medium (EFM), PB1 is supplemented with 1,2-preponediol (PROH) to a final concentration of 2.0 M. PROH is a small molecule that penetrates the cell membrane and substitutes intracellular water. Compared to other cryoprotectants, its toxicity is rather low [20–22].

Before loading the plastic straw with embryos, it is sealed with a metal bulb on one side and filled with 150 μL freezing medium (Figure 4.7.7). The embryos (approximately 20/straw) are then transferred to the centre of the EFM column by using a drawn-out Pasteur pipette (see the section 'Miscellaneous'). The straw is immediately sealed with a glass bulb.

The filled straws are placed into the precooled ($0\,°C$) programmable cooling bath. After an
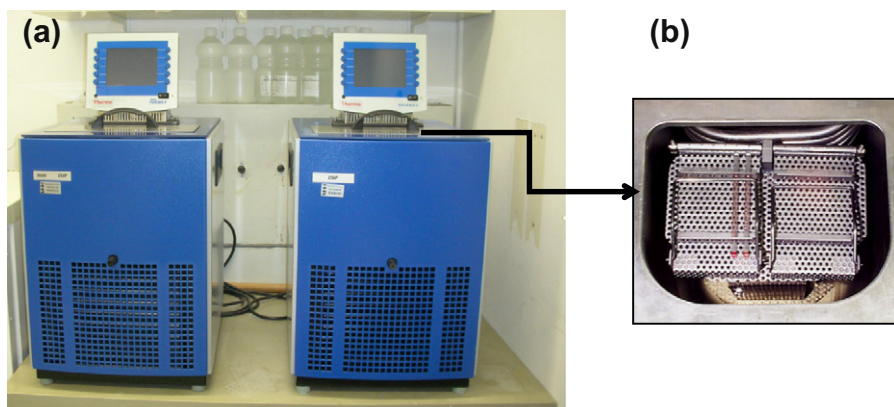
Figure 4.7.6 (a) Automatic programmable cooling bath (Haake C50P). (b) View into the cooling chamber with holding device for Minitübs.
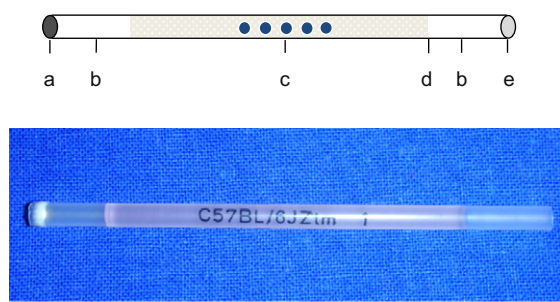
Figure 4.7.7 Plastic straws (Minitübs) used as cryocontainers for embryo freezing. Top: schematic view. a, glass bulb; b, air space; c, freezing medium containing the embryos; d, seeding point; e, metal bulb.

equilibration time of 10 min the freezing programme is started. The straws are cooled to −6 °C at 1 °C/min and mechanical seeding is induced with a precooled (−196 °C) metal punch. After 5 min cooling is continued with a cooling velocity of 0.4 °C/min down to −32 °C. After 10 min at −32 °C the straws are transferred to LN$_2$ for storage.

For thawing, the cryocontainer is taken from the storage tank (−196 °C) and placed at room temperature. The metal bulb is cut off. Within 20–30 s the ice melts, resulting in a warming rate of approximately 300 °C/min. The straw is now attached to a 2 mL syringe and the glass bulb is cut off. The content (including the embryos) is flushed into a 35 × 10 mm Petri dish and an equal volume of fresh PB1 is added immediately to dilute the cryoprotectant. After 10 min a further volume of PB1 is added. This

step is repeated until a final volume of 3 mL is reached. The embryos have to be washed at least three times with fresh buffer to remove the cryoprotectant entirely. Only morphologically intact embryos should be transferred to foster mothers, to increase the rate of development. It has been shown that two-cell embryos with only one intact blastomere can also develop to term, but with less success [23].

Cryopreservation of oocytes

The successful cryopreservation of phase II oocytes was first reported by Whittingham [24]. The structure of oocytes, with their cortical granules, is more complex than that of embryos [25]. This structure complicates an unhampered dehydration and therefore requires ultrarapid freezing (vitrification). Moreover, the spindle apparatus and the structure of the zona pellucida have to be preserved in a manner that enables IVF after thawing.

As only the haploid genome is preserved, all resulting animals are heterozygous for the modified gene or mutation after IVF, if the oocyte donor is homozygous. Should the oocyte donor be heterozygous, only 50% of the offspring carry the gene of interest. In any case, backcross programmes are necessary to produce animals with the desired genetic status. This additional time must be taken into account.

Collection of oocytes

Oocytes can be collected from unmated females 12-14 h after the second injection of the super-ovulation procedure. The preparation and collection is as described for zygotes.

Freezing and thawing of oocyctes

The method described here is based on a method developed by Nakagata [26, 27]. The oocyte freezing medium (OFM) is composed of 5 M dimethylsulfoxide (DMSO) in PB1, supplemented with 10% foetal calf serum (FCS). The medium is adjusted to pH 7.2-7.4 with HCl. Ten to 15 oocytes are gathered in 5 µL OFM and transferred to a 2 mL cryocontainer with screw cap. The container is incubated on ice for 5 min before adding 95 µL OFM. After another 5 min on ice, the cryocontainer is transferred directly to LN$_2$ for storage.

For thawing, the cryocontainer is placed at room temperature. As soon as the OFM is melted 900 µL of 250 mM sucrose in PB1 is added. The total content (1000 µL) is carefully transferred to a Petri dish (35 × 10 mm). Subsequently, the oocytes are washed several times with fresh PB1. They can now be prepared for IVF.

Cryopreservation of ovaries

In principle, ovaries of all postnatal stages, including ovaries from premature and very old females, can be cryopreserved as long as the ovaries contain sufficient primordial follicles. The ovary has a complex structure composed of fibrous and glandular tissue containing oocytes of all stages of folliculogenesis.

For freezing it is important that the cryo-preservation medium permeates the ovary completely. After thawing, reperfusion has to be rapid. Although Bouquet et al. [28] raise concern that the whole cycle of freezing and thawing might induce DNA damage, successful freezing of mouse ovaries can be achieved [29, 30].

Preparation of ovaries

The method described is adapted from Sztein et al. [29] and Harp et al. [31]. Ideally, donor females should be 3-7 weeks old. They are killed by cervical dislocation and the ovaries are aseptically removed. The ovaries are transferred to a 35 × 10 mm Petri dish containing 2 mL PB1 at room temperature. Fat pad and bursa are removed.

Freezing and thawing of ovaries

The ovaries are transferred to 2 mL cryotubes with screw caps containing 200 µL PB1 supplemented with PROH at a final concentration of 1.5 M and 10% FCS. After 10 min at room temperature the cryotubes are placed on ice for 45 min. The cryotubes are then transferred to the pre-cooled (−6 °C) programmable cooling bath. After 5 min, seeding is induced by touching the surface of the medium with a Pasteur pipette containing frozen medium (−196 °C). Cooling is continued to −80 °C at 0.5 °C/min. After 10 min at −80 °C the cryotubes are transferred to LN$_2$ for storage.

For thawing, the cryotubes are transferred from the storage tank to room temperature. As soon as the ice has melted, the freezing medium is removed immediately and replaced by 200 µL fresh PB1. After 10 min the ovaries can be used for transplantation.

Setting up frozen storage of mouse germplasm

Table 4.7.3 summarizes what can be stored in LN$_2$ and revitalized fairly successfully. The table also shows the additional steps that are needed to start a new breeding nucleus after thawing the respective germplasm. These additional steps have to be considered, as they might be very time consuming.

It is important to calculate the number of embryos or gametes that have to be frozen to ensure a high probability that a new breeding

TABLE 4.7.3: Cryopreservation of gametes and associated methods

Cryopreservation of:	Procedures prior to cryopreservation	Methods after thawing	Additional needs	Estimated risk for germ transmission
Sperm	Evaluation of sperm quantity and quality	IVF Superovulation Oocyte recovery Embryo culture Embryo transfer backcrossing Genetic monitoring	Oocyte donors Pseudopregnant dams Vasectomized males	High
Preimplantation embryos	Superovulation Embryo collection Embryo culture	Embryo transfer Embryo culture Genetic monitoring	Pseudopregnant dams Vasectomized males	Marginal
Oocytes	Superovulation Oocyte collection	Sperm preparation IVF Embryo culture Embryo transfer backcrossing Genetic monitoring	Sperm donors Pseudopregnant dams Vasectomized males	Marginal
Ovaries	Ovarectomy	Ovary transplantation backcrossing Genetic monitoring	Coisogen or immune-deficient recipients	High

colony can be built up after thawing. Successful revitalization, however, is strongly influenced by the genetic background, mutations and genetic modifications of the animals. The suggestions made below can therefore be given as guidance only.

Number of spermatozoa that should be frozen for back-up

The number of spermatozoa that can be collected from one male varies from one strain to another. In our experience, $3\text{-}4 \times 10^7$ spermatozoa can be collected from the epididymis of one fertile male of a good breeding strain. The fertilization ability of frozen-thawed sperm depends not only on the strain of the sperm donor but also on the oocyte donor used for IVF, and can vary greatly. The developmental ability of *in vitro* fertilized embryos can also be reduced. It is therefore recommended that control batches should also be frozen simultaneously. The control batches should be revitalized before the vital colony is terminated to test

whether the sperm can survive the whole cycle of cryopreservation, thawing and IVF. Freezing of sperm from two males is considered as sufficient to preserve a strain [32, 33]. As a safeguard one should at least freeze the sperm of 5-10 males with proven fertility to ensure a secure genetic back-up.

A further problem associated with the freezing of spermatozoa is the possibility of vertical transmission of microorganisms and viruses, such as mouse hepatitis virus (MHV), with the sperm suspension [19, 34]. Therefore, direct descendants of mice produced from male sperm with unknown microbiological status should be reared in quarantine to prevent contamination of healthy breeding colonies.

Number of preimplantation embryos that should be frozen for back-up

In our experience approximately 25-30% of Swiss-Webster embryos, ICR embryos or embryos from F1 hybrids frozen by the modified two-step

TABLE 4.7.4: Principles for embryo donor selection		
Type of strain	**Genetic requirements**	**Origin of embryos of one batch**
Outbred	Random sample of the strain	*n* embryos from n donors
Inbred	B × S pairs	All embryos from one B × S pair
Congenic	Maintenance of differentiating gene	Pool of *n* donors
Mutant and genetically modified	Maintenance of the mutation/ modification	Pool of *n* donors
B × S pairs: brother × sister pairs.		

method result in live offspring. Depending on these results, at least 10 batches containing 10 embryos each have to be frozen in order to have a 99.9% probability of obtaining one breeding pair after thawing. This probability may be much lower for other strains [35]. Control batches should be frozen and revitalized to calculate the number of embryos needed for a frozen back-up. As only the production of live offspring indicates successful freezing, this revitalization should include thawing and embryo transfer. We recommend that 200-400 embryos per strain should be frozen. The frozen back-up not only serves as a safeguard against loss, but also permits the restocking of a breeding nucleus several times.

A limiting factor in embryo banking is the availability of sufficient donor females. The number of embryos that can be collected from one female can range from 3-60 after superovulation (own data). The following equation can help to calculate the required number of donor females at an early time point of a freezing programme [36]:

Depending on the type and genetic requirements of the strain (inbred strains, outbred stocks, strains carrying a mutation or genetic modification, etc.), different selection principles for the embryo donors have to be followed. Table 4.7.4 summarizes these principles.

Number of oocytes that should be frozen for back-up

Currently oocytes are not routinely used for frozen storage. The success of revitalization depends on the strain and can vary widely. According to our own experience approximately half of the oocytes survive freezing and thawing and only 30-50% of these oocytes can be fertilized *in vitro*. We therefore recommend cryopreservation of 200-400 oocytes per strain. The equation in the previous section can be used to calculate the number of donors.

The risk of vertical germ transmission is the same as for embryos.

$$\text{Donor number} = \text{number of embryos to be frozen}/(\text{percentage of females with embryos} \times \text{mean number of embryos per female})$$

The risk of vertical germ transmission is quite low for embryos. Nevertheless, some viruses (e.g. mouse minute virus, MMV) might penetrate the zona pellucida [19, 34]. Therefore, direct descendants of embryo donors with unknown microbiological status should be separated from other healthy breeding colonies.

Number of ovaries that should be frozen for back-up

As the ovary is a complex structure composed of different cell types and containing follicles at various stages of folliculogenesis, a large proportion of follicles could be damaged during freezing and thawing of ovarian tissue. Sztein

et al. [36] showed that two out of four females receiving frozen–thawed grafts became pregnant compared to three out of four females receiving unfrozen ovaries. Based on published data and our own experience, for most strains it is sufficient for ovaries of 5–10 donors to be frozen.

Two problems associated with the transfer of ovaries remain unsolved. First, the possibility of vertical transmission or microorganisms cannot be precluded. Therefore, the same precautions as for the freezing and revitalization of sperm should be taken. The second problem is the need for immunodeficient or isogenic recipients to prevent graft rejection (see the section on 'Orthotopic ovary transplantation').

Associated methods for cryopreservation

Superovulation

To increase the number of embryos that can be collected from one donor female and to reduce the number of animals needed for producing embryos, exogenous gonadotrophins are used. Additionally, the reproductive cycle of the treated females is synchronized. This allows timed mating and the collection of embryos at a defined stage of development. Gates [37] suggested a scheme for superovulation of mice which works for most of the common mouse inbred strains and outbred stocks. Some inbred strains and some genetically modified strains, however, do not respond to exogenous hormones, whereas others produce a multiple of the normal amount [38]. Aside from genetic factors, others factors also influence the success of superovulation: age, body weight and state of health of the animals. Seasonal effects also influence the success of superovulation in spite of the animals being maintained under controlled and standardized conditions. The quality of embryos may decrease with increasing number and a great percentage of embryos do not develop beyond the first 2–3 days. The virgin females should be 4–8 weeks of age, but also older females may be effective. The optimal age depends on the strain.
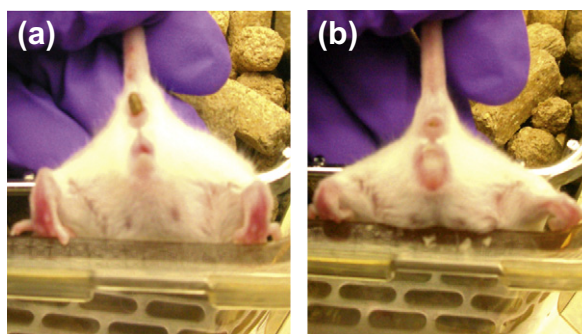
Figure 4.7.8 The vaginal plug. Female mouse (a) without and (b) with a vaginal plug on day 0.5 of pregnancy.

BALB/c mice give the best results when they are 4 weeks old; for the superovulation of C57BL/6 mice we use females at the age of 6 weeks. With a light/dark cycle of 12 h the females receive an intraperitoneal injection of 5–10 units pregnant mare's serum gonadotrophin (PMSG) at 4:00 p.m. and 5 units of human chorionic gonadotrophin (hCG) 48 h later. They are mated to males immediately after the second injection. On the following morning (day 0.5 of pregnancy) the females are checked for the presence of a vaginal plug (Figure 4.7.8). The pregnant females are sacrificed at the appropriate developmental stage of the embryos.

If hormones from natural sources are used, brand new samples may vary in hormone content and purity level. They should be tested before routine use to determine the optimal dose for superovulation. For this purpose three groups (n = 5) need to be tested with different doses (5, 7, 10 units). Strains with known response to superovulation should be used. The hormones are diluted in 0.9% NaCl to the optimal concentration. Aliquots can be stored at $-20\,°C$ for at least 1 year.

Embryo transfer

Efficient transfer of preimplantation embryos into pseudopregnant surrogate dams is an important step for the rederivation of cryopreserved or contaminated mouse stocks and for the generation of genetically modified mice, as well as for IVF programmes.

Preferably, a natural copulation stimulus by mating with sterile but sexually active males

should induce pseudopregnancy. Vasectomized males are used for this purpose. In principle, embryo transfer to pregnant females is possible, but the transferred embryos have to compete with the recipient's own embryos. The vaginal plug produced by the male during ejaculation is used as a sign that copulation has occurred. On average 30 females are needed to produce enough that are plug-positive.

Due to manipulations, the embryos are often delayed in their development. Therefore, embryos from the one-cell to the morula stage are transferred into the oviduct of 0.5 to 2.5 day pseudopregnant recipients; blastocysts have to be transferred into the uterus at day 2.5 or day 3.5 of pseudopregnancy at the latest. This means that the state of pseudopregnancy should be 1 day beyond the developmental stage of the embryo. This gives the embryo time to catch up with development [39].

Embryos transferred to the oviduct must be covered by the zona pellucida [40], whereas this is not a prerequisite for embryos transferred into the lumen of the uterus.

The number of transferred embryos depends on the donor strain and on the kind of previous manipulations. Embryos of some inbred strains are less viable [41], whereas most embryos of hybrid or outbred stocks complete their development (50-70%) upon transfer. Freshly collected and unmanipulated embryos develop more successfully than frozen-thawed or microinjected embryos. Sometimes the embryos from genetically modified mice are less viable than those of their genetic background. This might be due to unknown side effects of genetic manipulation [42]. Some experience is needed to transfer the ideal number of embryos to achieve reasonable litter sizes.

Outbred mice, such as Swiss-Webster or ICR and F1 hybrid females (e.g. C57BL/6 × DBA or BALB/c × DBA) have shown to serve as good surrogate dams. They should be 10-12 weeks old and have a body weight of 20-25 g.

Preparation of surrogate dams

Females are mated to vasectomized males in the afternoon. On the assumption of a 4-5 day oestrus cycle, 20-25% of the females should mate and have a vaginal plug the following morning

(day 0.5 of pseudopregnancy). However, in practice fewer females have copulated.

Whitten [43] found that the female oestrus cycle is strongly influenced by male odour. With this in mind, the copulation rate can be raised to 45% when the females can smell the males at least one night prior to mating (the so-called 'Whitten effect'). Unplugged females should not be mated again until a normal oestrus cycle has been re-established (approximately 10 days). This is necessary because the vaginal plug is sometimes lost and the female is judged as 'false-negative', despite her actually being pseudopregnant.

Anaesthesia and analgesia

Embryo transfer has to be performed under general anaesthesia. This can be achieved by intraperitoneal injection of a mixture of ketamine HCl 10% and xylazine HCl 2% (100 mg/kg ketamine and 4 mg/kg xylazine). Eye ointment is administered for corneal protection. In order to prevent hypothermia, cages with anaesthetized mice are placed on a warming plate (25-30 °C) until recovery. Despite the analgesic effect of this regimen we administer 0.2-0.4 mL metamizole sodium subcutaneously per animal directly after anaesthesia. Inhalation anaesthesia with isoflurane, using a vaporizer unit, is also possible. However, with this kind of anaesthesia no analgesic effect can be achieved. The advantage of inhalation anaesthesia is the fast recovery time.

Embryo transfer to the oviduct

Once the toe pinch reflex is lost, the lower back is shaved and disinfected. The mouse is placed under a stereomicroscope (6 × 20 magnification) with the tail facing towards the operator. A transverse incision (≤1 cm) is made across the lumbar area with fine dissection scissors and the skin is loosened from the tissue. The cavity is opened with a small transverse incision (0.5 cm) in the area of one ovary. The ovarian fat pad and oviduct are grasped with fine forceps and advanced and fixed using a small tissue clamp. The bursa ovarica is carefully pulled open with watchmaker's forceps. The embryo-transfer pipette is loaded with embryos (see the section 'Loading pipettes with embryos') and gently

inserted into the infundibular opening of the oviduct. The transfer capillary is held in place with the tips of watchmaker's forceps (Figure 4.7.9a). The embryos are blown into the oviduct with a minimum of fluid; the pipette has to be pulled out immediately and the infundibular opening squeezed with the forceps for a few seconds to prevent the embryos from flowing out. The ovary and oviduct are then replaced in the abdominal cavity. There is no need to suture the body wall if the incision is less than 0.5 cm. This procedure is repeated on the other side of the body. The skin is closed with wound clips, which should be removed 1 week after surgery.

Embryo transfer to the lumen of the uterus

The procedure is almost the same as for the oviduct transfer. The upper part of one uterine horn is extracted through the small incision in the body wall and the uterus is punctured with a 26G needle. Blood vessels should not be injured. After removing the needle, the transfer pipette is inserted into the hole and the embryos are blown into the uterine lumen (Figure 4.7.9b). The remainder of the procedure is as described in the previous section.

Vasectomy of mice

Outbred mice or F1 hybrid males (e.g. C57BL/6 × DBA or BALB/c × DBA) are ideal partners for the production of surrogate dams.

One efficient and fast method for vasectomy of mice is the cauterization of the vasa deferentia [44]. In addition to the surgical instruments a Bunsen burner and a pair of watchmaker's forceps are needed.

The male mice are between 8 and 10 weeks old. Anaesthesia can be achieved as described above. The mouse is positioned in a supine position and the abdomen shaved and disinfected. A small middle incision of the skin, 0.5 cm cranial to the preputial orifice, is made with fine dissection scissors. The abdomen is opened with a small longitudinal incision (0.5 cm). The fat pad surrounding the right testicle is grasped with forceps and pulled out until the vas deferens is visible. The tips of the watchmaker's forceps are made red hot under the flame of the Bunsen burner and a small piece of the vas deferens (0.5 cm) is removed by cauterization (Figure 4.7.10). Alternatively, a high temperature battery cautery set can be used. This procedure is repeated on the left side of the body. The ends of
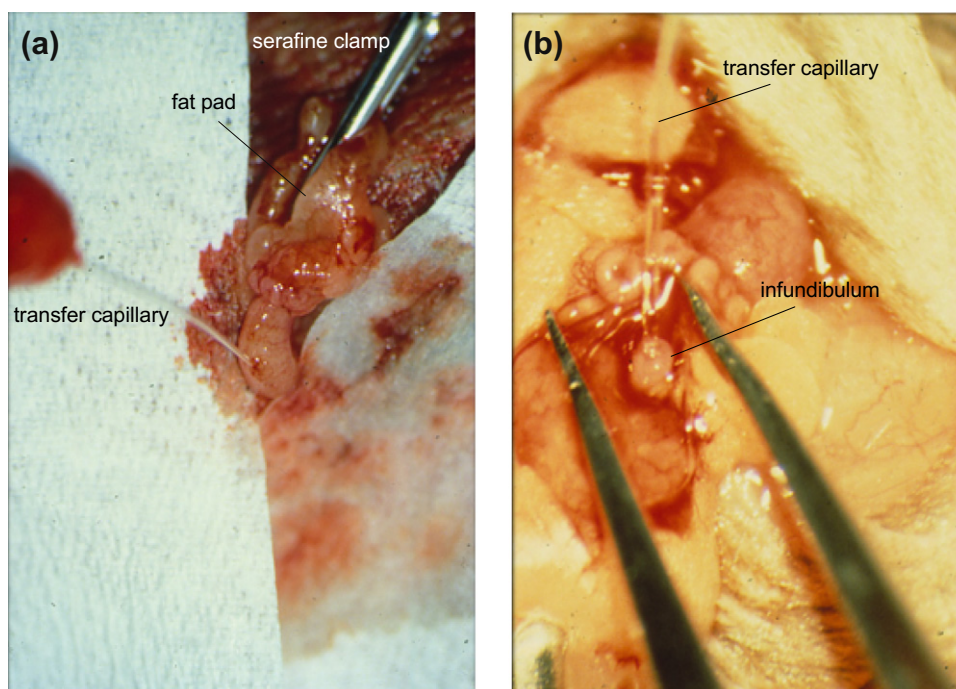
Figure 4.7.9 Embryo transfer. (a) Transfer to the uterus. (b) Transfer to the oviduct.
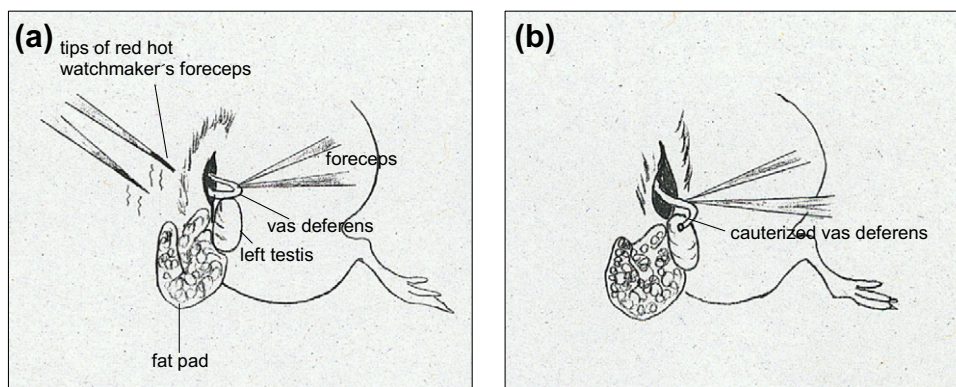
Figure 4.7.10 Vasectomy through cauterization. (a) Immobilization of the vas deferens. (b) Cauterization.

the vasa deferentia are returned to the cavity, which is closed with two or three sutures using an absorbable suture material. The skin is closed with non-absorbable material. During surgery, the testes are removed from the scrotum. By gently pressing the ventral abdomen with the thumbs, they slide back into the correct position. The males can be mated 1 week later. If possible, the vasectomized males should be mated twice, to ensure their sterility, before using them in the production of pseudopregnant surrogate dams.

The males can be used for about 1 year. For the permanent production of pseudopregnant surrogate dams, 30 sterile, sexually active males should be made available. To ensure this, 50% of the males should be exchanged every 6 months.

In vitro fertilization (IVF)

Freshly collected oocytes, or oocytes which have been frozen, can be fertilized by a method described by Hogan et al. [44]. For fertilization, unfrozen or freeze-thawed sperm can be used.

Media for IVF and in vitro culture

For IVF we suggest using HTF medium (human tubal fluid, Table 4.7.5) [45] and for *in vitro* culture M16 (Table 4.7.6). Media should be preincubated for at least 2 h before use (preferably overnight) at 37 °C, 95% humidity and 5% carbon dioxide. To prevent drying out, and to stabilize the pH, the medium should be covered with mineral oil (embryo culture tested).

Preparation of culture dishes

Four 450 μL drops of HTF medium are placed into small culture dishes (35 × 10 mm), and three 700 μL drops into 50 × 15 mm culture dishes. For culture after IVF four-well Petri dishes are prepared. The wells are filled with 150 μL M16 and covered with mineral oil and incubated as described above.

IVF using freshly collected oocytes and sperm

Approximately 12-14 h after hCG injection of the females, the males are sacrificed for sperm

TABLE 4.7.5: HTF medium [45] for *in vitro* fertilization

Substance	Final concentration (mM)
NaCl	101.60
KCl	4.96
$MgSO_4 \cdot 7H_2O$	0.20
KH_2PO_4	0.37
$CaCl_2 \cdot 2H_2O$	2.04
$NaHCO_3$	25.00
Glucose	5.56
Na pyruvate	0.33
Na lactate	21.40
Penicillin G (K salt)	100 U/mL
Streptomycin sulfate	50 mg/mL
Phenol red	0.01 g/L
BSA	4 g/L

Substances have to be mixed in the given order. Filter medium through a sterile filter (0.45 μm). Osmolarity should be 288–292 mOsm. HTF should not be stored longer than 1 week at 4 °C. HTF is also available commercially.

TABLE 4.7.6: M16 medium for *in vitro* embryo culture

Substance	Final concentration (mM)
H$_2$O	
NaCl	94.66
Na pyruvate	0.33
Na lactate	23.28
KCl	4.78
KH$_2$PO$_4$	1.19
MgSO$_4$·7H$_2$O	1.19
CaCl$_2$·2H$_2$O	1.71
NaHCO$_3$	25.00
Penicillin G, K salt	100 units/mL
Streptomycin sulfate	50 mg/mL
Phenol red in NaHCO$_3$	0.01 mg/mL
Glucose	1 mg/mL
BSA	

Substances have to be mixed in the given order. Filter medium through a sterile filter (0.45 μm). Osmolarity should be 288–292 mOsm. M16 should not be stored longer than 1 week at 4 °C. M16 is also available commercially.
Source: [44].

collection as described above in 500 μL PB1. An aliquot of 2×10^5 spermatozoa is transferred into each 450 μL drop of HTF in the small culture dish and incubated for 1.5 h at 37 °C and 5% carbon dioxide for capacitation.

The females are sacrificed for oocyte preparation 12.5 h after injection of hCG. The excised oviducts are transferred to a 700 μL drop of HTF and the cumulus–oocyte complexes are prepared as described above. The cumulus–oocyte complexes are directly pipetted into the capacitated sperm and incubated at 37 °C for 4 h. Cumulus cells should not be removed for IVF because these oocytes are more efficiently fertilized *in vitro* than cumulus-free eggs ([46], own experience). Four hours later the cumulus cell-free fertilized oocytes are transferred to M16 medium for further *in vitro* culture, or transferred to pseudopregnant surrogate dams. Fertilized oocytes can be recognized by a visible second polar body, which is generated during the second maturation division of the oocyte after sperm penetration.

IVF using frozen sperm

For sperm that has been frozen in French straws according to the JAX method [7], the content of the straw is pulled into the medium with the cumulus–oocyte complexes immediately after the sperm suspension has melted.

In case of sperm frozen by the pellet method, one sperm pellet is directly transferred into the medium with the cumulus–oocyte complexes.

IVF using frozen oocytes

After thawing and washing as described above, the oocytes are transferred to capacitated sperm in HTF medium.

Intracytoplasmatic sperm injection

Sometimes freeze-thawing of spermatozoa results in immotile sperm. In such cases intracytoplasmatic sperm injection (ICSI) is the method of choice to fertilize oocytes. ICSI can also be used if the concentration of motile spermatozoa is too low, for example, due to genetic modification. The technique described is adapted from Hirabayashi and Hochi [47]. We suggest learning and training the technique in a laboratory where ICSI is performed routinely.

Special equipment and media for ICSI

Specialized technical equipment is indispensible for ICSI. An inverse microscope equipped with a piezo-driven micromanipulator is ideal. In addition, capillaries for holding and injecting the oocytes are needed. These capillaries are commercially available in good and reproducible quality. The injection capillary is mounted on the side of the piezo micromanipulator. The lid of a Petri dish (35 × 10 mm) can be used as an injection chamber. This enables a nearly horizontal injection angle.

Two to three days prior to ICSI, PB1 (or M2 medium) is supplemented with 10% polyvinyl pyrrolidone (PVP). The PB1-PVP is kept at 4 °C, so that the PVP can dissolve completely.

One day (at least 2 h) prior to ICSI, HTF medium is prepared in culture dishes as described above.

On the day of ICSI, the PB1-PVP is brought to room temperature. The injection chamber is prepared by pipetting two 6 μL drops (drops I and II) of HTF medium and two drops (drops III and IV) of PB1-PVP into the injection chamber, and all drops are covered with mineral oil.

ICSI procedure

The whole procedure for ICSI has to be performed under the microscope. The cumulus cells have to be disaggregated from the oocytes. To do this hyaluronidase is added to the medium, reaching a final concentration of 10 000 units. Then 10-15 oocytes without cumulus cells are transferred to drop I, and 1 μL sperm suspension into drop III.

The injection capillary is washed several times by aspirating and blowing out PB1-PVP in drop IV. After washing, the injection capillary is moved to drop III and approximately 10 spermatozoa are soaked in the capillary. The injection capillary is now moved to drop I. After this, the sperm heads are deposited near the tip of the injection capillary and one oocyte immobilized with the holding capillary. The orientation of the equatorial plate should be from 12 to 6 o'clock. The zona pellucida is penetrated by the injection capillary with three to four piezo pulses. Then, one sperm is aspirated and the injection capillary is inserted through the hole. The vitelline membrane is pushed hard towards the holding capillary and penetrated by a single piezo pulse. The sperm head is then released into the cytoplasm. The injection capillary should be withdrawn immediately. The injected oocytes are deposited in drop II until all oocytes have been injected. All injected oocytes are then transferred to HTF medium for culture or are directly transferred to surrogate dams.

Orthotopic transplantation of ovaries

The ovary recipient has to be coisogenic to the ovary donor to prevent graft rejection. Should this option not be available, immunodeficient recipients have to be used. Mice carrying the *scid* mutation in the *Prkdc* gene ($Prkdc^{scid}$) can be used for this purpose. Recipient females should ideally be 4-6 weeks old.

The technique described here is adapted from Jones and Krohn [48] and Sztein et al. [29]. Recipient females are anaesthetized as described above. The surgical field is shaved and disinfected. A single transverse incision of the skin across the lumbar area gives access to the ovaries on both sides. First, the right ovary is removed. For this, the cavity is opened with a small transverse incision. The ovary is extracted from the cavity by grasping the surrounding fat with forceps. A single ligature with absorbable suture material is made proximal to the oviduct-uterus junction and the ovary is removed with a single cut. The remaining tissue is replaced in the cavity. The incision is closed with absorbable suture material.

To remove the left ovary, the cavity on the left side of the body is opened as described. The ovary is carefully removed. A small incision is made in the bursa ovarica with microsurgical scissors and the ovary is removed and replaced by the donor ovary. The cavity is closed as described. It is important that both of the recipient's ovaries are totally removed, to ensure that the offspring is from the transplanted organ.

Two weeks after surgery the reproductive cycle should be established and the recipients can be mated with appropriate males.

Miscellaneous

Record-keeping

Precise record-keeping should include the following data: type and physical conditions of the freezing procedure, temperature recording, results of viability tests from control batches, description of mouse strain (strain history, phenotype, reproductive performance, pedigree information, genotype, generation), identification and storage location, hygienic status of the donor animals. For embryos the number and developmental stage must also be recorded.

Pipettes for embryo handling and embryo transfer

Pipettes for embryo handling are pulled from Pasteur pipettes [49]. The narrow part of the pipette is held over the blue flame of a Bunsen burner. As soon as the glass begins to soften, it is removed from the flame and pulled out immediately. The narrow part should be 7.0 cm in length. The tip should have an inner diameter of 150-200 μm. To polish the tip, hold it under the blue flame of the Bunsen burner for less than a second. The pipettes are attached to a silicone tube connected to a mouthpiece (Figure 4.7.11).

Pipettes for embryo transfer are pulled out from the narrow end of a Pasteur pipette to an inner diameter of 120-150 μm. The resulting capillary (10 cm long) is inserted into the shaft of a Pasteur pipette (2 cm of the capillary should project). The capillary is fixed in position with sealing wax (Figure 4.7.12).

Loading pipettes with embryos

To prevent capillary forces, the micropipettes are dipped briefly into the medium and immediately removed. Then, a small air bubble is drawn in followed by a second column (0.5 cm) of medium and another air bubble. The embryos can now be easily collected from the medium. At least one more air bubble and a small amount of medium follow. When the embryos are expelled into the reproductive tract of a foster mother, the second air bubble allows the control of successful transfer. Figure 4.7.13 illustrates how the embryos
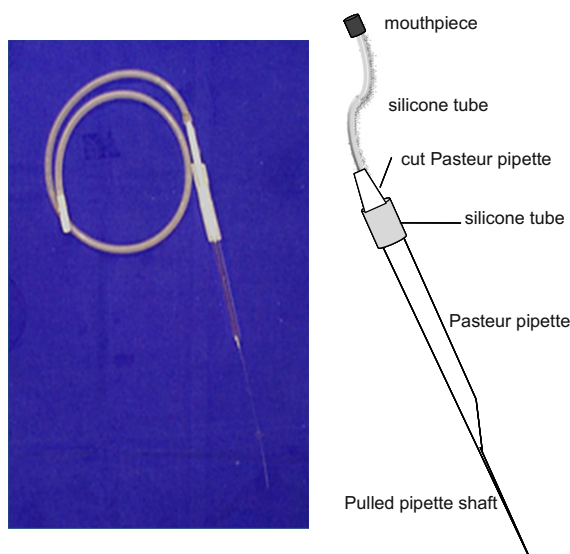
Figure 4.7.11 Drawn-out Pasteur pipette with a mouth-pipetting device for embryo handling. (Rafferty, 1970).
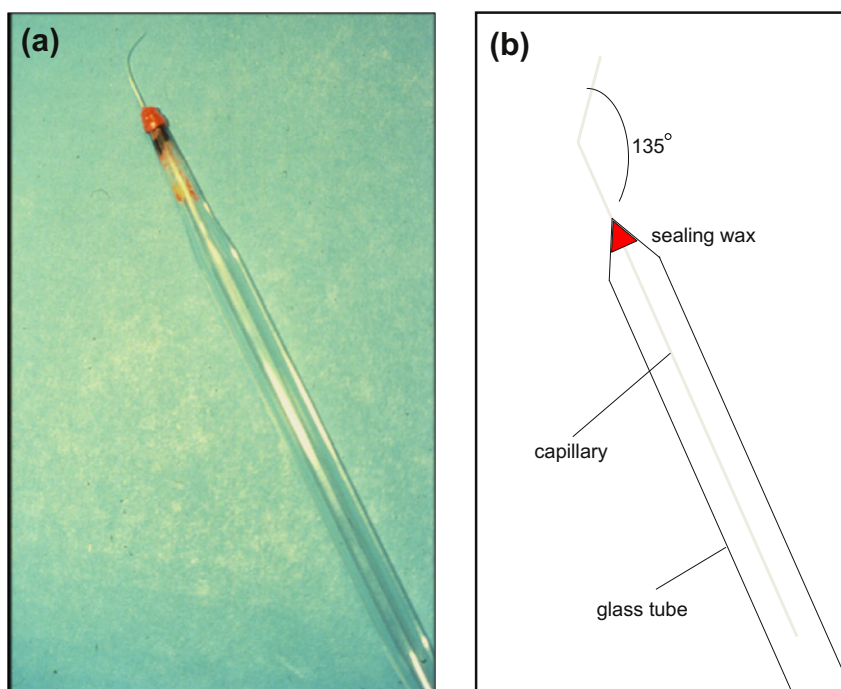
Figure 4.7.12 Embryo transfer capillary. (a) Photograph. (b) Schematic view.
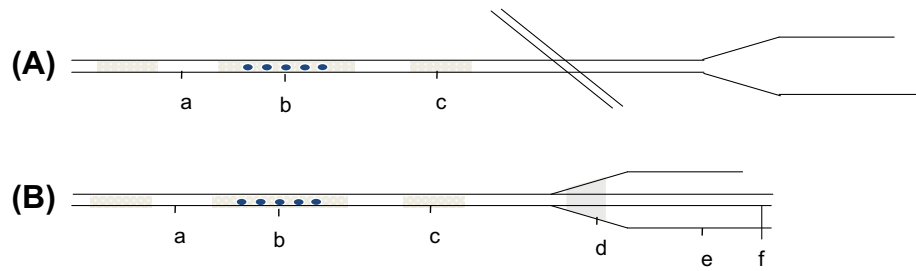
Figure 4.7.13 Tips of capillaries loaded with embryos. (A) Narrow part of a drawn-out Pasteur pipette for embryo handling. (B) Tip of a transfer capillary. a, air bubble; b, medium containing embryos; c, medium; d, sealing wax; e, glass tube; f, capillary.

must be sucked into the handling and transfer capillaries.

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Handling and Restraint

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Introduction

The use of animals in biomedical research can be traced back to the 1600s. Since then the mouse has contributed to a vast number of scientific findings and progress in basic biological and pharmaceutical research [1]. An enormous number of different inbred and outbred mouse strains, including genetically modified mouse lines, are available and are used in research laboratories worldwide.

Mice are not always regarded as a species with a strong drive to cooperate. Despite the lengthy period of selective breeding in captivity, the natural behavioural pattern of the wild mouse—although less prevalent—still persists. Laboratory mice have to be 'involved' by means of professional handling/restraint in order to perform all procedures necessary during husbandry and/or experimentation. Allowing for strain-related differences, mice are not usually very aggressive and can be handled or restrained without major problems. Correct handling is imperative during experimental work, but it should begin early, at breeding sites and be continued during daily husbandry, in order to familiarize the animals with people and manipulations. Little scientific information on handling and restraint of mice is available, but many general textbooks deal with the technical approach to this topic [2-9]. This chapter presents comprehensive information about handling and restraint, including the personal experience of the authors with this species.

Occupational health and risks

Injuries

Work with laboratory mice does not usually bear much risk of severe injuries. Minor incidents such as mouse bites, mainly to fingers, may occur if staff members are not very experienced and/or adequate protective measures are not properly used during handling and restraint. Mice can move extremely quickly and will usually tend to escape or defend themselves if they are given the opportunity to do so. In addition to proper handling and restraint, the wearing of single-layered synthetic hypo-allergenic gloves, or preferably a double layer of both cotton and synthetic gloves, has considerable potential to reduce the number of mouse bites perforating both gloves and skin. The initial reluctance of staff to use double gloves is eventually overcome because it can increase the comfort and well-being of people handling and restraining animals.

Human infection and disease

Most purpose-bred laboratory mice from defined sources are specified pathogen free (SPF), and repeated microbiological testing during housing and experimentation is recommended [10-12]. Despite those precautions, the laboratory mouse and its excretions still harbour the potential to transmit opportunistic agents and cause human disease. Infection of skin scratches and bite wounds with mouse- or human-borne opportunistic microbes demands attention through a strict occupational medical treatment programme [13]. Immediate cleansing and disinfection of the wound is the first step in order to prevent infection. Special attention should be given to mice experimentally infected with human-pathogenic or zoonotic agents or genetically modified mice which harbour receptors for human pathogens. Both cases require work in higher biosafety level containments, additional screening methods and special guidelines for the handling and restraint of such animals. Tumour cells that are implanted into mice should be microbiologically screened for human and mouse pathogens and excluded from use if found positive.

Allergies

The development of human allergy to mice has been observed for more than 25 years in people working with these animals. This phenomenon, also called laboratory animal allergy (LAA), is a form of occupational allergic disease which includes a great number of laboratory animal species to which people may develop allergic reactions. After the sensitization phase, resulting from complex processes within the immune system, allergy occurs and is usually represented by nasal symptoms (e.g. sneezing, watery discharge), eye reactions or skin rashes. Asthma and, rarely, a life-threatening allergic reaction due to bite-related anaphylaxis may occur.

The level of exposure to the laboratory animal allergen is crucial to the nature and intensity of the symptoms [14]. In mice, the most important allergen is Mus m 1, the major urinary protein (MUP) which is a prealbumin and may be found in urine as well as in hair follicles and dander. As the level of production of this protein in the liver is driven by testosterone, it is predominant in adult male mice. The second mouse allergen, Mus m 2, is a glycoprotein, also found in hair and dander and the third one is albumin, a serum protein. Mouse allergens can be distributed and found throughout an animal facility and even spread into separate buildings adjacent to the facility. This wide distribution of particles may also cause problems of sensitization and allergy to people not directly working with mice. However, the highest exposure to allergens has been reported in people dealing with cage cleaning and feeding of the animals [15].

In order to reduce exposure to mouse allergens and prevent LAA, personal protection measures should be taken. Street clothing should never come in direct contact with mice or their excretions. When working with animals, appropriate protective clothing is mandatory. This includes laboratory coats, long non-allergic gloves and adequate respiratory protective equipment to reduce skin contact with animal products such as urine, dander and serum. After finishing work with mice, protective clothing should be left within the animal facility.

Further measures can be taken for animal husbandry and handling: directing airflow away from workers, performing manipulations within ventilated hoods where possible, installing ventilated animal cage racks or filter-top cages, using absorbent pads for bedding, etc. helps to reduce the allergen load [16]. Combined use of ventilated micro-isolators, cage change stations and benches for procedures and robotics for automatic cage emptying and cleaning, together with the use of a centralized vacuum cleaning system, results in considerably lower exposure levels to allergens [17]. Detailed information about occupational health issues with regard to allergies is provided in a review about laboratory animal allergy including an outline LAA management programme with recommendations and questionnaires [18].

Definitions

Handling

Handling in this context is defined as dealing with a mouse by hand, in a direct or indirect way—with or without touching the animal. Handling should always be done in a species-specific, calm and firm way in order not to harm the animal and provide as much safety as possible to the experimenter. In order to reduce the stress component of any handling procedure to a minimum for both parties, the personnel involved should be dedicated to animals, motivated and well trained. The aims of training are attainment of sovereign handling skills as well as habituation of animals to people and manipulations with as little disturbance of their physical and psychological well-being as possible. In the best case, animals may even be motivated to cooperate with their trainers. This voluntary approach of the animals not only facilitates work and enhances safety for handlers but also helps to reduce stress-induced changes in the animals' physiological parameters under experimental conditions.

Restraint

Restraint is described as immobilization of an animal by keeping it, or parts of it, in a comfortable but safe hold by hand or by means of a physical device. Physical restraint is performed on conscious animals undergoing manipulations that do not require sedation or anaesthesia, but necessitate exact positioning and prevention of unexpected movements. Restraining measures are indispensable for performing experimental work. They help to avoid injuries in animals and also provide an adequate level of safety for the animal handler. In instances where unacceptable stress or pain may occur in the animal, physical restraining measures may be facilitated by sedation or general anaesthesia and analgesia.

Handling techniques

Despite the general ease of handling, only limited cooperation and voluntary approach can be expected in mice, even after a prolonged time of acclimatization to procedures. Therefore, positive reinforcement training is not widely distributed in this species. Handling is generally restricted to individual or group transfer during cage change or transfer of animals to and from the experimental environment. As with other species, hectic and jerky movements should be avoided. The animals should be given time to investigate the handler's hand and become adapted to the smell of the gloves. After manipulations on them are finished, mice should never be dropped but should be gently placed back into their cage.

Transfer of mice

Techniques without animal fixation

Individual or small groups of mice, often sitting together in a corner of the cage, can be surrounded with the cupped palms of both hands. Without exerting any pressure, the hands are then slid towards each other beneath the mice and the whole group is lifted up and transferred, e.g. to another cage, where they are gently placed on to the cage bottom (Figures 5.1.1 and 5.1.2). This method is very effective when animals are not used to being handled and/or the transfer must be time-efficient.

Figure 5.1.1 Grasping a group of C57BL/6 mice.

Figure 5.1.3 C57BL/6 mouse climbing into a glass beaker.

Another way of transferring individual or groups of mice is by using a glass or plastic bowl. The vessel is brought close to the mice with its open end directed towards the cage wall. Mice can then be encouraged to climb into the container (Figures 5.1.3 and 5.1.4).

A cardboard reel or polycarbonate tunnel may also serve as a means of transfer, as mice like to climb onto the device (Figure 5.1.5) or crawl into it. They can be replaced by being allowed to climb freely from the device into the cage (Figure 5.1.6).

Handling techniques without fixation of the animals are usually very well tolerated by mice.

Techniques with animal fixation

For a short transfer lasting less than 2-3 s, mice can be gripped by the base of the tail, lifted up and carried to the new destination. When heavy,

obese or pregnant mice are to be transferred, they have to be supported by the other hand. The tail should be held in one hand in order to prevent the animal from escaping (Figure 5.1.7). When transferring mice over longer distances, they should always be placed on the hand and must not be carried by the tail; otherwise the overlying skin of the tail may become detached from the body due to the force exerted on it. Again, mice are put back into the cage gently. After weighing, for example, they can be released directly from the scale pan into the cage.

In case of special hygienic precaution requirements (e.g. SPF or immunocompromised animals), where exposure of the animals to potential pathogens and opportunistic microbes should be kept to a minimum, mice can also be transferred by means of forceps (25-30 cm long, with rubber protected tips). The loose skin at the rear of the neck (neck fold) is grasped with the forceps. In order not to harm the animal, it is approached

Figure 5.1.2 Carrying a group of C57BL/6 mice in the cupped hand.
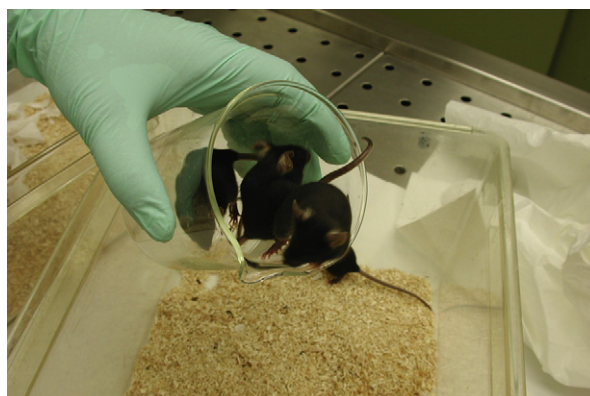
Figure 5.1.4 Transferring a group of C57BL/6 mice within a glass beaker.

Figure 5.1.5 C57BL/6 mouse climbing onto a cardboard reel for further transfer.
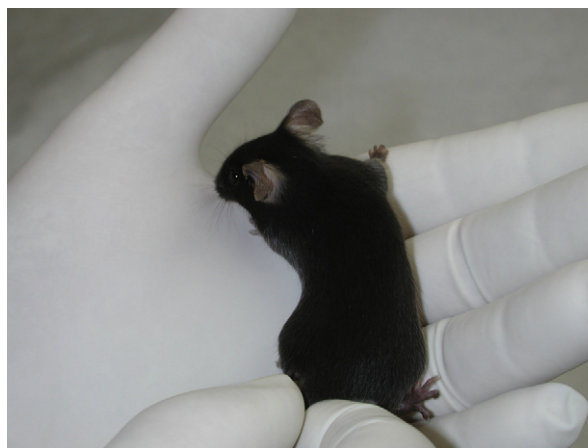
Figure 5.1.7 Carrying a C57BL/6 mouse in the hand while fixing the tail.

from behind with the forceps and lifted carefully (Figure 5.1.8). The animal is released gently by opening the forceps after placing it on the bottom of the new cage. This method mimics the behaviour of a mouse pup carried by its mother by gripping the pup's neck fold with its mouth. The resulting relaxation can still be seen in adult animals when being handled (Figure 5.1.9a, b).

Transfer of litters and mother

When transferring a mother with her litter, the mother is removed first, in order to reduce defensive reactions when the nest is taken out of the cage. The female is transferred according to the procedure described above and placed into the new cage. The litter, i.e. nesting material and pups together, is grasped by sliding cupped hands

beneath the nest. The whole nest with its contents is then lifted, carried to the new cage and gently replaced, preferably not touching the pups with unprotected hands. When litters are transferred in this way, the female usually immediately approaches the nest and returns to the pups without any problems (Figures 5.1.10 and 5.1.11).

Restraining techniques

The limited cooperation of the mouse, its unpredictable behaviour and continual readiness to bite when being restrained demand careful action and proper restraint of each individual animal. This includes secure immobilization that minimizes movements of the animal but still allows it to breath normally. Such action avoids casualties even in very sensitive strains and

Figure 5.1.6 BALB/c mice sitting inside a tunnel.
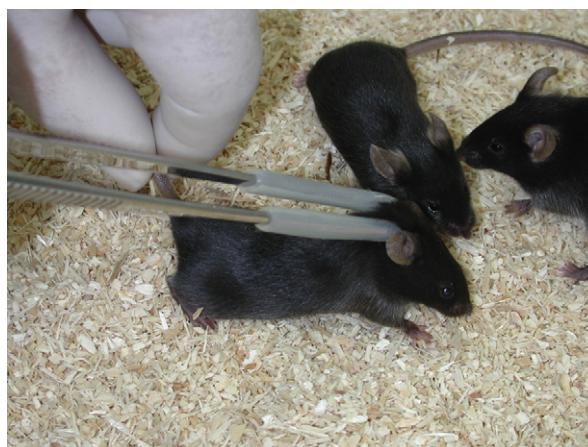
Figure 5.1.8 Gripping a C57BL/6 mouse with rubber-tipped forceps.
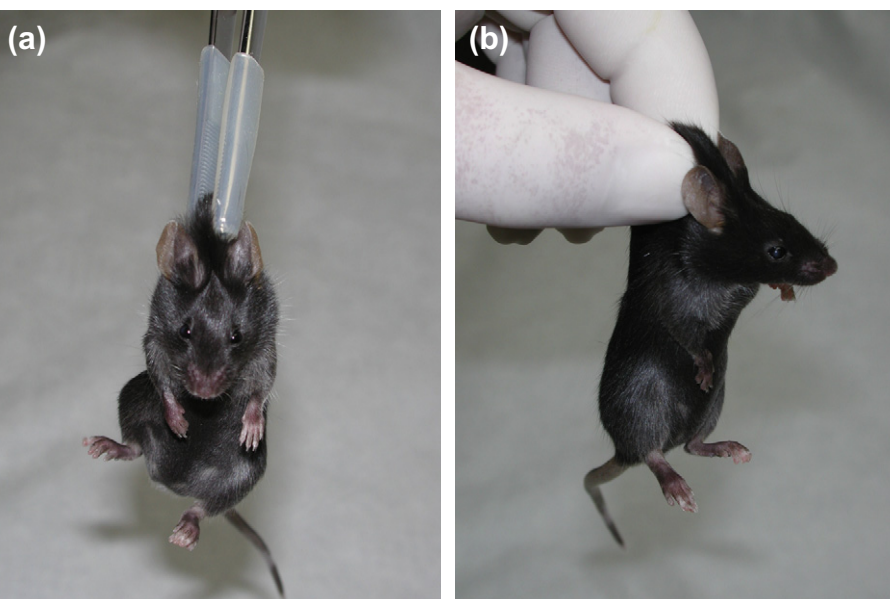
Figure 5.1.9 (a) Carrying a C57BL/6 mouse with rubber-tipped forceps in a relaxed position; (b) C57BL/6 mouse carried by hand at neck skin fold; mimics carriage by mother. Note the natural relaxation.

reduces the risk of the animals being harmed by the handler as a result of vigorous dropping of a mouse after a bite. Gentle release in the researcher's hands before returning the animal into the cage can contribute to the animals' adaptation to restraining procedures.

Restraining by hand

The tail of the mouse is gripped at its base and the mouse is lifted on to the grid cage top. As the tail is gently pulled backwards, the animal tends to move forward and to hold on to the grid with its forelegs. At this moment, the other hand approaches the rear of the neck and the skin fold is grasped with thumb and forefinger quite close to the ears, while the loose skin extending over the back is gripped with the other fingers. It is important to grip the loose skin at the rear of the neck properly, in order to prevent the animal from turning its head and biting the handler's fingers. At the same time, care must be taken not to impair the animal's breathing and venous blood backflow from the head to the chest. By turning the palm of the hand upwards, the mouse is positioned with its ventral side uppermost. The tail is then gripped between
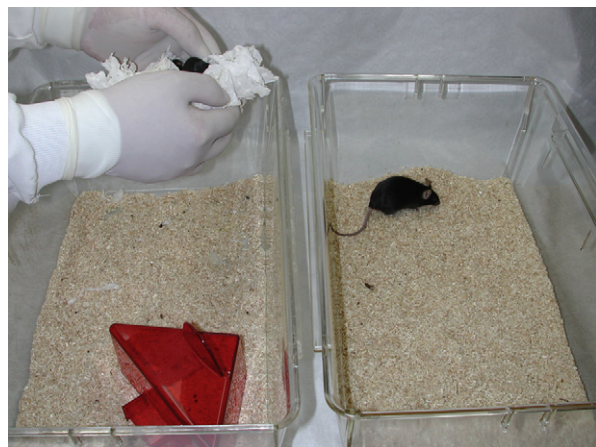
Figure 5.1.10 Grasping a nest with litter from the bottom of a cage. Note: C57BL/6 mother has been transferred first.

Figure 5.1.11 Placing a complete nest with litter and nesting material back into a cage.
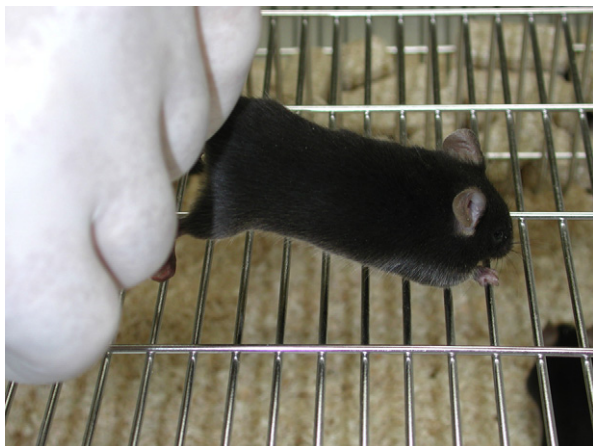
Figure 5.1.12 Fixation of a C57BL/6 mouse by the base of its tail.

Figure 5.1.14 Fixing loose skin along the back and tail of the C57BL/6 mouse.

the third finger and the ball of the thumb. The head and body of the animal are brought into a straight and comfortable position with its back being supported by the palm of the hand. In this position the mouse is held safely for any further manipulations (Figures 5.1.12–5.1.16).

Mouse pups can be restrained in two ways: (i) without any prior handling, the thumb and the first two fingers are placed around the shoulder and thorax region and the animal is picked up from the cage. It is then held in this way and can be positioned for physical examination or rectal temperature recording, for example (Figure 5.1.17); (b) a skin fold in the dorsal neck/shoulder region is first grasped between the thumb and the index finger. Special care has to be taken not to restrict the pup's breathing because of its small size. After positioning the

pup in the same way as described for the adult, drugs can be orally administered by means of metal or plastic gavage needles, for example (Figure 5.1.18).

Two further indications in which manual restraint is frequently used are tail marking and sexing. *Tail marking* is best done by lifting the mouse at the base of its tail and putting it on to the grid cage top. Then the tail is gently pulled backwards and different marks can be applied by means of a waterproof text marker. For *sexing*, the mouse is put on to the grid cage top and the tail is carefully pulled backwards in the same way as described for tail marking. When the animal reaches an extended position due to its attempt to move forward, its back is gently pushed downwards with the third and fourth finger while the tail base and rear legs are lifted

Figure 5.1.13 Grasping a skin fold at the rear of the neck with thumb and forefinger.

Figure 5.1.15 Final fixation for further manipulations.

Figure 5.1.16 Fixation of a C57BL/6 mouse and intraperitoneal injection into the left caudal abdomen.

Figure 5.1.18 Restraining a mouse pup during oral dosing.

up in order to expose the genitalia. The sex can then be determined by checking the anogenital distance, which is longer in male animals (Figures 5.1.19 and 5.1.20).

Restraining by means of a device

New short-term mouse restraining devices are continuously being developed. They may be either custom-built for special purposes or obtained from commercial sources. Materials used include soft leather or textile tissue, plastic, hard plexiglass or polycarbonate (e.g. Macrolon) and metal among others. The design and choice of material for such devices is restricted by hygienic requirements as well as avoidance of harm and stress for animals and humans. Optimally, the restraining device allows the

experimenter to have both hands free for the execution of procedures on the animal. Long-term restraining devices should allow the animal to fulfil its basic physiological needs and must be well ventilated in order not to cause heat discomfort for the animals. Commercial catalogues are available from various suppliers, or devices can be searched for on the internet [19, 20].

Some examples of commonly used restraining devices are shown in Figures 5.1.21-5.1.25. Various restraining devices for blood sampling from the lateral tail vein, or injections into it, are widely distributed and many of them are commercially available. An example of a 'restraining wall' which has been used and modified is given in Figure 5.1.21. To restrain the mouse, the animal is grasped at the base of the tail and lifted up. It is then positioned in front of the wall with its tail being placed in the slit and

Figure 5.1.17 Fixation of a C57BL/6 mouse during temperature recording. Note: this is an adult animal.
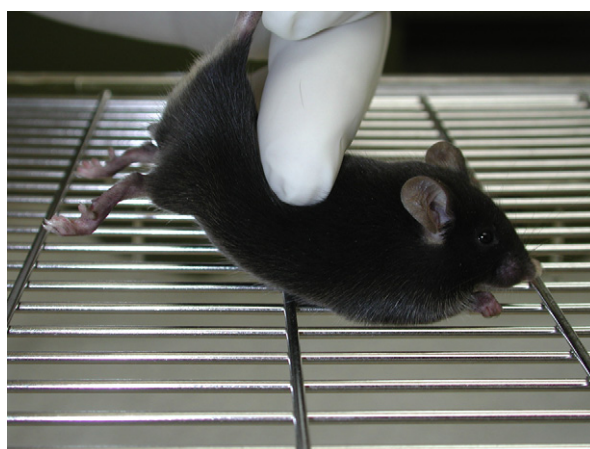
Figure 5.1.19 Animal positioning for sexing.

Figure 5.1.20 Exposing genitalia for gender determination of the animals by checking the anogenital distance; left: male, right: female.

Figure 5.1.22 Restraining conical cylinder for manipulations on the tail of a BALB/c mouse.

the mouse is lowered to the underlying platform. When the mouse has reached the bottom, the tail can be gently pulled backwards and blood can be taken from the tail vein with the animal not being squeezed into a narrow tube but allowed to move freely.

A conical cylinder (Harvard Apparatus, Holliston, USA) is shown in Figure 5.1.22. To restrain the mouse, it is grasped at the base of its tail, lifted up, supported and positioned at the front end of the cylindrical tube. It is then gently pulled backwards to the rear end of the cylinder by carefully keeping its tail outside the slit in the upper half of the device. When the mouse has reached the narrow end of the tube, the tail can be gently pulled backwards and blood can be taken from the tail vein.

Figure 5.1.23 shows a flat-sided cylinder (Harvard Apparatus, Holliston, USA) for exposing the tail of the animal or giving injections through openings in the upper and lower halves of the device. The mouse is placed at the open end of the tube, and then introduced forwards into the device. As soon as it has reached the front end, a divider is positioned through one of the slits in the upper half of the cylinder. Special care has to be taken to provide enough space lengthwise and around the head of the animal to ensure proper breathing. Now, for example, the tail can be slightly pulled backwards through an opening in the divider and the animal is ready for further manipulations. Other means of restraint include devices made of flexible material like the 'mouse snuggle'

Figure 5.1.21 Using a restraining wall for, e.g., blood sampling on the lateral tail vein of a BALB/c mouse.
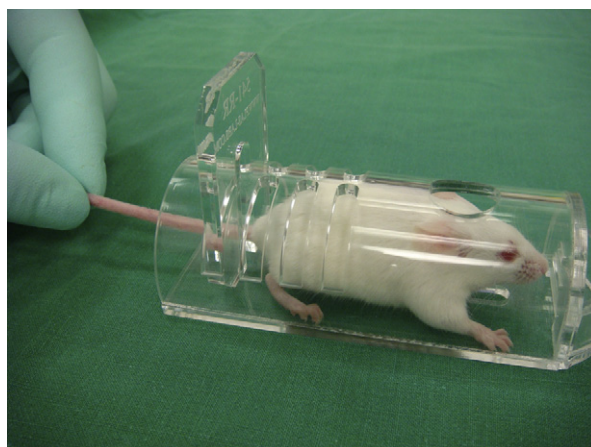
Figure 5.1.23 Restraining flat-shaped cylinder for manipulating a BALB/c mouse.
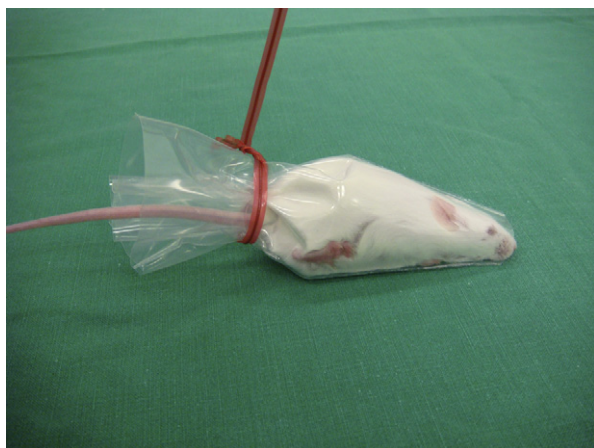
Figure 5.1.24 BALB/c mouse positioned inside a triangular plastic sleeve.

(LOMIR, Biomedical Inc., QC, Canada), a kind of cocoon with adjustable flaps in which the animal can be wrapped. Access to body parts for different procedures is provided by appropriate positioning of the flaps.

Figure 5.1.24 shows a triangular plastic sleeve for restraint. The animal is gripped at the base of the tail, lifted up and supported. After sliding the mouse into the sleeve, the animal is advanced to the narrow tip, orienting its nose in the small opening for smooth breathing. When the mouse is positioned correctly, the surplus material at the animal's hindquarters is gathered, drawn and secured with a 'twisty' wire around the base of the tail. By carefully cutting appropriate slots into the plastic, different parts of the animal can be accessed for subcutaneous or intraperitoneal injections or blood sampling (Figure 5.1.25).

Figure 5.1.25 BALB/c mouse positioned inside a body triangular plastic sleeve with hindleg exposed for possible blood sampling at the vena saphena.

Mice are usually well restrained and stay calm and relaxed in these sleeves.

Effect of handling and restraint on well-being of mice

Little basic research has been done on the stress-related impact of handling or short-term restraint on mice, with only limited scientific background information being available on this topic. Stress is considered to be influenced by the combination of restraint and procedure and to be dependent on the duration and frequency with which the animals are exposed to manipulations. The effects of continuous restraint stress can be manifold and range from temporary weight loss to restraint-induced pathology [21].

Further studies have revealed more subtle stress responses. It has been shown that mice that were restrained for 12-24 h in restraint cages and tubes showed reduction of lymphocyte cell numbers in lymphoid organs and suppression of *in vivo* antibody production [22], elevation of endogenous glucocorticoid and suppression of migration of granulocytes and macrophages to an inflammatory focus [23], delay of cutaneous wound healing [24] and impairment of bacterial clearance during wound healing [25]. In a study of strain-dependent stress responses in mice, a 1 h tube restraint induced an increase in prolactin and REM sleep in C57BL/6J but not in BALB/cJ mice [26]. A comparison of several studies showed that routine laboratory animal handling procedures such as lifting the animals or cage moving may cause increase of heart rate, body temperature and corticosterone levels in mice [27]. The impact of different restraint measures on plasma corticosterone, heart rate and body temperature was investigated in mice. Animals restrained in a tube restraint showed the highest increase in plasma corticosterone as well as the highest heart rate during the recovery period [28].

There is still some controversy as to whether frequent handling and restraint will reduce or increase stress in the mouse. Although repeated restraint has been found to cause significant

impairment of anti-tumour T cell responses [29], further studies are required to clarify the effect of repeated handling and restraint in the mouse.

Recently, the impact of different handling methods on anxiety and stress responses in three different mouse strains has been studied. This study has shown that capturing and picking up mice by the tail resulted in less voluntary approach towards the handler, more frequent urination and defecation, and more signs of anxiety in an elevated maze test. In contrast, mice which were handled without restraint, by means only of a tunnel or the cupped hand, were much more willing to enter these devices voluntarily. In a later phase, animals that were handled via tunnels or open hand showed no aversion to tail handling for abdominal inspection and even scruff restraint did not reverse the beneficial effects as no increase in avoiding to the handler was seen in these mice [30].

Handling and restraint should be carried out in a firm, confident and gentle manner and constant care should be taken not to crush or squeeze the animals [31]. The different temperament, adaptability and stress sensitivity of strains must be taken into account before any final conclusion regarding stress response to handling and restraint can be made.

Summary and recommendations

Despite its limited friendliness and cooperative behaviour the laboratory mouse is the most widely used species in the *in vivo* research laboratory because of the many other benefits it brings, e.g. its high reproductive rate, small size and vitality. The mouse shows a generally less positive response to good handling than the rat, but the risk of deep bite injuries is low. The animals should be approached, handled and restrained with care and deep respect. All measures should be taken to ensure competent and minimally stressful manipulation. This can be achieved by professional training of the experimenters and animal care staff. Whenever possible, latest findings about the impact of handling and restraint on the well-being of mice should be taken into account when working with these animals. Proper handling and restraint contribute to refinement of animal research and validity of research data.

Physical restraint may allow a range of safe and efficient manipulations in mice, such as subcutaneous, intraperitoneal and intramuscular injections or gavage applications. However, the procedure-related stress and pain of an animal should be evaluated carefully.

Safe and efficient anaesthetic agents providing fast onset of anaesthesia together with a short recovery phase may be used for chemical restraint of mice. They are indicated in situations where physical restraint of conscious animals may not be appropriate for certain procedures from an animal welfare point of view. Such instances may not only be surgical events but also injection of transponders, tattooing of tails, ear punching and injections of compounds (for chemical restraint see Chapter 5.4).

Acknowledgements

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C H A P T E R

5.2

Routes of Administration

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Introduction

Mice are the most widely used animals for a range of experiments including medical, chemical, pharmacological, toxicological, biological and genetic. The administration of test substances, such as chemical elements, compounds, drugs, antibodies, cells or other agents to mice is one of the major methods for evaluating their biological activity. A knowledge of available methods and techniques of administration, as well as knowledge of the deposition and fate of the administered substance, will help scientists to select the most appropriate route for their purpose. The administration route is largely dependent on the property of the test substance and the objective of the experiment. All administration should be performed with knowledge of the chemical and physical characteristics of the substance. All routes have both advantages and disadvantages, such as the absorption, bioavailability and metabolism of the substance. Consideration should be given to the pH, viscosity, concentration, sterility, pyrogenicity, irritancy and toxicity, as well as the existence of hazardous substances. In addition, animal welfare must be taken into consideration in deciding on the administration route, and the route must be selected before the start of any experiment.

Proper restraint is the most important technique when mice are treated as this decreases stress and increases successful treatment. Personnel using experimental animals should be well trained in handling and restraint; they should be qualified in the responsible use of experimental animals and attain a scientifically high standard [1, 2]. Further experience will lead to repeatable and reliable results (see Chapter 5.1). The foundation of animal welfare is that during administration mice should be protected from pain, suffering, distress or lasting harm—or at least pain and distress must be kept to a minimum [1]. Some injection sites (such as footpad injection) are strongly discouraged and if required must be justified on a case-by-case
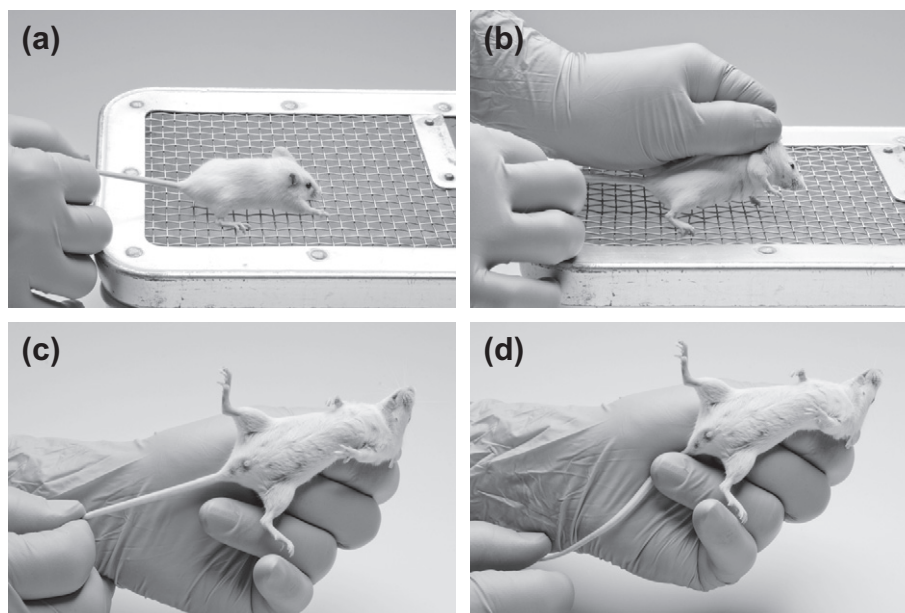
Figure 5.2.1 Manual restraint of a mouse using both hands. (a) The mouse is placed on the cage lid with the preferred hand. The hand pulls the tail gently back. (b) The mouse is quickly and firmly picked up by the scruff of the neck behind ears with thumb and index finger of the other hand. (c) The tail is transferred from the preferred hand to between palm and little or ring finger of the other hand, then held firmly. (d) The mouse is restrained.

basis [3]. The regulation of administration sites, methods, kind of substances and amount to be administered all have to be reconciled with the animal welfare board and permission has to be obtained.

Principles of administration

Handling and restraint

Good handling and restraint is the most important technique for correct administration. Proper restraint leads to successful administration and varies with the routes of administration. Disposable gloves must be worn, as manual restraint is frequently used for injections. There are two styles of manual restraint, one using both hands and the other single-handed (see Chapter 5.1; [4, 5]).

Two-handed manual restraint

The mouse is lifted by the base of the tail and placed on the cage lid or other solid surface with one hand and then its tail is pulled gently

back (Figure 5.2.1a). It is then quickly and firmly picked up by the scruff of the neck behind the ears with the thumb and index finger of the other hand (Figure 5.2.1b). The tail is transferred from the first hand to between the palm and little or ring finger of the other hand, then fixed (Figure 5.2.1c). The mouse is now restrained (Figure 5.2.1d).

Single-handed restraint

The tail is picked up using thumb and index finger of the chosen hand (Figure 5.2.2a), then the mouse is placed on the cage lid or other solid surface (Figure 5.2.2b). The tail is immediately grasped by the palm and middle finger, ring finger and/or little finger, and the thumb and index finger released (Figure 5.2.2c). The fold of skin from the scruff of neck down the back is immediately gripped using the thumb and index finger (Figure 5.2.2d, e). The mouse is then restrained (Figure 5.2.2f).

To prevent kicking by the hind legs, the tail is fixed using the palm and index finger and then the left hind leg is held firmly between the ring and little finger (where the mouse is restrained by the left hand) (Figure 5.2.3).
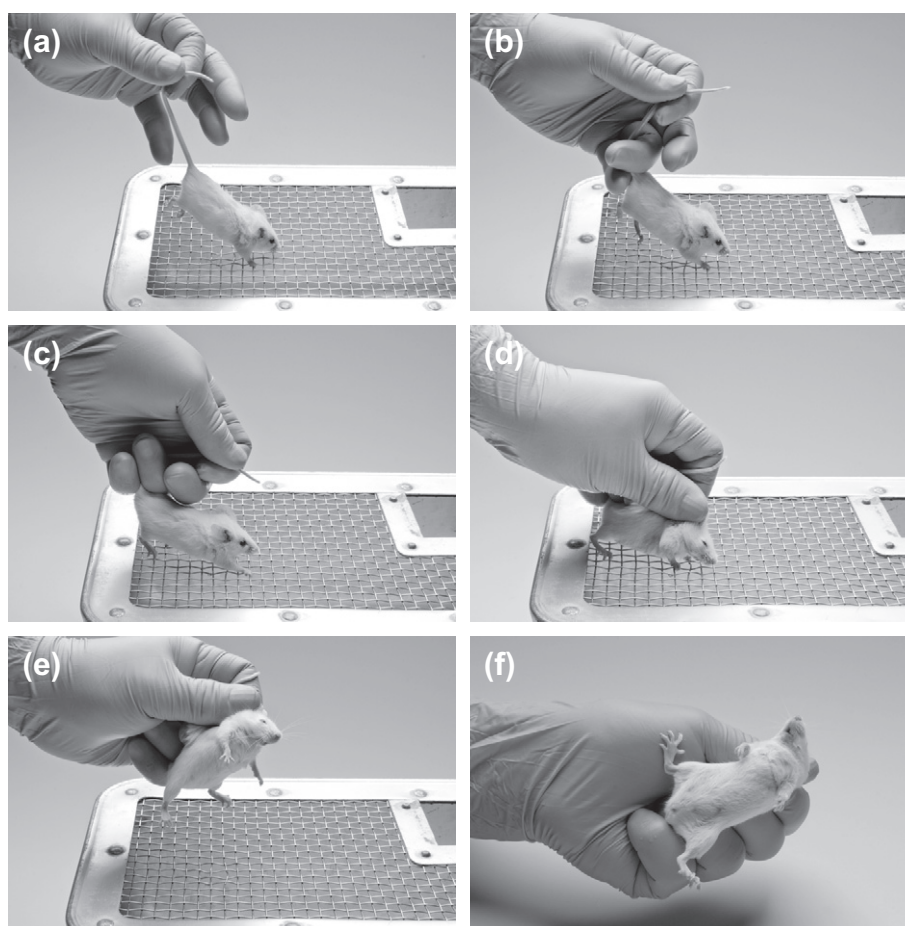
Figure 5.2.2 Single-handed restraint of the mouse. (a) The tail is picked up using thumb and index finger of preferred hand. (b) The mouse is placed on the cage lid or other solid surface pulling gently back by the hand. (c) The tail is immediately grasped by the palm and middle finger, ring finger and/or little finger and then the tail held between thumb and index finger is released. (d) and (e) The fold of skin from the scuff of the neck down the back is immediately gripped using the thumb and index finger. (f) The mouse is restrained.

Figure 5.2.3 Manual restraint of a mouse to prevent kicks from hind leg. The tail is held using the palm and index finger and then left hind leg is fixed between the ring and little finger (when the mouse is restrained by the left hand).

Site of administration

Among several possibilities for the administration of substances to mice, the most common are subcutaneous, intraperitoneal or intravenous injection. Intramuscular administration is not recommended, as the muscle of the mouse is too small. Some sites, such as footpad injection of Freund's complete adjuvant, intrasplenic injection and intralymph node injection are unacceptable nowadays [3], and should be restricted to cases where they are absolutely necessary.

Preparation of the site

The area for administration is clipped (Figure 5.2.4) or cleaned with warm water if necessary before cleaning the skin with cotton wool moistened with alcohol or disinfectant. Where aseptic skin is necessary the fur must be clipped, followed by a three-stage surgical preparation: surgical soap, alcoholic rinse and surgical preparation solution. The skin is dried immediately before administration [3]. In some cases a local anaesthetic may be applied first to prevent pain.

Preparation, solubility and safety of solutions

Test substances, solutions and equipment should be prepared aseptically and free from pyrogens, especially for parenteral injections. Solutions can be sterilized by filtration (0.22 μm). Living organisms or cells must be free from contaminants when administered. The toxicity of the substance, the volume and the route of administration should be considered to prevent tissue damage and to give precise dosage. The following solvents or vehicles have been found suitable in most instances and do not greatly affect drug action because of their own inherent properties:

- water
- water with 0.85% sodium chloride
- water with up to 50% polyethylene glycol
- water with not over 10% Tween 80
- water with up to 0.25% methylcellulose or carboxymethylcellulose
- corn oil, vegetable oil or peanut oil (oral and intramuscular route only).

A small percentage of the lower alcohols, glycols and acetone can also be used, provided the volume administered is kept small [6]. Phosphate buffered saline (PBS) or various culture media are also suitable vehicles [2]. Lipid-soluble substances can only be dissolved in oil but this delays absorption. Oil-soluble drugs have been successfully given intravenously in 15% oil–water emulsions using lecithin as an emulsifier [6]. Until experience indicates otherwise, solutions or suspensions should be prepared as near to the time of use as possible because some substances will deteriorate in solution within a few hours [6]. When administering drugs, the solvent should ideally be the same as the one in which the drug is normally formulated [2]. Although distilled water can be used under certain conditions, saline is preferable because water for injections injected subcutaneously causes pain and intravenous injection produces haemolysis. Oil and viscous fluids cannot be injected intravenously [2]. If suspended material is to be used for intravenous injection, the particles should be removed by filtration to prevent embolism [6]. The temperature of fluids must be raised at least to room
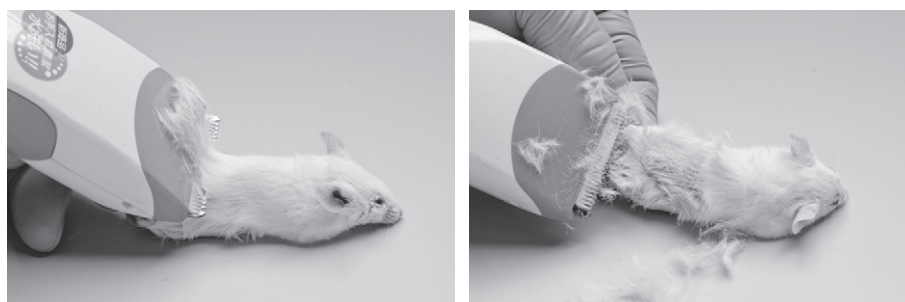
Figure 5.2.4 Clipping of hair on the back using cordless electric clipper.

Figure 5.2.5 Cell strainer (352350, Becton Dickinson).

temperature or better still up to body temperature before use, because the injection of cold fluids is painful [7].

Intravenous cell injection has been performed in many experiments, such as immunological or cancer research. In these cases, cells have to be suspended singly in solution because cell clumps cause embolism, and in some cases the mice would die. Consequently, cell suspensions for injection should be filtered using a cell strainer just before administration (Figure 5.2.5). Meanwhile, preintravenous administration of heparin before tumour cell injection is reported to be an effective method to decrease mortality caused by thromboembolism [8].

Concentration of substances

The concentration can vary over a fairly wide range without greatly influencing the end result of the experiment. Lower concentrations are clearly desirable [9]. Factors limiting the use of aqueous solutions for parenteral administration are probably related to their osmotic pressure. Low concentrations can be corrected by the addition of sodium chloride but ought not to be so high as to materially exceed the osmotic pressure of 0.15 M sodium chloride [6]. Highly concentrated solutions can be administered intravenously provided the rate of injection is kept slow and precautions are taken to avoid getting the solution outside the vein.

pH of the injected solution

For most administration routes, providing the solutions are not highly buffered, a pH range of 4.5–8.0 is satisfactory. For oral administration a pH as low as 3 can be tolerated, but alkaline solutions are very poorly tolerated. A rather wide range of pH is indicated for intravenous administration, because of the buffering effect of blood and dilution by blood flow following use of the intramuscular and subcutaneous routes. When solutions of low or high pH are injected intravenously the injection rate is kept slow and again precautions are taken to avoid getting solution outside the vein [6].

Volume and frequency of administration

The injection volume is limited by any toxicity of the substance and by the size of the mouse. It should be kept as small as possible. Excess volumes of solution can startle the animal. The frequency of administration should be kept to a minimum, to avoid unnecessary stress. If solutions are administered intravenously, haemodynamic changes and pulmonary oedema may occur, while very rapid injections can produce cardiovascular failure and be lethal [2]. Maximum volumes are shown in Table 5.2.1 [10–12]. For immunization, the maximum is lower still, because of the mixing

TABLE 5.2.1: Guidelines for maximal administration volumes and needle size

Administration route	Maximal administration volumes (mL)	Needle size
Oral	0.2	22G
Subcutaneous	2–3 (scruff) 0.2 (inguinal)	25G
Intraperitoneal	2–3	23G
Intravenous	0.2	25G
Intradermal	0.05	26G
Intramuscular	0.05	25–27G
Intracerebral	<0.03	27G
Intranasal	<0.02	

Source: *Flecknell [10], Reeves* et al. *[11], Wolfensohn and Lloyd [12]*.

with adjuvant. Maximum volumes for injection of antigen with or without adjuvant per route are indicated in the section on 'Immunization of mice' in this chapter.

Rate of absorption and distribution of administered substances

The blood flow to the site of administration, the nature of the substance and its concentration influence the rate of absorption [2, 12]. The time-course of the effect of the substance is an important factor in determining the dosage and is influenced by the rate of absorption [9]. Normally, injected substances must be absorbed from the site of administration into the blood. Therefore, the rate of absorption will be determined by the size of the absorbing surface, the blood flow and the solubility of the substance in the tissue fluid. The rate of absorption is also influenced by lipid solubility, physicochemical properties, degree of ionization and molecular

size of the substance [2]. Compounds that are highly soluble in the body fluids, will be absorbed quickly. Substances that are ionized and are not lipid soluble can only be absorbed if a specific carrier exists. In general, the rate of absorption is in the following order [12]:

$$iv > ip > im > sc > po.$$

Needles and syringes

Usually, 26-27G, 12.5-15.6 mm ($\frac{1}{2}$ to $\frac{5}{8}$ inch) needles are satisfactory for injection. The smallest gauge should be selected, as a fine needle prevents leakage of fluids and will help to minimize discomfort to the animal [2]. A 1-2 mL syringe is adequate for most injections. When a small volume (<1.0 mL) is administered, an insulin syringe plus needle is convenient (Figures 5.2.6 and 5.2.7; 27-30G, 8.0-12.5 mm ($\frac{5}{16}$ to $\frac{1}{2}$ inch)).

Figure 5.2.6 Insulin syringes. (a) 29G × $\frac{1}{2}$ in., 0.5 mL, Terumo; (b) 27G × $\frac{1}{2}$ in., 1.0 mL, Terumo; (c) 29G × $\frac{1}{2}$ in., 0.3 mL, Becton Dickinson; (d) 29G × $\frac{1}{2}$ in., 0.5 mL, Becton Dickinson; (e) 29G × $\frac{1}{2}$ in., 1.0 mL, Becton Dickinson.
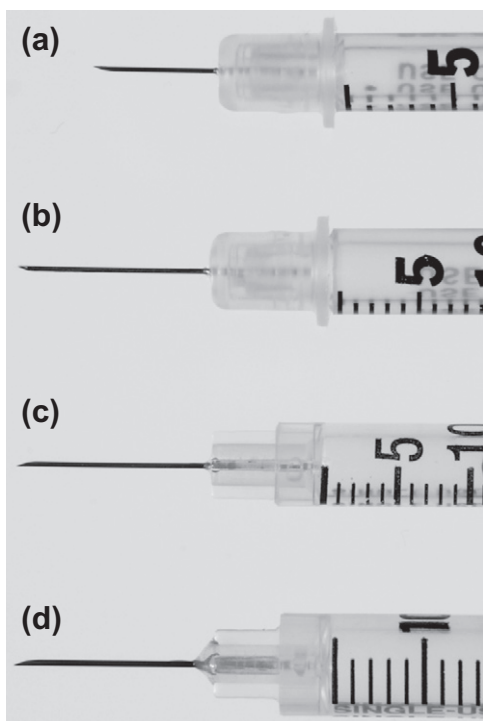
Figure 5.2.7 Needles for Insulin syringes. (a) 30G × $^3/_8$ in., 0.3 mL, Becton Dickinson; (b) 29G × $^1/_2$ in., 0.5 mL, Becton Dickinson; (c) 29G × $^1/_2$ in., 0.5 mL, Terumo; (d) 27G × $^1/_2$ in., 1.0 mL, Terumo.

These syringes can be obtained from various companies (e.g. Terumo, Japan; Becton Dickinson, USA). Intradermal needles are practical for intracerebral injections (Figure 5.2.8; Top, Tokyo, Japan). Plastic syringes cannot be used with solvents such as acetone.

The withdrawal of hazardous substances from bottles requires great care. An alcohol-moistened cotton pledget can be kept at the point where the needle enters the stopper in order to minimize the inadvertent formation of aerosols
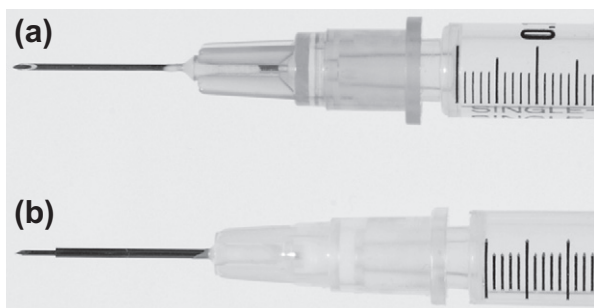
Figure 5.2.8 Intradermal needle. (a) 26G × $^3/_8$ in. needle (Terumo); (b) $^1/_2$ in. intradermal needle.

[13]. Because of the risk of embolism, air bubbles in fluid, syringe and needle must be purged. Gently tapping the side of the syringe and slowly expelling the air into absorbent tissue to prevent any dispersal of the contents until fluid appears at the tip of the needle can purge air bubbles.

The needle size will vary with the viscosity of the substance being used; the greater the viscosity, the larger the needle required [11]. If blood or body fluid flows back into the needle, it must be discarded and a fresh attempt made.

Enteral administration

Enteral administration has the advantages that it is possible to give quite large amounts of non-sterile substances or solution and that a pH as low as 3 can be administered by this route. On the other hand, alkaline solutions are very poorly tolerated by this route [6]. When using the oral route it should be understood that substances can be destroyed by the gastric juices and that the food content of the stomach influences both rate and order of the gastric emptying. The rate of absorption is markedly influenced by its time of residence in the stomach and is also directly related to the rate at which substances are passed from the stomach into the intestine [14]. Enzymes of the host and microflora of the digestive tract can also metabolize the substance. On the other hand, some insoluble substances may become solubilized as the result of enzymatic activity during their passage through the stomach and intestine, making absorption possible [2]. The two major methods for enteral administration are mixing the substance with food or water or direct administration using gavage. Rectal administration is also possible [6].

Oral administration

The simplest method for administration is giving the substance with food or drinking water. However, this is not practicable with substances that are unpalatable, insoluble or chemically unstable in drinking water or when they irritate the mucosa of the gastrointestinal tract [2]. The

daily food and water intake of the mice should be known before the experiment, to calculate the quantity of substance to be added. Because wastage of food and water happens all the time, it is difficult to determine the precise amount of food and water intake and therefore the precise intake of the substance. The only way this can be done is by keeping mice in metabolic cages and recording the wastage.

Intragastric administration

Direct administration by oral gavage is preferred to mixing substances with food or drinking water because the intake of the substances is precisely measured. A ball-tip needle is used to prevent damaging the oesophagus or passing through the glottal opening into the trachea (Figure 5.2.9). A 22G ball-tip needle is suitable for administration to adult mice and can be obtained from suppliers such as Cadence Inc., or Fine Science Tools Inc. The conscious mouse is manually restrained by firmly gripping a fold of skin from the scruff of the neck down the back (Figure 5.2.10a); immobilization of the head is essential for this procedure (Figure 5.2.10b). When the neck is extended, the position is vertical and there is a straight line from the mouth to the cardiac sphincter through the oesophageal orifice (Figure 5.2.10b). The needle is passed gently through the mouth and pharynx into the oesophagus (Figure 5.2.10c). The mouse usually swallows as the feeding needle approaches the pharynx, and these swallowing movements can help the probe to slip through the oesophageal opening. The substance is then administered slowly. If any obstruction is felt, if the mouse

Figure 5.2.9 Syringe with a gavage needle. (a) 1.0 mL syringe with 22G × 1.0 in. feeding needle; (b) 1.0 mL syringe with 22G × 1½ in. feeding needle; (c) 1.0 mL syringe with 20G × 1½ in. disposable feeding needle.

coughs, chokes or begins to struggle vigorously after the gavage begins, or if fluid is seen coming out of the nose, it may indicate that the needle has entered the lungs. Any of these signs necessitates immediate withdrawal of the needle, and the mouse must be observed very carefully. If there is any sign that fluid has got into the lungs, the mouse should be euthanized. As soon as administration is finished, the needle must be withdrawn [5, 15]. A volume of less than 0.2 mL is recommended.

Parenteral administration

Administration of substances to the body other than via the alimentary canal includes injection, infusion, topical application, inhalation and implantation of an osmotic pump or a controlled-release drug delivery pellet. Small amounts of solution are injected, and large volumes are infused. In both cases a needle must penetrate the skin. Subcutaneous, intraperitoneal and intravenous administration are the most common and important routes to inject substances in solution or suspension into the mouse. The rate of absorption is dependent on the administration route. Following intravenous injection the substance will disperse immediately; this route therefore achieves the most rapid absorption. The large surface area of the abdominal cavity and its abundant blood supply also facilitate rapid absorption; absorption from this route is usually 25-50% as rapid as that from the intravenous route [6].

Subcutaneous administration

Subcutaneous administration is easy. As it is rarely painful [12], a conscious mouse can usually be used. The rate of absorption is lower than with intraperitoneal or intramuscular injections [16]. Subcutaneous injections are made into the loose skin over the interscapular (Figure 5.2.11a) or inguinal area (Figure 5.2.11b). Subcutaneous administration
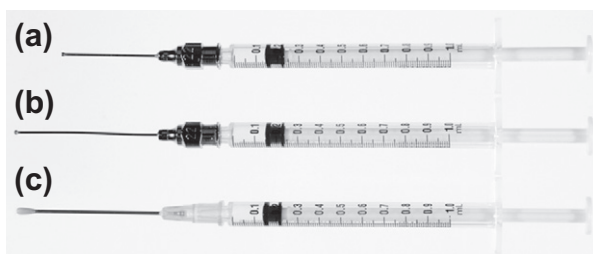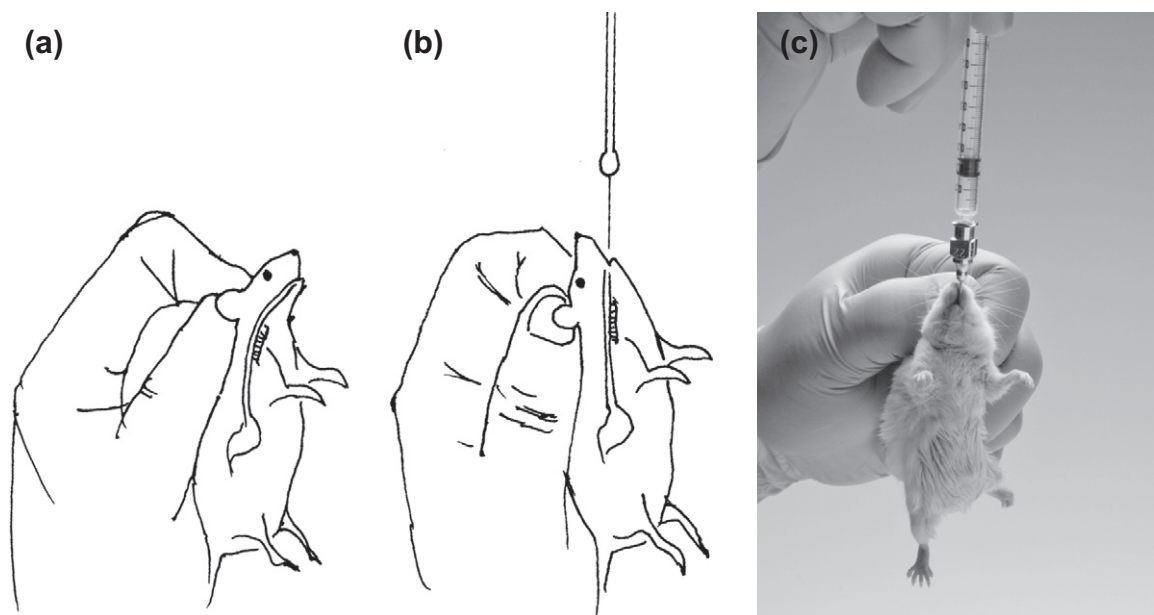
Figure 5.2.10 Procedure for intragastric administration using ball-tip needle. (a) Before extending mouse's neck; (b) A straight line is formed between mouth and stomach; (c) Intragastric injection is made using 1.0 mL syringe with 22G × 1.0 in. feeding needle.

over the interscapular area is performed as follows. The mouse is manually restrained and then placed on a clean towel or solid surface. The needle is inserted under the skin of the interscapular area tented by the thumb and index finger and the substance is then injected. A volume of less than 3 mL is recommended. Subcutaneous administration over the inguinal area is done as follows. The mouse is restrained manually and the head tilted downwards. Holding the hind leg firmly helps this procedure (Figure 5.2.3). The needle is inserted into the lower left or right quadrant of the abdomen, avoiding the abdominal midline, and the substance is injected. A volume of less than 0.2 mL per site is recommended. To minimize leakage, the needle should be advanced several millimetres through the subcutaneous tissue [5, 15].

Intraperitoneal administration

This is the most common route, being technically simple and easy. It allows quite long periods of absorption from the repository site. The rate of absorption by this route is usually 25–50% as rapid as by the intravenous route [6]. Its limitations are the sensitivity of the tissue to irritating
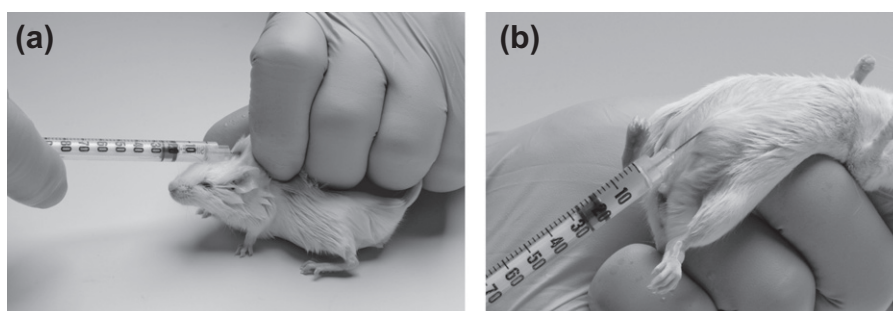
Figure 5.2.11 Subcutaneous injection. (a) Subcutaneous injection at the base of a fold of loose skin (area at the neck) using an insulin syringe: 27G × ½ in., 1.0 mL; (b) Subcutaneous injection at the lower left quadrant using an insulin syringe: 27G × ½ in., 1.0 mL.

Figure 5.2.12 Intraperitoneal injection to lower left quadrant using insulin syringe: 27G × ½ in., 1.0 mL.

substances and less tolerance to solutions of non-physiological pH. Solutions should be isotonic and quite large volumes can be administered via this route.

The conscious mouse is manually restrained [16] and held in a supine position with its posterior end slightly elevated, or the head can be tilted lower than the body (Figure 5.2.12). The needle and syringe should be kept almost parallel to the mouse's vertebral column in order to avoid accidental penetration of the viscera [17]. The needle is pushed in at an approximately 10° angle between the needle and the abdominal surface in the lower left quadrant of the abdomen [16]. To avoid leakage from the puncture point, the needle is run through subcutaneous tissue in a cranial direction for 2–3 mm and then inserted through the abdominal wall [15]. The recommended volume is less than 2.0 mL for a 40 g mouse.

Intravenous administration

Intravenous injection has advantages over other routes. There is one primary route, the tail vein; another possibility is the ophthalmic plexus route. Solutions that are highly concentrated, high or low pH, or irritating, can be administered intravenously provided that the rate of injection is kept slow and precautions are taken to avoid getting the solution outside the vein. Compounds that are poorly absorbed by the digestive tract may be given intravenously but intravenous administrations require technical expertise and skill. The syringe plus needle or the catheter must first be filled with the solution to remove air bubbles. Administration is usually into the lateral tail vein, not the dorsal tail vein (Figure 5.2.13a), as it is not straight. The lateral veins are readily visualized, but are quite small in diameter. If anaesthesia is not used, a restraining device is usually necessary (see Chapter 5.1; [5, 11, 18]).

The mouse is either placed in the restrainer or anaesthetized and the tail is then warmed with a lamp or warm towel, or immersed in warm water (40–45 °C) in order to dilate the vessels [10]. The tail is swabbed with 70% alcohol on a gauze sponge or swab. The needle is inserted parallel to the tail vein, penetrating 2–4 mm into the lumen, while keeping the bevel of the needle face upwards (Figure 5.2.13b). The solution is then injected slowly and no resistance should be felt if the solution is properly administered (Figure 5.2.13c). The injected solution temporarily replaces the blood, but should then be washed away by the blood stream. If this does not happen, the position of the needle is certainly not in the vein but in the surrounding tissue, so it must be either moved in the surrounding tissue in such a way that it then enters the vein, or a new attempt must be made. When the intravenous administration is finished or the cannula is pulled out, the injection site must be pressed firmly with a swab or fingers to prevent backflow of the administered solution and/or blood [2, 5]. If the same vein must be used several times the first administration should be made as distal as possible in relation to the heart and subsequent administrations should be placed progressively more proximally. Because venepuncture and the administration of substances can damage and/or block the vein, the distal part of the vein may no longer be used [2]. The recommended volume is less than 0.2 mL.

The ophthalmic plexus route is also used for fluid administration [19, 20], but this method of application is controversial and is under discussion or even forbidden in various countries for reasons of animal welfare. This route may be suitable in case of emergency for rescuing a mouse that shows signs of anaphylactic shock. Details of the technique are described in the section on 'Rescue from anaphylactics' later in this chapter.

Other routes for intravenous administration via the external jugular vein [21], the dorsal
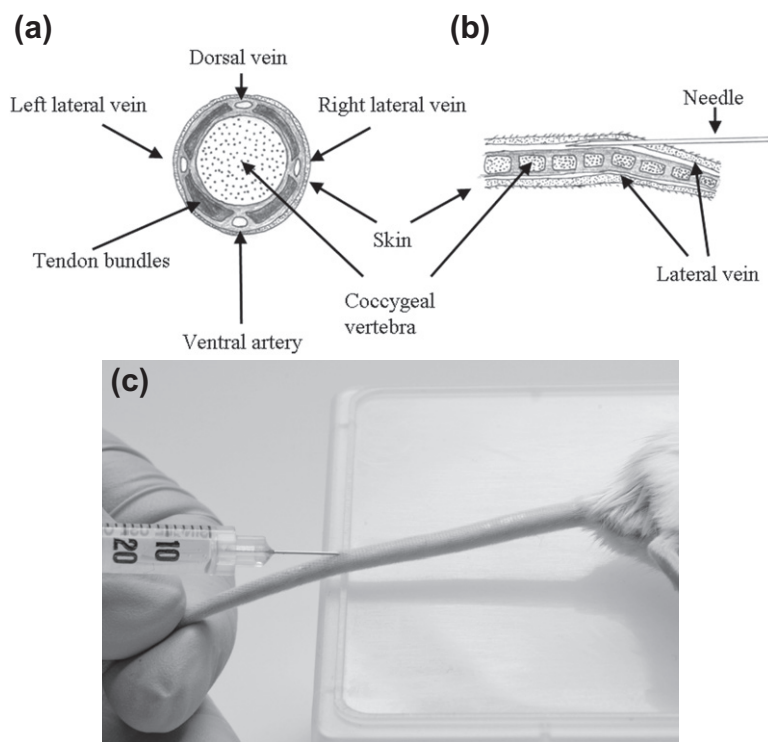
Figure 5.2.13 Intravenous administration. (a) Transverse section view of the mouse tail; (b) Sagittal view of the mouse tail (the tail is turned 90°); (c) Intravenous injection to lateral tail vein of an anaesthetized mouse using an insulin syringe: 27G × ½ in., 1.0 mL.

metatarsal vein [22] and the sublingual vein [23] have been reported.

Intramuscular administration

The intramuscular route should usually be avoided, as mouse muscles are small. If necessary,

Figure 5.2.14 Intramuscular injection into the leg muscle.

injections may be given into the thigh muscle with injection volumes of less than 0.05 mL. The tip of the needle should be directed away from the femur and sciatic nerve (Figure 5.2.14). The mouse is anaesthetized or is manually restrained by another person. The needle tip is inserted through the skin and into the muscle and aspirated briefly with the syringe before injection. If there is backflow of blood or body fluid, the procedure should be stopped; the needle must be moved or a fresh attempt must be made. Good technique and restraint are necessary and this method should only be performed by well-trained personnel [2, 4, 6, 15].

Intradermal administration

This route is not recommended in general and should be restricted to cases of absolute necessity [3, 24]. It is a very difficult route in the mouse because of the animal's very thin skin. A fine

Figure 5.2.15 Intradermal injection into the skin of the back.

needle (29G or less) is recommended. The mouse is anaesthetized, the fur clipped or hair removed from an area on the back, ventral abdomen, or hind footpad, which is wiped with 70% ethanol on a gauze sponge or swab. The skin is held taut with thumb and index finger and the needle inserted, bevel up and at a shallow angle, just under the superficial layer of epidermis. The volume should be less than 0.05 mL per site. Resistance should be felt both as the needle is advanced and as the compound is injected. A hard bleb will be seen upon successful intradermal injection of even a small quantity of fluid (Figure 5.2.15) [5]. If multiple sites are injected, adequate separation is necessary to prevent coalescing of lesions.

Intracerebral administration

For this procedure the mouse is anaesthetized and then restrained manually on a solid surface [25, 26].

The site of injection is approximately half way between the eye and ear and just off the midline (Figure 5.2.16a). The recommended maximum volume per suckling mouse is 0.01 mL and that for weanling or older mice is up to 0.03 mL. The needle directly pierces the cranium (Figure 5.2.16b). An intradermal needle (Figure 5.2.8) is convenient in order to prevent the needle from extending too deeply into the brain.

To inject into a specific region of brain, mice have to be restrained using a small animal stereotaxic instrument (Figure 5.2.17), as supplied for example by David Kopf Instruments, NARISHIGE or Stoelting. The injection region is decided with reference to a brain atlas [27]. This atlas
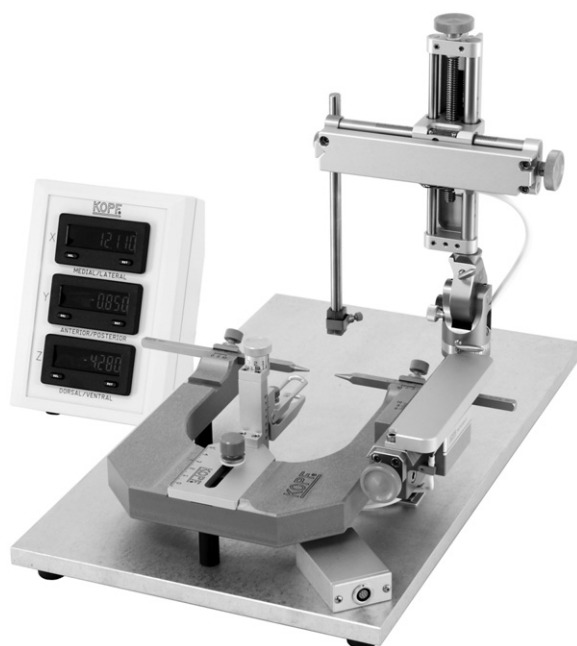
Figure 5.2.17 Stereotaxic alignment system (Model 940, David Kopf Instruments).
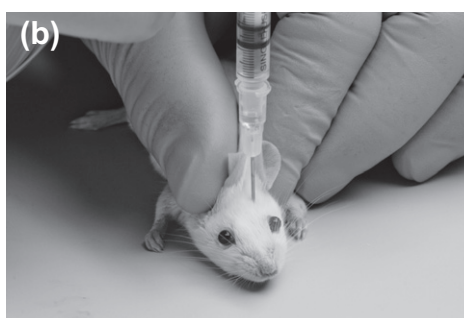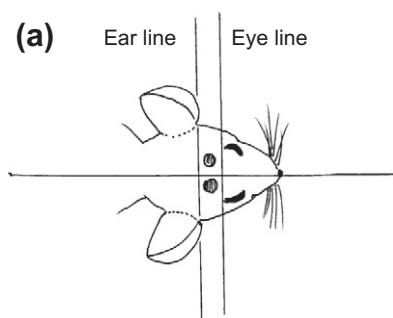
Figure 5.2.16 Intracerebral injection. (a) Injection site of the head for intracerebral injection; (b) Intracerebral injection into an anaesthetized mouse using an intradermal needle.

relates to the adult male C56BL/6J mouse, therefore some experiment is needed if other mouse strains are to be used.

Intrathoracic administration

Intrathoracic injection is restricted to special experiments. It can be made in mice with a slightly bent or curved needle, which should be inserted between the ribs at approximately the midpoint of the rib cage. Caution must be taken to insert the needle at an angle, thus preventing injection directly into lung tissue. The speed of absorption is similar to the intraperitoneal route [16].

Intranasal administration

This is usually done with the mouse lightly anaesthetized. The animal is manually restrained and the tail anchored between the small finger and the palm [16]. The mouse is held in a supine position with the head elevated. The end of the micropipette is placed at or in the external nares, and then the solution is poured in slowly (Figure 5.2.18) [25, 28, 29]. The volume should be less than 0.02 mL. Excess volume or rapid injection will induce suffocation and death.

Figure 5.2.18 Intranasal instillation into an anaesthetized mouse using a pipette (Gilson P-20).

Topical application

It is not often realized that the skin is the largest organ of the body and survival depends on its patency perhaps more than for most other organs. An animal (or human) can survive with only about one-seventh of its liver or one-fourth of its kidney functioning, but destruction of more than 50% of the skin usually results in death [6]. The skin is also a convenient site for the administration of drugs. Numerous factors, such as the physicochemical properties of the substance, the attributes of the vehicle and the permeability of the skin, can affect the degree of percutaneous absorption [30, 31]. The ability of a substance to be absorbed through the skin and enter the systemic circulation is determined by its ability to partition into both lipid and aqueous phases [2].

The usual site is the skin of the back or the abdomen. After clipping the hair for topical administration (Figure 5.2.4), the hairless area should be cleaned of any fat and grease and other debris. The substance to be administered should be dissolved in a volatile solvent or mixed in a suitable cream before application and then applied with a dropper or smeared onto the skin with a swab [2]. Some precautions are usually necessary to prevent the animal from licking or scratching the application sites [6].

Inhalation

This route is used for experiments on asthma, air pollution or respiration [32, 33]. The inhalation route is, incidentally, the most akin to an intravenous injection because of the relatively large area presented for absorption by a membrane that is separated from the blood by only one or two cell layers. Consequently, absorption of gases and aerosols that reach the alveoli is virtually complete. The greatest problems surrounding the use of the inhalation route are the generation of a suitable aerosol of the test substance, if it is not sufficiently volatile, a constant and suitable air level of the material under study and the determination of the dosage given. Particle sizes that are too small or too large are not suitable; it is generally believed that a particle size of 0.5–2.0 μm diameter

is optimum [6]. Equipment is available to purchase e.g. from Omuron or Buxco Electronics.

Other routes

Other routes of administration have been reported such as intra-arterial administration using the femoral artery [16] or the carotid artery [34], intrathymic injection [4], intraspinal injection [35], intrathecal injection [36] or intracardiac injection [8].

The dosing and treatment of newborn mice provides special problems not only because of their size or but also because the dam is apt to reject or cannibalize neonates that have been handled. Subcutaneous injections can be made over the neck and shoulders using a less than 30G $\times$ $^{5}/_{16}$ inch needle. Up to 0.1 mL (depending on the age of the infant mice) may be administered orally using a piece of plastic tubing inserted over a needle [15, 37]. Direct injection into the stomach of infant mice can be made through the abdominal wall [38]. Intravenous injection into infant mice has also been reported [15, 39, 40].

Implantable pumps, controlled-release drug delivery pellets and cannulas

The delivery of substances at a slow, steady rate over a period of days, weeks or months without the need for external connection or frequent animal handling can be accomplished by using an osmotic pump or controlled-release drug delivery pellets.

Osmotic pumps (ALZET pumps) can be used for systemic administration when implanted subcutaneously or intraperitoneally, or can be attached to a catheter for intravenous, intracerebral or intra-arterial infusion. The pumps have been used to target delivery to a wide variety of sites including the spinal cord, spleen, liver, organ or tissue transplants and wound healing sites. ALZET pumps are supplied by DURECT.

Controlled-release drug delivery pellets effectively and continuously release the active product into the animal. The pellets are intended for, although not limited to, simple subcutaneous implantation in laboratory animals. Pellets are available from Innovative Research of America. The dosage can be selected (from 1 µg to 200 mg per pellet), and the release period is also selectable (21, 60 or 90 days).

Implantable cannulas permit continuous access to the venous or arterial system for either intravenous substance administration or blood withdrawal. Using strict aseptic techniques, the cannula is inserted into a vein or artery (the femoral vessels, jugular vein and carotid artery are common sites) and secured in place. The other end of the cannula is attached to a small port that is secured in a subcutaneous location, most often over the shoulders [5]. See Desjardins [41] for more information on implantable cannulas.

Immunization

Mice are not used for the production of polyclonal antibodies because of the small amounts produced. On the other hand, they are a good source of antibody-producing lymphoid cells or hybridomas [42]. In general, immunization consists of two stages; primary and booster. The primary antigen is usually injected with adjuvant. Boosters are injected once or more with or without adjuvant depending on the immunogen. Footpad, intrasplenic [43] or intralymph node [44] injection is not recommended in general. If required, the investigators should provide scientific justification to ethics committees for such protocols (such as the need to use valuable, unique and irreplaceable antigens, or extremely small quantities of antigen). The injection of immunogens at the base of the tail or in the popliteal area substitutes for footpad injections with much less distress to the animal, because immunogens injected into the footpad are processed by the popliteal lymph node [3, 45]. The intraperitoneal route for injection of Freund's complete adjuvant (FCA) is permitted in small rodents only. FCA should be administered only once, and be limited to minimal volumes of up to 0.1 mL. In the mouse, up to 0.1 mL with adjuvant may be administered subcutaneously in the neck

TABLE 5.2.2: Maximum volumes of antigen, with or without adjuvant, per route		
Administration route	Maximum volume (mL)	
	With adjuvant	Without adjuvant
Subcutaneous	0.1	0.5
Intramuscular	Not recommended	0.05
Intraperitoneal	0.2	1
Intravenous	Not recommended	0.2
Intradermal	Not recommended	0.05

The intradermal route should be restricted to cases where it is absolutely necessary [3].
Source: *Warsson* et al. *[48]*; *Van Zutphen* et al. *[49]*.

region. Oil-based or viscous gel adjuvants should not be injected by the intramuscular route [46]. The intravenous route should also not be used for oil-based adjuvants, viscous gel adjuvants or large-particle antigens, due to the risk of pulmonary embolism [47]. Though FCA is the strongest adjuvant, use of other adjuvants can be recommended. Mice must be closely monitored immediately after injection for any anaphylactic reactions, both after the primary and any booster injections [3]. The recommended route and volumes are shown in Table 5.2.2.

Rescue from anaphylaxis

In some cases, anaphylactic shock happens in hyperimmunized mice. Intravenous fluid replacement of isotonic solution is effective to rescue mice, but the tail vein of mice in anaphylactic shock is often collapsed by the low blood pressure. In this case, peripheral intravenous access via the sublingual vein is the preferred route [23]. Alternatively, fluid replacement may also be achieved using the retro-orbital sinus route [19, 20]. In a normal-sized mouse, the injection of 0.5–1.0 mL of warmed isotonic solution is enough for rescue. In detail, the technique resembles blood collection by retro-orbital sinus puncture (see Chapter 5.3). The mouse is manually restrained on a solid surface, being held gently but firmly by the nape of the neck. By pressing down with the thumb and forefinger in the occipital area and pulling back the skin, the point of the needle can be directed toward the back of the orbit at a 20–40° angle. The needle is inserted medially through the conjunctiva on the inner side of the ocular cavity. If entry is blocked by bone, the needle is withdrawn slightly. Fluid is injected slowly, loosening the neck skin slightly. Mice in anaphylactic shock are unable to maintain their body temperature, therefore warming them is an effective means of recovery.

Acknowledgement

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Collection of Body Fluids

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Blood

Blood is composed of formed elements or blood cells consisting of erythrocytes, platelets and leukocytes, and fluid or plasma consisting of serum and coagulant. The alteration of blood cell components and plasma proteins such as albumin, globulin and fibrinogen reflects the health status of the animals. Therefore, blood collection and its analysis is indispensable for the maintenance of laboratory mice. Moreover, blood transports nutrients, metabolism residues, hormones, antibodies and enzymes. Antibodies indicate the history of infection of animals, while polymorphism of serum enzymes is utilized to identify inbred strains. In a breeding facility, blood is collected and analysed periodically to check on the microbial contamination of the facility and any accidental mating of an inbred strain with others.

Blood collection obtains either all the blood from sacrificed animals or a proportion of the blood from living animals. Blood is taken from mice for a wide variety of scientific purposes and the anatomical sites of blood collection are varied. The haematological values, such as haematocrit and cell count, may vary between different sampling sites [1]. Therefore, investigators should select a suitable blood collecting technique to fit the purpose of their examination.

Total blood collection from the heart via thoracotomy

Using this technique, a large amount of venous blood can be collected with certainty. A mouse is first anaesthetized and killed and its thorax is opened. Anaesthesia of mice for blood collection is usually achieved by ether inhalation, as ether is considered to have no effect on haematological findings [2]. A glass anaesthetizing jar with ether-soaked cotton and a wire mesh partition is used. After the mouse is put into the jar, it soon exhibits a rapid heartbeat which slowly decreases. By tilting the jar in order to change the position

of the mouse, the heart rate can be seen clearly. Once the rate has become slow, deep anaesthesia has been induced. If a mouse is left in the jar more than 1.5 min, it may die. The anaesthetized mouse is picked up and operated on for blood collection according to the following procedure. Should the animal wake up during the procedure, additional anaesthesia can be achieved by covering the snout of the mouse with a small cup containing ether-soaked cotton. Ether inhalation should take place in a draft chamber. In place of ether, isoflurane (CF3CHCl-O-CF2H) is recommended for light anaesthesia. For deep anaesthesia, 0.5% sodium pentobarbital solution is administered intraperitoneally at a dosage of 0.1 mL/10 g body weight.

1. The anaesthetized mouse should be laid on its back on an operating board, and restrained by adhesive tape or pins at the carpal and tarsal regions of its four limbs.
2. The xiphoid process is located, approximately at the midpoint of the head and body length. The skin around the xiphoid cartilage is cut off using scissors and the muscular wall of the thorax and abdomen is exposed.
3. The abdominal wall is incised just caudally to the xiphoid process, then the diaphragm and thoracic wall at both sides of sternum are cut with scissors (Figure 5.3.1). Paired blood vessels, the internal thoracic arteries, run on both sides of the sternum on the internal surface of the thoracic wall. Care should be taken to avoid

cutting these arteries. In deeply anaesthetized mice, bleeding from the cut thorax wall is minimal, though this will increase in insufficiently anaesthetized mice and disturb the following steps of the procedure.
4. The cut thoracic wall is gripped by a haemostat and pulled upwards, utilizing the weight of the haemostat. Through the cut wall, the rapidly beating heart is exposed. Although respiration stops quickly after the opening of the thorax, the heart continues to beat for several minutes. While the heart is still beating, blood can be collected successfully.
5. The investigator prepares a disposable 1 mL plastic syringe with a 21–23G needle. By inserting the needle into the right ventricle of the heart and withdrawing the plunger, blood will enter into the syringe (Figure 5.3.1).
6. The plunger should be withdrawn slowly, because rapid withdrawal leaves the ventricle empty and the tip of the needle sucks on the wall of the ventricle.

With this procedure, more than 1.3 mL of venous blood can be obtained from an adult mouse. Although the total blood volume is roughly estimated as being 7% of the body weight, complete collection of the blood is not possible. Quality is preferable to quantity. The collected blood should be transferred immediately into a test tube by removing the needle and depressing the plunger slowly so that the blood enters the test tube along its wall.

Total blood collection by bleeding from the femoral artery

This technique is easy to use for collection of arterial blood from mice. The mice must be anaesthetized deeply by intraperitoneal injection of 0.5% sodium pentobarbital solution as described above and then sacrificed.

1. An anaesthetized mouse is restrained on its back on the operating board. The skin around the inguinal region on either side of the mid line is cut off with scissors. The abdominal muscular wall and muscles of the medial side of the femur are exposed. The femoral artery and vein can be seen at the base of the thigh.

Figure 5.3.1 Venous blood collection from the right ventricles of the open thorax mouse.
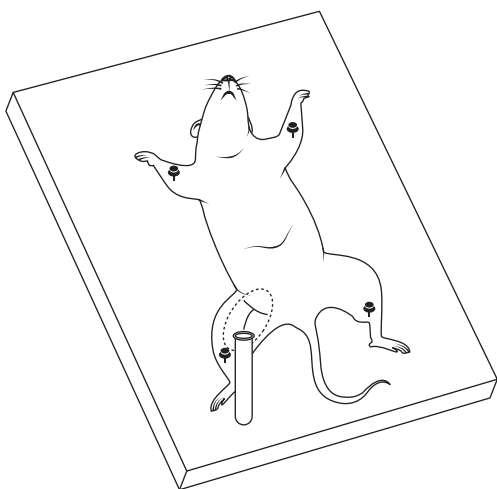
Figure 5.3.2 Arterial blood collection from the femoral artery. An operating board is tilted to collect blood effectively.

2. Before bleeding, the operating board should be tilted slightly so as to raise the head of the mouse in order to collect blood effectively. Place a test tube on the exposed femoral artery, then incise the artery just above the test tube (Figure 5.3.2).

3. Blood from the femoral artery then flows into test tube to give approximately 1 mL per adult mouse.

While collecting blood, bleeding sometimes stops before the animal dies. The investigator can then collect blood from the other side of the hindlimb. If a considerable volume has been obtained already, the mouse should be sacrificed quickly. Further collection may induce haemolysis.

Collecting arterial blood from the axillary artery is done using a similar procedure in the axillary region [3].

Bleeding from the abdominal aorta

The abdominal aorta is also used for arterial blood collection [4]. The deeply anaesthetized mouse should be restrained on its back on the board as before. Incision of the abdominal wall along the midline is started just below the xiphoid process, continued to near the pubic bone and then along the flank, so that the viscera are exposed. The intestine should be moved to the left side of the mouse, and the connective tissues removed along the midline of the dorsal wall of
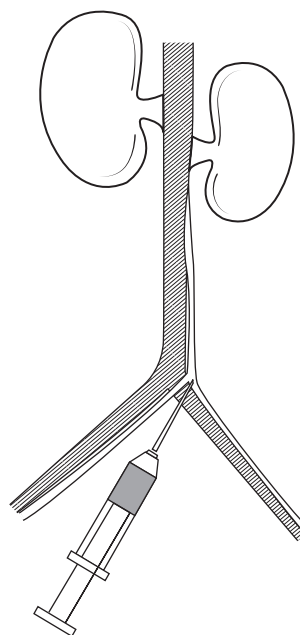
Figure 5.3.3 Abdominal aorta and caudal vena cava for blood collection. An incision is made in the abdominal aorta near to the divergence of the aorta into the right and left common iliac arteries, at which point a needle can be inserted for sampling.

the body. The caudal vena cava and the abdominal aorta can then be identified [5]. The abdominal aorta is incised at the level near the divergence of the right and left common iliac arteries (Figure 5.3.3). The blood is collected by a Pasteur pipette and transferred into a test tube.

A plastic disposable 1 mL syringe with a 26G needle can also be inserted into the abdominal aorta and aspirated to collect blood. In this case the best place to make the puncture is at the divergence of the right and left common iliac arteries (Figure 5.3.3). As the abdominal aorta of mice is very narrow at this level, the technique requires skill and experience. Incision of the abdominal aorta is easier than puncturing the artery with the needle.

Decapitation

For certain experiments, blood should be collected by decapitation. In this procedure, a decapitation device or guillotine is used (Figure 5.3.4). Mice must be lightly anaesthetized or stunned with a blow to the back of the head before they are placed on the guillotine, and their heads chopped off. The severed neck must be placed over a test tube for blood collection
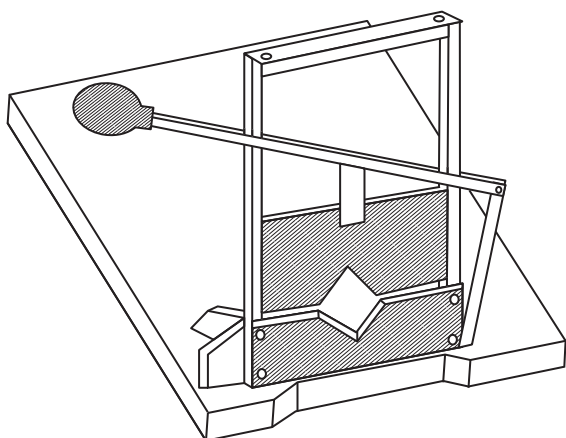
Figure 5.3.4 Use of a guillotine. The neck of an unconscious mouse is placed on the diamond-shaped slit of the device and its head is chopped off.

as quickly as possible. This is a fairly messy procedure; arterial blood is obtained, but may be contaminated with saliva and respiratory secretions.

To avoid contamination, the common carotid artery or jugular vein is surgically dissected, then either arterial or venous blood can be collected from the exposed vessels [6, 7].

Repeated blood collection from the retro-orbital venous plexus

In some experiments or during routine inspections, small amounts of blood may be collected repeatedly from the mice. For this purpose, the most popular technique is bleeding from the retro-orbital venous plexus [8]. However, nowadays this procedure is not recommended for blood collection, because histological damage to the eye and excessive bleeding can be induced, even by a skilful investigator [9]. Only if the blood sample obtained by another procedure is not

sufficient in quantity will the investigator be allowed to adopt this procedure.

Blood collection from the plexus can be repeated at intervals of 3–4 days. However, this technique requires training and experience, as untrained personnel cannot collect blood smoothly and may cause damage such as injured nerves. In order to collect blood, thin glass tubes, microhaematocrit capillary tubes (length 75 mm, internal diameter 1–1.2 mm, wall thickness 0.2 ± 0.02 mm), are used. The bleeding and collecting procedure for this technique is as follows.

1. A mouse should be anaesthetized lightly by intraperitoneal injection of 0.05% sodium pentobarbital solution. It is then laid on the operating board on its ventral surface. The investigator holds the mouse by the left hand and depresses it softly onto the board while holding a glass capillary tube by the thumb and forefinger of the right hand.
2. The head is depressed and the skin pulled by the thumb and forefinger of the left hand so as to extrude the eyeball as shown in Figure 5.3.5 a, b. Before bleeding, the eye should be wiped gently with sterile gauze to remove lacrimal fluids.
3. The glass capillary tube is inserted along the lower edge of the eyeball from the medial or nasal side towards the lateral side, and the tube pushed slightly deeper (3–4 mm) while being rotated by the thumb and forefinger of the right hand. As a result of this action, the blood vessel of the retro-orbital venous plexus is incised and bleeding occurs. When the capillary tube is withdrawn slightly, blood enters the tube by capillarity. Lowering of the opposite end of the tube promotes entrance of blood into it.
4. After blood collection, the investigator should depress the eyeball using sterile gauze until

(a)

(b)
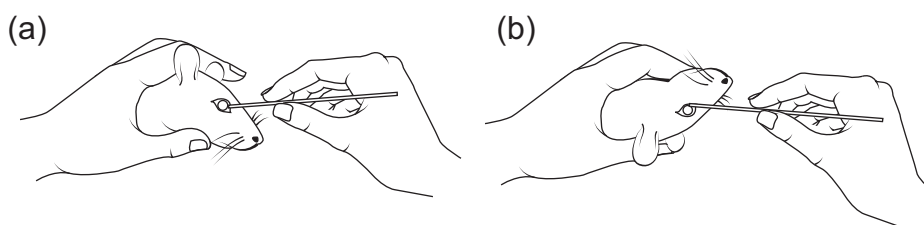
Figure 5.3.5 Collection of venous blood (a) from the right and (b) from the left retro-orbital venous plexuses. A microhaematocrit capillary tube is inserted along the lower edge of the eyeball from the nasal or medial side toward the side to a depth of 3–4 mm.

the bleeding stops. The mouse can then be returned to the cage.

With this procedure, two or three microhaematocrit capillary tubes can be filled with blood, i.e. a total of about 200 μL of blood per head obtained. Bleeding from the retro-orbital venous plexus can be done on either side of both eyes alternately, with an interval of 3 days, depending on the volume of sampling.

Bleeding from the surface veins of the hindlimb

For repeated blood collection from mice, bleeding from a surface vein of a hindlimb is also used. The dorsal metatarsal vein or medial saphenous vein may be incised. In this procedure the mouse does not have to be anaesthetized if there is an assistant available to hold the mouse or a restraining device is available (Figure 5.3.6). It is an easy procedure, but only very small volumes of blood are obtained. As the surface veins of the mouse hindlimb are very fine, the mouse or at least the hindlimb should be warmed before bleeding to dilate the blood vessels. Either the mouse is laid on a wire-mesh cage lid placed in warm water for 5–10 min or the limb itself is soaked in warm water at about 40 °C.

To collect blood from the dorsal tarsal vein, either foot is extended using the left hand. The surface is cleaned with alcohol-soaked cotton, wiped with dried sterilized gauze, and the vein pierced by a 21G needle or incised with a razor blade. Blood accumulates as a droplet on the dorsal side of the metatarsal region. By very quickly touching the droplet with a capillary tube it can

Figure 5.3.7 Puncture of the medial saphenous vein. About 80 μL of blood can be obtained.

be collected by capillarity before the droplet spreads out and becomes difficult to collect.

The procedure for bleeding from the medial saphenous vein is almost the same as that for the dorsal metatarsal vein. The mouse or the hindlimb should be warmed to dilate the blood vessel. The hindfoot of the restrained mouse is extended, the skin cleaned with alcohol-soaked cotton, the hair shaved and wiped with dry gauze, then the medial saphenous vein is pierced by a 21G needle or incised with a razor blade. The dripping blood or the droplet is again touched with a capillary tube to induce collection by capillarity (Figure 5.3.7).

Blood collection from the surface vein of the hindlimb gives only small quantities. The medial saphenous vein can yield about 80 μL, but less is obtained from the tarsal vein. If the vein is not dilated, blood flow is slow and little is obtained from bleeding. Therefore, warming up the mouse or its hindlimb is indispensable in these procedures.

Bleeding from the tail vein

Tail veins are frequently used for collection of venous blood and intravenous injections. In the tail there are three veins and one artery: paired lateral caudal veins, an unpaired dorsal caudal vein and the ventral caudal artery (Figure 5.3.8). As the tail veins are thicker than the dorsal metatarsal and medial saphenous veins, a larger quantity of venous blood can be obtained. The lateral caudal veins are preferentially used for both blood collection and intravenous injection. However, the tail should also be warmed up to

Figure 5.3.6 A mouse in a restraining device. The tail and a hindlimb are free for operation.
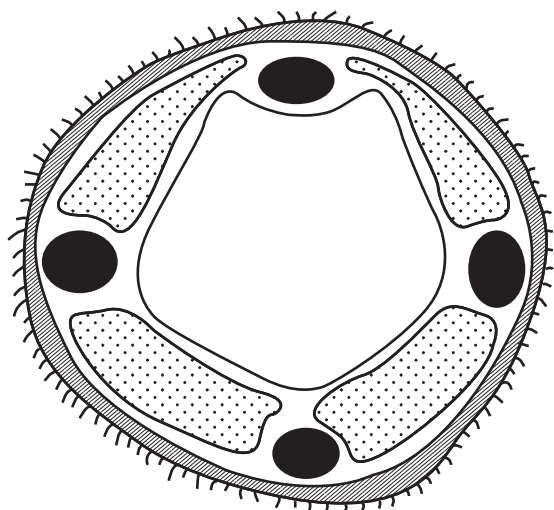
Figure 5.3.8 Schematic drawing of a transverse section of mouse tail showing the location of the dorsal caudal and lateral caudal veins and ventral caudal artery. Paired lateral caudal veins are used for venous blood collection and intravenous injections.

Figure 5.3.9 Another restraining device composed of transparent acrylic resin. (a) Only the tail is free for operation. (b) A winged needle is inserted into the lateral caudal vein to collect blood.

dilate the blood vessel before bleeding. The mouse is warmed up as before or its tail is soaked directly in warm water at 40 °C. The mouse need not be anaesthetized, if the assistant holds the mouse, or a restraining device is used. The device in Figure 5.3.6 is used for both hindlimb and caudal veins. For the tail vein only, another restraining device is preferably used without anaesthesia (Figure 5.3.9a). The investigator should grip near the tip of the tail of the restrained mouse with the left hand, and locate the veins running down both sides of the tail. Clean from one third to one half of the tail from its end with alcohol-soaked cotton, then wipe dry with sterile gauze. The vessel is then pierced with a needle (18–21G) or incised with a razor blade. A blood droplet will accumulate on the tail which can then be collected using a glass capillary tube as before. Two or three capillary tubes are filled, giving a total of 200–300 µL of venous blood. After bleeding the incision site should be pressed with sterile gauze until the bleeding stops.

Larger volumes can be obtained in this procedure, i.e. 0.5–1.0 mL [2] or 1.0–1.5 mL [10]. However, removal of this volume of blood will cause severe hypovolaemic shock or death of the animals.

A winged intravenous drip needle is used to collect blood in laboratory animals [11]. In mice a 25G winged needle is inserted into the lateral caudal vein. A disposable 1 mL plastic syringe is connected to the vinyl tube of the winged needle and the plunger is withdrawn, so that blood enters the vinyl tube (Figure 5.3.9b). Then the blood within the tube is transferred into a micro-haematocrit capillary tube by depressing the plunger. This procedure collects an adequate volume of blood (50–100 µL) and can be repeated with little damage to the blood vessel.

Bleeding from the tail artery

Small amounts of arterial blood can be collected by bleeding from a tail artery or ventral caudal artery. The mouse should be warmed up as before, the tail cleaned by alcohol-soaked cotton and wiped with dried sterile gauze. The artery is located on the ventral side of the tail, then pierced with a 21G needle. Incision with a razor blade is not appropriate for this artery, because bleeding from the incision site continues for longer. A blood droplet can be aspirated using a capillary tube.

Cardiac puncture from the closed thorax

Cardiac puncture is used for direct blood collection from the heart through the thoracic wall. The mouse must be deeply anaesthetized by intraperitoneal administration of sodium pentobarbital. This technique is executed repeatedly to obtain considerable amounts of blood from living mice. It is said that there is a slight difference between the components of blood obtained by open or closed thorax punctures [12].

For the cardiac puncture, there are three alternative approaches to the heart: (i) direct insertion of a needle into the heart perpendicular to the sternum; (ii) insertion from the thoracic inlet; and (iii) insertion below the xiphoid process towards the head (Figure 5.3.10). In any procedure, the investigator has to be thoroughly trained to perfect this technique before the experiment.

In the perpendicular approach to the thoracic wall, a deeply anaesthetized mouse is restrained on the operating board on its back. The xiphoid process is approximately at the mid-point of the head and body length. The length from the anterior thoracic inlet to the xiphoid process is about 20 mm. A point 9–10 mm caudally from the thoracic inlet on the midline is the site of the base of the heart. A 1 mL disposable plastic syringe with a 26G 11 mm ($^1/_2$ inch) needle is used. The needle is pushed in vertically to the thoracic wall just beside the sternum. The needle is depressed while the plunger of the syringe is withdrawn. When blood appears in the syringe, the position of the needle is kept steady and the slow withdrawal of the plunger continued. In this procedure, 300–400 μL of blood can be obtained. After removing the needle the blood
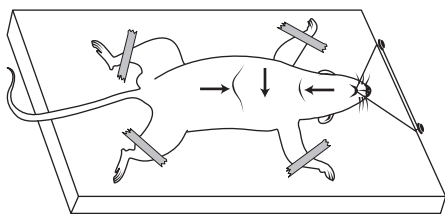
should be transferred into a test tube from the syringe by pouring slowly along the wall of the test tube to avoid haemolysis.

The approach from the thoracic inlet is more commonly used for cardiac puncture [13, 14]. The mouse is deeply anaesthetized and restrained on an operating board on its back. The neck must be fully extended by using a rubber band to pull on the upper incisors. A 1 mL plastic syringe with a 26G 11 mm ($^1/_2$ inch) needle is held ready. The anterior thoracic region is cleaned with alcohol-soaked cotton, the needle is pushed in from the thoracic inlet and then depressed toward the base of the tail, parallel to the sternum or slightly downward. The plunger is withdrawn to aspirate while the needle is depressed in the caudal direction, until blood appears in the syringe. When this occurs, the depression of the syringe is stopped but aspiration is continued until the expected amount of blood is obtained. Skilful and experienced investigators can grip an anaesthetized animal in the left hand, push in the needle and collect blood from the heart without restraining the mouse on a board.

The third is the cranial approach from just behind the xiphoid process [15]. A deeply anaesthetized mouse is restrained on the board and a 1 mL plastic syringe with a 26G 11 mm ($^1/_2$ inch) needle held ready. The needle is inserted just caudally of the xiphoid process slightly to the left of the midline and then the syringe is depressed cranially at an angle of 20° against the sternum and aspirated by withdrawing the plunger. When blood appears in the syringe, it is no longer depressed but aspiration is continued until the expected blood volume is obtained.

After collection of the blood, the mouse it put back into the cage and kept warm until it awakes. This procedure can be done repeatedly in living animals. However, heart puncture induces considerable damage. Intervals of at least 7 days between collections must be allowed even if the small sampling volume does not affect the health of the animal.

Figure 5.3.10 Three approaches for cardiac puncture of the closed thorax. (a) The perpendicular approach against the sternum at a point 9–10 mm caudally from the thoracic inlet; (b) the approach from the thorax inlet; and (c) the cranial approach from just behind the xiphoid cartilage.

Urine

Urine is examined for alterations in its components, the total volume, and the appearance of
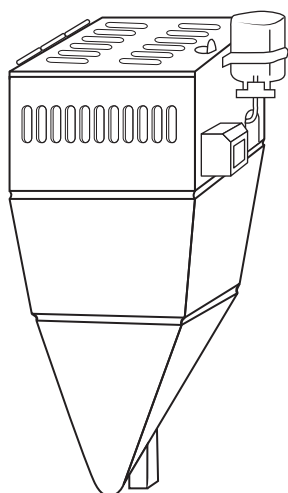
Figure 5.3.11 A steel metabolic cage for mice.

abnormal substances to check health status and to identify certain diseases. Consecutive urinalysis of an animal is adopted to chart the process of recovery from disease and the effect of an experiment.

Either the total urine produced over a certain length of time or fresh urine may be required. The first is executed using a metabolic cage (Figure 5.3.11). A mouse is placed in the metabolic cage and given food and water. The urine and faeces are collected separately (Figure 5.3.12). In

this method, the urine volume produced over a certain time is determined. However, the urine is not fresh and its components may change. Moreover, it may be contaminated with faeces or food residues. To avoid contamination of urine with faeces, anal cups or plastic bags are used [16, 17].

Fresh urine should be collected directly from the mice, which excrete urine easily when picked up from their cage, especially when gripped by hand. Preparations for collection of urine must therefore be made before taking the mouse out of the cage. A wire-mesh cage lid is placed on a clean steel plate or a piece of aluminium foil. A mouse is picked up gently from the cage by its tail, and placed on the wire-mesh lid. As the mouse will pull away by instinct when its tail is pulled back, the investigator places the palm of the free (usually left) hand on to the mouse's back and holds it. When the mouse is gripped, it excretes a little urine, especially in females. The mouse is then moved near to a test tube stand, the external urethra is placed over a test tube and the lower abdomen, where the urinary bladder is assumed to be located, is softly depressed using the forefinger of the right hand (Figure 5.3.13). The mouse then excretes urine into the test tube. In case the test tube collection causes a little trouble, the investigator

Figure 5.3.12 A mouse in a metabolic cage. Urine and faeces are collected separately.
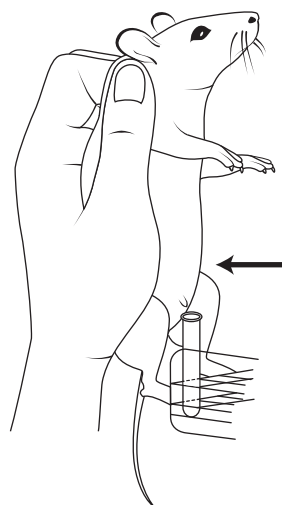
Figure 5.3.13 Collection of fresh urine. Grip the mouse firmly and place the external urethra over a test tube, then gently depress the lower abdomen with the forefinger of the right hand to induce excretion of urine.

may let the mouse urinate above a watchglass or hole slide by the same procedure. The urine on the glass is then collected by means of a capillary tube or Pasteur pipette. Usually the amount of urine produced in this procedure is between one drop and 0.4 mL at any one time. If this quantity is not sufficient, the investigator can try again at some point. Urine excreted on the wire-mesh lid can be collected from the steel plate or aluminium foil. In this technique the bladder is not completely emptied. However, depression of the lower abdomen by means of the forefinger is limited, because excessive pressure induces change in the urine components by entering the blood. The collected urine should be quickly stored in a freezer.

In the mouse, insertion of a catheter is not generally used for collecting urine. In case of laparotomy, the abdominal cavity is opened under anaesthesia and the urine in the bladder can then be aspirated directly after puncture, giving a total of 0.1–0.15 mL.

Milk

Milk is a complete food and indispensable for newly born pups. It is important for pups not only as a nutrient supply, but also because of the immunoglobulins it contains. Therefore, the milk is examined for its components and volume for nutritional and immunological studies. For the establishment of a first generation of clean mice, such as specific pathogen free (SPF) and germ free animals, the milk must be collected when a clean foster mother is not available.

To collect milk, there are two procedures; milking directly from the lactating mother or collecting milk from the stomach of suckling pups. In the case of a large litter, the pups can be sacrificed over several days after birth and any change in the components of the milk can be investigated. The lactating mother can be milked at certain days after parturition under anaesthesia. On the other hand, the total volume of the milk secretion can be estimated indirectly by the sum of the increments in the pup's body weight during suckling. In this section milking from lactating mice is described.

Milk from lactating mice

In mice the quantity of secreted milk from the mother reaches a maximum at 8-10 days after parturition. Therefore, lactating mice are used for milking 8-12 days after parturition. The ICR strain of mouse is frequently used, but other strains may also be used.

To collect milk, a milking device must be prepared. Some milking machines with single or multiple teat cups have been designed for this purpose [18–20]. The device with a single teat cup is made of two disposable syringe needles, a test tube, vinyl tubing, a silicon plug and an aspirator (or vacuum system). These parts are connected as shown in Figure 5.3.14. A disposable syringe needle is adequate as a teat cup for the milking device. The aspirator should be connected with the water supply. The procedure for milking is as follows.

1. Lactating mice 8 days after parturition should be prepared.
2. 6-8 h before milking the lactating mother must be separated from her pups by a wire-mesh partition, through which the mother is able to watch her pups and smell them.
3. The mouse is anaesthetized by intraperitoneal injection of sodium pentobarbital (0.5% sodium pentobarbital solution is administered at a dosage of 0.1 mL/10 g body weight).
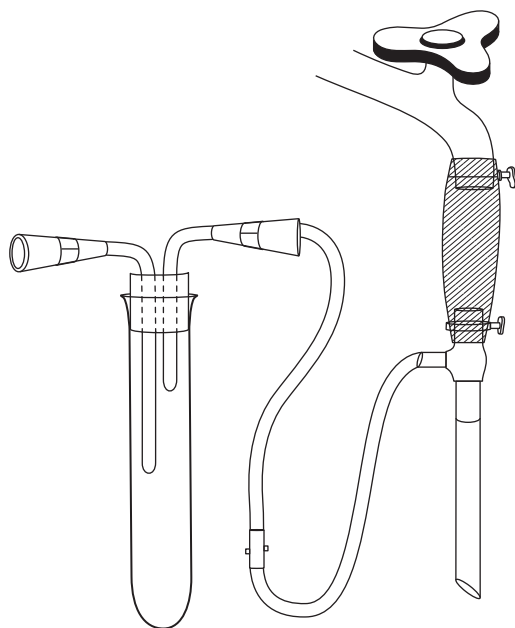
Figure 5.3.14 Milking device with single teat cup.

4. After anaesthesia, the mammary glands should be massaged gently with steam-warmed gauze. An injection of 0.1–0.18 IU oxytocin/kg body weight is given subcutaneously.

5. Milking should be started 2–3 min after oxytocin administration. Aspiration begins by turning on the tap. The investigator holds the mouse in the left hand and the milking device in the right hand.

6. In the mouse there are three pairs of pelvic mammary glands and two pairs of inguinal ones. The teat cup of the milking device is placed over the teat and aspirated with weak vacuum. Milk enters the test tube of the device (Figure 5.3.15).

7. Aspiration should be continued for 2–3 min from one teat and then changed to the next. Sometimes a teat itself may enter the lumen of the needle, or the needle may become blocked. In this case the entry of milk into the needle should be checked, then the angle of the teat cup adjusted, the vacuum pressure controlled and the blockage removed.

8. After milking the mouse should be returned gently into the cage of pups that were present when she was anaesthetized. Soon the pups will suck on the teats of the anaesthetized mother. This suckling stimulus is considered to have a good effect on the mother so she awakes early from the anaesthesia.

Skilful investigators can collect 1.0–1.5 mL of milk per mouse.

Oxytocin begins to exert its effect on milk secretion 10 min after injection, but milking should be started much earlier, about 2 or 3 min after injection.

Subsequent milking can be done at 3 day intervals, so that one lactating mother can be milked twice, 8 and 12 days after parturition. This milking is known not to affect the pups' growth. However, the investigator should handle lactating mice gently so as not to cause unnecessary stress, as nursing mothers are delicate.

Bile

Unlike rats, mice have a gallbladder in which bile collects. The gallbladder is identified clearly cranial to the liver at laparotomy (Figure 5.3.16). To collect the bile a mouse should be anaesthetized deeply with an intraperitoneal injection of 0.5% solution of sodium pentobarbital. The anaesthetized mouse is restrained on its back on the operating board. The abdominal wall is incised at the midline in the upper abdominal region. A small gallbladder is present at the cranial part of the liver between the left and right hepatic lobes. The base of the gallbladder is closed by a haemostatic clamp. Then the investigator

Figure 5.3.15 Milking from an anaesthetized lactating mother. A teat cup is put on an inguinal nipple and the milk aspirated.
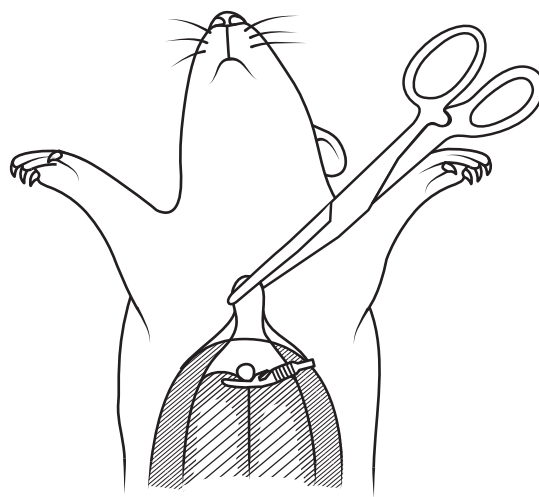
Figure 5.3.16 Gallbladder exposed at laparotomy of upper abdominal region. The gallbladder is clearly identified at the cranial side of the liver between the left and right hepatic lobes and secured by a haemostatic clamp closing off its base.

pierces the gallbladder with a 26G needle or incises it with fine iris scissors. The amount of bile in the gallbladder is very small, so it should be absorbed onto a small piece of filter paper, or collected into a microhaematocrit capillary tube by capillarity. In case of aspiration by a 1 mL syringe with a 26G needle, the bile is expressed onto a glass slide then collected into a capillary tube. The total amount of bile is 3–6 µL when the gallbladder is full.

Semen

For semen collection see Chapter 4.7.

Saliva

Saliva is excreted from the salivary glands, such as the mandibular, parotid and sublingual glands. Small amounts of saliva can be obtained by soaking it up with a piece of filter paper. To collect more, the salivary glands should be stimulated by pilocarpine hydrochroride. A mouse is anaesthetized lightly by intraperitoneal injection of 0.5% sodium pentobarbital solution, then injected subcutaneously with pilocarpine hydrochloride, at a dosage of 3–5 mg/kg body weight. Excreted saliva is collected from the oral cavity by a Pasteur pipette or a microhaematocrit tube.

As pilocarpine hydrochloride also stimulates the lacrimal glands and nasal mucous glands, the stimulated saliva is contaminated to some extent with lacrimal fluid and secretions from nasal glands. The stimulated saliva is also soaked up by filter paper and analysed.

Lacrimal fluid

The lacrimal fluid of mice is produced in very small amounts in intact animals. To collect lacrimal fluid, mice are stimulated by pilocarpine hydrochloride to induce excretion. A solution of 0.05% pilocarpine hydrochloride in saline is injected intraperitoneally at a dosage of 0.2 mL/animal. Several minutes after the injection, lacrimal fluid appears. This is collected by capillarity using a glass haematocrit capillary tube. Where small amounts are analysed, the fluid is soaked up by a small piece of filter paper. The contamination of saliva and secretion from the nasal glands must be carefully avoided.

Peritoneal fluids

Peritoneal exudate fluid is very small in quantity and contains some neutrophilic leukocytes, macrophages and lymphocytes. The peritoneal exudate is therefore usually collected to obtain the leukocytes, especially free macrophages. Resident peritoneal exuded cells in intact mice and the increased numbers of stimulated cells are collected and used for experimental purposes. In this section the general procedure for the collection of the stimulated peritoneal exudate is described. Stimulants, such as 5% glycogen, 2.4% fluid thyoglycolate medium and 10% proteose peptone, are used. The procedure for collection of stimulated peritoneal exudate cells is as follows.

1. 2 mL of thyoglycolate medium (or 0.5 mL of 5% glycogen) is administered intraperitoneally.
2. 3–4 days after administration, the mouse is sacrificed by cervical dislocation, and placed on its back on the operating board. The skin of the abdomen is incised and opened out in order to expose the abdominal muscular wall.
3. 10 mL of Eagle's MEM are injected intraperitoneally by a 10 mL plastic syringe with a 21G needle. The abdomen is then massaged gently with the fingers.
4. Removal and injection of the Eagle's MEM is repeated three times with the same syringe. Finally the medium is recovered. About 9 mL out of 10 mL of the medium is normally recovered.
5. The recovered medium is centrifuged slowly and the cell pellet resuspended in fresh Eagle's medium supplemented with 10% fetal calf serum, depending on subsequent treatments.

After stimulation, polymorphonuclear leukocytes, macrophages and lymphocytes appear in the peritoneal exudate in turn. Resident peritoneal exudate cells are also collected by the same procedure without stimulation.

Macrophages are separated utilizing their adhesive nature. Other immune competent cells are collected from the spleen.

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Anaesthesia, Analgesia and Euthanasia

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Introduction

Anaesthesia and analgesia in mice is particularly challenging because of a number of problems associated with the small body size of the animals. These include:

1. Mice are very susceptible to hypothermia since the high surface area relative to body mass results in rapid heat loss. Much of the mortality during anaesthesia may be due to hypothermia rather than to other physiological effects of anaesthetic agents.
2. Considerable strain-, sex and age-dependent variability in the effective dose of some injectable anaesthetics may result in inadequate depth of anaesthesia or lethal overdose.
3. Lack of reliable respiratory and cardiovascular monitors.
4. Rapid loss of even small amounts of blood (as little as 0.5 mL) may cause cardiovascular failure [1].
5. Lack of objective measurements of pain in mice.

Anaesthesia

Preanaesthetic considerations

Health conditions

Any animal subjected to an experiment must be in excellent health. Animals should be housed for a 1–2 week acclimatization period which

allows daily health control. It is undesirable to withhold food or water before anaesthetizing mice [1].

Hypothermia

Intra- and postoperative hypothermia in mice may be potentially lethal and needs to be a major concern before anaesthesia. Any suitable means of preventing a pronounced decrease in body temperature (e.g. heating blanket, infrared heat lamp) rather than treatment of hypothermia should be considered. In addition, body temperature in the mouse is closely tied to locomotor activity [2]. Therefore, the duration of anaesthesia and of the recovery period should be kept as short as possible in order to minimize the decrease in body temperature postoperatively.

Anticholinergic premedication

Mice may be treated with atropine (0.04 mg/kg subcutaneously (s.c.)) about 30 min before induction of anaesthesia [3]. Although atropine does not eliminate the copious secretions associated with ether or ketamine anaesthesia, the reduced secretion will help to maintain a patent airway [4].

Anaesthetic regimen

When selecting an anaesthetic regimen the type and length of the procedure, advantages and disadvantages of the various anaesthetics, and especially the aim of the study need to be considered in order to avoid interaction between the anaesthetic and the experimental protocol. The main choices to be made are between administration of injectable anaesthetics or inhalant anaesthetics. For major surgery and other long-term procedures a combination of an injectable anaesthetic with an inhalant anaesthetic or inhalation anaesthesia plus an opioid analgesic may be considered as well.

Most injectable anaesthetics are easy to administer by the intraperitoneal (i.p.) route in mice, but this technique is more likely to produce an unpredictable depth of anaesthesia and prolonged recovery periods. This is because drug absorption into the systemic circulation is slow after i.p compared with intravenous (i.v.) administration, making it impossible to titrate the anaesthetic to effect and overdosing may result.

Furthermore, i.p. administration of relatively large total quantities of a drug are required to produce anaesthesia, which in turn results in prolonged recovery [1].

On the other hand, for modern inhalation anaesthetics such as halothane, isoflurane or sevoflurane, more sophisticated and expensive equipment (e.g. calibrated vaporizer, breathing systems) are required. The major advantages of this technique include rapid recovery and immediate adjustment of the anaesthetic concentration to the animal's needs.

Injectable anaesthetics

Injectable anaesthetics (Table 5.4.1) can be administered i.p., s.c., intramuscularly (i.m.) or i.v. Acceptable volumes range from 0.1-0.5 mL for i.p. and s.c. injection, from 0.05-0.1 mL for i.v. and should not exceed 0.05 mL for i.m. injection for an adult mouse [1, 3].

Propofol

Propofol (2,6-diisopropylphenol) is a hypnotic agent that is used as an induction agent and as a maintenance anaesthetic delivered by continuous i.v. infusion or intermittent i.v. bolus [5]. In mice, a single bolus i.v. injection of 20-30 mg/kg produces about 2-3 min of surgical anaesthesia and loss of righting reflex for approximately 3-20.5 min (mean 7.1 min [1, 6, 7]). Advantages of propofol include rapid onset and short duration of anaesthesia, lack of accumulation after repetitive injections, no tissue damage following inadvertent perivascular infiltration and rapid, tranquil recovery after the cessation of i.v. administration [8]. Rapid bolus injection in rodents may be followed by 5-10 s of apnoea and hypotension, while continuous infusion causes only minimal changes in heart rate and respiratory rate [7].

Intravenous administration of propofol is followed by smooth induction and rapid recovery [9]. Therefore, propofol is useful for very short-term procedures (e.g. tattooing, blood sampling) owing to its rapid redistribution and metabolism after i.v. injection [10]. Other routes of administration (e.g. i.p.) may interfer with its rapid redistribution to body tissue and may thereby prevent anaesthetic concentrations being achieved in the brain.

TABLE 5.4.1: Anaesthetics and tranquilizers used in mice

Drug	Dosage (mg/kg)	Comments	Reference
α-Chloralose	114 i.p.	5% solution, only in combination with analgesics and/or other anaesthetic agents	White and Field [95]
Alphaxalone/alphadolone (*Saffan, Althesin*)	10—20 i.v.	Unpredictable anaesthetic effect following i.p., volume too large for i.m.	Green et al. [96], Flecknell [1]
Chloral hydrate	60—90 i.p.	Light surgical anaesthesia, considerable strain differences	Green [3]
	370—400 i.p.		Flecknell [1]
Fentanyl/fluanisone (*Hypnorm*)	0.4 mL/kg i.m.	Muscle rigidity, pronounced respiratory depression, 1:10 dilution	Flecknell [1]
Hypnorm/midazolam	0.1 mL/10g i.p.	1 mL Hypnorm + 1 mL midazolam (5 mg/mL) + 2 mL water for injection	Flecknell [10]
Fentanyl/droperidol (*Innovar Vet*)	0.5 i.m.	Irritant, tissue necrosis, self-trauma following i.m.	Flecknell [1]
Ketamine	80—100 i.m.	Sedation, muscle rigidity	Green et al. [15]
	100 i.p.		White and Field [95]
	100—200 i.m.		Flecknell [1]
Ketamine/acepromazine	44/0.75 i.p.	Sedation only	Gardner et al. [2]
	100/2.5 i.p.	Marked respiratory depression	Flecknell [1]
	100/5 i.p		Flecknell [1]
Ketamine/midazolam	100/5 i.p.	Only light anaesthesia	Flecknell [1]
Ketamine/medetomidine	75/1 i.p.	female mice	Flecknell [1], Cruz et al. [28]
	40/1 i.p.	Male mice	Cruz et al. [28]
	100—150/0.25 i.p.		Kilic et al. [29]
Ketamine/xylazine	100/5 i.m.	Excellent relaxation, sedation, analgesia	Erhardt et al. [20]
	100/10 i.p.		Flecknell [1]
	120/16 i.p.		Zeller et al. [19]
Ketamine/xylazine/acepromazine	100/10/3 i.p.		Buitrago et al. [26]
Methohexital (*Brevital, Brevimytal*)	10 i.v. 44 i.p.	Short-term anaesthesia	Flecknell [1] Dörr and Weber-Frisch [14]

(*Continued*)

TABLE 5.4.1: Anaesthetics and tranquilizers used in mice—cont'd			
Drug	**Dosage (mg/kg)**	**Comments**	**Reference**
Metomidate/fentanyl	60/0.06 s.c.		Green et al. [33], Flecknell [1]
Pentobarbital (*Nembutal*)	45 i.p.	1:10 dilution, narrow safety margin, marked strain differences in response	Flecknell [1]
	50 i.p.	Severe respiratory depression	Erhardt et al. [20]
	60 i.p.		Zeller et al. [19], Koizumi et al. [7]
Propofol (*Rapinovet, Diprivan*)	26 i.v.	Short-term anaesthesia, i.v. injection required	Flecknell [1]
	30 i.v.		Koizumi et al. [7]
Thiopental (*Penthotal, Trapanal*)	30 i.v.	Short-term anaesthesia, i.v. injection required, dose-dependent hypothermia and respiratory depression	Flecknell [1]
Tiletamine/ zolazepam (*Telazol*)	40 i.p. 80–100 i.p.		Flecknell [1] Silverman et al. [97]
Telazol/xylazine	7.5/45 i.m. 45/7.5 i.m.	Long-term anaesthesia	Gardner et al. [2] Gardner et al. [2]
Tribromoethanol (*Avertin*)	240 i.p.	Possible peritonitis, serositis	Zeller et al. [19], Flecknell [10]

i.p., intraperitoneally; i.m., intramuscularly; i.v., intravenously; s.c., subcutaneously.
Proprietary names are shown in *italics*.

The combination of propofol and a fast-acting opioid is a relatively safe and common total intravenous anaesthesia (TIVA) technique used in humans. Injections of 50, 75, 100 or 200 mg/kg i.p. of propofol were generally insufficient to achieve surgical anaesthesia [11]. The propofol-opioid combinations were associated with inconsistent effects between individuals receiving the same dose. Higher doses did not result in the expected predictable induction of surgical anaesthesia and were associated with high mortality rates. Thus the authors concluded that i.p. administration of propofol and fentanyl, sufentanil or remifentanil was associated with difficulties in finding and proposing an appropriate dose combination for anaesthesia in mice [11]. When the authors used a freshly prepared combination of 75/1/0.2 mg/kg of propofol, medetomidine and fentanyl they concluded

that this combination is a safe, easy and reversible technique to anaesthetize mice via the i.p. route. The combination provides a surgical window of 15 min and restraint for 30 min with a fast recovery [12]. However, lymphangiectasis and chyloperitoneum observed at necropsy may result from i.p. administration of propofol. Therefore, researchers should be strongly discouraged from administering propofol by routes other than intravenously [13].

Alphaxolone/alphadolone

Slow i.v. administration of 10-20 mg/kg of the steroid combination alphaxalone/alphadolone produces deep anaesthesia with analgesia and good muscle relaxation within 10 s and maintenance of the maximum effect for approximately 5-8 min [1, 3]. The mean sleep time is 10 min and

the time to full recovery ranges from 20–30 min. Prolonged anaesthesia may be achieved either by further increments (6 mg/kg i.v.) at 15 min intervals or by continuous infusion of 0.25–0.75 mg/kg per minute via an indwelling needle in the tail or jugular vein [1, 3]. When used in rodents, a 1:10 dilution of the commercial preparation is recommended. The wide therapeutic index and the lack of cumulative effects or tolerance with repetitive i.v. doses make this combination an excellent i.v. anaesthetic in mice [3]. However, the unpredictable anaesthetic effect following i.p. administration and the large volume required for i.m. injection limit its usefulness in this species [3].

Methohexital

Intravenous injection of 6–10 mg/kg of the ultra-short-acting barbiturate methohexital produces anaesthesia within 10 s for about 3–5 min [1, 3]. Time to full recovery is about 50 min. Repetitive dosing may, however, cause accumulation with subsequent prolongation of the recovery period. Methohexital produces moderate cardiovascular and respiratory depression. Intraperitoneal injection of 44 mg/kg methohexital (6.46 mg/mL) to female C3H/Neu mice induced anaesthesia within 3.3 min (mean; [14]). Complete immobilization lasted for 1.5 min (mean) and recovery was completed 10–15 min after injection. The study also revealed that methohexital at a dose less than 40 mg/kg did not result in chemical restraint while doses greater than 50 mg/kg caused considerable lethality. Although no gross pathomorphological changes were found after repeated i.p. injections of methohexital in this study, administration of methohexital by the i.p. route has not been recommended because of unpredictable effects [1].

Thiopental

Thiopental is irritating perivascularly and thus must be administered i.v. Slow injection of 30–40 mg/kg i.v. produces light surgical anaesthesia within 10–15 s and with a duration of about 10–12 min [1, 8]. Thiopental produces moderate respiratory and cardiovascular depression. Incremental doses should not be used for prolonging anaesthesia as accumulation is very marked with this agent [3].

Ketamine

When used as a sole agent (100–200 mg/kg i.m. or i.p.), ketamine may produce surgical anaesthesia for about 30 min, which may, however, be accompanied by insufficient analgesia, muscle rigidity and significant mortality [1, 8, 15]. Therefore, administration of ketamine with an accompanying tranquilizer (e.g. α_2-adrenoceptor agonist, phenothiazine) has been recommended [1]. When using ketamine, a reduction of endotoxin-induced production of proinflammatory cytokines, including tumour necrosis factor-alpha (TNF) in monocytes and macrophages [16, 17] need to be considered. Ketamine alone [18] or in combination with xylazine [19] may cause exophthalmus due to increased intracerebral and intraocular pressure.

Ketamine/xylazine

Ketamine plus the α_2-adrenoceptor agonist xylazine (100 mg/kg + 10 mg/kg i.p.) has become the most widely used ketamine combination [1, 8]. Xylazine has sedative and analgesic properties. Both drugs may be given as a mixture in a single injection. A combination of ketamine with a lower dose of xylazine (100 mg/kg + 5 mg/kg i.m.) produces calm, rapid (2–3 min) induction, 80 min surgical anaesthesia and a total anaesthesia time of 110 min [20]. Surgical anaesthesia was associated with excellent muscle relaxation, sedation and analgesia throughout the 80 min period of anaesthesia. Zeller [19] recommended a ketamine/xylazine combination (120 mg/kg + 16 mg/kg i.p.) for embryo transfer in female CD1-, OF-1- and NMRI mice. The combination provided adequate surgical anaesthesia. In addition, all animals survived and no disadvantages were detected regarding the result of the embryo transfer.

Adverse effects of this combination on cardiovascular function include bradycardia and increase in preload and left ventricular fractional shortening, along with a significant reduction in cardiac output and hypotension [20–23]. In addition, production of acute temporary cataracts in some animals were reported [24]. Ketamine/xylazine may cause injury to lymphocytes and hepatic Kupffer and endothelial cells within 3 h of administration accompanied by an increase in activity of hepatic aspartate transaminase (AST) [25].

The intraperitoneal injection of ketamine (100 mg/kg) combined with xylazine (10 mg/kg)

and acepromazine (3 mg/kg) resulted in safe and reliable anaesthesia in BALB/c mice requiring a surgical plane of anaesthetic depth [26]. In contrast to this anaesthetic protocol, other combinations of ketamine/xylazine (100 mg/kg + 10 mg/kg i.p.) used alone or including either buprenorphine (0.05 mg/kg i.p.) or carprofen (4 mg/kg s.c.) produced immobility while surgical depth of anaesthesia could not be confirmed.

Ketamine/medetomidine

Ketamine may be combined with the newer α_2-agonist medetomidine (75 mg/kg + 1.0 mg/kg i.p.) [1]. Medetomidine closely resembles xylazine in its effects, but possesses much higher affinity for the α_2-adrenoceptor [27]. It causes sedation, excellent muscle relaxation and analgesia but cardiovascular and respiratory depression, hypothermia, hyperglycaemia, diuresis and increased salivation may also be present [1, 28]. Cruz [28] used ketamine/medetomidine combinations for short-term (5–10 min) anaesthesia in Swiss-Webster mice of both sexes. Female mice needed a higher dose of ketamine (75 mg/kg + 1 mg/kg i.p.) than male mice (40 mg/kg + 1 mg/kg i.p.). When ketamine/medetomidine (100–150 mg/kg + 0.25 mg/kg i.p.) were given to NMRI mice, loss of righting reflex occurred within 5 min of drug administration and lasted for approximately 75–80 min (mean) [29].

Because of the depression of cardiovascular and respiratory function and the development of long-lasting hypothermia induced by these combinations, reversal of the xylazine or medetomidine effects by the α_2-adrenoceptor antagonist atipamezole is strongly recommended [1, 28]. Atipamezole (1 mg/kg i.m., i.p., s.c., i.v.) and, if needed, followed by increments of 0.25 mg/kg, also results in a much faster reappearance of righting reflex and a shorter total recovery time. However, the total dose of atipamezole required for satisfactory reversal was considerably higher (up to 2.5 mg/kg) in female mice than in male mice [28].

Ketamine/acepromazine

Ketamine combined with the phenothiazine tranquilizer acepromazine (44 mg/kg + 0.75 mg/kg i.p.) may fail to produce loss of righting reflex in some mice [2] while high doses of this combination (100 mg/kg + 2.5 mg/kg i.p.) can produce surgical anaesthesia in some strains of mice but this may be accompanied by marked respiratory depression [1].

Ketamine/midazolam

Administration of ketamine plus the water-soluble benzodiazepine midazolam produces effects similar to that of ketamine/acepromazine. Since neither midazolam nor acepromazine possesses detectable analgesic action in mice, low doses produce only light anaesthesia while high doses (100 mg/kg + 5 mg/kg i.p.) may cause deeper levels of anaesthesia with pronounced respiratory depression [1].

Telazol/xylazine

Telazol is a combination of the dissociative anaesthetic tiletamine with the benzodiazepine zolazepam [8]. Because Telazol alone is an adequate anaesthetic in rats, but not in mice, it has been combined with xylazine for anaesthesia in mice [2]. Following i.m. injection of Telazol/xylazine (7.5 mg/kg + 45 mg/kg i.m.) to male Hsd:ICR mice, onset of anaesthesia occurred within 0.6–1.3 min of injection and duration of surgical anaesthesia (absence of toe pinch reflex) ranged from approximately 46 to 164 min (mean 99 min), suggesting that this combination could be useful for long-term anaesthesia [2]. However, total recovery time may last up to 14 h, accompanied by respiratory depression and hypothermia. When 45 mg/kg i.m. of Telazol plus 7.5 mg/kg i.m. of xylazine were administered, mean duration of surgical anaesthesia was decreased to 36.1 min and some mice did not lose toe pinch reflex at all.

Fentanyl/droperidol

For neuroleptanalgesia in mice a combination of the potent opioid analgesic fentanyl and the butyrophenone tranquilizer droperidol (Innovar Vet) can be used. The usefulness of Innovar Vet in mice, however, is compromised by its irritant nature causing tissue necrosis and self-trauma to the digits following i.m. injection [30].

Fentanyl/fluanison

The combination of fentanyl and the butyrophenone fluanison (Hypnorm) produces immobilization and profound analgesia accompanied by

muscle rigidity and pronounced respiratory depression [1]. Furthermore, nervous twitching, paddling, extreme hyperacusia and hyperaesthesia has been reported for fluanison in mice [31]. The undesirable effects can be overcome if the dose of the neuroleptanalgesic is reduced and a benzodiazepine is incorporated in the combination [34]. A combination of 1 mL fluanison (0.135 mg fentanyl/mL; 10 mg fluanisone/mL) plus 1 mL midazolam (5 mg/mL) plus 2 mL water for injection, mixed in the same syringe and administered at a dose of 0.1 mL/10 g i.p., has been recommended [10]. With this combination good surgical anaesthesia lasting about 20-40 min can be achieved [31, 32]. Although the Hypnorm/benzodiazepine combination may cause respiratory depression, there is no cyanosis and mortality is very low provided due attention is paid to the body temperature and hydration [3].

Fentanyl/metomidate

The combination of the hypnotic metomidate plus fentanyl (60 mg/kg + 0.06 mg/kg s.c.) produces stable, surgical anaesthesia for 60-70 min [33].

Etorphine/methotrimeprazine

This neuroleptanalgesic combination of the opioid etorphine and the phenothiazine tranquilizer methotrimeprazine (Immobilon) can be used alone (1:10 dilution, 0.1-0.2 mL/mice s.c.) [34] or in combination with midazolam to produce deeper levels of anaesthesia (0.1 mL/10 g i.p. of the mixture composed of 0.3 mL methotrimeprazine + 1 mL midazolam + 8.3 mL sterile water for injection; [35]. This combination produces prolonged anaesthesia. The mean duration for absence of the righting reflex and pedal withdrawal reflex was 155 and 170 min, respectively. When compared with fluanison/midazolam, methotrimeprazine/midazolam produces a twofold increase in the duration of surgical anaesthesia accompanied by a severe respiratory depression and followed by a prolonged recovery of up to 6 h. Therefore, supplemental oxygen and, if necessary, positive pressure ventilation, should be provided.

Carfentanyl/etomidate

The use of this combination of the highly potent opioid analgesic carfentanyl and the hypnotic etomidate (0.003 mg/kg + 15 mg/kg i.m.) may cause strong excitation during induction and prolonged recovery from anaesthesia [20]. The recovery period may be accompanied by tonic-clonic spasms and trembling. Because of these adverse effects, the combination is not recommended for use in mice.

Chloral hydrate

Chloral hydrate (370-400 mg/kg i.p.) provides light surgical anaesthesia for 45-60 min [1]. There are, however, considerable strain differences in the depth of anaesthesia.

Tribromoethanol

Tribromoethanol (125-250 mg/kg i.p., 1.2% v/v solution) can produce excellent surgical anaesthesia in mice that is characterized by rapid induction, a 16 min duration of surgical anaesthesia, good skeletal muscle relaxation, moderate degree of respiratory depression and full recovery within 40-90 min [3, 8, 36]. However, while the use of tribromoethanol anaesthesia in mice has been recommended by a number of investigators [8, 36, 37], others advise against its use [1, 19].

Exposure of tribromoethanol to light or improper storage at room temperature may cause decomposition of the anaesthetic into its toxic byproducts dibromoacetaldehyde and hydrobromic acid, which are potent gastrointestinal (GI) irritants, leading to fibrinous peritonitis, ileus and fatalities [36]. Therefore, only freshly prepared (on the day of administration), properly stored (4 °C, dark conditions) solutions must be used [8, 36]. However, i.p. injection of even a freshly prepared solution of tribromoethanol to CDI, OF-1 or NMRI mice at either 240 mg/kg i.p., dissolved in tertiary amyl alcohol and distilled water to a solution of 1.2%, or 450 mg/kg i.p., diluted to a concentration of 2.5% in 0.9% NaCl, produced profound histopathological changes 24 h after injection [19]. Histopathological changes included focal to diffuse necrosis of subperitoneal muscle fibres associated with peritonitis and serositis in the spleen, liver, intestines and stomach. In mice treated with the higher concentration an increased severity of necrotic and inflammatory changes was noticed. Because the lesions were not related to the solvent or strain-specific differences, the authors concluded that the

histopathological changes were induced by tribromoethanol. In conclusion, the use of tribromoethanol anaesthesia in mice should be avoided because of the unpredictable adverse effects and it should therefore be replaced by newer injectable or inhalant anaesthetic techniques [10, 38].

Pentobarbital

The barbiturate pentobarbital has been widely used in mice because of its non-irritant nature and modest costs. Intraperitoneal injection of 45 mg/kg of a 1:10 dilution of the commercially available solution may result in surgical anaesthesia for 15-60 min and total sleep time of 2-4 h [1]. However, the appropriate dose depends on the strain and within-strain differences are also present for age, sex, dose level, litter size, diurnal periodicity, diet, fasting prior to anaesthesia and type of bedding material [3, 39-41]. Pentobarbital at 50-60 mg/kg i.p. may result in good sedation but inadequate surgical, moderate to severe circulatory and respiratory depression during anaesthesia in some strains (e.g. BALB/c, ICR; [2, 20]) while it can produce deep anaesthesia for up to 169 min in other strains (e.g. outbred ICR mice; [7]). For surgical anaesthesia, pentobarbital at 50-90 mg/kg i.p. may be required [3]. Anaesthesia may last for 20-40 min with a total sleep time of approximately 120 min and time to full recovery between 6 and 24 h. Thus, supplemental oxygen and a recovery incubator are mandatory for deeply anaesthetized mice.

Inhalation anaesthesia

Traditionally, a bell jar has been used for induction of inhalation anaesthesia. The liquid (diethyl ether, methoxyflurane) is volatilized by placing it on cotton balls or gauze squares in the bottom of the jar. The anaesthetic-impregnated cotton should be covered with a woven mesh grid to prevent local irritation of the feet of the animal by direct contact with the liquid anaesthetic. The animal is placed within the jar and visually observed for cessation of movement and recumbency, thereby signifying the onset of anaesthesia. There are, however, major disadvantages associated with this delivery system. The anaesthetic concentration within the jar cannot be controlled, and lethal concentrations can rapidly accumulate when volatile agents with high vapour pressures (e.g. halothane, isoflurane, sevoflurane) are used. For this reason, use of the bell jar should be reserved for inhalant agents such as methoxyflurane, which reaches a maximum concentration of approximately 3% after full vaporization at room temperature in contrast to levels of approximately 30% inhalant gas which can be reached upon volatilization of halothane or isoflurane with this method [8]. Furthermore, scavenging of waste anaesthetic gas is difficult. Hence, high vapour pressure agents (e.g. halothane) should be delivered at controlled concentrations (Table 5.4.2) into a transparent induction chamber using an anaesthesia machine with a calibrated vaporizer [1, 8]. The induction chamber should also have both an inlet for

TABLE 5.4.2: Inhalant anaesthetics used in mice

Anaesthetic	Induction[a] (Vol%)	Maintenance[a] (Vol%)	Reference
Methoxyflurane	3.5	0.4—1.0	Flecknell [1, 10]
(*Metofane, Penthrane*)	3.0	1.5—2.0	Sedgwick et al. [98]
Halothane	3—4	1—2	Flecknell [1]
(*Fluothane*)	2.4	1.0—1.5	Sedgwick et al. [98]
Isoflurane	3.5—4.5	1.5—3	Flecknell [1]
(*Forane, Aerrane*)	2.5	1.0—1.5	Sedgwick et al. [98]
Enflurane	3—5	0.5—2	Flecknell [1]
(*Ethrane*)	2.0	1.5—2.0	Sedgwick et al. [98]
Diethylether	10—20	4—5	Flecknell [1]
	5—10		Sedgwick et al. [98]

[a]To effect.
Proprietary names are shown in *italics*.

delivery of fresh gas and an outlet for effective removal of waste anaesthetic gases.

Following induction of anaesthesia, the mouse is removed from the induction chamber and very brief surgical procedures (<30 s) are possible. For longer periods of anaesthesia, reduced concentrations of the inhalant anaesthetic (Table 5.4.2) should be administered via a face mask/nose cone connected to the anaesthesia machine. Face or head masks can be purchased or can be easily made from the proximal end of a 20, 50 or 60 mL syringe [8]. Alternatively, orotracheal intubation of the mouse and controlled ventilation at a rate of 60-100 breaths/min and a tidal volume of 0.15 mL/10 g body weight (bwt) can be performed [1].

The concentration of a volatile anaesthetic required for induction and maintenance of anaesthesia is determined by the agent- and species-specific minimum alveolar concentration (MAC value) of the compound which is a measure of its potency [42]. Normally end-tidal anaesthetic concentrations will be used for MAC determination. It is assumed that at 1.0 MAC animals are unconscious and under anaesthesia, while end-tidal concentrations equal to approximately 1.25 to 1.50 MAC may be required to assure adequate surgical anaesthesia [43]. The MAC values of the most commonly used volatile anaesthetic agents in mice are summarized in Table 5.4.3.

Whenever MAC multiples are applied as measures of anaesthetic depth, the effect of strain differences on MAC requirements need to be considered [44, 45]. In 15 different mouse strains, mean (±SD) MAC values for desflurane, isoflurane and halothane ranged from 6.55 ± 0.54% to 9.12 ± 0.68%, 1.23 ± 0.17% to 1.77 ± 0.17%, and from 1.06 ± 0.09% to 1.64 ± 0.09%, respectively.

Ether

Ether (diethyl ether) is usually administered to mice by simple 'open-drop' methods using an ether-impregnated cotton ball in a bell jar for induction followed by inhalation via a simple face cone if prolonged anaesthesia is required [3]. The simple induction method can be used to provide 5-10 min of anaesthesia suitable for minor procedures. The advantages of ether anaesthesia, such as low costs, the wide margin of safety for the inexperienced investigator due to the slow induction and a lack of effect on haematological parameters (e.g. haematocrit, white and red blood cell count) [8] are offset by a number of disadvantages that need thorough consideration before ether is used. Induction of ether anaesthesia is unpleasant, is an irritant to the respiratory tract and may provoke excessive mucous secretions, pulmonary oedema and airway obstruction [4]. Furthermore, ether is

TABLE 5.4.3: Minimum alveolar concentration (MAC) values (%) of volatile anaesthetics in mice			
	1 MAC (mean ± SD)	**Mouse strain**	**Reference**
Halothane	1.00 ± 0.04 (SE)	CD-1, male	Deady et al. [99]
	0.95 ± 0.07[a]	Swiss-Webster	Mazze et al. [100]
	1.19 ± 0.13	129/J, male	Sonner et al. [44]
	1.39 ± 0.16	B6129F2/J, male	Sonner et al. [44]
Isoflurane	1.41 ± 0.03 (SE)	CD-1, male	Deady et al. [99]
	1.34 ± 0.10	Swiss-Webster	Mazze et al. [100]
	1.30 ± 0.11	C57BL6/J, male	Sonner et al. [44]
	1.77 ± 0.17	Spret/Ei, male	Sonner et al. [44]
Enflurane	2.19 ± 0.08 (SE)	CD-1, male	Deady et al. [99]
	1.95 ± 0.16	Swiss-Webster	Mazze et al. [100]
Sevoflurane	2.50	CBI, male	Puig et al. [101]
Desflurane	6.55 ± 0.54	129/J, male	Sonner et al. [44]
	9.12 ± 0.68	Spret/Ei, male	Sonner et al. [44]

[a]Neither the sex of the animals nor whether female mice were pregnant influenced the data.
SE, standard error of means.

flammable and explosive. Although Green [3] concluded that ether retains a useful place in mouse anaesthesia when measured volumes (2-4 mL) are used for induction of anaesthesia, other investigators considered ether to be highly unsuitable for this species [1, 4].

Methoxyflurane

Methoxyflurane combined with oxygen or air is the volatile agent of choice for inhalation anaesthesia in mice [3] and many investigators agreed that methoxyflurane is the safest agent to use whether by simple open-drop methods (bell jar) or with sophisticated apparatus [3, 4]. Induction of anaesthesia (time to loss of righting reflex) is smooth and rapid (1-3 min) if mice are introduced to an induction chamber in which methoxyflurane on gauze (0.5-1.0 mL in a 1 L chamber) has been allowed approximately 10 min at room temperature to evaporate. Methoxyflurane reaches a maximum concentration of approximately 3% after full volatilization at room temperature [2, 3, 8]. Thereafter, the mouse is removed and short procedures (10-20 min) such as retro-orbital bleeding or surgery in the head and neck region can be performed without the encumbrance of masks or tubes. For further maintenance of anaesthesia a methoxyflurane-soaked nose cone or a calibrated vaporizer with a nonrebreathing circuit at 100-500 mL/min fresh gas flow and 0.5-2% methoxyflurane can be used.

The advantages of methoxyflurane include the ease to maintain even prolonged surgical anaesthesia, no need for an assistant to act as anaesthetist, smooth recovery with some degree of analgesia and a postoperative survival rate of 100%. Furthermore, methoxyflurane produces less salivation than ether and less cardiovascular and respiratory depression than halothane [8]. However, animals must be monitored closely for maintenance of body temperature and depth of anaesthesia, and exposure must be quickly reduced in animals exhibiting slow and erratic respiration [2, 3].

Recovery may be very prolonged (up to 24 h) depending on the duration of administration [3]. About 40% of methoxyflurane is metabolized, resulting in inorganic fluoride ion release which can cause renal damage [46]. Therefore, scavenging of anaesthetic waste gases is important for personnel safety.

Halothane

Halothane is a non-flammable, non-explosive, non-irritating volatile anaesthetic but needs a calibrated vaporizer for delivery of controlled concentrations. It provides much more rapid induction and recovery than methoxyflurane, necessitating careful anaesthetic monitoring to prevent overdose and requires the use of a calibrated vaporizer [8]. Halothane provides excellent surgical conditions.

In newborn, spontaneously breathing mice, surgical anaesthesia can be safely induced by inhalation of 3% halothane in 1 L/min fresh gas flow composed of an equal $N_2O:O_2$ mixture and maintained at 1-1.5% halothane [47]. During recovery, supplemental oxygen (2 L/min) should be applied. When halothane (0.25-0.75%) was compared with ketamine/xylazine (80 mg/kg + 10 mg/kg i.p.) in CF-1 mice, halothane anaesthesia was more convenient and reliable with respect to rate of induction, reversal and control of anaesthetic depth and produced much less cardiac depression (heart rate, left ventricular fractional shortening, cardiac output) than the injectable regimen [21].

However, halothane causes a dose-dependent depression of the cardiovascular and respiratory system [1]. Furthermore, halothane, like isoflurane, may inhibit immune function (interferon stimulation of natural killer (NK) cell activity) in mice [48], and female CBI mice subjected to multiple exposures to halothane anaesthesia before mating may produce increased amounts of specific antibody-secreting B cells accompanied by microscopic fatty changes in the liver. Although halothane does not alter reproductive performance, offspring survival may be lowered [49].

Isoflurane

Isoflurane is the anaesthetic agent of choice particularly for procedures requiring low risk and reliable rapid recovery. Isoflurane produces stable haemodynamic conditions and has been recommended for anaesthesia in Swiss, CD-1 and C57BL/6 strains [50]. Its effects on cerebral metabolism and cerebral blood flow offer some degree of protection to ischaemic and hypoxic brains [51]. Unlike methoxyflurane and many injectable anaesthetics, only minimal amounts

of isoflurane are subjected to hepatic metabolism, biotransformation and excretion [8] and do not affect hepatic enzyme activities [25]. After induction of anaesthesia with 2% isoflurane in oxygen (700 mL/min) delivered to an induction chamber, long-term anaesthesia (6 h) was maintained at 1.7% isoflurane in oxygen delivered via a face mask to spontaneously breathing mice [52]. Although recovery was followed by a 12 h period of lethargy, all survivors (89%) returned to normal activities.

Potential disadvantages of isoflurane include transient postoperative immunosuppression [48], increased frequencies of cleft palate, skeletal variations and fetal growth retardation in pregnant mice exposed to light anaesthetic doses of isoflurane and an increased maternal blood loss secondary to depressed uterine muscle contractility [53]. Therefore, isoflurane should be avoided in surgical procedures directly preceding immunological research studies in mice [48].

Sevoflurane

Sevoflurane offers a significantly greater precision and control of maintenance of anaesthesia and potentially a much more rapid induction and recovery from anaesthesia than all the other inhalants [8]. Its blood solubility is one-half to one-third of that shown by isoflurane, approaching that of nitrous oxide [54]. Furthermore, sevoflurane is less pungent than isoflurane and rapid induction is not accompanied by struggling.

Sevoflurane resembles isoflurane in that it depresses ventilation and blood pressure in a dose-dependent manner, but maintains heart rate. This stability of heart rate provided by sevoflurane is desirable because it neither increases myocardial oxygen consumption nor decreases the time available for myocardial perfusion. Hypotension occurs due to a decrease in total peripheral resistance. At clinically relevant concentrations, sevoflurane preserves cardiac output, but excessive levels depress cardiac contractility and can produce cardiovascular collapse. It preserves splanchnic, including renal, blood flow. Sevoflurane can also decrease cerebral vascular resistance and cerebral metabolic rate.

Sevoflurane is degraded by soda lime or baralyme in a temperature-dependent manner [54]. Although this breakdown may not significantly affect the course of anaesthesia, the breakdown product known as compound A (an olefin) is lethal in rodents at high concentrations (400 ppm).

Desflurane

Desflurane, like sevoflurane, has a much lower blood/gas partition coefficient (PC) compared with isoflurane ($PC_{desflurane}$ 0.45, $PC_{sevoflurane}$ 0.65, $PC_{isoflurane}$ 1.4) resulting in a much faster induction of and recovery from anaesthesia [54]. One disadvantage of desflurane is that it is considerably less potent than isoflurane, resulting in a greater anaesthetic consumption rate and therefore greater cost [55]. In addition, the low boiling point of desflurane requires a special heated vaporizer to control its delivery [55].

Nitrous oxide

The use of nitrous oxide as the sole anaesthetic agent in rodents is discouraged because it is not a complete anaesthetic [34]. It is also a health hazard to humans.

Carbon dioxide

Carbon dioxide (CO_2) combined with oxygen (O_2) can be used to produce very short-term anaesthesia (e.g. retro-orbital bleeding, cardiac puncture) in adult mice. Carbon dioxide is readily available, inexpensive, safe for personnel and provides rapid and smooth recovery. When mice were exposed to a mixture of 80% CO_2 plus 20% O_2 for about 120 s, anaesthesia was induced within 10 s and surgical tolerance lasted for 19.5 s (mean) [56]. There has been, however, no consensus on the inspired CO_2 concentration and the duration of exposure to CO_2 in mice. Low (50%) inspiratory CO_2 concentrations may produce long induction times up to 16 min and may also lead to severe adverse effects including nasal bleeding, excessive salivation, seizures and death associated with moderate distress and discomfort for the animals [8]. In contrast, high (100%) inspiratory CO_2 concentrations produce rapid anaesthesia with fewer adverse effects but may not be reliable or appropriate for anaesthesia where recovery is planned. Therefore, 70% CO_2 in oxygen may be the optimal concentration, based on practicality and humane

acceptability [8]. Furthermore, it is not known how long CO_2/O_2 anaesthesia can be safely maintained, but exposure to CO_2 for no longer than 2 min may be appropriate [3].

Orotracheal intubation

In order to assure adequate alveolar ventilation during general anaesthesia, especially during inhalation anaesthesia or when controlled ventilation becomes mandatory (e.g. during thoracotomy), a technique for intubation in mice may be reviewed at http://www.hallowell.com/index.php?doc=2&pr=Video_Presentations.

Anaesthesia in neonates and pups

Anaesthesia in neonatal mice and pups is critical not just because of their small size but also because only a small amount of information exists on drug metabolism, excretion and biotransformation in these animals. Specific aspects of neonatal pharmacokinetics and pharmacodynamics have been reviewed more recently [57]. The authors do not agree with the widespread careful and restricted use of drugs in neonates because of the immaturity of the organ systems. Rather, careful attention should be paid to dosage and monitoring in animals at this age. The organ systems of the newborn animal show some special characteristics at birth. Their heart is less extensible, which makes the heart rate the determining factor for cardiac output [58]. The respiratory centre is not developed until the 15th postnatal day, which manifests in irregular breathing patterns [59]. Hypoxic conditions lead to a rapid depletion of glycogen stores [60]. Regarding the nervous system, Schaeffer [61] describes the effect of the divergent connection of peripheral nerve endings in the posterior horn of the neonatal spinal cord and the lack of protective mechanisms in the brainstem. Stimuli reach the cortex unfiltered in neonates and even a smooth touch of their skin can evoke intense reactions, thus making the developing nervous system of animals as well as humans very susceptible to painful interventions leading to long-term alterations like hyperalgesia and allodynia [62-64].

Despite the fact that the sensory system is intact at birth [65], data on anaesthetic regimes in neonatal rodents reveal very heterogenous combinations and dosages of drugs and, on the other hand, describe antiquated methods in either case with a lack of postoperative analgesia.

For more than 70 years hypothermia has been judged to be an appropriate form of 'analgesia/anaesthesia' in neonates less than 1 week old [3, 66, 67]. Newborn mice are regarded to be poikilothermic while adult thermoregulatory capabilities do not develop until the third week of life [8]. Because of their small body mass, rapid core cooling can be achieved by surface cooling. Infant rodents can tolerate extended periods of 1 °C body temperature. Using this method of immobilization neonatal mice are cooled in an ice-water slush at 1-2 °C for 20-30 min, and retained with elastic bands on a piece of sponge soaked in ice-cold water or an ice pack for the duration of the operation [3]. After about 30 min the animal should be warmed till slight reflexes are regained to 'recover from extended hypothermia' and 'survive the procedure'. Then the pups can be cooled again [68]. This technique is commonly still used for thymectomy of 1-2 day old pups [69], with a quoted mortality of 3% by Clifford [70]. On completion of the operation, the pups should be dried on paper tissue, warmed to 37 °C in an incubator, and returned to their original cage as a group to join a single unoperated littermate. Aggressive rewarming techniques such as heating pads or lamps, however, should not be used in order to avoid tissue damage [52]. The use of hypothermia as an 'anaesthetic' method has to be discussed as the excessive and non-physiological decrease in body temperature may be associated with an increased risk of ventricular fibrillation, tissue hypoxia and metabolic acidosis after rewarming, especially in older animals [67]. Moreover, the process of cooling and rewarming itself can be painful and the analgesic effect is stopped in warmed neurons, which places hypothermia in the category of 'non-analgesic anaesthetics' along with hypnotics like pentobarbital and halothane [71].

Alternative methods of anaesthesia in newborn rodents have been reviewed by Danneman and Mandrell [72]. However, the majority of reports have dealt almost exclusively with anaesthetic techniques applicable to neonatal rats rather

than to neonatal mice. The authors describe the effects of a combination of fentanyl and droperidol injected intraperitoneally. In contrast to Park et al. [73], who administered the combination subcutaneously in a 2.5 times lower dosage, Danneman and Mandrell could not observe satisfying surgical tolerance. Furthermore, the authors reported the use of ketamine (100 mg/kg) and pentobarbital (30, 35, and 40 mg/kg) in different doses. Under ketamine injection the withdrawal reflex ceased after 8 min, but mortality was around 70%. Pentobarbital in the lowest dose gained no surgical tolerance and the higher doses were associated with increased mortality rate. Clowry and Flecknell [74] achieved good results with fentanyl and fluanison in 7 day old rats for intracranial surgery. Cattano et al. [75] used propofol in a concentration between 150 and 200 mg/kg to anaesthetize 5-7 day old mice with an intraperitoneally injection for 90-120 min. First findings [76] in own studies with newborn mice between 1 and 2 days old reveal a stable anaesthesia for surgery with a combination of medetomidine (0.5 mg/kg), midazolam (5 mg/kg) and fentanyl (0.05 mg/kg) given subcutaneously as in anaesthesia of adult mice [77]. Animals can be positioned in dorsal recumbency after about 2 min. They are kept at physiological body temperature and oxygen is supplied during a 30 min duration of anaesthesia. Despite a decrease of respiratory and heart frequency no deaths were observed after anaesthesia of 21 neonates. The administered drugs can be antagonized completely by subcutaneous injection of atipamezole (2.5 mg/kg), flumazenile (0.5 mg/kg) and naloxone (1.2 mg/kg) after the intervention, or in emergency cases, and all pups can be returned to their dams a short time after reversal of anaesthesia.

More often inhalational anaesthesia is described when dealing with neonatal mice. Older studies, e.g. [78], indicate good results using ether but provide no information about mortality or reflex status of the animals. Dazert [47] carries out surgeries about 10-15 min in 1-16 day old mice with a mixture of oxygen, nitrous oxide and halothane, whereas Gotoh et al. [79] prefer isoflurane (2% for induction, 1.5% for maintenance) for their 20 min interventions in 6-7 day old mice. Moreover, Loepke et al. [80] describe a method of mechanical ventilation in 10 day old mice under isoflurane anaesthesia.

They intubate the pups orotracheally with a 24G catheter and ventilate them with 300 breaths/min and a tidal volume of 12 μL/g with a mixture of 1.8% isoflurane and oxygen. All three studies quote survival rates of 100%. Independent of the choice of anaesthesia for neonatal rodents, Jevtovic-Todorovic and Olney [81] outline an anaesthesia-induced developmental apoptosis of neurons shown by different authors over a 10 year period. This issue has to be kept in mind, especially when anaesthetizing neonates for neuronal or behavioural studies.

Monitoring of animals at this small size is a challenge, especially for the use of devices such as a pulse oximeter. Tan et al. [82] obtained reliable heart frequencies with transthoracal Doppler ultrasound. Ishii et al. [83] succeeded in measuring the blood pressure in neonatal mice with micropipettes in the femoral artery, whereas Loepke et al. [80] measured it in the carotid artery. Heier et al. [84] collected ECG data non-invasively in neonatal mice from postnatal day 2. But these techniques are usually not practicable for routine interventions, especially short ones. Rather, the breathing rate and skin colour of the nude pups is a good and quick indicator for a change regarding the function of respiration or cardiac cycle [73]. If the animals present cyanosis anaesthesia has to be stopped and oxygen has to be supplied if not already done. Moreover, survival rate can be improved if simple precautions are taken. To avoid rejection by the dam, working with neonates demands quiet surroundings during labour and careful and stress-free handling of the litter [73]. The pups should be rubbed in the original bedding to ensure that they acquire the dam's scent, thus minimizing the risk of cannibalism when they are returned to the cage [8, 85] and the pups should behave normally before being returned to their mothers [73]. As mentioned above, only a few authors describe the use of postoperative analgesia after surgery in preweaning rodents. Sternberg et al. [86] achieved good results with morphine sulfate (10 mg/kg) administered subcutaneously. Morphine significantly reduces the ultrasonic stress vocalizations induced by pain after laparotomy.

If neonates have to be euthanized, the AVMA [87] points out the resistance of newborns to hypoxia. Thus methods including inhalant agents

like isoflurane or CO_2 are inadvisable because it takes a long time for the young animal to die. More appropriate are injections of long-acting barbiturates (recommendation of the Swiss Federal Veterinary Office [88]) or physical methods for euthanasia such as decapitation with scissors, stunning, cervical dislocation or immersion of the neonates in liquid nitrogen [89, 90].

Monitoring anaesthesia

Because *hypothermia* may be the most important cause of anaesthetic death in mice, continuous monitoring of body temperature is mandatory and it is very important to prevent loss of body heat rather than to treat hypothermia.

For monitoring *respiratory function,* recording of the respiratory rate and pattern, colour of the muzzle and footpads for evidence of cyanosis, and pulse oximetry can be used [29, 52]. Marked depression of respiratory function may be treated by either removal of mucus or blood from the upper airway, extension of the head and neck and/or assisted ventilation by manually squeezing the chest between the finger and thumb at a rate of approximately 90 breaths/min [1]. In addition, administration of an α_2-adrenoceptor antagonist (atipamezole) or opioid receptor antagonist (naloxone) will help to reverse respiratory depression but will also decrease analgesia. In order to decrease mortality, oxygen supplementation via a face mask has been recommended for injectable and inhalation anaesthetic regimen.

Cardiovascular monitoring may include recording of the heart rate (ECG, pulse oximeter), pulse rate (apical pulse through the chest wall), arterial blood pressure and skin colour. However, many ECG monitors designed for use in humans may be unable to detect the low-amplitude ECG signals in mice. Additional fluid support is beneficial in terms of raising arterial blood pressure and s.c. or i.p. administration of warmed saline, lactated Ringer's solution or glucose 5% in normal saline solution at 0.5–1 mL per 30 g mouse or at 0.2 mL/h have been recommended [1, 8, 52]. Special care must be taken to avoid haemorrhagic shock, which may occur in mice after a rapid loss of as little as 0.5 mL of blood [1].

Assessment of *depth of anaesthesia* is based on a number of clinical signs such as loss of the righting reflex, recumbency and loss of purposeful movements, muscle relaxation, respiratory rate and loss of the tail-pinch, pinnae and pedal reflexes [3, 19, 50]. Of these reflexes, loss of the pedal reflex is the most reliable indication of the development of surgical anaesthesia in mice [3].

Analgesia

The detection of pain perception in individual rodents is based on subjective evaluation of behavioural and attitudinal changes as well as on objective analysis of physiological parameters [8, 91]. Behavioural signs indicative of pain perception include reluctance to move, abnormal posturing, facial expression, decreased appetite, vocalization, anxiety, apprehension, hypersensitivity, depression, aggression and polyphagia of bedding. The pain intensity of a given type of procedure and location of the lesion, based on the discomfort level of that procedure in larger companion animal species or in humans, should be considered for pain assessment in mice. This information may be also very helpful if investigators choose to design an analgesic protocol for a group of mice.

The recognition of attitudinal changes largely depends on pre-existing knowledge of the temperament and behaviour of each individual animal. Therefore, veterinary and animal husbandry technicians and research staff are crucial members of the veterinary care team.

In addition to behavioural changes physiological signs of pain perception include fluctuations in blood pressure, heart rate, respiratory rate, body temperature and food and water consumption resulting in changes in body weight.

These behavioural, attitudinal and physiological signs of pain perception are currently the best methods available to detect individual rodents that are in some degree of pain as well as to assess the success or failure of ongoing analgesic therapy. However, in mice (unlike rats) body weight and food and water consumption may not be greatly affected by invasive surgeries (e.g. thoracotomy), leading to the conclusion that it is doubtful that these parameters are useful as objective measures of pain [92].

For analgesic treatment in mice non-steroidal anti-inflammatory drugs (NSAIDs) and opioids are most commonly used (Table 5.4.4). While NSAIDs such as paracetamol (acetaminophen), aspirin, carprofen, diclofenac, flunixin, ibuprofen and piroxicam are primarily indicated for the treatment of low to moderate intensity inflammatory pain, opioids are used

TABLE 5.4.4: Analgesics used in mice

Drug	Dosage (mg/kg)	Route	Interval	Reference
Acetaminophen	300	i.p.		Jenkins [102]
Amitrytiline	5—10	i.p.	24 hourly	Abad et al. [94]
Aspirin	20	s.c.		Flecknell [103]
	120	p.o.	Once	Dobromylskyi et al. [104]
	120—300	p.o.		Jenkins [102]
Buprenorphine	0.01	s.c.		Liles and Flecknell [92]
	0.05	s.c.	8—12 hourly	Flecknell [1]
	0.05—0.10	s.c.	8—12 hourly	Dobromylskyi et al. [104]
	2.0	s.c.		Flecknell [34], Wiersema et al. [52]
	2.5	i.p.		Harvey and Walberg [105]
Butorphanol	0.05—5	s.c.		Jenkins [102]
	1—2	i.m., s.c.	4 hourly	Dobromylskyi et al. [104]
	1—5	s.c.	4 hourly	Flecknell [1]
	5.4	s.c.		Harvey and Walberg [105]
Carprofen	5	s.c., p.o.	Daily	Dobromylskyi et al. [104]
Codeine	20	s.c.	4 hourly	Flecknell [34], Jenkins [102]
	60—90	p.o.		Flecknell [34], Jenkins [102]
Diclofenac	8	p.o.	Daily	Dobromylskyi et al. [104]
Fentanyl	0.0125—1.0	i.p.		Thurmon et al. [106]
Flunixin	2.5	s.c.	12—24 hourly	Dobromylskyi et al. [104]
Ibuprofen	7.5	p.o.		Jenkins [102]
	30	p.o.	Daily	Dobromylskyi et al.[104]
Meperidine	20	s.c., i.m.	2—3 hourly	Jenkins [102]
	2—5	i.m., s.c.	2—4 hourly	Flecknell [1], Dobromylskyi et al. [104]
	10—20	s.c.	2—4 hourly	Flecknell[34,103]
Nalbuphine	1.0	s.c.		Liles and Flecknell [92]
	2—4	i.m.	4 hourly	Dobromylskyi et al. [104]
	4—8	i.p., s.c.		Flecknell [1]
Paracetamol	300	p.o.	4 hourly	Flecknell [34]
Pentazocine	5—10	s.c., i.m.	3—4 hourly	Flecknell [1], Dobromylskyi et al. [104]
	10	s.c.	3—4 hourly	Flecknell [34], Jenkins [102]
Pethidine (meperidine)	10—20	s.c., i.m.	2—3 hourly	Flecknell [1], Dobromylskyi et al. [104]
Phenacetin	200	p.o.	4 hourly	Flecknell [34]
Piroxicam	3	p.o.	Daily?	Dobromylskyi et al. [104]

i.p., intraperitoneally; i.m., intramuscularly; i.v., intravenously; s.c., subcutaneously; p.o., orally.

to combat moderate to severe pain. The opioid analgesics used in mice include the pure agonists morphine and meperidine, the partial agonist buprenorphine and the mixed agonist/antagonist pentazocine, nalbuphine and butorphanol. Opioid partial agonist and mixed agonist/antagonists may exhibit a ceiling effect such that further increases in dosage produce no further analgesia, but may escalate its adverse effects, such as respiratory depression. Administration of an opioid mixed agonist/antagonist to an animal initially receiving a potent opioid agonist may mitigate the remaining analgesic activity of the agonist. In rodents, buprenorphine was found to be 25–40 times more potent than morphine after parenteral (e.g. s.c.) injection [93]. Furthermore, buprenorphine may cause tolerance and positive Straub tail effect (tail elevated >45° to the horizontal; ED_{50} 0.06–0.75 mg/kg s.c.) in mice [93].

Tricyclic antidepressants (e.g. amitriptyline) are primarily indicated for the treatment of neuropathic pain. They may decrease behavioural signs of pain perception, including digital irritation and autotomy, for up to 2 weeks with no tolerance or overt adverse effects [94].

Analgesics may be administered to mice either parenterally or orally. Oral formulations are available for morphine, butorphanol, oxycodone, codeine, meperidine and pentazocine. However, when adding drugs to drinking water the risks are inaccurate dosing, lack of consumption due to palatability and degradation of the agent over time due to hydrolysis [8]. Furthermore, a marked first-pass metabolism may rapidly degrade oral opioids, thus making it difficult to achieve efficacious blood and tissue drug levels. Moreover, oral opioids are quite expensive and thus may not be cost-effective for 'herd' analgesic therapy.

Euthanasia

When euthanizing an animal the method used must be painless, provide rapid unconsciousness and death, require minimum restraint, avoid excitement, should be appropriate for the age, species and health of the animal, must minimize fear and psychological stress in the animal, must be reliable, reproducible, irreversible, simple to administer and safe for the operator [89].

Recognition and confirmation of death

The cessation of respiration and heartbeat and the absence of reflexes are good indicators of irreversible death in rodents. In addition, death may be confirmed by additional methods such as exsanguination, extraction of the heart or evisceration [90].

Embryos and neonates

If a fetus is removed from a deeply anaesthetized dam, then it may be killed by decapitation or removal of the heart [90]. Newborn rodents up to 10 days old may be killed by decapitation or concussion; CO_2 is *not* recommended [90].

Adult rodents

Recommended methods for euthanasia of adult rodents include the use of barbiturates, carbon dioxide, cervical dislocation, stunning and decapitation [43].

Barbiturates

Sodium pentobarbital, injected i.v. or i.p., is considered the agent of choice for most euthanasia. Barbiturates are safe and humane. Other barbiturates such as thiopental and thiamylal must be administered i.v. For nervous and intractable animals, sedation with xylazine or preinduction with ketamine plus xylazine might be appropriate [43].

Carbon dioxide

Carbon dioxide is a well-accepted, commonly used gas for euthanasia of laboratory animals but *not* neonates (see above). Inhalation of at least 70% CO_2 in oxygen has a rapid anaesthetic effect that proceeds to respiratory arrest and death if exposure is prolonged [90]. Animals become unconscious within 45–60 s but should remain in the chamber for at least 5–6 min and then examined closely to determine that all vital signs have ceased [43].

Cervical dislocation

This technique consists of a separation of the skull and brain from the spinal cord by anteriorly directed pressure applied to the base of the skull. Cervical dislocation causes almost immediate unconsciousness because of cerebral shock. All voluntary motor and sensory functions cease because of damage to the spinal cord. However, considerable involuntary muscle activity may occur [43].

Stunning

A sharp blow delivered to the central skull bones must be of sufficient force to produce massive haemorrhage and thus immediate depression of the central nervous system. When this is done properly, unconsciousness is immediate. Stunning should be used only by properly trained persons and when other means are inappropriate or unavailable. After stunning, the animal must be killed immediately by another procedure, such as exsanguination or decapitation [43].

Decapitation

Decapitation with a guillotine is used primarily when pharmacological agents and CO_2 are contraindicated (e.g. pharmacological and biochemical studies). This method causes rapid death if properly performed. The animal needs to be properly restrained, and its head must be completely severed from its body at the atlanto-occipital joint. The guillotine must be kept in good operating condition, and the blade must be sharp [43].

Other acceptable methods for euthanasia in rodents include the use of inhalation anaesthetics (halothane, enflurane, isoflurane), T-61 (only to be injected i.v.) and rapid freezing in liquid nitrogen (only for small neonates; [90]).

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Imaging the Laboratory Mouse *in vivo*

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Introduction

Non-invasive medical imaging technologies came to prevalence in the 1970s and 1980s, coming into widespread use as diagnostic tools. Imaging modalities such as MRI (magnetic resonance imaging), CT (computed tomography), PET (positron emission tomography), SPECT (single photon emission tomography) and ultrasound were developed on the basis of earlier technologies that were used to enable the first non-invasive glimpses inside the living body. Today these imaging modalities are used as standards for patient care across all of the major human diseases.

More recently, all of the above techniques, as well as an increasing number of more novel imaging technologies, have been adapted or developed specifically for mouse imaging. As

such, the resulting technology names are commonly differentiated slightly from the parental technologies (e.g. 'microCT', 'microPET' or 'small animal PET'). For the purposes of this review, however, these derivative terms will not be used. As the laboratory mouse has become one of the most impactful drivers of medical research discovery, imaging technology developers have successfully met the challenge of adapting their technologies to enable the higher resolution and sensitivity needed in imaging subjects 3000 times smaller than their human counterparts. These advancements are paving the way for image-based improvement in quantitative assessment of disease progression and prediction of response to therapies in mouse models. This chapter explores the why, what and how of imaging in laboratory mice, ending with a glimpse at the exciting future of imaging in mouse models.

Mouse imaging: why the fuss?

Clinical translation

One of the most powerful aspects of imaging in mice is the ability for clinical translation of the methods. In a mouse imaging protocol, the mouse acts as a model for the human body or a human disease condition. The interface between basic medical science research and medical advances for human patients becomes more seamless when translating methods that have become standards in clinical medicine. Technologies that include therapies, and biomarkers and probes for disease progression and response to therapy, can be developed and optimized through testing in mouse imaging studies before being applied in clinical trials. In other words, imaging in the laboratory mouse can be used to determine when and how to apply new therapies, or new imaging technologies and biomarkers themselves, in human patients.

Unique end-points

While medical imaging initially evolved as a diagnostic tool through *anatomical* imaging, today a broad range of imaging techniques, tools and protocols exist for obtaining spatially resolved *physiological* information. Commonly termed *functional imaging*, this has led to increased use of imaging to provide physiological biomarkers [1–3] that otherwise cannot be obtained non-invasively, or not as easily or repeatedly, in living subjects. Increasing validation of molecular imaging protocols [4, 5] that provide information at the molecular and cellular levels is providing even more unique image-based end-points, some of which are actively being translated for clinical use.

Improved accuracy and prediction

Non-invasive imaging provides an inherent opportunity for repeated longitudinal imaging in laboratory mice. This can enable more accurate data from studies compared with traditional, invasive procedures that cannot be repeated as extensively, or that require serial sacrifice cohorts. Since imaging technologies provide an opportunity for multiple anatomical, functional and molecular end-points from a single imaging session, each animal can provide richer and more powerful data to improve the potential of the preclinical study to predict clinical outcome.

Mouse imaging: the technology who's who

Mouse imaging technologies
MRI

MRI is based on the phenomenon of nuclear magnetic resonance (NMR), whereby certain atomic nuclei, when placed in a magnetic field, can be perturbed by radiofrequency (RF) electromagnetic waves. This perturbation results in magnetic resonance of the individual nuclei, which can be detected through RF receivers. Initially used to provide chemical information, the use of magnetic field gradients enabled determination of the position of resonating nuclei, and thereby reconstruction of a three-dimensional image [6–8], the resolution of which can be as good as around 10 µm.

Most commonly, the nuclei being imaged are hydrogen nuclei in water (protons or ^{1}H). Since the specific environment of the nuclei affects its magnetic resonance signature, an MRI experiment can be designed to differentiate water in different tissues or water in normal vs diseased tissue. Therefore, ^{1}H MRI can result not only in a three-dimensional image of water distribution, but also in an image that clearly delineates different tissues and diseased tissue (Figure 5.5.1). This provides an opportunity to study mouse anatomy, tissue-based disease progression and treatment response in mouse models.

Since the magnetic resonance properties of nuclei are affected by a variety of physiological parameters, MRI can be used to spatially encode

Figure 5.5.1 Magnetic resonance image of an adrenal tumour in a PTEN$^{-/-}$ mouse. Proton MRI enables high-resolution delineation of tissues and disease (e.g. a tumour). Different tissues have different MR 'signatures' as indicated by the different shades of grey (intensity) in this mouse image.

a variety of tissue properties including water content, cellular density, iron content, oxygenation, metabolite concentration and elasticity. MRI probes [9] such as gadolinium, iron oxide, manganese, nitrous oxide and molecules containing fluorine-19 (^{19}F) can additionally be used to improve diagnostic sensitivity or provide unique biomarkers for properties including blood flow, blood volume and tissue perfusion.

While MRI is one of the most flexible imaging modalities for the above reasons, a limitation is its low sensitivity compared to some of the other established imaging modalities. This limits throughput, affecting the feasibility of a given protocol type or study design, or the sensitivity and accuracy of the end-points.

PET

A PET image is a three-dimensional map of a positron-emitting radionuclide, introduced systemically as a tracer in a living subject. The inherent process of positron–electron annihilation in these radionuclides results in two gamma photons originating from the event site and

travelling in opposite directions. Certain crystals can detect the gamma energies and through highly time-resolved coincident counting of gamma photon pairs, a three-dimensional image of the spatial distribution of the radionuclide in the living subject can be obtained [10].

While the spatial resolution of PET is limited to the millimetre level, the power of this imaging modality is in the broad variety of tracers that can be used. Any molecule that can be chemically labelled by a PET isotope (e.g. ^{18}F, ^{13}N, ^{14}C, ^{15}O, ^{64}Cu, ^{68}Ga, ^{124}I, ^{89}Zr) can be used as a PET tracer. This has enabled the determination of uptake and biodistribution of exogenous and endogenous molecules for a variety of purposes. It has also enabled a rich variety of physiological, molecular and cellular PET-based end-points through the use of tracers linked to specific biological processes. As common examples, labelling of metabolites can provide a spatially resolved readout of tissue metabolism [11] and labelling of nucleosides can provide a spatially resolved readout of cell division [12].

While translation of PET technology, initially used in human patients, to mice has been highly challenging because of the limited resolution, recent successes have led to a number of state-of-the-art dedicated mouse PET scanners based on a variety of detector technologies and strategies becoming available today. These systems have launched a new era of mouse PET imaging research. Mouse models have greatly facilitated the development of new PET tracers, many of which are now in clinical development [13, 14].

SPECT

Similarly to PET, SPECT imaging provides a three-dimensional map of the spatial distribution of a radionuclide. SPECT detectors can detect a range of photon energies, allowing a large diversity of radionuclides to be used for SPECT imaging, many of which have seen long and widespread use in medicine for other purposes (e.g. ^{99m}Tc, ^{67}Ga, ^{111}In, ^{123}I and ^{125}I, ^{192}Ir, ^{201}Tl). The historical applications and availability of many of these radionuclides provide several advantages over PET, where production is typically more difficult and more expensive and availability is more limited. The recent introduction of dedicated mouse SPECT scanners is

facilitating development of new SPECT-based disease biomarkers [15] by the use of mouse models [16]. The higher resolution achievable with mouse SPECT scanners makes it generally more suited than PET to imaging of biodistribution in mice. For example, SPECT imaging of iodinated molecules such as antibodies has become prevalent for testing whole-body therapeutic pharmacodynamics *in vivo* [17, 18].

CT

CT is an X-ray based imaging technology, whereby highly spatially resolved (to ~10 μm) three-dimensional images that represent tissue density are generated by rotating an X-ray source ('tube') and detector in parallel around a subject. Although the properties of the X-rays produced can be adjusted to provide different windows for contrast and tissue discrimination, the relatively minor differences in X-ray opacities of different tissues generally limit the soft tissue contrast accessible through CT imaging. Contrast enhancement through the use of a variety of commercially available contrast agents (commonly iodine-containing) is one method that has been used to increase the soft tissue imaging ability of CT [19]. Despite these endeavours, CT is predominantly used for imaging bone and airways (where the contrast with soft tissue is very high), especially in mouse models where imaging systems and contrast agents are generally not as sophisticated as those developed for clinical use. One major advantage provided by modern CT is the speed of image acquisition, albeit generally at the expense of tissue radiation dose. The speed and relative simplicity of skeletal imaging by CT has led to a renaissance of the modality through integration with PET and SPECT in dual-modality systems. In these systems, CT provides a rapid anatomical image overlay that can be used to help overcome the resolution and contrast limitations of PET and SPECT, and aid in image interpretation. Dual-modality PET/CT and SPECT/CT systems have become clinical standards, and are becoming standards in mouse imaging as well.

Optical imaging

The term 'optical imaging' can be a confusing one since it technically pertains to a variety of imaging technologies, including traditional light microscopy. In live mouse imaging, however, optical imaging generally refers to *in vivo* fluorescence (FLI) or bioluminescence imaging (BLI). Unlike the modalities described so far, BLI and FLI became prominent as technologies developed more specifically for mouse imaging. Part of the reason for this was their reliance on the use of genetically manipulated cells that express bioluminescent proteins (light emission after systemic introduction of a substrate such as luciferin) or fluorescent proteins (light emission after excitation at a specific light wavelength). Additionally, the poor depth penetration of light has precluded widespread use of the technology in species larger than mice. More recently, the development of exogenous fluorescent probes has led to efforts for clinical translation [20, 21]. An increasing variety of probes that can be configured to target specific cells [22, 23] or lesions [24–26] or activate after specific molecular events [27, 28] has led to development of fluorescent imaging systems based on a variety of illumination, detection and image reconstruction technologies, including small fluorescence imaging probes that could be introduced systemically or during surgeries for clinical use [29–31]. While both BLI and FLI have largely been restricted to animal imaging (particularly the mouse), they have enabled many medical advancements through their unique ability to study molecular and cellular level events [32–34] and also transgenic mice manipulated to express light-emitting proteins dependent on molecular events [35–36].

Ultrasound

Ultrasound is an extremely prevalent and relatively low-cost clinical imaging modality that has seen recent translation for dedicated mouse imaging systems in which resolution to around 30 μm is possible [37]. Image generation occurs via introduction of high frequency sound waves into the subject that are then detected after reflection at tissue boundaries. Traditionally a two-dimensional imaging modality, the latest ultrasound technologies enable three-dimensional images. The power of ultrasound imaging in mice has so far been coupled to imaging of blood flow in a variety of diseases through power Doppler

ultrasound [38, 39], and imaging of specific cells or molecular processes by use of targeted microbubble-based ultrasound contrast agents [40].

Mouse handling, preparation and biosecurity for *in vivo* imaging

Mouse handling and preparation is one of the most important aspects of mouse imaging, as it has the ability to affect both the quality and reproducibility of images, as well as the efficiency of image generation. Yet it is also commonly overlooked or underestimated as an influencing variable. Mouse imaging requires significant financial and personnel investment for a preclinical imaging laboratory, but the full gain from the technology cannot be realized unless the investment in animal handling and preparation is sufficient. Simple handling itself has the ability to determine the level of stress a mouse experiences. This may affect its physiology and toleration of the imaging procedure. It will also drive the efficiency and throughput of the procedure which, in turn, sets limits on the design, size and scope of each study. Biosecurity for mice entering imaging facilities is an additional critical concern, particularly given the prevalence of immunocompromised mouse strains for medical research.

Animal positioning

For all imaging modalities, basic animal positioning drives the sensitivity and reproducibility of the data. Positioning directly affects sensitivity of MRI (through RF coil tuning and shimming), PET/SPECT (through position of the anatomy of interest relative to the centre of the field of view, where sensitivity is highest) and optical imaging (where the position of the anatomy of interest relative to other tissues can result in differential light scattering and depth of tissue penetration). Positioning indirectly affects the efficiency and accuracy of image segmentation and ease of interpretation during image reading across all modalities. The most consistent animal positioning possible is the most basic yet critical factor in ensuring quality datasets and maximization of study statistical power. For this reason, a variety of devices from basic mouse 'sleds' [41] and body

moulds [42, 43] to sophisticated stereotactic multimodality compatible positioning devices [44] are now commercially available. Figure 5.5.2 shows examples of such devices.

Anaesthesia

Mouse anaesthesia is discussed in detail in Chapter 5.4. In the context of imaging, anaesthesia is required in the vast majority of procedures [45], although devices for conscious imaging have been successfully demonstrated [46, 47]. Commonly, gas anaesthesia is used, mostly in the form of halogenated ethers such as isoflurane or sevoflurane (which are mixed with air or oxygen). These anaesthetics are used for their convenience, tolerance, adjustability and minimization of depth of effect. For both, there are few known side effects and they are not metabolized. Since they are relatively insoluble in blood, recovery is typically rapid (within minutes of the gas being turned off). The ability to adjust the dose in real time is also a major advantage, especially during longer imaging procedures, or in cases where specific mice in a study are experiencing adverse clinical symptoms, due to disease progression or a treatment.

However, injectable anaesthetics are also commonly used. Most commonly, the dissociative anaesthetic ketamine is used in combination with the sedative/relaxant xylazine. Injectable anaesthetics have the advantage of reducing exposure to laboratory personnel, and they can be combined with other injectables to increase efficiency. Injectable anaesthetics can also result in more relaxed breathing, compared with isoflurane, which may be critical during imaging examinations in the abdomen or thorax.

Physiological monitoring

Physiological monitoring is an important aspect of mouse imaging, both to ensure animal health during the imaging examination, as well as to characterize and control physiology during the procedure. Common needs for mouse physiological monitoring include body temperature, respiration and the cardiac cycle. Robust measurement of these parameters is typically challenging in the mouse, mainly due to the animal's size. Fortunately, there are now a variety
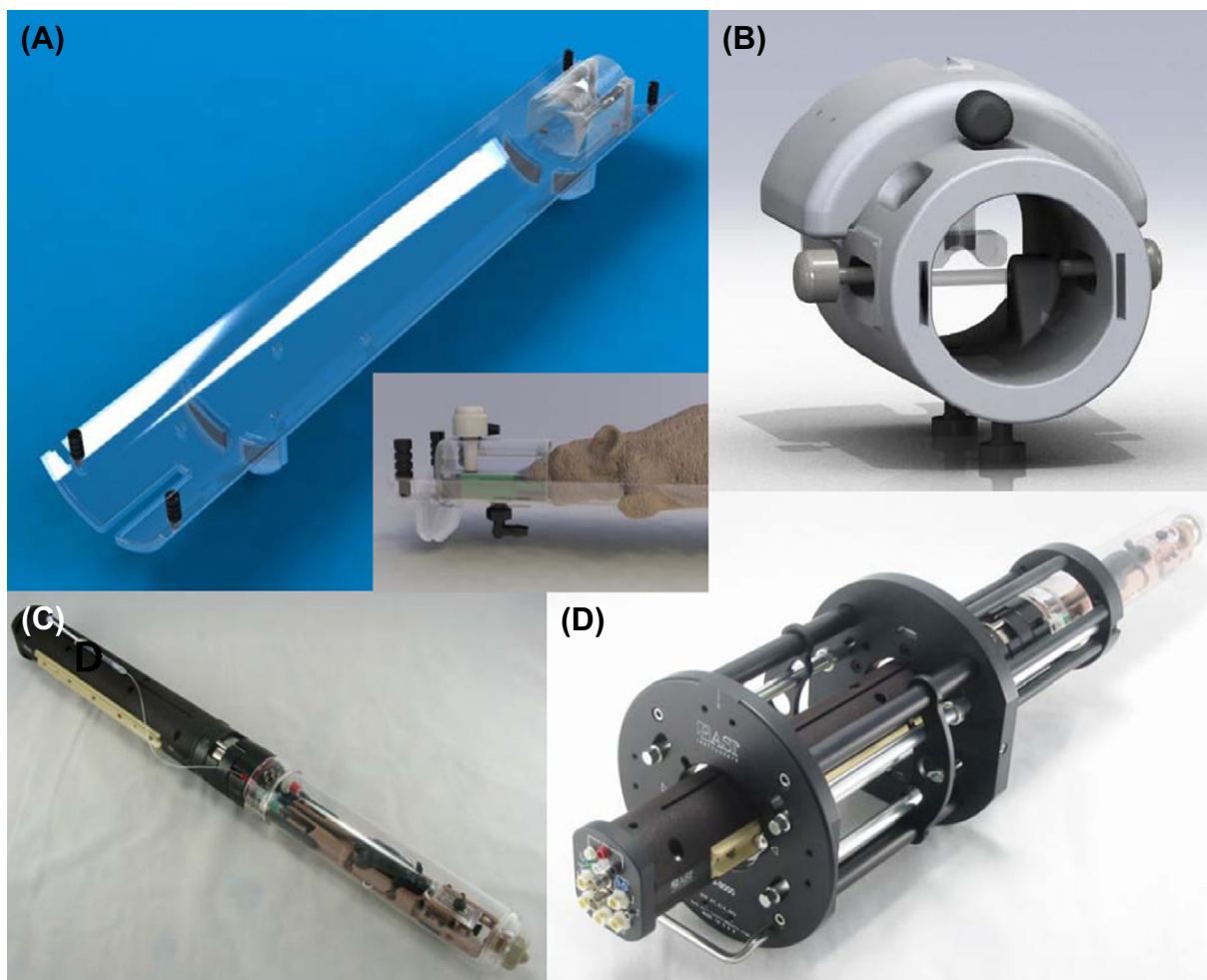
Figure 5.5.2 Examples of animal positioning and management devices. (A) A modular 'sled' device incorporating a head restraint system (m2m Imaging: www.m2mimaging.com). (B) An RF volume coil with integrated stereotactic functionality that can be used for awake animal brain imaging (EKAM Imaging: www.ekamimaging.com). (C) and (D) An advanced animal management system that incorporates modular technology for (C) the animal bed and (D) a mounting device that holds an RF volume coil and localizes it in the axial direction (ASI Instruments: www.asi-instruments.com).

of commercially available sensors and complete integrated systems that facilitate this. Many of these are compatible with multiple imaging modalities across the major manufacturers, and incorporate software to record multiple physiological parameters in parallel. Software can also be used to provide signals that can be sent to the scanners to trigger image data acquisition with the respiratory and/or cardiac cycles (termed image 'gating').

Imaging facility biosecurity

As the laboratory mouse has become one of the cornerstones of biomedical research, attention to facility biosecurity has also increased. This is particularly relevant with the increased use of immunocompromised mouse strains (e.g. in oncology research). The necessity for multiple animals from multiple studies to enter and exit imaging laboratories and contact imaging equipment makes it difficult or impossible to maintain the same level of biosecurity in imaging laboratories as in animal housing. If the laboratory is not well designed and regulated, imaging biosecurity can become such a weak point that an entire animal vivarium could become at risk. Typically, a combination of rigorous personnel gowning requirements, entry/exit procedures, HEPA filtering, appropriate disinfection of imaging

equipment and appropriate use of disposable supplies, can sufficiently reduce the biosecurity risks in mouse imaging laboratories.

Facilitating mouse modelling of human disease and therapeutic development through imaging

The laboratory mouse has provided models for almost all the major diseases known to humanity. In parallel, imaging technology has provided many carefully designed and validated end-points (biomarkers), which have facilitated the successful use of these models. Imaging-based biomarkers include any anatomical, physiological or molecular parameters detectable by imaging methods that are used to establish the presence and/or severity of disease [48]. The next few years will better define specific cases in which imaging protocols will generate surrogate markers. Surrogate markers are biomarkers that can be used as true substitutes for clinically meaningful disease end-points [48].

Anatomical imaging

Anatomical imaging refers to the use of imaging to define diseased or normal tissues based on volumetric or surface area extent. MRI has been classically used to generate high resolution, high tissue contrast images of the body *in vivo*. MRI-based atlases of the human body have helped build a foundation for anatomical medicine [49]. Similar MRI atlases now exist for the mouse [50], as it has become such a prevalent species in medical research. These atlases facilitate anatomical references for use in mouse surgeries and necropsies. However, they are also importantly used as references for functional images [51], whereby functional images can be coregistered with the MRI mouse atlas image data, in order to interpret functional signals in a tissue- or sub-tissue-specific manner.

The classic example for disease-based anatomical imaging is the use of imaging modalities such as MRI [52, 53] or CT [54] to define a tumour volume. Diseases that change image-sensitive tissue properties macroscopically can be tracked through volumetric biomarkers. Optical imaging methods can also be used in an anatomical sense. For example, detection of light from tumour cells engineered to express luciferase constitutively enables light-based detection and determination of tumour volume. This provides a convenient and accurate means of tracking tumours in deep tissue models of metastasis [55–57], or in which tumours are implanted in mice in their tissues of origin (orthotopic models) [58, 59].

Anatomical imaging can also provide a convenient means for assessing structural changes in diseases that have a bone component, including bone metastasis [60] and rheumatoid arthritis [61]. Mouse imaging has provided a powerful means for testing these approaches, and for testing therapeutics that are designed to inhibit the adverse bone changes. Figure 5.5.3 demonstrates this strategy in a mouse model (PC-3M-luc intracardiac injection model) of bone metastasis. CT imaging provides characterization of bone loss in the tibiae. The success of a treatment designed to inhibit this bone loss is demonstrated through imaging in the mouse model. Similarly, CT can be used to characterize bone erosion in mouse models of rheumatoid arthritis and to determine the effectiveness of therapies designed to inhibit this aspect of the disease (Figure 5.5.3).

Use of mouse models to provide functional imaging biomarkers

Functional imaging refers to imaging-based biomarkers that represent non-anatomical physiological parameters of tissue. As increasing numbers of unique image-based biomarkers are being demonstrated to couple with disease mechanisms, our ability to utilize more definitive tests for new therapeutics has increased. Mouse imaging has become the cornerstone for development of these image-based end-points. This includes validation of the biomarker, which broadly means proof in one or more
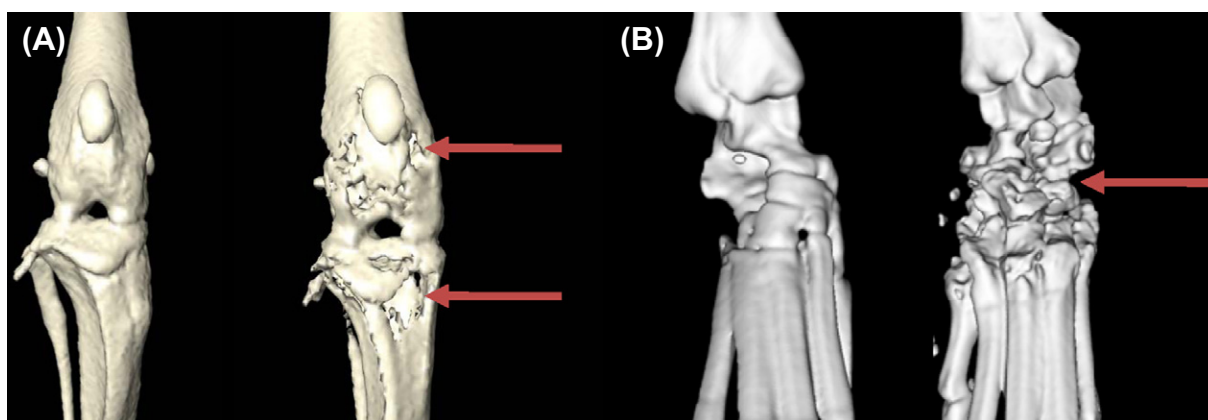
Figure 5.5.3 Anatomical CT images of (A) treated (left) and control (right) mouse hindlimbs in a peritibial implant model of bone metastasis and (B) naive (left) and diseased (right) rat hind paws in a rheumatoid arthritis model. The arrows indicate bone lesions where the extent and severity of erosion and surface roughening can be assessed at high three-dimensional resolution using CT.

circumstances that the biomarker is correlated with the biology it is intended to measure. This is typically obtained through parallel traditional gold standard measurements (e.g. histopathology). Importantly, the situations in which this correlation becomes uncoupled should also be determined. Once validated, the same mouse imaging methods can be used for testing therapeutics that target the underlying biology with which the image biomarker is correlated. This approach is depicted schematically in Figure 5.5.4. As medical imaging research delivers a deeper and broader array of validated image biomarkers and probes, disease researchers can increasingly tap the power of this approach, which can be broken down into three components:

1. A target biology or mechanism hypothesized to be critical to the disease progression
2. Choice of an appropriate mouse disease model that reciprocates the specific biology or mechanism through disease progression in the model
3. Choice of an imaging biomarker or probe that provides an end-point that can be used as a metric for the presence, magnitude, modulation or severity of the target biology or mechanism.

The above strategy lays the foundation for testing therapies designed to target the biology or mechanism, providing:

1. A screening assay for new therapies in the earlier stages of research
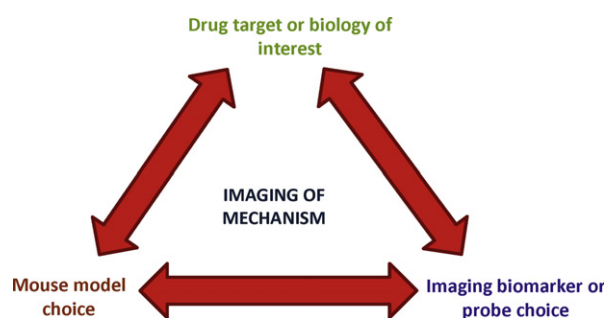
Figure 5.5.4 Schematic depiction of a broad strategy for imaging a disease or drug mechanism. The concept begins with a drug target or disease pathway biology under study (the question). For example, the mechanistic biology hypothesized to drive the disease or its response to therapy. Choice of (i) a relevant mouse model that incorporates this biology and (ii) an imaging biomarker or probe that has been demonstrated to generate end-points that correlate with modulation of the target or disease severity enables effective imaging of mechanism. This strategy can be used to provide information that is more predictive of outcome than traditional measures that do not access disease and/or drug mechanism as effectively.

2. A checkpoint for later stage therapies that can be directly compared to model therapies or standards of care
3. A method for testing hypotheses that can be used to optimize initial testing of the therapy in human patients
4. A method for addressing new unknowns or questions generated through initial testing in human patients.

Functional imaging in mouse models of human disease

Oncology

DCE MRI

Tissue permeability, vascular density and blood flow can be measured in mice and human patients through dynamic contrast-enhanced (DCE) MRI [62-64]. Translation of the clinically standard DCE MRI methodology to mice has posed many challenges, including how to introduce and control the rapid bolus of the contrast agent and how to characterize its vascular input function, both critical to the end-point determination. Preclinical MRI systems are also typically not as well equipped as their clinical counterparts with sophisticated imaging protocols and hardware that enhance the rapid imaging associated with DCE MRI. Despite these challenges, DCE MRI has been broadly and successfully applied in mouse models of human cancer, leading to facilitated development of a number of successful and promising antiangiogenic and antivascular therapies against human cancers [65-68].

FDG AND FLT PET

PET imaging has provided oncology research in particular with a new suite of tracers that enable imaging of tissue function, many of which were initially tested and developed through mouse imaging. ^{18}F-FDG [11] and ^{18}F-FLT [12] in particular have risen to clinical prominence through enabling of quantitative measures of tissue metabolism and cellular proliferation, respectively. Mouse PET imaging today is an essential part of drug development [69], oncology clinical trial design and optimization. There are an increasing number of examples of mouse PET imaging work that have been used to justify or drive the use of similar imaging in clinical trials with positive outcomes. For example, early testing of ^{18}F-FDG PET in the melanoma mouse tumour model demonstrated that ^{18}F-FDG uptake provided a robust biomarker for tumour progression and response to the B-Raf enzyme inhibitor PLX4032 (RG7204, vemurafenib). A subsequent clinical trial showed substantial inhibition of ^{18}F-FDG uptake after PLX4032 treatment in patients with advanced metastatic disease [70]. ^{18}F-FDG has also become a standard in the assessment of therapeutic response in gastrointenstinal stromal tumours (GIST) due to robustness of response in clinical trials [71]. ^{18}F-FDG PET has therefore seen increased prominence in mouse models of GIST [72, 73] facilitating development of future improved therapies against this disease.

DIFFUSION MRI

Diffusion MRI is an MRI-based technique that provides a measurement of the translational mobility of water. In tissue, the apparent diffusion coefficient (ADC) can be measured through diffusion MRI and is commonly used as a biomarker of response of tumours to anticancer therapies [74]. Generally, the driving hypothesis for this is that successful anticancer therapies lead to killing of tumour cells, and subsequently reduced tumour cell density and increased average water mobility and ADC. The earliest demonstrations of this approach by Ross and co-workers occurred in mouse and rat tumour models [75, 76], leading to the biomarker being used successfully in clinical trials [77-81]. Importantly, ADC has been shown to provide significantly earlier prediction of treatment success or failure, and thereby patient outcome, than currently standard measures.

Inflammatory disease

Medical imaging is playing an increasing role in assessment of inflammatory diseases. Early applications in rheumatoid arthritis (RA) have been centred in anatomical imaging such as CT of bone erosion (see above) and MRI-based assessment of joint anatomical changes [82, 83] and bone marrow oedema [84]. However, more promising image-based assessments are centred on characterization of the earlier mechanistic aspects of inflammation in these diseases. Clinical prognosis and treatment monitoring in inflammation diseases such as RA have been challenging. Commonly, patient pain is used as a primary clinical end-point for RA. However, pain does not correlate generally with disease severity or disease progression [85]. Imaging in mouse models is paving the way for the development and validation of new, more predictive biomarkers in RA and other inflammation diseases such as inflammatory bowel disease [86-88] and pulmonary fibrosis [89, 90].

OPTICAL PROBE IMAGING OF INFLAMMATION

New fluorescent probes that are activated by molecules mechanistically specific to early inflammation are showing promise as biomarkers that better reflect disease mechanism than traditional measures. Figure 5.5.5 shows an example in the mouse sponge granuloma model of acute inflammation. In this model, implanted polymeric sponges can be presoaked in inflammatory agents, or PBS as a control, then implanted subcutaneously. Fluorescent probes activated by cathepsins and matrix metalloproteinases (MMPs) (Prosense and MMP Sense, respectively, PerkinElmer, Boston, MA), both upregulated during acute inflammation, provide optical signals that correlate with inflammation. This was demonstrated through comparison of sponges presoaked with PBS (control) versus complete Freund's adjuvant (CFA; diseased) (Figure 5.5.5).

The same optical probes have been shown in a mouse model of RA to enable improved correlation with histopathology and more robust assessment of targeted therapies such as disease-modifying anti-rheumatic drugs (DMARDs), compared with traditional measures including paw swelling and clinical score [28]. Work like this will drive future RA drug development standards, as well as more predictive means for screening new therapies in preclinical testing.

Activatable fluorescent probes have also been demonstrated in mouse models of inflammatory bowel disease [91] and pulmonary fibrosis [92]. Importantly, these models are not easily assessed by traditional end-points, relying largely on serial
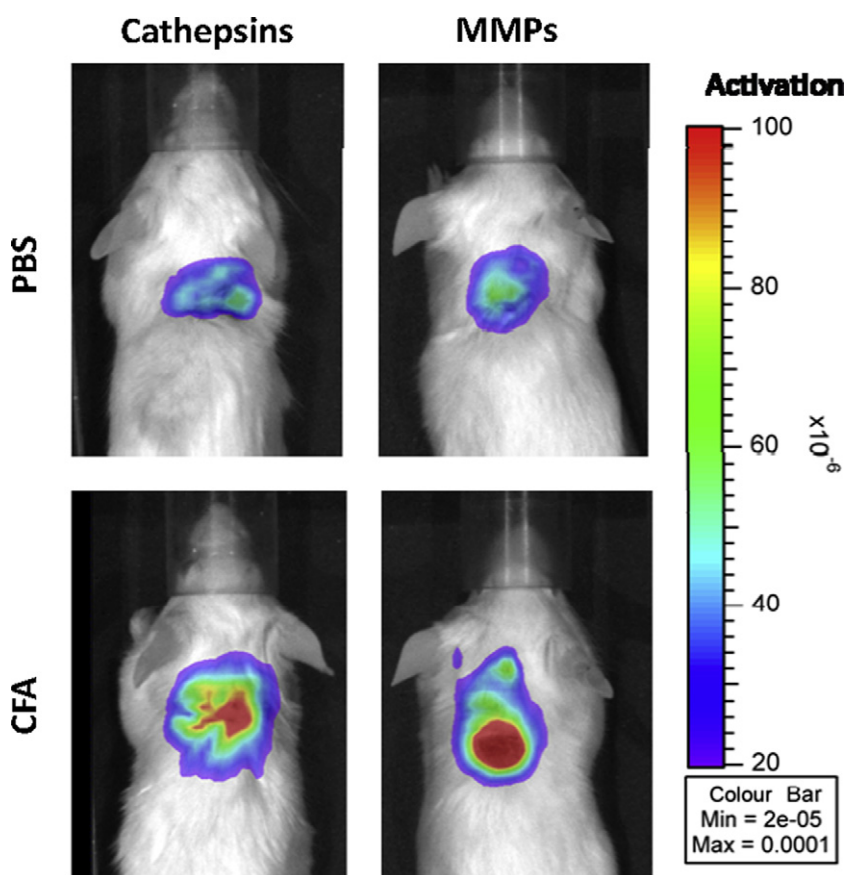
Figure 5.5.5 Fluorescence images of activatable probes in a subcutaneous sponge granuloma model of acute inflammation. The model involves implant of presoaked polymer sponges subcutaneously in mice (control: PBS; diseased: complete Freund's adjuvant, CFA). Probes activated by both cathepsins (left; ProSense, PerkinElmer, Boston, MA) and matrix metalloproteinases (MMPs, right; MMP Sense, PerkinElmer, Boston, MA) were sensitive to acute inflammation (induced by CFA), compared with controls. Both cathepsin and MMP activities are commonly upregulated in acute inflammatory disease. Activatable optical probes can therefore be used to detect and track acute inflammation.
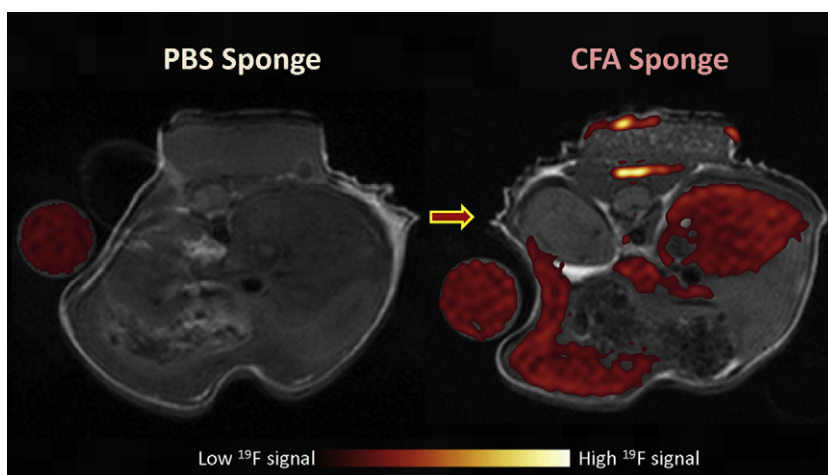
Figure 5.5.6 Fluorine-19 MR images displayed as ^{19}F signal (on 'hot' colour map) overlaid on ^{1}H T2-weighted anatomical images (greyscale). The images were acquired in the mouse sponge granuloma model of acute inflammation. Briefly, polymer sponges were presoaked in PBS (control) or CFA (inflammatory disease) and implanted subcutaneously on the dorsal aspect of mice. A ^{19}F nanoemulsion (Cell Sense, Celsense, Inc., Pittsburgh, PA, www.celsense.com) selectively taken up by macrophages was then administered intravenously and the mice were imaged 2 h later. The high ^{19}F signal around the CFA-soaked sponge (right), compared with no detectable signal in the control sponge (left) indicates accumulation of inflammatory cells. Activation of inflammatory cells in the liver and spleen was also observed. An external tube containing the ^{19}F reagent acts as a signal reference standard.

sacrifice methods for measurement of disease progression and response to therapy. Imaging probe-based methods may therefore enable more mechanistically relevant end-points and improved assessment of novel therapies against these diseases. Successful testing through mouse imaging may pave the way for clinical translation of these optical probe-based imaging approaches.

INFLAMMATORY CELL TRACKING

Cell tracking has become an important area of research in inflammation disease because of the critical role of inflammatory cells such as neutrophils, macrophages and monocytes in diseases such as RA. MRI has emerged as the leading modality for cell tracking due to successful combination of its relatively high resolution with the increased sensitivity that can be provided through MRI contrast agents. The most promising approaches for cell tracking have been through labelling of cells. For example, disease or therapeutically relevant cells can be labelled by iron oxide nanoparticles [93–95], then introduced into mice and tracked through the body. Single cells have been imaged using this approach [96]. ^{19}F-MRI incorporating fluorinated nanoparticles can also be used in a similar approach [97–104]. Commercially available ^{19}F nanoemulsions (e.g. Cell Sense,

Celsense Inc., Pittsburgh, PA; www.celsense.com) have also been successfully used via systemic delivery and preferential uptake by endogenous inflammatory cells [105]. Figure 5.5.6 demonstrates this approach in the sponge granuloma model. Resolved hypointense signals around the implanted sponge presoaked in CFA indicate acutely activated macrophage presence and accumulation over time.

IMAGING OF BIODISTRIBUTION

An important aspect of drug development is determination of the kinetics and pattern of drug accumulation. As the drug discovery paradigm continues to shift away from non-specific therapies toward targeted therapies, testing of successful targeting through imaging is becoming an increasingly powerful tool in drug research. The laboratory mouse has been used successfully for this purpose across most of the major imaging modalities, including MRI [106], PET [107], SPECT [108], ultrasound [109] and optical imaging [110, 111]. Antibody-based therapeutics are a major area of current focus for targeted therapies. Tagging of antibodies using radionuclides [112] and fluorophores [113, 114] provides a convenient and powerful means for testing biodistribution including tissue half-lives, clearance pathways and degree

of selective targeting of specific tissues or pathologies.

Spectroscopy and spectroscopic imaging

NMR offers the ability to distinguish nuclei that exist in differing chemical environments. For example, water protons resonate at a slightly different frequency from macromolecular protons, and macromolecular protons in different chemical groups may also resonate at different frequencies. This difference, known as *chemical shift*, has led to a variety of clinical applications for proton spectroscopy through quantification of levels of metabolites that are signatures of disease presence, progression and response to therapy (magnetic resonance spectroscopy, MRS). MRI additionally provides the ability to spatially resolve nuclei in a three-dimensional manner (spectroscopic imaging). Spectroscopy research in mouse models [115, 116] is helping to drive development of new approaches.

Proton MRS is most prevalent due to the natural abundance of ^{1}H (the MR-compatible hydrogen isotope) and the high sensitivity for this nucleus, compared with other nuclei. Of the other nuclei, carbon-13 (^{13}C) has been used extensively in MRS, but creates major challenges due to its low natural abundance and sensitivity (MR signal per nucleus). For this reason, metabolites purposely labelled with ^{13}C are used in measuring a variety of aspects of tissue metabolism. However, the very low sensitivity of ^{13}C MR limits the power of the technique and has largely precluded the ability to *image* ^{13}C metabolite distribution since imaging inherently reduces the sensitivity of the experiment even further. However, recent approaches that leverage a technique called *hyperpolarization* to artificially increase the MR signal per ^{13}C nucleus have led to a promising area of imaging technology expansion [117-119]. Development of this technology has largely occurred through mouse imaging, which is paving the way for future clinical use of this technology. Importantly, broad

validation and use of ^{13}C MRI would provide a means to measure the concentrations and tissue distributions of a broad array of metabolites. This would provide new ways in which to advance knowledge of disease at a mechanistic level, as well as facilitating development of therapeutic strategies that target specific metabolic pathways and mechanisms.

The future of mouse imaging

Mouse imaging has become so prominent and relied on in medical science today that significant effort is being applied in a variety of areas to either increase imaging applications (to new biology) or improve the sensitivity, speed, resolution or specificity of existing imaging applications. The following section provides a glimpse at some of the more prevalent areas of current mouse imaging technology development.

High-throughput mouse imaging

Mouse imaging (like human imaging) is traditionally relatively slow. For imaging modalities such as PET and MRI, 15-30 min sessions per subject tend to be the standard, leading to limitations on study size and scope. A typical preclinical drug test, for example, would require four test groups (control, positive control and two dosage levels for the test agent) and approximately 8-12 mice per group. This equates to a study size of 32-48 mice and total image acquisition time of 12-24 h. Although in reality these times depend on the model, effect size expected and the sensitivity of the end-point being used, they provide a frame of reference that highlights the need for efficiency gain in imaging. Adoption of mouse imaging by the pharmaceutical industry and supporting contract research organizations (CROs) is pushing the boundaries of mouse imaging throughput, while ensuring that end-point validation is not compromised. However, current research is investigating more novel approaches to improving mouse imaging throughput [120], including mouse handling and positioning devices (see Figure 5.5.2

[43, 44]), multiple mouse imaging [121-125] and rapid imaging techniques [126, 127].

Imaging probe development

Some examples of the more promising imaging probes were discussed previously. However, the increasing research effort and availability of imaging probes across all modalities is worth highlighting. While some imaging modalities can be executed successfully based on natural image contrast (e.g. MRI, CT), exogenously introduced or endogenously expressed imaging probes can be viewed as accessories that provide an increasingly broad and powerful toolkit for medical imaging. Mouse models play a key role in early testing and development of imaging probes, some of which are unlikely to ever translate to clinical use and may be designed for, or restricted to, facilitation of medical research through use in preclinical models. Imaging probes in use today include non-targeted perfusion and blood pooling agents [128], contrast agents targeted to specific tissues, receptors or cell surface markers [22, 23] and conditional or activatable agents that provide a readout for the presence of specific molecules or the degree or rate of a specific molecular process [24-27, 129]. An increasing number of suppliers offer commercial-grade access to imaging probes that can be used in mouse models.

Multimodality imaging

The shift away from 'modality-centric' imaging labs, toward core 'modality-agnostic' mouse imaging labs in the last 10 years has led to integrated use of multiple imaging modalities in single projects, studies or animals. The ability to call on the optimal modality for the question at hand provides a powerful means for biologists, pharmacologists, chemists and clinicians, as much as the imaging scientists and physicists themselves, to draw on imaging for the advancement of medicine.

The power of multimodality imaging has more recently led to the introduction of single imaging systems that combine one or more imaging modalities. For example, integrated PET/CT and SPECT/CT systems have become clinical standards and create increased power and efficiency for medical imaging. Newer PET/MRI [130] and SPECT/MRI [131] systems are now in use and are beginning to be offered by manufacturers. For mouse imaging, the combination of PET/SPECT/CT has become a standard [132]. This trend will continue for mouse imaging, due to the desire to address multiple questions in preclinical studies and the gain in efficiency that can result by integrated multimodality imaging.

New mouse imaging systems

A number of new approaches to mouse imaging are becoming available. One of the most promising new modalities is photoacoustic imaging [133, 134], which combines the excellent tissue penetration and resolution of acoustic imaging with the sensitivity and flexibility offered by optical imaging. Photoacoustic imaging technology is also potentially clinically translatable [135, 136].

New compact, bench-top versions of traditionally large and expensive imaging systems including MRI (e.g. Aspect MRI, http://www.aspectimaging.com) and PET (e.g. Sofie Biosciences, http://www.sofiebio.com/) [137] are also increasingly prevalent in mouse imaging. Providers of these technologies have created integrated turnkey systems that include attention to details of mouse handling, anaesthesia and monitoring. These convenient systems enable high imaging throughput, albeit with some sacrifice in imaging sensitivity and/or resolution. Importantly, these systems will likely increase adoption rates due to their greater affordability and relative ease of installation and use. This will help drive even greater rates of imaging technology development and validation.

Imaging of drug safety and toxicology

The successful translation of imaging technology from disease diagnosis to measurement of disease progression and therapeutic efficacy has led to a broad array of disease-relevant endpoints that can be provided through *in vivo* and/or clinical imaging. The future will see

a new translation, as imaging end-points are applied and validated for drug safety and toxicology [138, 139]. The ability to image drug toxicity in the same subjects in which efficacy is being tested would provide better ability to develop new therapies and predict outcome. Early advances in this area include the use of anatomical imaging approaches for teratology and reproductive toxicology [140-145]. New initiatives such as the HESI Project Committee on the Use of Imaging in Preclinical Safety Assessment (www.hesiglobal.org) are paving the way in this area.

Summary

Medical imaging technologies have revolutionized the field of medicine and patient care. The more recent translation of the major imaging modalities such as MRI, PET, SPECT, CT and ultrasound to analogous technologies dedicated to mouse imaging has broadened the field substantially and has led to rapid creation of new imaging protocols and facilitating technologies such as imaging probes. Most of the major medical research institutions around the world, including academia, pharmaceutical companies and CROs, have now introduced multiple modality preclinical imaging centres. These imaging centres rely on the laboratory mouse as the primary test subject in the imaging operation. Mouse imaging will continue to drive imaging technology development and facilitate knowledge of all of the major human diseases, and development of new therapies against them.

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Necropsy Methods

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Introduction

The methodology used to systematically evaluate bodies to determine the cause of death is commonly called the autopsy procedure. Use of the word 'autopsy' should, however, be limited to a postmortem examination performed on humans; *necropsy* is the correct term used in veterinary medicine for postmortem examination of non-human species. A necropsy is a highly technical procedure which, when done correctly, can yield a great deal of information. Proper handling of the animal antemortem and postmortem is necessary to optimize results. Detailed analysis of clinically ill or genetically engineered mice (GEMs) is necessary to obtain the correct diagnosis or characterization of the clinical features (phenotype). Simply looking at the organ of interest, limited selections of organs (e.g. lungs, kidney and/or liver), or biological fluids (blood, sera, etc.) are, by themselves, not adequate and result in many errors in the literature [1]. Although 'physiological phenotyping', the method in which a series of assays are run asking specific questions (blood chemistry, behavioural assays, etc.), codified by the EMPReSS system [2–5], provides a great deal of data, albeit out of context, the resulting information is obtained on specific parameters but no definitive diagnosis or specific criteria by which the mouse lesions can be specifically compared to a human disease. By contrast, a well-done necropsy with accompanying histopathology can do all of this and put the physiological phenotyping data into biological context [6].

Biological characterization of a new mutation

Individual mice of an inbred strain are essentially the same; therefore normal gross anatomy and histology should be the same, or at least very similar, for all mice within a strain. If maintained properly in controlled specific pathogen-free (SPF) facilities, only a few mutant and littermate control mice from a new line

need to be examined. In fact, when done by an experienced pathologist, it is possible to identify the major defects in tissues from one mouse; however, this is risky as lesions change over time and some organs cycle which affects onset and severity. Because pathogen status, diet, water, room conditions, and many other factors change over time, using appropriate controls for each study is imperative. The use of wild-type mice from the same strain as controls provides background context necessary for interpretation. For example, finding epicardial mineralization in mice carrying the *Prkdc^scid* mutation does not mean the mineralization is due to the mutation; rather, it is a strain-specific background lesion that would have been recognized had the investigator compared their findings with wild-type mice of the same strain [7, 8]. Regardless, strain-specific diseases are often relatively reproducible over time and historical documentation can be valuable for interpretation of results, as reviewed in Chapter 3.1 [9].

Preliminary studies can be done using small groups of animals to identify important lesions. Information obtained in this manner can be used to plan more in-depth studies. The numbers and ages of mice needed for any given study should be carefully considered. Some groups select ages based on convenience. A common age is 6-8 weeks because mice are fully developed (adult) and have been weaned. Discussions relative to the international knockout mouse project (KOMP) recommend routine screening of mice at 15 weeks of age [6]. Understanding the biology of the laboratory mouse provides a more logical approach. Major changes in the life of a mouse, as in all animals, provide reasons to choose specific ages for following the development and progression of lesions. Major life changes include birth (postpartum day 0), weaning (3-4 weeks), sexual maturity (6-8 weeks), sexual quiescence (6-8 months) and geriatric stages (1-2 years+) [10, 11]. Ranges for sexual quiescence are given to allow for differences between inbred strains [12]. It is important to understand these strain-specific differences and determine specific ages for the strain of interest at the onset of the study. The cost of maintaining mice through geriatric age is prohibitive for screening studies, so this is

rarely done on a routine basis, although more studies are in progress to address these issues [9]. Many mice with genetic mutations often do not live to geriatric age so it may also be impossible to obtain mutant mice for such a group.

Rodents, including mice, are *altricial*, meaning their young are born in a relatively undeveloped state. For example, hair shafts in mice do not emerge from the skin until 5 days of age because hair follicles have not completed development. Eyelids open around 12 days of age. Glomeruli in the kidneys do not fully develop for several weeks after birth. Each organ has a specific developmental process postpartum; as such, specific modifications in protocols must be made to conduct detailed studies on each organ. The skin is used as an example in this text. Readers should refer to books and review articles to find details on other organs [13-18]. During the first 3 weeks of a mouse's life the skin and hair undergo dramatic changes. The epidermis of a normal newborn mouse is relatively thick and becomes thinner by 2 weeks of age [19, 20]. Hair follicles develop completely during the first week after birth and the first hair fibres begin to emerge at around 5 days of age [21, 22]. The hair cycle is synchronous and short during the first 3 weeks after birth, making it easy to study details of the hair cycle in mice. To evaluate these cutaneous changes systematically, skin is collected from mice at 2-3 day intervals (birth, 3 days, 6 days, etc. to 21 days). Interval-specific postpartum collection ages can be defined for each organ using this approach.

The numbers of mice used in this type of study can rapidly become quite large. However, since most mutations occur, or are induced, on inbred mouse strains maintained in controlled environments, free of specific pathogens, it is possible to do a complete study on as few as two mutants and two controls of each sex at each of the time points mentioned above (N = 8 per age group) [6, 10]. Appropriate housing, as described elsewhere in this book, helps to ensure the accuracy of the study. Mice collected for the major life event points should have complete sets of organs harvested for study (Table 5.6.1). Injecting the mice with bromodeoxyuridine or tritiated thymidine before euthanasia makes it possible to use serial sections of tissues taken from individual mice for kinetic analysis. Mice with some

TABLE 5.6.1: Sample corresponding table	
Cassette	**Organs or tissues**
1—3	Small intestinal rolls
4	Colon roll with anus
5	Longitudinal section of stomach
6	Longitudinal section of cecum (or cecum and stomach)
7	Cross-section of medial lobes of liver with gallbladder Cross-section of left lateral lobe of liver (largest piece) Cross-section of spleen and attached pancreas
8	Longitudinal section of left kidney and adrenal Cross-section of right kidney and adrenal
9	Reproductive organs and urinary bladder
10	Longitudinal section of heart, showing all chambers
11	Longitudinal sections of lungs (centre of lobes on both sides)
12	Coronal sections of brain
13	Dorsal skin, tail skin, and ear
14	Ventral skin, muzzle, and eyelid
15	Longitudinal section of hind leg, showing long bones (foot removed)
16	Longitudinal section of front leg, showing long bones (foot removed)
17	Longitudinal section of hind foot, both halves, showing bones and nails
18	Longitudinal section of front foot, both halves, showing bones and nails
19	Foot pad from hind foot
20	Coronal sections of the skull
21	1—2 cross-sections of the tail, 1 longitudinal section of the tail
22	1 cross-section, 1 longitudinal section of the cervical and thoracic spine
23	1 cross-section (at the hip joint) and 1 longitudinal section of the lumbar spine
24	3 sections of the lower jaw: 1 cross-section at the caudal end including the thyroid and parathyroid, 1 longitudinal section showing the molars, 1 longitudinal section including the tongue and front teeth Other tissues such as salivary glands, thymus
25	Tumour or other abnormality

skin mutations exhibit a positive Köbner's reaction following injury, so the quality of multiple biopsies over time from the same mouse may be less than optimal [22, 23]. This is why individual mice are necropsied at specific time points rather than taking successive biopsies from the same animal.

In order to ensure optimal standardization, the same technician should collect tissues from the same anatomical sites from each mouse. Whenever possible, the same technician should be involved in a particular study, thus keeping the technique and any inherent errors consistent.

Clinical evaluation

Live mice should be carefully examined for behaviour and external abnormalities. Most homozygous recessive mutations (m/m) are available with heterozygous ($+/m$) or wild-type ($+/+$) age- and sex-matched controls on the same genetic background (where m is the mutant gene being studied, and $+$ is the normal or wild-type gene). Controls should be examined side by side with mutants as a basis for comparison. Familiarity with the normal phenotype of the inbred strain being used is essential in assessing

phenotypic variations of the mutant. Many infectious diseases in mice can present as behavioural abnormalities, such as circling or torticollis associated with middle ear infections (otitis media). These changes can be common in some strains, such as C3H/HeJ [9]. When they are present in both mutant and control mice, it is prudent to do infectious disease surveillance studies on the colony. Some mutations have clinical phenotypes that resemble infectious diseases such as cutaneous scaling (as in ringworm) or focal alopecia (hair loss potentially due to ectoparasite infestation). Disease issues are beyond the scope of this chapter and are covered in detail elsewhere [13, 14, 24].

Mice should be allowed to move around in their box so that their patterns of behaviour can be observed. Normal mice will constantly explore their environment and be alert and active. They should respond to external stimuli without abnormal reactions. Some inbred strains like DBA/2J respond to loud noises by developing sudden and sometimes prolonged seizures, often ending in death [25]. The mouse should have a uniform hair coat that lies flat. Vibrissae, the long hairs around the eyes and muzzle, should be straight and prominent. Ears should be erect and light pink in colour for albino mice. Pale white ears in albino mice are suggestive of anaemia. A blood sample can be taken to determine packed cell volume or haematocrit if needed. Eyes should be bilaterally symmetrical and clear. Incisors (front teeth) are commonly overgrown in many strains (malocclusion of incisors), but this may be part of the phenotype if it is consistently observed in association with a particular mouse mutation.

Body openings should be checked for any gross abnormalities, and any secretions or excretions produced should appear normal. For example, mouse faeces are about the size and shape of a rice grain, of firm consistency and dark brown in colour. Perianal matting of faeces or light yellow coloured faeces might indicate the phenotype of inflammatory bowel disease or other intestinal disease [26]. Several commonly used inbred strains of mice may have the appearance of extra testicles (three or four) due to cysts of the bulbourethral glands [27]. The extremities should be visually inspected for any obvious deformity or swelling. The nails should be short and slightly curved.
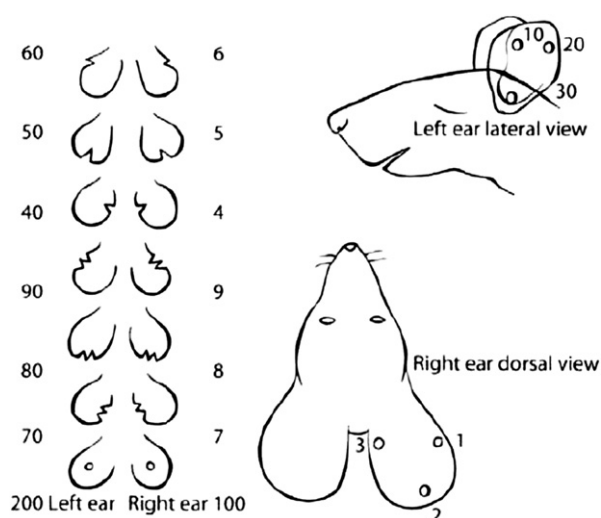
Figure 5.6.1 Mouse identification by ear notching.

Many abnormalities can be identified at the clinical and gross evaluation of the mouse. Thorough evaluation of the first mutants in a study will provide guidelines on what to look for more specifically as the study progresses.

Animal identification information should be recorded appropriately. This includes age (dates of birth and necropsy), sex, strain, genotype if known, pedigree numbers, animal identification numbers including ear tag or punch (Figure 5.6.1) or toenail amputation, source (room), reason for submission and name of submitting technician or scientist. If the mouse is part of an ongoing study, a special code number for that study should be assigned [28, 29].

Clinical pathology

Routine collection of biological fluids is done as part of physical examinations for humans and domestic animals in sickness or health. The methods are identical for mice but microassays have had to be developed [30]. Specimen collection and analysis can be done prospectively, as a routine procedure throughout all studies, or retrospectively, once a series of abnormalities are identified, to monitor or define the pathogenesis of the disease. The former approach requires a broad screen while the latter can focus on parameters specific to the organ of interest, potentially reducing costs. Since mutant mice usually provide a readily renewable population

to study, the latter approach is commonly used in most research laboratories.

Blood collection

Blood is collected by submandibular bleeding, retro-orbital bleeding, ventral coccygeal vein bleeding, tail tip amputation, cardiac puncture, or decapitation [31]. The reasons for using each method vary with age, purpose of the study, volume needed, and methods approved at each institution. These methods are covered in detail in Chapter 5.3.

Blood handling

For serum collection, blood can be held at room temperature for an hour and then centrifuged. The serum should be decanted and stored in plastic tubes (Eppendorf, Brinkmann Instruments, Inc., Westbury, NY, USA; Nunc, Nalge Nunc International, Fisher Scientific, Pittsburgh, PA, USA) and frozen at −80 °C until used. Blood may sit for several hours before centrifuging, if stored at 4 °C.

Plasma is obtained by collecting blood in tubes containing ethylenediaminetetraacetic acid (EDTA) or heparin to prevent clotting. The blood is then centrifuged and frozen for future use.

Whole blood should be handled according to the instructions provided by the diagnostic laboratory that you work with.

Faeces

Faeces are collected at the time of necropsy and can be frozen in plastic tubes for a variety of assays. Most mice defecate upon handling, so a few fresh samples can be obtained from a defined individual. This is a simple resource for *Helicobacter spp.* surveillance using polymerase chain reaction (PCR) methods [32] or for faecal IgA quantification [33].

Urine

Urine is usually expelled when a mouse is handled (for details see Chapter 5.3). Urine can be collected in a clean plastic tube or tests done directly with a variety of chemically impregnated strips. Chemstrip (Boehringer Mannheim Diagnostics,

Indianapolis, IN, USA) and Ames Multistix (Miles Inc. Diagnostic Division, Tarrytown, NY, USA) are two urine analysis reagent strips that test for numerous urine components including glucose, ketones, protein content and pH. For more specific tests that require larger volumes of urine or urine collected over defined intervals, metabolic cages are commercially available (Columbus Instruments, Columbus, OH, USA). Urine specific gravity is measured using a hand-held refractometer. Some companies offer refractometers especially made for urine testing, such as the Fisherbrand UriSystem Refractometer (Fisher, Pittsburgh, PA, USA).

Gross pathological examination

Abnormalities are phenotypic deviations from known traits. Any abnormalities should be recorded in as much detail as possible. Anatomical and pathological terms should be used, if known, but careful descriptions using lay terms can often be translated into anatomical nomenclature by a medically trained collaborator [34].

Simple and specific descriptive terms commonly used by pathologists are summarized in Boxes 5.6.1 and 5.6.2. Combined with detailed anatomical location, these descriptions provide a great deal of information on lesions observed. Gross anatomy of the mouse is detailed elsewhere in this book (Chapter 2.2).

Fixatives

Numerous chemical solutions are available to preserve tissues for histologic, immunohistochemical and ultrastructural studies (Table 5.6.2). Each has advantages and disadvantages. The choice depends on the goals of the study, tissue processing available in the histology laboratory, and preference of the investigators, especially of the pathologist who will interpret the results [35, 36]. Fixatives should be prepared in advance and be available in adequate volume in appropriately sized containers before the

necropsy is started. There are two general rules for histology: (i) fixatives have various penetrating abilities, and should be individually tested before use. Penetration is usually 1–2 mm on any cut surface, so specimens should generally be trimmed and kept small; (ii) approximately 20 times the volume of fixative should be used to the volume of tissues to obtain optimal preservation. Excessive amounts of blood and faeces will limit the usefulness of the fixative. At the end of the necropsy, the fixative can be drained and replaced with fresh fixative to minimize or eliminate this problem. If the solution is clear it is probably adequate. However, if the solution has a red or brown discoloration it should probably be replaced by fresh chemicals.

Examples of commonly used fixatives for mouse histopathology are described in the Appendix to this chapter.

Euthanasia

Laboratory mice are usually provided live for necropsy. Mice have a very high metabolic rate and decompose rapidly after death, making histology useless in most circumstances. A variety of euthanasia methods are approved by the American Veterinary Medical Association [37]. Care should be taken to ensure that humane treatment is provided. Commonly used methods are described in the following sections.

Carbon dioxide asphyxiation

The use of carbon dioxide (CO_2) is a rapid and humane form of euthanasia for mice over the age of 7 days. It utilizes a container designed to allow gas to enter rapidly and displace room air. These can be easily manufactured out of plexiglass sheets and tubing, or a large clear glass jar may be used. Adequate ventilation should be available for the technicians performing the necropsies. Gas is provided from a compressed gas cylinder attached to a wall or cabinet. The container is lined with a disposable plastic bag and prefilled with CO_2 by opening the valve on the attached cylinder to fill the container

TABLE 5.6.2: Formulations of commonly used fixatives	
TELLYESNICZKY/FEKETE	
70% Ethanol	100 mL
Glacial acetic acid	5 mL
37—40% Formalin	10 mL
BOUIN'S SOLUTION	
Sat. aq. picric acid	85 mL
Glacial acetic acid	5 mL
37—40% Formalin	10 mL
10% NEUTRAL BUFFERED FORMALIN	
37—40% Formalin	100 mL
Distilled water	900 mL
Sodium phosphate—monobasic	4 g
Sodium phosphate—dibasic	6.5 g
B5 FIXATIVE	
Mercuric chloride	6 g
Sodium acetate—anhydrous	1.25 g
Distilled water (hot)	90 mL
Just before use, add:	
37—40% Formalin	10 mL
Carnoy's fixative	
Absolute ethanol	60 mL
Chloroform	30 mL
Glacial acetic acid	10 mL
4% PARAFORMALDEHYDE FIXATIVE	
16% Paraformaldehyde	10 mL
PBS pH 7.2	30 mL
GLUTARALDEHYDE FIXATIVE	
25% Glutaraldehyde	10 mL
0.2 M phosphate buffer pH 7.2	50 mL
Distilled water	40 mL
KARNOVSKY'S FIXATIVE	
25% Glutaraldehyde	8 mL
16% Paraformaldehyde	12.5 mL
0.2 M phosphate buffer pH 7.4	50 mL
Distilled water	29.5 mL
JB4 FIXATIVE	
25% Glutaraldehyde	16 mL
16% Paraformaldehyde	12.5 mL
1 M cacodylate buffer	20 mL
Distilled water	151.5 mL
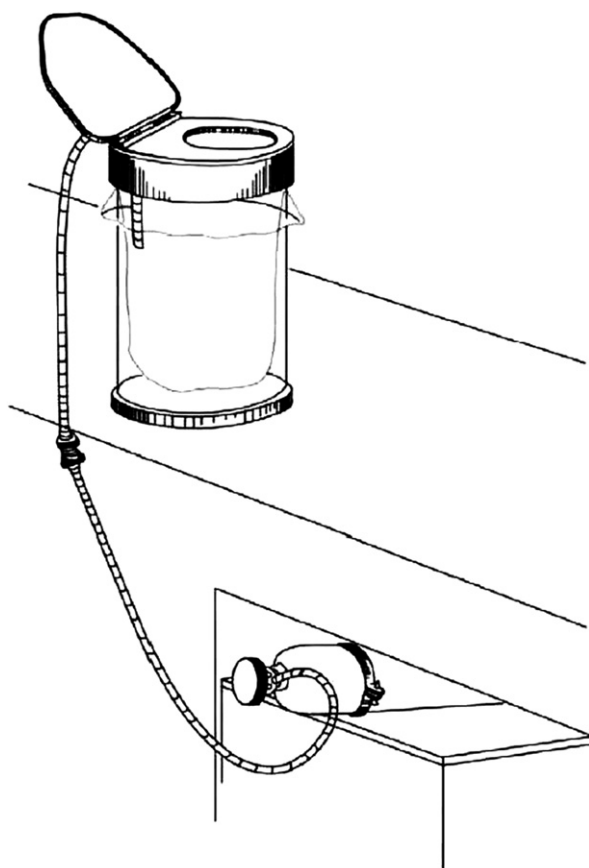
Figure 5.6.2 Plexiglass container attached to a CO₂ cylinder for euthanizing mice.

(Figure 5.6.2). The mouse is placed on the bottom of the container and the unit is refilled with CO_2. The mouse will die within 1–2 min. Only one mouse should be euthanized at a time; placing many mice in the container at the same time may result in those at the bottom not being euthanized. When the bag is removed and disposed of with the mice still in it, the mice at the bottom may revive. This is clearly an inhumane situation that must be prevented.

Neonatal mice placed in these containers filled with CO_2 will appear to be killed during the same time interval as for adults. However, neonatal mice are not euthanized by the gas. As such, most Institutional Animal Care and Use Committee guidelines now require decapitation with a pair of scissors to ensure euthanasia.

Dry ice is the solid form of CO_2. It undergoes sublimation to CO_2 gas. Dry ice should not be used to euthanize mice as adequate amounts of gas cannot be generated rapidly enough to ensure humane euthanasia. Furthermore, the

mice can suffer from thermal injury if they come into direct contact with the material.

Barbiturate overdose

This is an effective and humane method for adult mice that is described in detail under 'Perfusion Methods' below.

Decapitation of adults

This method should be avoided unless the experiment has very special requirements and the procedure has been approved by the Institutional Animal Care and Use Committee. For a description of the procedure, see Chapter 5.3.

Cervical dislocation

This method involves separating the vertebrae in the cervical area with a firm pinch to the neck and pull of the tail. It is quick and efficient, but this method is not often recommended since it results in damage to tissues in the cervical area as well as releasing large amounts of blood into the body cavity, which can make observation and collection of some organs more difficult. More importantly, if done improperly, the mid to lower vertebral column rather than the cervical region can be damaged creating an inhumane situation.

Perfusion methods

Perfusion combines euthanasia with fixation, providing the quickest way to get organs into fixative resulting in the freshest tissues for study with minimal autolytic changes. Special protocols must be followed to ensure this is done in a humane manner.

Materials needed include two 10 mL syringes with 23G needles, one 1 mL syringe with a 23G needle, stock solution of pentobarbital sodium (50 mg/mL; Nembutal, Abbott Laboratories, North Chicago, IL, USA), phosphate buffered saline (PBS), fixative of choice and 0.85% saline.

First, prepare the working solution of pentobarbital by diluting 1.6 mL of the stock solution with 8.4 mL PBS. Then fill one 10 mL syringe with 0.85% saline, and the other with your chosen

fixative. Label the syringes to avoid confusion during the procedure.

The mouse must be anaesthetized using an intraperitoneal injection of pentobarbital working solution (0.1 mL/10 g body weight) using the 1 mL syringe. After the mouse is completely anaesthetized, dip the mouse into a mixture of water and disinfectant and pin it to a dissection board, ventral side up (see section on 'Necropsy procedure'). Incise the ventral skin, undermine it using blunt dissection, and reflect it away from the incision site from the thorax to the mandible to reveal the jugular veins located under both salivary glands. Following instructions for the necropsy procedure, immediately open the thoracic cavity. If a blood sample is needed, a heart puncture must be done at this time (see Chapter 5.3, section on 'Blood collection'). After blood is collected, carefully cut the jugular veins. Blood will start to flow from the vessels. Insert the needle of the syringe containing the saline into the left ventricle of the heart. With gentle but constant pressure, perfuse the saline into the heart while observing the area where the blood vessels were severed. The saline should flush the blood out of the vascular system. It is important that the pressure exerted on the syringe is enough to push the blood through and out of the vascular system, but not so excessive as to cause damage to any of the organs. After injecting 4-8 mL of saline, depending on the size of the mouse, repeat the same procedure with the fixative. If fixation is successful, the body will stiffen from the tip of the tail to the nose and all organs will blanch. Tissue collection may then proceed.

Skin collection

For cryosectioning

After euthanasia the dorsal skin is shaved using electric clippers to remove the hair, as hair is very hard and can interfere with sectioning and quickly dull the knife blade. A rectangular portion of skin is surgically removed while maintaining orientation of the tissue, anterior to posterior. This is important as hair follicles grow at an angle and one objective of the skin sections is to take longitudinal sections of hair follicles. The skin is placed on a fine, firm, nylon mesh (Nitex fabric). The tissue is laid flat on the fabric and trimmed to 1.0 × 0.05 cm. Two embedding moulds (boats) (22 × 22 × 20 mm deep; VWR International, Bridgeport, NJ, USA) are placed in the work area. Fill both with O.C.T. Compound (Tissue-Tek, Sakura Finetek USA, Trannce, CA, USA). In the first boat 'wash' the sample for about a minute to saturate it with O.C.T. Compound and to remove any attached bubbles with fine, curved iris forceps. The purpose is to have a constant consistency between the block and tissue. Remove the sample from the boat and remove the skin from the nylon fabric. Place the skin on a small piece of aluminium foil with the cut section (anterior-posterior orientation edge) at the edge of the foil. Lightly cover the specimen with O.C.T. Compound and place on a thin metal platform that is lying on top of a block of dry ice or crushed dry ice. We found a galvanized cage card holder worked better than blocks of steel. At this point place the second boat into the dry ice container and watch until the O.C.T. Compound begins to turn white. Remove the frozen sample from the aluminium foil and quickly place it with the cut side into the semi-frozen O.C.T. Compound. Hold upright with chilled forceps until it is frozen in place. Holding skin longer than a few seconds will cause the forceps to stick to the skin and skin will come out of the mould. Fill the boat with O.C.T. Compound, close the top of the container, and allow the O.C.T. Compound to freeze *in toto*. As the sample freezes, remove bubbles with forceps. Remove the boat, wrap in aluminium foil and store at −80 °C until further use. Label the boat and aluminium foil for identification.

For RNA extraction

When collecting tissues for RNA extraction it is important to know that it is done in a very timely manner. The tissues begin to degrade as soon as the animal has been euthanized. Proper handling is important as well; wear gloves and change them often, especially between mice. The work area has to be thoroughly clean and wiped down with RNaseZap Wipes® (Ambion, Austin, TX). After euthanasia the dorsal skin is shaved using

electric clippers to remove all hair as it can contaminate the samples and thereby reduce the yield of RNA. The mouse is wiped with RNaseZap Wipes® to remove all remaining hair. Sterile instruments are used for removing a rectangular portion of skin and placing it on the Nitex fabric which has also been sterilized. Note that the work area, sterile instruments, and Nitex are sprayed with RNaseZap (Ambion) to guarantee an RNase-free environment. The tissue is laid flat on the Nitex fabric and cut to $0.5\,cm^2$. After the tissue has been cut, the Nitex is removed before placing the tissue into the tubes containing RNALater® (Ambion, Austin, TX, USA) to permeate the cells and stabilize the RNA for later use. How much sample is collected depends on the size of the tube needed. The amount of RNALater® to be added is 5 times the amount of tissue collected. It is important to have the correct tissue and RNALater® volume ratios as the consequence of too little RNALater® will be degraded RNA. However, make sure not to overfill the tube with RNALater® as it will freeze and possibly break the tube. The tissue is stored overnight at room temperature and then transferred to $-80\,°C$ until further use.

Necropsy procedure

Once the mouse has been euthanized it can be superficially disinfected by submersion in a dilute solution of a germicidal detergent such as Calgon Vestal Process NPD One Step Germicidal Detergent (ConvaTec, St. Louis, MO, USA), or a solution of 95% ethanol. When performing necropsy of a mouse with an abnormality of the skin or hair, it is important to collect samples of the hair before the mouse is dipped in the disinfectant. Hairs can be plucked and used to inoculate fungal culture media (Dermatophyte Test Medium, MG Scientific, Pleasant Prairie, WI, USA; Sabouraud's medium, MG Scientific). To examine hair fibres for structural abnormalities or for ectoparasites the hairs can be plucked manually using the thumb and forefinger. Forceps may damage the hair fibre. Gently plucking hairs will remove those hair fibres from follicles in the telogen (exogen) stage of the hair cycle. Anagen follicles are actively

growing, deep, and firmly attached to the dermis. Plucking hairs from follicles in anagen may result in damage or induction of abnormalities in fibres already weakened by structural defects. Plucking fibres from telogen follicles allows for examination of the whole hair shaft from root (club) to tip. Hair fibres should be stored in a clean cryopreservation tube (Nalge Nunc International, Denmark). When studying mutant mice, standardized collection techniques for skin and hair are as important as for other organs. Hair samples should be collected from the same area on every mouse in a study, such as from the left lateral skin surface from shoulder to pelvic region. Avoid areas where full-thickness skin will be collected for histological examination as plucking hairs will distort the inner root sheath and other structures in the hair follicle. If the vibrissae (very long straight hair fibres around the eyes and muzzle) appear abnormal, samples of these should be plucked as well, from the same side as the hair is plucked. The vibrissae should be stored in a separate cryopreservation tube. Other specialized hair fibre and follicle types can be collected and examined if necessary.

If a mouse has a skin abnormality, remove hair prior to disinfection. This is usually accomplished by shaving the mouse with electric hair clippers such as the Oster Finisher Trimmer (Oster Professional Products, McMinnville, TN, USA). These clippers are easy to handle and have a small blade that is ideal for mice or other small mammals. If complete hair removal is desired, there are commercially available depilatory products (Nair, Carter-Wallace Inc, New York, NY, USA; Neet, Reckitt & Coleman Inc, Wayne, NJ, USA) that can be applied to the mouse after shaving. These products should be left on for 2–3 min, then rinsed off under warm running water, which will wash away the hair as well.

At this point, the mouse may be disinfected. The disinfectant washes off loose hairs and mats hair down on an unshaven mouse for ease of examination. Allow the disinfectant to drain from the mouse, and then place the mouse on one to two layers of absorbent paper towel on a cork dissection board. The cork board should be approximately $14 \times 21.5\,cm$ in size and 1.0 cm thick.

Skin should be collected for histology at this point. With the mouse ventral surface down on the board, gently grasp a fold of dorsal skin

from the caudal region and make a small incision with the scissors. Carefully cut out a rectangular piece of skin along the dorsal midline from the thoracolumbar junction to the interscapular region. Place the skin on a piece of unlined index type card or aluminium foil. Orient the skin sample cranial-caudally and trim lengthwise across this axis to optimize the orientation of the hair follicles. Fix by immersion in the appropriate fixative.

After collecting dorsal skin, collect a sample of ventral skin in a similar fashion. Fresh samples of both dorsal and ventral skin may be frozen in O.C.T. Compound for immunofluorescence or *in situ* hybridization studies. The skin from the head should also be collected, including the pinnae (ears), eyelids and muzzle. Each of these sites includes specialized glands and/or hair follicles. There are mucocutaneous junctions present and the epidermis varies slightly from truncal skin in these sites as well. The skin can be carefully peeled and trimmed from the skull as a unit and mounted flat on a piece of index card or foil for fixation. Tail skin may be collected by severing the tail from the body and incising it lengthwise, using the tip of a pair of scissors. The loose skin is grasped from the base of the tail and stripped away from the bone and tendons. Tail skin is mounted flat on a piece of foil, as with other skin samples. Tail and head skins are often collected toward the end of the necropsy.

After skin is collected, place the mouse ventral side up and pin each limb firmly to the board. The rear feet may be pinned between the gastrocnemius tendon and the bone, while the front feet may be pierced, through the skin, between the metacarpal bones, in order to do the least damage to the tissues being collected. During the necropsy, the board may be rotated easily to adjust the position of the mouse, providing various angles of access for organ collection.

With a no. 12 scalpel blade and no. 3 handle, make a ventral midline longitudinal incision through the skin, from the external genitalia to the ramus of the mandible, then cut from the genitalia laterally toward the rear feet, along the medial surface of the rear legs. Avoid using scissors as they are quickly blunted. On female mice, this incision passes between the fourth and fifth nipples of each side. Grip the skin on either side of the incision and pull gently

outward, or use light strokes of the scalpel, to separate the skin from the abdominal muscles. Reflect the skin far enough so that it does not interfere with the rest of the necropsy.

With the skin reflected back, collect peripheral lymph nodes located on either side of the salivary gland (cervical lymph nodes), under each of the front legs (axillary lymph nodes) and on the medial side of each rear leg (inguinal lymph nodes). Unless they are enlarged due to disease, the peripheral lymph nodes may be difficult to locate, especially the axillary lymph nodes. Since the cervical lymph nodes are collected attached to the salivary gland, the cluster of glands and lymph nodes can be removed *in toto* and fixed. The inguinal lymph nodes are located in the fat pad on the medial surface of the rear leg. They are slightly darker in colour than the fat and are usually no larger than 0.1–0.2 cm. The easiest way to collect them is to remove the fat pad with the embedded lymph node and trim away as much of the fat as possible before placing the lymph node in the fixative.

Using a new set of sterile instruments, grip the abdominal muscles at the inguinal region with forceps, lift firmly and make a small incision to allow air into the abdomen. In this way the viscera will not be injured or contaminated. Cut through the abdominal muscles on each side, extending from the inguinal midline to the lateral thorax exposing the viscera. The coelom can be cultured with a sterile swab before proceeding further.

Another set of sterile instruments can be used at this point if it is important to culture visceral organs. The longer the animal is dead before tissues are collected and fixed the more autolysis will occur, which will make interpretation of histological sections more difficult. As a general rule, no more than 15 min should pass between euthanasia and collection of tissues. This should allow adequate time for photography, microbiological culture collection and other procedures. Throughout the necropsy, visceral organs should be evaluated to determine if they are in their proper anatomical orientation. Some mutations such as *situs inversus* (*Dnahc11^{iv}*) can cause the orientation to be reversed [38–40]. All tissues and organs should be carefully checked for abnormalities and all observations should be noted. Gross photographs of any external or

internal abnormalities are important to obtain in the study of a new mutant.

The intestines should be collected first because they undergo rapid autolysis. Separate the stomach from the oesophagus at the diaphragm. Gently retract the stomach while cutting the mesenteric attachment with scissors or scalpel. Before separating the duodenum, open it near the stomach and gently express the gallbladder (found in the liver). Bile should flow out of the ampulla of Vater, indicating that the common bile duct is patent. Continue to retract the intestine and cut the mesentery until the point is reached where the colon enters the pelvic girdle. Cut the pubic symphysis with a pair of heavy scissors.

Before the colon and attached caecum can be dealt with, one must first remove the reproductive organs, including the preputial gland (males)/clitoral gland (females) and urinary bladder.

If the bladder/urinary system is of interest, inflate the urinary bladder with fixative (0.5 mL) via a syringe using a 27G needle (Figure 5.6.3). The urethra should be bound tightly with string to prevent outflow of fixative from the bladder. This method is effective for evaluation of the bladder wall, especially in early neoplasia or epithelial hyperplasia.

In the male mouse, testes may be gently grasped by the inguinal fat pad, cut away from the other viscera and placed on a piece of card. Orient the testicles so that the epididymis and testis are in the same plane and may be trimmed simultaneously for histological presentation (Figure 5.6.4). The preputial gland is a paired organ located subcutaneously between the penis and anus. It may be collected by grasping one
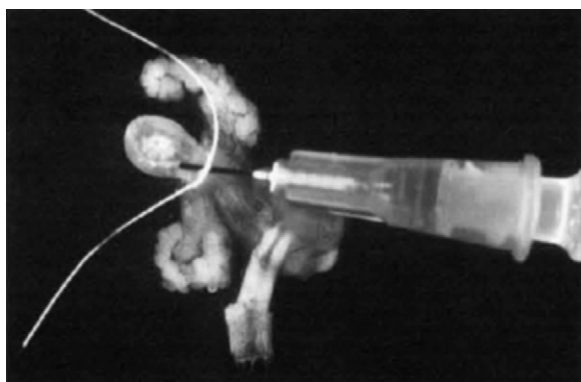
Figure 5.6.4 Orientation of the testes and epididymides.

edge of the gland with forceps, cutting it away from the abdominal wall, and placing it directly into fixative.

The seminal vesicles, coagulating gland, prostate gland, urinary bladder and penis may be removed as a unit by gently grasping the apex of the seminal vesicles with forceps and lifting it away from the colon. Insert the tip of heavy-duty scissors between the colon and the pelvis and cut the bone, on both sides, then lift the reproductive tract further and cut away the connective tissue between it and the colon. Remove the reproductive tract and arrange it on a card, then place it in the fixative (Figure 5.6.5).

The female reproductive tract is removed in a similar fashion. Clitoral glands are normally not easily visible, but are located subcutaneously, cranial to the vulva. The best way to collect these glands is to cut a small square of 0.5-0.8 cm of abdominal muscle and overlying skin immediately anterior to the clitoris. This section will include the clitoral glands. Smooth this piece of tissue gently on to a piece of white card and, using parallel pencil marks, indicate the area where the glands are expected to be. After placing the clitoral glands in fixative, grasp the fat pad of one ovary and cut it free from the mesentery, laying that ovary and uterine horn over on the other side of the colon. Remove the entire female reproductive tract as was done for the male organs, arrange it on a card to maintain orientation, then fix by immersion (Figure 5.6.6).

Retract the colon and sever the skin around the anus. The entire gastrointestinal tract can now be separated from the mouse, placed on a towel moistened with physiological saline, and

Figure 5.6.3 Urinary bladder fixation technique.
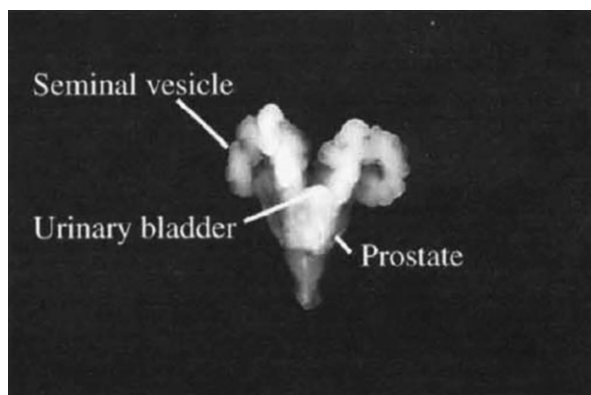
Figure 5.6.5 Male reproductive tract with urinary bladder.
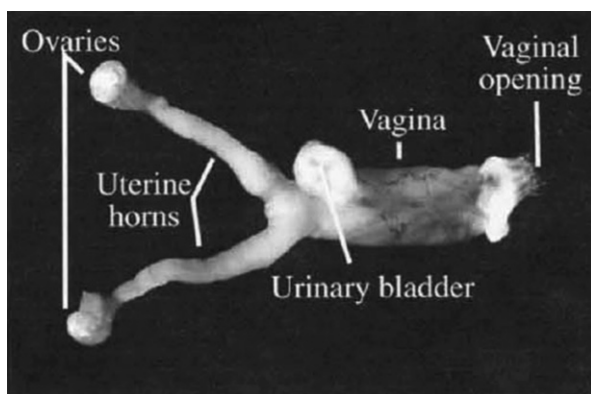
Figure 5.6.6 Female reproductive tract with urinary bladder.

examined. Mesenteric lymph nodes can be identified as small brown nodules at the root of the mesentery and along the intestine. These should be collected and fixed. At the duodenojejunal flexure, the pancreas is firmly adherent to both the small and large intestine. Care must be taken to separate the two parts of the intestine from the pancreas while preserving the integrity of all three. The spleen and pancreas are connected. They are often removed as a unit unless it is necessary to examine one or the other separately, as in the case of a mouse model for diabetes where special fixative (Bouin's solution) and stains (aldehyde fuchsin) are used to examine the beta cells of the pancreatic islets. Grasp the pancreas gently with the forceps and pull upward, cutting away any mesenteric tissue that adheres to the spleen or pancreas to free the structures, then place them directly in the fixative.

For proper fixation, the intestine must be inflated with fixative before it is rolled or otherwise prepared for histological preparation. This procedure is simple and rapid but runs the risk of personal injury if fixative splashes. Safety goggles and a fume hood are recommended since fixatives are noxious and toxic. Use a 10 mL syringe with a 17–22G needle. Fill the syringe with fixative and introduce the needle into either end of the intestine. Gently depress the plunger on the syringe and the intestine will slowly inflate (Figure 5.6.7). Make several injections along the length of the intestine if it does not fill completely due to the presence of faeces that form obstructions [34]. To make a second injection, simply pierce the wall of the intestine and clamp it gently with your fingers around the tip of the needle to prevent back-flow of the fixative and proceed as before [26]. An intestine that has been over-inflated is much more difficult to roll. Some laboratories prefer to open the entire intestine and remove digested food material and faeces prior to rolling [41]. This approach yields good mucosal fixation but may also cause damage to the mucosa.

Intestines can be rolled lengthwise for histological presentation [26, 41]. However, each roll must be able to fit comfortably into a cassette. For an average-sized adult mouse this requires the small intestine to be cut into three equal pieces (Figure 5.6.8 (a) and (b)).

Another approach to presenting intestines is similar to the way they are routinely collected in larger animals. Representative segments are cut and fixed by immersion. More precision comes by laying out the entire gastrointestinal tract and cutting segments out at specified distances from anatomical structures such as the
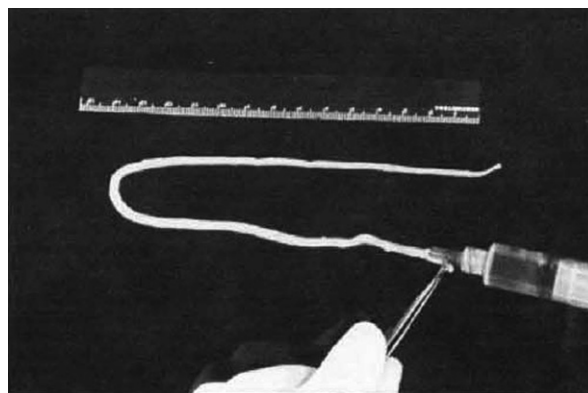
Figure 5.6.7 Intestines are removed and inflated by injection with fixative.
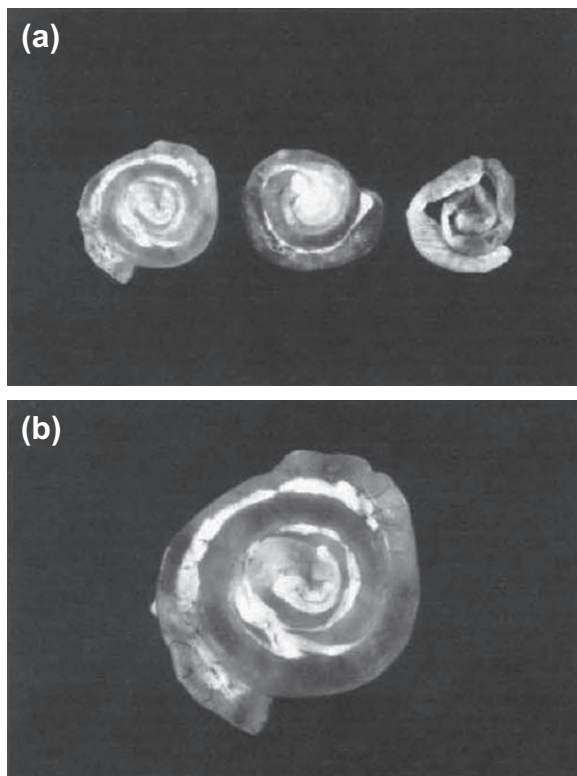
Figure 5.6.8 'Swiss roll' of intestines. It is important to make the rolls smaller than the cassettes (a), and minimize loss of fixative while rolling the segments (b).

anus, caecum or pylorus. However, as many lesions are segmental and not at reproducible locations, this approach misses many lesions.

To create so-called 'Swiss rolls' of intestine, roll the inflated intestine in concentric, centrifugal circles on a piece of white card [24]. If the fixative drains, the intestine may flatten, making it more difficult to roll. We use large index cards cut into strips approximately the width of cassettes to mount tissues. Orientation of the segments is important and must be agreed upon with the pathologist. It is commonly accepted to keep the end of the intestine proximal to the stomach toward the centre of the roll. Once the intestines have been rolled onto the paper, let them firm up to prevent the roll from unwinding, then fix by immersion. Intestinal rolls take practice to master. The important objectives are (i) do not over-inflate the intestine, (ii) maintain proper orientation, (iii) make the rolls smaller than the cassettes, and (iv) minimize loss of fixative while rolling the segments.

The stomach and caecum are collected separately, inflated with fixative and placed *in toto* in fixative. Modifications of this approach can be made to evaluate specific structures [26, 42]. Histological grading systems are available for the intestinal rolls and caecum [26, 43–45] , that cannot be done easily when only segmental cross-sections of bowel are available for examination, since lesions are often segmental and not uniformly distributed.

Kidneys may be removed by grasping the surrounding fat and pulling upwards while cutting around the organs. The adrenal gland is a small white structure that lies within the perirenal fat pad just anterior to the kidneys. It should be left within the fat that clings to the kidney and the two should be presented to histology as a unit. It may be important for the pathologist to be able to distinguish between the right and left kidneys. Since this may only become important after the fact, the right kidney should be cut transversely while the left is cut lengthwise (left/long) prior to fixation.

The liver is the last organ to be removed from the abdominal cavity. It can be manipulated using the diaphragm to avoid damage. The organ is cut from its vascular attachments with scissors and placed on a towel moistened with physiologic saline. Separate the right and left medial lobes of the liver as a unit along with the gallbladder. This may be accomplished by folding these lobes back onto the work surface and trimming the connections to the remaining liver lobes. The left lateral lobe is the largest and should be separated from all others in a similar fashion. The remaining smaller lobes may be placed in the fixative together. When separating the liver lobes, take care to handle them gently, by the edges, as they are easily damaged. Acid–alcohol–formalin fixatives penetrate quickly and deeply into this tissue. Other fixatives do not penetrate deeply even on cut surfaces so the liver lobes are best sectioned with a scalpel blade and separated to optimize fixation.

To enter the thorax, grasp the xiphoid process firmly with the forceps and lift upwards. If the chest cavity was not previously opened for blood collection or by cutting the diaphragm, this will create a negative pressure within the thorax, and cautiously cutting through the ribs and diaphragm on one side

allows air to enter the thoracic cavity. When negative pressure in the chest cavity is lost, the lungs will shrink away from the ribs and diaphragm. If a thoracic microbiological culture is to be taken, carefully extend the cut through the diaphragm and rib cage to expose the lungs, then use sterile scissors and forceps to take a tissue sample before proceeding. The thoracic cut may now be extended through the rest of the ribs at the costochondral junction to just short of the internal thoracic vein and artery on both sides. Often the mediastinum between the area of the heart and thymus continues pulling on the excised part of the rib cage, so it must be carefully trimmed away to prevent the rib cage from falling back over the thoracic cavity.

To remove the heart and lungs, turn the cork board around so that the head is facing you. Cut the mandibular symphysis, push the mandible laterally, retract the tongue and gently cut underlying soft tissue of the hyoid bones that extend dorsal to the larynx. Being careful not to puncture the trachea or the oesophagus, continue cutting on either side of the neck until the clavicles are encountered. Using the tips of the scissors, sever the bones and continue to dissect carefully through the ribs, avoiding the trachea, oesophagus and lung. Repeat this on the other side. Once all the surrounding tissue has been removed, gently retract the tongue, trachea and oesophagus, with the lungs, heart and thymus as a unit (generically referred to as the 'pluck'). The lungs should be inflated with fixative with a syringe to ensure proper histological preparation (Figure 5.6.9). Slip the tip of a needle into the trachea via the glottis, which is normally the most apparent opening at the base of the tongue. Clamp down around the needle with a pair of forceps and slowly depress the plunger of the syringe. The lungs should begin to expand and blanch. If the fixative flows out in a puddle between the lungs, try again. Inflate the lungs very slowly, stopping when they are about the size that they would normally be on inhalation. Overinflating the lungs can damage the alveoli.

The next organ to be collected is the brain. To access the brain, cut the vertebral column and spinal cord at the base of the skull. Slowly retract the skin from the skull if it has not already been
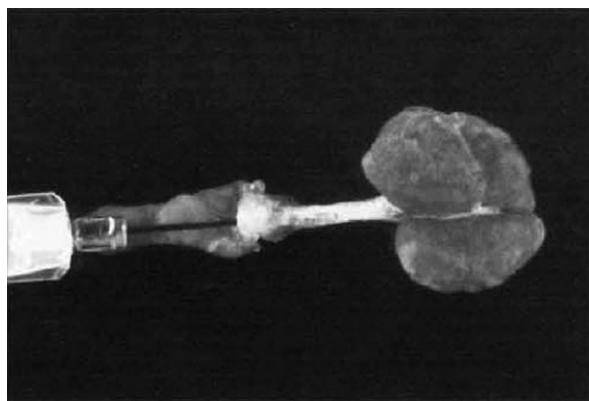
Figure 5.6.9 Lung inflation with fixative.

removed. If the eyes are of interest, you may wish to remove them from the skull. To remove the eye, insert a pair of curved forceps behind the globe, grasp the optic nerve and pull outward until the eye has been freed from the orbit. Eyes may be fixed and embedded separately. However, histological presentation of the eye within the skull is often sufficient for viewing many abnormalities.

Cut any remaining vertebrae away from the skull. The brain should be visible through the foramen magnum. This is the spinal medulla. Slip one blade of your scissors in between the neural tissue and the bone and make two small longitudinal cuts in the occipital bone, one on each side of the spinal medulla. Hold the skull between thumb and forefinger and, with forceps, grasp the edge of the occipital bone and pull upward to neatly break it off (Figure 5.6.10). Then gently insert the scissors tip between the brain and the skull to make a cut in the skull along the sagittal suture. Continue breaking
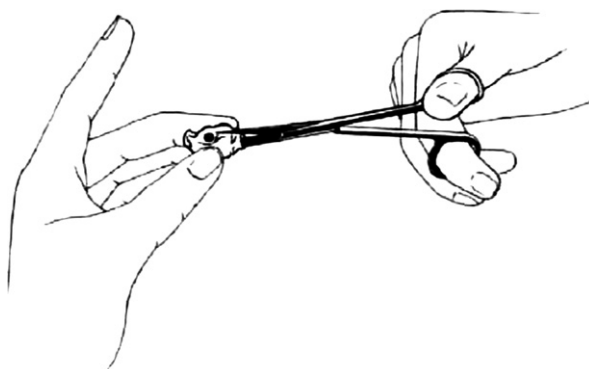
Figure 5.6.10 Initial cuts in the occipital bone to access the brain.

away the interparietal and the parietal bones in the same manner, being careful not to harm the delicate brain below. The frontal bone comes to a slight point at the intersection of the sagittal and coronal sutures. This area should be broken off as well, to allow the brain to be removed cleanly from the cranial vault. There may be a thin reddish membrane around the brain, particularly in the area of the cerebrum. This is the meninges, and must be removed carefully with forceps or it will cut into the brain as it is removed from the skull. Carefully separate the olfactory bulbs from the cerebrum to avoid damaging the turbinates (the olfactory bulbs will remain in the skull when the brain is removed). Turn the skull upside down over the jar of fixative. Gently work the forceps between brain and bone and pull away the connective tissues, freeing the brain from the cranial vault. The brain will fall into the fixative. Place the skull into the fixative as well.

The spinal column may now be collected. Grasp the proximal end of the spine and lift it away from the skin, cutting away the fascia that holds the two together. Cut through the pelvis to sever the hindlimbs from the distal vertebrae. Cut the tail away if this was not already done. Cut the ribs away from the spine, as closely as possible, without damaging the vertebrae. Place the spinal column in fixative, making sure to keep it straight so it is oriented correctly for trimming.

Trim the front and rear limbs from the skin and place them in fixative. If skin was not collected earlier in the necropsy, it may still be important to save it. Skin can be removed from a defined location for consistency. We usually remove a rectangular area over the thorax. The skin is flattened on a card or foil, cranial/caudal orientation is marked, and the tissue is fixed by immersion.

In summary, when evaluating a new mutation, it is important to collect total tissues in a methodical, standardized fashion to avoid diagnostic discrepancies. From these study sets, a more focused tissue collection protocol may be developed, concentrating on those tissues known to exhibit abnormalities in a particular mutant. Standard criteria for tissue collection, agreed upon by the technicians, researchers and the pathologist who will be reading the slides, are essential.

Trimming tissues for histology

After the tissues have been adequately fixed (see section on 'Fixatives'), they must be trimmed for histological processing. Proper tissue trimming will optimize interpretation by the pathologist. Presentation is critical when trying to identify any variation from normal. For most fixatives, tissues can be trimmed and processed after approximately 12 h (overnight) fixation. Before bones are trimmed they must be decalcified. Overnight soaking in a dilute hydrochloric acid-based decalcifying solution such as Cal-EX (Fisher, Pittsburgh, PA, USA) may be adequate. Bouin's and Fekete's solutions contain dilute acetic acid that also aids in decalcification when tissues are stored in them for days or weeks.

After decalcification, bones may be trimmed, following an initial rinse with water. However, the decalcified tissues must be continually rinsed in running water for at least 3-4 h before histological processing. Failure to rinse decalcified tissues thoroughly may result in inadequate staining of the tissue sections. Overuse of decalcifiers may also result in suboptimal staining of tissues. Optimal times for decalcification and washing should be customized for every laboratory and are often specific to particular organs or studies.

To trim tissues, use a firm clean cutting surface such as cork board, Teflon cutting board, or paraffin-filled petri dish. Each tissue, as it is trimmed, should be placed into a labelled and numbered histology cassette (OmniSette Tissue Cassettes, Fisher Scientific, Pittsburgh, PA, USA). Cassettes can be labelled with a #2 (HB) pencil, solvent resistant marker (HistoPrep Pen, Fisher Scientific), or mechanical labelling machine (Carousel Cassette MicroWriter, Thermo Shandon, Pittsburgh, PA, USA) to label the front and/or side of each cassette. Alternatively, identification information may be written on a small card and placed in the cassette. Indicate the mouse's accession number and, if there will be more than one cassette of tissue per mouse, number each cassette for that animal in sequential order. Sequential numbering will aid with identification and help determine whether all

tissues sent to histology are returned. A corresponding table (such as that shown in Table 5.6.1) can be used to standardize the combinations of tissues and numbering of cassettes.

Cassettes containing tissues that were fixed and not decalcified should be placed into a container of 70% ethanol, while those containing decalcified tissues not previously rinsed should be placed into water. After thorough rinsing, as described above, decalcified tissues may be placed into ethanol with other tissues and delivered to the histology laboratory for processing and embedding. Any remaining tissues not submitted for embedding may be stored in 70% ethanol for future use, if necessary. Tissues stored in fixatives for long periods may be altered (refer to section on 'Fixatives' and Appendix). When handling fixed tissues, it is still important to be gentle. Soft parenchymal organs, such as the liver, brain, lungs and kidneys, remain delicate after fixation and should be manipulated using a pair of wide wooden forceps or a similar tool. Trimming is best done with a sharp, single-edged razor blade to produce a clean cut with minimal tissue damage. Residual chemicals on the tissues can dull a razor blade rapidly, so it is important to change blades frequently while trimming. This will also help to minimize carryover of tissue remnants between specimens.

All tissues should be trimmed to a thickness of approximately 1–2 mm to permit adequate penetration of solvents and paraffin. If it is necessary to present a particular facet of a trimmed tissue to the pathologist, this is indicated by marking the side of the tissue one does *not* want presented. A blue pencil works well (Venus col-erase, #1276 blue, Eberhard Faber, Inc., Lewisburg, TN, USA). The blue pigment will not wash off in alcohol and will clearly indicate the desired orientation to the histologist embedding the tissue. If improperly included in the section, the blue pigment will contaminate the field, making it difficult for a pathologist to find clean fields for photography. Small, related tissues of similar densities may be placed together in the cassettes (i.e. kidneys with spleen and/or liver, reproductive tract tissues together, etc.; refer to Table 5.6.1). The heart, lungs, brain, bones and any possible

tumours found should each go into an individual cassette. Tumours or multiple lesions that need to be individually identified should be placed in different cassettes and documented.

The following is a description of trimming methods for each organ. After trimming, remaining tissues can be placed with 70% ethanol in heavy-duty, heat-sealed stand-alone plastic bags for archival purposes (Kapak/Scotchpak, Kapak Corporation, Minneapolis, MN, USA).

Large and small intestine

Intestines collected in rolls as described above need no further trimming at this point. Each segment should be carefully removed from its card backing, if rolled, and placed individually in a histocassette. If unrolled segments were collected, representative areas are cut in cross-section. These may be anatomical areas from each major section of the intestine or areas where there were grossly evident abnormalities (e.g. intestinal polyps from the multiple intestinal neoplasia mutant mouse, Apc^{Min}).

Stomach and caecum

These organs should be cut in half longitudinally. The stomach should be cut in a manner that presents both the oesophageal and duodenal openings. The caecum should be cut to show both the ileocaecal junction and the ampulla of the colon. Submit the half of each that best shows the desired features. If space allows, the caecum and stomach may be submitted in the same cassette. If lesions of the caecum or stomach are of interest, it may be important to submit both halves of these organs. If this is the case, each organ should be placed in its own cassette.

Liver (with gallbladder)

Cross-sections of only the left lateral lobe of the liver and the medial lobes with the gallbladder are sufficient for histology unless pathological changes are obvious in the accessory lobes. Lay the left lateral lobe flat on the trimming surface
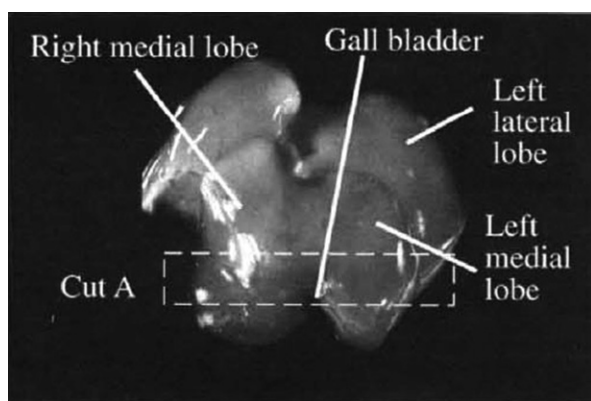
Figure 5.6.11 Example of trimming sites for a mouse liver. Left lateral lobe, left medial lobe, right medial lobe, gallbladder.

Figure 5.6.12 Anatomical location of kidneys and adrenal glands. Right kidney is trimmed transversely while left is cut longitudinally so that histologic sections can be identified. Lower panels illustrate features of cut sections.

and cut a crosswise section near the centre of the lobe. Trimming of the medial lobes must be a bit more precise, as they must be cross-sectioned to include a portion of the gallbladder in the section (Figure 5.6.11). This is usually accomplished by cutting across the medial lobes just below the juncture between the lobes, then just above that juncture, where the falciform and teres ligaments hold the two lobes together. The first cut should reveal a portion of the gallbladder. Mark the opposing side with a blue pencil and place both this section and the left lateral lobe section in the cassette.

Kidneys

The left kidney should be cut longitudinally down the centre and should include a segment of the adrenal gland, which was left attached to the kidney at the time of fixation. The right kidney will be identified by a small transverse incision, if handled properly at the time of fixation. It should be presented in a lateral cross-section, cut through the central area near the renal pelvis (Figure 5.6.12). Use the blue pencil to mark the side of this section furthest from the pelvis, as it is important to present the area nearest the centre of the kidney to the pathologist.

Spleen and pancreas

Unless the pancreas is collected separately for a focused study, the spleen and pancreas are fixed as a unit. Trim them together in cross-section at any point along the length of the spleen. To collect the

pancreas in order to view pancreatic beta cells stained by aldehyde fuchsin, remove the entire pancreas (from the head near the duodenum to the tail near the spleen) and spread the pancreas out. This is to keep the tissue extended during fixation. Set the pancreas in a labelled cassette, place it in a container with Bouin's solution and fix for 16–24 h at room temperature. After fixation, place cassettes in a large beaker with tap water and rinse gently overnight. It is important that most of the picric acid be diffused out of the tissue as failure to do so can result in acidification of the 70% ethanol when the tissue is being dehydrated when it is paraffin embedded. The picric acid-acidified alcohol extracts insulin from the beta cells, affecting the aldehyde fuchsin staining reaction and causing it to fail. Once the rinse is complete the tissues should be brought to the histology department for processing. If necessary tissues can be stored in 70% ethanol for a month.

Lungs

The lungs are collected with the heart and thymus as a unit at the time of necropsy, but are submitted separately for histology. Using a pair of wooden forceps, gently push apart the heart and lungs so that the lungs lie out flat.

Cut a longitudinal section from the centre of the lobes of the lung on each side. The individual lobes will separate, but should all be collected and placed in the same cassette. There is generally no need to separately identify each lobe of the lung, unless gross lesions are evident.

Heart and thymus

Carefully remove any remaining lung or tracheal tissue from around the heart, being certain not to separate the thymus from the heart. Place the heart on its base and begin the cut at the heart's apex. Angle the cut so that it bisects each of the four chambers of the heart. In some instances, the great vein of the heart (vena cordis magna) may be visible on the epicardium of the fixed heart. Making a cut along the line of this vein will often bisect the chambers properly (Figure 5.6.13). If space allows, both halves of the heart may be submitted. If only one half is submitted, ensure that it contains a portion of the thymus.

Salivary glands

Trim the base of the salivary glands to present a clean-cut edge. Make the second cut approximately 4 mm from this first cut. Mark the face of the second cut with a blue pencil and place the cross-section in the cassette.

Trachea and thyroid/parathyroid

Cut a cross-section of the trachea at the point where the thyroid and parathyroid glands are attached. This is located in an area 1–2 mm below the epiglottis.
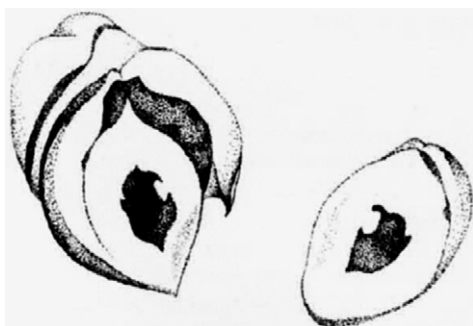
Figure 5.6.13 The heart is trimmed lengthwise to include all four chambers.

Lymph nodes

There are many lymph nodes located throughout the body, but it may not be necessary to submit all of these for processing unless involved in the disease process. Representative nodes from several areas may be chosen (i.e. mesenteric, axial, inguinal and cervical). If not enlarged, lymph nodes may be submitted whole, after the surrounding fat has been removed. Severely enlarged lymph nodes must be cut in cross-section.

Urinary bladder

The urinary bladder is often fixed as a unit with the reproductive organs. A lengthwise cut made down the centre of the urinary bladder should also include the uterine body, vagina and cervix in the female, and the prostate, bulbourethral glands, penis and prepuce in the male. After this first cut is made, trim the opposite side of one half of the tissue to the appropriate width for a cassette (approximately 4–5 mm), cutting off the uterine horn or seminal vesicle and any excess adipose tissue. Mark this side with a blue pencil and place the section in a cassette. If the urinary bladder is collected separately (see section on 'Necropsy procedure'), it should be trimmed in cross-section or longitudinally under water to prevent spraying of fixative and excess deformation of tissues.

Reproductive organs

Female

Cut one ovary away from either uterine horn, trim away any excess fat and place the entire ovary and its associated uterine tube in a cassette. Place a cross-section of one of the uterine horns into the same cassette. Trimming of the uterine body, vagina and cervix is discussed above with the urinary bladder (Figure 5.6.14).

Male

Separate the testes from the card on which they were fixed. The testes and epididymides should be slightly flattened and lie in the same plane on the surface that was attached to the card. Mark the rounded side of the testis with a blue
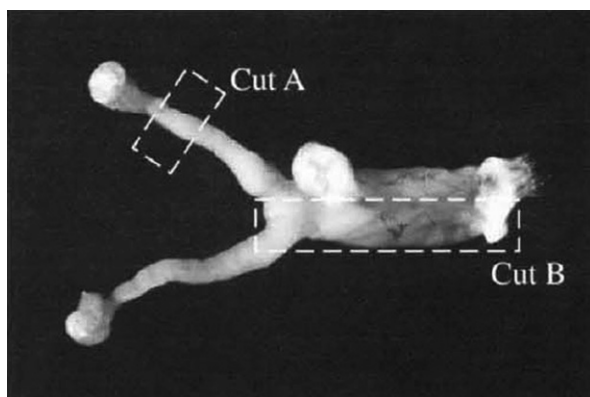
Figure 5.6.14 Female reproductive tract. Dotted lines indicate where to cut tissue for histological processing.

Figure 5.6.16 Trimming sites marked for sectioning a mouse brain.

pencil and place into a cassette. Place a cross-section from one side of the seminal vesicles into the same cassette. The penis, prepuce and accessory organs are discussed above with the urinary bladder (Figure 5.6.15).

In both male and female, all reproductive organs, as well as the urinary bladder, may be submitted in the same cassette.

Clitoral/preputial glands

The clitoral glands of the female mouse are normally small, and embedded in a segment of inguinal fascia and fat. This segment should be cut in cross-section and embedded on edge. It may be submitted in the same cassette as the reproductive organs. The preputial gland of the male mouse is larger and is collected individually. It should be cut in cross-section as well and submitted with the reproductive organs.

Figure 5.6.15 Male reproductive tract. Dotted lines indicate sampling sites for histology.

Brain

The brain is submitted to the histology laboratory cut in three cross-sections rostral to caudal (Figure 5.6.16). The first cut should be made through the cerebrum, approximately 1-2 mm from the most rostral surface. The second cut through the cerebrum, 2-4 mm from the first, will create the first section. This first section should present a view of the central portion of both cerebral hemispheres, so mark the face of the *first* cut with a blue pencil. The third cut should be made 2-4 mm from the second cut, just to the cerebral side of the confluence of sinuses. This will create the second cross-section, of which the face created by the *second* cut should be marked with a blue pencil. A fourth cut should be made at the transverse sinus to separate the remaining portions of the cerebral hemispheres from the cerebellum, after which a fifth cut is made which approximately bisects the cerebellum laterally to create the third section. On this section, the cut face closest to the cerebrum should be marked with a blue pencil. Alternatively, serial fine coronal sections can be obtained using one of a variety of Brain Matrices (Kent Scientific Corporation, Torrington, CT, USA). The brain should be submitted in an individual cassette.

Tongue

The tongue should be cut longitudinally down the midline. It is usually only necessary to submit half of the tongue to histology.
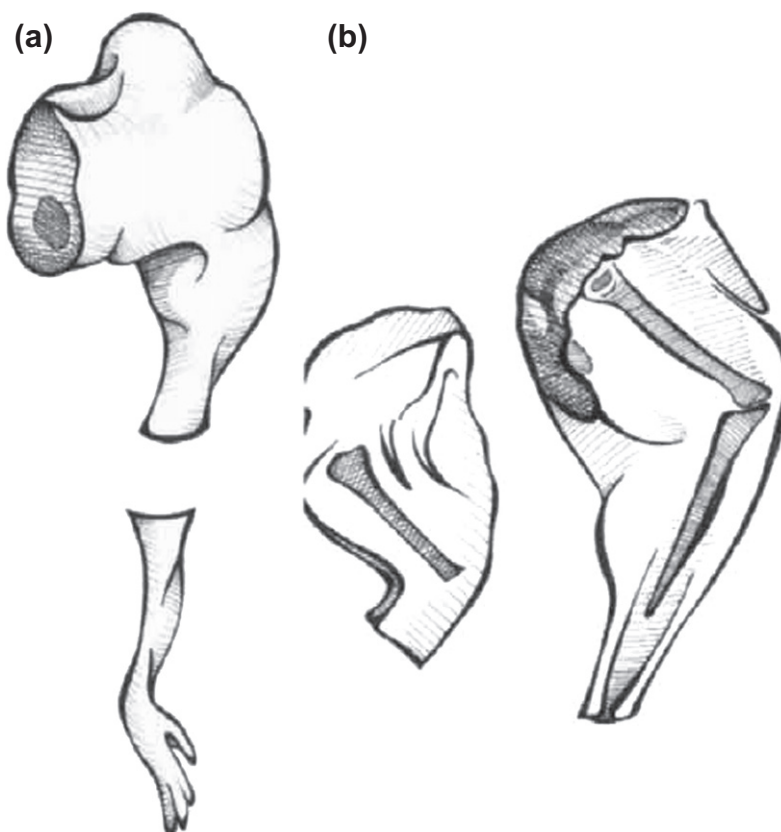
Figure 5.6.17 (a) Preliminary preparation of leg includes removal of skin and amputation of distal segment as indicated. (b) Decalcified limb cut lengthwise to expose joints.

Legs (long bones)

One each of the fore and hind legs should be cut longitudinally to show the long bones and major joints of each leg (Figure 5.6.17). Excess fat should be trimmed away; on the hindlimbs it may be necessary to trim away some of the bulk of the muscle in order to fit the section properly into the cassette. Feet should also be separated from the leg, and submitted separately. The longitudinal cut should be made using the major joint of each leg as a reference to bisect the long bones. Choose the half of each leg that best shows the desired view of the bones and place it in its own cassette.

Feet

One each of the front and back feet should be cut longitudinally to show the skin, foot pads and bones of the feet. The front foot should be cut so two toes are present on each half. The back foot should be cut directly through the middle toe on that foot. Each foot is placed into a separate cassette. Both halves of each foot may be sent to the histology laboratory.

Spinal column

The spinal column should be trimmed to present both the cross-section and longitudinal section of the thoracic and lumbar regions. First, cut the spine laterally (cross-section) between the 13th thoracic and first lumbar vertebrae (just below the 13th rib). Next, cut a lateral section 4-5 mm in width, from the distal end of each section. Bisect the remaining long segments longitudinally, placing the best half of each into a separate cassette with its related lateral section.

Skull

The skull should be cut into three cross-sections, similar to the brain. The sections should show the eyes, nasal passages, ear canals and pituitary gland (Figure 5.6.18). The first cut
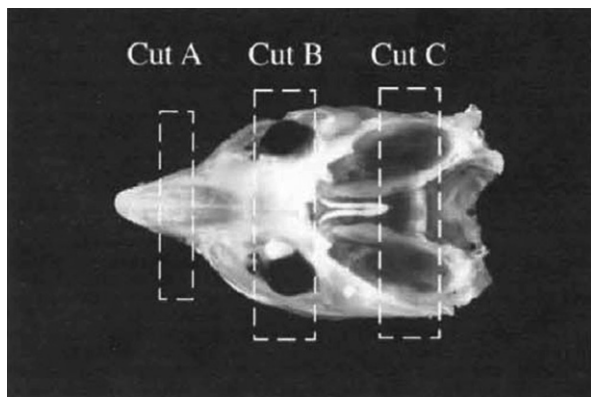
Figure 5.6.18 Three sections are cut in the decalcified skull. This exposes (A) the nasal cavity, (B) eyes and associated glands, and (C) inner, middle, and external ear as well as the pituitary gland.

Figure 5.6.19 Skin of the head trimmed to study (A) muzzle and vibrissae, (B) eyelids, cilia, meibomian gland, and conjunctiva, and (C) pinna of the ear.

should be made through the posterior edge of the pituitary, identified as a whitish mass located in the area between the occipital bone and basisphenoid bone on the inner surface of the skull. This is the crucial cut, and should present a view both of the pituitary and the middle ear. The second cut may be made 4–5 mm anterior to the first cut to create the first section. The third cut should be made through the posterior edge of the visible portion of the eyes, with the following cut made just anterior to the eyes. The third section should present a view of the sinuses, and may be cut from the approximate centre of the remaining portion of the snout. Each section should be marked with a blue pencil on its anterior surface. All sections of the skull may be submitted in the same cassette.

Skin

Trim portions of the dorsal and ventral skin longitudinally in the direction of the hair growth, into pieces approximately 0.3 × 1.0 cm (Figure 5.6.19). Cut 2–3 sections of both dorsal and ventral skin in this manner and mark one long edge with a blue pencil to indicate that the pieces should be embedded on the opposite edge. Depending on the focus of the study, you may also want to cut a piece of skin approximately 0.7 × 0.7 cm square to be submitted horizontally, haired side down, to view the hair follicles in horizontal section. Mark the

underside of the section with a blue pencil. Tail skin should be trimmed in the same orientation as the longer pieces of dorsal and ventral skin. Cut the section from an area that was not handled as the tail skin was removed from the bone, and mark one long edge with a blue pencil. Eyelids may be presented by making a cut bisecting both lids then making a second cut just posterior or anterior to the corners of the eyelids so that the upper and lower eyelids remain attached to one another. Mark the face of the second cut with a blue pencil. A section of the muzzle skin may be obtained by making a cut approximately 3–4 mm in from the front edge of the muzzle (Figure 5.6.19). Mark the outer, uncut, edge with a blue pencil. Trim a section out of one ear by first cutting one of the ears in half lengthwise and then cutting one of the halves completely off from the scalp. Lay this half flat on the cutting surface and make a second cut parallel to the first to obtain a section similar in size and shape to that of the dorsal and ventral skin sections. Put a blue mark on one of the long sides of the section of ear. The various skin sections may be combined in cassettes. However, the same sections should be combined every time (refer to Table 5.6.1) to aid with identification. We combine the long sections of dorsal skin with the sections of ear and tail skin in one cassette; long sections of ventral skin with the sections of eyelid and muzzle in a second cassette, and each of the square sections of dorsal and ventral skin in individual cassettes. Each cassette is also labelled with a 'D' or 'V' to aid in identification.

Routine histological staining

Haematoxylin and eosin (H&E) stain is usually requested on all tissues sent to the histology laboratory. Special stains may be requested on specific tissues if this is necessary to verify any suspected pathologic changes not sufficiently disclosed by the H&E stain. Protocols for various stains, what they stain, and what colours they stain are subjects of various histology and pathology textbooks [46, 47].

Skeletal staining of whole mice

The alizarin staining technique is an old technique [48] that has been extensively used to investigate skeletal abnormalities in various stocks of mutant [49, 50] and genetically engineered [51] mice. This technique has the advantage of being performed on whole bodies, which provides excellent three-dimensional visualization of skeletal lesions. The bones are stained red while the other tissues do not stain and are translucent to pale blue. Continual improvement in X-ray technology has reduced the usage of alizarin staining. However, this technique still provides incomparable three-dimensional representations of the skeleton and is still extensively used, especially for animals with a minimally mineralized skeleton, such as fetuses and young mice. Also, it has the potential to be used to assess the number and location of skeletal metastases. The techniques presented here are modified from P. B. Selby [52].

The mouse should be skinned, except for the skin below the digits to avoid damaging them. The mouse should be eviscerated. Any subcutaneous, retro-orbital, mesenteric, and mediastinal fat, as well as the trachea, oesophagus, salivary glands, tongue, and eyes should be removed. The mice should not be fixed but may be frozen.

Five working solutions are used (Table 5.6.3). Deionized water is used in all solutions. Solutions A, B, and E can be stored indefinitely. Solutions C and D should be discarded when they precipitate, which may start a few weeks after preparation.

The mice can be stained in 3 days (this works best when mice are 6–9 weeks old), or in 11–14 days (when mice are more than 12 weeks old). The bones of mice stained with the 3-day procedure may be brittle, especially with mice older than 12 weeks. The staining procedures are listed in Table 5.6.4.

Water rinses are with tap water. Room temperature should be 20–25 °C. Time in solution C should always be 2 days. Times in solutions A, B and E can be varied to accommodate the working schedule.

Conclusions

A research-quality necropsy and interpretation of gross and microscopic changes requires a great deal of skill on the part of both the technician and the pathologist. The two need to work together to coordinate their efforts and to optimize protocols to achieve consistent, high-quality results. This chapter provides an overview on how to achieve these results but practice is required to develop the skills.

Acknowledgements

TABLE 5.6.3: Working solutions for skeletal staining	
Solution A	1% (w/v) aqueous KOH
Solution B	2% (w/v) aqueous KOH
Solution C	1.9% (w/v) aqueous KOH containing 0.040 g/L alizarin red S
Solution D	1.6% (w/v) aqueous KOH containing 0.033 g/L alizarin red S
Solution E	Clearing solution made of 400 mL white glycerine, 200 mL benzyl alcohol, and 400 mL 70% ethanol
w/v, weight/volume.	

TABLE 5.6.4: Staining procedures

3 DAY PROCEDURE

Day 0	1. Skin and eviscerate specimen 2. Cover specimen with solution A. Use 0.5% aqueous KOH solution if mice are 3–5 weeks old, and 2% aqueous KOH solution for mice 10–17 weeks old
Day 1	1. Pour out solution; rinse jar and carcass with water 2. Remove any loose fat and muscle 3. Cover specimen with solution D
Day 3	1. Pour out solution; rinse jar and carcass with water 2. Clean off tail and immerse specimen in solution E 3. After at least 3 h, heat at 45 °C for 1 h in a water bath 4. Pour out solution; drain jar and specimen, do not rinse 5. Cover specimen with white glycerine

11–14 DAY PROCEDURE

Day 0	1. Skin and eviscerate specimen 2. Cover specimen with solution A
Day 1–2	1. Pour out solution. Rinse jar and carcass 2. Cover specimen with solution A
Day 4–7	1. Pour out solution. Rinse jar and carcass 2. Cover specimen with solution B
Day 6–8	1. Pour out solution. Rinse jar and carcass 2. Remove loose fat and muscles and clean tail 3. Cover specimen with solution C for 2 days
Day 8–10	1. Pour out solution. Rinse jar and carcass 2. Cover specimen with solution E
Day 11–14	1. Pour out solution; drain jar and specimen, do not rinse 2. Cover specimen with white glycerine

(AR047204, AR049288, AR054407, AR056635, CA034196, CA089713, and RR17436).

Appendix: Examples of commonly used fixatives for mouse histopathology

Fekete's acid alcohol formalin (Tellyesniczky's/Fekete's solution)

This is a commonly used fixative in mouse laboratories [10, 31, 36]. It provides rapid and surprisingly deep tissue fixation. Specimens are transferred to 70% ethanol following overnight fixation, after which they are trimmed and processed. This fixative yields high quality specimens in histological sections. Long-term storage can be a problem since ethanol is flammable and evaporates easily, which can render specimens useless. An artefact is that erythrocytes (red blood cells) are leached so that they appear only as pink ghosts within vessels. Of the commonly used fixatives, Fekete's acid alcohol formalin is the best general-purpose fixative for maintaining epitopes for immunohistochemistry [36].

Bouin's solution

This fixative uses picric acid (which stains everything permanently yellow), acetic acid and

formalin. Delicate detail is not often well preserved but this fixative is preferred by some pathologists and researchers. Penetration in tissue is moderate. Bouin's-fixed specimens must be washed in running tap water for 2–4 h after initial overnight fixation and stored in 70% ethanol or they become very brittle. Bouin's solution can be used for bone decalcification since the acids will demineralize specimens that are left in the fixative for several days or weeks. If used for decalcification, the Bouin's solution must be changed weekly to optimize demineralization.

Neutral buffered 10% formalin

This is the most commonly used fixative in most pathology laboratories and is the fixative of choice for participants in the Human Mouse Models Cancer Consortium [53]. Specimens can be fixed overnight and left indefinitely in the fixative. Specimens can be processed at any time as long as they remain wet, sometimes many years after initial collection. This fixative is particularly useful for retrospective evaluation of lipids or other substances that are soluble in ethanol and would be lost during tissue processing. For example, the presence of fat in adipocytes can be demonstrated if you take wet tissue fixed in neutral buffered 10% formalin, trim the tissue, cut frozen sections, and stain the sections with oil red O or other lipid histochemical stains. Most of the other fixatives are alcohol-based and remove lipid.

Long-term storage in neutral buffered 10% formalin causes continued cross-linking of amino groups resulting in changes in the tertiary structure of proteins. As a result, many antigenic epitopes are lost or changed, making immunohistochemistry problematic. Transfer of tissues into 70% ethanol after overnight (12 h) fixation can reduce but not eliminate this effect [36].

Neutral buffered 10% formalin is noxious and should be used in a fume hood. Buffering is needed to reduce acid haematin formation, an artefact of fixation.

B5 fixative

This fixative is used for immunohistochemistry and often yields the most accurate and reproducible results of any fixative. It is difficult to use because it has to be prepared immediately before use, is based on mercury salts (difficult to dispose of), and fine precipitates can form (a particular problem when using Gomori's methenamine silver or similar stains). Tissues fixed in B5 may be difficult to cut, and sections require treatment with Lugol's iodine to remove pigments.

Carnoy's fixative

This treatment fixes tissues rapidly. It preserves glycogen and enhances the staining of mast cell granules. Nissl granules are also well preserved. Because it is an alcohol/acid-based fixative, it lyses red blood cells and acid-soluble granules.

4% Paraformaldehyde

This is used as a fixative for electron microscopy and *in situ* hybridization and has become popular as a general histological fixative in laboratories that employ these techniques. The paraformaldehyde should be prepared in a buffered solution at pH 7 and refrigerated until use. It will keep for several weeks this way. It is one of the least useful fixatives for maintaining epitopes for immunohistochemistry [36].

Zinc-based fixatives

A variety of proprietary fixatives based on variations of the classic fixatives have been developed and marketed not so much as fixatives for routine, high-quality histopathology but rather to optimize antigenic epitopes for immunohistochemistry [54]. Such fixatives are the basis for large-scale tissue arrays that provide large numbers of small tissues on individual slides. However, a variety of proprietary zinc-based fixatives (such as IHC Zinc, BD Biosciences PharMingen, San Diego, CA, USA) yield good immunohistochemical results while providing adequate fixation for histopathology.

Glutaraldehyde

This fixative is commonly used for ultrastructural studies. Tissue penetration is minimal,

approximately 1 mm on any cut surface, so specimens have to be finely minced with a sharp razor blade to achieve adequate fixation. Several different buffers can be used. The most common are phosphate- and cacodylate-based buffers. Phosphate buffers are safe and yield good results when used fresh. Fine electron-dense precipitates may form that will render a specimen useless if the buffer used is old. Cacodylate buffer is arsenic-based, which is toxic and can be difficult to dispose of properly [55].

Karnovsky's fixative

This fixative is used for plastic embedding for transmission electron microscopy. Karnovsky's is a general term for any fixative combining glutaraldehyde and paraformaldehyde in a phosphate buffer. Glutaraldehyde has minimal penetration ability. Paraformaldehyde penetrates deeper, but fixation is unstable. Karnovsky's fixative combines the positive points of both these chemicals.

JB4 fixative

This fixative is used for plastic embedding for 1 μm sections used in light microscopy. It combines glutaraldehyde and paraformaldehyde in a cacodylate buffer [55].

O.C.T. Compound

O.C.T. Compound is an embedding medium for frozen sections. It is a thick, clear fluid used in conjunction with plastic base moulds such as CMS Tissue Path-Disposable Plastic Base Molds (Curtis Matheson Scientific, Inc. Houston, TX) to bind fresh tissues for freezing and sectioning. Tissues are floated in the mould and placed on dry ice. The O.C.T. solidifies and turns white as if freezes, after which the block has to be stored frozen at −80 °C until sectioning.

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Laws, Guidelines and Policies Governing the Use of Mice in Research

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Introduction

The mouse (*Mus musculus*) is the most commonly used mammal in the laboratory. This use has increased in recent years with the development of techniques to successfully modify the mouse genome. A number of laws, guidelines and policies exist to protect animals, including mice, from unnecessary pain or distress as part of the research process. Additionally, regulations exist which provide guidance on other aspects such as the transportation of mice, both locally and internationally, and the prevention of the spread of disease. It is very important for all those engaged in research with mice to be familiar with these instruments and to ensure full compliance.

General principles

A global framework

Over the last quarter of a century, two significant documents have created a global framework for

the regulation of the use of animals in research. The first, developed in 1985 by the Council for International Organizations of Medical Sciences (CIOMS), is the *International Guiding Principles for Biomedical Research Involving Animals* [1]. CIOMS is a non-governmental, non-profit organization established jointly by the World Health Organization and the United Nations in 1949 and represents a substantial proportion of the biomedical scientific community. These principles for animal experimentation were, in part, created because national and international ethical codes and laws mandated that new substances or devices should not be used for the first time in human beings unless previous tests on animals had provided a reasonable presumption of their safety. The principles have provided a framework for ethical animal use for the last 25 years.

The second document is Chapter 7.8 of the Terrestrial Code of the World Animal Health Organization (OIE), which covers the use of animals in research and education [2]. This chapter, approved by the OIE Code Commission and General Assembly in May 2010, provides advice and assistance to the 178 OIE member countries and territories when formulating regulatory requirements. It recommends that members should address all the essential elements identified in the chapter in formulating a regulatory framework that is appropriate to their local conditions and it accepts that the framework may be delivered through a combination of national, regional and institutional jurisdictions and that both public and private sector responsibilities should be clearly defined.

Meanwhile, the 1985 CIOMS International Guiding Principles are in the process of being revised by an *ad hoc* committee of experts which began by collecting statements, guidance and principles regarding the humane care and use of animals in research, testing and teaching from 33 national and professional societies. The resulting draft [3] is designed to assist ethics committees, animal care committees, organizations, societies and countries in developing programmes for the humane care and use of animals in research and education, especially those operating without federal or national regulations.

Hence the combination of the OIE chapter and the revised CIOMS principles can be expected to deliver sound guidance on the development of an appropriate regulatory framework for any country, no matter how developed its scientific expertise.

Alternatives: implementing the Three Rs

William M. S. Russell and Rex L. Burch first presented the concepts of the 'Three Rs' in a book entitled *The Principles of Humane Experimental Technique* [4]. Although the book was published in 1959, its principles were not universally accepted for over 30 years. Now, the Three Rs form the basis for the guidelines and regulations governing the use of laboratory animals throughout the world. The Three Rs represent replacement, reduction and refinement of animal use.

1. *Replacement* may be absolute, whereby non-animal methods such as *in vitro* methods, or computer models are used, or it may be relative, where animals on a lower phylogenetic scale are used, such as *C. elegans* or zebrafish rather than a mouse or a non-human primate.
2. *Reduction* describes methods for obtaining comparable amounts of information from fewer animals, or for obtaining more information from a given number of animals, so that in the long run fewer animals are needed for a given purpose. Reduction relies on proper experimental design and the use of appropriate statistics in data analysis.
3. *Refinement* pertains to methods that minimize or eliminate pain and distress and that enhance animal well-being. Such methods include appropriate use of anaesthesia and analgesia, optimal veterinary care and monitoring and environmental enrichment to promote species-specific behaviour.

The Three Rs govern the thought process by which animal experimentation is planned and by which protocols involving animals are reviewed.

Environmental enrichment

Greater knowledge of animal behaviour has increased the importance of environmental enrichment for laboratory animals. Environmental enrichment will differ depending on the species, but generally involves the inclusion of

sensory stimuli, or housing complexity, to foster species-specific behaviour. Examples may include foraging challenges for non-human primates, availability of nest-building materials for rodents, toys or bones for dogs, perches for cats, etc. These items promote psychological well-being and help to prevent boredom, stereotypic behaviour and fighting with conspecifics. They allow animals some control over their environment and may serve to help animals cope with environmental stressors [5] (see Chapter 4.3). In the last decade, more attention has been paid to environmental enrichment for mice and its effects on mouse physiological parameters. For example, mice housed in cages with a variety of enrichment devices, such as ladders, tunnels and running wheels, showed decreased anxiety-like behaviour and higher activity in behavioural tests, as well as attenuated stress responses and enhanced natural killer cell activity [6]. Several studies have also shown that including nesting material and deep bedding allows mice to better control their temperature (see [5] and Chapter 4.1).

Principles of ethical review

Many countries have regulations or guidelines relating to ethical review of proposed research projects. Usually these apply to the objectives of the review, legal requirements, the scope of work reviewed and general principles for the process including factors to be considered. The participants in the review process, and whether the review should be performed at a local institutional, regional or national level, are usually defined. The need for ongoing review after initial authorization is recognized and the concept of a formal retrospective review at the end of the project is becoming more common.

FELASA elicited information about ethical review in 20 European countries through a questionnaire and published a synopsis of the results with a series of recommendations [7, 8]. These included the concept of a cost-benefit assessment in which the harms likely to be caused to the animals should be considered with a view to reducing them, and that the quality of justification for such work should also be considered, including the potential and likely benefits.

Perhaps the most comprehensive review of this cost-benefit assessment was reported by the UK Animal Procedures Committee [9], which identifies the factors to be considered and how the practice and process can be enhanced so that it can be, and be seen to be, more critical and comprehensive. Nevertheless, the report acknowledges the challenge to all involved in the field to make progress in this difficult area.

A subsequent review by the Nuffield Council on Bioethics [10] acknowledged that the cost-benefit assessment was not merely a role for ethical review committees and regulators, but that all those involved in study design and implementation were responsible for setting out the costs and benefits of their research. There should be active and continued scrutiny of the costs and benefits from all those involved, before, during and after research.

The concept of postapproval monitoring was the topic of an *ILAR Journal* issue in 2008 [11] and the impact of regulatory burden on the research enterprise was also considered by Haywood and Greene [12].

Management of pain and distress

A key to achieving optimal welfare in laboratory animals is the minimization or elimination of pain and distress. Ensuring that alleviation of pain is a primary concern of those working with laboratory animals is key to engendering public support for animal research. Pain, as defined by the International Association for the Study of Pain, is 'an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage' [13]. Fundamental to alleviating pain in animals is the ability to recognize its occurrence. In mice, the signs of pain are often difficult to ascertain; thus close attention to their behaviour may be necessary to detect subtle changes. A recent controversial study [14] reported the development of a mouse grimace scale, which provides a standardized behavioural code to assess pain levels in mice. This system is similar to facial action evaluation systems in humans. While purposefully inflicting pain on mice to develop a system to assess pain has been criticized, the

mouse grimace approach may ultimately provide the best information about assessing pain in mice and may lead to major changes in the conduct of pain research and to animal husbandry in general [15]. However, until a more definitive system for assessing pain in mice is adopted, the fall-back position is that if a procedure is likely to cause pain in humans, it should be assumed that it would also cause pain in mice, e.g. postoperatively [16] (see Chapter 5.4).

Distress is more difficult both to define and to recognize. It may be considered an aversive state in which an animal fails to adapt to a significant level of stress and it may cause pathological or behavioural changes in the animal. Improper housing conditions, such as singly housing normally social animals for a prolonged period of time, may cause distress, as may chronic pain. Following the Three Rs is the best way to avoid distress in animals [17].

Regulatory balance

The regulatory balance is a guiding principle that can be illustrated as a Venn diagram of three overlapping circles [18]. In any regulatory system, it is essential to ensure that bureaucracy and rules do not become so burdensome as to inhibit scientists from developing good-quality scientific proposals that will address important research questions. However, it is also important to ensure that animals do not suffer unnecessarily in such

Figure 6.1.1 The regulatory balance for the use of animals in research [18].

research projects. Thus, there needs to be a balance between the needs of science and the needs of the animals (Figure 6.1.1). Furthermore, there is strong evidence to show that good animal welfare leads to good scientific outcomes (the overlapping area A).

It is this balance between science and welfare that provides the public with confidence in the regulatory system. The public wants to benefit from scientific advances, but also wants to be reassured that animals are not suffering unnecessarily (the overlapping areas B and C in Figure 6.1.1). The nature of this balance will differ between different countries, taking into consideration their diverse cultural, economic, religious and social factors. However the guiding principle of the regulatory balance can be fruitfully applied in any country in determining an appropriate approach to regulation of animal use in research.

Adequate veterinary care

Many governmental, professional and nongovernmental organizations have issued guidelines for adequate veterinary medical care for laboratory animals [19, 20]. Such care is generally considered to be an essential and integral part of animal care and use programmes. The guidelines generally provide standards for qualifications of veterinarians working in laboratory animal research programmes, their authority in oversight of facilities and experiments and their unique and special role in reviewing protocols involving laboratory animals. The training required for a qualified veterinarian to deliver adequate veterinary care has recently been reviewed [21]. The United States, Canada and the European Union (EU) all require that veterinarians working in laboratory animal facilities have training or experience in the management of the species maintained in the facility.

The primary role of veterinarians in animal research is to monitor the health and well-being of the animals under their care, provide appropriate treatment to sick animals and ensure that euthanasia is administered properly. Health monitoring includes preventive care as well as handling disease outbreaks in the facility. The veterinarian also oversees the appropriate use of anaesthetics, analgesics and other

pharmaceuticals, reviews and approves surgical, postsurgical and postprocedural care, and should have the authority to apply euthanasia to an animal that is experiencing severe pain or distress (in consultation with the investigator performing the study) [22, 23].

Inspection and compliance monitoring

The primary purpose of inspection is to monitor for non-compliance with the regulations and to take appropriate action. Hence, most inspection systems are operated by governmental bodies. However, some inspectorates play a more wide-ranging role within the regulatory system, offering advice on appropriate care and use of animals, on government policies for animal use, for the implementation of the Three Rs and dissemination of best practice. Depending on the skills and experience of those recruited to the inspectorate, this wider role can be enormously valuable.

In addition, an inspectorate can play a significant role in advising on the ethical evaluation and authorization of projects. This has the advantage that inspectors who have reviewed project applications are able to then inspect those projects for non-compliance with a great degree of understanding.

Generally the nature of inspections falls into two categories. Firstly, those which occur relatively infrequently and involve a major audit of all aspects of the animal care and use programme. To be effective, these inspections are often announced in advance so that the staff of the establishment to be inspected can prepare appropriate documentation. Usually the inspection is performed by a team of two or three inspectors who may have clearly defined and diverse roles. A detailed report will normally be produced at the end of such an inspection. The report provides a snapshot of the status of the establishment at the time of the inspection. However, much can change between inspections without the knowledge of the inspectorate.

The second model of inspection is less frequently practised but possibly more effective. In this model, an inspector is assigned to an establishment and becomes familiar with the work at that place through frequent visits. Where a specific purpose for the visit has been defined, a visit may be announced. However, a significant proportion of visits will be unannounced, giving the inspector the opportunity to view the place under normal working conditions. An advantage of this type of inspection may be that the inspector gains a genuine familiarity with the place and the research. Some elements of a visit can take an audit approach, for example, if inspecting animal health records or records of training. Other elements can be more discursive, discovering through discussion and observation.

Whichever model of inspection is adopted, it is appropriate to base the frequency of inspection on an objective assessment of the risk presented by the establishment. Assessing the risk might include consideration of objective measures such as the number and species of animals used, number and severity of projects and history of compliance. But a major aspect should be an assessment of the culture of the place and the quality of management. This will inevitably be subjective but should consider a list of factors including the effectiveness of local veterinary and animal care services, the operation of the local ethical review committee, the quality of communication between all those involved in the animal care programme, the quality of facility maintenance, the status of training plans and records, along with any contingency plans.

Accreditation schemes

There are two major bodies that accredit laboratory animal care programmes: the Canadian Council on Animal Care (CCAC) and the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). The CCAC, founded in 1968, is an accrediting body that limits itself to institutions located in Canada and will be described under Canadian regulations later in the chapter. AAALAC is a non-profit organization founded in 1965 that accredits programmes globally. AAALAC bases its accreditation process on compliance with the US *Guide for the Care and Use of Laboratory Animals* [5], national laws, regulations and policies and other scientifically based standards called 'reference resources' that address specific subject areas.

Accreditation, which is voluntary, includes an extensive review of the organization's laboratory animal care programme and an announced site visit performed by members of AAALAC's Council on Accreditation (or adjuncts) every 3 years. An annual report is also required. If an institution does not conform to AAALAC's standards, it will receive a notification that full accreditation has not been granted, and must provide a timeline for correcting deficiencies. If these deficiencies remain uncorrected, accreditation may be revoked. A full description of AAALAC's international programmes is available at their website [23].

Training

The importance of adequate training for all those involved in animal care and use is underscored by the emphasis it receives in regulations and policies in most jurisdictions. For example, training is a prominent theme in the European Directive (2010/63/EU) on the protection of animals used for scientific purposes (European Commission, 2010) and in the US *Guide for the Care and Use of Laboratory Animals* [5, 24].

Over the last decade there have been moves towards both regionalization and internationalization of training standards. A particularly strong example is the development of training guidelines across Europe. These were based on the Council of Europe Resolution on education and training of persons working with laboratory animals [25], which defined four categories of people working with laboratory animals and the broad principles of their training needs:

1. Category A: persons taking care of animals
2. Category B: persons carrying out procedures
3. Category C: persons responsible for directing or designing procedures
4. Category D: laboratory animal science specialists.

The Federation of European Laboratory Animal Science Associations (FELASA) has since elaborated on the training requirements for each of these categories to produce training guidelines together with an accreditation scheme for provision of this training [26]. However, the relatively inflexible nature of these guidelines has recently been questioned and, under Directive

2010/63/EU, proposals are emerging from bodies, including FELASA, for a more tailored approach to training which considers the individual needs of each trainee, and uses modern open-source methodologies to deliver quality training to meet those needs.

In the USA, the Animal Welfare Regulations and PHS Policy require institutions to ensure that people caring for, or using, animals are qualified to do so. The Animal Welfare Regulations stipulate a number of key topics that must be included in the institution's training programme.

The US *Guide* [5] urges that adequate training should also be provided to members serving on the Institutional Animal Care and Use Committee (IACUC) so that they can appropriately discharge their responsibilities. In addition, it recommends that the professional and technical personnel caring for animals should be trained, as should investigators, research technicians, trainees (including students) and visiting scientists. It also endorses training in occupational health and safety, in procedures that are specific to an employee's job, and in procedures specific to research (e.g. anaesthesia, surgery, euthanasia, recognition of the signs of pain and/or distress).

The focus on training standards with open-source access and flexible delivery is also seen in the training of those who care for animals. The UK Institute of Animal Technology (IAT) has revised its traditional certification programme to offer a unit-based framework for animal care training across Europe [27]. Diploma-level programmes in husbandry, as well as science and technology, are offered in four languages with open-source access and clear learning outcomes. With these modules, IAT is influencing training across Europe through the European Federation of Animal Technicians (EFAT). Similar approaches are being offered by the American Association for Laboratory Animal Science across the Americas.

Laboratory animal veterinarians have, for many years, recognized the speciality of laboratory animal medicine through the creation of specialist colleges such as the American, European, Japanese and Korean Colleges of Laboratory Animal Medicine. Each college sets standards for the achievement of specialist status through experience and examination. More recently, there have been moves to share

standards for specialization by the formation of an International Association of Colleges of Laboratory Animal Medicine (IACLAM) [28].

Regulations especially relevant to laboratory mice

Genetic modification

The widespread use of genetically modified mice presents special animal welfare concerns principally because of unanticipated outcomes. The insertion or deletion of a gene into or from mice may result in phenotypic characteristics that compromise the health or well-being of the animals, for example immunodeficiencies or physical deformities that could prevent mobility and hence access to food. When a new genetically modified animal is developed, the first generation of animals should be monitored regularly up to adulthood to detect any possible adverse phenotypes that might result in pain or distress. If the animals are determined to be experiencing pain or distress resulting from genetic manipulation, they should be euthanized. If a newly created animal model is known to be compromised, the earliest humane end-point should be established so that data may be collected, but also so that the animals are not subjected to unnecessary pain and/or distress.

Regulations for performing genetic modification, maintaining colonies of genetically altered animals, and importing and exporting such animals, are complex. They vary between different countries and may be related to environmental risks as well as welfare concerns. It is critically important to ensure full understanding and compliance with all the local regulations before embarking upon any of these procedures.

Transportation and quarantine

Laboratory mice comprise a small but important part of the global transport of live animals. This is partly due to the large-scale commercial production of mice in specialized facilities with rigorous disease control measures; the animals then need to be moved to the research facilities in which they will be used. However, small groups of mice that have been genetically modified are increasingly being moved for collaborative purposes between research facilities. The general principles of transportation are the same in both situations, though lack of experience may make the latter particularly challenging.

Animal transportation includes the entire period from packing through dispatch, carriage, receiving and unpacking at the final destination. It is important to understand the biological needs of the animals as well as the regulatory requirements for proper documentation (e.g. veterinary certification) for both the consigning and receiving countries. This means drawing up a comprehensive journey plan, which includes allowance for contingencies if needed. For those who do not routinely transport groups of mice, it is strongly recommended to engage the services of a professional adviser and to follow a written set of procedures and checklist to ensure all eventualities have been considered.

In general, animals being transported should be in good health with minimal phenotypic abnormalities that may impact their welfare. It is generally considered unwise to transport pregnant mice during the last 10% of gestation, nor nursing litters, since dams may reject their pups under the stress of transport. Appropriate containers should be used which ensure provision of sufficient ventilation, protection from microbial contamination and prevention of escape. The animals' needs in terms of bedding, food and hydration should be addressed, as well as protection from extreme temperatures.

The IATA Live Animals Regulations have been generally accepted as guidelines for the air transportation of laboratory animals [29]. Other useful guidance for all modes of transport is available from the Institute for Laboratory Animal Research [30] and a comprehensive guide to transporting all laboratory species, including mice, is provided by White et al. [31].

Correct documentation is essential to avoid delays in transit. An Export Health Certificate, the format of which will be defined by the competent authorities of both the exporting and the receiving countries, will normally need to be signed by an official veterinarian. Other

documents may include import licences issued by the state veterinary service, invoices for customs purposes, authorization for transfer from bodies specifically regulating laboratory animal use and, in the case of ground transportation, vehicle registration details and insurance. Most countries have a limited number of designated locations where animals can enter the country. Here, animal consignments are inspected by officials and therefore may need to arrive during defined working hours or with prior warning.

The receiving institute should be alerted to the planned arrival time so that cages can be prepared in advance. On arrival, animals should be removed from their transport containers and examined without delay. A thorough description of the health status of the consigning colony should have been provided in advance to ensure that appropriate measures are taken to avoid animals introducing unwanted infections into the receiving colony. This may include quarantine for a period usually defined by the local veterinarian, during which animals will be observed for clinical signs and possibly tested for evidence of infections.

Regional laws, guidelines and policies

North America

USA

The earliest legislation protecting laboratory animals in the USA is the Animal Welfare Act, first passed by Congress as the Laboratory Animal Welfare Act in 1966, primarily to prevent the theft of pets and their sale to research laboratories. Authority for oversight of laboratory animals under this law was given to the US Department of Agriculture (USDA) [32]. The Animal Welfare Act was amended in 1970, 1976, 1985 and 1990 to broaden the scope of the law, including the mandate for establishing Institutional Animal Care and Use Committees to review and approve all protocols for laboratory animals. The 1970 amendment to the Animal

Welfare Act stated that an animal was defined as: 'any live or dead dog, cat, monkey (non-human primate animal), guinea-pig, hamster, rabbit, or other such warm-blooded animal as the Secretary may determine is being used, or is intended for use, for research, testing, experimentation, or exhibition purposes, or as a pet.' In this way, the Secretary of the Department of Agriculture was provided with the authority to determine which animals would be covered by the Act.

In 1977 the USDA promulgated regulations that specifically excluded rats, mice and birds from the definition of 'animal.' The Helms amendment to the 2002 Farm Bill specifically excluded rats (of the genus *Rattus*), mice (of the genus *Mus*) and birds from the Act. Because the USDA regulates only those species covered by the Animal Welfare Act, the passage of this bill into law removed USDA oversight of these species. Thus, for the purposes of this book, which is dedicated to the laboratory mouse primarily of the genus *Mus*, the Animal Welfare Act is not applicable.

Another piece of legislation safeguarding laboratory animals in the US is the Health Research Extension Act of 1985 that put into law the Public Health Service [33] Policy on the care and use of *all* vertebrate animals (including rats, mice and birds). This law was initially passed in 1973 and modified in 1979 and applies to all institutions that are funded by any branch of the PHS, including the National Institutes of Health (NIH), Food and Drug Administration (FDA) and the Centers for Disease Control and Prevention (CDC). Any institution receiving funding from the PHS is required to hold on file an Animal Welfare Assurance Statement with the Office of Laboratory Animal Welfare (OLAW) at the NIH. This statement avers that the institution abides by the *US Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research and Training* [34], which are largely based on the CIOMS principles [1] and the US *Guide* [5].

Like the Animal Welfare Act, PHS policy requires an institution to have an Institutional Animal Care and Use Committee (IACUC). The Assurance Statement must describe in detail: the animal care and use programme for the institution and include a list of all components of the institution including satellite facilities; the clear

lines of authority and responsibility for institutional oversight of the work, inclusive of a designated 'institutional official' (IO) who is ultimately responsible for the animal care and use programme; identification of a veterinarian(s) involved in the programme and his/her qualifications; a description of the occupational health and safety programme for relevant personnel; a synopsis of mandated training in humane animal care and use; and a description of the facility.

Organizations with an Assurance Statement must submit an annual report to OLAW that indicates any change in their status and the dates on which the semi-annual review of the programme and animal facilities occurred. The report must also detail any serious or continuing issue of non-compliance with PHS policy, any serious deviations from provisions in the *Guide* and/or any suspension of an activity by the IACUC. The Assurance is renegotiated with OLAW every 5 years. OLAW can approve, disapprove, restrict or withdraw approval of the Assurance. Approval by OLAW is often achieved by an institution obtaining AAALAC accreditation. NIH provides the oversight of the PHS policy, but does not perform regular inspections. An assured institution is required to self-report any issues of non-compliance to OLAW and continued non-compliance with the policy may result in withdrawal of funding to the institution.

PHS policy also describes in detail the characteristics of the IACUC. The IACUC must consist of at least five members, include one veterinarian with training and experience in laboratory animal science and medicine, one practicing scientist experienced in animal research, one non-scientist, and one individual not affiliated with the institution in any way except as an IACUC member (who may not be a member of the immediate family of anyone affiliated with the institution). The IACUC must review the institution's animal care and use programme every 6 months and perform a site visit of all animal facilities in the institution. The committee must provide a summary report and evaluation of these reviews to submit to the IO. They must also review any concerns they may have about animal welfare at the institution and make recommendations to the IO regarding any aspect of the programme.

The IACUC must also review and approve any proposed work that involves the use of animals. Specifically, the proposed protocol must contain a detailed description of the proposed work, including species, strain, sex, age and number of animals to be used; a justification for the use of the specified animals; information on the veterinary care of the animals; documentation that all those who will work with the animals are appropriately trained; an explanation of how pain, distress, discomfort and/or injury will be eliminated or minimized; and a description of the method of euthanasia that will be used as well as the reason for the chosen method. With regard to euthanasia, a justification must be provided if the proposed method does not conform to the current American Veterinary Medical Association's euthanasia guidelines [35].

The USA has been criticized for not regulating the animals that constitute the majority of research animals, i.e. rats and mice. Although there are undoubtedly some institutions that escape any regulation (i.e. those that only have rats and mice and do not receive any funds from the PHS), an estimated 95% of these animals are subjected to oversight by PHS policy.

There are other guidelines for animal use related to safety testing for drugs and chemicals, but these are more focused on standardization of protocols (Good Laboratory Practice or GLP) rather than specifying conditions related to animal husbandry and welfare [36, 37].

Canada

Canada has a quasi-regulatory peer review system for overseeing the use of animals in science administered through the Canadian Council on Animal Care (CCAC). CCAC, which was created in 1968 in response to public concerns about the use of animals in research, provides guidelines for the use of animals primarily through the two-volume CCAC *Guide to Care and Use of Experimental Animals* [38], but also through other specific guidelines and policy statements. At the local level, the Animal Care Committee (ACC) at each institution provides primary oversight for animal use. In addition to its guidelines programme, the CCAC also has an assessment and certification programme, somewhat similar to that of AAALAC International

whereby panels of experts conduct on-site reviews on a 3-year basis. Institutions that achieve a CCAC status of Compliance or Conditional Compliance are awarded a CCAC Certificate of Good Animal Practice (GAP). In addition to guidelines, assessment and certification, CCAC also has programmes in the Three Rs and in education, training and communications. The Three Rs programme promotes the development and implementation of reduction, refinement and replacement of animals, and the education, training and communications programme supports the development and implementation of education and training opportunities for CCAC constituents.

Europe

The use of animals in scientific procedures in Europe was, until recently, covered by two similar legal instruments. The first is the Council of Europe Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes [39]. However, ratification of the Convention is not mandatory and, thus far, less than half of the 47 members of the Council of Europe have ratified it. Perhaps the greatest impact of the Convention has been Appendix A, which describes standards in animal care and husbandry of research animals. This was updated in June 2006 [40] following several years of discussions informed by working parties comprising representatives of science, industry and animal welfare groups.

European Union

The second instrument, applicable in the member states of the European Union, is the EU Council Directive 86/609/EEC on the protection of animals used for experimental and other scientific purposes [41]. Directives must be implemented through national law by all the Member States of the EU (currently 27) and adopted by any new State that joins the EU. Annex II to Directive 86/609/EEC adopted much of the content of Appendix A of the Convention as guidance. In June 2007, following revision of Appendix A, a Commission Recommendation (2007 526 EC) replaced Annex II guidance with the revised Council of Europe guidelines

(Appendix A of Convention ETS 123) on accommodation and care of laboratory animals [42].

Directive 86/609/EEC provides a framework that Member States may choose to develop into more demanding regulations and guidance when implementing their own national provisions. This has led to considerable variation in the implementation across Europe and is perceived to have placed some Member States at a competitive disadvantage within the internal market. Consequently, in November 2008 the European Commission published its proposals for a new Directive with three principal aims:

1. To increase the focus on the Three Rs achieved through the regulatory system
2. To raise standards of animal welfare applied across Europe
3. To harmonize the regulations implemented in all Member States [43].

The Commission's 2008 draft was extensively debated in both the European Parliament and Council of Ministers, resulting in a text agreed by all three parties in November 2010 [44]. Among the key provisions of the new Directive (2010/63/EU) are the following requirements:

1. All places where animal research is to be done must be authorized by the national competent authority (CA), normally a government department. At each place a specified individual is responsible for ensuring compliance.
2. Other specified individuals include those responsible: for overseeing animal care and welfare; for access to information about relevant species; for ensuring appropriate training, supervision and competence; and a designated veterinarian who will advise on the well-being and treatment of animals.
3. Each proposed project must be authorized by the CA applying a harm-benefit analysis and for a maximum of 5 years. Many projects will require a non-technical summary to be published and a retrospective review to be performed on conclusion.
4. Project applicants must show they have applied the Three Rs, and humane end-points to avoid death as an end-point wherever possible, in all types of research. Within regulatory testing, alternatives to using animals are mandatory where an appropriate non-animal test is legally recognized in the EU.

5. Procedures, unless performed entirely under anaesthesia, must be classified according to severity: mild, moderate or severe. An upper pain threshold will apply which can only be exceeded with specific authority from the Commission.

6. Each place must appoint an Animal Welfare Body whose role includes a strong emphasis on ongoing implementation of the Three Rs.

7. The use of non-human primates is especially restricted, and only those bred in captivity will, in due course, be permitted for use. The use of great apes is banned unless specifically authorized by the Commission.

8. Reuse of animals in more than one procedure is restricted based upon the actual severity that has been experienced in earlier procedures.

9. Statistics on animal use will be collected annually and will be based upon retrospective reporting of the actual severity experienced by animals.

10. All Member States will have an inspectorate who will carry out inspections based upon a risk-assessed frequency. A minimum frequency is defined.

11. Minimum standards of care and accommodation will become mandatory in 2017. Thereafter, exemptions will only be permitted for scientific, animal welfare or animal health reasons.

12. Approved methods of humane killing are listed. Other methods must either be shown to be equally humane or justified in the project authorization.

13. All vertebrates are protected, as well as some invertebrates. Protection for mammals generally starts from two thirds of gestation.

14. Minimum requirements for education and training, including continuing professional development, are likely to be harmonized as the Commission has stated that free movement of staff throughout the EU is an objective.

15. Member States may apply stricter national measures, provided these were already in force in November 2010, when the new Directive entered into force. However they may not use these to impede the internal market.

The new Directive (2010/63/EU) requires all Member States to transpose its provisions into national legislation within 2 years and to commence implementation by January 2013. Hence this Directive will shortly become the regulation for animal care and use throughout the EU.

Meanwhile, the standards of accommodation and care in Appendix A of the Convention ETS123, in so far as they have been adopted into Annex III of the new Directive, will become the mandatory minimum standards for animal care and husbandry, possibly with extended Codes of Practice prepared by individual Member States to include some of the additional advisory text in Appendix A not transposed into Annex III.

Russia

Russian regulations (Sanitary Regulations for the Organization, Equipment and Maintenance of Animal Facilities for Experimental Biology) were established in 1973 [45] and describe the location and design of animal facilities, sanitation requirements, housing and husbandry requirements, acquisition and quarantine of animals, and standards for personal hygiene. The regulations also describe standards for the humane treatment of animals, including the requirement to minimize pain experienced by an animal through the use of anaesthetics and analgesics.

Additional regulations [46] specify some further controls but these are largely to provide for human safety in the vivarium. The US *Guide* [24] has been translated into Russian and is used by some facilities on a voluntary basis. In addition, a number of the larger institutes also have committees similar to IACUCs.

Although all of these regulations tend to focus on facilities, hygiene, husbandry and human safety, in practice, establishments are becoming increasingly aware of the broader issues, through translations of key publications into Russian and international accreditation schemes, and are applying ethical and welfare considerations voluntarily.

Asia
China

In China, the Statute on the Administration of Laboratory Animals, which was passed in 1988, provides general oversight over laboratory

animal procedures for experimental and other scientific purposes. Compliance with this Statute is encouraged by the Ministry of Science and Technology (MOST) nationally, by the provincial departments of science and technology regionally, and by the IACUCs institutionally. A series of other laws have been passed since then, establishing a laboratory animal quality control network, a programme for quarantine and infectious disease control, a licensing system for laboratory animal users and breeders and the establishment of national laboratory animal seed centres to breed laboratory animals.

The first regulation dealing with animal welfare and focusing on possible problems in husbandry, feeding, use and transport was passed in 2006 by MOST entitled *Guidelines on the Humane Treatment of Laboratory Animals*. These guidelines function similarly to the US *Guide for the Care and Use of Laboratory Animals* [5]. At the regional level, some provinces have strengthened their laboratory animal administration policies. In particular, *Guidelines of Beijing Municipality on the Review of Welfare and Ethics of Laboratory Animals* was passed in 2005 and several other provinces have passed, or are developing, similar guidelines. In general, China is working to improve its animal care policies to meet the requirements of those companies that are establishing research or testing laboratories [47]. Efforts include more emphasis on AAALAC accreditation; at the time of writing, there are 32 institutions whose animal care programmes have attained AAALAC accreditation [23].

India

The Animal Welfare Board of India was set up in 1962 under the Prevention of Cruelty to Animals Act, 1960 [48]. Since 1998, the Board has been the responsibility of the Ministry of Social Justice and Empowerment and, amongst its functions, advises the government on the prevention of unnecessary pain or suffering in captive animals, including experimental animals. The Act also includes the authority for government to appoint a national Committee for the Purpose of Control and Supervision of Experiments on Animals.

The Committee must ensure that animals are not subjected to unnecessary pain or suffering before, during or after experiments and develops

rules regarding animal experimentation. These rules pertain to: minimization of animal pain by the use of anaesthesia and euthanasia; consideration of alternatives to animals; ensuring that pre- and postprocedural care is provided; ensuring appropriate qualifications of individuals conducting experiments; and maintenance of suitable records. The Committee can authorize inspection of animal facilities and can suspend animal work by an individual or an institution.

Permission to conduct research on larger animals must be obtained from a subcommittee of the Committee. However most research is conducted on small laboratory animals (e.g. mice, rats, guinea-pigs, rabbits), proposals for which are reviewed and authorized at a local level by the Institutional Animal Ethics Committee (IAEC). IAECs operate according to guidelines developed by the Indian National Science Academy and each IAEC must include a member of the national Committee in its membership.

Japan

In 2006, the Science Council of Japan [49] issued guidelines for proper conduct of animal experiments following amendment of the Law for the Humane Treatment and Management of Animals in 2005. Subsequently, the Ministry of the Environment issued regulatory standards relating to the care and management of laboratory animals and relief of pain in 2006. Regulatory guidelines and policy statements were then prepared for universities by the Ministry of Education, Culture, Sports, Science and Technology, and for hospitals by the Ministry of Health, Labour and Welfare [50].

Ultimate responsibility for all experiments lies with the director of the research institution who is required to form a local committee for protocol review. The committee provides advice to the director who must approve the protocol. The size of the committee varies according to the size and complexity of the institution, but should include researchers who conduct animal experiments, laboratory animal specialists, and 'other persons of knowledge and experience'. The primary role of the committee is to evaluate the scientific merit of a proposed study, taking into consideration the relevant law, standards, guidelines and policies. The committee is also

charged with reviewing and making recommendations on the education and training of the investigator.

The amended law and standards require, for the first time, that attention be given to the Three Rs in the planning and conduct of research, with particular emphasis placed on refinement. The guidelines provide general considerations for reviewing protocols including the facility and equipment, animal restraint, food and water restriction, surgical procedures, analgesics and anaesthetics, humane end-points, euthanasia, education and training, and retrospective reporting. Other topics include animal selection and receipt, animal health, care and management including cage space—which should consider the animal's characteristics and behaviour in determining appropriate cage size, or should use the US *Guide* [5]. A government inspection system does not exist to validate conformance but a third-party audit system is encouraged.

Korea

The first Korean Animal Protection Law that formally permitted the use of animals for teaching, research or other scientific study was passed in 1991 and was amended in 2007, with a 1 year period for institutions to come into compliance [51]. The amended law addresses several key principles including harm-benefit analysis, the Three Rs, pain mitigation, euthanasia, and ensuring appropriate training of investigators. It also requires the appointment of an Animal Experimentation Ethics Committee at each facility to oversee the protection and ethical treatment of research animals. The composition of this committee is specified as a chair and 3-15 members, at least one third of whom must be independent of the institution. The Committee must include a veterinarian, a representative of an animal welfare group and a lawyer. The Committee is appointed by the director of the facility and must submit an annual report to the Minister of Agriculture and Forestry.

Singapore

In 2004, Singapore developed *Guidelines on the Care and Use of Animals for Scientific Purposes* [52]. These guidelines carry legal authority and cover animal care and use for scientific purposes based on ethical, legal, and scientific considerations. They bind the institution via the IACUC, Institutional Official (IO) and Attending Veterinarian (AV) to function similarly to the system in the USA, drawing heavily on the 7th edition of the US *Guide* [24]. Implementation is institution based, and draws heavily on US, Australian and Canadian standards for husbandry, care and protocol authorization, and on US and European standards for training guidelines.

The programme is evaluated at least annually by the Agri-Food Veterinary Authority. Oversight includes training at all levels, protocol approval, various institutional policies, disaster/emergency planning, and shared responsibilities (with the AV taking leadership) on animal husbandry, veterinary care, and physical plant matters. The IO is ultimately responsible although the IACUC holds authority over many aspects of the programme and can thus ensure appropriate and ethical animal care and use. AAALAC accreditation is strongly encouraged.

Taiwan

The Taiwan Animal Protection Law of 1998 [53] addresses the use of animals for commercial purposes (e.g. meat, milk, fur, etc.), science (teaching and research) and for animals kept as pets. The law precludes the killing of animals, with certain exceptions, such as killing for scientific purposes. It also specifies the conditions for the scientific use of animals including the requirement that the minimum number of animals necessary will be used in ways that cause the minimum amount of pain or injury. Each research institution must form an Animal Experimentation Management Unit and must also establish an Ethics Committee, which must include a veterinarian and a representative of a private animal protection group. The institution may employ an Animal Protection Inspector, or use voluntary Animal Protectors to assist with the supervision of animal use, including facility inspections. The law is administered by the Council of Agriculture which, in 2001, announced regulations for institutions using vertebrate animals to establish Laboratory Animal Care and Use Panels and developed guidelines for the care and use of laboratory animals, for use by these panels.

Australia and New Zealand

Australia

The *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes* (the Code), published by the National Health and Medical Research Council (NHMRC), is currently in its 7th edition, having been revised last in 2004 [54]. It is currently undergoing another major revision at the time of writing. The Code is a national standard of practice that requires justification for the use of animals in research and teaching, identifies responsibilities of investigators, teachers and institutions, including the functions of the Animal Ethics Committees, and specifies that the acquisition, care and use of animals for all scientific purposes in Australia must be done in compliance with the Code, as well as with Commonwealth, State or Territory legislation. The Three Rs form the basis for consideration of animal use in the Code. In 2008, the NHMRC published a supplementary set of guidelines—*Guidelines to Promote the Well-being of Animals Used for Scientific Purposes: the Assessment and Alleviation of Pain and Distress in Research Animals* [55]. Part of the rationale for these guidelines is to address the effects of animal well-being on scientific outcomes. The guidelines include factsheets with guidance on specific, potentially painful or distressful procedures, as well as information on environmental enrichment for common laboratory species. Animal welfare guidance has also been strengthened by the Australian Animal Welfare Strategy (AAWS) [56], a national effort to provide direction for the development of future animal welfare policies in Australia. The AAWS covers the uses of all animals throughout Australia and was developed in conjunction with state and territory governments, animal industry organizations, animal welfare groups and the general public.

New Zealand

The Animal Welfare Act of New Zealand was originally passed as the Animal Protection Act in 1960 and revised in 1999 through the Ministry of Agriculture and Forestry [57]. It covers most animals capable of feeling pain, either domesticated or wild. Its main tenets are to provide for animals' physical, health and behavioural needs, and to alleviate pain and distress. The Act provides core obligations of people, codes of welfare and specific regulations. Part 6 of the Act is specific for the use of animals in research, testing and teaching and requires that any person (or organization) who uses animals for these purposes must have an approved code of ethical conduct (CEC). The Director-General of the MAF must approve the CEC. The CEC is a document that describes the policies and procedures to be adopted by the code holder, and the Animal Ethics Committee (AEC) that will oversee the use of animals in research. (Part 6 of the Animal Welfare Act describes the criteria by which the committee reviews animal use protocols, and the composition of the committee.) The CEC also assures that all persons working with the animals are sufficiently trained. The CEC may be approved for 5 years, and then may be reviewed and renewed by the National Animal Ethics Advisory Committee (NAEAC). A guidance document to aid investigators in the preparation of a CEC was published in 2006 [58]. In order to help investigators to comply with the goals of the Animal Welfare Act to provide for animals' physical, health and behavioural needs, the NAEAC and MAF published guidelines for good practice that address the key issues relating to the maintenance of laboratory animal care and welfare [59].

Latin America

Brazil

A Brazilian federal law on the scientific use of animals was passed in 2008. It established the National Council for the Control of Animal Experimentation (CONCEA), which is a governing and advisory body under the Ministry of Science and Technology, and is empowered to accredit registered institutions and licence activities that use animals for scientific purposes. The law also requires that each institution establishes an Ethics Committee to oversee activities in which animals are used. Even with the passage of this law, many challenges remain regarding its implementation. CONCEA is faced with harmonizing the country's many legal provisions while not impeding research activities. And, while refinement is specified in the law, there is little

mention of reduction or replacement. CONCEA is thus also faced with promoting the Three Rs in practice. These activities will be important as Brazil has the most dynamic research enterprise in Latin America [60].

Mexico

The Norma Oficial Mexicana NOM-062-ZOO-1999 (technical specifications for the production, care and use of laboratory animals) was last updated in 2008. The Ministry of Agriculture, Livestock, Rural Development, Fisheries and Food promulgates this law, which covers rodents, rabbits, carnivores, non-human primates and pigs. The law provides guidelines for housing and husbandry as well as requirements for an Internal Committee for the Care and Use of Laboratory Animals [61].

Other significant regions

Israel

The Prevention of Cruelty to Animals Law (Experiments on Animals) was passed in 1994 and amended in 2001 [62]. The law establishes a review system and standards that are similar to the US institution-based system. The main control body is a 23-member National Council for Animal Experimentation, which includes scientists and animal welfare representatives. The Council mandates that the smallest number of animals be used while mitigating animal suffering. It oversees the institutional committees, approves applications (either directly or through the institutional committees) and visits animal facilities, often unannounced. Researchers are also required to undergo appropriate training.

Africa

In most parts of Africa there are no specific laws that regulate animal research, apart from some old and poorly enforced guidelines and regulations. General animal protection laws, wildlife protection laws and animal disease laws provide peripheral oversight. However, voluntary requirements for ethical review (e.g. [63]) and international accreditation are now emerging and are leading to consideration of regulation,

particularly for non-human primates, in a number of countries such as Kenya and Uganda. Often these are based upon the relevant chapter in the OIE Terrestrial Code [2].

Acknowledgements

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