Ratan Kumar Choudhary
Shanti Choudhary *Editors*

Stem Cells in Veterinary Science

Springer

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Preface

Stem cells have high hopes for regenerative medicine in human health and treating animal diseases. In recent years, it has gained significant momentum. Stem cells are unspecialized cells with profound self-renewal capability and differentiation ability into cells with specialized functions. Therefore, stem cell therapies are used to repair the body's inability to regenerate damaged tissues after acute or chronic ailments. Among the various stem cell types, mesenchymal stem cells hold promising therapeutic applications in treating many diseases because of the simplicity of isolation from various tissue sources and the lack of ethical concern regarding their usage. With the paucity of universal stem cell markers and the high plasticity of stem cells, biomarker-based identification may not precisely quantify stem cells. Administering incorrect doses of stem cells often results in unsuccessful outcomes both in human and veterinary medicine. This book provides the best and most updated information to students, clinicians, young budding veterinary scientists, professionals, and researchers. Reaching out current information to the young mind is critical for scientific research and teaching. Merits of stem cell therapy for the animal disease are naïve but poorly understood; therefore, they have not been practiced widely. The importance of stem cell research should not be underestimated. This book reviews the principle, practices, and up-to-date knowledge of animal stem cells therapeutic applications in veterinary medicine. We hope that our efforts will provide readers updated information about the application of stem cells in regenerative medicine of the animal diseases and techniques of characterizing them. It may provide a reference book of stem cell applications in veterinary and animal sciences.

Ludhiana, Punjab, India — Ratan Kumar Choudhary
Ludhiana, Punjab, India — Shanti Choudhary
2021

Contents

Part I Overview and Introduction

1 Overview of Stem Cells and Their Applications in Veterinary Medicine 3
Ratan K. Choudhary

2 Introduction to Mammary Gland and Its Cell Types 25
Tajeshwar Preet Kaur, Ramneek Verma, and Ratan K. Choudhary

3 Mammary Stem Cells: How Much Do We Know? 39
Alok Kumar and Satish Kumar

4 Methods of Identification and Characterization of Stem Cells 51
Shanti Choudhary

5 Potential of Stem Cell Therapy to Combat Mastitis in Dairy Animals 63
Neelesh Sharma, Sapna Devi, and Goran Bacic

Part II Stem Cells and Veterinary Research

6 Fatty Liver Disease and Utility of Stem Cells in Developing the Disease Model 79
Shanti Choudhary, Michelle LaCasse, Donald C. Beitz, and Eric D. Testroet

7 Mammary Epithelial Cells: A Potential Cellular Model to Understand the Impact of Heat Stress on Mammary Gland and Milk Production in Dairy Animals 97
Manishi Mukesh, Nampher Masharing, Preeti Verma, Manish Tiwari, Prince Vivek, and Monika Sodhi

8 Milk and Milk-Derived Stem Cells 111
Ratan K. Choudhary

9 Cryopreservation of Testicular Stem Cells and Its Application in Veterinary Science 125
Tanushree Patra, Rakesh Bhaskar, and Mukesh Kumar Gupta

10 Testicular Stem Cell Niche 161
Devendra Pathak, Kritima Kapoor, and Mukesh Kumar Gupta

11 Proteomics of Mammary Gland and Mammary Stem Cells 183
Sudarshan Kumar, Nikunj Tyagi, Ashok Kumar Mohanty, and Jai Kumar Kaushik

Part III Therapeutic Applications

12 Advancing Quantitative Stem Cell Dosing for Veterinary Stem Cell Medicine 207
Samuel R. Boutin and James L. Sherley

13 Mesenchymal Stem Cells: A Novel Therapy for the Treatment of Bovine Mastitis 223
Oscar A. Peralta

14 Therapeutic Applications of Mesenchymal Stem Cells in Canine Diseases 241
Mudasir Bashir Gugjoo, Amarpal, A. C. Saxena, Rohit Kumar, P. Kinjavdekar, A. M. Pawde, and G. Taru Sharma

15 Biomaterials and Scaffolds in Stem Cell Therapy 255
Mukesh Kumar Bharti, Vikash Chandra, and G. Taru Sharma

16 Prospects of Mesenchymal Stem Cell Secretome in Veterinary Regenerative Therapy 271
Vikash Chandra and G. Taru Sharma

17 Reprogramming and Induced Pluripotent Stem Cells in Porcine 289
Sujoy K. Dhara, Basavaraj K. Sajjanar, and Jyotirmoy Ghosh

18 CRISPR/Cas System and Stem Cell Editing: Prospects and Possibilities in Veterinary Sciences 323
Md Saddam Hussain and Manish Kumar

Part IV Issues and Perspectives

19 Identification of Species-Specific Stem Cells and Challenges 357
Ratan Kumar Choudhary

20 Regulations of Animal Cell-Based Drugs in Veterinary Regenerative Medicine 367
Ratan Kumar Choudhary and Shanti Choudhary

Editors and Contributors

About the Editors

Ratan Kumar Choudhary is an Assistant Professor at the College of Animal Biotechnology, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab, India. He holds a master's degree in veterinary sciences and a doctoral degree in animal science with a specialization in bovine mammary stem cells and their characterization. His research chiefly focuses on the development of stem cell therapy for the dry period and mastitis in dairy animals. He worked at the United States Department of Agriculture (USDA) for six years (2007–2012), followed by two post-doctoral trainings at the University of Kentucky (2012–2013) and the University of Vermont (2018–2020), USA. He received many awards and fellowships during his Ph.D. including the Dean's Fellowship, R. F. Davis Scholarship and Jacob K. Goldhaber Travel Award, Beltsville Agricultural Research Center, USDA poster awards, first prize winner of the National Milk Producers Federation, Graduate Student Paper Presentation Award in Dairy Production, and Novus International Inc. Travel Award. He is currently serving as a reviewer of the international journals. He has published more than 40 research articles in peer-reviewed international journals, attended/presented research papers at more than 30 conferences, and authored one book. He is a member of various international scientific societies and organizations, e.g., the American Dairy Science Association, the European Association for Animal Production (EAAP), the Indian Society for the Study of Reproduction and Fertility, and the Veterinary Council of India.

Shanti Choudhary served at the University of Vermont, Burlington, the USA, as a Lab Manager and Lab Safety Officer for more than a year. She has extensive research experience in many aspects of molecular biology, including mammary stem cell physiology. She worked at the USDA in Beltsville, USA, as a visiting scientist for more than two years, where her focus was on parasitic diseases in animals. She has also worked as a Research Associate at Guru Angad Dev Veterinary and Animal Sciences University. She received a National Post-doctoral Fellowship (N-PDF; SERB-DST, India) for a project on developing an in vitro

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Part I

Overview and Introduction

1 Overview of Stem Cells and Their Applications in Veterinary Medicine

Ratan K. Choudhary

Abstract

Stem cell therapy has high hopes, and several veterinary diseases may be treated using stem cells. Recent advances in stem cell isolation, characterization, and studies parallel in human medicine have advanced this field. Stem cells possess self-renewal ability and infinite capacity to divide and differentiated into functional cells. The therapeutic potential of either autologous or allogeneic transplantation—the regeneration potential of stem cells gained through immunomodulatory functions by secreting growth factors and many cytokines. Many studies demonstrated in various animal models that stem cell transplantation is beneficial, especially in chronic and problematic diseases. This chapter describes different types of animal stem cells, mechanisms of their action, and some of the diseases in which treatment has shown promising effects in curing the ailments. The application of mesenchymal stem cells, derived from variety of tissues, in diseases of livestock species and pet animals is noteworthy.

Keywords

Stem cells · Types · Application · Mechanism · Veterinary medicine

1.1 History of Stem Cells

Stem cells (SCs) are blank or non-specialized cells capable of differentiating into various types of cells of the body. Self-replication, the capacity to differentiate into several cell types, and unlimited proliferation are the specialized properties of a stem

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cell. The existence of stem cells was shown long back in 1959 when DeOme and his colleagues characterized hyperplastic nodular lesion of mouse mammary tumour virus (MMTV) in mouse mammary gland (DeOme et al. 1959). By that time, investigators had developed the technology of removing endogenously developing epithelial cells form fat pad (called "cleared pad") under the mammary gland of mice and the ability to develop epithelium from exogenously infused cells in the cleared pad. There were many other investigators during 1960s who discovered similar regeneration properties of specialized cells in spleen and other organs. Later, slow proliferating clonogenic precursor cells of hemopoietic tissue were discovered where the successive passage of initial colonies of precursors cells retained the capacity to form stromal tissue like that of their hemopoietic organs (Friedenstein et al. 1974). Discovery of the pluripotent embryonic stem-like cells from inner cell mass (ICM) (Martin 1981; Evans and Kaufman 1981), where cells could form descents of all the tissue layers of the adult, has set a new promises of stem cell research. In the year 2007, the discovery of induced pluripotent stem cells (iPSCs) independently by two scientists namely, Shinya Yamanaka from Kyoto University, Japan (Takahashi and Yamanaka 2006) and James Thomson of University of Wisconsin-Madison, USA (Yu et al. 2007) led the revolution of stem cell research for therapeutic applications.

Though, it is hard to pinpoint when, where, and who discovered the foundation work of stem cells but the first blood transfusion attempted soon after the discovery of blood circulation system in 1628 by William Harvey. Key properties of stem cells defined by McCulloch and till in 1960s in mice spleen appears to be the one of the earliest discoveries of blood forming cells, hematopoietic stem cells or HSCs (Becker et al. 1963). Thus, the foundation of stem cell research lies with adult stem cells (HSCs) but not with the embryonic stem cells. Finally, the discovery of iPSCs has fuelled tremendous impetus to the regenerative medicine.

1.2 Types of Stem Cells

Stem cells, based on the source, has been classified into three types: (1) embryonic stem cells (ESCs), (2) adult stem cells (ASCs), and (3) artificially induced pluripotent stem cells (iPSCs). Another classification based on potency and differentiation capacity includes (1) totipotent, (2) pluripotent, (3) multipotent, (4) unipotent, and (5) progeny of stem cells called progenitor cells (Fig. 1.1). Cells derived from inner cell mass (ICM) of blastocysts are the source of ESCs. Somatic or adult stem cells are the undifferentiated cells present in various organs after its development. The key role of ASCs is the healing, growth, and repair of tissue in the event of damage. Among the various types of ASCs, the name of these cells depends on the tissue from where cells have been derived. They are mesenchymal stem cells (MSCs) of bone marrow, neural stem cell (NSCs), hematopoietic stem cells, skin stem cells, mammary stem cells, and so on. ESCs and ASCs are obtained from the body, but iPSCs are created or manmade stem cells that are induced from differentiated cells by rewiring various pluripotency transcription factors (OCT4, SOX2, c-Myc, and KLF4) of the genome of differentiated cells to behave like embryonic stem cells.

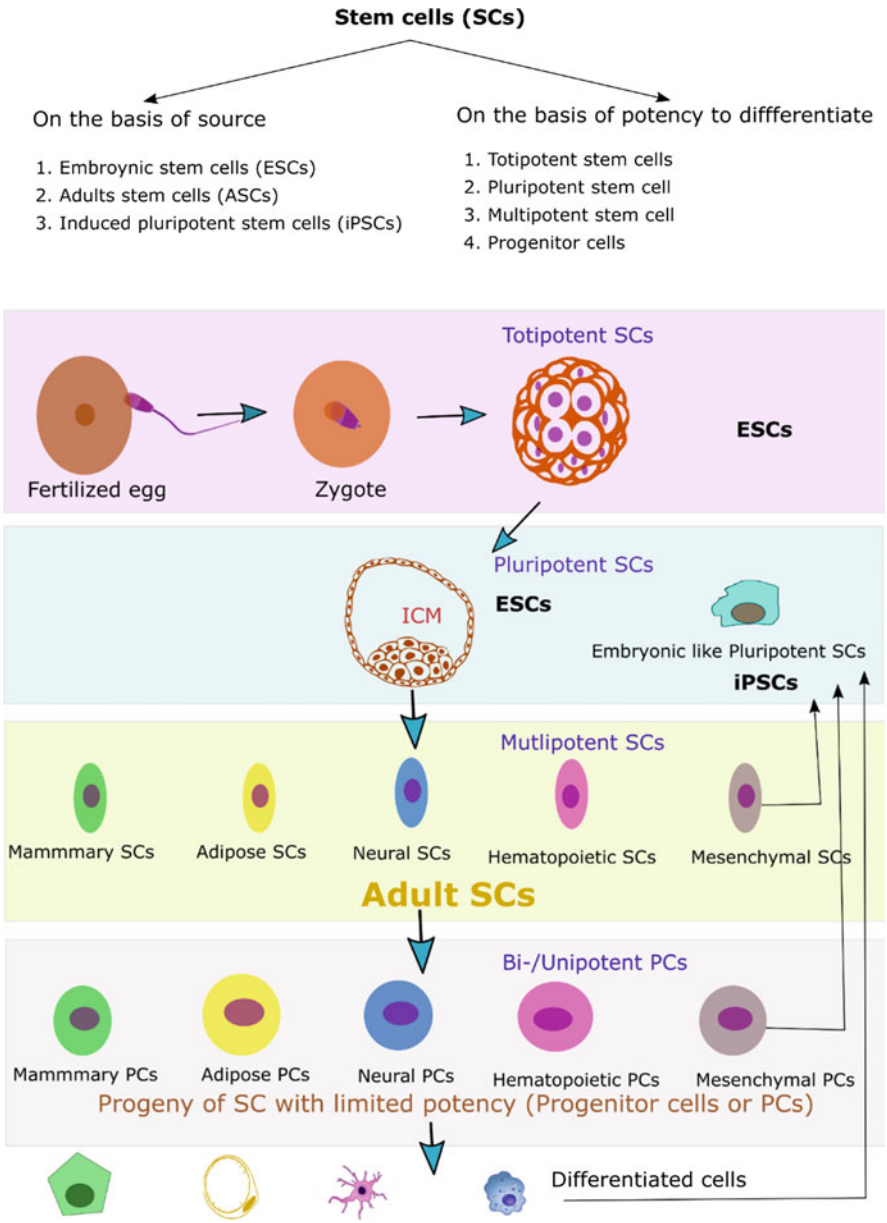

Fig. 1.1 Various types of stem cells and their differentiation

Capacity of totipotent stem cells includes the generation of embryonic (embryo proper) plus extra-embryonic tissues such as placenta. Cells of zygote and up to morula stage (solid balls of 16–32 celled stage) are totipotent. Cells derived from

inner cell mass (ICM) of blastocysts are pluripotent and are the source of ESCs. Pluripotent stem cells classified into naïve and primed ESC based on their in vitro growth characteristics and potency to give rise to all somatic cell linages. In mouse embryo proper, naive ESC are the cells' pre-implanted blastocysts whereas primed ESCs are of post-implanted blastocysts (Takahashi et al. 2018). In comparison to toti- and multi-potent stem cells, proliferative potential and differentiation ability of multi-potent stem cells are limited and produces only few varieties of cells. Generally, variety of cells are derived from closely related lineages. Adult stem cells (ASCs) are the good examples of multi-potent stem cells. Unipotent stem cells have very limited differentiation potential but have a unique repeated divisional capacity (Zakrzewski et al. 2019). Features of repeated divisional capacity make unipotent stem cell a good candidate for therapeutic application. Stem cell of skin that divides and produces dermatocytes is the good example of unipotent stem cell.

1.3 Stem Cells of Veterinary Importance

Embryonic Stem Cells Isolation of human ESCs in 1998 provided the first evidence of pluripotency and given rise to more than 200 cell types (Thomson 1998). Identification of pluripotency transcription factors namely OCT4, SOX2, NANOG, c-Myc, and KLF4 in ESCs provided the core internal circuit of pluripotency. Later, it was demonstrated how interplay between these pluripotency transcription factors determines differentiation of cells during transition phase in progenitor cell. For example, transcriptional circuit dynamic analysis showed OCT4 and SOX2 proteins maintain ESC identity by suppressing neural ectodermal differentiation while SOX2 promotes neural differentiation by suppressing mesodermal differentiation in progenitor cells (Thomson et al. 2011).

Use of ESCs in therapeutic applications is still controversial due to ethical concerns especially with human ESCs. In animals, in addition to ethical concern, the use of animal ESCs also involves religious issues. Therefore, the exploration of animal ESCs is yet to be explored fully. Moreover, lack of specific marker to identify ESCs in various animal species, the vulnerability of ESCs for teratoma formation (Solter 2006), and genomic instability of ESCs occurring during in vitro passaging (Tosca et al. 2015) caused to explore the alternate source of creating embryonic-like stem cells called induced pluripotent stem cells.

1.4 Adult Stem Cells

Mesenchymal Stem Cells (MSCs) MSCs are the alternative source of stem cells present in adult tissue. Though the origin of MSCs is not fully understood and hypothesized to derive from pericytes (Crisan et al. 2008), perivascular cells of microvasculature present in every organ and thus MSCs are harvested from bone marrow, adipose tissue, umbilical cords, dental pulp, and other organs including tonsils. MSCs are not only available in plenty but also lack ethical issues of tissue

harvest and least immunogenic upon autologous and heterologous application. MSCs are multipotent and give rise to bone, cartilage, tendon, ligaments, fat cells, skin, muscle, and connective tissues. Thus, the application of MSCs received the greatest attention in regenerative medicine. In companion animals, dogs, cats, and horse MSCs were harvested from (1) bone marrow, (2) adipose tissues, (3) placenta, (4) Wharton's jelly, (5) synovial fluids, (6) peripheral blood, (7) muscle and periosteum, (8) umbilical cord blood, and (9) muscle (reviewed in Voga et al. 2020). With the graded success of MSC therapy, various animals were depended on the source of stem cells and the species. Conventionally, MSCs are collected from either bone marrow or from adipose tissues. Cells from bone marrow are aspirated from femur of dogs and cats and from sternum of horse for the prospective isolation of MSCs (Marx et al. 2015). Bone marrow-derived MSCs are brought to the lab in special condition and proceed with either direct applications (after centrifugation to collect mononuclear cells) into the patients or culture for further purification and expansion and later applied to the patients. While the direct application of bone marrow-derived MSCs is not the pure population of MSCs but the mixtures of other cell types, growth factors and hormones and hence may provide quicker and better results. However, the culture and expansion of MSCs in the laboratory provides enriched population and abundant quantity for heterologous transplantations of cells into more patients. After fourth passages, MSCs are usually ready for clinical use and up to tenth passage cells can be harvested (Lee et al. 2014; Markoski 2016). In the later passage, MSCs loose in vivo characteristics and tends to differentiate, evidenced by the morphological characteristics and gene expression studies.

Applications of adipose-derived MSC for the treatment of inflammation of mammary gland, mastitis are noteworthy. Intra-mammary infusion of fetal adipose tissue-derived MSC into *S. aureus*-induced mastitis reduced somatic cell counts in bovine milk, indicating the possibility of allogenic transplantation of MSCs in the cow is safe for therapeutic application (Peralta et al. 2020). Fetal bovine MSC secretes antiproliferative factors and antibacterial peptides. In vitro studies indicated conditioned media of MSC decreased about 30% of bacterial population (Cahuascanco et al. 2019) and contained proangiogenic factors that supports neovascularization (Tao et al. 2016). One report on the application of adipose tissue-derived MSCs to treat goat mastitis indicated the efficacy of somatic tissue-derived stem cells in morphofunctional differentiation of cells into milk-producing epithelial cells (Costa et al. 2019). Such studies are indicating promising capabilities of adult stem cell's multipotentiality and immunomodulation properties as an alternative to the treatment of mastitis. Biotechnological and clinical potential of MSCs in livestock species and prospectus of its therapeutic applications has been described here (Hill et al. 2019).

Hematopoietic Stem Cells (HSCs) Similar to MSCs, HSCs are also available in plenty of amount from the bone mammary of developed (adult) tissue. Like that of MSCs, HSCs are multipotent stem cells which can give rise to different cells of immune systems, red and white blood corpuscles (RBCs and WBCs), and platelets.

Mammary Stem Cells (MaSCs) Application of MaSC for the use of treatment of mastitis is yet to be explored. Based on the work of secretome of MSC that enabled identification of healing processes by participating in inflammation, cell proliferation, remodelling of tissue repair and bacterial clearance (Krasnodembskaya et al. 2010; Cortés-Araya et al. 2018), MaSC-derived mammosphere has led the identification of factors and molecules that promoted angiogenesis, cell migration, and enhance cellular defence in the secretome (Ledet et al. 2018).

iPSCs The possibilities of use of iPSCs in veterinary science may bring great possibilities. In comparison to adult stem cells, use of iPSCs involves more safety challenges as all the induced cells are pluripotent and hence liable to uncontrolled division and tumour formation. Additionally, embryonic-like stem cells divide symmetrically (2 identical daughter cells) rather than asymmetrical (2 non-identical daughter cells, say one differentiated and the other stem cell). For the safety and tissue regeneration point of view, asymmetrical cells division is advantageous than the symmetrical division because it repairs the tissue while maintaining the stem cell pool. Moreover, upon receiving the differentiation signal, iPSCs tends to differentiate on all and none principle meaning all iPSCs will either terminally differentiated or none of them will differentiate. Adult stem cells have default asymmetrical divisional capacity in which they divide, differentiate, and maintain the stem cell pool in the tissues.

Remarkable works have been done on exploring the possibilities of ESCs and later iPSCs in pigs and other ungulates by Bhanu and his team. Difficulty in the identification of ICM (the source o ESCs) due to lack of ICM cell marker in ungulates hampered the research in those biomedically important species (Telugu et al. 2010). However, later the group successfully developed iPSCs from pig and other ungulates and showed capabilities of iPSCs to produce differentiated cells (Zhao et al. 2016). Potential of ESCs and iPSCs for committed progenitor cells and finally terminally differentiated cells for tissue or organ development is yet to come. In conclusions, a number of diseases can be treated with the applications of ESCs and ASCs (Fig. 1.2).

1.5 Mechanisms of Stem Cell Actions

Regeneration potentials of stem cells are achieved through re-epithelization, immunomodulations, remodelling of extracellular matrix and proangiogenic factors. Stem cells secrete growth factors, cytokines, growth regulators, anti-inflammatory factors, and some antibacterial peptides (Wangler et al. 2021). Initially, it was thought that stem cells, upon proliferation, provides (1) differentiated cells at the site of injection, or (2) migrate to distantly located tissue sites when injected into the circulation. Under the hypoxic condition, which is the microenvironment where stem cells maintain ‘stemness’ and the cell releases angiogenic growth factors like SDF-1, VEGF, FGF-2, angiopoietin-1, growth suppressor like TGF-alpha and

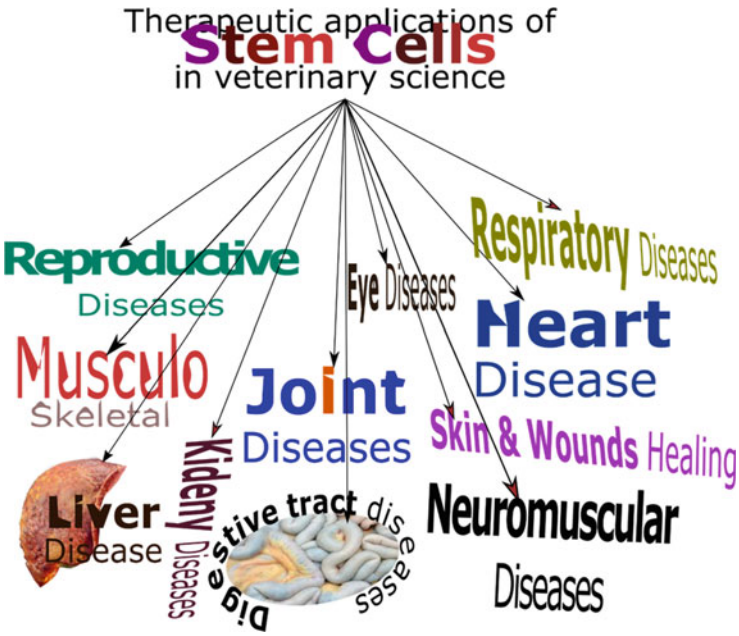

Fig. 1.2 Applications of stem cells for therapeutic purposes in various diseases of animals

TGF-beta and many cytokines like IL-1, IL-6, IL-10 (Spees et al. 2016) and even miRNAs loaded in extracellular vesicles (Asgarpour et al. 2020).

Transfer of Extracellular Vesicles (EVs) EVs include microvesicles, exosomes, and ectosomes that are generated either inward or outward budding of cellular plasma membrane. EVs are the lipid bilayer structures loaded with the cargos like proteins, fats, lipids, metabolites, nuclei acid (miRNA, mRNA), and other molecules has emerged as a significant cellular communicator (Raposo and Stoorvogel 2013). These EVs travel long distances inside the body and release from the healthy and diseased cells. Thus, stem cells also release EVs and transfer cargos to the other cell as a means of cellular communication. EVs was shown to increase the concentration of cytokine IL-10 (Park et al. 2019), IL-22, IL-23 (Hyvarinen et al. 2018), and TFG-beta (Crain et al. 2019). Communications between MSCs and cancer cells induces hybrid cell formation, indirectly evidenced by the presence of exosomes and micro-vesicles, which either promote or inhibit tumour (Melzer et al. 2018). Roles of EVs in stem cell biology has been discussed in detail elsewhere (Hur et al. 2020).

Paracrine Signalling Initial efforts to use stem cell therapy for the purpose of tissue regeneration was centred on the directed differentiation of these cells to the intended cell types. More recently, however, it is becoming apparent stem cells secrete factors which mediate action via paracrine fashion, rather than stem cell differentiation and repopulation. Paracrine mode of signalling is the cell-to-cell

communication method where, signalling molecule is released by one cell and the changes are induced in neighbouring cells. A resultant shift in research and the emergence of studies aiming to elucidate the paracrine mechanisms of stem cell action underlies tissue repair and regeneration mechanisms. Release of TGF-beta causes cell proliferation, differentiation, wound healing and angiogenesis. Appropriate regulation of TGFβ by the osteocytes in the tissue homeostasis has been emphasized for the management of bone diseases (Xu et al. 2018). This was based on the hypothesis that the eroded bone matrix of diseased patient releases TGFβ that has mitogenic and pro-survival activities, thus contributing to tumorigenesis (Portela et al. 2014). In addition, cardiac levels of IL1β and TNFα, factors implicated in angiogenesis, following bone marrow-MSC transplantation into ischemic myocardium showed optimal therapeuptic potential of stem cell (Kamihata et al. 2001).

Immunomodulation There are number of evidences showed that MSCs interact with innate and adaptive immune cells and bring immunomodulatory activities. MSCs inhibited CD^{4+} Th1 and Th2 cells, CD^{8+} T cells and NK cells proliferation and functions. Inhibition of T-cell was mediated by soluble cytokines and factors including TGFβ and human growth factors (Zhao et al. 2016). By understanding mechanisms by which MSCs modulate inflammation and contribute to healing will benefit the investigation of cellular therapy of animals. Animal models like pigs for cardiovascular diseases (Murphy et al. 2003) and goats for osteoarthritis (Shake et al. 2002) are currently used in understanding human diseases. It is a well-known fact that MSCs are hypoallergic due to low expression of HLA class I and no expression of HLA class II molecules and hence devoid of allogenic tissue rejection (Ryan et al. 2005). In addition to the release of soluble cytokines by MSCs, apoptotic and metabolically inactive MSCs also possess immunomodulation properties (Song et al. 2020), indicating cytokine independent mechanism. Apoptotic tissues-derived MSCs reduced rat mortality after sepsis induction, resulting in significantly low levels of circulating TNFα and T-cells (Chang et al. 2012). The current status of cellular and molecular mechanisms of MSC-mediated immunomodulation by live MSCs, dead MSCs and apoptotic MSCs has been discussed in the literature (Weiss and Dahlke 2019).

Mitochondrial Transfer Dysfunction of mitochondria has been associated with ageing and certain diseases like Alzheimer's disease, Parkison's disease, and type 2 diabetes. Mitochondrial dysfunction contributes to reactive oxygen species (ROS) production which ultimately disturbs homeostatic mechanism. Apart from the therapeutics mechanisms of stem cells, directly by cell differentiation, or indirectly *via* paracrine signalling, EVs, and immunomodulation, stem cells have also been shown to transfer mitochondria to the diseased cells. Mitochondrial transfer mediated in bone (Guo et al. 2020) and renal proximal tubular epithelial cells (Konari et al. 2019) enhanced cell proliferation, migration, and cellular differentiation in the tissue. Co-culture experiments of PC12 cells (neuronal cell line) with bone marrow-derived MSCs showed protective effects of neuronal cells under ischemic condition (Sarmah et al. 2020). Increasing evidences suggest that stem cells can directly donate

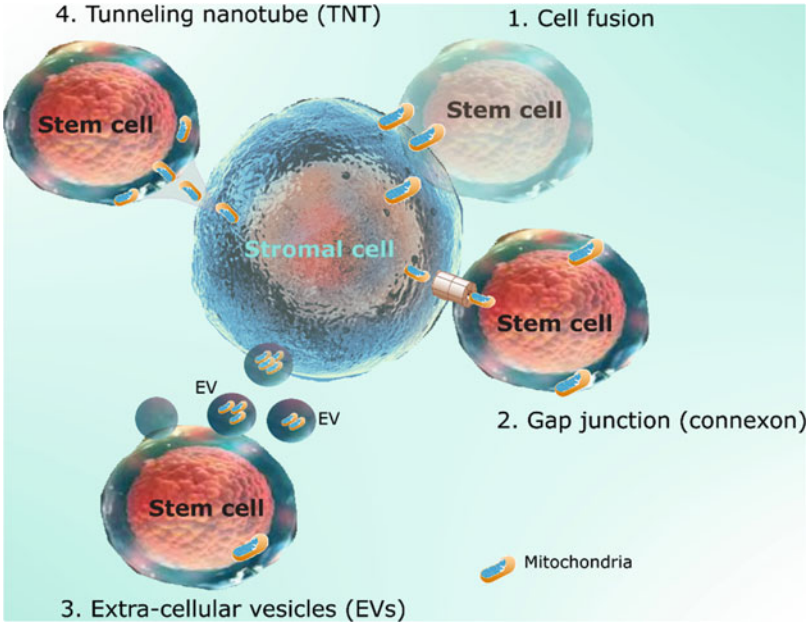

Fig. 1.3 Patterns of mitochondrial transfer from stem cell to stromal cell or diseased cell. These mechanisms include, (1) fusion of stem cell with the stromal cell, (2) transfer of mitochondria through gap junction (connexons, a transmembrane tunnel protein), (3) via extracellular vesicles (EVs) and (4) intracellular tunnelling nanotube (TNT) formation. Formation of TNT is regulated by TNFα/NFKβ and TNFαip2 signalling pathway (Jiang et al. 2016)

mitochondria in order to recover from cell injury, thus cells rescue mitochondrial damage-provoked tissue degeneration (Li et al. 2019). Mitochondrial transfer by stem cells thus could represent an emerging therapeutic approach for tissue regeneration of treating animal diseases. Various possible patterns of mitochondrial transfer from stem cell to somatic or diseased cells are presented (Fig. 1.3).

Homing Mechanisms of Stem Cells Homing is the process where stem cells migrate to specific tissue sites in response to chemoattractant gradients, released by the damaged tissue. Chemotactic factors like cytokines released from the damaged tissue. Understanding homing of stem cells is essential to enhance and refine its clinical significance in therapeutic settings. Chemo-attractive signals are perceived by the receptors present on the surface of stem cells. For example, stromal cell-derived factor 1 (SDF-1) and osteopontin (OPN) are the chemokine released by the damaged tissue sends chemoattractive signals to the cells expressing CXCR4 (Wynn et al. 2004) and integrin-β1 (Chen et al. 2014), respectively. There are many other factors like hepatocyte growth factor, insulin-like growth factor, TGF-beta1, dimers of vascular cell adhesion molecule 1 and very late antigen 4 (VCAM 1-CLA 4), matrix metalloproteinases 2 (MMP-2) and tissue inhibitor of metalloproteinases 3 (TIMP-1), PDGFR, and others (Becker et al. 1963).

Homing of stem cells depends on the route of administration of the cell. Local injection of stem cells is preferred but an invasive method like intraperitoneal injection. Intravenous injection of stem cells is the least invasive but suffers with many limitations. The limitations of intravenous injections of stem cells are (Voga et al. 2020): (1) stem cells have to exit circulation in order to reach the site of injury, (2) cells are squeezed in dimension to the size of narrowest capillary, particularly in lungs where the average diameter of capillary is 14 μm in comparison to the cell's diameter (25–30 μm), (3) high expression of integrin-beta 1 by lung epithelium attracts more number MSCs which blocks the availability of MSCs by cell entrapment, (4) systemic injection of stem cells may require high dose of stem cells to have a sufficient number of stem cells reaching to the site of injury, and (5) circulation of stem cells in the blood for a longer period (more than 24 h) may not provide viable MSCs to the damaged tissue. However, long-term effects of MSCs have been reported which likely to be mediated through non-viable and apoptotic MSCs.

1.6 Clinical Applications of Stem Cells in Regenerative Veterinary Medicine

Worldwide, investigators and veterinary clinicians are working on applications of stem cell for therapeutic potential in autologous and allogenic fashions either freshly isolated or cultured and expanded stem cell in the laboratory for the treatment of various diseases of animals. This indicates that the stem cell therapy in veterinary science is a reality. Existing literatures support that the stem cell transplantation is safe and beneficial to the animal health.

Diseases of Reproductive System Reproductive diseases in animals causes great loss to farmer's profit. Inflammation of uterus (endometritis), poorly developed ovarian follicles in female, and testicular degenerative disease in males are some of the reproductive disorders. In mares, endometritis is the main cause of reproductive failure and an incurable disease. Application of bone marrow-derived MSC did not restore ovarian functions in aged mares (Grady et al. 2019). However, in human MSCs therapy appears to restore ovarian functions (Yoon 2019). One may argue that the failure of MSC therapy in mare could be (1) due to old age of mare as fertility is naturally reduced in aged animals, (2) source of stem cells as MSCs can be derived from various tissue namely adipose, bone marrow, umbilical cord, menstrual blood in human, amniotic fluid, and (3) insufficient dose of MSCs. Study showed that, in human, multiple doses of MSCs ($1–5 \times 10^6$ cells/kg body weight) improved ovarian functions in primary ovarian insufficiency (Gadkari et al. 2014), whereas in mice 1×10^6 to 5×10^8 cells (Kalhori et al. 2018) were used for clinical applications. Mechanisms of stem cell action in restoring ovarian functions have been shown to help in folliculogenesis, prevention of granulosa cell apoptosis, regulation of reproductive hormones and vasculogenesis.

Stem cells treatment could offer solutions to infertility related disease. Testicular degeneration in dogs often leads to the production of defective sperms and affects fertility. Viability and fertility of frozen-thawed canine sperm were improved using adipose tissue-derived MSCs (Qamar et al. 2020) and exosomes derived from conditioned media of MSCs (Qamar et al. 2019). These results showed that like the availability of frozen semen from cattle, we may use freeze pure breed canine sperm for artificial insemination of female dogs. It is well known that during cryopreservation, cellular injury to organelles like plasma membrane, mitochondria, and nucleus occurs that affects post-thaw sperm motility and hence impaired fertility. The possible mechanisms of improved fertility of canine were due to the secretion of proteins like annexin, dysferlin, and fibronectin by MSCs (Qamar et al. 2020). These proteins are involved in the repairing process at a cellular level at many ways like prevention of cells from apoptosis and improvement of membrane integrity. Other possible mechanisms of restoring canine fertility are also possible like regulating glucose metabolism of sperm (Hsiao et al. 2019) that provides more energy to the cell for enhanced motility.

Musculoskeletal Disease Treatment of horse musculoskeletal disease using MSCs has been fairy a good success. Many reports with varying successful results have been reported since more than a decade (Wilke et al. 2007; Broeckx et al. 2014, 2019). Intra-articular administration of adipose tissue-derived MSCs in horse resulted no lameness after 3 months of treatment (Nicpon et al. 2013). Properties, cultural characteristic, and potential applications of MSCs in the management of equine diseases have been described (Gugjoo et al. 2019a, b). Ready-to-use stem cell containing products (like Arti-Cell Forte from Boehringer Ingehlheim and Investigational Veterinary Product or IVP from Dechra Limited, UK) are also available for the clinically applications in treating horse's degenerative disease.

Autologous stem cell therapy using adipose tissue-derived MSCs treated chronic osteoarthritis in canine (Black et al. 2007). MSCs derived from umbilical cord (UMSCs) have also shown promising results in treating osteoarthritis in dogs. No adverse reactions were reported in using UMSCs (Kim et al. 2019). Interestingly, allogenic transplantation of MSCs appears to be safe in dogs for osteoarthritis (Shah et al. 2018). In vivo studies summarizing the clinical effects of MSCs in canine osteoarthritis are given in Table 1.1. Majority of the results of stem cell applications for canine disease therapy are positive; however, investigators consider the results inconclusive (Gugjoo et al. 2019a, b), suggesting more basic research are needed to understand the biology of MSCs or other stem cell types for positive and conclusive results.

Liver Diseases Liver diseases (acute and chronic) are common in canines. Viral or bacterial infection, cancer, trauma, ingestion of toxic substances, and endocrine disturbances can cause liver disease in dogs. Many studies have tried the administration of autologous and allogeneic MSCs treatment with varied success. Mechanisms by which MSCs repair liver tissue is the generation of different cell

Table 1.1 Various studies and their results of canine mesenchymal stem cell therapy for osteoarthritis (table adapted from Sasaki et al. 2019)

Source	Injection/trans-plantation	Combination use	Cell number (million)	Model	Evaluation method
Bone marrow	Single intraarticular, autologous	–	7–8	Partial thickness articular cartilage defect	Histology
Adipose tissue	Single intraarticular, autologous	–	7–8	Partial thickness articular cartilage defect	Morphology, histology, fluorescence analysis
Adipose tissue	Four times intraarticular, allogeneic	–	5 (three times), 66 (once)	Intact	Pain and lameness scoring, immunohistochemistry
Bone marrow	Transplantation with scaffold, allogeneic	Scaffold	0.01	Full-thickness cartilage defect	Histology, immunohistochemistry, micro-CT
Synovium	Single intraarticular, autologous	Hyaluronic acid	0.05, 5 or 50	Partial thickness articular cartilage defect	Histology
Adipose tissue	Single intraarticular, allogeneic	Platelet-rich plasma	10	Cranial cruciate ligament transection	Lameness scoring, focal compression strength, extracellular matrix composition, histopathology, real-time PCR
Bone marrow	Single intraarticular, allogeneic	Hyaluronic acid	10	Partial thickness articular cartilage defect	Gross appearance, magnetic resonance imaging, histology, immunohistochemistry
Umbilical cord	Single intraarticular, allogeneic	–	1	Surgical manipulation of articular cartilage	Magnetic resonance imaging, radiography, ultrasonography, blood test, scanning electron microscope

types of the liver (hepatocytes, Kuffer cells, and hepatic stellate cells) and the secretion of various cytokines that modulates the immune system, apoptosis, and vasculogenesis. In canines, the liver fibrosis model to establish with autologous transplantation of bone-marrow-derived MSCs and documented improved liver fibrosis without adverse effect (Matsuda et al. 2017). However, caution should be kept in mind that prolongs immunomodulation and anti-inflammatory conditions to treat liver fibrosis may have adverse effects and thus, alternative strategies must be taken to improve outcomes of stem cell therapy for prolong use in treating chronic conditions. Strategies to improve liver stem cell success may include modification of stem cell niche, pretreatment or co-treatment of the recipient and gene modifications (Hu et al. 2019). Intravenous injection of adipose tissue-derived MSCs experimentally induced liver injury and caused restoration of liver function (Teshima et al. 2017), indicating the possibility of stem cell therapy for hepatic disease in dogs.

Kidney Diseases Chronic diseases of kidney is one of the common issues of old aged cats and dogs. The disease is manifested by fibrosis, nephritis, and tubular atopy. Currently, only the renal transplantation is the therapeutic solution to the chronic kidney disease (Voga et al. 2020). Very few studies conducted the same investigator (Quimby et al. 2016; Quimby and Dow 2015) and reported various strategies to look critically for MSCs-based therapy for the feline chronic kidney diseases. More research is required to know about the best source of MSCs (bone-marrow vs adipose tissue-derived cells), route of cell administration, dose of cells, stages of MSCs including the impact of age/health/status of tissue donor animals (SAGE 2018).

Joint Diseases To study the effects of stem cells therapy in bovine, caprine could be the alternative model animal. Bovines are heavy and especially grazing animals have locomotor issues. One such study aiming to test the efficacy of autologous MSCs for superior cartilage repair, bone marrow harvested cells were injected intra-articularly and demonstrated hyaline like cartilage regeneration in goats (Nam et al. 2013), suggesting potential applicability of stem cell therapy in livestock animals. In horse, bone spavin is a degenerative joint disease of horse and cattle evidence by bony outgrowth within the lower hock joint caused by osteoarthritis. Result of a study conducted in horses with intraarticular autologous transplantation of adipose tissue-derived MSCs suggested positive and long-lasting effect of stem cell therapy and had no signs of lameness after 180 days of the treatment (Nicpon et al. 2013), indicating long-lasting effects of stem cell therapy. There are many other studies in pet animals showing improved and promising results of stem cell treatment in joint diseases.

Digestive Tract Diseases Application of stem cell therapy has gained importance in treating inflammatory conditions of the digestive disorders especially inflammatory bowel diseases (IBD). In canine, a single dose (at two million/kg body weight) of adipose tissue-derived MSCs has reduced gastrointestinal inflammation (Pérez-Merino et al. 2015). Not much work has been done on this aspect in other animals.

Robust stem cell isolation and in vitro propagations protocol, characterization of different subsets of stem cells for various diseases of digestive system and understanding of digestive disorders are the keys to establish stem cell therapy.

Eye Diseases Dry eye disease or keratoconjunctivitis sicca (KCS) is one of the prevalent eye diseases affecting up 35% of human and 4–20% of dogs. The mechanisms of the development of the disease are not really understood. However, the most common cause of KCS immune-mediated inflammatory response targeted against the production of tear by affecting lachrymal glands. It causes discomforts, visual disturbances, and damage to the ocular surface. Treatment of KCS is difficult and effective and safe treatment of this disease is lacking (Villatoro et al. 2015). However, in 2016 Bittencourt et al. reported that even a single dose of allogenic administration of MSCs in canine has positive effects on KCS over the long period (up to a year).

Heart Diseases Cardiac diseases in animal include myocardial infarction, dilated cardiomyopathy, degenerative valvular disease, congestive heart failure, and other heart problems. Research has indicated the ability of cardiomyocytes (cells of the heart) to regenerate after myocardial injury, indicating the possibility of therapeutic interventions. In an interesting study, one study claims for improved heart functions in dogs suffered with chronic valvular disease when administered with puppy's deciduous teeth stem cells (Petchdee and Sompeewond 2016). In a study conducted on Dobermans with retrograde coronary venous, allogeneic administration of adipose tissue-derived MSCs did not increase days of survival but the method of stem cell treatment was safe (Pogue et al. 2013). In addition to intracoronary infusion and intramyocardial injection of stem cells, retrograde coronary venous infusion (RCVI) is one of the methods of stem cell administration aimed for improved cardiac functions (Gathier et al. 2018). A combination of two stem cells—mesenchymal (MSCs) and cardiac stem cells (CSC)—administered intra-cardially to immunosuppressed swine (model animal for human research) showed (Quijada et al. 2015) twofold reduction in scar tissue formation improved left ventricular compliance and contractibility in comparison to placebo animals.

Skin and Wound Healing Skin wound healing is a complex process and well-orchestration of inflammation, matrix formation and tissue remodelling. Cell therapies bring combined actions of immune modulation, growth factors, angiogenesis, extracellular matrix production, and cell differentiation (Giles et al. 2015). Chronic wounds are difficult to heal, as they are associated with other underlying diseases. Generally, in a normal wound healing process, skin infection is prevented and tissue integrity and functions are restored. Faster rate of healing to prevent infection, but compromised healing process resulting in scar formation. Cell-based therapy has actively been used for the treatment of dermal wounds in dogs. Multipotent stem cells and progenitor cells have high proliferative potential, ability to differentiate and secrete different types of growth factors and cytokines that improves wound healing. Cells like endothelial progenitor cells, bone marrow-

derived mesenchymal stem cells (BM-MSCs), and adipose-derived stem cells (ASCs) are being used for cellular therapy. Interestingly, allogenic transplantation of ASCs in canine is possible and results in improved wound healing (Enciso et al. 2020). In a recent study conducted using BM-MSCs, both fresh and frozen cells were capable of promoting wound healing (Bharti et al. 2020), suggesting stem cells have promising potential to treat the cutaneous wound in clinical cases.

Mastitis Economic loss due to mastitis is huge. In the United States alone, mastitis causes approximately $2 billion dollars annual loss to the dairy industry (Donovan et al. 2005). The current treatment of mastitis is to use broad-spectrum antibiotics to control bacterial infection but do not address the tissue regeneration. In vitro study suggests antibiotics induce hypoxia inducing factor 1 alpha (HIF1A) and causes oxidative damage (Elliott and Jiang 2019) that may even slow down the growth of mammary epithelial cells and tissue regeneration. Although antibiotic is the best available method to control mastitis, but has been associated with antibiotic resistance and milk residue. Recent study showed the potential application of stem cells in treating bovine mastitis. *S. aureus* induced mastitis in Holstein Friesian cows treated intramammarily with antibiotics (control group: days 4 and 5) or a suspension of adipose tissue-derived stem cells (2.5×10^7 cells: day 4 and 5) showed reduced colony formation unit (CFU/ml of milk) of bacterial in stem cells quarters (Peralta et al. 2020). Interestedly inoculation of repeated doses of allogenic stem cells was safe to use in healthy cows demonstrating possible cell-based therapy for mastitis treatment. In another study, conditioned DPBS from amniotic membrane stem cells injected into the induced mastitis mammary gland of cow showed improved milk quality of milk of the treated cow in comparison to the antibiotic-treated cow. Upon injection of conditioned media into the teat canal, pH value and titratable acidity of milk were significantly different in the experimental group (Ting et al. 2020), indicating another possibility of using AMSC-derived secretome as an alternative therapy in replacing antibiotics in treating bovine mastitis.

Neuromuscular Diseases Spinal cord injury due to trauma is one of the common injuries in dogs. Sometimes, naturally occurring intervertebral disc disease in larger breeds of dogs like German Shepherd has also been observed whose conventional treatment of the disease has very limited success. Injury to the spinal cord is because neuromuscular disease is manifested by the altered gait, pain, and sometimes permanent locomotor disability. Autologous and allogenic bone marrow MSCs therapy alone or in combination with conventional therapy showed some locomotor recovery in dogs. However, naturally occurring degenerative disc disease in German Shepherd dogs did not show positive clinical outcome after 1, 6, and 12 months of MSCs treatment (Steffen et al. 2017).

1.7 Conclusions

With advancement in understanding biology of stem cells and their applications in animal diseases and trail results, it seems possible to use healing properties, immunomodulatory activities, and low immunogenicity of adult stem cells, in particular mesenchymal stem cells, for therapeutic applications in various diseases of livestock species and pet animals.

References

Asgarpour K, Shojaei Z, Amiri F, Ai J, Mahjoubin-Tehran M, Ghasemi F, ArefNezhad R, Hamblin MR, Mirzaei H (2020) Exosomal microRNAs derived from mesenchymal stem cells: cell-to-cell messages. Cell Commun Signal 18(1):149. https://doi.org/10.1186/s12964-020-00650-6

Becker AJ, McCulloch EA, Till JE (1963) Cytological demonstration of the clonal nature of spleen colonies derived from transplanted mouse marrow cells. Nature 197:452–454

Bharti MK, Bhat IA, Pandey S, Shabir U, Peer BA, Indu B, Bhat AR, Kumar GS, Amarpal CV, Sharma GT (2020) Effect of cryopreservation on therapeutic potential of canine bone marrow derived mesenchymal stem cells augmented mesh scaffold for wound healing in guinea pig. Biomed Pharmacother 121:109573

Bittencourt MK, Barros MA, Martins JF et al (2016) Allogeneic mesenchymal stem cell transplantation in dogs with keratoconjunctivitis sicca. Cell Med 8(3):63–77. https://doi.org/10.3727/215517916X693366

Black LL, Gaynor J, Gahring C, Adams C, Aron D, Harman S, Gingerich DA, Harman R (2007) Effect of adipose-derived mesenchymal stem and regenerative cells on lameness in doges with chronic osteoarthritis of the coxofermoal joints: a randomized, double-blinded, multicenter, controlled trial. Vet Ther 8(4):272–284

Broeckx S, Zimmerman M, Crocetti S, Suls M, Mariën T, Ferguson SJ, Chiers K, Duchateau L, Franco-Obregón A, Wuertz K, Spaas JH (2014) Regenerative therapies for equine degenerative joint disease: a preliminary study. PLoS One 9:e85917

Broeckx SY, Seys B, Suls M, Vandenberghe A, Mariën T, Adriaensen E, Declercq J, Van Hecke L, Braun G, Hellmann K, Spaas JH (2019) Equine allogeneic chondrogenic induced mesenchymal stem cells are an effective treatment for degenerative joint disease in horses. Stem Cells Dev 28 (6):410–422. https://doi.org/10.1089/scd.2018.006

Cahuascanco B, Bahamonde J, Huaman O et al (2019) Bovine fetal mesenchymal stem cells exert antiproliferative effect against mastitis causing pathogen Staphylococcus aureus. Vet Res 50:25. https://doi.org/10.1186/s13567-019-0643-1

Chang CL, Leu S, Sung HC, Zhen YY, Cho CL, Chen A et al (2012) Impact of apoptotic adipose-derived mesenchymal stem cells on attenuating organ damage and reducing mortality in rat sepsis syndrome induced by cecal puncture and ligation. J Transl Med 10:244. https://doi.org/10.1186/1479-5876-10-244

Chen Q, Shou P, Zhang L, Xu C, Zheng C, Han Y, Li W, Huang Y, Zhang X, Shao C, Roberts AI, Rabson AB, Ren G, Zhang Y, Wang Y, Denhardt DT, Shi Y (2014) An osteopontin-integrin interaction plays a critical role in directing adipogenesis and osteogenesis by mesenchymal stem cells. Stem Cells 32(2):327–337. https://doi.org/10.1002/stem.1567

Cortés-Araya Y, Amilon K, Rink BE, Black G, Lisowski Z, Donadeu FX, Esteve CL (2018) Comparison of antibacterial and immunological properties of mesenchymal stem/stromal cells from equine bone marrow, endometrium, and adipose tissue. Stem Cells Dev 27 (21):1518–1525. https://doi.org/10.1089/scd.2017.0241

Costa CRM, Feitosa MLT, Rocha AR, Bezerra DO, Leite YKC, Argolo Neto NM et al (2019) Adipose stem cells in reparative goat mastitis mammary gland. PLoS One 14:e0223751. https://doi.org/10.1371/journal.pone.0223751

Crain SK, Robinson SR, Thane KE, Davis AM, Meola DM, Barton BA et al (2019) Extracellular vesicles from Wharton's Jelly mesenchymal stem cells suppress CD4 expressing T cells through transforming growth factor beta and adenosine signaling in a canine model. Stem Cells Dev 28:212–226. https://doi.org/10.1089/scd.2018.0097

Crisan M, Yap S, Casteilla L, Chen CW, Corselli M, Park TS et al (2008) A perivascular origin for mesenchymal stem cells in multiple human organs. Cell Stem Cell 3(3):301–313

Deome KB, Faulkin LJ Jr, Bern HA, Blair PB (1959) Development of mammary tumors from hyperplastic alveolar nodules transplanted into gland-free mammary fat pads of female C3H mice. Cancer Res 19(5):515–520

Donovan DV, Kerr DE, Wall RJ (2005) Engineering disease resistant cattle. Transgenic Res 14:563–567

Elliott RL, Jiang X-P (2019) The adverse effect of gentamicin on cell metabolism in three cultured mammary cell lines: "are cell culture data skewed?". PLoS One 14(4):e0214586. https://doi.org/10.1371/journal.pone.0214586

Enciso N, Avedillo L, Fermín ML et al (2020) Cutaneous wound healing: canine allogeneic ASC therapy. Stem Cell Res Ther 11:261

Evans MJ, Kaufman M (1981) Establishment in culture of pluripotential stem cells from mouse embryos. Nature 292:151–156

Friedenstein AJ, Chailakhyan RK, Latsinik NV, Panasyuk AF, Keiliss-Borok IV (1974) Stromal cells responsible for transferring the microenvironment of the hemopoietic tissues: cloning in vitro and retransplantation in vivo. Transplantation 17:331–340

Gadkari R, Zhao L, Teklemariam T, Hantash BM (2014) Human embryonic stem cell derived-mesenchymal stem cells: an alternative mesenchymal stem cell source for regenerative medicine therapy. Regen Med 9(4):453–465. https://doi.org/10.2217/rme.14.13

Gathier WA, van Ginkel DJ, van der Naald M, van Slochteren FJ, Doevendans PA, Chamuleau SAJ (2018) Retrograde coronary venous infusion as a delivery strategy in regenerative cardiac therapy: an overview of preclinical and clinical data. J Cardiovasc Transl Res 11(3):173–181. https://doi.org/10.1007/s12265-018-9785-1

Giles TS, Kirby MSJ, Cowin AJ, Smith LE (2015) Stem cells for cutaneous wound healing. Biomed Res Int 2015:285869

Grady ST, Watts AE, Thompson JA, Penedo MCT, Konganti K, Hinrichs K (2019) Effect of intra-ovarian injection of mesenchymal stem cells in aged mares. J Assist Reprod Genet 36:543–556. https://doi.org/10.1007/s10815-018-1371-6

Gugjoo MB, Amarpal, Makhdoomi DM, Sharma GT (2019a) Equine mesenchymal stem cells: properties, sources, characterization, and potential therapeutic applications. J Equine Vet Sci 72:16–27. https://doi.org/10.1016/j.jevs.2018.10.007

Gugjoo MB, Amarpal, Sharam GT (2019b) Mesenchymal stem cell basic research and applications in dog medicine. J Cell Physiol 234(10):16779–16811. https://doi.org/10.1002/jcp.28348

Guo Y, Chi X, Heng BC, Wei Y, Zhang X, Zhao H, Yin Y, Deng X (2020) Mitochondria transfer enhances proliferation, migration, and osteogenic differentiation of bone marrow mesenchymal stem cell and promotes bone defect healing. Stem Cell Res Ther 11:245. https://doi.org/10.1186/s13287-020-01704-9

Hill ABT, Bressan FF, Murphy BD, Garcia JM (2019) Applications of mesenchymal stem cell technology in bovine species. Stem Cell Res Ther 10(44):1145. https://doi.org/10.1186/s13287-019-1145-9

Hsiao C, Ji AT, Chang C et al (2019) Mesenchymal stem cells restore the sperm motility from testicular torsion-detorsion injury by regulation of glucose metabolism in sperm. Stem Cell Res Ther 10:270. https://doi.org/10.1186/s13287-019-1351-5

Hu C, Zhao L, Duan J, Li L (2019) Strategies to improve the efficiency of mesenchymal stem cell transplantation for reversal of liver fibrosis. J Cell Mol Med 23(3):1657–1670. https://doi.org/10.1111/jcmm.14115

Hur YH, Cerione RA, Antonyak MA (2020) Extracellular vesicles and their roles in stem cell biology. Stem Cells 38(4):469–476. https://doi.org/10.1002/stem.314

Hyvarinen K, Holopainen M, Skirdenko V, Ruhanen H, Lehenkari P, Korhonen M et al (2018) Mesenchymal stromal cells and their extracellular vesicles enhance the anti-inflammatory phenotype of regulatory macrophages by downregulating the production of interleukin (IL)-23 and IL-22. Front Immunol 9:771. https://doi.org/10.3389/fimmu.2018.00771

Jiang D, Gao F, Zhang Y, Wong DS, Li Q, Tse HF et al (2016) Mitochondrial transfer of mesenchymal stem cells effectively protects corneal epithelial cells from mitochondrial damage. Cell Death Dis 7:e2467

Kalhori Z, Azadbakht M, Soleimani MM, Shariatzadeh MA (2018) Improvement of the folliculogenesis by transplantation of bone marrow mesenchymal stromal cells in mice with induced polycystic ovary syndrome. Cytotherapy 20:1445–1458

Kamihata H, Matsubara H, Nishiue T et al (2001) Implantation of bone marrow mononuclear cells into ischemic myocardium enhances collateral perfusion and regional function via side supply of angioblasts, angiogenic ligands, and cytokines. Circulation 104(9):1046–1052

Kim SE, Pozzi A, Yeh J-C, Lopez-Velazquez M, Yong JAA, Townsend S, Dunlap AE, Christopher SA, Lewis DD, Johnson MD, Petrucci K (2019) Intra-Articular umbilical cord derived mesenchymal stem cell therapy for chronic elbow osteoarthritis in dogs: A double-blinded, placebo-controlled clinical trial. Front Vet Sci 6:474. https://doi.org/10.3389/fvets.2019.00474

Konari N, Nagaishi K, Kikuchi S, Fujimiya M (2019) Mitochondria transfer from mesenchymal stem cells structurally and functionally repairs renal proximal tubular epithelial cells in diabetic nephropathy *in vivo*. Sci Rep 9:5184. https://doi.org/10.1038/s41598-019-40163-y

Krasnodembskaya A, Song Y, Feng X, Gupta N, Serikov V, Lee J-W, Matthay MA (2010) Antibacterial effect of human mesenchymal stem cells is mediated in part from secretion of the antimicrobial peptide LL-37. Stem Cells 28:2229–2238

Ledet MM, Vasquez AK, Rauner G, Bichoupan AA, Moroni P, Nydam DV et al (2018) The secretome from bovine mammosphere-derived cells (MDC) promotes angiogenesis, epithelial cell migration, and contains factors associated with defense and immunity. Sci Rep 8(1):5378

Lee KS, Kang HW, Lee HT, Kim HJ, Kim CL, Song JY, Lee KW, Cha SH (2014) Sequential sub-passage decreases the differentiation potential of canine adipose-derived mesenchymal stem cells. Res Vet Sci 96(2):267–275

Li C, Cheung MKH, Han S, Zhang Z, Chen L, Chen J, Zeng H, Qiu J (2019) Mesenchymal stem cells and their mitochondrial transfer: a double-edged sword. Biosci Rep 39(5):BSR20182417

Markoski MM (2016) Advances in the use of stem cells in veterinary medicine: from basic research to clinical practice. Scientifica. https://doi.org/10.1155/2016/4516920

Martin GR (1981) Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci U S A 78:7634–7638

Marx C, Silveira MD, Nardi NB (2015) Adipose-derived stem cells in veterinary medicine: characterization and therapeutic applications. Stem Cells Dev 24(7):803–813

Matsuda T, Takami T, Sasaki R, Nishimura T, Aibe Y, Paredes BD, Quintanilha LF, Matsumoto T, Ishikawa T, Yamamoto N, Tani K, Terai S, Taura Y, Sakaida I (2017) A canine liver fibrosis model to develop a therapy for liver cirrhosis using cultured bone marrow-derived cells. Hepatol Commun 1(7):691–703. https://doi.org/10.1002/hep4.1071

Melzer C, Ohe J, Hass R (2018) Concise Review: Crosstalk of mesenchymal stroma/stem-like cells with cancer cells provides therapeutic potential. Stem Cells 36(7):951–968. https://doi.org/10.1002/stem.2829

Murphy JM, Fink DJ, Hunziker EB, Barry FP (2003) Stem cell therapy in a caprine model of osteoarthritis. Arthritis Rheum 48:3464–3474

Nam HY, Karunanithi P, Loo WC, Naveen S, Chen H, Hussin P, Chan L, Kamarul T (2013) The effects of staged intra-articular injection of cultured autologous mesenchymal stromal cells on

the repair of damaged cartilage: a pilot study in caprine model. Arthritis Res Ther 15(5):R129. https://doi.org/10.1186/ar4309

Nicpon J, Marycz K, Grzesiak J (2013) Therapeutic effect of adipose-derived mesenchymal stem cell injection in horses suffering from bone spavin. Pol J Vet Sci 16:753–754. https://doi.org/10.2478/pjvs-2013-0107

Park KS, Svennerholm K, Shelke GV, Bandeira E, Lasser C, Jang SC et al (2019) Mesenchymal stromal cell-derived nanovesicles ameliorate bacterial outer membrane vesicle-induced sepsis *via* IL-10. Stem Cell Res Ther 10:231. https://doi.org/10.1186/s13287-019-1352-4

Peralta OA, Carrasco C, Vieytes C, Tamayo MJ, Muñoz I, Sepulveda S, Tadich T, Duchens M, Melendez P, Mella A, Torres C (2020) Safety and efficacy of a mesenchymal stem cell intramammary therapy in dairy cows with experimentally induced *Staphylococcus aureus* clinical mastitis. Sci Rep 10:2843. https://doi.org/10.1038/s41598-020-59724-7

Pérez-Merino EM, Usón-Casaús JM, Duque-Carrasco J, Zaragoza-Bayle C, Mariñas-Pardo L, Hermida-Prieto M, Vilafranca-Compte M, Barrera-Chacón R, Gualtieri M (2015) Safety and efficacy of allogeneic adipose tissue-derived mesenchymal stem cells for treatment of dogs with inflammatory bowel disease: Endoscopic and histological outcomes. Vet J 206(3):391–397. https://doi.org/10.1016/j.tvjl.2015.07.023

Petchdee S, Sompeewond S (2016) Intravenous administration of puppy deciduous teeth stem cells in degenerative valve disease. Vet World 9(12):1429–1434. https://doi.org/10.14202/vetworld.2016.1429-1434

Pogue B, Estrada AH, Sosa-Samper I, Maisenbacher HW, Lamb KE, Mincey BD et al (2013) Stem-cell therapy for dilated cardiomyopathy: a pilot study evaluating retrograde coronary venous delivery. J Small Anim Pract 54:361–366. https://doi.org/10.1111/jsap.12098

Portela RF, Fadl-Alla BB, Pondenis HC, Byrun ML, Garrett LD, Wycislo KL, Borst LB, Fan TM (2014) Pro-tumorigenic effects of transforming growth factor beta 1 in canine osteosarcoma. J Vet Intern Med 28(3):894–904

Qamar AY, Fang X, Kim MJ, Cho J (2019) Improved post-thaw quality of canine semen after treatment with exosomes from conditioned medium of adipose-derived mesenchymal stem cells. Animals 9(11):865. https://doi.org/10.3390/ani9110865

Qamar AY, Fang X, Kim MJ, Cho J (2020) Improved viability and fertility of frozen-thawed dog sperm using adipose-derived mesenchymal stem cells. Sci Rep 10:7034. https://doi.org/10.1038/s41598-020-61803-8

Quijada P, Salunga HT, Hariharan N, Cubillo JD, El-Sayed FG, Moshref M, Bala KM, Emathinger JM, De La Torre A, Ormachea L, Alvarez R, Gude NA, Sussman MA (2015) Cardiac stem cell hybrids enhance myocardial repair. Circ Res 117:695–706. https://doi.org/10.1161/CIRCRESAHA.115.306838

Quimby JM, Dow SW (2015) Novel treatment strategies for feline chronic kidney disease: a critical look at the potential of mesenchymal stem cell therapy. Vet J 204(3):241–246. https://doi.org/10.1016/j.tvjl.2015.04.007

Quimby JM, Webb TL, Randall E, Marolf A, Valdes-Martinez A, Dow SW (2016) Assessment of intravenous adipose-derived allogeneic mesenchymal stem cells for the treatment of feline chronic kidney disease: a randomized, placebo-controlled clinical trial in eight cats. J Feline Med Surg 18:165–171. https://doi.org/10.1177/1098612X15576980

Raposo G, Stoorvogel W (2013) Extracellular vesicles: exosomes, microvesicles and friends. J Cell Biol 200(4):373–383. https://doi.org/10.1083/jcb.201211138

Ryan JM, Barry FP, Murphy JM, Mahon BP (2005) Mesenchymal stem cells avoid allogeneic rejection. J Inflamm 2:8. https://doi.org/10.1186/1476-9255-2-8

SAGE (2018) Mesenchymal stem cell therapy: holding promise for feline inflammatory diseases. ScienceDaily. Accessed Oct 27, 2020. www.sciencedaily.com/releases/2018/03/180313113501

Sarmah D, Rodriguez A-M, Kalia K, Yavagal DR, Bhatacharya P (2020) Mesenchymal stem cells (MSCs) protects hypoxic neuronal cells by mitochondrial transfer. Neurology 94(15):1749

Sasaki A, Mizuno M, Mochizuki M, Sekiya I (2019) Mesenchymal stem cells for cartilage regeneration in dogs. World J Stem Cells 11(5):254–269. https://doi.org/10.4252/wjsc.v11.i5.254

Shah K, Drury T, Roic I, Hansen P, Malin M, Boyd R, Sumer H, Ferguson R (2018) Outcome of allogeneic adult stem cell therapy in dogs suffering from osteoarthritis and other joint defects. Stem Cells Int 2018:7309201. https://doi.org/10.1155/2018/7309201

Shake JG, Gruber PJ, Baumgartner WA, Senechal G, Meyers J, Redmond JM, Pittenger MF, Martin BJ (2002) Mesenchymal stem cell implantation in a swine myocardial infarct model: engraftment and functional effects. Ann Thorac Surg 73:1919–1925

Solter D (2006) From teratocarcinomas to embryonic stem cells and beyond: a history of embryonic stem cell research. Nat Rev Genet 7(4):319–327

Song N, Scholtemeijer M, Shan K (2020) Mesenchymal stem cell immunomodulation: mechanisms and therapeutic potential. Trends Pharmacol Sci 41(9):653–664. https://doi.org/10.1016/j.tips.2020.06.009

Spees JL, Lee RH, Gregory CA (2016) Mechanisms of mesenchymal stem/stromal cell function. Stem Cell Res Ther 7:125. https://doi.org/10.1186/s13287-016-0363-7

Steffen F, Smolders LA, Roentgen AM, Bertolo A, Stoyanov J (2017) Bone marrow-derived mesenchymal stem cells as autologous therapy in dogs with naturally occurring intervertebral disc disease: feasibility, safety, and preliminary results. Tissue Eng Part C Methods 23(11):33. https://doi.org/10.1089/ten.tec.2017.0033

Takahashi K, Yamanaka S (2006) Cell 126:663–676

Takahashi S, Kobayashi S, Hirantani I (2018) Epigenetic differences between naïve and primed pluripotent stem cells. Cell Mol Life Sci 75(7):1191–1203

Tao H, Zhibo H, Zhong CH, Li Z (2016) Proangiogenic features of mesenchymal stem cells and their therapeutic applications. Stem Cells Int 2016:11. https://doi.org/10.1155/2016/1314709

Telugu B, Ezashi T, Roberts RM (2010) The promise of stem cell research in pigs and other ungulate species. Stem Cell Rev Rep 6(1):31–41. https://doi.org/10.1007/s12015-009-9101-1

Teshima T, Masumoto H, Michishita M, Matsuoka A, Shiba M, Nagashima T, Koyama H (2017) Allogenic adipose tissue-derived mesenchymal stem cells ameliorate acute hepatic injury in dogs. Stem Cells Int 2017:3892514. https://doi.org/10.1155/2017/3892514

Thomson JA (1998) Embryonic stem cell lines derived from human blastocysts. Science 282 (5391):1145–1147. https://doi.org/10.1126/science.282.5391.1145

Thomson M, Liu SJ, Zou L-N, Smith Z, Meissner A, Ramananahan S (2011) Pluripotency factors in embryonic stem cells regulate differentiation into germ layers. Cell 145(6):875–889. https://doi.org/10.1016/j.cell.2011.05.017

Ting W-J, Shaw SW, Hii L-Y et al (2020) Therapeutic effects of conditioned – DPBS from amniotic stem cells on lactating cow mastitis. Taiwanese J Obs Gynecol 59(4):520–526

Tosca L, Feraud O, Magniez A et al (2015) Genomic instability of human embryonic stem cell lines using different passaging culture methods. Mol Cytogenet 8(1):30

Villatoro AJ, Fernandez V, Claros S, Rico-Llanos G, Becerra J, Andrades JA (2015) Use of adipose-derived mesenchymal stem cells in keratoconjunctivitis sicca in a canine model. Biomed Res Int 2015:527926. https://doi.org/10.1155/2015/527926

Voga M, Adamic N, Vengust M, Majdic G (2020) Stem cells in veterinary medicine-current state and treatment options. Front Vet Sci 7:278

Wangler S, Kamali A, Wapp C et al (2021) Uncovering the secretome of mesenchymal stromal cells exposed to healthy, traumatic, and degenerative intervertebral discs: a proteomic analysis. Stem Cell Res Ther 12:11

Weiss ARR, Dahlke MH (2019) Immunomodulation by mesenchymal stem cells (MSCs): Mechanisms of action of living, apoptotic, and dead MSCs. Front Immunol 10:1191. https://doi.org/10.3389/fimmu.2019.01191

Wilke MM, Nydam DV, Nixon AJ (2007) Enhanced early chondrogenesis in particular defects following arthroscopic mesenchymal stem cell implantation in an equine model. J Orthop Res 25(7):913–925. https://doi.org/10.1002/jor.20382

Wynn RF, Hart CA, Corradi-Perini C, O'Neill L, Evans CA, Wraith JE et al (2004) A small proportion of mesenchymal stem cells strongly expresses functionally active CXCR4 receptor capable of promoting migration to bone marrow. Blood 104:2643–2645. https://doi.org/10.1182/blood-2004-02-0526

Xu X, Zheng L, Yuan Q, Xhen G, Crane JL, Zhou X, Cao X (2018) Transforming growth factor-β in stem cells and tissue homeostasis. Bone Res 6:2. https://doi.org/10.1038/s41413-017-0005-4

Yoon SY (2019) Mesenchymal stem cells for restoration of ovarian function. Clin Exp Reprod Med 46(1):1–7. https://doi.org/10.5653/cerm.2019.46.1.1

Yu J, Vodyanik M, Smuga-Otto K, Frane J, Antosiewicz-Bourget J, Frane J, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, Slukvin II, Thomson JA (2007) Induced pluripotent stem cell lines derived from human somatic cells. Science 318:1917–1920

Zakrzewski W, Dobrzynski M, Szymonowicz M, Rybak Z (2019) Stem cells: past, present, and future. Stem Cell Res Ther 10:68

Zhao Q, Ren H, Han Z (2016) Mesenchymal stem cells: Immunomodulatory capability and clinical potential in immune diseases. J Cell Immunother 2(1):3–20. https://doi.org/10.1016/j.jocit.2014.12.001

Introduction to Mammary Gland and Its Cell Types

2

Tajeshwar Preet Kaur, Ramneek Verma, and Ratan K. Choudhary

Abstract

The mammary gland is a highly dynamic organ. Histostructural composition of mammary tissue varies with the animal's physiology determined by the hormones produced at the different physiological stages. Mammary parenchyma contains various cell types: adipocytes, fibroblasts, epithelial cells, myoepithelial cells, inflammatory cells, and mammary stem cells (MaSCs). Among these various cell types, MaSC regulates mammary gland development and has a pivotal role in tissue regeneration. This chapter aims to introduce various cell types of the mammary gland and their roles in gland development and milk production. This chapter also discusses mammary stem cells, which explain the dynamic behavior of the gland.

2.1 Prenatal Development of the Mammary Gland

The development of the mammary gland starts when the animal is in the fetal stage. The first discernable structure of cells during the early developmental stage is the "mammary band." In bovine, the mammary band appears as early as 32 days after conception and ectodermal in origin. During this developmental process, epithelial-mesenchymal interactions play a role where mammary bud develops from the ectodermal cell. The mesenchymal signaling network brings modifications (expansion, placing cells to mammary line, and elongation) to mammary bud. These modifications confer sexual dimorphism of the mammary glands in a species-specific manner (Rowson et al. 2012). Transcription factors and other signaling

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molecules that are involved in early embryonic mammary developments are fibroblast growth factors, wingless-related integration site (WNT), Homeobox gene family (HOX), and T-Box family (TBX) (Carroll and Capecchi 2015).

2.2 Postnatal Development of the Mammary Gland

2.2.1 Early Development

In newborn female calves, mammary parenchyma is barely palpable as mammary cells exist in negligible quantities. Mammary parenchyma extends threadlike mass dorsally above each teat (Meyer et al. 2006). The mammary parenchyma consists of a gland cistern and a ductal system lined with 2–3 layers of epithelium accompanied by terminal alveolar structures (Daniels et al. 2009). Mammary stem cells (MaSCs), present in the mammary gland, are vital for maintaining mammary homeostasis and repair. The mammary gland develops significantly postnatally from heifers to lactating stages and again back to prepubertal-like stages, which emphasizes the pivotal roles of adult stem and progenitor cells on the mammary gland (Zhou et al. 2019).

2.2.2 Prepubertal Development

From birth to the time until estrous is attained is called the prepubertal phase of mammary development. This stage is essential because the amount of mammary parenchym formed during this stage will mark the performance of the first lactation and the future lactations. During this period, mammary epithelial cells grow in the mammary fat pad, and ductal branching and elongation occur. Classical ovariectomy experiments led to the conclusion that the development of the mammary gland heavily relies on steroid hormones (estrogen and progesterone) produced in the ovary (Velayudhan et al. 2015). In addition, there are other hormones like growth hormone (GH), insulin-like growth factor (IGF-1), and IGF-1/GH axis that bring proliferation to mammary parenchyma (Berry et al. 2003). Positive allometric mammary growth in heifers commences at 2–3 months of age (Sinha and Tucker 1969). As an estimate, this growth corresponds to a 60-fold increase in mammary parenchymal mass at 3 months of age in comparison to the mammary mass at birth (Meyer et al. 2006). Progesterone receptors are expressed in the embedded layer of mammary epithelium in prepubertal bovine (Choudhary et al. 2013), indicating that progesterone has a role in the proliferation of mammary epithelium during the prepubertal stage. Following ovariectomy, PR becomes undetectable (Velayudhan et al. 2015).

Other tissue compartments and factors are impacting prepubertal mammary gland development, and they are: (1) plane of nutrition, (2) stromal compartment, (3) fat pad, (4) myoepithelial cells, and (5) other factors like prolactin, epidermal growth factor, transforming growth factor-β, and insulin.

2.3 Mammary Growth During Pregnancy

The mammary gland undergoes an exponential phase of growth during the pubertal stage and then during pregnancy. These dynamic morphological changes of the mammary gland during various physiological stages of animals are brought by the hormones and the stem cells. For example, during puberty, mammary gland development occurs through unipotent progenitor cells, such that all basal cells arise from basal progenitor cells alone and all luminal cells from luminal progenitors (Van Keymeulen et al. 2011; van Amerongen et al. 2012). The pubertal gland comprises diverse stem and progenitor cell populations, including bipotent stem cells that generate luminal and myoepithelial cells of the duct (Rios et al. 2014). The mammary epithelium shows dramatic regenerative potential. It can undergo several cycles of growth and involution, signifying that the mammary gland epithelial compartment comprises mammary epithelial stem cells are single epithelial cells capable of generating the entire epithelial architecture (Inman et al. 2015). The entire functional mammary gland can be derived from a single cell (Kordon and Smith 1998; Shackleton et al. 2006; Stingl et al. 2006), supporting the idea that mammary epithelium comprises a stem cell population. Various investigators showed the existence of stem-like cells in the mammary gland of various species and established hierarchical organization of cells and their molecular players.

The majority of mammary growth happens throughout pregnancy. Mammary growth is an unceasing, exponential process from conception to parturition. There is the highest increase in parenchymal tissue mass during late pregnancy, evident by increased udder size. The elongation of mammary ducts, the development of alveoli, and the lessening of identifiable fat cells in the fat pad result increased udder size. MEC complete differentiation during pregnancy and milk synthesis commences. The alveoli show secretory activities in the last month of pregnancy, and the increment in udder size begins due to the accumulation of the secretory material. The simultaneous rise in blood concentrations of steroid hormones, namely estrogen and progesterone, is the primary cause of mammary growth during pregnancy. High levels of nutrition help increase future lactation potential and improve mammary development. It was found that with each pound rise in body weight at first calving, there is an upsurge in milk yield during the first lactation. A study showed that feeding high energy levels during pregnancy causes considerable enhancements in subsequent milk production (Macias and Hinck 2012).

2.4 Mammary Growth During Lactation

Mammary gland growth continues in early lactation, which may account for less than 10% of total mammary gland development in ruminants. A gradual decline in milk yield is seen after peak lactation (45–60 days after calving). Peak milk production relies on the number of differentiated alveolar cells secreting milk during lactation. Moreover, lactation (maintaining peak milk yield) is a function of the continued survival of those milk-secreting cells. There has been comparatively little

concentrated exploration on mammary growth during lactation in dairy cattle. However, numerous studies are observing the growth in rats and swine. An increase in total mammary DNA has been seen from parturition until weaning in rats. The rise in DNA represents an upsurge in cell number, and this growth is significant in determining milk production.

2.5 Post-lactational Mammary Gland Involution

Drying off period and lactation are routine management practices for optimal milk production in ensuing lactation. Various methods have been described to stop milking without affecting gland healthy and prepare cow to cease lactation. These methods of stopping milk secretion include reducing milking frequency, modulating the plane of nutrition from high to low plane, and antibiotic dry cow therapy (Vilar and Rajala-Schultz 2020). Dairy cows undergo a "regenerative involution" (Capuco et al. 1997; Capuco and Ellis 2013), where cell death and proliferation co-occur. Post-lactational regression has been established to be a two-stage process through murine models. The initial stage is reversible, while the later stage is irreversible. Suckling by pups can successfully recommence milking if the offspring are returned during the initial phase despite extensive cell death. The second irreversible stage sets in about 48 h onwards, causing further cell death and tissue remodeling (Hughes and Watson 2012).

Interestingly, in cows having abrupt discontinuation of milking at mid-lactation for seven days, milking reinitiates (with comparable milk yield and composition) to that observed before the onset of involution (Singh et al. 2015). At the point of involution or "drying off," animals exhibit "parallel pregnancy" involution signature (Hughes and Watson 2018), indicating cell death and proliferation co-occurs. The extent of apoptosis of mammary cells is reduced during concurrent pregnancy (Capuco et al. 2002a, b). However, there is an earlier onset of apoptosis in pregnant mice while undergoing natural weaning (Quarrie et al. 1996). The involution process in mice that are concurrently pregnant differs notably in non-pregnant mice in terms of cell death dynamics, histo-morphology, and patterns of gene expression (Quarrie et al. 1996).

2.6 Gross Morphology of Ruminant Mammary Gland

The first recognizable sign of mammary development that occurs during prenatal life is the mammary line's development. Postnatal development of mammary gland involves a series of complex physiological processes occurring during distinct physiological stages of the animal, namely, prepubertal, pubertal, pregnancy, lactation, and involution. These processes involves interactions of hormones, growth factors, and suppressors act in an orchestrated fashion to bring desired growth of the gland. Understanding the mammary gland's cellular and molecular development is imperative because its gross structure and tissue architecture differ among species.

The mammary gland develops in a tree-like fashion composed of hollow branches of ducts—tubuloalveolar structure. At the end of the ductal branches, a bunch of grape-like structures of mammary alveoli is visualized. It is composed of many histological lobes separated from each other by interlobular connective tissue. The lobes are subdivided into lobules and are lined with connective tissue strands (intralobular connective tissue). Lobules contain many alveolar structures. Thus, mammary glands parenchyma comprises alveoli, a network of ducts and bundles of connective tissue. The alveolus is the elementary secretory unit. It is sac-like or vesicle of unequal size lined by a single layer of mammary epithelial cells (MEC). They are made of flattened or cuboidal epithelial cells with a round nucleus. Myoepithelial cells present the basal side of MEC are responsible for squeezing of alveoli at the time of milk production, also hypothesized to contain mammary stem cells. Myoepithelial cells (Myo) are surrounded by the basement membrane separating epithelial cells (Myo and MEC) from the stroma (Fig. 2.1).

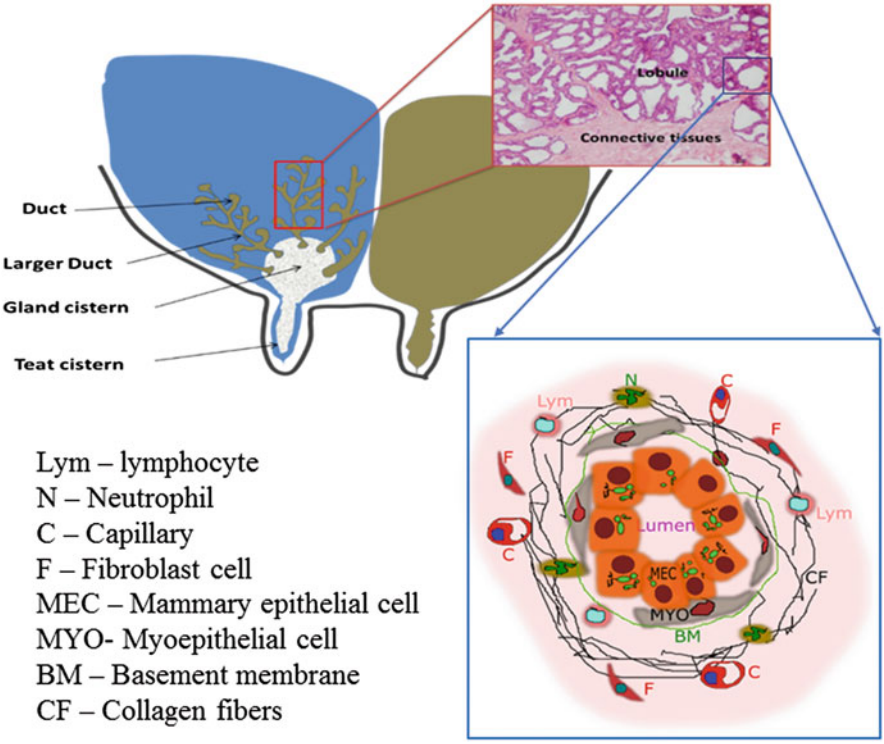

Fig. 2.1 Tree-like structure of the mammary glands of a ruminant with developed alveolar structure

2.7 Histomorphology of Mammary Gland

Gross morphology: The mammary gland's histological structure comprises two types of tissues: parenchyma and stroma. The parenchyma originates from the embryo's ectoderm and corresponds to the secretory part of the gland. The stroma is constituted of lymph, blood vessels, adipose, connective, and nervous tissues (Delouis and Richard 1991). Histological study reveals that the mammary gland's structure differs during various physiological stages. Following the difference in the mammary glands during various physiological stages, we can say that during the prepubertal stage, MEC are arranged in 2–3 layers of cells, and they are called terminal ductal lobular unit (TDLU) (Choudhary et al. 2018) (Fig. 2.2). While during the non-lactating stage, gland structure is similar to the prepubertal stage, but instead of TDLU, shrunken and inactive alveoli are present. Milk is synthesized in the secretory cells only during the active lactation stage. MECs are arranged in a single layer surrounded by the basement membrane in a spherical structure called alveoli (Fig. 2.1). Thus mammary gland is a very dynamic and complex organ whose histomorphology changes drastically depending upon the animal's stage. The mammary gland's dynamism is regulated by the presence of undifferentiated mammary epithelial cells called mammary stem cells (MaSCs) (Choudhary et al. 2018).

The mammary gland undergoes several alterations in its volume, internal structure, and composition during gestation and lactation. Structural alterations have a substantial impact on the quantity and quality of milk production. During pregnancy, no differences in udder weights are found until day 120, after which weight increases significantly. The majority of udder growth is observed between the last 30 days of pregnancy and the first 10 days of lactation. The modification of mammary gland tissue composition is apparent during gestation and lactation and during the first 15 days of gestation. During this time, parenchymal tissue modification occurs with a declination of the amount of fatty tissue and fluid-rich tissue upsurges. After that, constant change in mammary gland composition is observed throughout late gestation and the entire lactation period. The alterations in parenchyma composition can

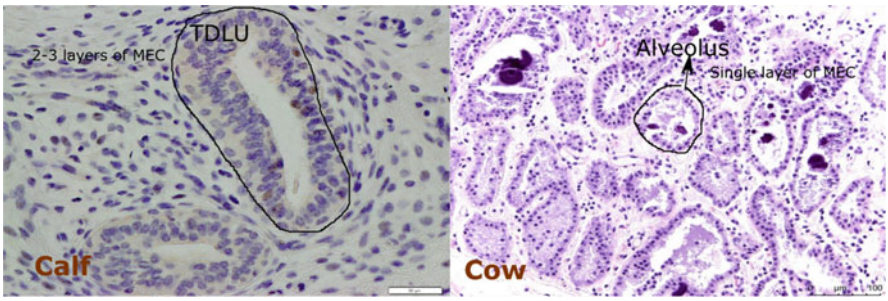

Fig. 2.2 Prepubertal and lactating glands of bovine. Histomorphology of the prepubertal (left panel) and lactating (right panel) bovine mammary tissue section stained with hematoxylin

be directly related to fluid accumulation in the gland due to the increment of milk secretion.

Morphology of mammary epithelial cells is polarized and becomes columnar. During polarization, the cell's nucleus is pushed toward the basal side (plasma membrane close to the basement membrane). In contrast, the terminal differentiation of cells results in the development of microvilli on the apical surface (side close to the lumen of alveoli). Mammary alveoli are surrounded by a thin layer of fibroblasts and capillaries. All these alterations can be related to the mammary gland's preparation for milk production. Indeed, the lobular-alveolar system's development is essential to produce and store milk. The number of secretory alveolar epithelial cells increases during early and mid-lactation and diminishes during late lactation (Elsayed et al. 2009). Likewise, the surface of alveoli is smaller during late lactation compared to early and mid-stages of lactation.

Additionally, alveoli numbers are reduced, and stroma tissue increases during late lactation. Also, during mid and late lactation, the alveoli number and sizes are reduced, and stromal tissue increases. There are immature alveoli with a thin lumen and cuboidal epithelial cells with a large nucleus during late gestation. Some alveoli are filled with secretion and lipid droplets in the epithelial cells (Colitti and Farinacci 2009). During late gestation and onset of lactation, alveoli size/number and lumen area progressively increase.

On the other hand, the stroma tissue is substituted to accommodate adequate secretory cells to produce milk. These changes are due to the secretion of matrix metalloproteinases that reorganize stromal tissue. The modification of the epithelial cells' shapes and the presence of secretory vesicles is also a variation essential for milk production. In conclusion, the mammary gland undergoes massive structural alterations in its cell shape, differentiation, secretory capacity, number of secretory cells, and the area of alveoli during the various stages of lactation, including from immature to mature stage at early lactation (Akers 2002; Cadar et al. 2012).

2.8 Types of Cells in the Mammary Gland

The mammary gland consists of secretory tissue, mammary epithelial cells (MEC), and connective tissue (stromal cells, adipose tissue, nerve cells, blood vessels, and inflammatory cells). The amount of secretory tissue or the number of secreting MEC is the limiting factor for the udder's milk-producing capacity. It is a consensus that a big udder produces more milk due to the high capacity of the gland. However, this is not true because a big udder might comprise more adipose and connective tissues instead of milk-secreting cells.

2.9 Mammary Epithelial Cell (MEC)

In the mammary gland, epithelial cell is the main component and considered the cause of the mammary gland's maximum activity. Mammary epithelial cells (MEC) can broadly be classified into basal and luminal cells. Luminal epithelial cells line mammary ducts and alveoli. MEC contains cytoplasm, nucleus, endoplasmic reticulum, Golgi apparatus, mitochondria, and lysosome. The ribosomes attached to the outside of the rough endoplasmic reticulum are the sites of protein synthesis (Tyler and Ensminger 2006). The Golgi apparatus releases casein and lactose-containing secretory vesicles as well as the site of protein modifications. There are numerous mitochondria present, the organelles that house the energy-releasing reactions, and their number increases as energy demand during lactation increases. The purpose of the lysosome is to destroy old nonfunctional MEC to make an area for new cells. Tight junctions create a milk/blood barrier limiting transportation between the cells to separate each separate component. In the ducts, tight junctions are present in continuity, but their distribution is somewhat discontinuous around alveoli. Luminal epithelial cells play a role in milk synthesis, storage, and secretion. The primary role of epithelial cells is to remove nutrients from adjacent blood capillaries and use them to synthesize milk (Bauman et al. 2006; Tyler and Ensminger 2006). During the transition/periparturient period (14 days before and after parturition), cows may experience excessive metabolic load and decrease the immune system and hence vulnerable to metabolic disorders and mammary infection. An increasing amount of milk production from early lactation to peak lactation (usually 2–3 months after the parturition in cow) is accounted by the increasing rate of MEC proliferation. Estrogen and progesterone hormones stimulate MEC proliferation (Capuco et al. 2001). MEC differentiation depends on the hormones prolactin and glucocorticoids. Prolactin is involved with the development of the Golgi apparatus and secretory vesicles. Glucocorticoids, the primary one cortisol, develop the rough endoplasmic reticulum (Akers 2002).

Upon reaching lactation at peak, milk production decreases due to an increased rate of programmed cell death and decreased cell proliferation. During the dry period, the MEC components regress, proliferate, and differentiate. In bovine, regression and proliferation of MEC coincide, with the ultimate goal of maximizing milk production in the subsequent lactation (Martignani et al. 2014). Surprisingly, during this period, there is no MEC loss, and hence this period is called "regenerative involution." During the dry period, increasing cell proliferation rate is hypothesized to enhance regeneration of the mammary gland and likely to reduce the non-lactating and non-productive dry period (60 days). Mammary stem cell manipulation in cows after peak lactation may increase cell proliferation or decrease the apoptosis of MEC and hence likely to improve lactation persistency (Stelwagen et al. 2013; Choudhary 2014). Therefore, the productivity of dairy cows can be changed by changing MEC numbers by understanding the homeostasis of mammary stem cells during the dry period and during the late lactation.

2.10 Myoepithelial Cells

Dyce et al. (2002) and Samuelson (2006) described that the mammary gland secretory units are surrounded by myoepithelial cells. Frandson et al. (2009) described that the alveoli and ducts are surrounded by contractile myoepithelial cells, also called basket cells. The alveoli are surrounded by branched, stellate, myoepithelial cells, the contraction of which aids in the emptying of the alveoli. The myoepithelial cells also are located along the ducts and alveoli. Morphologically, myoepithelial cells resemble smooth muscle cells and present on the outer side of MEC. Myoepithelial cells separate epithelial cell compartments from the stroma and are mesenchymal in nature. However, they also exhibit some features of epithelial cells, like an expression of specific cytokeratins. When contracted, myoepithelial cells provide compression on the alveoli and ducts milk to be directed toward the lactiferous sinus. There is a contraction of alveoli when the hormone oxytocin circulates and brings milk let down. In addition to alveolar contractions, cells also do a variety of functions, namely, modulation of cell proliferation, mammary gland morphogenesis during all the developmental stages, differentiation of luminal cells, and also protects from mammary cancer. Thus, myoepithelial cells act as a nature suppressor of mammary tumors (Goodwin and Nelson 2018).

2.11 Adipocytes

The mammary gland is exclusive in its requirement to develop a close association with a depot of adipose tissue called "mammary fat pad." The mammary fat pad represents mainly two types of adipocytes—white and brown fat cells, depending on the number of cellular compartments that are unilocular and multilocular, respectively. White fat cells are unilocular, while the brown fat cells are multilocular. It makes a complex stromal microenvironment and plays a role in tissue homeostasis and gland growth (Hovey and Aimo 2010). Mammary adipose tissue forms a local environment and provides signaling cues to mammary parenchyma. Hormones like prolactin are synthesized by mammary adipose tissues and play a role in prolactin-mediated mammary growth and lactation. Fat cells and associated stromal cells of mammary tissue are the target site for estrogen hormone. Estrogen receptor (ER) has been localized in the mammary adipocytes and fibroblasts of heifers (Meyer et al. 2006). The mammary fat pad also provides local IGF-1-medicated growth of ductal epithelium. IGF-I mRNA expression was observed in the fat pad in the proximity of the parenchymal tissue in the ewe (Hovey et al. 1998). In addition to this, mammary adipocytes secrete vascular endothelial growth factor (VEGF), hepatocyte growth factor (HCF), leptin, and other metabolites (Hovey and Aimo 2010). In bovine, distinct expression of the gene expression profile of mammary fat pad and stromal tissue was detected and identified 124 estrogen-responsive genes (Li et al. 2006). A recent study indicated that adipocytes (PDFGRα-positive) might serve as the reservoir of mammary epithelial progenitor cells (Joshi et al. 2019), indicating the role of adipocytes in mesenchymal to epithelial transition in the development of the

mammary gland. Thus, mammary adipocytes are the complex microenvironment and provide signaling cues to the mammary gland's development and functions.

2.12 Mammary Stem/Progenitor Cell

Stem cells are specialized cells showing the self-renewal capacity and the ability to proliferate and differentiate into various cell types. Mammary stem cells (MaSCs) were shown to generate a semi-functional mammary gland (functional alveolar subunits without a complete excretory network) in mouse (Shackleton et al. 2006) and human (Stingl et al. 2006). The proliferation of mammary stem cells in the bovine mammary gland may also influence milk production (Capuco et al. 2009). Mammary stem cells differentiate into cells of the myoepithelial and luminal lineages (Choudhary 2014). The mammary stem cells are epithelial in origin and exist in conjunction with cells of the connective tissue and fat pad, which are of mesenchymal origin (Choudhary 2014). Precise identification and then the characterization of MaSCs are conflicting and need further investigation in terms of their dynamics and complexity (Joshi et al. 2010; Kaimala et al. 2012). Identifying different MaSC subtypes will allow precise targets to be found for optimal manipulation for increased milk production in the dairy animals and hypogalactia mother. Mammary stem cells serve as a source of cells for the mammary gland's growth during puberty and gestation. MaSCs have been isolated from human and mouse tissues and cell lines derived from mammary epithelial cells. Single cells can give rise to both the luminal and myoepithelial cell types of the gland and have the ability to produce the entire organ in mice (Liu et al. 2007). Transplantation studies showed the existence of MaSC in enzymatically dissociated mouse mammary epithelial tissue when seeded into the cleared (epithelium-free) fat pads of immunodeficient mice (Wagner et al. 2002). Due to their self-renewal property, stem cells divide in two different fashions—symmetric and asymmetric cell division. Symmetric division gives rise to two identical daughter stem cells or two differentiated cells. The asymmetric division produces two asymmetrical cell types—one stem cell and the other progenitor cell or differentiated cells. Progenitors can undergo cell division several times before finally differentiating into a mature cell. The first evidence of mammary stem and progenitor cells' presence was suggested by a tissue fragment transplantation experiment when investigators transplanted epithelial tissue fragments into mammary fat pads cleared of the epithelium. Cell surface protein markers have been used to identify the MaSCs population using multiparameter cell sorting. It is seen that virtually any part of the mammary gland could reprise the entire glandular structure on transplanting into cleared mammary fat pads, indicating a candidate stem cell population's localization along the entire ductal tree (Stingl et al. 2006). A study on the human female mammary gland reveals that the gland undergoes dramatic changes during pregnancy. Even though lobules are present before pregnancy, the gland's complete development and differentiation occur only after the end of the first full-time pregnancy. Proliferating cells morphologically similar to pale staining cells observed in the murine mammary gland were observed

in the human breast, signifying stem cells' presence. These cells were pale-stained, with a round shape, and possessed large nuclei and clear cytoplasm. The distribution of organelles like mitochondria and endoplasmic reticulum was scanty, and these cells possessed terminal bars and tight junctions. In the pubertal bovine mammary gland, four-cell sub-populations were characterized by multiparameter cells sorting. They were, (1) basal lineages committed cells—CD49fhigh, CD24neg, $CD10^{+}$, $KRT14^{+}$, $vimentin^{+}$ and $PROCR^{+}$; (2) luminal cells ($CD49f^{low}$)—$CD24^{neg}$; (3) luminal cells ($CD49f^{low}$)—$CD24^{pos}$; and (4) mammary stem cells—$CD49f^{high}CD24^{pos}$. Mammary stem cells were not pure but a mixture of cell types with varying expression levels of CD10, KRT14, and KRT7 (Finot et al. 2018). In-depth characterization of bovine mammary epithelial sub-populations provided insights into the lineages and cell commitment in mammary gland development. The emerging diversity among MaSC and progenitor populations of the mammary epithelium in humans is likely to underpin breast cancer characteristics. Incidences of mammary cancer in ruminant are rare.

References

Akers RM (2002) Lactation and the mammary gland. Iowa State University Press, Ames, p 278

Bauman DE, Mather IH, Wall RJ, Lock AL (2006) Major advances associated with the biosynthesis of milk. J Dairy Sci 89(4):1235

Berry SD, Howard RD, Jobst PM, Jiang H, Akers RM (2003) Interactions between the ovary and the local IGF-1 axis modulate mammary development in prepubertal heifers. J Endocrinol 177:295

Cadar M, Miresan V, Lujerdean A, Raducu C (2012) Mammary gland histological structure in relation with milk production in sheep. J Anim Sci Biotechnol 45:3

Capuco AV, Ellis SE (2013) Comparative aspects of mammary gland development and homeostasis. Annu Rev Anim Biosci 1:179

Capuco AV, Akers RM, Smith JJ (1997) Mammary growth in Holstein cows during the dry period: quantification of nucleic acids and histology. J Dairy Sci 80(3):477

Capuco AV, Wood DL, Baldwin R, Mcleod K, Paape MJ (2001) Mammary cell number, proliferation, and apoptosis during a bovine lactation: Relation to milk production and effect of bST. J Dairy Sci 84:2177

Capuco AV, Ellis S, Wood DL, Akers RM, Garrett W (2002a) Postnatal mammary ductal growth: three-dimensional imaging of cell proliferation, effects of estrogen treatment, and expression of steroid receptors in prepubertal calves. Tissue Cell 34:143

Capuco AV, Li M, Long E, Ren S, Hruska KS, Schorr K, Furth PA (2002b) Concurrent pregnancy retards mammary involution: effects on apoptosis and proliferation of the mammary epithelium after forced weaning of mice. Biol Reprod 66(5):1471

Capuco AV, Evock-Clover CM, Minuti A, Wood DL (2009) *In vivo* expansion of the mammary stem/progenitor cell population by xanthosine infusion. Exp Biol Med 234:475

Carroll LS, Capecchi MR (2015) Hoxc8 initiates an ectopic mammary program by regulating Fgf10 and Tbx3 expression and Wnt/β-catenin signaling. Development 142:4056–4067

Choudhary RK (2014) Mammary stem cells: expansion and animal productivity. J Anim Sci Biotechnol 5:36

Choudhary RK, Li RW, Evock-Clover CM, Capuco AV (2013) Comparison of the transcriptomes of long-term label retaining-cells and control cells microdissected from mammary epithelium: an initial study to characterize potential stem/progenitor cells. Front Oncol 3:21

Choudhary RK, Pathak D, Choudhary S, Verma R (2018) Immunolocalization of estrogen alpha and progesterone beta receptors in goat mammary gland. Indian J Anim Sci 88:31

Colitti M, Farinacci M (2009) Cell turnover and gene activities in sheep mammary glands prior to lambing to involution. Tissue Cell 41:326

Daniels KM, Capuco AV, McGilliard ML, James RE, Akers RM (2009) Effects of milk replacer formulation on measures of mammary growth and composition in holstein heifers. J Dairy Sci 92:5937

Delouis C, Richard PH (1991) La lactation. In: Thibault C, Levasseur MC (eds) La reproduction chezlesmammifères et l'homme. INRA, Paris, p 487

Dyce KM, Sack WO, Wensing CJG (2002) Text book of veterinary anatomy, 3rd edn. Saunders, Philadelphia, p 723

Elsayed EH, ElShafie MH, Saifelnasr EOH, Abu El-Ella AA (2009) Histological and histochemical study on mammary gland of Damascus goats through stages of lactation. Small Rumin Res 85:11

Finot L, Chanat E, Dessauge F (2018) Molecular signature of the putative stem/progenitor cells committed to the development of the bovine mammary gland at puberty. Sci Rep 8:16194

Frandson DR, Wilke WL, Fails AD (2009) Anatomy and physiology of farm animals, 7th edn. Wiley, New York, pp 449–456

Goodwin K, Nelson CM (2018) Myoepithelial crow control of cancer cells. J Cell Biol 217 (10):3319–3321

Hovey RC, Aimo L (2010) Diverse and active roles for adipocytes during mammary gland growth and function. J Mammary Gland Biol Neoplasia 15:279–290

Hovey RC, Davey HW, Mackenzie DD, McFadden TB (1998) Ontogeny and epithelial-stromal interactions regulate IGF expression in the ovine mammary gland. Mol Cell Endocrinol 136 (2):139–144

Hughes K, Watson CJ (2012) The spectrum of STAT functions in mammary gland development. JAKSTAT 1(3):151

Hughes K, Watson CJ (2018) The mammary microenvironment in mastitis in humans, dairy ruminants, rabbits and rodents: a one health focus. J Mammary Gland Biol Neoplasia 23 (1–2):27

Inman JL, Robertson C, Mott JD, Bissell MJ (2015) Mammary gland development: cell fate specification, stem cells and the microenvironment. Development 142:1028

Joshi PA, Jackson HW, Beristain AG, Di Grappa MA, Mote PA, Clarke CL, Stingl J, Waterhouse PD, Khokha R (2010) Progesterone induces adult mammary stem cell expansion. Nature 465:803

Joshi PA, Waterhouse PD, Kasaian K et al (2019) PDGFRα+ stromal adipocyte progenitors transition into epithelial cells during lobulo-alveologenesis in the murine mammary gland. Nat Commun 10:1760

Kaimala S, Bisana S, Kumar S (2012) Mammary gland stem cells: more puzzles than explanations. J Biosci 37:349

Kordon EC, Smith GH (1998) An entire functional mammary gland may comprise the progeny from a single cell. Development 125:1921

Li RW, Meyer MJ, Van Tassell CP, Sonstegard TS, Connor EE, Van Amburgh ME, Boisclair YR, Capuco AV (2006) Identification of estrogen-responsive genes in the parenchyma and fat pad of the bovine mammary gland by microarray analysis. Physiol Genomics 27(1):42–53

Liu TM, Martina M, Hutmacher DW, Hui JHP, Lee EH, Lim B (2007) Identification of common pathways mediating differentiation of bone marrow and adipose tissue derived human mesenchymal stem cells into three mesenchymal lineages. Stem Cells 25(3):750

Macias H, Hinck L (2012) Mammary gland development. Dev Biol 1(4):533

Martignani E, Cravero D, Miretti S, Accornero P, Baratta M (2014) Bovine mammary stem cells: new perspective for dairy science. Vet Q 34:52

Meyer MJ, Capuco AV, Ross DA, Lintault LM, Van Amburgh ME (2006) Developmental and nutritional regulation of the prepubertal bovine mammary gland. I. Epithelial cell proliferation, parenchymal accretion rate, and allometric growth. J Dairy Sci 89:4298

Quarrie LH, Addey CV, Wilde CJ (1996) Programmed cell death during mammary tissue involution induced by weaning, litter removal, and milk stasis. J Cell Physiol 168(3):559

Rios AC, Fu NY, Lindeman GJ, Visvader JE (2014) In situ identification of bipotent stem cells in the mammary gland. Nature 506:322

Rowson AR, Daniels KM, Ellis SE, Hovey RC (2012) Growth and development of the mammary glands of livestock: a veritable barnyard of opportunities. Semin Cell Dev Biol 23(5):557

Samuelson DA (2006) Textbook of veterinary histology. Saunders, Township, p 474

Shackleton M, Vaillant F, Simpson KJ, Stingl J, Smyth GK, Asselin-Labat ML, Wu L, Lindeman GJ, Visvader JE (2006) Generation of a functional mammary gland from a single stem cell. Nature 439:84

Singh K, Swanson KM, Henderson HV, Erdman RA, Stelwagen K (2015) The effect of milking reinitiation following extended nonmilking periods on lactation in primiparous dairy cows. J Dairy Sci 98(11):7666

Sinha YN, Tucker HA (1969) Mammary development and pituitary prolactin level of heifers from birth through puberty and during the estrous cycle. J Dairy Sci 52:507

Stelwagen K, Phyn CVC, Davis SR, Guinard-Flament J, Pomiès D, Roche JR, Kay JK (2013) Invited review: reduced milking frequency: milk production and management implications. J Dairy Sci 96:3401

Stingl J, Eirew P, Ricketson I, Shackleton M, Vaillant F, Choi D, Li HI, Eaves CJ (2006) Purification and unique properties of mammary epithelial stem cells. Nature 439:993

Tyler HD, Ensminger ME (2006) Dairy cattle science. Pearson Prentice Hall, London

van Amerongen R, Bowman AN, Nusse R (2012) Developmental stage and time dictate the fate of Wnt/β-catenin-responsive stem cells in the mammary gland. Cell Stem Cell 11:387

Van Keymeulen A, Rocha AS, Ousset M, Beck B, Bouvencourt G, Rock J, Sharma N, Dekoninck S, Blanpain C (2011) Distinct stem cells contribute to mammary gland development and maintenance. Nature 479:189

Velayudhan BT, Huderson BP, Ellis SE, Parsons CL, Hovey RC, Rowson AR, Akers RM (2015) Ovariectomy in young prepubertal dairy heifers causes complete suppression of mammary progesterone receptors. Domest Anim Endocrinol 51:8–18

Vilar MJ, Rajala-Schultz PJ (2020) Dry-off and dairy cow udder health and welfare: effects of different milk cessation methods. Vet J 262:105503

Wagner KU, Boulanger CA, Henry MD, Sgagias M, Hennighausen L, Smith GH (2002) An adjunct mammary epithelial cell population in parous females: its role in functional adaptation and tissue renewal. Development 12(9):137786

Zhou J, Chen Q, Zou Y, Zheng S, Chen Y (2019) Stem cells and cellular origins of mammary gland: updates in rationale, controversies, and cancer relevance. Stem Cells Int 2019:4247168

Mammary Stem Cells: How Much Do We Know?

3

Alok Kumar and Satish Kumar

Abstract

Mammary stem cells are fundamental to the process of glandular development and homeostasis. Understanding the mammary gland biology is essential to delineate the processes that, in sync with the female's systemic hormones, maintain the supply of stem cells with multilineage differentiation potential. Like hematopoietic stem cells, the mammary stem cells also differentiate hierarchically through asymmetrical divisions and give rise to a daughter stem cell and a progenitor cell with lineage-restricted differentiation capabilities. Identification of such stem cells and the key signaling events that may initiate malignant transformations or lead to the mammary tissue's functional recovery will be the desired outcomes of the ongoing endeavors. These overwhelming possibilities assure that decoding the associated molecular factors/pathways that tightly regulate MaSCs activities will substantially add to our current understanding of breast oncogenesis, dairy animal productivity, and post-mastitis management.

Keywords

Mammary stem cells · MaSCs · Estrogen receptor · ER · Progesterone receptor · PR · Cluster of differentiation · CD

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3.1 Introduction

The mammary gland is unique to the class Mammalia. As one of the critical factors for their reproductive success, it has been instrumental in contributing to the mammalian species' remarkable survival fitness. This unique organ contributed significantly to the establishment of placentation in mammals, which led to the transition from oviparity to viviparity. This tissue had also impacted human history as it evolved and transitioned from hunters and gatherers to practicing agriculture and domesticating dairy animals. All of this had a strong correlation with the functional properties of this organ. The mammary gland's distinctive ability to secrete milk for the nourishment and the well-being of the young ones adds a new dimension to the maternal mode of investment into reproduction. This encouraged a generation of researchers to study the physiology of the gland.

Post puberty, the mammary gland goes through distinctive phases of growth, development, and functional activity well spread across the female individuals' reproductive age. The ability to lactate is a transient functionality that begins at each postpartum event and ceases once the infant's requirements for the milk decreases, reverting the gland to its pre-pregnancy state. This repetitive event of functional development and regression in the mammary gland during each cycle of a successful pregnancy and parturition event supported and strengthened the concept of mammary stem cells (MaSCs) in the gland. Inside a normal mammary gland, the MaSCs are a population of histologically undifferentiated epithelial cells that either forms two identical stem cells or stem cells and a progenitor cell with a committed cell lineage identity following a round of symmetrical or asymmetrical cell division, respectively. These stem cells and a few progenitor cells, and the rest of the somatic cells respond and proliferate in synchrony with the cyclical changes of the female's reproductive cycle (Capuco and Choudhary 2020).

The presence of MaSCs and the potential progenitor cells with single or bi-lineage differentiation capabilities (ductal and lobular) has been demonstrated through multiple studies that involved cell transplantation techniques in mice models (Smith and Medina 1988; Kordon and Smith 1998). The coordinated activity of these cells in response to systemic female hormones supports growth, development, and homeostasis maintenance in the gland (Fig. 3.1).

Thus, MaSCs, which are uniformly distributed across the mammary parenchyma, participate in the cyclic event of development and involution during each cycle of pregnancy and lactation. Such cells with self-renewing capacity and the ability to differentiate into multiple lineages are well accepted, but such cells' exact identity is still elusive. In this book chapter, we will discuss in some detail the identity of MaSCs.

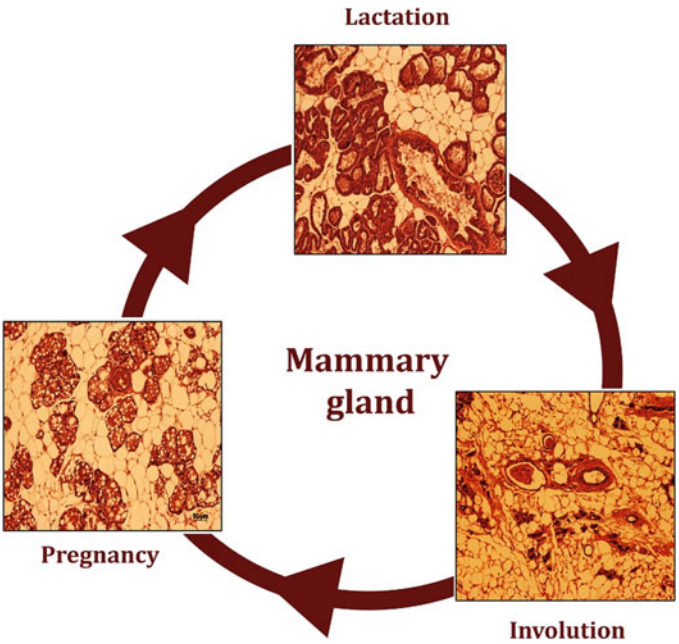

Fig. 3.1 The cycling phases of mammary epithelial cells development and regression during each session of pregnancy, lactation, and involution. The H&E sections of the mouse mammary gland at three different time points show how drastically the mammary epithelial turnover occurs during a single cycle of pregnancy, lactation, and involution

3.2 Discovery, Isolation, and Characterization of MaSCs

The development of new cellular assays and techniques over a couple of decades had led to a significant increase in our understanding of the MaSCs and their biology. The successful isolation and characterization of MaSCs population, mostly in rodent models and to some extent in humans, had added much to our current understanding of mammary tissue homeostasis and the causes of oncogenesis. The transplantation studies where the multipotent stem cell population was isolated from the mouse mammary gland using the specific cell-surface markers showed that a single cell, when provided the stromal microenvironment, can reconstitute and develop in vivo into a mammary gland. Such observations indicated that an independent stem cell has the required potential to regenerate the entire gland. The initial experiment by DeOme et al. (1959), where he transplanted tissue fragments into the cleared mouse mammary fat pads, established an excellent in vivo model for future studies. Through his pioneering work, DeOme et al. showed that any portion of the mammary tissue has a specific population of cells that can contribute to tissue regeneration and homeostasis akin to the one observed during the successive cycles of pregnancy and lactation. Follow-up studies by Smith and Medina (1988)) also

showed that epithelial cells that can form the entire ductal tree upon transplantation exist in the whole life span of the adult female mouse. Using the retrovirally marked mammary epithelial cells, Kordon and Smith (1998) later confirmed stem cells' existence in the mammary tissue. However, the isolation of a refined population of MaSCs is still elusive due to the lack of defined markers that characterize the MaSCs population.

Attempts like depleting the freshly dissociated mammary cell populations of hematopoietic and endothelial cells while selecting specific cell-surface markers like CD24, CD29, or CD49f was some of the initial approaches used for MaSCs isolation by Shackleton et al. (2006) and Stingl et al. (2006). These integrin markers in vivo form a functional alpha 6-beta1 integrin heterodimeric complex and facilitate interaction between the epithelial cells and the mammary stroma. So, transplantation experiments with these double-positive cell populations that express high levels of CD24 and CD29 or CD49f into the cleared fat pad at limiting dilutions showed that these subsets are enriched for mammary stem cell populations. Later it was also revealed that most of these double-positive cells express markers for myoepithelial cells, have low levels of Sca-1, and acquire a basal position in the mammary parenchyma (Stingl et al. 2006). Interestingly, among these are small proportions of cells that do not express myoepithelial lineage markers but have substantial potential to contribute to the development of lobuloalveolar structures during pregnancy. Such groups of cells that exhibit this multilineage differentiation potential are considered MaSCs (Shackleton et al. 2006; Asselin-Labat et al. 2006).

3.3 Single MaSC Can Form an Entire Mammary Gland

The in vivo labeling studies using analogs like bromodeoxyuridine (BrdU) and [3H] thymidine had shown that inside the mammary gland exists an actively cycling population of cells that retain a template of their immortal DNA strand (Smith 2005). This capability of asymmetrical cell division helps track the long-term label-retaining cells that are also enriched for stem cell populations. Although the correlation between the long-term label-retaining cells and MaSCs is not well understood, studies using Hoechst33342 and pyronin Y staining showed that most of such cycling cells constitute the G1 or S-G2-M fractions (Shackleton et al. 2006).

Shackleton et al. and Stingl et al. through their studies, showed that a single cell from the population sorted for $CD29^{hi}/CD24^{+}$ (Shackleton et al. 2006) or $CD49f^{hi}/CD24^{+}$ (Stingl et al. 2006) markers can repopulate a cleared fat pad and can in vivo reconstitute a functional glandular structure. The evidence for this multilineage differentiation potential of the cells with the above-described markers was derived from the clonal outgrowth of the transplanted cells in the recipient mice (Shackleton et al. 2006) and from the in vitro colony-forming assays (Stingl et al. 2006). This asymmetrical division of the transplanted cells into a daughter progenitor cell and a stem cell points to a probable epithelial lineage hierarchy within the gland, which may add to the understanding if delineated precisely step by step of the dynamics of the residing stem cell population. Accordingly, in the mouse mammary gland, based

on earlier transplantation experiments, multipotent cell types capable of forming three distinct lineages have been identified (Smith 1996). But the precise identity of such cells that give rise to ductal or lobular or the entire mammary outgrowth is still elusive. It is hypothesized that, like hematopoietic stem cells, broadly, the MaSCs may also consist of multiple bi- and unipotent progenitor cells with long-term and short-term repopulating capabilities in addition to the expected multipotent stem cells.

Inside the mammary parenchyma cell adhesion molecules of the integrin family-like α6-integrin and β1-integrin play crucial roles in mediating epithelial cells' attachment to the stroma and cell maintenance polarity. During pregnancy, alveolar architecture formation in the mammary gland of β1-integrin conditional mutants is severely compromised even though no effect was observed on the ductal morphogenesis and branching pattern. The attempts to repopulate the recipient mice cleared fat pads using mammary tissues from the above mutant mice were also unsuccessful (Li et al. 2005). However, no such anomalies were observed in CD24-deficient mice. Together these two cell adhesion markers seem to have deterministic roles in maintaining the MaSC population and its self-renewal. So, sorting of the dissociated mammary epithelial cells for these cell adhesion molecules yield MaSCs-enriched population. Even though the expression of CD24 marker is widespread across all sorts of epithelial cells present inside the mammary gland, the findings of Sleeman et al. (2006) showed that this specific cell adhesion molecule (CD24) could be useful for segregating luminal, myoepithelial, and non-epithelial cells from the freshly dissociated gland. Mostly, the epithelial cells that show high stem cell activity is low for CD24 and are quickly sorted out as a group of $CD24^{lo}$ population of cells. This study holds much significance compared to the similar findings described above because Sleeman et al. showed that based on the level of expression of this single cell surface marker CD24, mammary epithelial cells could be sorted into distinct subsets of varying potency. Likewise, a subset of epithelial cells characterized by low levels of either CD29 or CD49f but high levels of CD24 were enriched for progenitor cells that are committed to a luminal fate (Shackleton et al. 2006; Stingl et al. 2006). Still, the above subset of cells fails to yield an outgrowth during the transplantation experiments.

3.4 Endocrine Regulation of MaSCs

The studies to locate and identify the MaSCs populations within the mammary gland will undoubtedly have a significant impact on breast cancer biology and veterinary sciences. The considerable expansion in the mammary stem cell populations in the MMTV-wnt-1 transgenic mice during the preneoplastic phase (Shackleton et al. 2006) suggests that wnt-1 plays a crucial role in MaSCs self-renewal. The absence of a similar phenomenon of expansion in stem cell activity in MMTV-neu/erb2 transgenic mice signifies that in this transgenic model, a progenitor cell, down in the epithelial hierarchy, is the target. To further understand the complex regulation of MaSCs activity and study its effect on mammogenesis by systemic endocrine

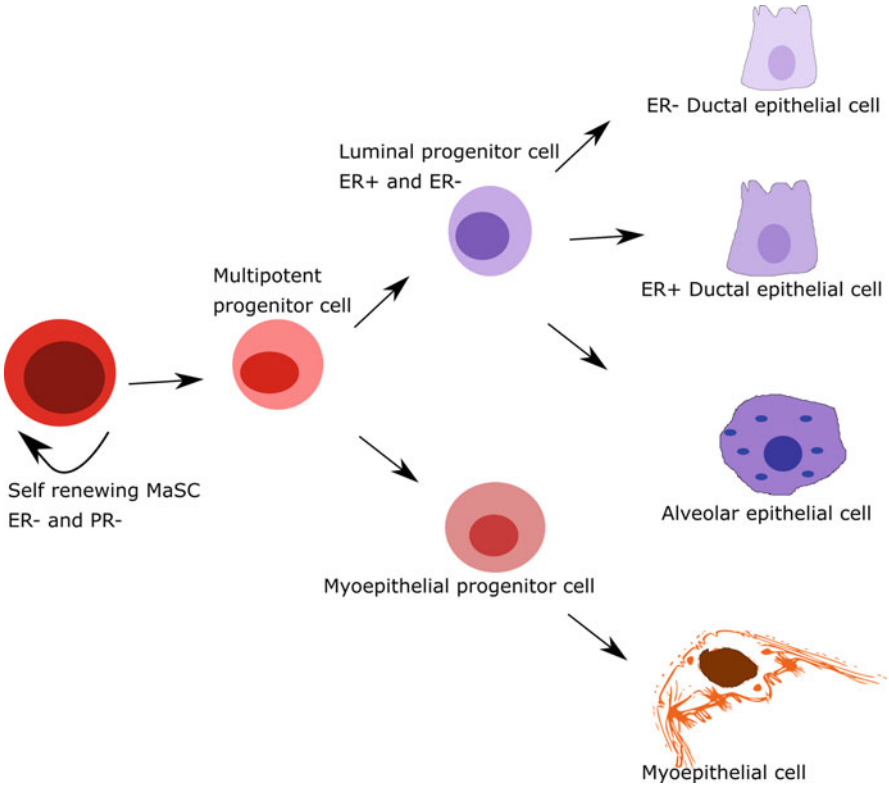

Fig. 3.2 The proposed hierarchy of mammary epithelial cells. Adapted from Visvader and Lindeman (2006) and created with BioRender.com

factors, ovariectomy experiments were done. The determination of ERα and PR expression patterns in the mammary gland revealed that MaSCs had an ER-α-negative and PR-negative identity (Asselin-Labat et al. 2006). The luminal progenitor populations had a significant number of cells having receptors for steroid hormones. Such distribution of steroid hormone receptors meant that the epithelial cells would vary remarkably in their repopulating potential. So, a hierarchy of stem cells should exist inside the mammary gland. Based on this understanding, it was hypothesized that an ERα-MaSC at the top of this hierarchy goes through the process of self-renewal or differentiation to form either luminal or myoepithelial restricted progenitor cells. Next, these predominantly ERα-luminal progenitor cells down the hierarchy commit either to ductal or alveolar epithelial cells. During this process, a transition to ERα+ phenotype happens, and the resulting ductal cells are now ERα+. Later, during puberty or pregnancy, these ERα+ cells in response to increased estrogen activity direct the expansion of mammary epithelial cells and MaSCs in paracrine fashion leading to complete glandular morphogenesis (Fig. 3.2).

The findings from the ovariectomy experiments thus revealed that estrogen and other systemic ovarian factors are critical for mammary development and stem cell

activation, all mediated in a paracrine or juxtacrine fashion because most of these proliferating cells lack estrogen receptor alpha (ERα). Apart from Erα that functions as a mediator, studies by Cheng et al. (2004) in mouse models had shown that ERβ might also participate in estrogen-mediated mammogenesis. The role of these mediators (ERα and ERβ) varies across the mammalian species as Schams et al. (2003) had shown that bovine mammary glands are very low on ERα expression. Localization studies by Capuco et al. (2002) and Berry et al. (2003) had demonstrated that ERα expression in the parenchymal portion of the bovine mammary gland is very much confined to the constituent epithelial populations rather than the stromal ones, and among these embedded epithelial cells, approximately one-third do express ERα. This ERα expression pattern is similar to the one reported in humans and rats (Anderson et al. 1998; Russo et al. 1999) but different from the patterns reported in murine mammary glands (Shyamala 1997; Haslam and Levely 1985). The marked increase in the percentage of these proliferating ER-negative cells estimated either through BrdU labeling or Ki67 staining from 85–90% to 99% (Capuco et al. 2002) post estrogen treatment probably supports the initial observation that the progenitor and the stem cells that constitute a majority of the proliferating epithelial cell populations are mostly ERα-negative. Together these findings suggest that there are two distinct populations of cells inside the mammary gland, one that detects an estrogen signal and another that responds by proliferating, both connected via a paracrine mode of communication between the two cell populations. Contrary to this, Zeps et al. (1996) reported that in mouse mammary glands are present a small population of slowly proliferating ERα-positive cells. A similar study by Dontu et al. (2003) shows that around 40% of the existing stem cell populations in the human mammary gland expresses ERα. Later, Clarke et al. (2003) and Dontu et al. (2004) came up with an explanation that these ERα-positive stem/progenitor cells are mostly quiescent cells that give rise to rapidly dividing ER-α-negative cells, which also acts as the leading population of proliferating cells in the gland.

3.5 Significance and Relevance to Dairy Animals and Humans Health

The maintenance of persistent lactation in dairy animals is an economically desirable trait for the dairy industry. However, attempts to achieve this feat are mostly unmet due to the majority of reasons and factors that affect mammary parenchyma growth and maintenance. The damage to the mammary parenchyma due to bacterial infections during mastitis severely affects milk productivity. In mastitis, the bacterial endotoxin leads to oxidative stress, and Spitzer et al., had shown that the observed reduction in the milk production capacity is due to the induction of apoptosis in the mammary epithelial cells (Spitzer et al. 2020). The development of antibiotic-resistant pathogenic bacterial strains has further compromised the conventional measures meant to prevent and treat mastitis. Alternative approaches, like using

antimicrobial proteins (Kumar et al. 2019; Neerukonda et al. 2019) and our current understanding of mammary stem cell biology, if exploited, may address this issue.

The studies on the bovine mammary stem and progenitor cells provide multiple challenges, avenues, and research targets in the field. Issues like reduced milk production during lactation, which happens due to the apoptotic cell death of mammary epithelial cells, are a significant cause of concern in dairy animals (Capuco et al. 2001; Boutinaud et al. 2004). The ability to alter the epithelial cell's proliferation/apoptosis rates in such cases will have a long-lasting effect on the persistence of lactation. Understanding the crucial events that control MaSCs activity and the corresponding epithelial cell dynamics would help mitigate the impact of processes that negatively affect epithelial homeostasis. The development of a scientific approach and methods to increase epithelial cell turnover inside the mammary gland by stimulating the MaSCs activity will substantially add to the secretory capacity per cell during lactation.

The incidence of mammary oncogenesis is pretty high in humans than the ruminants (Acland and Gillette 1982; Foreman et al. 1990; Kato et al. 1998). This striking contrast makes it attractive to understand why mammary carcinoma is so rare in herbivorous mammals. Multiple hypotheses were drafted to explain this notable difference between the humans the ruminants. The first and the most prominent argument is that most of these ruminant females undergo prolonged lactation phases or repetitive cycles of pregnancy and lactation, while human females do not. Pregnancy and lactation in females had been shown to confer protection against the occurrence of breast cancer (Rudas et al. 1994). However, the mammary gland biology in humans is so complex that many a time, a majority of mammary tumors are first reported during pregnancy (Chiedozi 1985; Donegan 1979; Kuerer et al. 1997). Another argument is that majority of these female ruminants do not live for more than ten years, so the incidences might get underreported. One impressive view is that the product of fermentation in ruminant mammals protects against mammary carcinoma. Butyrate for long has been known to act as a histone deacetylase and shown to promote apoptosis in tumor cells when introduced to the culture medium (Heerdt et al. 1999; Gaschott et al. 2001). The increased update of dietary fibers may lead to an increase in the production of butyrate inside the human gut. But rarely, the butyrate produced inside the human gut is able to reach peripheral circulation because a majority of it either gets metabolized by the gut epithelium or gets processed in the liver. A study in support of this hypothesis was conducted on rats. It was discovered that there was a 50% reduction in the incidences of mammary tumors in the group that was fed to sustain an increased level of butyrate in their circulation (Belobrajdic and McIntosh 2000). A detailed investigation into the differences in mechanism between different mammalian species would thus be useful to test novel approaches and get a deeper understanding of the mammary stem cells' physiology and dynamics and alteration of which results in mammary tumorigenesis.

3.6 Conclusion

Mammary stem cells are directly related to the observed dynamics of epithelial cells and their turnover rate inside the mammary gland. Therefore, MaSCs are of immense interest to the current scientific community. Strategies that can boost and regulate the MaSCs activity can benefit animal husbandry and regenerative medicine. Candidate molecules like xanthosine and inosine (Capuco et al. 2012; Choudhary and Capuco 2012) that promote the resident stem cell populations via increased symmetric division need further elucidation as this may add to ongoing mammary epithelial cell proliferation. Together these findings offer a promise of improving lactation efficiency and well-being of the dairy animals.

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References

Acland HM, Gillette DM (1982) Mammary carcinoma in a mare. Vet Pathol 19:93–95

Anderson E, Clarke RB, Howell A (1998) Estrogen responsiveness and control of normal human breast proliferation. J Mammary Gland Biol Neoplasia 3:23–35

Asselin-Labat ML, Shackleton M, Stingl J et al (2006) Steroid hormone receptor status of mouse mammary stem cells. J Natl Cancer Inst 98:1011–1014

Belobrajdic DP, McIntosh GH (2000) Dietary butyrate inhibits NMU-induced mammary cancer in rats. Nutr Cancer 36:217–223

Berry SDK, Jobst PM, Ellis SE, Howard RD, Capuco AV, Akers RM (2003) Mammary epithelial proliferation and estrogen receptor alpha expression in prepubertal heifers: effects of ovariectomy and growth hormone. J Dairy Sci 86:2098–2105

Boutinaud M, Guinard-Flamenta J, Jammes H (2004) The number and activity of mammary epithelial cells, determining factors for milk production. Reprod Nutr Dev 44:499–508

Capuco AV, Choudhary RK (2020) Symposium review: determinants of milk production: understanding population dynamics in the bovine mammary epithelium. J Dairy Sci 103:1–13

Capuco AV, Wood DL, Baldwin R, McLeod K, Paape MJ (2001) Mammary cell number, proliferation, and apoptosis during a bovine lactation: relation to milk production and effect of bST. J Dairy Sci 84:2177–2187

Capuco AV, Ellis S, Wood DL, Akers RM, Garrett W (2002) Postnatal mammary ductal growth: three-dimensional imaging of cell proliferation, effects of estrogen treatment and expression of steroid receptors in prepubertal calves. Tissue Cell 34:9–20

Capuco AV, Choudhary RK, Daniels KM, Li RW, Evock-Clover CM (2012) Bovine mammary stem cells: cell biology meets production agriculture. Animal 6:382–393. https://doi.org/10.1017/S1751731111002369

Cheng G, Weihua Z, Warner M, Gustafsson JA (2004) Estrogen receptors ER alpha and ER beta in proliferation in the rodent mammary gland. Proc Natl Acad Sci U S A 101:3739–3746

Chiedozi LC (1985) Breast cancer in Nigeria. Cancer 55:653–657

Choudhary RK, Capuco AV (2012) *In vitro* expansion of the mammary stem/ progenitor cell population by xanthosine treatment. BMC Cell Biol 13:14

Clarke RB, Anderson E, Howell A, Potten CS (2003) Regulation of human breast epithelial stem cells. Cell Prolif 36(Suppl1):45–58

DeOme KB, Faulkin LJ Jr, Bern HA, Blair PB (1959) Development of mammary tumors from hyperplastic alveolar nodules transplanted into gland-free mammary fat pads of female C3H mice. Cancer Res 19:515–520

Donegan WL (1979) Mammary carcinoma and pregnancy. Major Probl Clin Surg 5:448–463

Dontu G, Al-Hajj M, Abdallah WM, Clarke MF, Wicha MS (2003) Stem cells in normal breast development and breast cancer. Cell Prolif 36(Suppl 1):59–72

Dontu G, El-Ashry D, Wicha MS (2004) Breast cancer, stem/progenitor cells and the estrogen receptor. Trends Endocrinol Metab 15:193–197

Foreman JH, Weidner JP, Parry BW, Hargis A (1990) Pleural effusion secondary to thoracic metastatic mammary adenocarcinoma in a mare. J Am Vet Med Assoc 197:1193–1195

Gaschott T, Maassen CU, Stein J (2001) Tributyrin, a butyrate precursor, impairs growth and induces apoptosis and differentiation in pancreatic cancer cells. Anticancer Res 21:2815–2819

Haslam SZ, Levely ML (1985) Estrogen responsiveness of normal mouse mammary cells in primary cell culture: association of mammary fibroblasts with estrogenic regulation of progesterone receptors. Endocrinology 116:1835–1844

Heerdt BG, Houston MA, Anthony GM, Augenlicht LH (1999) Initiation of growth arrest and apoptosis of MCF-7 mammary carcinoma cells by tributyrin, a triglyceride analogue of the short-chain fatty acid butyrate, is associated with mitochondrial activity. Cancer Res 59:1584–1591

Kato M, Higuchi T, Hata H, Ishikawa Y, Kadota K (1998) Lactalbumin-positive mammary carcinoma in a mare. Equine Vet J 30:358–360

Kordon EC, Smith GH (1998) An entire functional mammary gland may comprise the progeny from a single cell. Development 125:1921–1930

Kuerer HM, Cunningham JD, Brower ST, Tartter PI (1997) Breast carcinoma associated with pregnancy and lactation. Surg Oncol 6:93–98

Kumar A, Parveen S, Sharma I, Pathak H, Deshmukh MV, Sharp JA, Kumar S (2019) Structural and Mechanistic insights into EchAMP: a antimicrobial protein from the Echidna milk. BBA-Biomembranes 1861(6):1260–1274

Li N, Zhang Y, Naylor MJ et al (2005) β1 integrins regulate mammary gland proliferation and maintain the integrity of mammary alveoli. EMBO J 24:1942–1953

Neerukonda M, Pavuluri S, Sharma I, Kumar A, Sailasree SP, Jyothi Lakshmi B, Sharp JA, Kumar S (2019) Functional evaluation of a monotreme specific antimicrobial protein, EchAMP in transgenic mice against experimentally induced mastitis. Transgenic Res 28(5-6):573–587

Rudas P, Bartha T, Toth J, Frenyo VI (1994) Impaired local deiodination of thyroxine to triiodothyronine in dogs with symmetrical truncal alopecia. Vet Res Commun 18:175–182

Russo J, Ao X, Grill C, Russo IH (1999) Pattern of distribution of cells positive for estrogen receptor alpha and progesterone receptor in relation to proliferating cells in the mammary gland. Breast Cancer Res Treat 53:217–227

Schams D, Kohlenberg S, Amselgruber W, Berisha B, Pfaffl MW, Sinowatz F (2003) Expression and localisation of oestrogen and progesterone receptors in the bovine mammary gland during development, function and involution. J Endocrinol 177:305–317

Shackleton M, Vaillant F, Simpson KJ et al (2006) Generation of a functional mammary gland from a single stem cell. Nature 439:84–88

Shyamala G (1997) Roles of estrogen and progesterone in normal mammary gland development - Insights from progesterone receptor null mutant mice and in situ localization of receptor. Trends Endocrinol Metab 8:34–39

Sleeman KE, Kendrick H, Ashworth A, Isacke CM, Smalley MJ (2006) CD24 staining of mouse mammary gland cells defines luminal epithelial, myoepithelial/basal and non-epithelial cells. Breast Cancer Res 8:R7

Smith GH (1996) Experimental mammary epithelial morphogenesis in an in vivo model: evidence for distinct cellular progenitors of the ductal and lobular phenotype. Breast Cancer Res Treat 39:21–31

Smith GH (2005) Label-retaining epithelial cells in mouse mammary gland divide asymmetrically and retain their template DNA strands. Development 132:681–687

Smith GH, Medina D (1988) A morphologically distinct candidate for an epithelial stem cell in mouse mammary gland. J Cell Sci 90:173–183

Spitzer AJ, Tian Q, Choudhary RK, Zhao FQ (2020) Bacterial endotoxin induces oxidative stress and reduces milk protein expression and hypoxia in the mouse mammary gland. Oxidative Med Cell Longev 2020:1–16

Stingl J, Eirew P, Ricketson I et al (2006) Purification and unique properties of mammary epithelial stem cells. Nature 439:993–997

Visvader JE, Lindeman GJ (2006) Mammary stem cells and mammopoiesis. Cancer Res 66:9798–9801. https://doi.org/10.1158/0008-5472.CAN-06-2254

Zeps N, Dawkins HJ, Papadimitriou JM, Redmond SL, Walters MI (1996) Detection of a population of long-lived cells in mammary epithelium of the mouse. Cell Tissue Res 286:525–536

Methods of Identification and Characterization of Stem Cells

4

Shanti Choudhary

Abstract

Stem cells are identified and characterized by their prolonged divisional capacity and capacity to differentiate into functional cell types. Moreover, these cells generate several lineages by transdifferentiation (called plasticity) due to their pluripotency and multipotency state. Identification of correct stem cell types from various tissues, followed by their characterization, will provide a way to investigate their mechanisms of self-renewal and differentiation potential. Increasing the efficiency of characterizing and expanding adult stem cells is the most challenging field of biology and veterinary regenerative medicine.

Keywords

Stem cell · Identification · Characterization

4.1 Introduction

Stem cells are rapidly dividing undifferentiated cells and are unique in their properties from other differentiated cells. The presence of stem cells in any tissue depends on their renewal rate and hence are found in large numbers in tissue with high renewal rates like the vascular tissue, blood, and epithelia. On the other hand, tissues with less or little renewal state, such as the central nervous system (CNS) or myocardial muscle, will have fewer stem cells (Lemoli et al. 2005). Before becoming fully differentiated, stem cells generate progenitors and more differentiated precursors, also called intermediate cell types. Progenitors and precursor cells are

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the medical field's future because of their potential to treat many incurable diseases, such as heart diseases and diabetes. Stem cells co-exist in a population with other cells. So, it is essential to know and identify the stem cells among other cells, and for the identification, there are some methods available for the screening of stem cells from other cells. Stem cells are potent and can transform into any cell type according to need. They divide asymmetrically, resulting in one differentiated and one stem cell, a key and unique feature of stem cells to keep their number growing and maintaining their population.

Based on the potency, stem cells are divided into four groups as follows:

Totipotent cell: Totipotent cells are the cells that can differentiate into all types of cells including embryonic and extraembryonic cell types, such as the fertilized egg. These cells can successfully make a whole viable organism (Bindu and Srilatha 2011).

Pluripotent cells: Pluripotency refers to the ability of cells to differentiate into any cell type of the three germ layers, namely, ectoderm, endoderm, and mesoderm. They are also termed as descendants of totipotent stem cells that can develop into almost all types of cells. A single pluripotent stem cell can produce cells of all three germ layers. Examples include embryonic stem cells, cells formed during early differentiation of stem cells, and cells derived from the germ layers viz, mesoderm, ectoderm, and endoderm.

Multipotent, oligopotent, and unipotent cells: Multipotent stem cells can successfully differentiate into all closely related types of cells. The limiting factor for multipotent stem cells is their only specificity for home tissue. For example, the multipotent stem cells from bone marrow can give rise to all types of blood cells (RBCs, WBCs, and platelets). Oligopotent stem cells differentiate into only a few cells, like lymphoid or myeloid stem cells. As the word "uni" suggests, unipotent stem cells can differentiate into only one cell type or lineage. They can be found in adult tissues.

Based on the origin, stem cells are divided into three groups as follows:

Embryonic stem cells (ESCs): Embryonic stem cells are derived from the blastocyst stage and are pluripotent. ESCs can be isolated from the embryo's inner cell mass from the developmental blastocyst stage. ESC is self-replicating in nature, and they are capable of forming all cells derived from all three germ layers. The term "embryonic stem cell" was coined by Martin (1981) and the characteristics feature of "pluripotency" can be a treatment for many incurable diseases like Parkinson's disease, diabetes, liver diseases, and cardiovascular disease (Khan et al. 2018). Embryonic stem cells (ESC) are a distinct category of stem cells that can divide indefinitely and have self-renewal capacity. ESCs can give rise to all somatic cell types present in the embryo (Vazin and Freed 2010). ESCs role in initiating organ structures and developing specialized cells is complex, and ESC can be a useful tool in understanding the underlying mechanism. ESCs can be a valuable tool for understanding the complex mechanisms involved in tissue regeneration and growth. Because of their unique properties of self-renewal and plasticity, they can be

Table 4.1 Viral and non-viral mediated methods for generating induced pluripotent stem cells (iPSCs) in various species

Species of which iPSCs were generated		References
Viral mediated reprogramming		
Rat		Liao et al. (2009)
Dog		Shimada et al. (2010)
Pig		Esteban et al. (2009)
Monkey		Liu et al. (2008)
Sheep		Bao et al. (2011)
goat		Ren et al. (2011)
Horse		Nagy et al. (2011)
Cattle		Han et al. (2011)
Buffalo		Deng et al. (2012)
Non-viral mediated reprogramming		
Human	Using plasmid	Yu et al. (2009)
Mouse	Using recombinant proteins	Zhou et al. (2009)
Human	Using modified mRNA, called RNA-iPS cells (RiPS)	Warren et al. (2010)
Mouse	Using small molecules - BIX-01294, BayK8644	Shi et al. (2008)
Bovine	Transposon mediated	Talluri et al. (2015)

harvested and grown in vitro, and this way, they can be used in regenerative medicine.

Adult stem cells (ASCs): Stem cells of fully differentiated tissue are called adult stem cells. The critical function of the adult stem cells is to maintain the adult tissue specificity by tissue regeneration and homeostatic cell replacement (Wagers and Weissman 2004).

Induced pluripotent stem cells (iPSCs): In the year 2006, Yamanaka and his group, for the first time, showed that a cocktail of four transcription factors, namely, Oct4, Sox2, Klf4, and c-Myc, were able to reprogram fibroblast to gain a pluripotency state leading to the generation of induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka 2006). Subsequently, in 2007 human iPSCs were derived (Takahashi et al. 2007) as an alternative source of human ESCs.

Viral and non-viral mediated delivery of core reprogramming factors and other molecules allowed the generation of iPSCs from various animals (Table 4.1).

The generation of reproducible protocols in different animal species is yet to be explored. The low efficiency of iPSC generation is another issue that scientists are dealing with in the successful creation of iPSCs. The iPSCs generation protocol may provide these cells with transgenic technology to create new breeding and therapeutic applications.

4.2 Identification and Isolation of Stem Cells

4.2.1 Embryonic Stem Cells

Martin pioneered the first isolation of embryonic stem cells from mice embryo in 1981. Martin noticed that the subclonal cultures derived from isolated single cells were able to differentiate into a broad category of cell types. However, ESCs came into light in 1998 with Thomson and his team's innovative discovery, who showed a technique for the first time to successfully isolate ESCs from human embryos (Thomson et al. 1998). This was a revolution in science and research. Because of their potential to differentiate into all types of cells present in the body, including cardiomyocytes (Pal et al. 2013), hepatocyte-like cells (Cho et al. 2007), neural cells (Sundberg et al. 2010), and islets of Langerhans (Baharvand et al. 2006), ESCs are targets for many incurable human diseases such as liver disease, diabetes, and cardiovascular diseases. The most crucial part of using them as therapeutic agents is to isolate them, and isolation is the biggest challenge. Further propagation can be done only after successful isolation of human embryonic stem cells (hESC) from the ICM of the human embryo (Khan et al. 2018; Löser et al. 2010) (Fig. 4.1). ESC can also be isolated from the frozen or cryopreserved embryos, used cryopreserved human zygotes, cultured them to the blastocyst stage, and isolated inner cell mass (ICM) via microsurgery (Meng et al. 2010).

After the isolation of the first embryonic cells from the mouse by Martin, several other methods were developed to isolate inner cell mass from a single human embryo. The following methods are being used for the isolation of ICM:

1. *In vitro cultivation*: In general, most of the ESC lines available today are from mice, not humans, and the fundamental protocol for the generation of ESCs is similar for all species. ESCs are pluripotent stem cells generated from early-stage embryos. The cells are harvested from the inner cell mass of 10–20 cells from blastocyst 4–5 days post-fertilization. The blastocyst consists of an outer layer called the trophoblast, a fluid-filled cavity called the blastocoele, and an inner cell mass of 10–20 cells. The inner cell mass, also called the embryoblast, is removed for culture. The inner mass cells are placed in the suitable culture medium, and the viable cells are selected for further expansion. However, the generation of ESCs in the culture medium is inefficient; thus, many cells do not adapt to in vitro conditions and do not survive.
2. *Identification using ESC markers*: The common embryonic stem cell markers, such as pluripotency transcription factors (OCT4, SOX2, NANOG, KLF4) and other markers like stage-specific embryonic antigen (SSEA)-3, SSEA-4, LIN28, and TRA-1-60. TRA-1-60 is a cell surface antigen of human ESC, and is expressed along with other two stem cell surface antigens SSEA-3 and SSEA-4.
3. *Mechanical dissection of ICM:* In addition to that, ESCs can be harvested from inner cell mass (ICM) of blastocysts using mechanical dissection, laser dissection, and immunosurgical procedures (Khan et al. 2018). Mechanical dissection of ICM can be an effective way to derive new hESC lines, but laborious and time-

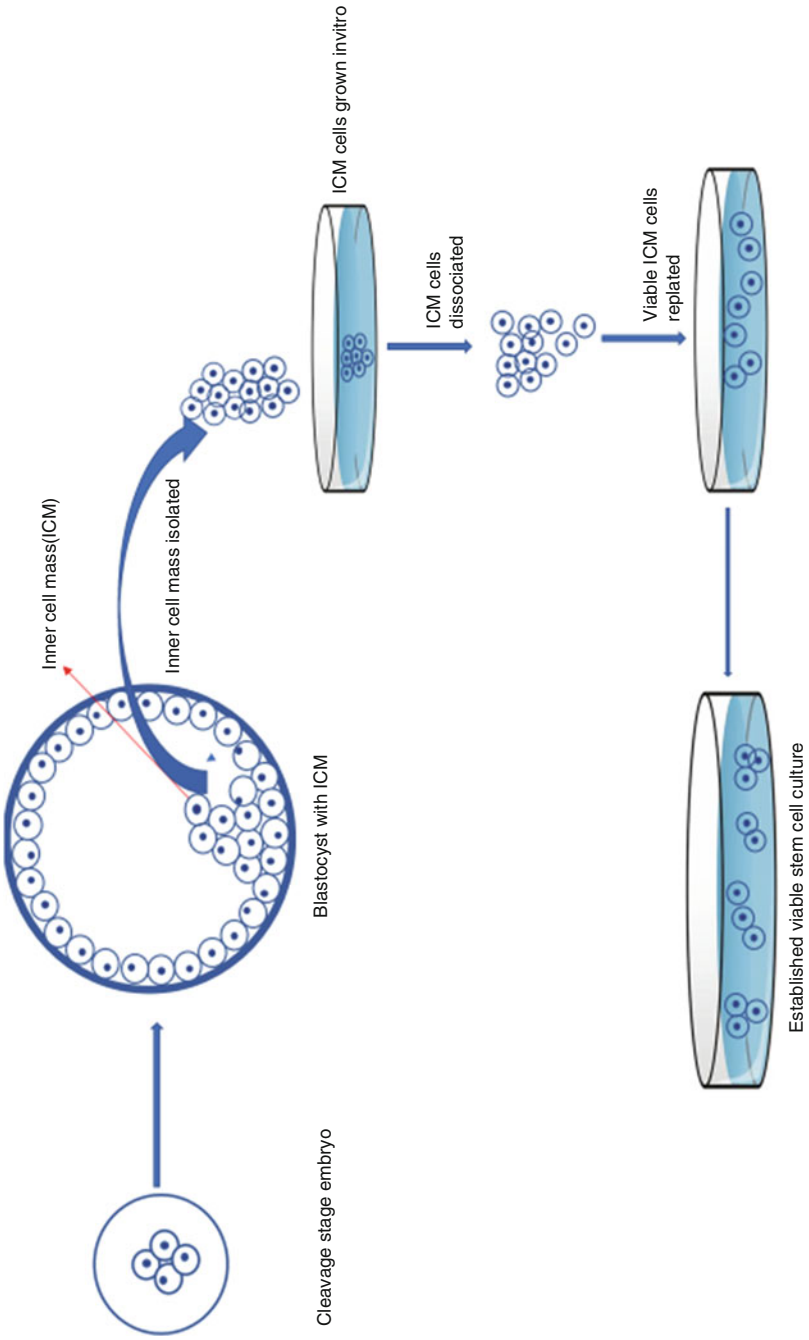

Fig. 4.1 Derivation of ESCs from the embryonic blastocyst's inner cell mass

Table 4.2 Small peptides can also be used as markers of ESCs

ESCs	Peptides	References
Mouse ESCs	GTYNLPNPPPPL, KHMHWHPPALNT, SAHGTSTGVPWP, VPTATLMGASAR, WAETWPLAQRPP, LLADTTHHRPWT	Zhao et al. (2010)
Macaca monkey ESCs	APWHLSSQYSRT, LDVRPWYVTPLP, TPLINMNALTVT GYPHPWTLWHLN, WAPEKDYMQLMK	Lu et al. (2010)
Human ESCs	APWHLSSQYSRT, DLNYFTLSSKRE, HGEVPRFHAVHL, NRQSNWPIHKTI, QLSEECSYLISRP, SNPQPYTILPPV QLTKNVPTYKSS, SPLITSTLIPQR, TALATSSTYDPH, TTLVSTGQRTHP	Derda et al. (2010)

consuming exercises. Laser dissection is an expensive procedure but the most promising technique for isolating the pure population of ESCs. Interestingly, a laser-based system for the isolation of the inner cell mass (ICM) allowed the harvest of live human ESCs that can be used to develop cell lines (Turetsky et al. 2008). Other ESCs isolation procedures like immunosurgery and microdissection have a limited success rate.

4. *Enzymatic and other markers*: Several different marker systems have been used for the stem cell identification and isolation of ESCs. These additional markers are enzymes like alkaline phosphatase and telomerase-based reactions. Lectins and small peptides which bind to ESC via affinity and specificity can also serve as markers for ESCs identification (Zhao et al. 2012). Many developmentally regulated glycans, displayed on cell surfaces, have been identified as lectin receptors of mouse ESCs (Mandai et al. 2010). Lectin is being used as a marker to define the developmental stages of mouse embryogenesis. A series of small peptides, conjugated with fluorescent probes, were able to target mouse, monkey, and human ESCs (Table 4.2).

4.2.2 Adult Stem Cells

Adult stem cells (ASCs) are the stem cells present in various developed tissues. ASCs have two essential properties, long-term self-renewal and the capacity to differentiate into mature cells with a distinct morphology. Before terminally differentiating into mature cells, ASCs produce intermediate cells having profound proliferation capacities called progenitor cells. Progenitor cells finally differentiate into functional mature cells.

Unlike that of ESCs, ASCs are rare. The prime functions of ASCs are to maintain the steady-state function of cells by replacing dead and apoptotic cells and maintaining a constant stem cell pool in the tissue. ASCs have no actual locale within the tissue. Moreover, there are no definite means of characterization of ASCs; various types of ASCs are harvested from various tissues. The list of ASCs present in various tissues is growing and includes mammary parenchyma, bone marrow,

adipose tissue, peripheral blood, brain, dental pulp, muscles, blood vessels, gut, skin, cornea, retina, umbilical cord, placenta, pancreas, and liver. Recently, pluripotent and multipotent stem cells have been found in milk (Ninkina et al. 2019; Cregan et al. 2007). Examples of ASCs are mesenchymal stem cell (MSC), mammary stem cell (MaSC), neuronal stem cell (NSC), hematopoietic stem cell (HSC), endothelial stem cell, pancreatic stem cell, liver stem cell, germinal stem cell, intestinal stem cell, cardiac stem cell, renal stem cell, corneal stem cell, retinal stem cell, dental stem cell, hair follicle stem cell, skin stem cell, and others.

Specific cell types have distinct morphological forms that can be examined under a microscope. For example, fully mature cardiomyocytes are elongated, rod-shaped mononuclear cells, whereas ESCs are oval and with round and prominent nucleus and a thin rim of cytoplasm. Stem cells have a poorly developed rough endoplasmic reticulum. Stem cells lack desmosomes and gap junctions, whereas differentiated cells have well-developed junctional complexes. The majority of the investigators rely on morphological evidence and cell surface markers' expression. Some investigators also showed functional assays by differentiation of ASCs into three germ layers if they claim cells to be pluripotent or many cell types if it is multipotent.

Isolation and adult stem cells culture varies with their types and tissue origin. Compared to ESCs, adult stem cells' frequency in different tissues varies dramatically but remained intrinsically low. Isolation of ASCs thus requires in vitro expansion before therapeutic applications. Primitive ASCs are slowly cycling under normal physiological conditions demonstrated by label-retaining experiments (Liu et al. 2016; Lu et al. 2010; Capuco 2007) and cell sorting experiments based on metabolic activities (Spangrude and Johnson 1990). Ideally, an ASC should repopulate genetically identical types of cells (clonogenic), which ultimately give rise to differentiated cell types. Demonstration of clonogenic potential of ASC is difficult, where the fate of stem cells is followed throughout the entire lifetime. With recent advancements in lineage tracing experiments, a stochastic genetic-labeling strategy is being utilized to mark a single cell and its progeny in situ in the mammary gland. Studies revealed clonal expansion of progenitor cells contribute either luminal or basal cell lineages and sporadically gave rise to cells of branching ducts or alveoli (Davis et al. 2016; Lloyd-Lewis et al. 2018). This study showed the utility of genetic lineage tracing of a single cell (stem cell/progenitor cell) is proliferative and gives rise to differentiated cells throughout the mammary epithelium.

4.3 Characterization of Stem Cells

Teratoma formation assay: The standard gold test for characterization of pluripotent stem cells is the teratoma formation and chimera formation assays. When pluripotent stem cells are injected into immunocompromised mice, they form teratoma, consisting of cells of all three germ layers of ectodermal, mesodermal, and endodermal origins (Wesselschmidt 2011). Pluripotent stem cells relies on the chimera approach, where pluripotent stem cells are microinjected into pre-implanted mouse blastocysts. They were able to make various cell types during normal developmental

Table 4.3 Major and novel types of ASCs and markers

Types of ASC	Localization	Markers
Hematopoietic stem cell (bovine)	Bone marrow, blood, and other tissue (?)	Lin-CD34+CD11b-CD14 (Pessa-Morikawa et al. 2021)
Mesenchymal stem cell (human)	Bone marrow	CD45+, CD34+, CD14+, CD19+, HLA-DR+ (Dominici et al. 2006)
Mesenchymal stem cell (canine)	Bone marrow	CD73+, CD45+, CD34+ (Hodgkiss-Geere et al. 2012)
Mesenchymal stem cell (bovine)	Endometrium	CD29+, CD166+, CD105+, CD73+, CD44+ and CD90+ (de Moraes et al. 2016)
Hepatic stem cell (mouse)	Liver	CDCP1+CD90+CD66− (Zhang et al. 2018)
Mammary stem cell (bovine)	Mammary gland	CD24medCD49f+ (Rauner and Barash 2012); CD49fhighCD24+ (Finot et al. 2018) NR5A2, HNF4A, FNDC3B (Choudhary et al. 2013; Choudhary and Capuco 2021)
Mammary stem cell (human)	Milk	Nestin+ (Cregan et al. 2007) OCT4, SOX2, NANOG (Hassiotou et al. 2012; Ninkina et al. 2019)
Mammary stem cell (bovine)	Milk	CD90+, CD73+, and CD105+, SOX2, OCT4 (Pipino et al. 2018)

processes. Thus, mouse embryonic chimera remained a valuable tool for studying the mammalian developmental process (Tam and Rossant 2003).

Colony formation assay: ESC grows in tightly packed colonies and maintains defined borders at the colonies' periphery. High nucleus to cytoplasm ratio and prominent nucleoli is typical features of ESCs.

Expression of cell surface markers: Another exciting feature of ESCs is the expression of cell surface marker and high expression of pluripotency transcription factors in the nucleus. Identification of stage-specific embryonic antigen-4 (SSEA-4), SSEA-3, and TRA-1-60 antigens, along with the nuclear expression of Oct4, Nanog, Sox2, and Klf4 (Vazin and Freed 2010), can be evaluated using RT-qPCR at the transcript level and Western blot and immunohistochemistry at the protein level. The absence of lineage-specific cell differentiation markers is considered ESC negative marker. Multiparameter cell sorting is used to identify various types of ASCs from enzymatically digested tissue. Markers to identify various ASC are listed in Table 4.3.

Analysis of telomerase enzyme activity: The ability of stem cells to grow in culture for a prolonged period with no differentiation of cells is called the self-renewal capacity of ESC. The ability is evaluated by estimating the telomere length and measuring the telomerase activity. ESCs have long telomere length and high telomerase activity (Carpenter et al. 2004). A repetitive sequence present at the end of the chromosome is called a telomere, which gets shortened after each cell cycle division. Telomerase is the enzyme that adds a species-dependent telomere repeat sequence to the 3′ end of telomeres and thus maintains telomere length constant even after many cell divisions.

Differentiation potential: Functional confirmation of pluripotent and multipotent nature of stem cell are examined by testing their potential to differentiate into ectoderm, mesoderm, and endoderm cells in vitro and in vivo. In vitro, stem cells form an embryoid body (EB) which are aggregate of cells in suspension culture (Reubinoff et al. 2000). Examining all the three germ layers in EB is performed either immunocytochemically or by measuring mRNA expression of germ layers markers by RT-qPCR. The in vivo test for pluripotency of ESCs is evaluated by teratoma formation in immunocompromised mice (Nelakanti et al. 2015).

Adult stem cells present in postnatal tissues are of various types. They possess two essential stem cells: the extended self-renewal and differentiation ability. Due to a great diversity of ASCs, isolation of mesenchymal stem cells from bone marrow is done and finds use in many therapeutic applications. Knowledge of true isolation and characterization of ASCs from various tissue types and their difference in potency will help in the future to understand their more comprehensive application.

References

Amit M, Itskovitz-Eldor J (2009) Embryonic stem cells: isolation, characterization, and culture. Adv Biochem Eng Biotechnol 114:173–184

Baharvand H, Jafary H, Massumi M, Ashtiani SK (2006) Generation of insulin-secreting cells from human embryonic stem cells. Develop Growth Differ 48(5):323–332

Bao L, He L, Chen J, Wu Z, Liao J, Rao L, Ren J, Li H, Zhu H, Qian L, Gu Y, Dai H, Xu X, Zhou J, Wang W, Cui C, Xiao L (2011) Reprogramming of ovine adult fibroblasts to pluripotency via drug-inducible expression of defined factors. Cell Res 21:600–608

Bindu H, Srilatha (2011) Potency of various types of stem cells and their transplantation. J Stem Cell Res Ther 2011:1–3

Capuco AV (2007) Identification of putative bovine mammary epithelial stem cells by their retention of labeled DNA strands. Exp Biol Med 232(10):1381–1390

Carpenter MK, Rosler ES, Fisk GJ, Brandenberger R, Ares X, Miura T et al (2004) Properties of four human embryonic stem cell lines maintained in a feeder-free culture system. Dev Dyn 229 (2):243–258

Cho CH, Parashurama N, Park EYH et al (2007) Homogeneous differentiation of hepatocyte-like cells from embryonic stem cells: applications for the treatment of liver failure. FASEB J 22 (3):898–909

Choudhary RK, Capuco AV (2021) Expression of NR5A2, NUP153, HNF4A, USP15 and FNDC3B is consistent with their use as novel biomarkers for bovine mammary stem/progenitor cells. J Mol Histol. https://doi.org/10.1007/s10735-020-09948-8

Choudhary RK, Li RW, Evock-Clover CM, Capuco AV (2013) Comparison of the transcriptomes of long-term label retaining-cells and control cells microdissected from mammary epithelium: an initial study to characterize potential stem/progenitor cells. Front Oncol 3:21

Cregan MD, Fan Y, Appelbee A, Brown ML, Klopcic B, Koppen J, Mitoulas LR, Piper KM, Choolani MA, Chong YS, Hartmann PE (2007) Cell Tissue Res 329:129–136

Davis F, Lloyd-Lewis B, Harris O et al (2016) Single-cell lineage tracing in the mammary gland reveals stochastic clonal dispersion of stem/progenitor cell progeny. Nat Commun 7:13053

de Moraes CN, Maia L, Dias MC, Dell'Aqua CPF, da Mota LSLS, Chapwanya A, Landim-Alvarenga FDC, Oba E (2016) Bovine endometrial cells: a source of mesenchymal stem/progenitor cells. Cell Biol Int 40(12):1332–1339

Deng Y, Liu Q, Luo C, Chen S, Li X, Wang C, Liu Z, Lei X, Zhang H, Sun H, Lu F, Jiang J, Shi D (2012) Stem Cells Dev:2485–2494. https://doi.org/10.1089/scd.2012.0018

Derda R, Musah S, Orner BP, Klim JR, Li LY, Kiessling LL (2010) High-throughput discovery of synthetic surfaces that support proliferation of pluripotent cells. J Am Chem Soc 132:1289–1295

Dominici M, LeBlanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop DJ, Horwitz E (2006) Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8 (4):315–317

Esteban MA, Xu J, Yang J, Peng M, Qin D, Li W, Jiang Z, Chen J, Deng K, Zhong M, Cai J, Lai L, Pei D (2009) Generation of induced pluripotent stem cell lines from Tibetan miniature pig. J Biol Chem 284:17634–17640

Finot L, Chanat E, Dessauge F (2018) Molecular signature of the putative stem/progenitor cells committed to the development of the bovine mammary gland at puberty. Sci Rep 8:16194

Han X, Han J, Ding F, Cao S, Lim SS, Dai Y, Zhang R, Zhang Y, Lim B, Li N (2011) Generation of induced pluripotent stem cells from bovine embryonic fibroblast cells. Cell Res 21:1509–1512

Hassiotou F, Beltran A, Chetwynd E, Stuebe AM, Twigger AJ, Metzger P, Trengove N, Lai CT, Filgueira L, Blancafort P, Hartmann PE (2012) Breastmilk is a novel source of stem cells with multilineage differentiation potential. Stem Cells 30(10):2164–2174

Hodgkiss-Geere HM, Argyle DJ, Corcoran BM, Whitelaw B, Milne E, Bennett D, Argyle SA (2012) Characterisation and differentiation potential of bone marrow derived canine mesenchymal stem cells. Vet J 194:361–368

Khan FA, Almohazey D, Alomari M, Almofty SA (2018) Isolation, culture, and functional characterization of human embryonic stem cells: current trends and challenges. Stem Cells Int 26:1429351

Lemoli RM, Bertolini F, Cancedda R, De Luca M et al (2005) Stem cell plasticity: time for a reappraisal? Haematologica 90(3):360–381

Li N, Nakauka-Ddamba A, Tobias J, Jensen ST, Lengner CJ (2016) Mouse label-retaining cells are molecularly and functionally distinct from reserve intestinal stem cells. Gastroenterology 151 (2):298–310

Liao J, Cui C, Chen S, Ren J, Chen J, Gao Y, Li H, Jia N, Cheng L, Xiao H, Xiao L (2009) Generation of induced pluripotent stem cell lines from adult rat cells. Cell Stem Cell 4:11–15

Liu H, Zhu F, Yong J, Zhang P, Hou P, Li H, Jiang W, Cai J, Liu M, Cui K, Qu X, Xiang T, Lu D, Chi X, Gao G, Ji W, Ding M, Deng H (2008) Generation of induced pluripotent stem cells from adult rhesus monkey fibroblasts. Cell Stem Cell 3:587–590

Liu X, Liu H, Sun L, Chen Z, Nie H, Sun A, Liu G, Guan G (2016) The role of long-term label-retaining cells in the regeneration of adult mouse kidney after ischemia/reperfusion injury. Stem Cell Res Ther 7(1):68

Lloyd-Lewis B, Davis FM, Harris OB, Hitchcock JR, Watson CJ (2018) Neutral lineage tracing of proliferative embryonic and adult mammary stem/progenitor cells. Development 145:164079

Löser P, Schirm J, Guhr A, Wobus AM, Kurtz A (2010) Human embryonic stem cell lines and their use in international research. Stem Cells 28(2):240–246

Lu S, Xu X, Zhao WX, Wu WW, Yuan H, Shen HB, Zhou CH, Li S, Ma L (2010) Targeting of embryonic stem cells by peptide-conjugated quantum dots. PLoS One 5:e12075

Mandai M, Ikeda H, Jin ZB, Iseki K, Ishigami C, Takahashi M (2010) Use of lectins to enrich mouse es-derived retinal progenitor cells for the purpose of transplantation therapy. Cell Transplant 19:9–19

Martin GR (1981) Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci U S A 78(12):7634–7638

Meng G, Liu S, Li X, Krawetz R, Rancourt DE (2010) Derivation of human embryonic stem cell lines after blastocyst microsurgery. Biochem Cell Biol 88(3):479–490

Miki T, Ring A, Gerlach J (2011) Hepatic differentiation of human embryonic stem cells is promoted by three-dimensional dynamic perfusion culture conditions. Tissue Eng Part C Methods 17(5):557–568

Nagy K, Sung HK, Zhang P, Laflamme S, Vincent P, Agha-Mohammadi S, Woltjen K, Monetti C, Michael IP, Smith LC, Nagy A (2011) Induced pluripotent stem cell lines derived from equine fibroblasts. Stem Cell Rev 7:693–702

Nelakanti RV, Kooreman NG, Wu JC (2015) Teratoma formation: a tool for monitoring pluripotency in stem cell research. Curr Protoc Stem Cell Biol 32:4

Ng TK, Pelaez D, Fortino VR, Greenberg J, Cheung HS (2013) Pluripotent adult stem cells: a potential revolution in regenerative medicine and tissue engineering. IntechOpen, London. https://doi.org/10.5772/54366

Ninkina N, Kukharsky MS, Hewitt MV et al (2019) Stem cells in human breast milk. Hum Cell 32:223–230

Pal R, Mamidi MK, Das AK, Bhonde R (2013) Comparative analysis of cardiomyocyte differentiation from human embryonic stem cells under 3-D and 2-D culture conditions. J Biosci Bioeng 115(2):200–206

Pessa-Morikawa T, Niku M, Iivanainen A (2021) Fetal bovine bone marrow is a rich source of CD34+ hematopoietic progenitors with myelo-monocytic colony-forming activity. Dev Comp Immunol 36(3):572–577

Pipino C, Mandatori D, Buccella F, Lanuti P, Preziuso A, Castellani F et al (2018) Identification and characterization of a stem cell-like population in bovine milk: a potential new source for regenerative medicine in veterinary. Stem Cells Dev 27(22):1587–1597

Rauner G, Barash I (2012) Cell hierarchy and lineage commitment in the bovine mammary gland. PLoS One 7:e30113

Ren J, Pak Y, He L, Qian L, Gu Y, Li H, Rao L, Liao J, Cui C, Xu X, Zhou J, Ri H, Xiao L (2011) Generation of hircine-induced pluripotent stem cells by somatic cell reprogramming. Cell Res 21:849–853

Reubinoff BE, Pera MF, Fong CY, Trounson A, Bongso A (2000) Embryonic stem cell lines from human blastocysts: somatic differentiation in vitro. Nat Biotechnol 18(4):399–404

Shi Y, Desponts C, Do JT, Hahm HS, Scholer HR, Ding S (2008) Induction of pluripotent stem cells from mouse embryonic fibroblasts by Oct4 and Klf4 with small-molecule compounds. Cell Stem Cell 3:568–574

Shimada H, Nakada A, Hashimoto Y, Shigeno K, Shionoya Y, Nakamura T (2010) Generation of canine induced pluripotent stem cells by retroviral transduction and chemical inhibitors. Mol Reprod Dev 77:2

Spangrude GJ, Johnson GR (1990) Resting and activated subsets of mouse multipotent hematopoietic stem cells. Proc Natl Acad Sci U S A 87:7433e7437

Strom S, Inzunza J, Grinnemo GH et al (2007) Mechanical isolation of the inner cell mass is effective in derivation of new human embryonic stem cell lines. Hum Reprod 22 (12):3051–3058

Sundberg M, Andersson PH, Åkesson E, Odeberg J, Holmberg L, Inzunza J, Falci S, Öhman J, Suuronen R, Skottman H, Lehtimäki K, Hovatta O, Narkilahti S, Sundström E (2010) Markers of pluripotency and differentiation in human neural precursor cells derived from embryonic stem cells and CNS tissue. Cell Transplant 20(2):177–191

Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126:663–676

Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131:861–872

Talluri TR, Kumar D, Glage S, Garrels W, Ivics Z, Debowski K, Behr R, Niemann H, Kues WA (2015) Derivation and characterization of bovine induced pluripotent stem cells by transposon-mediated reprogramming. Cell Reprogram 17(2):131–140

Tam PP, Rossant J (2003) Mouse embryonic chimeras: tools for studying mammalian development. Development 130(25):6155–6163

Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM (1998) Embryonic stem cell lines derived from human blastocysts. Science 282:1145–1147

Turetsky T, Aizenman E, Gil Y, Weinberg N, Shufaro Y, Revel A, Laufer N, Simon A, Abeliovich D, Reubinoff BE (2008) Laser-assisted derivation of human embryonic stem cell lines from IVF embryos after preimplantation genetic diagnosis. Hum Reprod 23(1):46–53

Vazin T, Freed WJ (2010) Human embryonic stem cells: derivation, culture, and differentiation: a review. Restor Neurol Neurosci 28(4):589–603

Wagers AJ, Weissman IL (2004) Review: plasticity of adult stem cells. Cell 116(5):639–648

Warren L, Manos PD, Ahfeldt T, Loh YH, Li H, Lau F, Ebina W, Mandal PK, Smith ZD, Meissner A, Daley GQ, Brack AS, Collins JJ, Cowan C, Schlaeger TM, Rossi DJ (2010) Highly efficient reprogramming to pluripotency and directed differentiation of human cells with synthetic modified mRNA. Cell Stem Cell 7:618–630

Wesselschmidt RL (2011) The teratoma assay: an in vivo assessment of pluripotency. Methods Mol Biol 767:231–241

Yu J, Hu K, Smuga-Otto K, Tian S, Stewart R, Slukvin II, Thomson JA (2009) Human-induced pluripotent stem cells are free of vector and transgene sequences. Science 324:797–801

Zhang RR, Zheng YW, Li B et al (2018) Hepatic stem cells with self-renewal and liver repopulation potential are harbored in CDCP1-positive subpopulations of human fetal liver cells. Stem Cell Res Ther 9:29

Zhao SJ, Zhao WX, Ma L (2010) Novel peptide ligands that bind specifically to mouse embryonic stem cells. Peptides 31:2027–2034

Zhao W, Ji X, Zhang F, Li L, Ma L (2012) Embryonic stem cell markers. Molecules 17 (6):6196–6236

Zhou H, Wu S, Joo JY, Zhu S, Han DW, Lin T, Trauger S, Bien G, Yao S, Zhu Y, Siuzdak G, Scholer HR, Duan L, Ding S (2009) Generation of induced pluripotent stem cells using recombinant proteins. Cell Stem Cell 4:381–384

Potential of Stem Cell Therapy to Combat Mastitis in Dairy Animals

5

Neelesh Sharma, Sapna Devi, and Goran Bacic

Abstract

Mastitis is the inflammation of the secretory or alveolar part of the udder and is the most expensive disease of the dairy industry. The estimated economic loss in the US dairy industry alone is approximately $2 billion annually. The substantial increase in economic losses, owing to the high occurrence rate and low recovery rate, is alarming for the dairy industry, which allures the heed of researchers, policymakers, veterinarians, and dairy owners. Therefore, there is an increasing necessity to treat and prevent the highly prevalent disease in dairy animals using the most efficacious protocol. Moreover, since the last more than 70 years, numerous pharmacological and animal husbandry-based approaches are being practiced to control mastitis in dairy herds, but results are below the expectations. This chapter discusses causative agents of mastitis, the pathophysiology of its development, and the potential application of stem cells as regenerative medicine.

Keywords

Mastitis · Mesenchymal stem cells · Mammary stem cells · Regenerative therapy

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5.1 Mastitis

Bovine mastitis can be defined as "inflammation of mammary parenchyma" and is one of the most common dairy cows' diseases and poses heavy economic loss to the dairy industry. It is one of the most challenging diseases of dairy animals because it causes numerous complications to milk processing, milk production, and quality of milk and milk products, resulting in huge economic losses to the dairy industry. The quality of milk, like physical, chemical and bacteriological, and other properties, is negatively affected by mastitis. Mastitis is a dairy affliction that represents an impediment to the development of the dairy industry. Mastitis milk contains pathogenic organisms and their toxin; the disease is also important from the consumer's standpoint. Bovine mastitis is presenting majorly in two forms: clinical and subclinical mastitis. The clinical form of mastitis shows several visible signs such as changes in the udder, including swelling, redness, pain, heat, and disturbed function or changes in milk such as clots or watery secretions and systemic reaction in varying degrees. The major changes in mastitis milk include discoloration, the presence of clots, a large number of leukocytes, etc. Mastitis subclinical form is characterized by not showing visible changes in milk appearance and is the most prevalent form in a dairy herd.

Mastitis is responsible for approximately $2 billion losses in the US dairy industry, which is similar to the impact in Europe (Donovan et al. 2005). Due to the high occurrence rate and low recovery rate, the substantial increase in economic losses is alarming for the dairy industry, which allures the heed of researchers, policymakers, veterinarians, and dairy owners. Therefore, there is an increasing necessity to treat and prevent the highly prevalent disease in dairy animals using the most efficacious protocol. Moreover, since the last more than 70 years, numerous pharmacological and animal husbandry-based approaches are being practiced to control mastitis in dairy herds, but results are below the expectations (Halasa et al. 2007, 2009; McDougall et al. 2009; Nickerson 2009). Although antibiotics treatment is effective in managing mastitis, antibiotic therapy is unable to address the repair of mammary secretory tissue. Moreover, blind use of antibiotics is responsible for antimicrobial resistance worldwide (Peralta et al. 2020).

Based on data available and clinicians' feedback, there is presently no single therapeutic strategy to revert more than 50% of tissue damage caused by mastitis. Improving the mammary gland's structural defects can be done using adult stem/progenitor cells (Sharma and Jeong 2013).

5.2 Etiology of Mastitis

Most cases of intramammary inflammations are due to bacterial pathogens. Although most of the bacteria in milk are harmless or often beneficial, several are pathogenic. Pathogenic bacteria are capable of causing disease in animals as well as in humans. Bovine mastitis is characterized by mammary gland inflammation and is caused mainly by microorganisms that attack the udder, multiply, and produce toxins

harmful to the mammary tissue. The major mastitis-causing organisms are Staphylococci (*Staphylococcus aureus* and *Staphylococcus epidermidis*), Streptococci (*Streptococcus agalactiae*, *Streptococcus dysgalactiae*, *Streptococcus uberis*, and *Streptococcus bovis*), and coliforms (mainly *Escherichia coli* and *Klebsiella pneumoniae*) (Sharma et al. 2007, 2018). Coagulase Negative Staphylococcus (CNS) is also a prevalent bacterial pathogen in udder infections, particularly in heifers mastitis (Kour et al. 2020). Mastitis caused by CNS is extremely insignificant and typically remains subclinical. Moreover, *S. aureus* is predominating bacteria among all mastitis-causing microorganisms.

5.3 Mammary Gland

The mammary gland is a secretory reproductive organ exclusive to the class Mammalia. The mammary gland has the property of milk synthesis and secretion of the milk components, which is well nutritious and supplies nutrition to their young. Milk is the whole shebang and easily digestible nutrient source, provide all required nutrients, including sugars, proteins, lipids, minerals, and vitamins, for growth. Besides nutrients, milk also offers immunological constituents, such as immunoglobulins and white blood cells, which support the protecting neonate from various diseases. The mammary gland passes through four different developmental stages: mammogenesis, lactogenesis, galactopoiesis, and involution. It is a must to understand the basic information about mammary gland structure and its functional anatomy before studying the mammary stem cells' pathophysiology.

5.4 Structure

The mammary gland structure varies from species to species, with some having udder, breasts, rows of teats, etc. The mammary gland is a more complex productive organ that consists of secretory epithelium with cells of the stroma. It has an atypical development and takes place at diverse stages of the lifecycle of the animal. The mammary gland's growth occurs in the manner of branching morphogenesis, in which the distal epithelial bud outspreads and divides to form an extensive network of ducts. It is well established that most of the mammary gland develops after birth, mainly the fundamental ductal part develops before puberty and the lobulo-alveolar part during pregnancy. During lactation, animal performance depends on the organized process of cell proliferation and the mammary gland's growth during pregnancy and cell repair during the involution period. Mammary cell development, differentiation, and their secretory functions have been explored in vitro. Numerous studies on rats and mice have been conducted to study the effect of hormones in mammary gland development and their milk production ability.

5.5 Effect of Mastitis on Mammary Gland Structure

The infection enters the mammary gland through the teat canal. Upon overcoming the anatomical barriers, bacteria evade the mammary gland cellular and humoral defense mechanisms to establish disease. Infection is when the pathogens multiply rapidly and invade the mammary tissue. After the invasion, the pathogen population may be established in the teat canal and, with this as a base, a series of multiplications and extensions into mammary tissue may occur, with infection of mammary tissue occurring frequently or occasionally depending on its susceptibility (Fig. 5.1). An inflammatory response is initiated upon the bacteria's entrance into the mammary gland, which is the body's second line of defense (leukocytes). Bacteria initially affect tissues lining the large milk collecting ducts and cisterns by inflicting damage to small areas of mammary tissues. Then bacteria enter small ducts and alveolar regions of the lower portions of the gland, probably by multiplication and milk currents produced by cow movement. Bacteria multiply and produce toxins, enzymes, and cell-wall components which stimulate the production of numerous mediators of inflammation by cells (Jones 2009). The bacterial products and other irritants cause swelling and death of milk-producing cells and leukocytes. In the event of non-elimination of infection, bacterial levels in the mammary gland rise to a level and cause damage to the mammary epithelial cells and sometimes complete loss of the affected quarter (Fig. 5.2). Intramammary infection severely damages secretory cells, which results in a significant decrease in milk production.

Polymorphonuclear leukocytes (PMNs) are the most critical cellular elements of the antimicrobial self-defense mechanism. PMN can potentially harm the mammary gland, which exact mechanism is still unknown. Neutrophils may promote tissue injury and disturb mammary function via reactive oxygen metabolite generation (i.e., the respiratory burst) and granular enzyme release (i.e., degranulation) (Paape

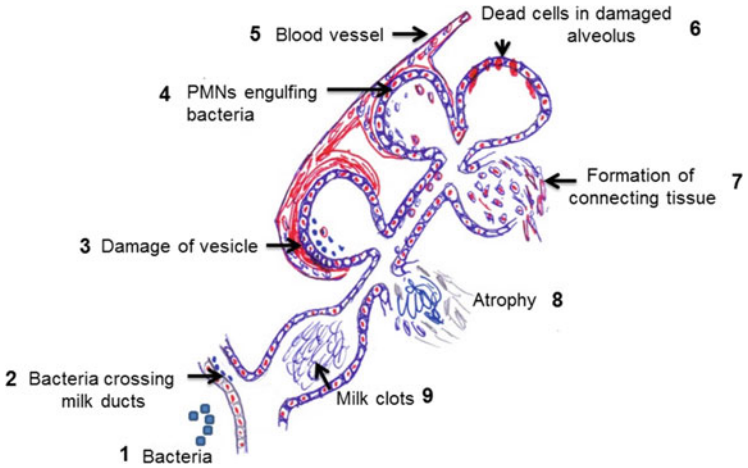

Fig. 5.1 Stepwise progression of infection and damage of mammary parenchyma in the mastitis

Fig. 5.2 Complete loss of left forequarter in a cow after intramammary infection

et al. 2002). After leukocytes cross blood vessels and move through udder tissues toward damaged tissue sites, they accumulate around alveoli, ducts, and cisterns before entering milk. During migration, the leukocytes may release enzymes that cause local destruction of milk-producing cells.

The last stage of inflammation is the "chronic proliferative phase," which is characterized by tissue degeneration, regeneration, and formation of fibrotic tissue. On account of persistent infection, mammary ducts remain clogged, milk accumulates in the alveoli, exerting pressure on milk-producing cells. After the destruction, alveolar structures are permanently replaced by scar tissue (fibrosis), leading to reduced milk yield in the current and subsequent lactation. After the destruction of mammary parenchyma, the affected mammary gland gets atrophied.

Gangrenous mastitis is caused by *Staphylococcus aureus,* which is highly damaging to milk-producing tissues due to the release of injurious toxins and enzymes. *S. aureus* produces α, β, γ and δ toxins, leukocidins, hemolysins, exfoliative toxins, enterotoxin, toxic shock syndrome toxin, and coagulase. Of these, α-toxin appears to be the most toxic. Gangrenous mastitis is characterized by a patchy blue discoloration, coldness of the affected tissue, and leaking of blood serum through the teat skin and sometimes eventually sloughs.

Streptococcal mastitis is caused by *Streptococcus agalactiae*, which inhabits ducts and cisterns of the gland. This organism primarily infects the cisterns and the duct system of the lower portion of the udder. It causes an inflammation that blocks the ducts, leading to decreased milk production or produce agalactia, increased stem cell count, and eventually to involution.

Coliform mastitis is caused by coliform bacteria, mainly *E. coli*. Once the infection has occurred, the *E. coli* bacteria proliferate in great numbers and die and lyse; endotoxin is released from the cell wall. This massive and sudden liberation of endotoxin results in severe, life-threatening toxemia. Tissue damage caused by coliform toxins can be found in the teat cistern, gland cistern, and large ducts within 1 h after the invasion.

5.6 Stem Cell Biology

Stem cells are the undifferentiated cells that have the capability to continuously divide and differentiate into various kinds of cell(s). Stem cells are founder cells that act as a foundation for other types of cells. Stem cells have important characteristics, such as they are unspecialized and can renew themselves for a longer period of time. Stem cells are capable of giving rise to specialized cell types under certain conditions. There are two types of stem cells from animals and humans: embryonic and adult stem cells (Fig. 5.3). Stem cell biology offers promise for revealing the next generation of molecular targets for various diseases.

5.7 How Does Stem Cell Technology Work to Combat?

Regenerative medicine is an emergent area in medicine that uses stem cells or their secreted products to treat a variety of diseases in animals and humans. Stem cells are commonly defined as cells having the capability of self-renewal through replication

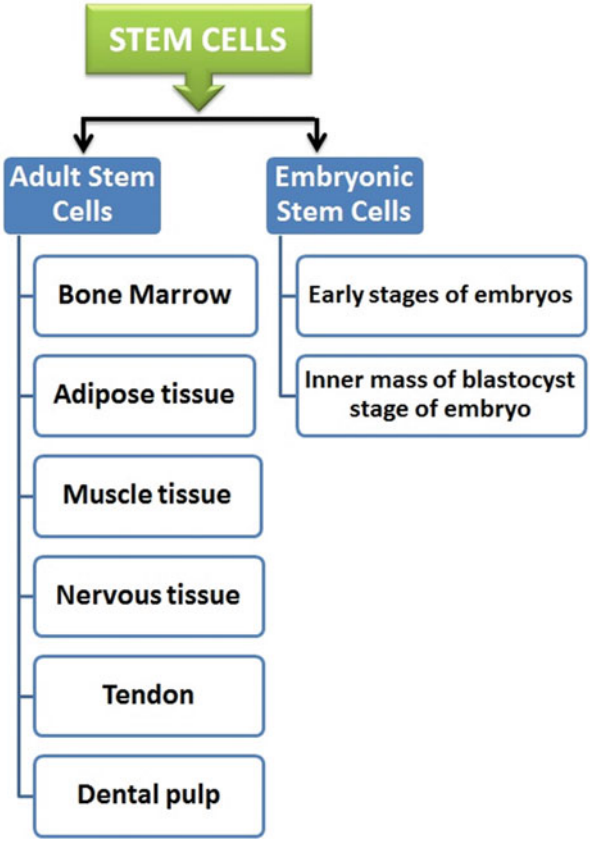

Fig. 5.3 Major types of stem cells and various sources of stem cells

and differentiating into specific lineages. The progeny of stem cells is called progenitor cells that can self-renew and generate differentiated progenies. Besides this, stem cells have the important property of unlimited divisional capacity that helps restore internal tissue repair. In human and veterinary research, adult stem cells have promising features for disease treatment where plasticity and low immunogenicity have the potential for their high anti-inflammatory (Gao et al. 2015).

Bovine mammary stem cell therapy offers significant application in repairing damaged udder with minimal side effects (Sharma and Jeong 2013). Many investigators are working on adult stem cells and are trying to invent simple protocols to grow immense quantities of adult stem cells in laboratories for further clinical applications. They attempt to manipulate stem cells to generate specific cell types for further possible applications to treat specific disease conditions or repair post mastitis mammary parenchymal tissue injury. An undifferentiated cell, present in the organ tissue, has an ability to self-renew and differentiate into specialized cell types called adult stem cells. Their primary function is to maintain the tissue growth and repair in which they are found. There are plenty of data on human and mouse mammary gland stem cells from normal biological process to cancer studies (Jiang et al. 2010; Bruno and Smith 2011; VanKeymeulen et al. 2011; Pond et al. 2013). On the contrary, the information on other species, including large animals, mammary gland stem cells, and their progeny, is very limited. It has been reported that mammary epithelial/stem cells play an important role in the udder defense against intramammary infection (Wellnitz and Kerr 2004). The research on bovine stem cells is very meager. Applications of stem/progenitor cells have future opportunities in the post-mastitis mammary tissue repair in dairy animals. Foundations of works on bovine mammary stem cells are available in the literature (Capuco et al. 2012; Choudhary et al. 2012, 2013). A recent study showed the application of MSCs on bovine mastitis (Peralta et al. 2020).

Mammary gland biology has an important feature of regenerating ability of functional mammary epithelium during the involution period in the successive cycles of lactation. It has been reported that upon implantation of any part of the mouse mammary epithelial tree into a syngeneic gland-free mammary fat pad can reproduce as an entire or functional mammary gland. A study on transgenic mouse models has been recommended that pre-committed mammary epithelial progenitors can regenerate mammary ducts and secretory mammary lobules.

Transplantation of transgene-marked mammary epithelial cells derived from primary cell culture was carried out under conditions of limiting dilution, which varied the results. Interestingly, it is likely that the entire cellular section of the mammary gland is formed from the single antecedent cell. The research on the possible role and use of a stem cell-based approach for repairing mammary parenchyma in dairy animals is in the fancy stage. There are few recent reports on the possible use of varied stem cells in the management of mastitis in dairy animals.

5.8 Bovine Mammary Stem/Progenitor Cells

It has been discovered that murine mammary gland has a strong regeneration ability, which may be due to the presence of distinct cells that can proliferate and generate both lobular and ductal structures of the mammary gland, which are called "mammary adult stem cells". Several studies have endorsed the division of the mammary epithelial cells (MEC) into several sub-divisions. Bovine mammary epithelial/stem cells have been established for further applied research to manage mastitis and improve milk production (Kaushik et al. 2013; Sharma et al. 2015). The major function of mammary stem/progenitor cells is in the growth and maintenance of mammary epithelium (Capuco and Ellis 2013).

The mammary gland has the regenerative capacity, which is evidenced during the regression phase in successive reproductive cycles (Smith and Chepko 2001). The development of the mammary gland during the next cycle of pregnancy and lactation occurs due to the existence of self-renewing stem cells (Capuco 2007).

The mammary gland tissue is a well-arranged branched ductal network comprising of the bilayered system, including luminal (secretory epithelial cells) and basal (myoepithelial) (Muschler and Streuli 2010). The internal layer of columnar/luminal cells of bovine mammary epithelial cells (bMECs) is characterized by cytokeratin 18 (CK18), cytokeratin 19 (CK19), and external layer like myoepithelial cells, which are categorized by CK14, α-smooth muscle actin (α-SMA) and p63. Several studies have been done in mice and humans, on mammary gland stem cell research, predominantly in cancer therapy. However, stem cell research in bovine is still in its early stages. Several studies in stem/progenitor cells in human and mouse mammary glands fetch some expectations for use in bovines and other families of animals. These progenitors may be therapeutically executed to correct the structural/cytological defects in the bovine udder due to mastitis. Concealed factors of stem cells carry multiple positive effects. They show a distinct role in the formation of the latest blood vessels and arouse the relocation of cells; both cells are integral in healing tissue damaged by mastitis. Some secreted factors defend epithelial cells from damaged tissue that occurred due to bacterial toxins; others proved to be antimicrobial peptides. A recent study by Rauner and Barash (2012) has described the bovine mammary stem cell/progenitor hierarchy with their specific markers.

In a recent study, researchers have been working on the stem cell-based therapeutic management of mastitis through transfection of bMECs with antibacterial peptide (Lactoferricin) and found good leads in the in-vitro study (Sharma et al. 2017). They have successfully transfected the antibacterial peptide in the bMECs using the PiggyBac transposon system. It offers evidence that the PiggyBac transposon system has the potential to carry antibacterial peptides into bMESCs.

Ledet et al. (2018) studied the MSCs-derived secretome and found that the mammosphere-derived cells (MDC)-derived secretome can stimulate angiogenesis, epithelial cell migration, and having immunity enhance proteins; those are required for damaged mammary tissue repair. Furthermore, it has also been reported that MDC-derived secretome maintains their effectiveness even after freezing and

thawing, which is important for better therapeutic potential. This approach opens new avenues in the use of stem cell-based therapy for the management of mastitis.

5.9 Caprine Mammary Stem Cells

The anatomical structure of the goat mammary gland is a tubule-alveolar that consists of two lineages of epithelial cells, which consists of luminal cells (alveolar and ductal) and myoepithelial cells. Their ductal and terminal ductal lobular parts are similar to the human mammary gland. In rodents, the epithelium is in straight contact with adipose stromal cells. In contrast, in adult humans and in ruminants, adipocytes are not closely associated with the epithelium, which normally grows enclosed by a loose connective tissue (Hovey et al. 1999). Goat mammary tissue has been utilized in lactation studies, host-pathogen interactions, and the establishment of epithelial cell lines (Pantschenko et al. 2000). The expression of cytokeratins in goat mammary gland tissue has been proven to vary consistent with developmental status. The authors of this chapter are performing on the goat model to develop the stem cell-based approach to manage mastitis.

5.10 Murine Mammary Stem Cells

In the murine mammary gland, Kordon and Smith (1998) employed retroviral tagging to demonstrate the existence of multipotent MaSCs capable of regenerating a functional mammary gland. In mice, epithelium of mammary cells stained with Blue-Azure II dye identified various populations of pale-staining mammary epithelial cells and hypothesized to represent MaSCs. These pale cells were categorized by light staining of the cytoplasm, an outsized round nucleus, sparse cytoplasmic organelles, and tight junctions (Smith and Medina 1988). The more darkly staining cells were reflected more differentiated cells. These pale staining cells were refractory to differentiation signals, evidenced by their lack of casein immunostaining in mammary explants that were cultured in the presence of lactogenic hormones. On the idea of quantitative and morphological properties (cell frequency, shape, size, organelle distribution, staining characteristics, and nuclear morphology), Chepko and Smith (1997) established a model for epithelial cell lineage in rat and mouse mammary glands. Pale staining cells were sectioned into small light cells (SLC) and enormous light cells (LLC). The richness of SLC was unaltered across physiological developmental stages, and these were concluded to be stem cells. These SLC (putative MaSCs) were characterized by morphological features, including small cell size, high nucleus to cytoplasm ratio, condensed chromosomes, and unspecialized cytoplasmic organelles. In the human breast, a study of proliferating cells provided proof for the existence of cells that are morphologically almost like the pale staining cells in rat and mouse mammary glands (Ferguson 1985).

5.11 Mesenchymal Stem Cells (MSCs)

Stem cells have anti-inflammatory properties, and paracrine expression of cytokines accelerates wound healing and suppresses inflammatory reactions in mastitis (Ting et al. 2020). Ting et al. (2020) have used conditioned-Dulbecco's pluripotent stem cells (DPBS) from amniotic membrane stem cells (AMSCs) in treating bovine mastitis and found good results in comparison with the antibiotic-treated group.

Stem cells modified with therapeutic agents can also be used to combat mastitis. Cloning the bovine lactoferricin gene (LFcinB) into the PiggyBac transposon vector was feasible for making MSCs having a heterologous expression of the hybrid antimicrobial peptide LfcinB (Sharma et al. 2017). These cells would then confer high antibacterial activities against bovine mastitis (*S. aureus* and *E. coli*) directly into the mammary gland, providing strong innate udder immunity (Sharma et al. 2017).

MSCs-derived secretome encompassed all secretory factors and plays a crucial role in various physiological processes, including tissue regeneration and cellular cross-talk (Hathout 2007; Toh et al. 2014). The MSCs secretome dower in healing processes through inflammatory reactions, propagative, and remodeling stages of tissue repair. They can also increase the bacterial clearance by the assembly of antimicrobial peptides (AMP) (Krasnodembskaya et al. 2010; Harman et al. 2017).

In a recent study, adipocyte-derived MSCs are wont to study the security and efficacy of MSCs in *S. aureus* mastitis (Peralta et al. 2020). They have also reported that cows didn't persuade changes in clinical or hematological parameters, and organic phenomenon silhouettes in peripheral blood leukocytes (PBLs) linked to activation (CD4, CD8, CD25, CD62L, and CD69) and pro-inflammatory cytokines (IL2, CXCL3, CCL2, CCL5, IFNγ, and TNFα) (Peralta et al. 2020).

5.12 Adipose Stem Cells

The fat is a crucial and abundant source of stromal cells/mesenchymal stem cells, low invasive collecting with an outsized amount of cells, which magnetizes those cells' clinical application in the various disease conditions. Adipose-derived stem cells (ASCs) exist in various fat depots of the animal body, and culture looks like fibroblast-like cells. The International Fat Applied Technology Society has stated that the term ASC should be used for committed adipogenic progenitors (pre-adipocytes). Pre-adipocytes have the capability of multilineage differentiation into myocytes, osteoblasts, chondrocytes, and adipocytes (Zuk et al. 2002). The multipotency property of ASCs has opened the window for more research on ASCs (Sampaio et al. 2015). These cells have potential applications in the tissue repair and regeneration ability of acute and chronically affected tissues (Mizuno et al. 2012).

Costa et al. (2019) have been worked on the therapeutic potential and regenerative ability of ASCs in the repair of mastitis udder and milk production in goats. They have suggested that ASCs have possible regeneration potential of fibrotic mastitis lesions in the mammary gland. It has also recommended that ASCs be

used to rehabilitate milk productivity in the chronically damaged udder tissue due to mastitis in goats, which is suggestive of promising clinical alternative in chronic mastitis and angiogenesis property (Casteilla et al. 2011). On intramammary injection of ASCs in the chronic mastitis udder, they are able to restore its functionality, since it's a richly vascularized organ.

5.13 Limitations

The prevalent hurdles that are averting more frequent use of stem cell therapy are the lack of accessibility and very expensive technique. All of those procedures require expertise and enormous well-equipped hospitals or labs for the preparation of stem cell lines and administration of stem cells in the mammary gland. Additionally, the prices related to stem cell therapy are often in the thousands. If stem cell therapy is resolute to be the simplest course of action for a specific animal, setting realistic expectations with clients is of the utmost significance and can be among the toughest discussions to possess. Stem cell therapy remains in its initial stages, and positive outcomes can't be guaranteed and should even be elusive or temporary for several that undergo the procedure. Additionally, albeit contrary events are rare, the procedure requires anesthesia, which brings associated risks.

5.14 Conclusion and Future Needs

Mastitis is one of the foremost widespread and expensive diseases affecting the dairy business worldwide: mastitis, a multi-etiological complex disease related to dairy production, and economic losses related to mastitis. Unluckily, the present therapeutic strategies are unable to enhance the post-mastitis physical impairment quite 50% in the mammary gland. The most important concern is that currently available therapeutic strategies are not able to revert the mammary tissue damage due to mastitis. For a previous couple of years, stem cell technologies are being applied as a therapeutic tool for regenerative medicine in human, but it's still deficient in the treatment of varied, stimulating infirmities in animals due to the self-renewal capability and, therefore, the successive generations with mutable grades of differentiation capabilities, the powerful effect of those cells in research, and the application of the therapeutic approach. Hence the main factor of those cells might be helpful to get the tissue that will effectively repair damaged tissue in the udder. Two main cell types, epithelial and myoepithelial stem/progenitor cells, are of serious therapeutic interest in mammary gland tissue.

These can sustain a vascular network's development (endothelial and smooth muscle cells) in the mammary gland. Furthermore, an in-depth study is needed to seek out stem/progenitor cells markers of bovine for the healing of udder damage, and consequently, easy and precise technology should be there to isolate the bovine mammary epithelial/stem cells, and their further culture and storage protocols are needed to develop.

References

Bruno RD, Smith GH (2011) Role of epithelial stem/progenitor cells in mammary cancer. Gene Expr 15(3):133–140

Capuco AV (2007) Identification of putative bovine mammary epithelial stem cells by their retention of labeled DNA strands. Exp Biol Med 232:1381–1390

Capuco AV, Ellis SE (2013) Comparative aspects of mammary gland development and homeostasis. Annu Rev Anim Biosci 1:179–202

Capuco AV, Choudhary RK, Daniels KM et al (2012) Bovine mammary stem cells: cell biology meets production Agriculture. Animal 6(3):382–393

Casteilla L, Planat-Benard V, Laharrague P, Cousin B (2011) Adipose-derived stromal cells: their identity and uses in clinical trials, an update. World J Stem Cells 26(3):25–33

Chepko G, Smith GH (1997) Three division-competent, structurally-distinct cell populations contribute to murine mammary epithelial renewal. Tissue Cell 29:239–253

Choudhary RK, Evock-Clover CM, Capuco AV (2012) Expression of novel, putative stem cell markers in prepubertal and lactating bovine Mammary glands. J Anim Sci 89(1):180–181

Choudhary RK, Li RW, Evock-Clover CM et al (2013) Comparison of the transcriptomes of long-term label retaining-cells and control cells microdissected from mammary epithelium: an initial study to characterize potential stem/progenitor cells. Front Oncol 3:21. https://doi.org/10.3389/fonc.2013.00021

Costa CRM, Feitosa MLT, Rocha AR, Bezerra DO, Leite YKC, Argolo Neto NM et al (2019) Adipose stem cells in reparative goat mastitis mammary gland. PLoS ONE 14(10):e0223751

Donovan DV, Kerr DE, Wall RJ (2005) Engineering disease resistant cattle. Transgenic Res 14:563–567

Ferguson DJ (1985) Ultrastructural characterisation of the proliferative (stem?) cells in the parenchyma of the normal resting breast. Virchows Arch A Pathol Anat Histopathol 407:379–385

Gao Y, Zhu Z, Zhao Y, Hua J, Ma Y, Guan W (2015) Multilineage potential research of bovine amniotic fluid mesenchymal stem cells. Int J Mol Sci 15(3):3698–3710

Halasa T, Huijps K, Osteras O et al (2007) Economic effects of bovine mastitis and mastitis management: a review. Vet Q 29:18–31

Halasa T, Nielen M, De Roos APW et al (2009) Production loss due to new subclinical mastitis in Dutch dairy cows estimated with a test-day model. J Dairy Sci 92(2):599–606

Harman RM, Yang S, He MK, Van de Walle GR (2017) Antimicrobial peptides secreted by equine mesenchymal stromal cells inhibit the growth of bacteria commonly found in skin wounds. Stem Cell Res Ther 8:157

Hathout Y (2007) Approaches to the study of the cell secretome. Expert Rev Proteomics 4:239–248

Hovey RC, McFadden TB, Akers RM (1999) Regulation of mammary gland growth and morphogenesis by the mammary fat pad: a species comparison. J Mammary Gland Biol Neoplasia 4(1):53–68

Jiang S, Lee BC, Fu Y et al (2010) Reconstitution of mammary epithelial morphogenesis by murine embryonic stem cells undergoing hematopoietic stem cell differentiation. PLoS ONE 5(3):e9707

Jones GM (2009) Understanding the basics of mastitis. VCE Publications. https://www.pubs.ext.vt.edu/content/dam/pubs_ext_vt_edu/404/404-233/404-233_pdf.pdf. Accessed 10 Oct 2020

Kaushik R, Singh KP, Kumari A et al (2013) Isolation, characterization, and EGFP expression in the buffalo (Bubalus bubalis) mammary gland epithelial cell line. In Vitro Cell Dev Biol Anim 49(1):1–7

Kordon EC, Smith GH (1998) An entire functional mammary gland may comprise the progeny from a single cell. Development 125:1921–1930

Kour S, Neelesh S, Huma ZI, Devi S, Ahmed T, Singh R, Kumar A (2020) Prevalence of mastitis in cow heifers and associated risk factors in Himalayan region, India. J Environ Biol 41:796–802

Krasnodembskaya A et al (2010) Antibacterial effect of human mesenchymal stem cells is mediated in part from secretion of the antimicrobial peptide LL-37. Stem Cells 28:2229–2238

Ledet MM, Vasquez AK, Rauner G, Bichoupan AA, Moroni P, Nydam DV, Van de Walle GR (2018) The secretome from bovine mammosphere-derived cells (MDC) promotes angiogenesis, epithelial cell migration, and contains factors associated with defense and immunity. Sci Rep 8:5378

McDougall S, Parker KI, Heuer C et al (2009) A review of prevention and control of heifer mastitis via non-antibiotic strategies. Vet Microbiol 134:177–185

Mizuno H, Tobita M, Uysal AC (2012) Concise review: adipose-derived stem cells as a novel tool for future regenerative medicine. Stem Cells 30:804–810

Muschler J, Streuli CH (2010) Cell –matrix interactions in mammary gland development and breast cancer. Cold Spring Harb Perspect Biol 2:a003202

Nickerson SC (2009) Control of heifer mastitis: antimicrobial treatment-an overview. Vet Microbiol 134:128–135

Paape M, Mehrzad J, Zhao X, Detilleux J, Burvenich C (2002) Defense of the bovine mammary gland by polymorphonuclear neutrophil leukocytes. J Mammary Gland Biol Neoplasia 7:109–121

Pantschenko AG, Woodcock-Mitchell J, Bushmich SL, Yang TJ (2000) Establishment and characterization of caprine mammary epithelial cell (CMEC). In Vitro Cell Dev Biol Anim 36 (1):26–37

Peralta OA, Carrasco C, Vieytes C, Tamayo MJ, Muñoz I, Sepulveda S, Tadich T, Duchens M (2020) Safety and efficacy of a mesenchymal stem cell intramammary therapy in dairy cows with experimentally induced Staphylococcus aureus clinical mastitis. Sci Rep 10:2843

Pond AC, Bin X, Batts T et al (2013) Fibroblast growth factor receptor signaling is essential for normal mammary gland development and stem cell function. Stem Cells 31:178–189

Rauner G, Barash I (2012) Cell hierarchy and lineage commitment in the bovine mammary gland. PLoS ONE 7(1):e30113

Sampaio RV, Chiaratti MR, Santos DCN et al (2015) Generation of bovine (Bos indicus) and buffalo (Bubalus bubalis) adipose tissue derived stem cells: isolation, characterization, and multipotentiality. Genet Mol Res 14:53–62

Sharma N, Jeong DK (2013) Stem cell research: a novel boulevard towards improved bovine mastitis management. Int J Biol Sci 9(8):818–829

Sharma N, Gupta SK, Sharma U et al (2007) Treatment of clinical mastitis in buffalo-A case report. Buffalo Bull 26(2):56–58

Sharma N, Hyun KJ, Sodhi SS, Do Huynh L, Sung-Woo K, Jong OS, Dong-Kee J (2015) Differentiation dynamics of mammary epithelial stem cells from Korean Holstein dairy cattle under ECM-free conditions. J Biomol Struct Dyn 33(12):2633–2654

Sharma N, Huynh DL, Kim SW, Ghosh M, Sodhi SS, Singh A, Kim NE, Lee SJ, Hussain K, Oh SJ, Jeong DK (2017) A PiggyBac mediated approach for lactoferricin gene transfer in bovine mammary epithelial stem cells for management of bovine mastitis. Oncotarget 8 (61):104272–104285

Sharma N, Huma ZI, Singh SG, Navjot SS, Gupta SK, Upadhyay SR (2018) Prevalence of clinical and subclinical mastitis in buffaloes of Jammu region. Int J Agric Environ Biotechnol 11 (2):415–420

Smith GH, Chepko AG (2001) Mammary epithelial stem cells. Microsc Res Tech 52:190–203

Smith GH, Medina D (1988) A morphologically distinct candidate for an epithelial stem cell in mouse mammary gland. J Cell Sci 90:173–183

Ting WJ, Shaw SW, Hii LY, Lin T-Y, Chang S-C, Liu K-Y, Shen P-C, Chen T-J, Peng S-Y (2020) Therapeutic effects of conditioned e DPBS from amniotic stem cells on lactating cow mastitis. Taiwan J Obstet Gynecol 59:520–526

Toh WS, Foldager CB, Pei M, Hui JHP (2014) Advances in mesenchymal stem cell-based strategies for cartilage repair and regeneration. Stem Cell Rev Rep 10:686–696

VanKeymeulen A, Rocha AS, Ousset M et al (2011) Distinct stem cells contribute to mammary gland development and maintenance. Nature 479:189–193

Wellnitz O, Kerr DE (2004) Cryopreserved bovine mammary cells to model epithelial response to infection. Vet Immunol Immunopathol 101:191–202

Zuk PA, Zhu M, Ashjian P et al (2002) Human adipose tissue is a source of multipotent stem cells. Mol Biol Cell 13:4279–4295

Part II

Stem Cells and Veterinary Research

Fatty Liver Disease and Utility of Stem Cells in Developing the Disease Model

6

Shanti Choudhary, Michelle LaCasse, Donald C. Beitz, and Eric D. Testroet

Abstract

Fatty liver disease (FLD) is one of the most prevalent disorders of the periparturient dairy cow. The deleterious effects of FLD are multifaceted. First, FLD decreases milk production, and second, co-morbidities (e.g., ketosis, displaced abomasum) associated with FLD contribute to the loss in production. Fatty liver disease is a common disorder with approximately half of all dairy cattle accumulating lipid in the liver to some degree. Treatment options are limited, and diagnosis rarely occurs outside of research settings because a liver biopsy is the most reliable method for detection currently available. Therefore, management of FLD is focused on decreasing further adipose lipid mobilization, in part through enhancing gluconeogenesis to promote insulin secretion and suppression of lipolysis. A second strategy is to promote hepatic lipoprotein synthesis, but this synthetic pathway is limited in ruminants. A final strategy to promote the resolution of FLD is to enhance mitochondrial function and increase disposal of fatty acids through oxidative metabolism. However, FLD in bovine is not fully understood, and thus prevention of the disease through nutrition and metabolism is the only viable option for disease mitigation currently available to producers. This chapter describes the basic histological structure of liver tissue and describes various in vitro methods to study fatty liver disease. Recent advances in understanding the human liver's non-alcoholic fatty liver disease (NAFLD) have been discussed and the potential application of bovine liver for the modelling of human liver disease has been advocated. The application of

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these recent tools and techniques will transform basic biological research and understanding of bovine FLD.

6.1 Histology of Liver

The liver, one of the body's largest organs, is involved in a number of roles such as synthesis of several plasma proteins, storage of vitamins and iron, production of bile, carbohydrate metabolism, and detoxification of many toxins and drugs. Histologically, the liver consists of many functional units, viz., *hepatic lobule*, *portal lobule*, *and liver acinus*. These functional units work in unison to perform a variety of functions. In hepatic lobules, liver epithelial cells (hepatocytes) are arranged radially outside of the central vein, just like the spokes of a bicycle wheel, and forming hexagonal plates. These hexagonal plates are packed on top of each other, and hepatic sinusoids traverse the space between the hepatocytes, ultimately draining into a centrally located central vein. The portal lobule is the liver's polygonal mass containing three adjacent hepatic lobules with a centrally located portal vein. This triangular area of the hepatic region performs exocrine functions by secreting bile juice that empties into the common bile duct—the third functional unit of the liver—called acinus, which is oval. The short axis of liver acinus is represented by the distance between two adjacent hepatic lobules with the portal triad, and the long axis is represented by the imaginary line between the two central veins (Fig. 6.1).

The liver is comprised of four distinct components that are called the parenchyma, stroma, sinusoids, and the perisinusoidal spaces (or space of Disse). The parenchyma of the liver consists mainly of hepatocytes. Hepatocytes are hexahedral (six surfaces) shaped cells, making 80% of the liver tissue. They are often polynucleated cells containing 4–6 nuclei per cell. The space between the hepatocytes is called bile canaliculi and is about 1–2 μm in diameter. Hepatic cells contain various organelles like smooth endoplasmic reticulum, rough endoplasmic reticulum, Golgi apparatus, mitochondria, peroxisomes (contains catalase and oxidase enzymes), glycogen deposits, lysosome (iron storage in the form of ferritin), and lipid droplets. The stroma is made of connective tissue (type III collagen) and blood vessels and is the continuation of Glisson's capsule. It provides integrity and support to liver parenchyma and sinusoidal space. Sinusoids are the capillary that travels between hepatocytes. The space between hepatocytes and vein (sinusoids) is the perisinusoidal space. Hepatocytes extend villi into perisinusoidal space to exchange materials with the blood. Hepatic stellate cells (star-shaped cells) are present in the perisinusoidal space, and are the primary storage site for vitamin A inside the cytoplasmic lipid droplet. Hepatic stellate cells are also responsible for collagen deposition at the time of tissue injury and cause fibrosis. Hepatic stellate cells originate from mesenchymal stem cells (MSCs) during embryo formation (Cassiman et al. 2006). Other types of cells present in the liver are Kupffer cell, fibrocyte, hepatic stem cells (HSCs), and HSC-derived myofibroblasts. The localization of the various cell types is illustrated in Fig. 6.2.

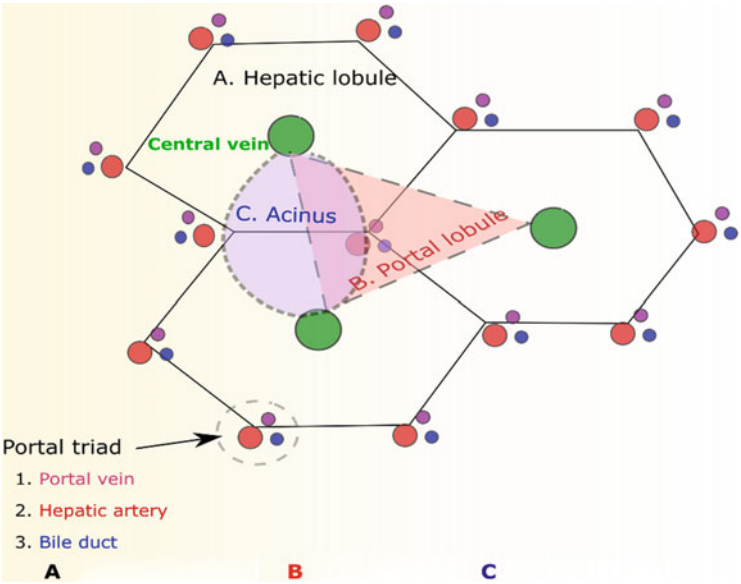


Fig. 6.1 The three functional liver units. The hexagonal shape represents the haptic or classical lobule having a central vein in the middle. The portal lobule is indicated by a triangular red colour shadow marked by dotted lines with the portal triad in the middle. Hepatic acinus is demonstrated in a blue colour oval shaped by central veins and portal triads

6.2 Pathology of the Liver and Altered Histology

Liver Fibrosis Hepatic stellate cells (HSCs) constitute 10% of the liver mass and are originated from MSCs. Under normal physiological conditions, MSCs are quiescent and primarily function as vitamin A storage sites. Hepatic stellate cells are located in the space of Disse. In the event of fibrogenic liver injury, these tissues, including stellate cells and fibroblasts release the profibrogenic cytokine TGFβ, then activates quiescent HSCs (Xu et al. 2014) that down-regulate the formation of lipid droplets containing vitamin A and differentiate into Type 1 collage and alpha-smooth muscle actin (A-SMA) expressing myofibroblasts (Forbes and Parola 2011).

Bovine Fatty Liver Diseases (FLD) Fatty liver disease or hepatic lipidosis is a significant metabolic disease of the cow during the first four weeks after parturition. FLD develops when the accumulation of lipid exceeds oxidation; then, lipids are stored in the form of triacylglycerol (TAG) and secreted out from the liver. As TAG accumulates in the liver, hepatic and, consequently, metabolic functions are impaired (Drackley 1999). During this transition period, increased demand for the nutrients for both lactation and maintenance is insufficient, and thus to balance this need, non-esterified fatty acids (NEFAs) are mobilized from the adipose tissue (e.g., subcutaneous fat). The degree of NEFA mobilization is usually far exceeded the

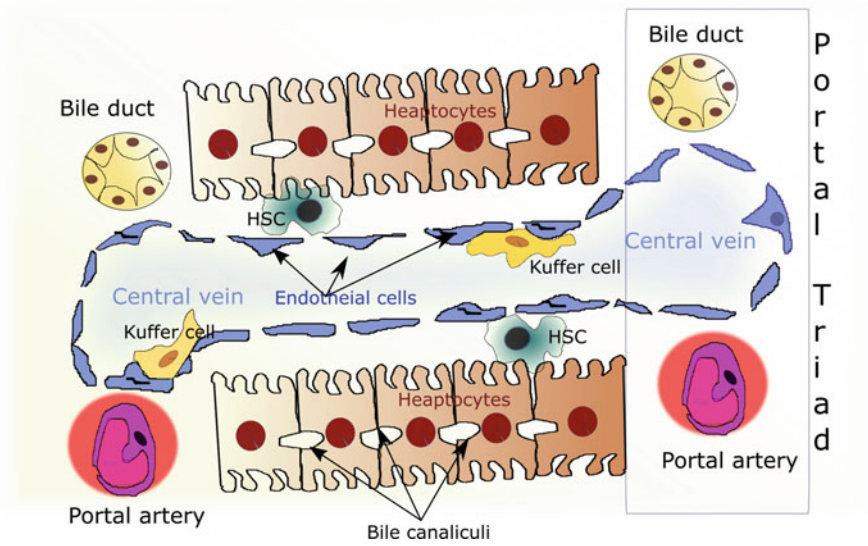


Fig. 6.2 Structure of hepatic lobule. Various liver cells, including hepatocytes, hepatic stem cells (HSCs), Kuffer cell, bile canaliculi, and central vein, are shown

needs of the cow needs, particularly in over-conditioned cattle, because of the relatively abundant supply of energy reserves (McNamara 2000). Acutely, particularly after parturition, the concentration of TAGs can dramatically increase, reaching liver lipid percentages from 5% to >25%, in cattle can happen within 38–48 h from the onset of lactation, and thus resulting in hepatic steatosis or fatty liver disease (Haass and Paul 1984).

When an excess accumulation of TAG occurs in the liver, the physiology of the cell becomes dominated by the growing lipid droplet. This phenomenon is readily observable at the microscopic level and also to a lesser extent at the macroscopic level when observing the gross morphology of the liver, which becomes round, swollen (ballooning), and pale to yellow with enlarged size and prominent lipid infiltration resulting in a soft consistency of the liver that is easily mechanically disrupted. Furthermore, white discolouration of peripheral adipose depots may be evident due to accelerated lipolysis. In acute cases, other pathological findings include necrosis of the kidney, myocarditis, inflammation, ulceration of the gastrointestinal tract, and involution of the pancreas (Bobe et al. 2004). Non-specific signs of FLD are anorexia, weight loss, depression, weakness associated with recumbency, decreased milk production, and reduced rumen motility.

The manifestation of FLD in liver histology includes altered cellular architecture and gross morphology as well as impaired functionality of cellular organelles as evidenced by the formation of fatty cysts in the liver parenchyma, hypertrophic

expansion of hepatocyte volume, and decreased volume of cell organelles, including damaged mitochondria, rough endoplasmic reticulum, sinusoids, and reduced nucleus to cytoplasmic ratio (Bobe et al. 2004).

6.3 Characteristics of the Transition Period

The transition period is a time of severe metabolic strain for the dairy cow that is commonly defined as three weeks before calving and three weeks after calving (Drackley 1999). Roughly speaking, the transition period is the period before and after parturition when metabolic adaptations must occur to support a substantial shift in energetic partitioning from supplying energy to the gestating calf to producing milk that in normal circumstances would be consumed by the calf and support growth in early life before rumen development. Dairy cattle have, however, been genetically selected to maximize the production of milk at the expense of all other metabolic processes. Thus, this periparturient period is when the limits of metabolic flexibility are seen. The cow either adapts to partition energy towards milk production by the mammary or will not adapt and suffer metabolic disorders of calving such as fatty liver disease, ketosis, displaced abomasum, and various infectious diseases.

The defining feature of the transition period is that the cow can only consume enough energy to meet about 75% of her requirements, with most of that energy going towards supporting lactation at the expense of body condition (Bell 1995; Drackley 1999). To adapt to this negative energy balance (NEB), the cow will utilize the energetic reserves of her body (i.e., adipose and muscle tissue) to supplement the functional deficiency created by her limited intake. Herein lies the origin of the metabolic disorders' characteristic of the transition period. For purposes of this chapter, we will focus on the interaction of adipose tissue and the liver. However, it is worth noting that the proteolysis of muscle is also a contributor to the overall energy supply to the cow in glucogenic and ketogenic amino acids.

6.4 Homoerotic Shifts in Glucose Partitioning Support Lactation

Plasma insulin and glucose concentrations decrease after calving (Locher et al. 2011), as would be expected in a period of NEB, similar to the onset of starvation. Glucose is preferentially shunted to the mammary gland to produce lactose, the primary osmolyte of milk. Because milk and blood must be equal in osmolarity, increased lactose production results in increased milk yield (Drackley 1999).

Shunting of glucose to the mammary gland is accomplished via whole body alterations in glucose metabolism. After calving, insulin sensitivity of peripheral tissue other than the mammary gland decreases, resulting in adipose and muscle tissues utilizing less glucose. This adaptation allows for the limited glucose supply to be taken up by the mammary gland and a decrease in the expression of insulin-responsive glucose transporter GLUT4 in those same tissues (Bell and Bauman

1997). Because insulin suppresses lipolysis (Chakrabarti et al. 2013), decreased insulin sensitivity and overall decreased circulating insulin concentrations support the mobilization of triacylglycerol stores (Janovick et al. 2011). Decreased peripheral tissue sensitivity to insulin also supports the facilitated uptake of glucose via the GLUT1 and GLUT12 transporters and transport by the sodium co-transporters SGLT1 and SGLT2 (Zhao and Keating 2007).

6.5 Altered Glucose Partitioning Promotes Mobilization of Energetic Reserves in Adipose Tissue

The ruminant obtains virtually no glucose from the diet; glucose is supplied exclusively by hepatic gluconeogenesis. Because glucose is being removed from circulation to support the production of lactose, glucose concentrations are low in the periparturient period, which results in low circulating insulin and elevated circulating glucagon. As previously mentioned, insulin blocks lipolysis, and with insulin concentrations low and glucagon high, extensive lipolysis occurs, helping to supplement the energetic deficit of the transition period (Fig. 6.3). The amount of triacylglycerols mobilized through conversion to non-esterified fatty acids (NEFAs) is largely dependent on the severity of the negative energy balance, and the body condition score (BCS) of the cow, and the quantity and composition of feed given during the dry period, which all indicate the amount of NEFA available for mobilization. Fatty liver disease (hepatic steatosis), known historically as "fat-cow syndrome" (Morrow 1976), is a disorder that results when the mobilization and transport of NEFAs from adipose stores to the liver overwhelms the capacity of the liver to dispose of those fatty acids through repackaging and distribution through the body via very-low-density lipoproteins (VLDLs) or by oxidation in the mitochondria of the liver. The severity of the fatty liver disease can be expressed in a variety of ways, one of which is the percentage triacylglycerol (TAG) in the liver.

6.6 Mobilization of TAG from Adipose Tissue in the Periparturient Period

Blood NEFA concentrations steadily decrease from the beginning of the dry period (approximately 60 days before expected calving) to 15 days before calving and then steadily increase, reaching peak concentrations between 7 and 14 days in milk (DIM) (Weber et al. 2013). The majority of NEFAs are mobilized from white adipose tissue. About 1/3 of the entire energetic requirements between calving and the third week of lactation are met by mobilization of stored triacylglycerols (Block et al. 2001). Adipocytes have β-adrenergic receptors responsive to glucagon and epinephrine that, when signalled, convert AMP to cAMP. An increase in the concentration of cAMP phosphorylates and activates protein kinase A (PKA). Increased activity of PKA activates phosphorylation of perilipin. Triacylglycerols then are hydrolysed to diacylglycerol and monoacylglycerol and then glycerol sequentially, releasing

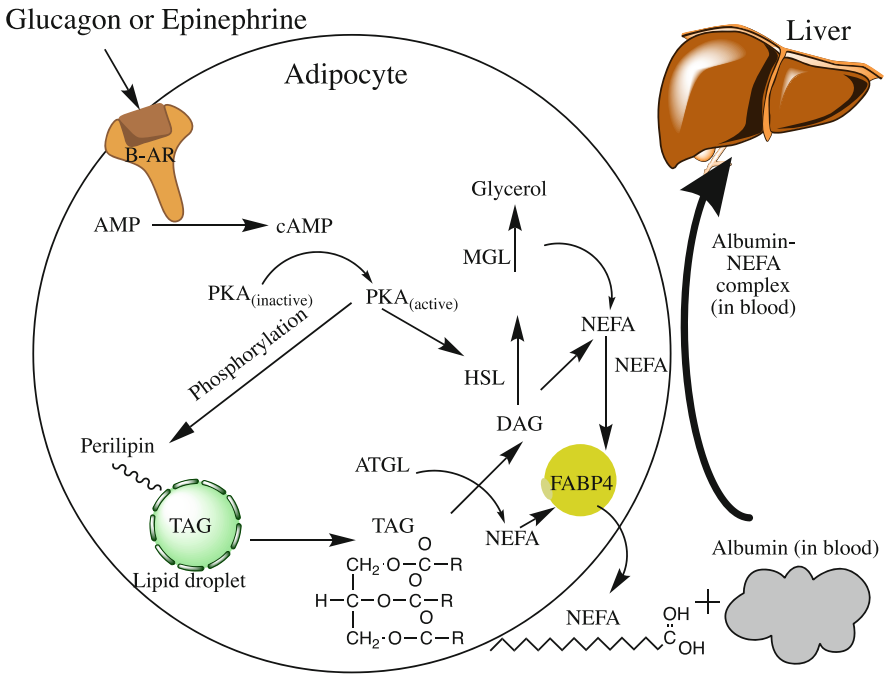


Fig. 6.3 Lipolysis in adipose tissue. NEFAs are released from the hydrolysis of TAG to DAG, MAG, and glycerol after glucagon or epinephrine (β-adrenergic) signal stimulated the G-protein coupled receptor-mediated conversion of AMP to cAMP activates PKA. Fatty acids are hydrolysed off the glycerol backbone in a stepwise fashion using specialized lipases. Mobilized NEFA are trafficked out of the cell by FABP4, where they are complexed with serum albumin and shuttled to the liver for distribution as cargo of VLDL. *NEFA* Non-esterified fatty acid, *TAG* triacylglycerol, *DAG* diacylglycerol, *MAG* monoacylglycerol, *PKA* protein kinase A, *β-AR* β-adrenergic receptor, *ATGL* adipose triglycerol lipase, *HSL* hormone-sensitive lipase, *MGL* mono-glycerol lipase, *FABP4* fatty acid-binding protein 4

NEFAs at each step (Duncan et al. 2007). Because NEFAs are not water-soluble, their transport to the liver is facilitated by complexing with the blood protein albumin.

6.7 Limited Protein Intake Results in Impaired VLDL Secretion and Mitochondrial Dysfunction

During the transition period, overall dry matter intake is limited, and therefore, protein intake is insufficient. Ruminants are limited in their capacity to synthesize and secrete VLDL because of a limited capacity to synthesize apolipoproteins necessary to assemble the VLDL (Oikawa et al. 1997; Wu et al. 2020). The other route of lipid disposal by the liver is through β-oxidation of fatty acids by the mitochondria. β-oxidation by the mitochondria is also impaired in the transition

dairy cow, firstly because protein intake is limited, and thereby limiting carnitine supply. Carnitine is an essential component of the carrier system responsible for importing fatty acids into the mitochondria to undergo β-oxidation and is limited during times of feed restriction. Feed-restricted lactating cattle supplemented with carnitine accumulated less lipid in the liver and had a greater capacity for β-oxidation (Cassiman et al. 2006). Because mitochondria are dysfunctional during the transition period, considerable oxidative stress occurs during this period from the formation of reactive oxygen species and other radical species. Oxidative stress depletes NADPH (generated from glucose in the pentose phosphate pathway) to regenerate reduced glutathione. Besides, mitochondria that are not functioning to their capacity tend to not wholly oxidize fatty acids, resulting in ketone bodies like beta-hydroxybutyrate and acetoacetate. Mitochondrial metabolism predicts a dairy cow's productive lifespan, with decreased inflammation and increased mitochondrial function being positively correlated with lifespan (Huber et al. 2016). Impaired mitochondrial function can be measured by lysine acetylation and is positively correlated with liver triacylglycerol content and serum beta-hydroxybutyrate concentrations (García-Roche et al. 2019).

Peroxisome proliferator-activated receptor alpha (PPARα) is a transcription factor that is activated during times of energy deficit. It is a downstream target of the AMPK and PKA pathways—key nutrient-sensing pathways responsible for adaptation to energy deficit. This transcription factor is a significant enhancer of lipid catabolism (acting opposite of PPARγ) and regulates the expression of fibroblast growth factor 21 (FGF-21) (Lundåsen et al. 2007). During the periparturient period, PPARα and co-activator peroxisome proliferator-activated receptor gamma co-activator 1-alpha (PGC-1α) expression are significantly increased (Louet et al. 2002; Liang and Ward 2006; Schlegel et al. 2013), which result in up-regulation of FGF-21 expression (amongst other changes promoting catabolism and fatty acid disposal). In cows with ketosis, FGF-21 concentration in blood is 41 times higher than that in non-ketotic, cows indicating FGF-21 plays a large role in the catabolism of fatty acids, although it seems that FGF-21 alone does not enhance complete oxidation of fatty acids (Akbar et al. 2015). Furthermore, PPARα expression enhances the expression of carnitine-palmitoyltransferase 1A (CPT1α), which directly facilitates the entry of fatty acids to the mitochondria by carnitine-mediated transport increasing β-oxidation (Brown et al. 2018; Akbar et al. 2015).

6.8 The Immune System is Impaired in the Transition Period Because of Inadequate Glucose and Elevated NEFA

An increase in circulating NEFA also can impair immune function (Goff 2006; Lacetera et al. 2004) as adipose tissue mobilization and inflammation are closely linked. In early lactation, it is well established that elevated circulating NEFA and immune system function are inversely correlated. Specifically, neutrophil and lymphocyte function is significantly decreased, as are proliferation and oxidative burst of peripheral blood mononuclear cells (PBMCs (Ster et al. 2012)) during early lactation

(Goff 2006). Another aspect of the transition period is impaired gluconeogenesis. It has been estimated that the transition dairy cow is approximately 500 g of glucose deficient per day (Drackley 1999), which corresponds precisely with the estimated glucose requirements of the activated immune system (Kvidera et al. 2017), indicating that, in addition to inflammation, limited glucose supply during the transition period is likely partially responsible for the increased incidence of infectious diseases.

6.9 Impaired Hepatic Function Results in Impaired Gluconeogenesis

Ruminants obtain little glucose from dietary sources, but rather glucose is produced from the hepatic conversion of gluconeogenic precursors such as propionate, amino acids, and glycerol to glucose. In the early lactation cow, glucose is limited for the reasons already mentioned (preferential use by mammary, impaired hepatic function, increased oxidative stress, increased demand for glucose by the immune system); so, triacylglycerol-induced impairment of hepatic function is exceptionally detrimental because the low glucose concentrations do not stimulate insulin secretion allowing lipolysis to proceed unchecked (Contreras et al. 2017).

6.10 Early Lactation Requires Coordinated Shifts in Lipid and Glucose Metabolism

Successful adaptation to early lactation is made by a coordinated suppression of hepatic TAG accumulation (enhanced disposal) and by enhancing gluconeogenesis (elevated insulin inhibits lipolysis (Contreras et al. 2017)). Enhanced gluconeogenesis is the result of a combination of increased availability of gluconeogenic precursors and through upregulation of expression and activity of phosphoenolpyruvate carboxykinase (cytosolic), which converts oxaloacetate to phosphoenolpyruvate and ultimately glucose (She et al. 2000).

6.11 MCJ and Mitochondrial Function: Decreased Triacylglycerol Accumulation

Methylation-controlled J protein (MCJ/DNAJC15) is a methylation-controlled J protein present in the mitochondria that regulates the electron transport chain in response to impaired metabolic function. In MCJ knockout mouse models, the absence of MCJ prevents the accumulation of lipids in the liver (Hatle et al. 2013). Mouse models also have shown increased β-oxidation of fatty acids and lower concentrations of liver lipids when MCJ expression is decreased (Barbier-Torres et al. 2020). MCJ has been found in the bovine liver (Choudhary et al. 2020) and is a

promising target for increasing β-oxidation of FA and preventing lipid accumulation in dairy cows.

6.12 Bovine FLD as a Model of NASH and NAFLD

Because dairy cattle are readily available and experience severe metabolic stress during the periparturient period, cattle could be used as a model organism for studying metabolic disorders, emphasizing hepatic disorders. For example, with the rising rate of obesity in humans, non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) are common co-morbidities. The incidence rate of fatty liver disease in humans is estimated to be 25% worldwide (Younossi et al. 2019; Younossi et al. 2020). This increased FLD prevalence is estimated to cost $245 billion annually (Younossi et al. 2019). Most FLD research involving dietary interventions has been done in mice. However, FLD progression to fibrosis is challenging to accomplish in the mouse due to their high metabolism rates relative to humans, often requiring extreme diets such as the methionine-choline-deficient diet or long feeding periods (Anstee and Goldin 2006). Additionally, the ability of rodent models to recapitulate human disease is inconsistent (Takao and Miyakawa 2015). Finally, humans are more closely related genetically and in terms of physiology to farm animals, including cattle, than they are with rodents (Humphray et al. 2007; Tellam et al. 2009), making them a potentially important biomedical model of human disease (Hamernik 2019).

6.13 In Vitro Models of NAFLD

To understand FLD's mechanistic regulation and to intervene in the occurrence of the disease, several in vitro studies have been conducted. Isolation of bovine hepatocytes from the liver tissue has been presented but is complicated. Perfusion technique is the established method of hepatic cell isolation in the cow (Ehrhardt and Schmicke 2016; Donkin and Armentano 1993) and buffalo (Panda et al. 2015), human (Shulman and Nahmias 2013), and mice (Hewitt et al. 2007). Using a non-perfusion technique with disaggregating liver tissue in a collagenase solution, a primary hepatocytes culture was established (Spotorno et al. 2006). However, such studies were performed on either calves or steers' liver tissues, and thus actual physiology of fatty liver might not be addressed. Our lab established a mixed culture of bovine primary hepatic cells from adult cows to mimic in vivo conditions and modelled for bovine hepatic lipidosis (LaCasse et al. 2020). Our group successfully isolated primary hepatic cells from cow liver, and FLD was induced in the presence of 0.4 mM palmitate, 20 ng/mL TNFα, and a cocktail of 0.4 mM palmitate and 20 ng/mL TNFα. The control cells were grown in complete growth media (DMEM + 10% FBS +1× antibiotics) without treatments. We aimed to induce FLD mixed hepatic culture and investigate the mitochondrial complex I inhibitor (iC1) gene's

functional role. First, we successfully showed the in vivo expression of *iC1* in the bovine liver and then in vitro expression of *iC1* in primary hepatic cells in aliquots of conditioned media collected 24 h after the treatment of primary hepatic culture. Results of RT-qPCR, immunocytochemistry, and capillary-based Western blot suggested successful expression of iC1 in bovine. Altogether, we showed the expression of mitochondrial complex 1 inhibitor gene and protein in bovine liver tissue, primary hepatic cells, and the conditioned media (Choudhary et al. 2020). Our future research is to find a way to mitigate the effect of induced FLD by altering the expression of iC1 in dairy cows.

Isolation of donor-derived hepatic cells and 2D culture of hepatocytes is considered 'gold standard' short-term methods to study the human liver's functionality. However, hepatocytes' short-term culture and survival issue variability in donor tissue are the major drawbacks. The difference in age, sex of donor tissue, isolation procedures, and differences in proportions of cells isolated from tissue leads to variations in experimental results and poor reproducibility. Nevertheless, the free fatty acid dose-dependent response of lipid accumulation in 2D hepatic culture was studied. Results showed hepatic cells' fibronectin activation, TGFβ increased expression, and proliferation of endoplasmic reticulum (ER) stress protein in NAFLD events (Wanninger et al. 1811; Muller and Sturla 2019).

The disadvantages of 2D culture include the lack of other cell types and dimensions. The liver comprises hepatocytes and other cells like stellate cells and Kupffer cells. The inclusion of different cell types in 2D culture—called the 2D co-culture model, increases the complexity of in vitro model and overcomes some drawbacks of the 2D system. Exposure of human hepatic cell line (HuH 7) co-culture with human stellate cells with free fatty acids for 24 h produced lipid accumulation in both the cell types (Xu et al. 2005). Our laboratory showed the mixed culture of murine primary hepatic cells treated with palmitate and TNFα and a cocktail of palmitate plus TNFα induced lipid accumulation in hepatocytes and stellate cells (Fig. 6.4; unpublished photomicrograph).

Furthermore, significant advances in 3D monoculture cell models and 3D co-culture cell models have further complexity to the in vitro hepatic models. The 3D cell culture system mimics in vivo situations and successfully been validated by producing spheroid from human hepatocytes and showing up-regulation lipogenic genes in steatotic phenotypes (Kozyra et al. 2018).

Microfluidic-based models to study the pathophysiology of liver disease have also been investigated. Liver-on-a-chip takes advantage of microfluidic technology and integrates biology and engineering on a small device to mimic biological fluids' flow and improve experimental conditions. This device allows live-cell imaging, computation modelling, and flow-through analysis in the temporal dimension. Human hepatoma cells were cultured in a quasi-3D fashion analogous to a liver sinusoid's endothelial barrier. This unique arrangement of microfluidic perfusion allowed continuous diffusion of nutrients to grow cells and removing their end products. Long-chain FFAs (palmitic acid and oleic acid) were added to the medium, and intracellular lipid accumulation, cell viability, oxidative stress was measured. Results showed higher cell viability and minimal oxidative stress than the 2D

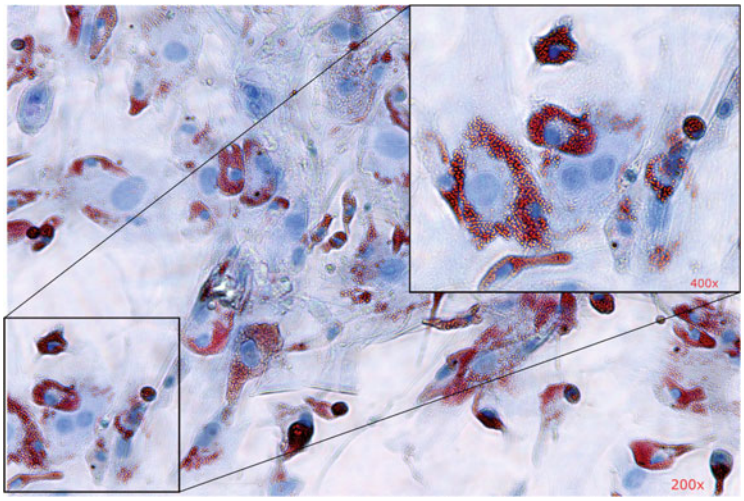


Fig. 6.4 Murine primary hepatic cell culture (P1) treated with TNFα-induced lipid accumulation in the cells. Oil red staining of lipid droplets shown in red colour. (Photo source: Eric's lab; unpublished data)

culture. Surprisingly, the intracellular lipid accumulation is gradual and lower than 2D culture (Gori et al. 2016). Recently, FLD's pathophysiology studied liver-on-a-chips, which opens a promising integrated multidisciplinary research, including biology, pharmacology, and clinical medicine (Hassan et al. 2020).

6.14 Stem Cells as an Alternative Source of Individual-specific Hepatic Cells

Hepatic cells can be derived from stem cells like induced pluripotent stem cells (iPSCs). Somatic cells are reprogrammed by the ectopic coexpression of pluripotency transcription factors (OCT4, SOX2, c-Myc, Nanog) to iPSCs, which further can be used to generate patient-specific hepatocyte-like cells (HLCs) (Sauer et al. 2014). HLCs derived from iPSCs are being used to study pazopanib-mediated hepatotoxicity (Choudhury et al. 2017) and lethal acute liver toxicity (Park et al. 2019). Small molecule-derived differentiation of iPSCs into HLCs demonstrated fundamental haptic marker expression and hepatic functions in differentiated HLCs (Gao et al. 2020). Recent reports on iPSC-derived HLCs with zone-specific properties of hepatocytes have also been identified. Acquisition of zone-specific properties of HLCs was achieved by WNT signal modulators like WNT7B and WNT8B secreted from hepatocytes and cholangiocytes (Mitani et al. 2017). iPSC-derived HLCs have the potential to increase survival rate in mice when implanted in acute liver failure, indicating functional differentiation of HLCs into hepatocytes (Nagamoto et al. 2016).

The lipid profile of hepatocytes and fatty acid (FA) composition of the cell culture media at different stages of iPSCs-derived HLCs was described using biochemical, functional, and gene expression analyses. Results demonstrated an improved understanding of exogenous metabolite supply and cellular biosynthesis essential for iPSC-HLC differentiation and function (Kiamehr et al. 2017). At the later stage of cellular differentiation, polyunsaturated phospholipids and sphingolipids with a very long FA were observed, suggesting a differential expression of lipid-associated genes during hepatic cell differentiation. Thus, HLCs differentiated from iPSCs may offer an alternative model to primary hepatic cells to study aberrations in lipid metabolism and associated alternations in gene expression profiling. In a recent study, in vitro experimental model of NAFLD and non-alcoholic steatohepatitis has been characterized and validated in iPSCs derived HLC in humans. Normal dermal fibroblasts were reprogrammed to generate iPSCs and then induced differentiation into HLCs using hepatocyte maturation medium containing non-essential amino acids and dexamethasone and supplemental oncostatin M. On day 5, iPSCs were differentiated into HLCs, evidenced by hepatic gene expression (Cyp3A4, Cyp1A2, glucose transporters—Glut2, Glut4). Functional characterization of HLCs for glucose uptake assay (insulin-dependent glucose uptake) was similar in both primary human hepatocytes and iPSC-derived HLC (Deepak et al. 2020), demonstrating the utility of HLCs as an alternate in vitro cellular model for studying fatty liver disease. Interestingly, iPSCs-derived hepatocytes harvested from patients of NAFLD liver displayed distinct disease-specific gene signature in comparison to the healthy controls (Duwaerts et al. 2020) and identified hepatic markers and gene pathways of steatosis (Parafati et al. 2018). These results demonstrate that iPSCs-derived hepatocytes are like tissue-derived hepatocytes and may serve as an alternative source of cells.

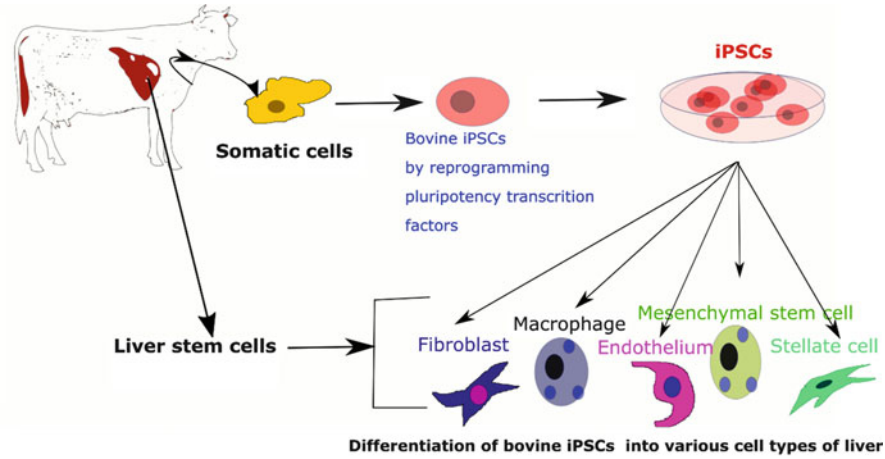
Like the mitochondrial complex I inhibitor (iC1) gene, sirtuin 1 (*SIRT1*) is another key gene that can be therapeutically targeted for fatty liver disease. SIRT1 appears to be a negative inflammatory regulator protein in a high-fat diet or fatty liver disease. SIRT1 down-regulate NF-κB transcription activity by deacetylating NF-κB (Ding et al. 2017). By activating *SIRT1* using the SIRT1 activator (SRT1720) for four weeks, modulated NF-κB pathways and protected high-fat diet-induced liver injury in mice (Niu et al. 2018). In a unique approach, human livers were genetically bio-fabricated using rat livers. Rat livers were repopulated with human mesenchymal cells, fibroblasts, macrophages, and human *SIRT1* knockdown iPSC-derived hepatocytes modelled human fatty liver conditions. Results showed the development of macrosteotosis and inflammatory phenotype of iPSC-derived fatty liver whose metabolic profile was akin to human non-alcoholic steatohepatitis (NASH) liver (de l'Hortet et al. 2019). Biofabrication of livers of other species using stem cells could be a novel and vital tool to investigate liver diseases' mechanisms.

6.15 Other Possible Hepatic Cell Culture Models of Bovine FLD

In addition to in vivo models and existing in vitro models being utilized to understand bovine FLD, it pertinent to adopt some new methods to investigate and elucidate the mechanisms using advances in understanding human liver diseases. New approaches would aid novel functions of FLD biomarkers. A complex in vitro model would better validate drugs and prevent occurrences of the disease. Stem cell-based methods these new methods can include understanding FLD even in genetically susceptible cows (Fig. 6.5).

6.16 Conclusions

Recent promising results of iPSC-derived HLCs and co-culture models include 2D, 3D, liver-on-a-chip, and fabrication; investigators found encouraging information on NASH and NAFLD. We propose using FLD-in-a-dish-models on hepatocytes



Various *in vitro* approaches to study bovine Fatty liver diasese

1. 2D hepatocyte culture
2. 2D hepatocyte co-culture
3. 2D hepatocyte mixed culture
4. 3D hepatocyte culture
5. 3D hepatocyte coculture
6. Microfluidic coculture
7. iPSCs derived cultures and co-cultures
8. Liver stem cell derived hepatocyte cultures
9. Biofabrication of bovine liver with disease inducing gene knockout model

Fig. 6.5 Stem cell-based methods and novel approaches to study bovine fatty liver disease

derived from genetically susceptible cow-specific iPSCs, which considers individual differences in this complex metabolic disease and propose that future studies utilize, at the least, 2D and 3D mixed culture models rather than the conventional 2D monoculture bovine hepatocyte model which is less physiologically relevant.

References

Akbar H, Batistel F, Drackley JK, Loor JJ (2015) Alterations in hepatic fgf21, co-regulated genes, and upstream metabolic genes in response to nutrition, ketosis, and inflammation in peripartal holstein cows. PLoS ONE 10(10):e0139963. https://doi.org/10.1371/journal.pone.0139963

Anstee QM, Goldin RD (2006) Mouse models in non-alcoholic fatty liver disease and steatohepatitis research. Int J Exp Pathol 87(1):1–16. https://doi.org/10.1111/j.0959-9673.2006.00465.x

Barbier-Torres L, Fortner KA, Iruzubieta P, Delgado TC, Giddings E, Chen Y, Champagne D, Fernández-Ramos D, Mestre D, Gomez-Santos B, Varela-Rey M, de Juan VG, Fernández-Tussy P, Zubiete-Franco I, García-Monzón C, González-Rodríguez Á, Oza D, Valença-Pereira F, Fang Q, Rincón M (2020) Silencing hepatic MCJ attenuates non-alcoholic fatty liver disease (NAFLD) by increasing mitochondrial fatty acid oxidation. Nat Commun 11(1):3360. https://doi.org/10.1038/s41467-020-16991-2

Bell AW (1995) Regulation of organic nutrient metabolism during transition from late pregnancy to early lactation. J Anim Sci *73*(9):2804–2819. https://doi.org/10.2527/1995.7392804x

Bell AW, Bauman DE (1997) Adaptations of glucose metabolism during pregnancy and lactation. J Mammary Gland Biol Neoplasia 2(3):265–278

Block SS, Butler WR, Ehrhardt RA, Bell AW, Van Amburgh ME, Boisclair YR (2001) Decreased concentration of plasma leptin in periparturient dairy cows is caused by negative energy balance. J Endocrinol 171(2):339–348. https://doi.org/10.1677/joe.0.1710339

Bobe G, Young JW, Beitz DC (2004) Invited Review: Pathology, etiology, prevention, and treatment of fatty liver in dairy cows. J Dairy Sci 87(10):3105–3124. https://doi.org/10.3168/jds.S0022-0302(04)73446-3

Brown ZJ, Fu Q, Ma C et al (2018) Carnitine palmitoyltransferase gene upregulation by linoleic acid induces CD4+ T cell apoptosis promoting HCC development. Cell Death Dis 9(6):620

Cassiman D, Barlow A, Vander BS, Libbrecht L, Pachnis V (2006) Hepatic stellate cells do not derive from the neural crest. J Hepatol 44:1098–1104. https://doi.org/10.1016/j.jhep.2005.09.023

Chakrabarti P, Kim JY, Singh M, Shin YK, Kim J, Kumbrink J, Wu Y, Lee MJ, Kirsch KH, Fried SK, Kandror KV (2013) Insulin inhibits lipolysis in adipocytes via the evolutionarily conserved mTORC1-Egr1-ATGL-mediated pathway. Mol Cell Biol 33(18):3659–3666

Choudhary S, LaCasse M, Choudhary RK, Rincon, M, Deitz DC Testroet ED (2020) Expression of mitochondrial complex 1 inhibitor in bovine liver tissue, primary hepatic cells and detection of its transcript in conditioned media mimicking fatty liver disease. FASEB J 34(S1):05160. DOI: https://doi.org/10.1096/fasebj.2020.34.s1.05160

Choudhury Y, Toh Y, Xing J et al (2017) Patient-specific hepatocyte-like cells derived from induced pluripotent stem cells model pazopanib-mediated hepatotoxicity. Sci Rep 7:41238. https://doi.org/10.1038/srep41238

Contreras GA, Strieder-Barboza C, Raphael W (2017) Adipose tissue lipolysis and remodeling during the transition period of dairy cows. J Anim Sci Biotechnol 5(8):41. https://doi.org/10.1186/s40104-017-0174-4. PMID: 28484594; PMCID: PMC5420123

de l'Hortet AC, Takeishi K, Guzman-Lepe J, Morita K et al (2019) Generation of human fatty livers using custom-engineered induced pluripotent stem cells with modifiable SIRT1 metabolism. Cell Metab 30(2):385–340. https://doi.org/10.1016/j.cmet.2019.06.017

Deepak HB, Shreekrishna N, Sameermahmood Z, Anand NN, Hulgi R, Suresh J, Khare S, Dhakshinamoorty S (2020) An in vitro model of hepatic steatosis using lipid loaded induced pluripotent stem cell derived hepatocyte like cells. J Biol Methods 7(3):e135. https://doi.org/10.14440/jbm.2020.330

Ding RB, Bao J, Deng CX (2017) Emerging roles of SIRT1 in fatty liver diseases. Int J Biol Sci 13 (7):852–867. https://doi.org/10.7150/ijbs.19370

Donkin SS, Armentano LE (1993) Preparation of extended in vitro cultures of bovine hepatocytes that are hormonally responsive. J Anim Sci 71:2218–2227

Drackley JK (1999) Biology of dairy cows during the transition period: the final frontier? J Dairy Sci *82*(11):2259–2273. https://doi.org/10.3168/jds.S0022-0302(99)75474-3

Duncan RE, Ahmadian M, Jaworski K, Sarkadi-Nagy E, Sul HS (2007) Regulation of lipolysis in adipocytes. Annu Rev Nutr 27:79–101. https://doi.org/10.1146/annurev.nutr.27.061406.093734

Duwaerts CC, Her CL, Phillips NJ, Willenbring H, Mattis AN, Maher JJ (2020) iPSC-derived hepatocytes from patients with non-alcoholic fatty liver disease display a disease-specific gene expression profile. bioRxiv 052001. https://doi.org/10.1101/2020.04.20.052001

Ehrhardt S, Schmicke M (2016) Isolation and cultivation of adult primary bovine hepatocytes from abattoir derived liver. EXCLI J 15:858–866

Forbes SJ, Parola M (2011) Liver fibrogenic cells. Best Pract Res Clin Gastroenterol 25:207–217. https://doi.org/10.1016/j.bpg.2011.02.006

Gao X, Li R, Cahan P, Zhao Y, Yourick JJ, Sprando RL (2020) Hepatocyte-like cells derived from human induced pluripotent stem cells using small molecules: implications of a transcriptomic study. Stem Cell Res Ther 11:393. https://doi.org/10.1186/s13287-020-01914-1

García-Roche M, Casal A, Mattiauda DA, Ceriani M, Jasinsky A, Mastrogiovanni M, Trostchansky A, Carriquiry M, Cassina A, Quijano C (2019) Impaired hepatic mitochondrial function during early lactation in dairy cows: association with protein lysine acetylation. PLoS One 14(3):e0213780

Goff JP (2006) Major advances in our understanding of nutritional influences on bovine health. J Dairy Sci *89*(4):1292–1301. https://doi.org/10.3168/jds.S0022-0302(06)72197-X

Gori M, Simonelli MC, Giannitelli SM, Businaro L, Trombetta M, Rainer A (2016) Investigating non-alcoholic fatty liver disease in a liver-on-a-chip microfluidic device. PLoS One 11(7): e0159729. https://doi.org/10.1371/journal.pone.0159729

Haass CL, Paul EG (1984) Bovine fatty liver syndrome. Iowa State Univ Vet 46(2):7

Hassan S, Sebastian S, Maharjan S, Lesha A, Carpenter AM, Liu X, Xie X, Livermore C, Zhang YS, Zarrinpar A (2020) Liver-on-a-chip models of fatty liver disease. Hepatology 71 (2):733–740. https://doi.org/10.1002/hep.31106

Hamernik DL (2019) Farm animals are important biomedical models. Anim Front 9(3):3–5

Hatle KM, Gummadidala P, Navasa N, Bernardo E, Dodge J, Silverstrim B, Fortner K, Burg E, Suratt BT, Hammer J, Radermacher M, Taatjes DJ, Thornton T, Anguita J, Rincon M (2013) MCJ/DnaJC15, an endogenous mitochondrial repressor of the respiratory chain that controls metabolic alterations. Mol Cell Biol 33(11):2302–2314. https://doi.org/10.1128/MCB.00189-13

Hewitt NJ, Lechón MJG, Houston JB, Hallifax D, Brown HS, Maurel P et al (2007) Primary hepatocytes: current understanding of the regulation of metabolic enzymes and transporter proteins, and pharmaceutical practice for the use of hepatocytes in metabolism, enzyme induction, transporter, clearance, and hepatotoxicity studies. Drug Metab Rev 39:159–234

Huber K, Dänicke S, Rehage J et al (2016) Metabotypes with properly functioning mitochondria and anti-inflammation predict extended productive life span in dairy cows. Sci Rep 6:24642. https://doi.org/10.1038/srep24642

Humphray SJ, Scott CE, Clark R, Marron B, Bender C, Camm N, Davis J, Jenks A, Noon A, Patel M et al (2007) A high utility integrated map of the pig genome. Genome Biol 8:R139. https://doi.org/10.1186/gb-2007-8-7-r139

Janovick NA, Boisclair YR, Drackley JK (2011) Prepartum dietary energy intake affects metabolism and health during the periparturient period in primiparous and multiparous Holstein cows1. J Dairy Sci 94(3):1385–1400. https://doi.org/10.3168/jds.2010-3303

Kiamehr M, Viiri LE, Vihervaara T, Koistinen KM, Hilvo M, Ekroos K, Käkelä R, Aalto-Setälä K (2017) Lipidomic profiling of patient-specific iPSC-derived hepatocyte-like cells. Dis Model Mech 10(9):1141–1153. https://doi.org/10.1242/dmm.030841

Kozyra M, Johansson I, Nordling Å, Ullah S, Lauschke VM, Ingelman-Sundberg M (2018) Human hepatic 3D spheroids as a model for steatosis and insulin resistance. Sci Rep 8:14297. https://doi.org/10.1038/s41598-018-32722-6

Kvidera SK, Horst EA, Abuajamieh M, Mayorga EJ, Fernandez MV, Baumgard LH (2017) Glucose requirements of an activated immune system in lactating Holstein cows. J Dairy Sci 100(3):2360–2374. https://doi.org/10.3168/jds.2016-12001. Epub 2016 Dec 29. PMID: 28041733

LaCasse M, Choudhary S, Choudhary R, de Avila J, Beitz D, Du M, Testroet E (2020) A nonperfusion-based method of hepatic cell isolation and development of fatty liver disease model for dairy cattle. FASEB J 34(S1):06887. https://doi.org/10.1096/fasebj.2020.34.s1.06887

Lacetera N, Scalia D, Franci O, Bernabucci U, Ronchi B, Nardone A (2004) Short Communication: Effects of nonesterified fatty acids on lymphocyte function in dairy heifers. J Dairy Sci 87 (4):1012–1014. https://doi.org/10.3168/jds.S0022-0302(04)73246-4

Liang H, Ward WF (2006) PGC-1α: a key regulator of energy metabolism. Adv Physiol Educ 30 (4):145–151

Locher LF, Meyer N, Weber E-M, Rehage J, Meyer U, Dänicke S, Huber K (2011) Hormone-sensitive lipase protein expression and extent of phosphorylation in subcutaneous and retroperitoneal adipose tissues in the periparturient dairy cow. J Dairy Sci 94(9):4514–4523. https://doi.org/10.3168/jds.2011-4145

Louet J-F, Hayhurst G, Gonzalez FJ, Girard J, Decaux F-J (2002) The co-activator PGC-1 is involved in the regulation of the liver carnitine palmitoyltransferase I gene expression by cAMP in combination with HNF4 alpha and cAMP-response element-binding protein (CREB). J Biol Chem 277(41):37991–38000

Lundåsen T, Hunt MC, Nilsson LM, Sanyal S, Angelin B, Alexson SE, Rudling M (2007) PPARalpha is a key regulator of hepatic FGF21. Biochem Biophys Res Commun 360 (2):437–440

McNamara JP (2000) Integrating genotype and nutrition on utilization of body reserves during lactation of dairy cattle. In: Cronje B (ed) Ruminant physiology: digestion, metabolism, growth and reproduction. CAB International, London, pp 353–369

Mitani S, Takayama K, Nagamoto Y, Imagawa K, Sakurai F, Tachibana M, Sumazaki R, Mizuguchi H (2017) Human ESC/iPSC-derived hepatocyte-like cells achieve zone-specific hepatic properties by modulation of WNT signaling. Mol Ther 25(6):1420–1433

Morrow DA (1976) Fat cow syndrome. J Dairy Sci 59(9):1625–1629

Muller FA, Sturla SJ (2019) Human in vitro models of non-alcoholic fatty liver disease. Curr Opin Toxicol 2019(16):9–16

Nagamoto Y, Takayama K, Ohashi K, Okamoto R, Sakurai F et al (2016) Transplantation of a human iPSC-derived hepatocyte sheet increases survival in mice with acute liver failure. J Hepatol 64:1068–1075. https://doi.org/10.1016/j.jhep.2016.01.004

Niu B, He K, Li P, Gong J, Zhu X, Ye S (2018) SIRT1 upregulation protects against liver injury induced by a HFD through inhibiting CD36 and the NF-κB pathway in mouse kupffer cells. Mol Med Rep 18(2):1609–1615. https://doi.org/10.3892/mmr.2018.9088

Oikawa S, Katoh N, Kawawa F, Ono Y (1997) Decreased serum apolipoprotein B-100 and A-I concentrations in cows with ketosis and left displacement of the abomasum. Am J Vet Res 58 (2):121–125

Panda S, Bisht S, Malakar D, Mohanty AK, Kaushik JK (2015) In vitro culture of functionally active buffalo hepatocytes isolated by using a simplified manual perfusion method. PLoS One 10:e0118841

Parafati M, Kirby RJ, Khorasanizadeh S, Rastinejad F, Malany S (2018) A non-alcoholic fatty liver disease model in human induced pluripotent stem cell-derived hepatocytes, created by endoplasmic reticulum stress-induced steatosis. Dis Model Mech 11(9):033530. https://doi.org/10.1242/dmm.033530

Park S, In Hwang S, Kim J et al (2019) The therapeutic potential of induced hepatocyte-like cells generated by direct reprogramming on hepatic fibrosis. Stem Cell Res Ther 10:21. https://doi.org/10.1186/s13287-018-1127-3

Sauer V, Roy-Chowdhury N, Guha C, Roy-Chowdhury J (2014) Induced pluripotent stem cells as a source of hepatocytes. Curr Pathobiol Rep 2(1):11–20. https://doi.org/10.1007/s40139-013-0039-2

Schlegel G, Ringseis R, Keller J, Schwarz FJ, Windisch W, Eder K (2013) Expression of fibroblast growth factor 21 in the liver of dairy cows in the transition period and during lactation. J Anim Physiol Anim Nutr 97(5):820–829

She P, Shiota M, Shelton KD, Chalkley R, Postic C, Magnuson MA (2000) Phosphoenolpyruvate carboxykinase is necessary for the integration of hepatic energy metabolism. Mol Cell Biol 20(17):6508–6517. https://doi.org/10.1128/MCB.20.17.6508-6517.2000

Shulman M, Nahmias Y (2013) Long-term culture and co-culture of primary rat and human hepatocytes. Methods Mol Biol 945:287–302

Spotorno VG, Hidalgo A, Barbich M, Lorenti A, Zabal O (2006) Culture of bovine hepatocytes: a non-perfusion technique for cell isolation. Cytotechnology 51(2):51–56. https://doi.org/10.1007/s10616-006-9000-0

Ster C, Loiselle MC, Lacasse P (2012) Effect of postcalving serum nonesterified fatty acids concentration on the functionality of bovine immune cells. J Dairy Sci 95(2):708–717. https://doi.org/10.3168/jds.2011-4695. PMID: 22281335

Takao K, Miyakawa T (2015) Correction for Takao and Miyakawa, genomic responses in mouse models greatly mimic human inflammatory diseases. Proc Natl Acad Sci [Internet]. National Academy of Sciences

Tellam RL, Lemay DG, Van Tassell CP, Lewin HA, Worley KC, Elsik CG (2009) Unlocking the bovine genome. BMC Genomics 10:193. https://doi.org/10.1186/1471-2164-10-193

Wanninger J, Neumeier M, Hellerbrand C, Schacherer D, Bauer S, Weiss TS, Huber H, Schäffler A, Aslanidis C, Schölmerich J, Buechler C (1811) Lipid accumulation impairs adiponectin-mediated induction of activin Aby increasing TGFbeta in primary human hepatocytes. Biochim Biophys Acta 2011:626–633

Weber C, Hametner C, Tuchscherer A, Losand B, Kanitz E, Otten W, Singh SP, Bruckmaier RM, Becker F, Kanitz W, Hammon HM (2013) Variation in fat mobilization during early lactation differently affects feed intake, body condition, and lipid and glucose metabolism in high-yielding dairy cows. J Dairy Sci 96(1):165–180. https://doi.org/10.3168/jds.2012-5574

Wu J, Liu J, Wang D (2020) Effects of body condition on the insulin resistance, lipid metabolism and oxidative stress of lactating dairy cows. Lipids Health Dis 19(1):56

Xu L, Hui AY, Albanis E, Arthur MJ, O'Byrne SM, Blaner WS, Mukherjee P, Friedman SL, Eng FJ (2005) Human hepatic stellate cell lines, LX-1 and LX-2: new tools for analysis of hepatic fibrosis. Gut 54:142–151. https://doi.org/10.1136/gut.2004.042127

Xu J, Liu X, Koyama Y, Wang P, Lan T, Kim IG, Kim IH, Ma HY, Kisseleva T (2014) The types of hepatic myofibroblasts contributing to liver fibrosis of different etiologies. Front Pharmacol 22(5):167. https://doi.org/10.3389/fphar.2014.00167

Younossi ZM, Tacke F, Arrese M, Chander Sharma B, Mostafa I, Bugianesi E, Wai-Sun Wong V, Yilmaz Y, George J, Fan J, Vos MB (2019) Global perspectives on nonalcoholic fatty liver disease and nonalcoholic steatohepatitis. Hepatology 69(6):2672–2682

Younossi ZM, Corey KE, Alkhouri N, Noureddin M, Jacobson I, Lam B et al (2020) Clinical assessment for high-risk patients with non-alcoholic fatty liver disease in primary care and diabetology practices. Aliment Pharmacol Ther

Zhao FQ, Keating AF (2007) Expression and regulation of glucose transporters in the bovine mammary gland. J Dairy Sci 90(Suppl 1):E76

7 Mammary Epithelial Cells: A Potential Cellular Model to Understand the Impact of Heat Stress on Mammary Gland and Milk Production in Dairy Animals

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Abstract

Heat stress affects the production potential of the dairy animals. Elevated temperature of the barn and high humidity negatively affects feed intake thus, affects milk production. Alveolar epithelial cells are the major cell types of mammary gland and responsible for the milk secretion. Therefore, analyzing changes of mammary epithelial cells (MECs) during pre- and post-heat stress can provide insight into the protective mechanism. Enhanced expression of heat shock proteins (HSP) 70 during heat stress is critical for developing tolerance and protection. This chapter examines the responsiveness of MECs to heat stress in various studies and reviews its suitability in developing an in vitro cellular model to examine cellular heat stress response.

Keywords

Heat stress · Mammary epithelial cell · In vitro model

7.1 Introduction

The heat stress phenomenon induced by global warming has become a significant challenge for the world dairy sector as it affects all the aspects of dairy animals such as production, reproduction, growth, immunity, physiology, and overall well-beingness (Chase 2013; Salak-Johnson and McGlone 2007; Marai and Haeeb 2010; Hansen 2013; Wohlgemuth et al. 2016; Tao et al. 2018; Liu et al. 2007). Heat stress is now a leading environmental factor that is expected to have substantial economic implications in times to come for the dairy industry worldwide. There has

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been a consensus among the livestock researchers that excessive heat load directly affects animal performance. Its impact has to be minimized for optimal production, especially in tropical and subtropical regions. The exact mechanism associated with heat stress-induced impairment in dairy animals' functional traits is not fully understood because of the complexity and involvement of multiple animal response factors. One of the simplest explanations of the negative impact of heat stress on milk yield is a reduction in feed and nutrient intake by the animal (Gaughan et al. 2010). The milk synthesis in dairy animals is believed to be reduced when the temperature-humidity index (THI) crosses 72 (Du Preez et al. 1990; Armstrong 1994). At higher THI, the animal experiences heat stress because it cannot maintain the body's thermal balance by dissipating adequate heat. The dairy animals tend to maintain their core body temperature within a narrow range to control normal physiological and metabolic processes. Therefore, the optimal genetic potential of dairy animals is generally achieved within their thermo-neutral environmental conditions (THI < 72), wherein dairy animals maintain the equilibrium between heat production and heat loss (Roenfeldt 1998). The thermoneutral zone (TNZ) of the dairy animals' ranges from 16 °C to 25 °C, where they maintain a normal physiological body temperature of 38.4 °C to 39.1 °C. The higher ambient temperature (above an animal's upper critical temperature) dramatically alters the behavior, health, and productivity of dairy animals.

7.2 Economic Consequences of Heat Stress on Livestock Production

The livestock-based economy is an essential component of the world's economy. It provides milk, dairy products, meat, manure, skin, horn, and bone to sustain millions of people (Herrero and Thornton 2010). The dependencies on livestock products are expected to increase by 70%, especially when the world population is expected to reach 9.6 billion by 2050 (FAO 2015). However, the livestock sector, mainly the dairy sub-sector, faces enormous challenges due to changing climate patterns, resulting in producers' economic burdens and levitating serious animal welfare apprehensions (Baumgard and Rhoads 2013; Rhoads et al. 2013). The dairy sector is continuously changing over time globally, especially with the replacement of well-adapted local breeds with high-yielding exotic breeds. It is a known fact that elevated temperature and humidity negatively affect livestock feed intake, leading to decreased milk production (Gaughan et al. 2010). The heat stress phenomenon is causing substantial economic loss to the dairy industry (St-Pierre et al. 2003; Rhoads et al. 2013; Smith et al. 2013). The decrease in milk production caused by heat stress results in an annual loss of $1.2 billion to the US dairy sector (Key and Sneeringer 2014). Even India, a humid tropic country, loses about 1.8 million tons of milk production to heat stress (Upadhyay et al. 2009). Per projected estimate, global warming will negatively impact India's milk production further by 15 million tons by 2050 (Sreenivasaiah 2016).

7.3 Heat Stress and Milk Yield

In many studies, heat stress has been established as a significant environmental factor that reduces milk yield in dairy animals (West 2003; Brouček and Šoch 2009; Zhu et al. 2016). Elevated core body temperature reduces milk output and changes the milk composition like percentages of milk protein, fat, solids, and lactose (Key and Sneeringer 2014). Above 35 °C, heat stress directly affects the feed intake, thereby creating a negative energy balance, which ultimately affects milk synthesis (Wheelock et al. 2010; Das et al. 2016). Studies have shown that with a per unit increase in THI beyond 72, there is a reduction of 0.2 kg in milk yield in dairy cows (West 2003; Ravagnolo et al. 2000). About 1 °C increase in air temperature beyond TNZ has been shown to reduce feed intake by 0.85 kg, which results in an approximately 36% decline in milk production (West 2003; Rhoads et al. 2009). It has been recognized that only 35% of the reduction in milk yield is due to decreased feed intake, while the remaining 65% reduction is due to the direct physiological effect of heat stress (Rhoads et al. 2009). The decline in milk production during heat stress is contributed by decreased nutrient absorption, hormonal imbalance, and the rumen's alteration (Bernabucci et al. 2010). The consequences of heat stress are far more damaging to high-yielding cows, which are already under substantial metabolic strain (Bajagai 2011). Reports have also revealed that cows conceived during heat stress conditions also experience a decline in milk production (Ominski et al. 2002; Nardone et al. 2010). Interestingly, Dikmen et al. (2014) observed that cows calved during the summer season produced lesser milk production than the cows calved during winter. Holstein cows with high rectal temperature during summer stress also showed a reduction in milk yield (Dikmen et al. 2015). A report has also shown that heat stress also affects the cow's udder, making them more susceptible to infection like mastitis (Igono et al. 1988).

7.4 Impact of Heat Stress on Mammary Gland of Dairy Animals

The mammary gland is a complex organ made up of a tubulo-alveolar ductal structure of secretory epithelium and diverse cells of the supportive stroma with different types of accessory cells, such as adipocytes, fibroblasts, myoepithelial cells, and endothelial cells. The mammary gland of dairy animals has always drawn researchers' attention because of its unique role in milk synthesis and secretion. The development of the mammary gland in dairy animals is a complex process that involves a series of structural and functional changes from early lactation to the involution period. These coordinated alterations are responsible for the difference in composition and yield of milk and several developmental and hormonal changes (Neville et al. 2002; Bionaz and Loor 2007). During different physiological stages, the mammary gland exhibits marked variation in its milk-producing capability.

Furthermore, the expression of a defined set of transcripts associated with milk production increased substantially. There was a dramatic rise in milk production during early lactation in bovine (Neville et al. 2002; Sorensen et al. 2006). A gradual

decrease follows the peak lactation period until the mammary gland involutes before entering the non-lactating or dry period.

The heat stress or environmental heat load affects mammary gland functioning in dairy animals (Tao et al. 2018). Also, severe HS mammary glands use a negative regulatory feedback mechanism to reduce milk production (Silanikove et al. 2009; Baumgard and Rhoads 2012). The negative effect of heat stress on mammary gland function is mainly characterized by a reduction in mammary epithelial cell proliferation, reduced macrophages and lymphocytes, and an increase in neutrophils and apoptotic cells (Tao et al. 2011). Such changes affect immunity and make dairy animals more susceptible to infection, mostly by mastitis-causing pathogens (Wohlgemuth et al. 2016). Heat stress is reported to reduce glucose uptake by the mammary gland, limiting glucose availability and lactose synthesis (Wheelock et al. 2010). The induction of acetyl CoA carboxylase and fatty acid synthetase genes expression in the mammary gland during heat stress reflects that more energy is being partitioned toward fatty acid synthesis rather than mammary cell proliferation (Adin et al. 2009). Besides, heat stress has also been shown to affect the synthesis and secretion of key hormones that, in turn, alter mammary gland development and, subsequently, milk yield. Interestingly, reports have also shown that heat stress affects mammary gland involution, characterized by autophagy (Zarzynska and Motyl 2008) and apoptosis (Wilde et al. 1997; Sorensen et al. 2006).

For a long time now, it is known that a cow's core body temperature directly affects the mammary gland's internal temperature (Bitman et al. 1984). The higher core body temperature during heat stress also elevates the mammary gland temperature (West 2003). The mammary gland's internal temperature also affects mammary cells, especially mammary epithelial cells (MECs), by inducing a heat shock response. The series of studies have shown the induction of heat shock proteins (HSPs) in MECs (Kapila et al. 2016; Kapila et al. 2013; Collier et al. 2006a, b) as well as in other cell types like peripheral blood mononuclear cells (Sharma et al. 2019; Lacetera et al. 2006) and skin fibroblast (Shandilya et al. 2020) in dairy animals. The induction of HSPs in different cell types during heat stress indicates the primary response of cells to maintain homeostasis and cell survival. These HSPs act like a molecular chaperone and help in the folding of denatured protein during heat stress. Additionally, studies have also shown that heat stress affects the gene expression or folding/denaturation of proteins and affects cellular morphology. Shyy et al. (1989) have reported that the cytoskeleton of mouse primary MECs gets reorganized during heat stress. The exposure of mouse primary MECs to heat stress conditions (43–45 °C) even for 15–30 min degraded the actin filaments and retracted the keratin filaments. Interestingly when the same cell type was exposed to relatively milder temperature (41 °C), there was no change in cytoskeleton structure (Shyy et al. 1989), which provided an important clue that the cellular response of MECs depends upon the degree of heat shock treatment. In a significant study by Collier et al. (2006a, b), the exposure of MECs to 42 °C resulted in reduced ductal structure.

7.5 Mammary Epithelial Cells as a Cellular Model to Understand the Heat Stress Response

While conducting any biological research on the mammary gland, the critical issue has always been the availability of a suitable cellular model that could mimic the mammary gland's functioning (Jedrzejczak and Szatkowska 2014). Many times, while conducting in vivo studies, it is difficult to get a mammary-specific response as the experimental condition or treatment will cause other systemic effects on animals (Ogorevc et al. 2009; Rose et al. 2002). Therefore, to understand the mammary specific changes to any external stimuli or experimental conditions, there had been a widespread consensus to establish an appropriate in vitro cellular model to understand the physiological, biochemical, and immunological functions of the mammary gland in a more precise manner (Zhao et al. 2010; Tong et al. 2012). In the past, mammary tissues or explants have been widely used as in vitro models during various experimental settings (Rabot et al. 2007). However, with mammary explants, the major limitation is distinguishing between primary mitogens and secondary regulators of mammary gland function and development. To study cMECs several cell culture methodologies were developed. These cells are the predominant cell types in the lactating mammary gland and thus crucial in synthesizing and secreting milk into the mammary lumen. Any change in cell function of MECs due to experimental conditions could provide an insight into the mechanism happening in the mammary gland. Several cell lines of MECs across different species such as human (Gaffney et al. 1976), mouse (Danielson et al. 1984; Kittrell et al. 1992), cow (Jedrzejczak and Szatkowska 2014; Rose et al. 2002; German and Barash 2002), pig (Kumura et al. 2001; Zheng et al. 2010), buffalo (Anand et al. 2012; Kaushik et al. 2013), sheep (Düchler et al. 1998; Ilan et al. 1998), and goat (Tong et al. 2012; Ke et al. 2012; Zheng et al. 2010) have been established. These cell lines have been successfully utilized in understanding the mammary gland functioning of respective species under wide arrays of experimental conditions. Subsequent studies were also designed to record mammary epithelial cells' responses to heat stress (Collier et al. 2006a, b; Du et al. 2008; Du et al. 2008; Liu et al. 2007; Li et al. 2015; Chen et al. 2020). These studies led to the development of an in vitro cellular model to delineate molecular mechanisms, which was otherwise not possible because of the complexity of mammary gland tissue. With the advent of an appropriate cellular model of the mammary gland, several studies related to cell differentiation during lactation, the immune response to infections, and the response to hormonal induction were conducted. Because MECs are primary secretary cells involved in milk production and sensitive to environmental changes, these cells were utilized successfully in various studies to understand mammary gland and lactation biology (Liu et al. 2007; Du et al. 2008; Lauzon et al. 2005; Qian et al. 2004).

Li et al. (2015) studied the transcriptional response of cultured cow MECs to heat stress. They successfully delineated the transcriptomic differences in global gene expression data between heat-stressed and unstressed MECs. A total of 2716 differentially expressed genes involved in regulating the cytoskeleton, cell cycle, and stress response processes were identified in heat-stressed MEC (Li et al. 2015).

Following a similar approach, Kapila et al. (2016) identified several heat-responsive genes and biological pathways in heat-stressed MECs of riverine buffaloes (*Bubalus bubalis*). The impact of heat stress was reflected in inhibition of cell proliferation ability, cellular viability, and induction of cellular apoptosis, necrosis, and lactate dehydrogenase activity. The specifically up-regulated genes belonged to the heat shock family, *viz*., HSPA6, HSPB8, DNAJB2, and HSPA1A.

Along with HSPs, genes like BOLA, MRPL55, PFKFB3, PSMC2, ENDODD1, ARID5A, and SENP3 were also up-regulated. Majorly, the heat-responsive genes belonged to chaperons, immune system, cell proliferation, and metabolism categories. Even though these studies were performed in cell culture environments, such studies helped provide a better understanding of heat stress's impact on MECs and the mammary gland in general. The effect of heat stress was also studied on milk-derived somatic cells in Holstein Friesian cows (Garner et al. 2020). The transcriptome data of somatic cells purified directly from the milk of animals provided a vital clue about the impact of heat stress in vivo, which was not well defined in dairy animals. Genes that were affected in milk somatic cells were associated with the cellular stress response, apoptosis, oxidative stress, and glucose metabolism. Their findings have also provided new insights into BDKRB1 and SNORA19 candidate genes' potential role during heat stress response in dairy cows.

The response of cow MECs to heat stress was also captured in another cell culture-based study conducted by Han et al. (2010). The HSPs, Bcl-2 family, caspases (3, −7, and − 8), and apoptosis-regulated genes (Bcl-2, Bcl-2A, and Mcl-1) increased sharply post heat stress treatment. Further, the genes that act as signaling factor for apoptosis-like; tumor necrosis factor receptor, p53, Apaf-1 genes were also up-regulated. However, the caspase-6, nine, and pro-apoptotic genes like Bax, Bak, and Bid were down-regulated in heat-stressed MECs. Based on the overall data, Han et al. (2010) concluded that heat stress conditions promote cellular apoptosis and de-regulate regular cellular activity in MECs of dairy cows.

Recently, Chen et al. (2020) have used MECs as an in vitro model to evaluate the impact of heat stress on mitochondrial functioning. Through a series of cell culture parameters, they successfully validated that heat stress disrupts the mitochondrial functioning by inducing subtle changes in its morphology. The mitochondria generally undergo fusion and fission in a regulated manner. Few previous studies have highlighted the importance of mitochondrial fusion in maintaining the healthy mitochondrial population inside cells. The presence of a healthy mitochondrial population helps to circumvent the effect of stress on cellular functioning (Westermann 2010). It is now a well-established fact that heat stress causes an imbalance in the fission and fusion process of mitochondria, leading to a change in its morphology and induces fragmentation. Chen et al. (2020) have shown that mitochondria in heat-stressed MECs become more fragmented, disrupting mitochondria function. Their study reported many other cellular changes like reduction in ATP level, decreased mitochondrial membrane potential, antioxidant enzyme activity, and increased respiratory chain complex I activity. Earlier, Qian et al. (2004) have also reported the destruction of mitochondrial structure and function in rats' cardiomyocytes cells after heat stress. Similar findings have also been

reported in neuronal cells, skeletal muscle, etc., suggesting that heat stress grossly disrupts the mitochondrial function in different cell types. These studies have concluded that heat stress causes morphological and functional damage to mitochondria.

Accumulation of reactive oxygen species (ROS) inside mitochondria is another cause of oxidative stress during heat stress (Andreyev et al. 2005). The accumulation of ROS is likely to damage mitochondria's functionality by inducing mutations or reducing the mitochondrial membrane potential through lipid peroxidation (Dodson et al. 2014). The opening of mitochondrial permeability transition pore (MPTP) and the inner membrane anion channel (IMAC) reduces the mitochondrial membrane potential and increases the ROS production by the electron transport chain. This phenomenon is known as ROS-induced ROS release (RIRR) and a critical factor in causing mitochondrial dysfunction. RIRR leads to superoxide ions, further causes oxidative damage to the cell.

An increase in cytosolic Ca^{2+} concentration during heat stress was another critical observation made by several researchers (Chen et al. 2020; Xu et al. 2013; Han et al. 2010). This sort of Ca^{2+} accumulation and loss of calcium homeostasis in the cell triggers the mitochondria's free radical production. Recently, Chen et al. (2020) investigated whether the mitochondrial fragmentation in MECs is linked with loss of calcium homeostasis. The large increase in intracellular Ca^{2+} concentration in response to heat stress confirmed their hypothesis. Previously, Xu et al. (2013) and Han et al. (2010) also showed disruption in calcium homeostasis, potentially disrupting the cellular process, including mitochondrial fission and fusion under heat stress (Hom et al. 2010).

Additionally, cytochrome c expression (Cyto-c) level has also shown to be increased substantially in heat-stressed MECs (Chen et al. 2020). During heat stress, mitochondria participate actively in inducing oxidative stress and apoptosis by promoting the release of Cyto-c from mitochondria to the cytoplasm. Cyto-c, a component of the electron transport chain, is known to initiate apoptosis. The Cyto-c release and mitochondria fragmentation, the two key factors for apoptosis induction, is directly linked with each other (Estaquier and Arnoult 2007). Interestingly, Chen et al. (2020) reported an increase in Cyto-c level only in the cytoplasm and not in the mitochondria, which suggested that during heat stress, the Cyto-c released from mitochondria induces cellular apoptosis.

The disruption in the mitochondrial network during heat stress has also been reported through changes in the expression pattern of mitofusin1/2 (Mfn1/2), optic atrophy protein-1 (*Opa1*), dynamin-related protein 1 (*Drp1*), and fission 1 (*Fis1*) genes. Post heat stress, the high expression of *Drp1* and *Fis1* genes in MECs associated with mitochondria fission induces mitochondrial fragmentation (Chen et al. 2020). The up-regulation of the *Drp1* gene has also been linked to mitochondrial fragmentation and cellular apoptosis by several other workers (Frank et al. 2001; Liot et al. 2009). In contrast, down-regulation of Mfn1 and Mfn2 genes in heat-stressed MECs that provide protection and promote the fusion of outer mitochondrial membranes suggests a defect in Mfn1 *Mfn2* function. This phenomenon can further boost the fragmentation and apoptosis in mitochondria post heat stress. The down-regulation of the *Opa1* gene is another important indication of a defect in the mitochondrial fusion process. The changes in expression of these genes could

reflect the underlying mechanism of heat stress-induced disruption of mitochondrial networks and fragmentation in MECs. Based on the available data from various studies, heat stress-induced dysfunction of mitochondria is associated with the disruption of different physiological homeostasis. They are the imbalance of mitochondrial fission/fusion process, increased intracellular Ca^{2+} concentration, reduction in mitochondria membrane potential, increased ROS information, changes in mitochondrial dynamic networks, and induction of apoptosis. These studies suggested that the response of MECs to heat stress plays a vital role in regulating mammary gland function. The negative effect of heat stress on mammary gland cells' secretary activity is the primary factor for the decline in milk synthesis. Some of the critical studies that have utilized MECs as a cellular model to understand mammary gland functioning during heat stress are summarized in Table 7.1.

Table 7.1 Bovine mammary epithelial cell as in vitro models for studying heat stress

S. No.	Title of the study	Livestock species	References
1	Use of gene expression microarrays for evaluating environmental stress tolerance at the cellular level in cattle	Cow	Collier et al. (2006a, b)
2	Establishment of a bovine epithelial mammary cell line and its ultrastructural changes when exposed to heat stress	Cow	Du et al. (2008)
3	Hyperthermia causes bovine mammary epithelial cell death by a mitochondrial-induced pathway	Cow	Du et al. (2008)
4	In vitro culture and characterization of a mammary epithelial cell line from Chinese Holstein dairy cow	Cow	Hu et al. (2016)
5	Cytoprotection of vitamin E on hyperthermia-induced damage in bovine mammary epithelial cells	Cow	Liu et al. (2007)
6	The global effect of heat on gene expression in cultured bovine mammary epithelial cells	Cow	Li et al. (2015)
7	Effect of heat stress on markers of autophagy in the mammary gland during the dry period	Cow	Wohlgemuth et al. (2016)
8	Impact of heat stress on cellular and transcriptional adaptation of mammary epithelial cells in Riverine Buffalo (*Bubalus Bubalis*)	Buffalo	Kapila et al. (2016)
9	The effect of heat stress on gene expression and synthesis of heat-shock and milk proteins in bovine mammary epithelial cells	Cow	Han et al. (2010)
10	The influences of heat stress on bovine mammary gland function	Cow	Tao et al. (2018)
11	Gene expression of the heat stress response in bovine peripheral white blood cells and milk somatic cells in vivo	Cow	Garner et al. (2020)
12	Heat stress induces apoptosis through disruption of dynamic mitochondrial networks in dairy cow mammary epithelial cells	Cow	Chen et al. (2020)
13	Milk yield, milk composition, and milk metabolomics of dairy goats intramammary-challenged with lipopolysaccharide under heat stress conditions	Goat	Salama et al. (2020)

7.6 Conclusion

Mammary epithelial cells (MECs), the secretory cells of the mammary gland of dairy animals, are directly affected by heat stress. The MECs harvested from the mammary gland of dairy animals have been successfully utilized in numerous studies to understand the cellular response to heat stress. Since MECs are the predominant cell types of the mammary gland, its analysis during pre- and post-heat stress can provide insight into the mechanism giving protection. The enhanced synthesis of heat shock proteins like HSP70 post heat stress is critical for developing tolerance and heat stress protection. Recent studies have confirmed that heat stress disrupts mitochondrial morphology and functioning in MECs. Responsiveness of MECs to heat stress in various studies has suggested its suitability is an attractive in vitro cellular model to delineate the genes, pathways, metabolic and cellular response associated with the heat stress response. In the future, an extension of similar kinds of approaches will also delineate the impact of other stressors on the mammary gland of dairy animals.

References

Adin G, Gelman A, Solomon R, Flamenbaum I, Nikbachat M, Yosef E, Zenou A, Shamay A, Feuermann Y, Mabjeesh SJ, Miron J (2009) Effects of cooling dry cows under heat load conditions on mammary gland enzymatic activity, intake of food and water, and performance during the dry period and after parturition. Livest Sci 124:189–195. https://doi.org/10.1016/j.livsci.2009.01.014

Anand V, Dogra N, Singh S, Kumar SN, Jena MK, Malakar D, Dang AK, Mishra BP, Mukhopadhyay TK, Kaushik JK, Mohanty AK (2012) Establishment and characterization of a buffalo (bubalus bubalis) mammary epithelial cell line. PLoS One 4:e7636. https://doi.org/10.1371/journal.pone.0040469

Andreyev AY, Kushnareva YE, Starkov AA (2005) Mitochondrial metabolism of reactive oxygen species. Biokhimiya 70:246–264

Armstrong DV (1994) Heat Stress Interaction with Shade and Cooling. J Dairy Sci 77:2044–2050. https://doi.org/10.3168/jds.S0022-0302(94)77149-6

Bajagai YS (2011) Global climate change and its impacts on dairy cattle. NVJ 30:2–16

Baumgard LH, Rhoads RP (2012) Ruminant nutrition symposium: Ruminant production and metabolic responses to heat stress. J Anim Sci 90:1855–1865. https://doi.org/10.2527/jas.2011-4675

Baumgard LH, Rhoads RP (2013) Effects of heat stress on postabsorptive metabolism and energetics. Annu Rev Anim Biosci 1:311–337. https://doi.org/10.1146/annurev-animal-031412-103644

Bernabucci U, Lacetera N, Baumgard LH, Rhoads RP, Ronchi B, Nardone A (2010) Metabolic and hormonal acclimation to heat stress in domesticated ruminants. Animal 4:1167–1183. https://doi.org/10.1017/S175173111000090X

Bionaz M, Loor JJ (2007) Gene networks regulating lipid synthesis in bovine mammary gland during the lactation cycle. FASEB J 21(6):A1106

Bitman J, Lefcourt A, Wood DL, Stroud B (1984) Circadian and ultradian temperature rhythms of lactating dairy cows. J Dairy Sci 67:1014–1023. https://doi.org/10.3168/jds.S0022-0302(84)81400-9

Brouček J, Šoch M (2009) Effect of high temperature on milk production of cows from free-stall housing with natural ventilation. Slovak J Anim Sci 42:167–173

Chase LE (2013) Climate change impacts on dairy cattle, pp 17–23

Chen KL, Wang HL, Jiang LZ, Qian Y, Yang CX, Chang WW, Zhong JF, Xing GD (2020) Heat stress induces apoptosis through disruption of dynamic mitochondrial networks in dairy cow mammary epithelial cells. In Vitro Cell Dev Biol Anim 56:322–331. https://doi.org/10.1007/s11626-020-00446-5

Collier RJ, Dahl GE, Vanbaale MJ (2006a) Major advances associated with environmental effects on dairy cattle. J Dairy Sci 89:1244–1253. https://doi.org/10.3168/jds.S0022-0302(06)72193-2

Collier RJ, Stiening CM, Pollard BC, VanBaale MJ, Baumgard LH, Gentry PC, Coussens PM (2006b) Use of gene expression microarrays for evaluating environmental stress tolerance at the cellular level in cattle. J Anim Sci 84(Suppl):1–13. https://doi.org/10.2527/2006.8413_supplE1x

Danielson KG, Oborn CJ, Durban EM, Butel JS, Medina D (1984) Epithelial mouse mammary cell line exhibiting normal morphogenesis in vivo and functional differentiation in vitro. Proc Natl Acad Sci U S A 81:3756–3760. https://doi.org/10.1073/pnas.81.12.3756

Das R, Sailo L, Verma N, Bharti P, Saikia J, Kumar R (2016) Impact of heat stress on health and performance of dairy animals: a review. Vet World 9:260–268. https://doi.org/10.14202/vetworld.2016.260-268

Dikmen S, Khan FA, Huson HJ, Sonstegard TS, Moss JI, Dahl GE, Hansen PJ (2014) The SLICK hair locus derived from Senepol cattle confers thermotolerance to intensively managed lactating Holstein cows. J Dairy Sci 97:5508–5520. https://doi.org/10.3168/jds.2014-8087

Dikmen S, Wang XZ, Ortega MS, Cole JB, Null DJ, Hansen PJ (2015) Single nucleotide polymorphisms associated with thermoregulation in lactating dairy cows exposed to heat stress. J Anim Breed Genet 132:409–419. https://doi.org/10.1111/jbg.12176

Dodson M, Darley-Usmar V, Zhang J (2014) Cellular metabolic and autophagic pathways, pp 207–221. https://doi.org/10.1016/j.freeradbiomed.2013.05.014.Cellular

Du Preez JH, Giesecke WH, Hattingh PJ (1990) Heat stress in dairy cattle and other livestock under southern African conditions. I. Temperature-humidity index mean values during the four main seasons. Onderstepoort J Vet Res 57:77–87

Du J, Di HS, Guo L, Li ZH, Wang GL (2008) Hyperthermia causes bovine mammary epithelial cell death by a mitochondrial-induced pathway. J Therm Biol 33:37–47. https://doi.org/10.1016/j.jtherbio.2007.06.002

Düchler M, Schmoll F, Pfneisl F, Brem G, Schellander K (1998) OMEC II: A new ovine mammary epithelial cell line. Biol Cell 90:199–205. https://doi.org/10.1016/S0248-4900(98)80340-7

Estaquier J, Arnoult D (2007) Inhibiting Drp1-mediated mitochondrial fission selectively prevents the release of cytochrome c during apoptosis. Cell Death Differ 14:1086–1094. https://doi.org/10.1038/sj.cdd.4402107

FAO (2015) Livestock and the environment. http://www.fao.org/livestock-environment/en/

Frank S, Gaume B, Bergmann-Leitner ES, Leitner WW, Robert EG, Catez F, Smith CL, Youle RJ (2001) The role of dynamin-related protein 1, a mediator of mitochondrial fission, in apoptosis. Dev Cell 1:515–525. https://doi.org/10.1016/S1534-5807(01)00055-7

Gaffney EV, Polanowski FP, Blackburn SE, Lambiase JP (1976) Origin, concentration and structural features of human mammary gland cells cultured from breast secretions. Cell Tissue Res 172(2):269–279

Garner JB, Chamberlain AJ, Vander Jagt C, Nguyen TTT, Mason BA, Marett LC, Leury BJ, Wales WJ, Hayes BJ (2020) Gene expression of the heat stress response in bovine peripheral white blood cells and milk somatic cells in vivo. Sci Rep 10:1–12. https://doi.org/10.1038/s41598-020-75438-2

Gaughan JB, Mader TL, Holt SM, Sullivan ML, Hahn GL (2010) Assessing the heat tolerance of 17 beef cattle genotypes. Int J Biometeorol 54:617–627. https://doi.org/10.1007/s00484-009-0233-4

German T, Barash I (2002) Characterization of an epithelial cell line from bovine mammary gland. In Vitro Cell Dev Biol Anim 38:282–292. https://doi.org/10.1290/1071-2690(2002)038<0282:COAECL>2.0.CO;2

Han AY, Zhang MH, Zuo XL, Zheng SS, Zhao CF, Feng JH, Cheng C (2010) Effect of acute heat stress on calcium concentration, proliferation, cell cycle, and interleukin-2 production in splenic lymphocytes from broiler chickens. Poult Sci 89:2063–2070. https://doi.org/10.3382/ps.2010-00715

Hansen PJ (2013) Antecedents of mammalian fertility: lessons from the heat-stressed cow regarding the importance of oocyte competence for fertilization and embryonic development. Anim Front 3(4):34–39

Herrero M, Thornton PK (2010) Mixed crop livestock systems in the developing world: present and future. Adv Anim Biosci. https://doi.org/10.1017/s2040470010001019

Hom J, Yu T, Yoon Y, Porter G, Sheu SS (2010) Regulation of mitochondrial fission by intracellular Ca2+ in rat ventricular myocytes. Biochim Biophys Acta Bioenerg 1797:913–921. https://doi.org/10.1016/j.bbabio.2010.03.018

Hu H, Wang J, Gao H, Li S, Zhang Y, Zheng N (2016) Heat-induced apoptosis and gene expression in bovine mammary epithelial cells. Anim Prod Sci 56:918–926. https://doi.org/10.1071/AN14420

Igono MO, Johnson HD, Steevens BJ, Hainen WA, Shanklin MD (1988) Effect of season on milk temperature, milk growth hormone, prolactin, and somatic cell counts of lactating cattle. Int J Biometeorol 32:194–120. https://doi.org/10.1007/BF01045279

Ilan N, Barash I, Gootwine E, Sham M (1998) Establishment and initial characterization of the ovine jiamimary epithelial cell line nish. In Vitro Cell Dev Biol Anim 34:326–332. https://doi.org/10.1007/s11626-998-0009-x

Jedrzejczak M, Szatkowska I (2014) Bovine mammary epithelial cell cultures for the study of mammary gland functions. In Vitro Cell Dev Biol Anim 50:389–398. https://doi.org/10.1007/s11626-013-9711-4

Kapila N, Kishore A, Sodhi M, Sharma A, Kumar P, Mohanty AK, Jerath T, Mukesh M (2013) Identification of appropriate reference genes for qRT-PCR analysis of heat-stressed mammary epithelial cells in riverine buffaloes (*Bubalus bubalis*). ISRN Biotechnol 2013:1–9. https://doi.org/10.5402/2013/735053

Kapila N, Sharma A, Kishore A, Sodhi M, Tripathi PK, Mohanty AK, Mukesh M (2016) Impact of heat stress on cellular and transcriptional adaptation of mammary epithelial cells in riverine buffalo (*Bubalus bubalis*). PLoS One 11:1–28. https://doi.org/10.1371/journal.pone.0157237

Kaushik R, Singh KP, Kumari A, Singh MK, Manik RS, Palta P, Singla SK, Chauhan MS (2013) Isolation, characterization, and EGFP expression in the buffalo (Bubalus bubalis) mammary gland epithelial cell line. In Vitro Cell Dev Biol Anim 49:1–7. https://doi.org/10.1007/s11626-012-9557-1

Ke MW, Hsu JT, Jiang YN, Cheng WTK, Ju YT (2012) Characterization of hTERT-immortalized caprine mammary epithelial cells. Reprod Domest Anim 47:553–561. https://doi.org/10.1111/j.1439-0531.2011.01916.x

Key N, Sneeringer S (2014) Potential effects of climate change on the productivity of U.S. dairies. Am J Agric Econ 96:1136–1156. https://doi.org/10.1093/ajae/aau002

Kittrell FS, Oborn CJ, Medina D (1992) Development of mammary preneoplasias in vivo from mouse mammary epithelial cell lines in vitro. Cancer Res 52:1924–1932

Kumura H, Tanaka A, Abo Y, Yui S, Shimazaki KI, Kobayashi E, Sayama K (2001) Primary culture of porcine mammary epithelial cells as a model system for evaluation of milk protein expression. Biosci Biotechnol Biochem 65:2098–2101. https://doi.org/10.1271/bbb.65.2098

Lacetera N, Bernabucci U, Scalla D, Basirico L, Morera P, Nardone A (2006) Heat stress elicits different responses in peripheral blood mononuclear cells from Brown Swiss and Holstein cows. J Dairy Sci 89:4606–4612. https://doi.org/10.3168/jds.S0022-0302(06)72510-3

Lauzon K, Zhao X, Bouetard A, Delbecchi L, Paquette B, Lacasse P (2005) Antioxidants to prevent bovine neutrophil-induced mammary epithelial cell damage. J Dairy Sci 88:4295–4303. https://doi.org/10.3168/jds.S0022-0302(05)73115-5

Li L, Sun Y, Wu J, Li X, Luo M, Wang G (2015) The global effect of heat on gene expression in cultured bovine mammary epithelial cells. Cell Stress Chaperones 20:381–389. https://doi.org/10.1007/s12192-014-0559-7

Liot G, Bossy B, Lubitz S, Kushnareva Y, Sejbuk N, Bossy-Wetzel E (2009) Complex II inhibition by 3-NP causes mitochondrial fragmentation and neuronal cell death via an NMDA-and ROS-dependent pathway. Cell Death Differ 16(6):899–909

Liu HY, Yang JY, Wu HH, Wu YM, Liu JX (2007) Effects of methionine and its ratio to lysine on expression of α. J Anim Feed Sci 16(2):330–334

Marai IFM, Haeeb AAM (2010) Buffalo's biological functions as affected by heat stress - a review. Livest Sci 127:89–109. https://doi.org/10.1016/j.livsci.2009.08.001

Nardone A, Ronchi B, Lacetera N, Ranieri MS, Bernabucci U (2010) Effects of climate changes on animal production and sustainability of livestock systems. Livest Sci 130:57–69. https://doi.org/10.1016/j.livsci.2010.02.011

Neville MC, McFadden TB, Forsyth I (2002) Hormonal regulation of mammary differentiation and milk secretion. J Mammary Gland Biol Neoplasia 7(1):49–66

Ogorevc J, Prpar S, Dovč P (2009) In vitro mammary gland model: establishment and characterization of a caprine mammary epithelial cell line. Acta Agric Slov 2:133–138

Ominski KH, Kennedy AD, Wittenberg KM, Moshtaghi Nia SA (2002) Physiological and production responses to feeding schedule in lactating dairy cows exposed to short-term, moderate heat stress. J Dairy Sci 85:730–737. https://doi.org/10.3168/jds.S0022-0302(02)74130-1

Qian L, Song X, Ren H, Gong J, Cheng S (2004) Mitochondrial mechanism of heat stress-induced injury in rat cardiomyocyte. Cell Stress Chaperones 9:281–293. https://doi.org/10.1379/CSC-20R.1

Rabot A, Wellnitz O, Meyer HH, Bruckmaier RM (2007) Use and relevance of a bovine mammary gland explant model to study infection responses in bovine mammary tissue. J Dairy Res 74 (1):93–99. https://doi.org/10.1017/S0022029906002147

Ravagnolo O, Misztal I, Hoogenboom G (2000) Genetic component of heat stress in dairy cattle, development of heat index function. J Dairy Sci 83:2120–2125. https://doi.org/10.3168/jds.S0022-0302(00)75094-6

Rhoads ML, Rhoads RP, VanBaale MJ, Collier RJ, Sanders SR, Weber WJ, Crooker BA, Baumgard LH (2009) Effects of heat stress and plane of nutrition on lactating Holstein cows: I. Production, metabolism, and aspects of circulating somatotropin. J Dairy Sci 92:1986–1997. https://doi.org/10.3168/jds.2008-1641

Rhoads RP, Baumgard LH, Suagee JK, Sanders SR (2013) Nutritional interventions to alleviate the negative consequences of heat stress. Adv Nutr 4:267–276. https://doi.org/10.3945/an.112.003376

Roenfeldt S (1998) You can't afford to ignore heat stress. Dairy Herd Manage 35:6–12

Rose MT, Aso H, Yonekura S, Komatsu T, Hagino A, Ozutsumi K, Obara Y (2002) In vitro differentiation of a cloned bovine mammary epithelial cell. J Dairy Res 69:345–355. https://doi.org/10.1017/S0022029902005551

Salak-Johnson JL, McGlone JJ (2007) Making sense of apparently conflicting data: stress and immunity in swine and cattle. J Anim Sci 85:81–88. https://doi.org/10.2527/jas.2006-538

Salama AAK, Contreras-Jodar A, Love S, Mehaba N, Such X, Caja G (2020) Milk yield, milk composition, and milk metabolomics of dairy goats intramammary-challenged with lipopolysaccharide under heat stress conditions. Sci Rep 10:1–12. https://doi.org/10.1038/s41598-020-61900-8

Shandilya UK, Sharma A, Sodhi M, Mukesh M (2020) Heat stress modulates differential response in skin fibroblast cells of native cattle (Bos indicus) and riverine buffaloes (Bubalus bubalis). Biosci Rep 40:1–14. https://doi.org/10.1042/BSR20191544

Sharma P, Sharma A, Sodhi M, Verma P, Parvesh K, Swami SK, Jast A, Shandilya UK, Mukesh M (2019) Characterizing binding sites of heat responsive microRNAs and their expression pattern in heat stressed PBMCs of native cattle, exotic cattle and riverine buffaloes. Mol Biol Rep 46(6):6513–6524. https://doi.org/10.1007/s11033-019-05097-8

Shyy TT, Asch BB, Asch HL (1989) Concurrent collapse of keratin filaments, aggregation of organelles, and inhibition of protein synthesis during the heat shock response in mammary epithelial cells. J Cell Biol 108:997–1008. https://doi.org/10.1083/jcb.108.3.997

Silanikove N, Shapiro F, Shinder D (2009) Acute heat stress brings down milk secretion in dairy cows by up-regulating the activity of the milk-borne negative feedback regulatory system. BMC Physiol 9:1–9. https://doi.org/10.1186/1472-6793-9-13

Smith DL, Smith T, Rude BJ, Ward SH (2013) Short communication: comparison of the effects of heat stress on milk and component yields and somatic cell score in Holstein and Jersey cows. J Dairy Sci 96:3028–3033. https://doi.org/10.3168/jds.2012-5737

Sorensen MT, Nørgaard JV, Theil PK, Vestergaard M, Sejrsen K (2006) Cell turnover and activity in mammary tissue during lactation and the dry period in dairy cows. J Dairy Sci 89:4632–4639. https://doi.org/10.3168/jds.S0022-0302(06)72513-9

Sreenivasaiah K (2016) Climate change and its impact on milk production in India. In: Climate change challenge (3C) and social-economic-ecological interface-building. Springer, Cham, pp 531–547

St-Pierre NR, Cobanov B, Schnitkey G (2003) Economic losses from heat stress by US livestock industries. J Dairy Sci 86:E52–E77. https://doi.org/10.3168/jds.S0022-0302(03)74040-5

Tao S, Bubolz JW, do Amaral BC, Thompson IM, Hayen MJ, Johnson SE, Dahl GE (2011) Effect of heat stress during the dry period on mammary gland development. J Dairy Sci 94:5976–5986. https://doi.org/10.3168/jds.2011-4329

Tao S, Orellana RM, Weng X, Marins TN, Dahl GE, Bernard JK (2018) Symposium review: the influences of heat stress on bovine mammary gland function. J Dairy Sci 101:5642–5654. https://doi.org/10.3168/jds.2017-13727

Tong HL, Li QZ, Gao XJ, Yin EY (2012) Establishment and characterization of a lactating dairy goat mammary gland epithelial cell line. In Vitro Cell Dev Biol Anim 48:149–155. https://doi.org/10.1007/s11626-012-9481-4

Upadhyay RC, Ashutosh RV, Singh SV, Aggarwal P (2009) Impact of Climate Change on reproductive functions of cattle and buffaloes. Global climate change and Indian agriculture. ICAR, New Delhi, pp 107–110

West JW (2003) Effects of heat-stress on production in dairy cattle. J Dairy Sci 86:2131–2144. https://doi.org/10.3168/jds.S0022-0302(03)73803-X

Westermann B (2010) Mitochondrial fusion and fission in cell life and death. Nat Rev Mol Cell Biol 11:872–884. https://doi.org/10.1038/nrm3013

Wheelock JB, Rhoads RP, VanBaale MJ, Sanders SR, Baumgard LH (2010) Effects of heat stress on energetic metabolism in lactating Holstein cows. J Dairy Sci 93:644–655. https://doi.org/10.3168/jds.2009-2295

Wilde CJ, Addey CVP, Li P, Fernig DG (1997) Programmed cell death in bovine mammary tissue during lactation and involution. Exp Physiol 82:943–953. https://doi.org/10.1113/expphysiol.1997.sp004075

Wohlgemuth SE, Ramirez-Lee Y, Tao S, Monteiro APA, Ahmed BM, Dahl GE (2016) Short communication: effect of heat stress on markers of autophagy in the mammary gland during the dry period. J Dairy Sci 99:4875–4880. https://doi.org/10.3168/jds.2015-10649

Xu S, Pi H, Chen Y, Zhang N, Guo P, Lu Y, He M, Xie J, Zhong M, Zhang Y, Yu Z, Zhou Z (2013) Cadmium induced Drp1-dependent mitochondrial fragmentation by disturbing calcium homeostasis in its hepatotoxicity. Cell Death Dis 4:1–10. https://doi.org/10.1038/cddis.2013.7

Zarzynska J, Motyl T (2008) Apoptosis and autophagy in involuting bovine mammary gland. J Physiol Pharmacol 59:275–288

Zhao K, Liu HY, Zhou MM, Liu JX (2010) Establishment and characterization of a lactating bovine mammary epithelial cell model for the study of milk synthesis. Cell Biol Int 34(7):717–721

Zheng YM, He XY, Zhang Y (2010) Characteristics and EGFP expression of goat mammary gland epithelial cells. Reprod Domest Anim 45:323–331. https://doi.org/10.1111/j.1439-0531.2009.01568.x

Zhu W, Zhang BX, Yao KY, Yoon I, Chung YH, Wang JK, Liu JX (2016) Effects of supplemental levels of Saccharomyces cerevisiae fermentation product on lactation performance in dairy cows under heat stress. Asian-Australasian J Anim Sci 29:801–806. https://doi.org/10.5713/ajas.15.0440

Milk and Milk-Derived Stem Cells

8

Ratan K. Choudhary

Abstract

Milk is a wholesome food not only for infants but also for adults. Except for fiber, milk is composed of cellular and non-cellular components. Recent studies have demonstrated that milk contains a population of cells displaying multipotent stem cells' features. This chapter briefly summarizes milk's composition and outlines the progress made in this newly emerging field of stem cell biology that is pertinent to the investigators. This chapter covers milk-derived stem cells of humans and discusses the perspective of the application of milk-derived stem cells in regenerative medicine. Non-invasive source and unique properties of milk-derived stem cells with low tumorigenic potential are likely to make these stem or stem-like cells regenerative therapy in the future.

8.1 Milk Composition

Milk is a homogenous mixture of water, fat, proteins, minerals, vitamins, antibodies, and cellular components like live cells, dead cells of hematopoietic and mammary origins, exosomes, and stem cells. Exocrine secretions of mammary glands are initially meant for the babies to support complete growth and development, and thus milk is a nutrient-dense food. Milk's composition and flavors vary greatly depending on the species, age, diet, stage of lactation, environment, and seasons (FAO technical bulletin). Cow milk contains 12–14% solid matter composed of 2.5–4% of proteins, 3–5% of fat, and about 5% of lactose—the milk sugar.

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Table 8.1 Nutritional composition of cow milk vs. human milk (FAO of the US n.d., accessed through www.vivahealth.org.uk)

Nutrients (per 100 g of milk)	Cow's milk (semi-skimmed, pasteurized)	Human milk (mature)
Sodium (mg)	43	15
Potassium (mg)	156	58
Calcium (mg)	120	34
Magnesium (mg)	11	3
Phosphorus (mg)	94	15
Iron (mg)	0.02	0.07
Copper (mg)	Trace	0.04
Zinc (mg)	0.4	0.3
Chloride (mg)	87	42
Manganese (mg)	Trace	Trace
Selenium (μg)	1	1
Iodine (μg)	30	7
Retinol (μg)	19	58
Carotene (μg)	9	24 (estimated value)
Vitamin D (μg)	Trace	Trace
Vitamin E (mg)	0.04	0.34
Thiamin (mg)	0.03	0.02
Riboflavin (mg)	0.24	0.03
Niacin (mg)	0.1	0.2
Vitamin B6 (mg)	0.06	0.01
Vitamin B12 (μg)	0.9	Trace
Folate (μg)	9	5
Pantothenate (mg)	0.68	0.25
Biotin (μg)	3.0	0.7
Vitamin C (mg)	2	4

Composition of one cup (240 mL) of cow's milk (USDA food composition database) is

- Calories: 149.
- Water: 88%.
- Protein: 7.7 g.
- Carbs: 11.7 g.
- Sugar: 12.3 g.
- Fiber: 0 g.
- Fat: 8 g.

The nutritional composition of cow milk is compared with human milk is given (Table 8.1).

8.2 Milk Fat

Milk lipids are predominantly composed of triacylglycerol (TAG) and are about 98% of total milk fat (Dils et al. 1977) and contain short (C4–10), intermediate (C12–16), and long-chain (C18) fatty acids (FAs). Synthesis of short-chain FAs occurs within the mammary epithelial cells (MEC) from precursors like acetate, butyrate, and pyruvate. Long-chain fatty acids are derived from the blood, meaning they are directly coming from the diet. The endoplasmic reticulum (ER) synthesized TAGs, which then migrated to the apical plasma membrane of the MEC, where they are encapsulated by the membrane and pinched off from the cytoplasm into the lumen. Secretion of milk fat by the MEC is not a continuous process but develops during pregnancy under the influence of various reproductive hormones. The first stage of differentiation occurs around mid-pregnancy, where branching of mammary ducts followed by alveolar development occurs with the rise in progesterone levels. Mammary alveoli consist of a single MEC layer, which upon functional differentiation secrets milk protein. The second stage of differentiation, initiated at the time of calving, reduces serum progesterone levels. The biosynthetic process of copious secretion of milk starts at the second stage of differentiation. Lipogenesis in MEC occurs by the two independently regulated, and temporarily distinct processes called the "bipartite model of lipogenesis". The first phase is characterized by an increased adipophilin expression and other related genes that impair triglyceride lipolysis. Increased expression of adipophilin and other molecules stabilizes the formation of TAGs. The second phase is characterized by the dramatic increases in the rate of diacylglycerol acyltransferase 1-dependent TAG synthesis (McManaman 2009). The percentage of milk fat varies in different animals. Total fat % in the milk of cow, water buffalo, sheep, and goats are 3.55, 6.2, 6.91, and 3.03%, respectively (Rasheed et al. 2016). In general, lipid composition in cow milk varies between 3 and 4%.

There are no unequivocal opinions, and investigators defer in their opinion on the effect of milk fat on human health. Traditionally, milk fat consumption, which is composed of saturated fat, was shown to raise "bad" cholesterol (low-density lipoprotein or LDL) and cause blockage of an artery leading to coronary heart disease (Artaud-Wild et al. 1993). Based on such studies, dietary guidelines in many countries recommend consuming low-fat dairy foods. On the other hand, contrasting reports have emerged recently stating the benefits rather than dairy fat harm. Fat of milk and dairy do not increase total and "bad" cholesterol levels (Lee and Cho 2017; Chen et al. 2015). Whole milk significantly increases the level of "good" cholesterol (high-density lipoprotein or HDL) in comparison to skimmed milk (Engel et al. 2018; Chen et al. 2015). In addition to this, the fatty acids of milk have anti-inflammatory properties. Multiple studies examined the effects of fatty acids on various cell lines and found the opposite association with inflammation and exhibited pro-inflammatory effects. Long-chain saturated fatty acids such as palmitic and stearic may show pro-inflammatory effects in cell lines and mouse models. However, in humans, no such pro-inflammatory effects of these saturated fatty acids have been reported despite the abundance of these fatty acids in milk and dairy

products (Lordan et al. 2018). Contrarily, saturated fatty acids such as lauric acid (C12:0) may have neutral or anti-inflammatory effects; however, further research is required to conduct humans (Da Silva and Rudkowska 2015).

8.3 Milk Protein

The next important component of milk is protein. Milk protein is a cheap source of high-quality animal origin proteins in the human diet. Therefore, milk's high protein content is nutritionally beneficial, and it increases the milk quality. Biosynthesis of protein occurs during lactation in MEC. Protein synthesis machinery requires amino acids as primers and a large energy supply. Constituents of protein synthesis are brought inside the cells with the help of ion and protein transporters, an active process that requires 30–40% of the basal energy for the ion transport and about 10% of the basal life of the protein re-synthesis (Baldwin et al. 1980). There is an inversely opposite relationship between milk's fat and protein contents. Administration of medium-chain and long-chain FA to the lactating animal negatively affected milk fat content while the positive effect on milk protein content (Bauman et al. 2011). Although protein synthesis is reduced by anoxia and restricted calorie intake, the quantity and quality of milk protein cannot be increased. The amount and composition of milk protein are primarily determined by the animal's genetic makeup, breed, and species (Bionaz and Loor 2011). The importance of a large supply of energy for protein synthesis is understood from the fact that the abomasal infusion of casein protein failed to increase milk protein yield. In contrast, starch did increase (Rius et al. 2010). Other factors that may affect protein synthesis included specific amino acids, insulin (Griinari et al. 1977; Herbein et al. 1985), and the increasing availability of metabolizable energy (Bionaz and Loor 2011).

8.4 Minerals, Antioxidants, and Vitamins in Milk

Calcium, selenium, iron, iodine, magnesium, and zinc are essential minerals present in cow milk. The concentration of calcium in cow milk is about 1 g/L and provides most of the daily calcium requirement to the population. For example, cow milk provides more than 50% of the daily calcium requirement to most Americans (Insel et al. 2003). A report that analyzed the global calcium intake of people in India suggested low consumption of daily calcium intake than the recommended dose of calcium. Though there are no current guidelines in India regarding calcium supplementation in adults, 0.6 g of daily calcium is hypothesized as recommended dietary allowance (RDA) of adult men (Raj et al. 2018). A meta-analysis of calcium intake data of 74 countries revealed astonishing results. Except for European countries whose daily calcium intake was more significant than 1 g, all other countries, including India, had daily calcium intake <0.5 g (Balk et al. 2017). A low level of calcium in the diet can increase bone-related diseases like fractures and osteoporosis. Revisiting RDA guidelines for calcium and public health measures were suggested

as a required step to tackle the morbidity of nutritional deficiency diseases in India (Harinarayan and Akhila 2019).

Selenium is an essential micronutrient of human diets required for proper immune functions, antioxidant system, DNA repair, and DNA synthesis. The concentration of selenium is usually low and varies from 11 to 37 μg/L of cow milk (Haug et al. 2007). Role of selenium in some human diseases like cancer and asthma has been suggested. However, selenium's role in metabolism is undeniable because selenium is a component of the enzyme involved in the thyroid hormone's metabolism.

The antioxidants have a role in the prevention of oxidation of the milk. They also provide protective effects to the milk-producing mammary epithelial cell during the lactation process. The most critical antioxidants in milk are selenium and vitamins E and A. Milk is a good source of a few vitamins, mainly vitamin A, vitamin B2 (riboflavin), and B12. Milk contains 1.83 mg riboflavin/L milk (Haug et al. 2007). The daily recommended dose of riboflavin is 1.1 and 1.3 mg for women and men, respectively (Insel et al. 2003). It plays a vital role in anti-oxidation and DNA repair. The bioavailability of B2 and B12 vitamins in cow milk is good. Hence, increasing proportions of cow milk in the diet can be an excellent alternative source to meet B vitamins' dietary guidelines.

8.5 Immunoglobulins in Milk

Immunoglobulins (Igs) or antibodies are the glycoproteins produced by plasm cells and play a critical role in recognizing and biding specific pathogens toward their destruction. Igs are present abundantly in colostrum and low amount in the milk of all lactating species. About 70–80% of colostrum's total protein content consists of Igs, whereas in milk, they account for roughly 1–2% of the complete protein. There are five known classes of Igs: IgA, IgD, IgE, IgG, and IgM. The major Ig classes in both bovine and human milk are IgA, IgG, and IgM. IgG1 is the predominant Ig class in bovine milk compared to IgA in human milk (reviewed in Kohronen and Marnila 2009). Maternal colostrum and milk provide Igs and bioactive peptides to newborn infants and provide passive protection against enteric pathogens. Neonates of ruminant are born with virtually no Igs and therefore very susceptible to enteric infections and other associated pathogens. Thus, the colostrum feeding within half an hour of birth is essential to transfer colostral Igs to neonates for survival. Various studies showed a complex supplementation mechanism of direct supply of mothers' immune cells to the nursing babies that helps develop and functional regulation of infant's immunity-innate and adaptive immune systems. The effects of bovine Igs on human health have also been studied. The stability and functional results of orally ingested bovine Igs in milk products showed functionally active bovine Igs in the human gut. A large volume of research has provided sufficient evidence of bovine Igs in preventing rotavirus gastrointestinal tract infection (den Hartog et al. 2014; Rump et al. 1992), upper respiratory tract infection (Loss et al. 2011), lipopolysaccharide-induced inflammation, and even allergy (Collins et al. 1991).

8.6 Milk Exosomes

These exosomes are present in various biological fluids like plasma, urine, saliva, and other body fluids, including milk. Milk exosomes and milk microvesicles (MVs) have recently been the focus of an investigation. These are released from mammary epithelial cells of all mammals, including dairy cows (Zempleni et al. 2017; Melnik and Schmitz 2019). Inward budding of cell membranes forms exosomes resulting in membrane-surrounded multivesicular bodies secreted by fusion with the cell membrane like milk fat globules. Milk has been called not just a nutritious food but also a genetic transfection system that transfer miRNA to neonates and babies for postnatal growth through the mechanistic target of rapamycin complex 1 (mTORC1) signaling called "functional hypothesis of milk signaling" initially proposed by the Melnik group in 2013 (Melnik et al. 2013). Exosomes communicate intracellularly via messengers like RNA, miRNA, DNA, and proteins. Milk exosomes are viewed as a culprit in the pathogenesis of many human diseases. Authors claim that dairy milk-derived exosomes are hardy and resistant to heating and cooling. The cargo of exosomes contributes to the pathogenesis of common diseases like allergy, fetal macrosomia, obesity, type 2 diabetes, hyperphagia, and others. Hence milk exosomes can be viewed as "critical pathogens" (Melnik and Schmitz 2019). The cargo of Milk exosomes is available to the milk consumers as bovine exosomes are resistant to pasteurization, cold, and any other methods of treatment (Fig. 8.1). As an estimate, bovine milk exosomes contain nearly 400 different miRNA (Hata et al. 2010). These miRNAs and some selected proteins remain bioavailable to humans when they consume milk.

Expression of miR-148a enhances mammary epithelial cell expression upon a genetic selection of cows for increased milk production. Thereby, the potential increase in milk secretion also increases milk exosome miR-148a content. miR-148 is linked with cancer in human beings. Upregulated expression of miR-148a occurs in glioma and osteosarcoma (Li et al. 2016). Milk exosomes also contain miR-155 and transforming growth factor-β (TGF-β) and have been associated with cancer-related inflammation (Guo et al. 2016). Since pasteurization and other treatments have no significant effect on milk exosome integrity and their cargo bioavailability, exosomes provided persistent entry of these cargoes into the human food chain (Melnik and Schmitz 2019). Milk is the only biological fluid used commercially to obtain natural exosomes (Sedykh et al. 2020). These exosomes are harvested and being utilized in pharmaceutical industries.

Aside from milk being nutritionally dense food, it provides other biologically active compounds, namely antimicrobial peptides, vitamins, minerals, fats, high-quality proteins, probiotic bacteria, antioxidants, and others) which have crucial impacts on human health (Górska-Warsewicz et al. 2019).

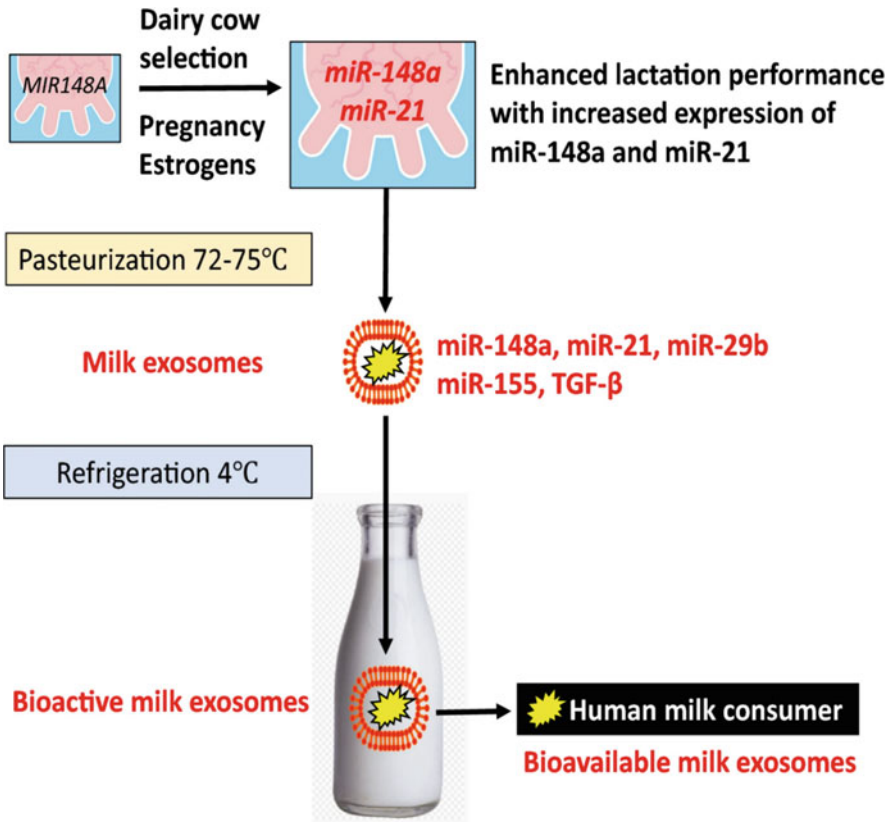

Fig. 8.1 Genesis of miR-148, miR-21, and another miRNA in bovine milk exosomes and their constant availability in the human food chain. (Adapted from Melnik and Schmitz 2019)

8.7 Milk Probiotics

It is now realized that milk is a wholesome food and an excellent source of other biologically active components, including probiotic bacteria, immune cells, and stem cells (Fig. 8.2).

The presence of probiotic bacteria has been realized before, but its functional role in milk is very recent and has not been underdeveloped. Experimental evidence showed that lactating mother's gut bacteria could enter the mammary gland via an entero-mammary pathway. *Lactobacillus salivarius* (strain CECT 5713) isolated from human milk was carried by the dendritic cells and β_2 integrin ($CD18^+$) cells from the gut lumen to the mammary gland (Langa et al. 2012). It is worth to mention here that CD18 is expressed on leukocyte pairs with several α integrin (CD11) subunits, and heterodimer of these can bind a variety of ligands, including fibrinogen and polysaccharides (Harris et al. 2000). Investigators started believing that mothers'

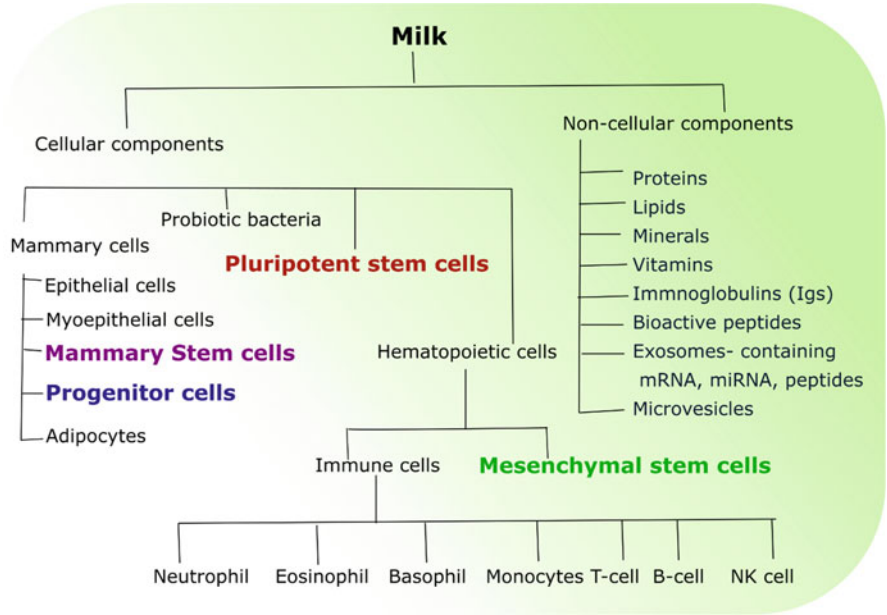

Fig. 8.2 Cellular and non-cellular components of milk

gut bacteria (such as *L. salivarius* and others) can be considered probiotic supplement food for infants. They concluded that infants consume about 800 mL of milk on a single day, which could comprise 10–100 million probiotic bacteria per day (Witkowska-Zimny and Kaminska-El-Hassan 2017; Ninkina et al. 2019). It is a well-known fact that good health of infants is provided by the availability of initial milk of mother within half an hour after birth, especially mother's milk. Though the initial milk (colostrum) contains abundant immunoglobulins and another active biological compound, the initial colonization of probiotic bacteria from mothers' milk can be considered an essential factor in developing healthy physiology. These commensal bacteria, including gut-associated anaerobic bacteria *Bifidobacterium breve*, *B. adolescentis,* and others, can be beneficial to the young ones (Martin et al. 2009; Cong et al. 2016). Likewise, cow milk might contain other commensal bacteria suitable for calf and not for human babies may contribute to cow milk allergies in some infants. Infants fed with hydrolyzed casein and probiotics showed no to fewer server allergies with the cow milk. Such cow milk treatments could have altered infants' gut microbiome, which has helped them become resistant to cow milk allergy (Berni et al. 2016).

8.8 Milk-Derived Stem Cells

Various types of cells found in the milk can be broadly divided into two major groups: blood-origin and mammary tissue origin cells, and in both these pools live progenitor, and stem cells have also been identified in human (Cregan et al. 2007; Hassiotou et al. 2012) and cow milk (Pipino et al. 2018). The presence of undifferentiated cells (like stem cells or stem-like cells) and partially differentiated cells (like progenitor cells) in milk remain enigmatic. Excluding blood-derived cells, the largest population of epithelial cells in human milk was $CK18^{+}$ and beta-casein positive luminal/alveolar epithelial cells. In estimation, the total percentage of mature luminal epithelial and myoepithelial cells together constitute almost 98% (Hassiotou et al. 2013), indicating the remaining 2% of the cells are immature stem and progenitor cells. Identify these immature cells, and their origin remains elusive. Mammary stem cells are bipotent (van Amerongen 2014) or multipotent stem cells (Smith and Medina 2018). These cells can give rise to the mammary epithelium and myoepithelium cells or could produce various cell types, respectively. One of the most critical questions that remains a conundrum is mammary stem cells' nature—are these cells unipotent or multipotent? (Joshi and Khokha 2012; Kaimala et al. 2012). Lineage tracing experiments addressed the elasticity of mammary epithelial cells. Results have indicated that multipotent mammary stem cells present in embryonic placode after birth (postnatal) become unipotent and responsible for stage-specific development and mammary tissue homeostasis (Seldin et al. 2017).

In the cow, stem cells were identified in milk. These cells showed a heterogenous group of cell populations ranging from epithelial-like (E-type) to spindle-shaped (F-type) colonies. Immunophenotype characterization of bovine milk-derived stem cells (bmdSC) were positive with epithelial markers E-cadherin, cytokeratin-14 (KRT14), KRT18, and smooth muscle actin (SMA). E-cadherin marks the epithelial cells, KRT14 and SMA are the markers of basal mammary epithelial cells, while KRT18 is the marker of luminal milk secreting epithelial cells. This clearly shows that milk-derived stem cells are partially differentiated and probably committed to epithelial cell lineage (progenitor cells).

Interestingly, a subset (30–40%) of these heterogeneous cells showed typical mesenchymal stem cell (MSC) surface markers, namely, CD90, CD73, and CD105. Some MSCs showed co-expression of pluripotent stem cell markers (SOX2 and OCT4) in the nuclei, indicating the multilineage potential of these subsets of MSCs. Multilineage potential of MSCs shows nuclear expression of pluripotency transcription factor demonstrated by cells' differentiation ability into osteoblasts, chondroblasts, and adipocytes (Pipino et al. 2018). Inconsistent with stem cells' findings in human milk and bovine milk also contained multipotent stem cells. It can easily be the source of multipotent stem cells used for regenerative medicine in veterinary and animal science to improve animal health and milk production.

Breast milk-derived stem cells were multipotent. Gene expression analysis of these multipotent stem cells revealed up-regulation of transcripts of pluripotency transcription factors OCT4, SOX2, NANOG, and KLF4. These cells form all the three germ layers—mesoderm, endoderm, and ectoderm, upon receiving

differentiation signals. Differentiation of cells into cells of mesodermal (osteoblast-like cells, chondrocytes, adipocytes, cardiomyocytes), endodermal (pancreatic beta-like cells, hepatocyte-like cells), and endodermal origin (neuron-like cells) was reported (Hassiotou et al. 2013; Twigger et al. 2013).

Phenotypic and genotypic expression of milk-derived stem cells are not the same across the different lactation stages. A comparison of milk genetic signature from the milk of preterm mother and full-term mother revealed intriguing differences. The proportion of stem-like cells in these two types of milk samples were different, along with the difference in the gene signature (Briere et al. 2017). The author speculated that these differences could arise due to a symbiotic relationship between feeding infants and nursing mothers. Though similar studies revealed different proportions of stem-like cells in milk, highest proportions of stem cells were found in concurrent lactating and pregnant mothers (Hassiotou et al. 2012). Experimental evidence of cross-talk between the infant and mother via circulating cells was established. The miRNAs appeared to be the critical regulators of milk components. The mRNA controlled the body fluid balance, thirst, appetite, immune response, and infants' development, implicating their functional role and cross-talk between infants and mother (Alsaweed et al. 2016).

Progenitor cells seem to be present in milk and could be available for therapeutic applications. Progenitor cells are seen as the critical source of adult stem cells for medicinal purposes (Lloyd-Lewis et al. 2017; Visvader and Stingl 2014; Choudhary 2014). These cells have a profound proliferative capacity and could be advantageous for cancer research. These cells may harbor clues of the understanding role of proliferation-responsive factors. Mammary stem cells reside in a hypoxic state in a niche in the basal layer and remain quiescent. Upon proliferative signal, stem cells escape from the niche and the control mechanisms that hold them in quiescence state (Choudhary et al. 2013; Tiede and Kang 2011; Capuco 2007; Hassiotou et al. 2012). An early report on the presence of progenitor cells in mammary tissue was demonstrated in humans where cytokeratin (KRT) 5^{-} positive cells were able to differentiate into two different types of cells, namely, alveolar epithelial cell or myoepithelial cell lineage (Böcker et al. 2005).

Interestingly, such KRT5-positive cells were obtained from the donor mother's breast milk and remain an enigma for the investigators (Cregan et al. 2007). Immunohistochemical evidence showed that KRT5-positive epithelial cells are in the basal layer of mammary tissue, suggesting the possibility of cells coming from the basal layer of epithelium during the milk secretion. However, the presence of these progenitor cells in mammary alveoli can also be possible and subject to further investigation.

8.9 Therapeutic Applications of Milk-Derived Stem Cells

Multilineage potentials of milk-derived stem cells might pave the way for a non-invasive source of stem cell isolation and therapeutic applications. Milk-derived stem cells possess low immunogenic and negligible teratoma formation upon

transplantation (Hassiotou et al. 2012). Another significant advantage of harvesting stem cells from milk is the patient-specific, repeated harvesting time, stage-specific, and liable for autologous transplantation. A potential application of milk-derived stem cells was explored and in vitro study showed differentiation of such cells into neural stem cells, neuroglia, and neurons (Hassiotou et al. 2013; Hassiotou and Hartmann 2014). Later in an in vivo study using a murine model, the presence of breast milk stem cells has been demonstrated in blood and differentiation of cells into neuronal cells and glial cell in the brain of suckling pup (Aydin et al. 2018). This phenomenon of integration of mothers' milk stem cells into the brain of suckling mice pups has been referred to as microchimerism. Microchimerism is a phenomenon similar to the exchange of stem cells between the mother and the fetus in utero, and transferred cells survived in the chimeras for many years (Kinder et al. 2017).

Many animal studies are required before finding milk-derived stem cells' applications in therapy for diseases like degenerative neuronal diseases and other diseases like heart strokes. Three important aspects that need to be emphasized while utilizing milk-derived stem cells are: (1) characterization of injected stem cells, (2) divisional kinetics and differentiation ability, and (3) safety potential of injected cannot induce teratoma.

In coming years, the development of new and improved stem cell isolation methods from milk and harvesting a pure population of cells will improve its utility rather than a heterogeneous one.

References

Alsaweed M, Lai CT, Hartmann PE, Geddes DT, Kakulas F (2016) Human milk cells contain numerous miRNAs that may change with milk removal and regulate multiple physiological processes. Int J Mol Sci 17(6):956

Artaud-Wild SM, Connor S, Sexton G, Connor WE (1993) Differences in coronary mortality can be explained by differences in cholesterol and saturated fat intakes in 40 countries but not in France and Finland. A paradox. Circulation 88:2771–2779

Aydin MS, Yigit EN, Vatandaslar E, Erdogan E, Ozturk G (2018) Transfer and integration of breast milk stem cells to the brain of suckling pups. Sci Rep 8(1):14289

Baldwin RL, Smith NE, Taylor J, Sharp M (1980) Manipulating metabolic parameters to improve growth rate and milk secretion. J Anim Sci 51:1416–1428

Balk EM, Adam GP, Langberg VN et al (2017) Global dietary calcium intake among adults: a systematic review. Osteoporos Int 28:3315–3324

Bauman DE, Harvatine KJ, Lock AL (2011) Nutrigenomics, rumen-derived bioactive fatty acids, and the regulation of milk fat synthesis. Annu Rev Nutr 31:299–319

Berni CR, Sangwan N, Stefka A et al (2016) Lactobacillus rhamnosus GG-supplemented formula expands butyrate-producing bacterial strains in food allergic infants. ISME J 10:742–750

Bionaz M, Loor JJ (2011) Gene networks driving bovine mammary protein synthesis during the lactation cycle. Bioinform Biol Insights 5:83–98

Böcker W, Bürger H, Buchwalow IB, Decker T (2005) Ck5-positive cells are precursor cells of glandular and myoepithelial cell lineages in the human breast epithelium. A new cell concept as a basis for a better understanding of proliferative breast disease? Verh Dtsch Ges Pathol 89:45–47

Briere CE, Jensen T, McGrath JM, Young EE, Finck C (2017) Stem-like cell characteristics from breast milk of mothers with preterm infants as compared to mothers with term infants. Breastfeed Med 12:174–179

Capuco AV (2007) Identification of putative bovine mammary epithelial stem cells by their retention of labeled DNA strands. Exp Biol Med 232:1381–1390

Chen GC, Szeto I, Chen L, Han S, Li Y, van Hekezen R, Qin L (2015) Dairy products consumption and metabolic syndrome in adults: systematic review and meta-analysis of observational studies. Sci Rep 5:14606

Choudhary RK (2014) Mammary stem cells: expansion and animal productivity. J Animal Sci Biotechnol 5:36

Choudhary RK, Li RW, Evock-Clover CM, Capuco AV (2013) Comparison of the transcriptomes of long-term label retaining-cells and control cells microdissected from mammary epithelium: an initial study to characterize potential stem/progenitor cells. Front Oncol 3:21

Collins AM, Roberton DM, Hosking CS, Flannery GR (1991) Bovine milk, including pasteurised milk, contains antibodies directed against allergens of clinical importance to man. Int Arch Allergy Appl Immunol 96:362–367

Cong X, Xu W, Janton S, Henderson WA, Matson A, McGrath JM et al (2016) Gut microbiome developmental patterns in early life of preterm infants: impacts of feeding and gender. PLoS One 11(4):e0152751

Cregan MD, Fan Y, Appelbee A, Brown ML, Klopcic B, Koppen J, Mitoulas LR, Piper KM, Choolani MA, Chong YS, Hartmann PE (2007) Identification of nestin-positive putative mammary stem cells in human breastmilk. Cell Tissue Res 329(1):129–136

Da Silva MS, Rudkowska I (2015) Dairy nutrients and their effect on inflammatory profile in molecular studies. Mol Nutr Food Res 59:1249–1263

den Hartog G, Jacobino S, Bont L, Cox L, Ulfman LH, Leusen JHW et al (2014) Specificity and effector functions of human RSV-specific IgG from bovine milk. PLoS One 9:e112047

Dils R, Clark S, Knudsen J (1977) Comparative aspects of milk fat synthesis. In: Peaker M (ed) Comparative aspects of lactation. Academic, London, pp 43–55

Engel S, Elhauge M, Tholstrup T (2018) Effect of whole Milk compared with skimmed milk on fasting blood lipids in healthy adults: a 3-week randomized crossover study. Eur J Clin Nutr 72 (2):249–254

FAO of the US (n.d.) Gateway to dairy production and products. Milk composition. http://www.fao.org/dairy-production-products/products/milk-composition/en/. Accessed Nov 30, 2020

Górska-Warsewicz H, Rejman K, Laskowski W, Czeczotko M (2019) Milk and dairy products and their nutritional contribution to the average polish diet. Nutrients 11(8):1771

Griinari JM, Dwyer DA, Bauman DE, Barbano DM, House WA (1977) The role of insulin in the regulation of milk protein synthesis in dairy cows. J Dairy Sci 80(10):2361–2371

Guo L, Zhang Y, Zhang L et al (2016) MicroRNAs, TGF-β signaling, and the inflammatory microenvironment in cancer. Tumor Biol 37:115–125

Harinarayan CV, Akhila H (2019) Modern India and the tale of twin nutrient deficiency-calcium and vitamin D-nutrition trend data 50 years-retrospect, introspect, and prospect. Front Endocrinol 10:493

Harris ES, McIntyre TM, Prescott SM, Zimmerman GA (2000) J Biol Chem 275(31):23409–23412

Hassiotou F, Hartmann PE (2014) At the dawn of a new discovery: the potential of breastmilk stem cells. Adv Nutr 5(6):770–778

Hassiotou F, Beltran A, Chetwynd E, Stuebe AM, Twigger AJ, Metzger P, Trengove N, Lai CT, Filgueira L, Blancafort P, Hartmann PE (2012) Breastmilk is a novel source of stem cells with multilineage differentiation potential. Stem Cells 30(10):2164–2174

Hassiotou F, Geddes DT, Hartmann PE (2013) Cells in human milk: state of the science. J Hum Lact 29(2):171–182

Hata T, Murakami K, Nakatani H, Yamamoto Y, Matsuda T, Aoki N (2010) Isolation of bovine milk-derived microvesicles carrying mRNAs and microRNAs. Biochem Biophys Res Commun 396:528–533

Haug A, Høstmark AT, Harstad OM (2007) Bovine milk in human nutrition--a review. Lipids Health Dis 6:25

Herbein JH, Aiello RJ, Eckler LI, Pearson RE, Akers RM (1985) Glucagon, insulin, growth hormone, and glucose concentrations in blood plasma of lactating dairy cows. J Dairy Sci 68:320–325

Insel P, Turner RE, Ross D (2003) Discovering nutrition. American Dietetic Association, Boston

Joshi PA, Khokha R (2012) The mammary stem cell conundrum: is it unipotent or multipotent? Breast Cancer Res 14:305

Kaimala S, Bisana S, Kumar S (2012) Mammary gland stem cells: more puzzles than explanations. J Biosci 37(2):349–358

Kinder JM, Stelzer IA, Arck PC, Way SS (2017) Immunological implications of pregnancy-induced microchimerism. Nat Rev Immunol 17(8):483–494

Kohronen HJ, Marnila P (2009) 10 - bovine milk immunoglobulins against microbial human diseases. In: Corredig M (ed) Woodhead publishing series in food science, technology and nutrition, dairy-derived ingredients. Woodhead Publishing, Sawston, pp 269–289

Langa S, Maldonado-Barragan A, Delgado S, Martin R, Martin V, Jimenez E et al (2012) Characterization of lactobacillus salivarius CECT 5713, a strain isolated from human milk: from genotype to phenotype. Appl Microbiol Biotechnol 94(5):1279–1287

Lee K, Cho W (2017) The consumption of dairy products is associated with reduced risks of obesity and metabolic syndrome in Korean women but not in men. Nutrients 9:630

Li Y, Deng X, Zeng X, Peng X (2016) The role of mir-148a in cancer. J Cancer 7(10):1233–1241

Lloyd-Lewis B, Harris OB, Watson CJ, Davis FM (2017) Mammary stem cells: premise, properties, and perspectives. Trends Cell Biol 27(8):556–567

Lordan R, Tsoupras A, Mitra B, Zabetakis I (2018) Dairy fats and cardiovascular disease: do we really need to be concerned? Foods 7(3):29

Loss G, Apprich S, Waser M, Kneifel W, Genuneit J, Buchele G et al (2011) The protective effect of farm milk consumption on childhood asthma and atopy: the GABRIELA study. J Allergy Clin Immunol 128:766–773

Martin R, Jimenez E, Heilig H, Fernandez L, Marin ML, Zoetendal EG et al (2009) Isolation of bifidobacteria from breast milk and assessment of the bifidobacterial population by PCR-denaturing gradient gel electrophoresis and quantitative real-time PCR. Appl Environ Microbiol 75(4):965–969

McManaman JL (2009) Formation of milk lipids: a molecular perspective. Clin Lipidol 4 (3):391–401

Melnik BC, Schmitz G (2019) Exosomes of pasteurized Milk: potential pathogens of Western diseases. J Transl Med 17:3

Melnik BC, John SM, Schmitz G (2013) Milk is not just food but most likely a genetic transfection system activating mTORC1 signaling for postnatal growth. Nutr J 12:103

Ninkina N, Kukharsky MS, Hewitt MV, Lysikova EA, Skuratovska LN, Deykin AV, Buchman VL (2019) Stem cells in human breast milk. Hum Cell 32(3):223–230

Pipino C, Mandatori D, Buccella F, Lanuti P, Preziuso A, Castellani F, Grotta L et al (2018) Cell-like population in bovine milk: a potential new source of regenerative medicine in veterinary. Stem Cells Dev 27:1587–1597

Raj JP, Venkatachalam S, Shekoba M, Norris JJ, Amaravati RS (2018) Dietary calcium intake and physical activity levels among people living in Karnataka, India - an observational hospital-based study. J Fam Med Prim Care 7(6):1411–1416

Rasheed S, Qazi IM, Ahmed I, Durrani Y, Azmat Z (2016) Comparative study of cottage cheese prepared from various sources of Milk. Proc Pak Acad Sci 53(4):269–282

Rius AG, Appuhamy JN, Cyriac J, Kirovski D, Becvar O, Escobar J, McGilliard ML, Bequette BJ, Akers RM, Hanigan HD (2010) Regulation of protein synthesis in mammary glands of lactating dairy cows by starch and amino acids. J Dairy Sci 93:3114–3127

Rump JA, Arndt R, Arnold A, Bendick C, Dichtelmuller H, Franke M et al (1992) Treatment of diarrhoea in human immunodeficiency virus-infected patients with immunoglobulins from bovine colostrum. Clin Investig 70:588–594

Sedykh S, Kuleshova A, Nevinsky G (2020) Milk exosomes: perspective agents for anticancer drug delivery. Int J Mol Sci 21(18):6646

Seldin L, Le Guelte A, Macara IG (2017) Epithelial plasticity in the mammary gland. Curr Opin Cell Biol 49:59–63

Smith GH, Medina D (2018) Does the mouse mammary gland arise from unipotent or multipotent mammary stem/progenitor cells? J Mammary Gland Biol Neoplasia 23(1–2):1–3

Tiede B, Kang Y (2011) From milk to malignancy: the role of mammary stem cells in development, pregnancy and breast cancer. Cell Res 21(2):245–257

Twigger AJ, Hodgetts S, Filgueira L, Hartmann PE, Hassiotou F (2013) From breast Milk to brains: the potential of stem cells in human Milk. J Hum Lact 29(2):136–139

van Amerongen R (2014) Bipotent mammary stem cells: now in amazing 3D. Breast Cancer Res 16 (6):480

Visvader JE, Stingl J (2014) Mammary stem cells and the differentiation hierarchy: current status and perspectives. Genes Dev 28(11):1143–1158

Witkowska-Zimny M, Kaminska-El-Hassan E (2017) Cells of human breast milk. Cell Mol Biol Lett 22:11

Zempleni J, Aguilar-Lozano A, Sadri M, Sukreet S, Manca S, Wu D et al (2017) Biological activities of extracellular vesicles and their cargos from bovine and human milk in humans and implications for infants. J Nutr 147:3–10

Cryopreservation of Testicular Stem Cells and Its Application in Veterinary Science

9

Tanushree Patra, Rakesh Bhaskar, and Mukesh Kumar Gupta

Abstract

Testis-derived spermatogonial stem cells (SSCs) and its in vitro counterpart, male germline stem (GS) cells, can repopulate the empty seminiferous tubules of infertile males and, therefore, are rapidly emerging as newer biotechnological tools for the treatment of male infertility, posthumous reproduction, preservation of elite germplasm and animal transgenesis. It also has potential application in the preservation and restoration of fertility in males with diseases/treatments affecting spermatogenesis. Consequently, the cryopreservation of SSC and GS cells is becoming pivotal in assisted reproductive technology (ART) for male reproduction. Cryopreservation of testis-derived stem cells is particularly indispensable for fertility preservation in pre-pubertal males, who have not yet begun their sperm production. This chapter provides an overview of the indications, applications, and strategies of fertility preservation through the cryopreservation of testicular stem cells. Recent advances on freezing and vitrification techniques, assessment of cryopreservation-induced injuries, and methods of their amelioration are also discussed.

Keywords

Cryopreservation · Vitrification · Freezing · Spermatogonial stem cells · Germline stem cells · GS cells · SSV

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9.1 Introduction

Mammalian testis is known to contain a pool of stem cells, known as spermatogonial stem cells (SSCs), which maintains spermatogenesis throughout the adulthood. These cells can be isolated and propagated in vitro as male germline stem (GS) cells and are capable of initiating donor-derived spermatogenesis in germ cell-depleted testis of infertile males for restoration of fertility. The GS cells can also undergo in vitro spermatogenesis in an organ culture system to produce haploid germ cells that can be used for the production of offspring by in vitro fertilization (IVF), intracytoplasmic sperm injection (ICSI) or round spermatid injection (ROSI). Under an appropriate culture system, the unipotent GSCs can also undergo reprogramming events to generate multipotent adult germline stem (maGS) cells or germline pluripotent stem (GPS) cells. Thus, testicular stem cells have potential application in both infertility management and regenerative medicine. Further, the testicular stem cells, combined with gene manipulation tools, can also be exploited for production of transgenic animal.

The testicular stem cells can be cryopreserved as a purified single-cell suspension or testicular tissue fragments that house SSCs in their niche (Jung et al. 2020a; Onofre et al. 2020). The cryopreservation of purified testicular stem cells can be conducted by conventional freezing or vitrification protocols used for somatic cells and are technically easier. However, SSCs are present in a very low number in adult tissues [~0.03%; (Nakagawa et al. 2007)] and may be difficult to isolate from biopsied specimens of testis. Consequently, newer and effective methods of cryopreservation for these novel cells are necessary. On the other hand, cryopreservation of testicular tissue fragments, enriched in SSC, is more challenging and depends on various factors such as the size of the tissue, permeation efficiency of cryoprotective agents (CPA) and variations in cryobiological properties of their cellular and extracellular matrix (ECM). The cryopreservation of testicular tissue fragments does not require tissue dissociation steps for isolation of cells and can maintain complex interaction between SSCs and testicular somatic cells (Leydig cells, Sertoli cells, and Myoid cells) and cell-to-ECM, which are essential for SSC-derived spermatogenesis. The testicular tissue cryopreservation can also be combined with in vitro spermatogenesis system for in vitro production of spermatozoa and eliminating the need for spermatogonial stem cell transplantation (SSCT). By preserving supporting somatic cells and ECM, the testicular tissue cryopreservation may provide the physical and molecular microenvironment required for survival, self-renewal, proliferation, and differentiation of germ cells to haploid germ cells. However, the superiority of cryopreserving testicular tissue fragments over testicular stem cells remains debatable. Moreover, although in vitro spermatogenesis system has shown promising results in rodents and primates for generating live offspring from cryopreserved testicular tissue fragments, it is still under developing phase for livestock animals and human.

This chapter provides an overview of various strategies for cryopreservation of testicular stem cells, including GS, maGS and GPS cells, and discuss their indications and applications in veterinary science. Suitable references are also

drawn from human applications in which the technology is rapidly finding its clinical applications. The details on cryopreservation of testicular tissue fragments is discussed elsewhere (Patra et al. 2021).

9.2 Methods of Cryopreservation of Testicular Stem Cells

Semen freezing is a well-established procedure in animals for breed improvement programs through artificial insemination. However, cryopreservation of testicular stem cells is relatively recent technology and is still under experimental stage in livestock animals and human. There are very limited systematic studies on the development and optimization of cryopreservation protocols of testicular stem cells. Most of the initial studies have used conventional somatic cell freezing protocols with empirical modifications to cryopreserve the SSC, GS and GPS cells. The post-thaw viability varied from lab-to-lab although the functionality of frozen-thawed SSCs was confirmed by testicular transplantation assays as early as 1996 (Avarbock et al. 1996). Initial studies have also shown that the success of freeze-thawing also varies with animal species, type, and concentration of cryoprotective agents (CPAs), method of freezing and type of cryocontainers (Kanatsu-Shinohara et al. 2003; Wu et al. 2012). However, attempts to directly relate the post-thaw viability of testicular stem cells with variable factors have been very limited.

More recently, ice-free vitrification has been explored as simpler, faster, and cost-effective method for cryopreservation of testicular stem cells. Several strategies ranging from conventional vitrification in plastic straws (PS) to ultrarapid vitrification in microdroplets (MD), electron microscopy (EM) grid, nylon membranes, open pulled straws (OPS), solid surface vitrification (SSV), etc. have been attempted and were reported to support the cryo-survival of both somatic and stem cells in testis (Patra and Gupta 2019, 2020). However, like stem cell freezing, systematic studies on the vitrification of testicular stem cells have been limited. Some studies have suggested vitrification to be superior to freezing due to its simplicity, cost-effectiveness, and better post-warming viability. However, the superiority of one over the other remains debatable (Pukazhenthi et al. 2015). Systematic and controlled studies are needed to optimize the freezing and vitrification protocols by analysing the influence of variable factors such as rate of cooling, type of CPA, size or volume of the sample, cryopreservation device, storage conditions, etc.

9.3 Freezing of Testicular Stem Cells

Freezing of testicular stem cells involves controlled crystallization of water by their slow cooling in the presence of CPA such as glycerol, dimethyl sulphoxide (DMSO), ethylene glycol (EG) or 1,2-propanediol (PROH). The rate of cooling is so chosen to crystallize the extracellular water with minimal or no intracellular ice

crystallization. The optimal rate of cooling varies with the cell type and species but generally range between 0.3 °C and 10 °C/min in most mammalian cell types. In testicular cells and stem cells, a cooling rate of 1 °C/min is considered critical during their cooling from 4 °C to (−)40 °C or lower temperature (optimal window of cooling rate). During this window of cooling, the extracellular water super cool and starts crystallizing between (−)5 °C to (−)10 °C, which results in a gradual increase in extracellular solute concentration and thereby, dehydrates the cells by osmotic-driven efflux of intracellular water. Between (−)10 °C to (−)15 °C, the extracellular ice expands and the sample become increasingly supercooled below (−)40 °C. When the sample temperature reaches (−)40 °C or lower, it can be plunged into a cryogenic agent such as liquid nitrogen or helium for intracellular vitrification without ice crystallization. The frozen samples can then be stored in liquid nitrogen for long-term storage. A working freezing-thawing protocol for cryopreservation of mice SSCs is provided in Box 9.1. In the hands of the authors, the protocol also works for the freezing of putative SSCs in goat.

Box 9.1 A Suggested Protocol for Slow Freezing of Testicular Stem Cells in Mechanical Freezer

Uncontrolled slow freezing of SSCs:

1. Trypsinize the SSC clumps by using trypsin-EDTA to prepare single cell suspension. *Note:* SSCs survive better in cell clumps of ~15 to 20 cells.
2. Centrifuge the cells at 500 g for 8–10 min and wash the pellets in a serum-free SSC culture medium twice.
3. Count the cell concentration and dilute to a concentration of 2×10^6 cells per ml.
4. Aliquot 0.5 ml of SSC cell suspension in 1.5 ml cryovials and slowly add 0.5 ml of SSC culture medium having 20% (v:v) DMSO.
5. Close the cryovials, rack on an insulated container (e.g. Stryrofoam rack) and keep at −80 °C freezer for overnight (at least 4 h).
6. Quickly plunge the cryovials into liquid nitrogen for cryostorage.

Thawing of SSCs:

1. Identify and retrieve the cryovial from the liquid nitrogen container and place it in a water bath set at 37 °C.
2. Transfer the thawed cell suspension into 9 ml of pre-warmed (37 °C) SSC culture medium in a 15 ml centrifuge tube and centrifuge at 500 g for 8–10 min.
3. Wash the cell pellet with 10 ml of SSC culture medium twice.
4. Count the cell concentration and re-suspend the cells in complete SSC culture medium (supplemented with GDNF) to the desired concentration and plate in culture plates.

The desired rate of cooling for freezing of testicular cells and stem cells can be achieved by uncontrolled slow freezing in a vapour or mechanical freezer or by controlled slow freezing in a programmable controlled rate freezer. Uncontrolled slow freezing can be performed using simple devices that are usually available in most laboratories and does not require expensive programmable freezers. Further, in contrast to controlled rate freezing, it requires a much less volume of liquid nitrogen (cryogenic agent) and the time required for the processing and freezing of the samples. Consequently, uncontrolled slow freezing has been conventionally used in most laboratories for cryopreservation of testicular stem cell suspensions in both human and animals (Pacchiarotti et al. 2013; Sá et al. 2012; Yango et al. 2014). In most conventional protocols, the cells are trypsinized to single-cell suspension, treated with CPAs such as glycerol or DMSO, loaded in cryocontainers such as plastic straws, cryovials or cryobags and cooled at 1 °C/min to (−)70 °C to (−) 80 °C, achievable by exposure to liquid nitrogen vapour or (−)80 °C freezer, which is usually available in most laboratories. A number of devices such as Mr. Frosty™, Cryo Cool™, CoolCell™, etc. are also available for maintaining uniform heat transfer during the cooling process, although they are not absolutely necessary. After freezing to (−)70 °C, which typically takes 5–20 min in liquid nitrogen vapour and 4–8 h in the deep freezer, the samples are directly plunged into the liquid nitrogen for storage until further use (Frederickx et al. 2004; Izadyar et al. 2002; Redden et al. 2009). Some researchers have also used multistep slow freezing by equilibration with CPA at 4 °C for 2 h, cooling at (−)20 °C for 2 h and freezing at (−)80 °C before storage in liquid nitrogen (Zhang et al. 2015) but is not considered essential. At the time of use, the cells can be thawed in water bath at 37 °C and cultured in complete culture medium after removal of the CPA. In most freeze-thaw protocols, the post-thaw viability of testicular spermatogonial cells was reported to be as low as 50–60% (Izadyar et al. 2002). Nevertheless, frozen-thawed stem cells retained their self-renewing capability and could restore spermatogenic function to produce normal donor-derived offspring upon transplantation recipient testis without any apparent genetic or epigenetic errors (Wu et al. 2012; Yuan et al. 2009).

In controlled rate slow freezing, the CPA-treated cells are cooled at a controlled rate using a programmable controlled rate freezer. The rates of cooling vary with the species, the volume of cell suspension, and the type and concentration of CPA and may require optimization. Typically, controlled rate freezing of testicular stem cells is performed in 0.25 ml plastic French straws and cooling is done at the rate of (−) 1 °C/min to (−)5 °C/min to cool the sample to (−)7 °C to (−)9 °C, following which they are seeded for 7–15 min and then cooled at the rate of (−)0.3 °C/min to (−) 0.5 °C/min to (−)40 °C to (−)80 °C before plunging to liquid nitrogen for storage (Frederickx et al. 2004). Seeding induces the ice nucleation by mechanical vibration or rapid temperature reduction in the programmable controlled rate freezer. Some experimenters have found that seeding step is not necessary for controlled rate freezing of testicular stem cells when they were cooled at the rate of (−)1 °C/min to (−)5 °C/min to (−)80 °C, and subsequently at the rate of (−)50 °C/min to (−)

120 °C followed by plunging into liquid nitrogen for storage (Izadyar et al. 2002; Pacchiarotti et al. 2013).

Rapid freezing has also been attempted for cryopreservation of testicular cells by treating them with high concentration of CPA in step-wise manner and plunging into liquid nitrogen (Gouk et al. 2011). However, it has met with limited success in terms of post-thaw viability. Besides, rapid freezing lead to random intracellular ice crystal formation that caused extensive cryoinjuries and high cell death. Thus, rapid freezing is not normally used for cryopreservation of testicular cells and stem cells.

9.4 Vitrification of Testicular Stem Cells

Vitrification is a rapid and simple method of cryopreserving testicular stem cells. It involves the solidification of both extra- and intra-cellular water into amorphous glass-like vitreous state without ice crystal formation. Vitrification of living cells can be achieved by ultrafast cooling ($>10^6$ °C/min) below their glass-transition temperature (T_g) to cause an extreme elevation of viscosity to greater than 10^{15} poise and result in molecular stasis and solidification without ice crystallization of water. Typically, the cells are treated with a very high concentration of CPA for cryoprotective effect, loaded into a cryocontainer such as PS, cryovial, EM grid, OPS, etc. and directly plunged into a cryogenic agent such as liquid nitrogen, liquid helium or nitrogen slush for vitrification and storage. Since high concentration of CPA required for vitrification is toxic to cells, an equilibration step of treating the cells with low concentration of CPA (typically, half of the final concentration) is usually necessary before their exposure to the full concentration of CPA (Gupta and Lee 2010; Jung et al. 2020b). The equilibration period is usually 1–3 min whereas the incubation period in vitrification solution is usually restricted to 25–45 s and are to be optimized for the type and concentration of CPAs, temperature of the solution and volume of the sample (Gupta et al. 2007). A longer (5–15 min) equilibration time with considerably low concentration of permeable CPA in the equilibration solution may be used for high volume samples. At the time of use, the vitrified cells can be warmed and rehydrated in a step-wise manner in the presence of a non-permeating disaccharide (e.g. sucrose, trehalose, etc.) to remove the CPA. The non-permeating disaccharides act as osmotic buffers and prevent the swelling and rupture of cells during cell re-hydration. A working vitrification-warming protocol for cryopreservation of mice SSCs is provided in Box 9.2. In the hands of the authors, the protocol also works for the vitrification of putative SSCs in goat.

Box 9.2 A Suggested Protocol for Vitrification of Testicular Stem Cells by SSV

Vitrification of SSCs:

1. Trypsinize the SSC clumps by using trypsin-EDTA to prepare single cell suspension. *Note:* SSCs survive better in cell clumps of ~15 to 20 cells.
2. Centrifuge the cells at 500 g for 8–10 min and wash the pellets in a serum-free SSC culture medium twice.
3. Count the cell concentration and dilute to a concentration of 2×10^6 cells per ml in equilibration solution [20% (v:v) EG in calcium- and magnesium-free phosphate buffered saline (PBS)] for 90 s at 37 °C.
4. Expose the equilibrated cells to vitrification solution [40% (v:v) DMSO, 18% (w:v) Ficoll 70 (Sigma) and, 0.3 M Sucrose (Sigma) in calcium- and magnesium-free PBS] at 37 °C.
5. Using a micropipette swiftly drop the cell laden-vitrification solution as microdrop (~10 μl) on the pre-chilled dry surface of a metal block partially (3/4th) submerged into liquid nitrogen. Ensure that the surface of metal block is dry.
6. Upon visual observation of the vitrification, move the vitrified microdrops (complete transparent microdrops) into 1.8 ml cryovials (Nunc cat. No. 343958) using liquid nitrogen-cooled forceps and stored into liquid nitrogen tanks until further analysis. The total duration between treatment with vitrification solution and plunging in liquid nitrogen should be limited to 30-45 s.

Warming and rehydration of vitrified SSCs:

1. Identify and retrieve the cryovial from the liquid nitrogen container and allow the liquid nitrogen, if any, to evaporate.
2. Drop the vitrified microdrops from the cryovial directly into a petri dish containing 1 ml of WS1 solution (0.5 M Sucrose in PBS) at 37 °C for 5 min.
3. Wash the cells through 10 ml of WS2 solution (0.25 M Sucrose in PBS) for 5 min and centrifuged at 300× g for 5 min.
4. Wash the cells through 10 ml of WS3 solution (PBS) for 5 min and centrifuged at 300× g for 5 min.
5. Count the cell concentration and re-suspend the cells in complete SSC culture medium (supplemented with GDNF) to the desired concentration and plate in culture plates.

Vitrification of cells requires a shorter duration of CPA exposure, fewer instruments and therefore is a rapid method of cryopreservation. Further, it does not require any expensive equipment such as programmable controlled rate freezer and therefore, is economical. Moreover, although debatable, the efficiency of

vitrification is generally considered superior, or at least similar, to those of slow freezing. Several studies have reported that lack of ice crystal formation reduced cellular damage and improved the post-warming viability of testicular stem cells (Poels et al. 2013). The vitrified-warmed testicular stem cells were shown to be capable of undergoing complete spermatogenesis upon transplantation. Consequently, it has been successfully used for cryopreservation of testicular cells and stem cells in both human and animals (Patra and Gupta 2019, 2020; Yokonishi et al. 2014). However, although the results are encouraging, the vitrification procedure for testicular cells and stem cells remain sub-optimal. Further, the success of vitrification may vary with the experimenter. Several efforts are therefore being made to improve the vitrification of testicular stem cells through optimization of sample volume, type of cryocontainers, type and concentration of CPA, duration of CPA-exposure, use of hydrostatic pressure, optimization of post-warming culture with additives such as antioxidant, etc. (Patra and Gupta 2019, 2020; Saragusty et al. 2010).

9.4.1 Cryocontainers for Vitrification

Plastic cryovials and straws are the most used cryocontainers for holding the cells during freezing. The same were later used to hold the cell suspension during vitrification. However, it was soon discovered that minimizing the volume of cell suspension, or increasing the surface area, can dramatically improve the post-warming viability of cells. Consequently, several minimum volumes cryocontainers such as EM grid, cryoloop, cryotop, OPS, cryotip, nylop loop, hemi-straw, vitrification spatula, plastic blade, metal mesh, nylon mesh, paper container, etc. became the containers of choice for vitrification for various cell types (Gupta and Lee 2010; Kim et al. 2012; Patra and Gupta 2019). Some of these cryocontainers such as EM grid and OPS allowed direct contact of the sample with cryogenic agent for rapid heat exchange whereas other cryocontainers such as cryotop and closed pulled straw protected the samples from direct contact with cryogenic agent for safety. Accordingly, depending on contact or non-contact of the samples with cryogenic agent during vitrification, the methods of vitrification can be classified as “open vitrification” and “closed vitrification”, respectively (Kumari et al. 2016). The open vitrification methods favour faster heat transfer than closed vitrification methods but poses a risk of contamination with pathogens such as hepatitis B, *Brucella* spp., which can survive in liquid nitrogen (Bielanski et al. 2000; Fountain et al. 1997; Kumari et al. 2016; Tedder et al. 1995).

While vitrification of testicular stem cells can theoretically be performed by both open and closed vitrification methods using a variety of cryocontainers, most studies have used OPS for their vitrification. In this method, the SSCs, suspended in vitrification solution, are loaded into thin OPS by capillary action and plunged into liquid nitrogen for vitrification and storage. This method of vitrification was found to be superior to both uncontrolled and controlled freezing of testicular cell suspensions (Sá et al. 2012). It was also suggested that the risk of microbial contamination due to direct contact with liquid nitrogen can be minimized using UV-C irradiated liquid

nitrogen (Poels et al. 2013) and storing them in a pre-cooled cryotubes (Curaba et al. 2011).

Container-less vitrification methods such as solid surface vitrification (SSV), nylon mesh and MD methods have also been used for open vitrification of testicular cells and tissues (Abrishami et al. 2010; Higaki et al. 2017). The SSV method is a relatively simple method of vitrification wherein the CPA-treated samples are placed on the surface of sterile metal block, kept half-submerged in the liquid nitrogen, for vitrification. Following vitrification, the vitrified samples can be transferred into pre-cooled cryovials for storage in liquid nitrogen. A modified SSV (mSSV) method has also been developed wherein the solid metal block is replaced with a readily available aluminium foil that can be used like a floating boat on the surface of liquid nitrogen (Gupta et al. 2007, 2010). The mSSV method was demonstrated to be successful for vitrification of both testicular cells and tissues (Patra and Gupta 2019; Abrishami et al. 2010; da Silva et al. 2019; Kaneko et al. 2013). The testicular cells, cryopreserved by SSV or mSSV, were viable and could successfully re-initiate the spermatogenesis process upon transplantation (Onofre et al. 2016). In a comparative study, SSV was reported to be superior to OPS and MD vitrification as well as controlled rate freezing for vitrification of testicular tissue (Dumont et al. 2015). Thus, SSV and mSSV may offer excellent alternatives to both slow freezing and OPS vitrification. However, studies on SSV of testicular stem cells are less explored.

More recently, encapsulation-vitrification of testicular cells in alginate hydrogels have also been reported. We have shown that the testicular cells can be encapsulated into alginate microdroplets by mixing them with sodium alginate solution and allowing them to cross-link by dropping into calcium solution. The encapsulated cells can then be vitrified by closed or open vitrification such as SSV or MD (Patra and Gupta 2020). Following vitrification, the encapsulated cells can be retrieved by vortexing them in citric acid solution. We have further shown that the post-warming viability of encapsulation-vitrification varies with the size of microdroplets and can be regulated by adjusting the concentration and flow rate of alginate solution and calcium ion (Noguchi et al. 2006; Patra and Gupta 2020). The encapsulation-vitrification offers several advantages over other methods: (1) encapsulation provides a 3D structure which allows easy handling of cells during vitrification, dilution and rehydration process without requiring a centrifugation step (Patra and Gupta 2020); (2) encapsulation into alginate protects the SSCs against mechanical damages and oxidative stress during vitrification (Pirnia et al. 2017; Poels et al. 2016); (3) encapsulation may support the stemness of SSCs (Pirnia et al. 2017); (4) vitrified-warmed testicular stem cells, encapsulated in alginate microbeads, can be directly cultured in bioreactors for their large-scale expansion for clinical use and; (5) encapsulated cells can be used directly for transplantation (Gül et al. 2020). However, studies on encapsulation-vitrification of testicular cells are very limited. Furthermore, all studies on encapsulation-vitrification of testicular cells so far have used alginate biomaterial for hydrogel formation. A number of other natural protein (fibrin, collagen, gelatine, etc.) and polysaccharides (e.g. chitosan, dextran, etc.) or synthetic organic (e.g. polycaprolactone, polytetrafluoroethylene, etc.) and inorganic (e.g. Titania, silicon, etc.) biocompatible materials may be suitable for encapsulation of testicular cells and are yet to be explored (Vermeulen et al. 2017).

9.5 Cryoprotective agents (CPA) for Freezing and Vitrification

CPAs are the integral part of freezing and vitrification protocols for prevention of cryoinjury. Two classes *viz.* permeating (e.g. glycerol, DMSO, EG, PROH) and non-permeating (e.g. sucrose, trehalose, polyethylene glycol, hydroxyethyl starch) CPAs are commonly used for protecting the cells from cryoinjury and improving the cryosurvival during freezing and vitrification. The permeating CPAs can permeate into the cells and modify their cryobiological properties to protect against chilling injury by regulating ice crystallization. On the other hand, non-permeating CPAs are generally used to reduce the requirement of high concentration of permeating CPA and for increasing the membrane stability during dehydration (Lee et al. 2014a). Among various CPAs, glycerol and DMSO have been the most used as CPA for slow freezing whereas DMSO, EG and PROH have been the commonest CPAs for vitrification of testicular stem cells.

In early studies, slow freezing of testicular cells and tissues was achieved using glycerol at a concentration of 1 M, with or without non-permeating CPA such as 0.03–0.1 M sucrose and 10–80% (v:v) serum (Abrishami et al. 2010; Izadyar et al. 2002). However, it required a long duration of glycerization at low temperature and hence, its use became less frequent with the development of more effective permeating CPAs such as DMSO, EG and PROH (Keros et al. 2005; Travers et al. 2011). Among these permeating CPAs, 0.5–1.5 M DMSO (usually, 5–10%, v:v) has been the most commonly used permeating CPA and is considered least cytotoxic for better cell survival during slow freezing. Several studies have shown that SSCs frozen in the presence of DMSO could retain their self-renewing, proliferating and engraftment potential upon transplantation (Hermann et al. 2007). Combined use of DMSO with 200 mM trehalose could further improve the success of SSC cryopreservation (Jung et al. 2020b) and was superior to those of 2.5% polyethylene glycol (PEG) and 200 mM trehalose although number of spermatogonial colonies were higher from SSCs that were frozen thawed in 2.5% PEG with 200 mM trehalose (Lee et al. 2013a, b). Additive effect of trehalose with DMSO was also shown in bovine species (Kim et al. 2015).

The DMSO has also been used very commonly for vitrification of testicular cells at a concentration of 3.0–5.5 M (usually, 30–40%, v:v) (Curaba et al. 2011; Frederickx et al. 2004; Keros et al. 2007; Lee et al. 2014a; Pietzak 3rd et al. 2015; Unni et al. 2012; Wu et al. 2012; Wyns et al. 2007; Yango et al. 2014). DMSO forms transient pores in the plasma membrane, which favours cell dehydration and reduces ice crystal formation during the cooling (Gurtovenko and Anwar 2007). It can also interact with lipid bilayer of plasma membrane to increase its fluidity and protect from mechanical injury caused by ice crystals. Moreover, DMSO can improve the cryosurvival of cells by allowing supercooling of cells below (−)40 °C and preventing intracellular ice crystallization (Mazur 1984). However, high concentration or longer exposure duration of DMSO can be cytotoxic and induce cell differentiation, aberrant DNA methylation and histone modification and apoptosis (Huang et al. 2008; Iwatani et al. 2006; Kawai et al. 2010). Consequently, several alternative CPAs such as EG, PROH, formamide, HES, dextran, etc. have been tried,

either alone or in combination to DMSO for reducing its cytotoxicity in various cell types, including testicular cells. The EG has higher cell permeability that DMSO and can weaken the hydrogen bond formation between solutes and water molecules (Weng et al. 2011). It also reduces the osmotic shock during ice crystallization. Thus, EG at a concentration of 1.5–3.0 M or 5.5–6.5 M has been viewed as a good alternative to DMSO for freezing and vitrification, respectively (Frederickx et al. 2004). Nevertheless, till date, DMSO remains to be the most used CPA for both freezing and vitrification of testicular cells. The DMSO and EG can also be used at 1:1 ratio to reduce the toxicity of individual CPAs by reducing their concentration to half.

Natural antifreeze proteins and glycoproteins found in Antarctic fish and plants that have adapted to extreme cold conditions have also been explored for use as CPA (Cheung et al. 2017; Kim et al. 2017). These proteins can cause freezing point depression and prevent the intracellular ice crystallization to reduce cryoinjury. They can also maintain the stability of phospholipids and unsaturated fatty acids in plasma membranes. However, studies on use of antifreeze proteins and glycoproteins have been limited and, their usefulness for cryopreservation of testicular stem cells remains to be explored.

9.6 CPA Toxicity and Strategies for Its Amelioration

All permeating CPAs are known to have cytotoxicity depending on their concentration and the duration of exposure. Commonly used CPAs such as DMSO, EG and PROH have been reported to cause abnormal spindle morphology and aneuploidy in oocytes and embryos (Huang et al. 2008), although such damage was not observed in testicular cells and tissues (Li et al. 2009; Oblette et al. 2017; Song et al. 2016). DMSO is also known to cause epigenetic aberrations in DNA methylation in testicular cells and tissues (Iwatani et al. 2006; Kawai et al. 2010). Thus, titration of concentration and duration of exposure is required to be optimized for effective cryopreservation with minimal cytotoxicity. Toxicity of CPAs can also be reduced by combining two or more permeating or non-permeating CPAs and reducing the concentration of individual CPA. Experiments have shown that excellent vitrification solutions can be formed by combining a strong glass former such as DMSO with a weak glass former such as EG or formamide (Fahy et al. 2004). Thus, the mixture of DMSO and EG in 1:1 ratio is occasionally used for reducing the concentration of DMSO and its toxicity during vitrification of testicular stem cells and tissues (Abrishami et al. 2010; Baert et al. 2012; Curaba et al. 2011; da Silva et al. 2019; Dumont et al. 2015; Gholami et al. 2013).

Replacing the part of permeating CPA with non-permeating CPAs such as mono- or di-saccharides, polyvinylpyrrolidone (PVP), PEG, Ficoll, dextran and polyvinyl alcohol (PVA) offers another possibility for minimizing the CPA toxicity. In several studies, DMSO was combined with sucrose (Curaba et al. 2011; Goossens et al. 2008; Gouk et al. 2011; Izadyar et al. 2002; Poels et al. 2014; Wyns et al. 2008), trehalose (Lee et al. 2014b, b; Zhang et al. 2015) and dextran (Pacchiarotti et al.

2013) to reduce its cytotoxicity and improve the cryosurvival of testicular cells and tissues during freezing and vitrification. The EG has also been combined with sucrose, trehalose and PVP to reduce its cytotoxicity and improve cryosurvival of testicular cells and tissue (Kaneko et al. 2013; Poels et al. 2012). High concentrations of blood serum, serum albumin, hyaluronan, anti-oxidants, etc. are the other additives that may be used in freezing and vitrification media to improve the success rates of cryopreservation. Since CPAs are known to induce apoptotic cascade, inhibitors such as Z-VAD-FMK, Z-DEVD-FMK, sphingosine-1-phosphate, etc. may also be useful in reducing the CPA-induced apoptosis (Men et al. 2006; Onions et al. 2008).

9.7 Cryoinjury and Strategies for Its Amelioration

In addition to CPA, the chilling injury during freezing and vitrification procedures can also cause thermal and biochemical stress to the cells and reduce their viability. The chilling injury can occur due to improper cooling rate, osmotic stress and mechanical damage caused by sharp ice crystals. The immediate outcome of these chilling injuries is apoptosis and cell death, which may be observed in the form of cellular degeneration, rupture of cell membrane and leakage of cytoplasmic contents. Mechanism by which freezing and vitrification induce cell death is not fully understood but both canonical and mitochondrial-mediated apoptosis pathways and oxidative stress pathways have been shown to be involved. It may also cause transcriptional stress to cells resulting in increased expression of responsive genes such as Bax, Cirbp, Hsp90ab1 and Sod1 (Bebbere et al. 2019). Further, cryopreservation may also cause dysregulated gene expression and stress resulting in epigenetic aberrations of DNA methylation (Iwatani et al. 2006; Kawai et al. 2010). However, despite these cryoinjuries, the birth of normal and fertile offspring has been reported from the cryopreserved SSCs and testicular cell suspensions (Goossens et al. 2011; Tanaka et al. 2015, 2018; Wu et al. 2012; Yuan et al. 2009). Studies have also shown that cryopreservation had little or no effect on meiotic recombination and fidelity of synapsis formation in testicular cells (Li et al. 2009; Song et al. 2016).

Reactive oxygen species (ROS) such as superoxide anion radical ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$) and hydrogen peroxide (H_2O_2) are normally generated during mitochondrial respiration and are important for cellular homeostasis. At physiological levels, the ROS also stimulates the self-renewal of testicular stem cells via MAPK and JNK signalling pathways (Morimoto et al. 2013). However, excessive ROS generation or its intracellular accumulation can cause cell death by modifying the functional groups of proteins, lipids and nucleic acids (Finkel and Holbrook 2000). Several studies have shown that the cryopreservation can induce excessive ROS generation to cause oxidative stress and leads to DNA fragmentation, altered cellular metabolism, dysregulation of cell signalling pathways and cell death (Gupta et al. 2010; Thomson et al. 2009; Zhang et al. 2015). Consequently, use of antioxidants in cryopreservation and in vitro culture media has been explored by

various researchers to ameliorate the adverse effects of ROS. Several studies have shown the beneficial effects of several antioxidants such as vitamin E, α-tocopherol (an active form of vitamin E), catalase (CAT), reduced glutathione (GSH), β-mercaptoethanol (βME), superoxide dismutase (SOD), melatonin, caffeic acid, selenium, hypotaurine and cysteine on the viability of sperm, oocytes, somatic cells and stem cells, due to their ROS scavenging activity (Aliakbari et al. 2016; Boroujeni et al. 2019; Gupta et al. 2007, 2010; Ha et al. 2016; Navid et al. 2017; Sayed Mahdi et al. 2018). Studies have also shown that supplementation of antioxidants such as vitamin E into the cryopreservation and in vitro culture media reduced the ROS accumulation in frozen-thawed testicular tissue of pre-pubertal mice and lead to better in vitro spermatogenesis (Arkoun et al. 2019). Thus, use of antioxidants in cryopreservation protocols of testicular cells and tissue is generally recommended (Patra and Gupta 2019; Zhang et al. 2015).

Many studies have also shown that apoptosis and necrosis is a common observation during cryopreservation of testicular stem cells by freezing or vitrification. Although the cause of apoptosis could not be precisely ascertained, both canonical and mitochondrial-mediated pathways were found to be involved in cryopreserved testicular stem cells. Studies have also shown that CPA such as DMSO and DM can themselves cause apoptosis. Thus, several studies have also analysed the use of apoptotic inhibitors such as Z-VAD-FMK, Z-DEVD-FMK, sphingosine-1-phosphate, RIPA-56, etc. and reported them to be useful in reducing the CPA- or cryopreservation-induced apoptosis (Men et al. 2006; Onions et al. 2008; Xie et al. 2020).

9.8 Assessing the Viability of Cryopreserved Testicular Stem Cells

The success of a cryopreservation protocol can be assessed by multiple morphological, biochemical, and molecular methods for assessment of cell viability, apoptosis, ROS activity, ability to initiate spermatogenesis by short-term in vitro culture, and production of fertile and healthy offspring upon SSCT or through in vitro spermatogenesis followed by IVF/ICSI/ROSI. Several biochemical and molecular analytical methods can also be utilized to assess the extent of cellular damage such as chromosomal anomalies, reduced mitochondrial membrane potential, aberrant DNA methylation or histone modifications, free radical formation, etc. Most studies analyse and report the percentage of living cells in frozen-thawed or vitrified-warmed cells as a measure of success in cryopreservation. However, the presence of living cells not necessarily indicates the functionality of the cells. The functionality of cryopreserved testicular cells needs to be verified by transplantation assay or in vitro culture. Successful in vitro spermatogenesis of motile sperm, which could lead to the birth of live offspring by artificial insemination, IVF or ICSI is also a sufficient proof for the success of testicular cell cryopreservation. Alternatively, the success of testicular cryopreservation can be assessed by transplantation of the cryopreserved cells into recipient testis or grafting into a heterologous location

such as under skin or orthotropic grafting for monitoring the serum testosterone level or spermatogenesis process (Baert et al. 2012; Kirpatovskii et al. 2018).

9.9 Biochemical Assays

Biochemical methods are routinely used for evaluating the viability of cryopreserved cells by dye-exclusion Trypan Blue assay, fluorescein diacetate (FDA) assay and lactate dehydrogenase (LDH) release assay. On the other hand, apoptosis in cryopreserved cells can be evaluated by analysing the caspase activity, Annexin V staining or TUNEL assay. Staining for nucleolar organizing region (NOR) and immunocytochemistry for PCNA staining or Ki67 staining can also be utilized to get an idea about the cell proliferation ability of cells. Further, cryoinjuries can also be assessed by evaluating mitochondrial damage by MitoTracker™ or MTT assay, oxidative stress by ROS activity, lipid peroxidation by malondialdehyde (MDA) assay, etc. More recently, Fourier transformed infrared (FTIR) spectroscopy has also been found to be suitable for analysing the general biochemical profile and membrane lipid transition in cryopreserved cells (Meneghel et al. 2019; Wang et al. 2019), although its potential has not yet been fully utilized for testicular stem cells.

9.10 Molecular Methods

Conventional molecular tools such as polymerase chain reaction (PCR), reverse transcriptase PCR (RT-PCR) and Western blotting can be used for analysis of genes and proteins related to cell proliferation, cell viability, apoptosis, etc. in cryopreserved cells. The stemness of viable SSCs can also be evaluated by analysing the expression of marker genes and proteins such as Oct4, Pgp9.5, VASA, boule, UTF1, UCHL1, GFRα-1, PLZF, etc. (Li et al. 2018). Immunohistochemistry and RNA sequencing (RNAseq) can also be used to evaluate the transcriptome, proteome and epigenome status of the cryopreserved cells. On the other hand, karyotyping and fluorescence in situ hybridization (FISH) can be done to assess the chromosomal configuration of cells and haploidy of spermatozoa obtained from cryopreserved testicular stem cells.

9.11 Transplantation Assays

Testicular transplantation and donor-derived spermatogenesis is the 'gold standard' for true assessment of viability in cryopreserved testicular stem cells. The cells ($0.5–2.0 \times 10^5$ cells/ml) can be mixed with a dye such as Trypan Blue to visualize the microinjection (Kaul et al. 2010; Onofre et al. 2020) and microinjected into recipient testes prepared by busulfan treatment or gamma irradiation to deplete of endogenous SSCs. The microinjection can be done *via* rete testes or vas deference or directly into the seminiferous tubules using borosilicate pipettes under microscope.

The transplanted SSCs colonize at the basal membrane and re-populate the seminiferous tubules to initiate spermatogenesis and produce fertile spermatozoa. The proof of principle for SSC transplantation (SSCT) came from studies on transplantation of SSCs from infertile Steel (SI/SI) mice to infertile dominant white spotting (W/Wv). The SI mutants are infertile due to Sertoli cell dysfunction whereas W mutant male mice are infertile due to germ cells dysfunction but with normal Sertoli cell function. The testicular transplantation of SSCs from SI mice into W mice restored fertility with donor-derived offspring (Ogawa et al. 2000). Several studies have subsequently shown that fertile donor-derived spermatozoa can be obtained by SSCT in several animal species (Herrid et al. 2019; Shetty et al. 2020).

The transplantation assay for evaluation of cellular viability of testicular stem cells can also be done at an orthotropic location either directly or after encapsulation into alginate matrix (Del Vento et al. 2019; Gül et al. 2020). The functionality of transplanted cells can be evaluated by histological analysis of the transplanted cells for spermatogenesis progression and generation of donor-derived sperm. Studies have also shown that mouse testicular cells, encapsulated into Matrigel™, could self-organize into seminiferous tubules upon subcutaneous injection into the dorsum of the nude mice (Gao et al. 2020). The functioning of testicular cells and SSCs can be tracked by pre-labelling the cells with PKH67 or DiI stain, which retains in the loaded cells for nearly two months (Dong et al. 2019; Mohaqiq et al. 2019). Under experimental settings, genetically modified cells with marker proteins such as enhanced green fluorescent protein (EGFP) under the control of sperm-specific promoters such as Acrosin (Acr) can also be utilized. Transgenic mice expressing EGFP under the control of Acr is already available commercially. Xeno-grafting is also an attractive strategy for fertility preservation in endangered animals and resurrection of extinct animals (Pukazhenthi et al. 2006).

In vitro generation of three-dimensional (3D) testicular organoids (TO) from cryopreserved testicular cells has also emerged as a novel strategy for assessment of cell viability (de Michele et al. 2017; Pendergraft et al. 2017; Vermeulen et al. 2019). The TOs consists of SSCs, germ cells and somatic cells (Sertoli cells, Leydig cells, peritubular Myoid cells) to resemble the testicular structure with interstitial compartments separated by basement membrane and can respond to various environmental cues (de Michele et al. 2017; Sakib et al. 2019; Vermeulen et al. 2018). The functionality of 3D TOs in terms of testosterone production and spermatogenesis events in testicular cells could be a good indicator for the success of cryopreservation (de Michele et al. 2017; Pendergraft et al. 2017).

9.12 In Vitro Spermatogenesis

In vitro spermatogenesis from SSC could be an excellent alternative to the in vivo SSCT. It was initially applied for mice testicular tissue (Sato et al. 2011) and was found to be useful for verifying the post-thaw viability of frozen-thawed mice testis (Yokonishi et al. 2014). The method involved organ culture of testicular tissue fragments at air-liquid interphase following which the in vitro produced sperm

could be used for production of offspring by IVF, ICSI or ROSI. The organotypic air-liquid interphase culture was also proven to be useful for in vitro spermatogenesis from testicular stem cells. The protocol required the introduction of SSCs into an allogenic or cadaver-derived testicular tissue before their organ culture at air-liquid interphase (Gül et al. 2020; Sato et al. 2011). Studies have shown that xenoxenic recipient testis can also be used for in vitro spermatogenesis from testicular stem cells (Mohaqiq et al. 2019) but complete spermatogenesis depends on the compatibility of spermatogenic cycle and phylogenetic distance between the species (Ntemou et al. 2019). Moreover, xenoxenic transplantation of SSCs for in vitro spermatogenesis may pose possible risks of zoonotic disease transmission and immunoreactions (Kaneko et al. 2013; Liu et al. 2016). An alternative to the requirement of cadaver-derived or xenogenic testis could be the use of tissue-engineered testicular constructs (Mohaqiq et al. 2019; Perrard et al. 2016; Rezaei Topraggaleh et al. 2019; Vermeulen et al. 2018). It is possible that the SSCs can be seeded into biomaterial-derived scaffolds and cultured ex vivo in an organotypic air-liquid interphase to achieve in vitro spermatogenesis. Macroporous 3D scaffolds have been developed using decellularized testicular matrix (DTM), which supported the differentiation of spermatogonial cells up to post-meiotic stage (Rezaei Topraggaleh et al. 2019). However, the testicular engineering approach for in vitro spermatogenesis is still at its infancy and, till date, no study has reported the production of fertile spermatozoa in both human and animals. A detailed review on in vitro spermatogenesis can be found elsewhere (Ibtisham and Honaramooz 2020; Pelzman et al. 2020).

9.13 Application of Testicular Stem Cell Cryopreservation

Semen cryopreservation is the most convenient and the first choice for fertility preservation in both human and animals. However, in instances wherein semen cryopreservation is not possible due to non-availability of sperm (e.g. pre-pubertal males, non-obstructive azoospermia etc.) or non-feasibility (e.g. posthumous reproduction, pre-meiotic barriers to spermatogenesis etc.), cryopreservation of testicular stem cells, or testicular tissue enriched in SSCs, offers alternate and attractive options. The latter is still under the experimental stage with limited demonstrated clinical applications in large animals and human (Valli-Pulaski et al. 2019). Nevertheless, fertility preservation through cryopreservation of testicular stem cells is indicated in both medical and non-medical conditions and, occasionally the only choice (Table 9.1). Testicular stem cell cryopreservation can also be used to preserve genetic resources of wild animals if sperm is not available (e.g. testis from morbid wild animal) or are difficult to cryopreserve. Furthermore, cryopreservation is of utmost importance for preservation of genetically modified testicular stem cells that are meant for transgenic animal production by SSCT or ARTs. The complexity and diversity of important applications of the testicular stem cell cryopreservation are discussed below.

Table 9.1 Indications and applications of testicular stem cells and their cryopreservation

Indications	Applications	Reasons
Banking of animal genetic resources	– Rapid dissemination of elite germplasm. – International transportation at low cost. – Preservation of near-threatened, vulnerable or endangered species.	– Introduction of genetic variation, minimization of inbreeding depression in captive breeding and resurrection of extinct and threatened animals.
Transgenesis and gene targeting	– Transgenic animal production. – Knock out animals. – Knock in animals.	– Genetic manipulation of testicular stem cells for production of transgenic animals by SSCT.
Regenerative medicine	– Deriving patient-specific stem cells. – Androgen reconstitution.	– Isolation of pluripotent maGS and GPS cells. – SSCT.
In vitro spermatogenesis	– Azoospermia.	– Pre-meiotic barriers to spermatogenesis.
Fertility restoration in males with impaired spermatogenesis	– Azoospermia. – Cryptorchidism. – Vasectomized individuals.	– Pre- or post-meiotic barriers to spermatogenesis.
Posthumous reproduction	– Reproduction from testicular tissue of deceased animals or human.	– Preservation of animal genetic resources. – Resurrection of extinct animal. – Surgical removal of testicular cells and tissues upon the death of patients.
Ageing	– Aged males.	– Reduced sperm count. – Impaired fertility and fecundity. – Accumulation of spontaneous mutations. – Pregnancy complications, birth defects and neurological disorders in offspring.
Gender reassignment	– Males undergoing gender reassignment.	– Gender dysphoria or failure of the process.
Fertility preservation in pre-pubertal males expecting gonadotoxic treatment	– Cancer.	– Gonadotoxicity of radiation and chemotherapy.
Fertility restoration in males expecting bone marrow transplantation	– Sickle cell anaemia. – Thalassemia. – Drepanocytosis. – Idiopathic medulla aplasia. – Chronic granulomatous disease.	– Gonadotoxicity of pre-conditioning radiation and chemotherapy.

(continued)

Table 9.1 (continued)

Indications	Applications	Reasons
Fertility preservation and restoration in males with infectious diseases	– Immunodeficiency viruses (e.g. HIV, CIV, FIV etc.).	– Impaired fertility. – Sexual transmission of pathogen.
Fertility preservation and restoration in males with autoimmune diseases	– Systemic sclerosis. – Systemic lupus erythematosus. – Crohn's disease.	– Abnormal semen parameters, erectile dysfunction and ejaculation failures. – Gonadotoxicity of radiation and chemotherapy before bone marrow transplantation.
Fertility restoration in males with genetic diseases	– Klinefelter syndrome.	– Progressive degeneration of SSCs and impaired spermatogenesis at a later stage of life.

9.14 Fertility Restoration in Males with Pre- and Post-meiotic Barriers to Spermatogenesis

Among various causes of male infertility such as obstruction to vas deference, orchitis, hormonal disorders, etc., spermatogenic defects contribute ~70 to 90% to male infertility. In livestock animals such as cattle and buffalo, this is generally handled by use of donor-derived frozen-thawed sperm for artificial insemination. However, preservation of male infertility become pertinent in elite bulls, race horses of high genetic merit, pets, and wild animals. In such cases, cryopreservation of testicular stem cells can be envisaged as a powerful tool for preservation and restoration of male fertility. The testicular stem cells may be isolated from the testicular biopsy and cryopreserved by freezing or vitrification to restore the fertility by SSCT or other ARTs. More importantly, cryobanking of testicular stem cells may offer fertility solutions for azoospermic males with defective or immature spermatogenesis due to congenital or acquired conditions. In such cases, the testicular stem cells can be isolated from biopsied testicular tissue and differentiated in vitro to produced spermatozoa for ICSI or ROSI.

Cryptorchidism could be yet another reason that may necessitate cryopreservation of testicular stem cells in males of high genetic merit or transgenic animals. Testis that fails to descent into extra-abdominal scrotum undergoes a progressive reduction in germ cells, defective germ cell maturation and testicular dysfunction due to thermal stress (Docampo and Hadziselimovic 2015; Lee and Coughlin 2001). In such cases, testicular stem cells may be isolated from testis at an early stage, before the herald of the germ cell degeneration, and cryopreserved for restoration of fertility by in vitro spermatogenesis or ectopic grafting (Makala et al. 2015; Wyns et al. 2007). In cryptorchidism, the possibility of fertility restoration by transplantation of cryopreserved testicular stem cells is very bleak because of simultaneous impairment

of somatic cell (Leydig cells, Sertoli cells) functionality. Thus, in vitro spermatogenesis is necessary to restore fertility from cryopreserved testicular stem cells.

Castration and vasectomy are effective methods of neutering in animals for making them docile. Vasectomy is also practiced in adult human for sterilization. There may be instances (e.g. death of the offspring) wherein reversal of male fertility is desired. However, the success of vasovasostomy or epididymovasostomy reversal procedure varies with the duration of vasectomy and may even fail (Fuchs and Burt 2002). Semen or testicular tissue freezing before vasectomy could be an option in such instances. The testicular stem cells, isolated from cryopreserved testicular tissue, can be used for restoration of fertility.

9.15 Male Fertility in Ageing

Cryopreservation of testicular stem cells may also be warranted in non-medical reasons such as ageing. It can offer the opportunity for restoration of fertility in males who have become aged or in cases of postponed parenthood. The later can also be applied for obtaining offspring from animals that have stopped ejaculation or are unsuitable for breeding due to ageing. Recent studies have shown that reduction in sperm count can occur with increasing age (Brandt et al. 2019; Levine et al. 2017) and therefore, can reduce the fertility and fecundity. Sperm from aged individuals also increases the risk of pregnancy complications, accumulation of spontaneous mutations, birth defects and neurological disorders in offspring (Su et al. 2015; Taylor et al. 2019). Thus, preservation of testicular stem cells may become necessary in such cases.

9.16 Posthumous Reproduction

Cryopreservation of testicular cells offers an opportunity to obtain live offspring from deceased individuals in both human and animals. The sperm or testicular tissue can be collected from peri- or post-mortem males and cryopreserved for future procreation of offspring (Check et al. 2002). It human, it has been applied in rare circumstances of unexpected death of the male partner, and birth of live offspring have been reported (Batzer et al. 2003; Check et al. 2002) although it poses several ethical and legal challenges to both the family and the society at large (Hurwitz and Batzer 2004).

In animals, cryopreservation of testicular specimens offers opportunity for posthumous reproduction in elite bulls or from testicular tissue samples that may have been found accidently from wild animals. The testicular stem cells, combined with in vitro spermatogenesis or testicular transplantation in recipient testis, can restore fertility. The success of such procedure, however, depends on the availability of live testicular stem cells and/or precursor cells and reduces with the increase in the duration of sample collection (Batzer et al. 2003). Depending on the availability of

the cell type and their viability, the collected sample may be used for IVF, ICSI, ROSI or in vitro spermatogenesis.

9.17 Fertility Preservation and Restoration in Pre-pubertals with Oncological Conditions

Testicular dysfunction can occur due to testicular cancer as well as non-testicular neoplasm such as leukaemia. The oncological conditions may either have a direct effect on gonadal function or may indirectly cause testicular dysfunction due to prolonged hyperthermia, cytokine storm, cachexia and nutritional deficiencies of vitamins, minerals and trace elements, etc. (Dohle 2010). While most cancer cases (e.g. leukaemia) remain to have high mortality rate, many neoplastic conditions (e.g. Hodgkin's lymphoma, testicular tumours) may eventually result in the long-term survival of patients upon treatment (Crha et al. 2009). Unfortunately, radiation and chemotherapy for cancer treatment are generally cytotoxic to rapidly dividing cells, including testicular stem cells, and predispose the surviving males to testicular failure and infertility (Duca et al. 2019). In human, radiation and chemotherapy have been reported to result in azoospermia in ~25% of cancer cases (Green et al. 2010) although such systematic studies on infertility analysis in animals are lacking. Thus, cryopreservation of germ cells is crucial for fertility preservation and restoration in cancer patients expecting gonadotoxic treatment. The semen, cryopreserved prior to initiation of gonadotoxic treatment could be used for treatment of infertility. However, this option is not available in pre-pubertal and paediatric males, which have not yet begun their sperm production. In such cases, cryopreservation of testicular stem cells, or testicular tissue enriched in SSCs, are the only options for fertility preservation and restoration (Lakhoo et al. 2019). The transplantation of cryopreserved testicular stem cells, isolated from the patient's own testis, allows autologous transplantation, and thereby, not only avoids potential immune rejection but also is ethically acceptable. Thus, isolation, cryopreservation, and testicular transplantation of SSCs are increasingly proposed as a method of choice for restoration of fertility in cancerous individuals in which SSC populations is expected to deplete due to radiation or chemotherapy. Several studies have shown very encouraging results of autologous SSCT in rodents and primates (Ginsberg et al. 2010). However, it is yet to be applied in large animals and human due to several unresolved technical and safety concerns.

One of the biggest safety concerns with SSC cryopreservation and autologous transplantation in cancer cases has been the potential risk of reintroducing cancerous cells upon SSCT. Experiments have shown that presence of 20 cancerous cells in cryopreserved testicular cells from leukemic rats was enough to re-establish malignancy in 3 out of 5 non-leukemic recipient rats (Jahnukainen et al. 2001). Thus, purification of SSCs from contaminating cancerous cells is necessary prior to their SSCT in treated and cured cancer cases (Hermann et al. 2011). Purification of SSCs may be achieved by fluorescence-activated cell sorting (FACS) or magnetic-assisted cell sorting (MACS) using specific antibodies for cancerous cells (e.g. CD4 for

T-cell leukaemia) (Fujita et al. 2006; Geens et al. 2007; Hou et al. 2007, 2009; Tian et al. 2019). Long-term culture of testicular cells in SSC-selective media that does not support cancer cell growth has also been tried for elimination of cancerous cells (Sadri-Ardekani and Atala 2014). However, sorting of cells by FACS or MACS or their selective culture in SSC-selective media were met with limited success for clinical use (Geens et al. 2007; Hou et al. 2007, 2009).

A practical and feasible alternative to FACS or MACS-based elimination of cancerous cells for SSCT could be in vitro spermatogenesis of spermatozoa and production of offspring by IVF, ICSI or ROSI (Yokonishi et al. 2014). The cryopreserved SSCs can be seeded into allogenic, xenogenic or a cadaver-derived testicular tissue and organ cultured at air-liquid interphase for in vitro spermatogenesis (Gül et al. 2020; Ntemou et al. 2019; Sato et al. 2011). Unfortunately, this later strategy has not been optimized in most animal species and human. An alternative to the requirement of donor-derived testicular tissue for in vitro spermatogenesis could be the use of biomaterial-derived tissue-engineered testes (Mohaqiq et al. 2019; Perrard et al. 2016; Rezaei Topraggaleh et al. 2019). However, complete in vitro spermatogenesis in tissue engineered testis remains to be demonstrated (Mohaqiq et al. 2019; Portela et al. 2019).

9.18 Fertility Preservation and Restoration in Non-oncological Diseases

Non-oncological diseases such as autoimmune diseases, haematological disorders, spinal cord injuries, severe trauma to testis, etc. may result in testicular dysfunction and ejaculatory failure. Autoimmune diseases such as systemic lupus erythematosus, multiple sclerosis, and Crohn's disease are also often associated with abnormal semen parameters, erectile dysfunction and ejaculation failures (Fode et al. 2012). Further, preconditioning chemotherapy or whole-body irradiation before bone marrow transplantation in these patients entails a high risk of gonadal dysfunction (Snowden et al. 2017). In human, ~85% of patients experienced azoospermia after preconditioning chemotherapy or radiation for bone marrow transplantation (Anserini et al. 2002). Similarly, 3 out of 5 pre-pubertal individuals, who underwent hematopoietic stem cell transplantation for sickle cell anaemia, experienced azoospermia (Lukusa et al. 2009). Thus, in such cases, in the absence of semen for cryopreservation, banking of testicular stem cells, or testicular tissue enriched in SSCs, is recommended prior to corresponding therapies.

Cryopreservation of testicular stem cells is also an alternative to semen preservation in individuals affected with serious infectious diseases with potential of transmission *via* semen or during mating (e.g. immunodeficiency viruses). The repeat sperm washing procedure may reduce the pathogen load but remains a risk to the offspring as well as sero-negative partner, even from males with undetectable serum load of pathogen (Nicopoullos et al. 2011). Further, the duration of infection, patient's age, immune status, treatment regimen, etc. also reduces the semen volume, sperm concentration, sperm viability and their fertilizability (Bujan et al. 2007;

Wang et al. 2014). On the other hand, unlike semen, the testicular stem cells can be screened extensively before in vitro spermatogenesis or SSCT to reduce the risk of disease transmission. The repeat sub-culture steps, differentiation protocols and advanced molecular tools for detection and sorting of purified population of testicular stem cells can significantly reduce or remove the risk of disease transmission altogether.

Cryopreservation of testicular stem cells may also be warranted for fertility preservation prior to pre-conditioning radiation or chemotherapy for bone marrow transplantation in non-infectious genetic diseases such as sickle cell anaemia, thalassemia, Drepanocytosis, idiopathic medulla aplasia, chronic granulomatous disease, etc. It may also be required for early preservation of male fertility in genetic diseases such as Klinefelter syndrome in which degeneration of germ cells is seen at a later stage of life. In Klinefelter syndrome, the loss of SSCs may start at pre-pubertal stage and meiotic arrest is seen at the later stage of life due to aneuploid spermatogonia (Vialard et al. 2012; Wikström and Dunkel 2008). Thus, in such genetic diseases, it becomes imperative to cryopreserve testicular stem cells at an early stage of life. Since individuals with Klinefelter syndrome have fibrosed and hyalinized seminiferous tubules, which may not support spermatogenesis upon SSCT, in vitro spermatogenesis of cryopreserved testicular stem cells will be of immense utility (Braye et al. 2019; Wikström and Dunkel 2008). Importantly, transplantation of autologous cryopreserved testicular stem cells in genetic diseases such as Klinefelter syndrome of AZF mutations will not prevent the vertical transmission of mutations or genetic diseases to the progeny per se. However, genetic correction of GS cells using gene editing technologies such as CRISPR-Cas9 is a possibility in future for generation of mutation-free spermatozoa and healthy fertile offspring.

9.19 Animal Transgenesis and Genome Editing

Manipulation of testicular stem cells has immense potential for transgenic animal production. This is of particular importance in livestock animal species such as cattle and pigs in which existing methods of transgenesis are not efficient. Moreover, it can be combined with newer genome editing tools such as Zinc Finger Nucleases (ZFNs), Transcription Activator-Like Effector Nucleases (TALENs) and Clustered Regularly Interspaced Short Palindromic Repeats-Cas9 (CRISPR-Cas9) system for knock-in and knock-out animal production for understanding the gene function through gain-of-function and loss-of-loss function studies (Koppes et al. 2020; Park et al. 2017; Zhang et al. 2020). These genetically engineered stem cells are created through a lengthy and cumbersome process and are novel to be cryopreserved for later use. An improper cryopreservation protocol for these genetically modified testicular stem cells can ruin the work of several months and years.

The application of genome editing tools is also expected to broaden the application of testicular stem cell cryopreservation to radically change our approach for infertility treatment. The CRISPR-Cas9 system has been successfully applied to

testicular stem cells for genetic correction and production of disease-free individuals or for treatment of infertility through correction of underlying genetic mutation (Wu et al. 2015). Cryopreservation of these gene edited cells is essential for preservation of mutation-free testicular stem cells until the individuals are ready for transplantation. Nevertheless, the genome editing tools require refinements and remain a farfetched goal for both animal and human. The current application of these genome editing is restricted to their application in the generation of model animals for unravelling the gene function.

Gene targeting and genome editing tools can also be used for differentiation and in vitro spermatogenesis from cryopreserved testicular stem cells. Several molecules such as SCF/c-kit system, Dazl RNA binding protein, cyclin D2 and D3 and retinoic acid have been shown to promote differentiation of testicular stem cells. The SCF/c-kit system upregulates cyclin D3 and promotes cell cycle progression in spermatogonia through a rapamycin-sensitive PI3K/p70 S6 kinase pathway (Feng et al. 2000). On the other hand, targeted disruption of Dazl (deleted in azoospermia-like) RNA-binding protein inhibited the differentiation of Aal spermatogonia into A1 (Schrans-Stassen et al. 2001) while transfection of ES cells with Daz gene family (Dazl, Daz and Boule) promoted their in vitro differentiation into germ cell lineage to form haploid gamete in both mouse (Yu et al. 2009) and human (Kee et al. 2009). In another study, in vitro production of haploid sperm cells was shown to occur from male germ cells of fetal cattle when they were treated with retinoic acid (Dong et al. 2010). There are several other factors such as cyclin-dependent kinases (cdks) and cyclins (Sicinski et al. 1996; Tsutsui et al. 1999), Notch-1 signalling molecules (Hofmann et al. 2005), which are involved in the differentiation of spermatogonia but need further investigation for their application in establishing a system for in vitro spermatogenesis from cryopreserved testicular stem cells.

9.20 Banking for Preservation and International Movement of Animal Genetic Resources

Cryopreservation of testicular stem cells has immense utility in the banking of animal genetic resources, rapid dissemination of elite germplasm and their international transportation at low cost. In last few decades, the genetic diversity of both domestic and wild animals has considerably declined due to rapid urbanization, intensification of agriculture and deforestation (Kristensen et al. 2015). Therefore, several countries have established cryobanks for storage of animal genetic resources wherein cryopreservation of testicular stem cells has been viewed as an important option when semen cryopreservation was not possible. Testicular cell and tissue cryopreservation have also been used for preservation of near-threatened, vulnerable or endangered species (Bashawat et al. 2020; da Silva et al. 2019). Use of sperm from cryopreserved testes allows introduction of genetic variation and minimizes the inbreeding depression in captive breeding. The testicular cells and tissue for cryopreservation can be obtained from living organisms as well as from accidently found dead cadavers for resurrection of the species (Higaki et al. 2017). It has been

successfully applied in preserving the testis of few endangered species such as Cheetah (*Acinonyx jubatus*), Asiatic golden cat (*Catopuma temminckii*) (Bashawat et al. 2020), Collared peccary (*Pecari tajacu*) (da Silva et al. 2019), Cyprinid honmoroko (*Gnathopogon caerulescens*) (Higaki et al. 2017), Hog deer (*Hyelaphus porcinus*), Barking deer (*Muntiacus muntjak*), Sambar deer (*Rusa unicolor*) (Pothana et al. 2017) and Indian spotted mouse deer (*Moschiola indica*) (Pothana et al. 2015).

9.21 Challenges and Future Perspectives

Cryopreservation of testicular stem cells suffers from sub-optimal cryopreservation protocols. Most laboratories have borrowed and used the freezing or vitrification protocols from somatic cells or embryonic stem cells to testicular stem cells. Systematic studies on optimization of freezing and vitrification protocols have been very limited and therefore, the viability remains to be low (typically, 50-60%) in most cases. While various factors such as rate of cooling, volume of sample, hydrostatic pressure, type and concentration of CPA, duration of CPA-exposure, use of ROS scavengers or anti-apoptotic agents, epigenetic modifiers, etc. have been reported to affect the cryoinjuries to influence the success of cryopreservation in various cell types, such studies on testicular stem cells have been very limited. Furthermore, fertility preservation and restoration through cryopreservation of testicular stem cells also require costly and lengthy processes of stem cell isolation, characterization and SSCT or in vitro spermatogenesis to be followed by IVF, ICSI or ROSI (Costa et al. 2017; Hermann et al. 2007; Kim et al. 2015; Pacchiarotti et al. 2013; Wu et al. 2012). Several studies have shown successful cryopreservation of testicular stem cells, which could colonize in the empty seminiferous tubules of the recipient testis for donor-derived spermatogenesis and restoration of male fertility (Kanatsu-Shinohara et al. 2003; Wu et al. 2012). However, such studies are yet to be optimized for large animal species such as cattle, buffaloes, pigs, sheep, goats, horses, dogs, cats, and most of the wild animals.

Another challenge with SSC-based fertility preservation is the low number of viable cells in cryopreserved samples, which might be insufficient for restoration of fertility by SSCT. Gonadal ablation of recipient testis by busulfan treatment or irradiation can improve the colonization of transplanted SSCs into recipient testis by depletion of endogenous SSCs (Shetty et al. 2018). However, it not only compromises the viability and functionality of endogenous Sertoli and Leydig cells in recipient testis but also may be lethal to animals due to possible aplacia in bone marrow. Gonadal ablation of endogenous germ cells is also possible by intratesticular injection of less toxic Dolichos biflorus agglutinin or aplantlectin, which specifically binds to spermatogonia and eliminates endogenous germ cells (Herrid et al. 2019) but remains less explored. Currently, gonadal ablation is practiced only in cases where the goal is to verify the stemness of SSCs or to produce transgenic animals *via* donor-derived spermatogenesis. In other studies, attempts have also been made to improve the success of SSCT by specifically

eliminating the endogenous germ cells using gene editing tools, without affecting the stem cell niche. Knock out of Nanos2 by CRISPR/Cas9 system in boars resulted in loss of germ cells without any adverse effect on interstitial cells (Park et al. 2017). However, such knock out-mediated germ cell depletion requires extensive skills and cost, and are not feasible in large animals and human.

In fertility restoration, a possible solution to low number of viable SSCs could be their in vitro expansion prior to SSCT. However, in vitro cultured SSC may pose a risk of genetic or epigenetic instability. Co-transplantation of SSCs with niche components (e.g. Sertoli cells, Leydig cells) or mesenchymal stem cells (MSCs) could also be an alternative to improve the efficiency of SSCT (Kadam et al. 2018, 2019). It has been shown that co-transplantation of SSCs with TGFβ1-treated MSCs could improve the success of SSCT. In fact, co-transplantation of SSCs and MSCs reduced the requirement of SSCs to half of its number (Kadam et al. 2019).

De novo formation of seminiferous tubules and in vitro spermatogenesis are also emerging as a potential alternative to SSCT from cryopreserved testicular stem cells (Patra et al. 2021; Shetty et al. 2018). In vitro spermatogenesis of sperm from cryopreserved testicular stem cells has been demonstrated in rodents and primates (Sato et al. 2011; Yokonishi et al. 2014). However, its usefulness and long-term safety is yet to be confirmed in most of the other large animals and in human.

9.22 Conclusions

Cryopreservation of testicular stem cells is rapidly emerging as a potential approach for preservation of fertility in both human and animals. It has also shown promising results in preservation of germplasm in endangered wild animals for posthumous reproduction and has become indispensable for preservation of fertility in pre-pubertal or paediatric males. Recent development of air-liquid interphase culture of testicular stem cells has resulted in in vitro spermatogenesis and may eliminate the need of SSCT and potential risk of re-introducing malignant cells into pre-pubertal individuals expecting gonadotoxic treatments. The combination of cryopreservation protocols with in vitro spermatogenesis of testicular stem cells is thus, expected to provide their bench top application in clinics. Although very few systematic studies have been conducted for optimization of freezing or vitrification of testicular stem cells, newer methods and strategies of cryopreservation are rapidly evolving. Results from rodent studies are very promising and future research and clinical trials are expected to provide better results in livestock animals and human. Further, rapid advancements in genome editing tools such as CRISPR-Cas9 will broaden the application of testicular stem cell cryopreservation to radically change our approach for infertility treatment. However, cryopreservation protocols for testicular cells and tissues are still sub-optimal and their success depends on several variable factors, including species variation and lab-to-lab variations. The protocol for in vitro spermatogenesis has also not been validated in human and large animals. Thus, while promising results have been obtained in experimental animals, birth of live offspring from pre-pubertal testes is not yet reported in human and large animals.

Further studies are required for optimization of freezing and vitrification protocols including the optimization of cooling rates, finding new substitutes for non-toxic CPAs, standardization of solutions for freezing, equilibration and vitrification, and to assess the long-term safety of the procedures in offspring derived from cryopreserved testicular stem cells.

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References

Abrishami M, Anzar M, Yang Y, Honaramooz A (2010) Cryopreservation of immature porcine testis tissue to maintain its developmental potential after xenografting into recipient mice. Theriogenology 73:86–96

Aliakbari F, Gilani MA, Amidi F, Baazm M, Korouji M, Izadyar F, Yazdekhasti H, Abbasi M (2016) Improving the efficacy of cryopreservation of spermatogonia stem cells by antioxidant supplements. Cell Reprogram 18:87–95

Anserini P, Chiodi S, Spinelli S, Costa M, Conte N, Copello F, Bacigalupo A (2002) Semen analysis following allogeneic bone marrow transplantation. Additional data for evidence-based counselling. Bone Marrow Transplant 30:447–451

Arkoun B, Galas L, Dumont L, Rives A, Saulnier J, Delessard M, Rondanino C, Rives N (2019) Vitamin E but not GSH decreases reactive oxygen species accumulation and enhances sperm production during in vitro maturation of frozen-thawed prepubertal mouse testicular tissue. Int J Mol Sci 20:5380

Avarbock MR, Brinster CJ, Brinster RL (1996) Reconstitution of spermatogenesis from frozen spermatogonial stem cells. Nat Med 2:693–696

Baert Y, Goossens E, van Saen D, Ning L, Tournaye H (2012) Orthotopic grafting of cryopreserved prepubertal testicular tissue: in search of a simple yet effective cryopreservation protocol. Fertil Steril 97:1151–1152

Bashawat M, Braun BC, Müller K (2020) Cell survival after cryopreservation of dissociated testicular cells from feline species. Cryobiology 97:191–197

Batzer FR, Hurwitz JM, Caplan A (2003) Postmortem parenthood and the need for a protocol with posthumous sperm procurement. Fertil Steril 79:1263–1269

Bebbere D, Pinna S, Nieddu S, Natan D, Arav A, Ledda S (2019) Gene expression analysis of ovine prepubertal testicular tissue vitrified with a novel cryodevice (E.Vit). J Assist Reprod Genet 36:2145–2154

Bielanski A, Nadin-Davis S, Sapp T, Lutze-Wallace C (2000) Viral contamination of embryos cryopreserved in liquid nitrogen. Cryobiology 40:110–116

Boroujeni MB, Peidayesh F, Pirnia A, Boroujeni NB, Ahmadi SAY, Gholami M (2019) Effect of selenium on freezing-thawing damage of mice spermatogonial stem cell: a model to preserve fertility in childhood cancers. Stem Cell Investig 6:36

Brandt JS, Cruz Ithier MA, Rosen T, Ashkinadze E (2019) Advanced paternal age, infertility, and reproductive risks: a review of the literature. Prenat Diagn 39:81–87

Braye A, Tournaye H, Goossens E (2019) Setting up a cryopreservation programme for immature testicular tissue: lessons learned after more than 15 years of experience. Clin Med Insights Reprod Health 13:1179558119886342

Bujan L, Sergerie M, Moinard N, Martinet S, Porte L, Massip P, Pasquier C, Daudin M (2007) Decreased semen volume and spermatozoa motility in HIV-1-infected patients under antiretroviral treatment. J Androl 28:444–452

Check ML, Check JH, Summers-Chase D, Choe JK, Check DJ, Nazari A (2002) Live birth after posthumous testicular sperm aspiration and intracytoplasmic sperm injection with cryopreserved sperm: case report. Clin Exp Obstet Gynecol 29:95–96

Cheung RCF, Ng TB, Wong JH (2017) Antifreeze proteins from diverse organisms and their applications: an overview. Curr Protein Pept Sci 18:262–283

Costa GMJ, Avelar GF, Lacerda S, Figueiredo AFA, Tavares AO, Rezende-Neto JV, Martins FGP, França LR (2017) Horse spermatogonial stem cell cryopreservation: feasible protocols and potential biotechnological applications. Cell Tissue Res 370:489–500

Crha I, Ventruba P, Zakova J, Huser M, Kubesova B, Hudecek R, Jarkovsky J (2009) Survival and infertility treatment in male cancer patients after sperm banking. Fertil Steril 91:2344–2348

Curaba M, Verleysen M, Amorim CA, Dolmans MM, Van Langendonckt A, Hovatta O, Wyns C, Donnez J (2011) Cryopreservation of prepubertal mouse testicular tissue by vitrification. Fertil Steril 95:1229–1234

da Silva AM, Bezerra LGP, Praxedes ECG, Moreira SSJ, de Souza CMP, de Oliveira MF, Pereira AF, Comizzoli P, Silva AR (2019) Combination of intracellular cryoprotectants preserves the structure and the cells proliferative capacity potential of adult collared peccary testicular tissue subjected to solid surface vitrification. Cryobiology 91:53–60

de Michele F, Poels J, Weerens L, Petit C, Evrard Z, Ambroise J, Gruson D, Wyns C (2017) Preserved seminiferous tubule integrity with spermatogonial survival and induction of Sertoli and Leydig cell maturation after long-term organotypic culture of prepubertal human testicular tissue. Hum Reprod 32:32–45

Del Vento F, Vermeulen M, Ucakar B, Poels J, Rieux A, Wyns C (2019) Significant benefits of nanoparticles containing a necrosis inhibitor on mice testicular tissue autografts outcomes. Int J Mol Sci 20:5833

Docampo MJ, Hadziselimovic F (2015) Molecular pathology of cryptorchidism-induced infertility. Sex Dev 9:269–278

Dohle GR (2010) Male infertility in cancer patients: review of the literature. Int J Urol 17:327–331

Dong WZ, Hua JL, Shen WZ, Dou ZY (2010) In vitro production of haploid sperm cells from male germ cells of foetal cattle. Anim Reprod Sci 118:103–109

Dong L, Gul M, Hildorf S, Pors SE, Kristensen SG, Hoffmann ER, Cortes D, Thorup J, Andersen CY (2019) Xeno-free propagation of spermatogonial stem cells from infant boys. Int J Mol Sci 20:5390

Duca Y, Di Cataldo A, Russo G, Cannata E, Burgio G, Compagnone M, Alamo A, Condorelli RA, La Vignera S, Calogero AE (2019) Testicular function of childhood cancer survivors: who is worse? J Clin Med 8:2204

Dumont L, Arkoun B, Jumeau F, Milazzo JP, Bironneau A, Liot D, Wils J, Rondanino C, Rives N (2015) Assessment of the optimal vitrification protocol for pre-pubertal mice testes leading to successful in vitro production of flagellated spermatozoa. Andrology 3:611–625

Fahy GM, Wowk B, Wu J, Paynter S (2004) Improved vitrification solutions based on the predictability of vitrification solution toxicity. Cryobiology 48:22–35

Feng LX, Ravindranath N, Dym M (2000) Stem cell factor/c-kit up-regulates cyclin D3 and promotes cell cycle progression via the phosphoinositide 3-kinase/p70 S6 kinase pathway in spermatogonia. J Biol Chem 275:25572–25576

Finkel T, Holbrook NJ (2000) Oxidants, oxidative stress and the biology of ageing. Nature 408:239–247

Fode M, Krogh-Jespersen S, Brackett NL, Ohl DA, Lynne CM, Sønksen J (2012) Male sexual dysfunction and infertility associated with neurological disorders. Asian J Androl 14:61–68

Fountain D, Ralston M, Higgins N, Gorlin JB, Uhl L, Wheeler C, Antin JH, Churchill WH, Benjamin RJ (1997) Liquid nitrogen freezers: a potential source of microbial contamination of hematopoietic stem cell components. Transfusion 37:585–591

Frederickx V, Michiels A, Goossens E, De Block G, Van Steirteghem AC, Tournaye H (2004) Recovery, survival and functional evaluation by transplantation of frozen-thawed mouse germ cells. Hum Reprod 19:948–953

Fuchs EF, Burt RA (2002) Vasectomy reversal performed 15 years or more after vasectomy: correlation of pregnancy outcome with partner age and with pregnancy results of in vitro fertilization with intracytoplasmic sperm injection. Fertil Steril 77:516–519

Fujita K, Tsujimura A, Miyagawa Y, Kiuchi H, Matsuoka Y, Takao T, Takada S, Nonomura N, Okuyama A (2006) Isolation of germ cells from leukemia and lymphoma cells in a human in vitro model: potential clinical application for restoring human fertility after anticancer therapy. Cancer Res 66:11166–11171

Gao H, Liu C, Wu B, Cui H, Zhao Y, Duan Y, Gao F, Gu Q, Wang H, Li W (2020) Effects of different biomaterials and cellular status on testicular cell self-organization. Adv Biosyst 2020:1900292

Geens M, Van de Velde H, De Block G, Goossens E, Van Steirteghem A, Tournaye H (2007) The efficiency of magnetic-activated cell sorting and fluorescence-activated cell sorting in the decontamination of testicular cell suspensions in cancer patients. Hum Reprod 22:733–742

Gholami M, Saki G, Hemadi M, Khodadadi A, Mohamma-di-Asl J (2013) Effect of melatonin on the expression of apoptotic genes in vitrified-thawed spermatogonia stem cells type A of 6-day-old mice. Iran J Basic Med Sci 16:906–909

Ginsberg JP, Carlson CA, Lin K, Hobbie WL, Wigo E, Wu X, Brinster RL, Kolon TF (2010) An experimental protocol for fertility preservation in prepubertal boys recently diagnosed with cancer: a report of acceptability and safety. Hum Reprod 25:37–41

Goossens E, Frederickx V, Geens M, De Block G, Tournaye H (2008) Cryosurvival and spermatogenesis after allografting prepubertal mouse tissue: comparison of two cryopreservation protocols. Fertil Steril 89:725–727

Goossens E, Bilgec T, Van Saen D, Tournaye H (2011) Mouse germ cells go through typical epigenetic modifications after intratesticular tissue grafting. Hum Reprod 26:3388–3400

Gouk SS, Loh YF, Kumar SD, Watson PF, Kuleshova LL (2011) Cryopreservation of mouse testicular tissue: prospect for harvesting spermatogonial stem cells for fertility preservation. Fertil Steril 95:2399–2403

Green DM, Kawashima T, Stovall M, Leisenring W, Sklar CA, Mertens AC, Donaldson SS, Byrne J, Robison LL (2010) Fertility of male survivors of childhood cancer: a report from the Childhood Cancer Survivor Study. J Clin Oncol 28:332–339

Gül M, Dong L, Wang D, Diri MA, Andersen CY (2020) Surrogate testes: allogeneic spermatogonial stem cell transplantation within an encapsulation device may restore male fertility. Med Hypotheses 139:109634

Gupta MK, Lee HT (2010) Cryopreservation of oocytes and embryos by vitrification. Clin Exp Reprod Med 37:267–291

Gupta MK, Uhm SJ, Lee HT (2007) Cryopreservation of immature and in vitro matured porcine oocytes by solid surface vitrification. Theriogenology 67:238–248

Gupta MK, Uhm SJ, Lee HT (2010) Effect of vitrification and beta-mercaptoethanol on reactive oxygen species activity and in vitro development of oocytes vitrified before or after in vitro fertilization. Fertil Steril 93:2602–2607

Gurtovenko AA, Anwar J (2007) Modulating the structure and properties of cell membranes: the molecular mechanism of action of dimethyl sulfoxide. J Phys Chem B 111:10453–10460

Ha SJ, Kim BG, Lee YA, Kim YH, Kim BJ, Jung SE, Pang MG, Ryu BY (2016) Effect of antioxidants and apoptosis inhibitors on cryopreservation of murine germ cells enriched for spermatogonial stem cells. PLoS One 11:e0161372

Hermann BP, Sukhwani M, Lin CC, Sheng Y, Tomko J, Rodriguez M, Shuttleworth JJ, McFarland D, Hobbs RM, Pandolfi PP, Schatten GP, Orwig KE (2007) Characterization, cryopreservation, and ablation of spermatogonial stem cells in adult rhesus macaques. Stem Cells 25:2330–2338

Hermann BP, Sukhwani M, Salati J, Sheng Y, Chu T, Orwig KE (2011) Separating spermatogonia from cancer cells in contaminated prepubertal primate testis cell suspensions. Hum Reprod 26:3222–3231

Herrid M, Nagy P, Juhasz J, Morrell JM, Billah M, Khazanehdari K, Skidmore JA (2019) Donor sperm production in heterologous recipients by testis germ cell transplantation in the dromedary camel. Reprod Fertil Dev 31:538–546

Higaki S, Kuwata N, Tanaka K, Tooyama I, Fujioka Y, Sakai N, Takada T (2017) Successful vitrification of whole juvenile testis in the critically endangered cyprinid honmoroko (Gnathopogon caerulescens). Zygote 25:652–661

Hofmann MC, Braydich-Stolle L, Dym M (2005) Isolation of male germ-line stem cells; influence of GDNF. Dev Biol 279:114–124

Hou M, Andersson M, Zheng C, Sundblad A, Söder O, Jahnukainen K (2007) Decontamination of leukemic cells and enrichment of germ cells from testicular samples from rats with Roser's T-cell leukemia by flow cytometric sorting. Reproduction 134:767–779

Hou M, Andersson M, Zheng C, Sundblad A, Söder O, Jahnukainen K (2009) Immunomagnetic separation of normal rat testicular cells from Roser's T-cell leukaemia cells is ineffective. Int J Androl 32:66–73

Huang JY, Chen HY, Park JY, Tan SL, Chian RC (2008) Comparison of spindle and chromosome configuration in in vitro- and in vivo-matured mouse oocytes after vitrification. Fertil Steril 90:1424–1432

Hurwitz JM, Batzer FR (2004) Posthumous sperm procurement: demand and concerns. Obstet Gynecol Surv 59:806–808

Ibtisham F, Honaramooz A (2020) Spermatogonial stem cells for in vitro spermatogenesis and in vivo restoration of fertility. Cell 9:745

Iwatani M, Ikegami K, Kremenska Y, Hattori N, Tanaka S, Yagi S, Shiota K (2006) Dimethyl sulfoxide has an impact on epigenetic profile in mouse embryoid body. Stem Cells 24:2549–2556

Izadyar F, Matthijs-Rijsenbilt JJ, den Ouden K, Creemers LB, Woelders H, de Rooij DG (2002) Development of a cryopreservation protocol for type A spermatogonia. J Androl 23:537–545

Jahnukainen K, Hou M, Petersen C, Setchell B, Söder O (2001) Intratesticular transplantation of testicular cells from leukemic rats causes transmission of leukemia. Cancer Res 61:706–710

Jung SE, Ahn JS, Kim YH, Kim BJ, Won JH, Ryu BY (2020a) Effective cryopreservation protocol for preservation of male primate (Macaca fascicularis) prepubertal fertility. Reprod Biomed Online 41(6):1070–1083

Jung SE, Kim M, Ahn JS, Kim YH, Kim BJ, Yun MH, Auh JH, Ryu BY (2020b) Effect of equilibration time and temperature on murine spermatogonial stem cell cryopreservation. Biopreserv Biobank 18:213–221

Kadam P, Ntemou E, Baert Y, Van Laere S, Van Saen D, Goossens E (2018) Co-transplantation of mesenchymal stem cells improves spermatogonial stem cell transplantation efficiency in mice. Stem Cell Res Ther 9:317

Kadam P, Ntemou E, Onofre J, Van Saen D, Goossens E (2019) Does co-transplantation of mesenchymal and spermatogonial stem cells improve reproductive efficiency and safety in mice? Stem Cell Res Ther 10:310

Kanatsu-Shinohara M, Ogonuki N, Inoue K, Ogura A, Toyokuni S, Shinohara T (2003) Restoration of fertility in infertile mice by transplantation of cryopreserved male germline stem cells. Hum Reprod 18:2660–2667

Kaneko H, Kikuchi K, Nakai M, Somfai T, Noguchi J, Tanihara F, Ito J, Kashiwazaki N (2013) Generation of live piglets for the first time using sperm retrieved from immature testicular tissue cryopreserved and grafted into nude mice. PLoS One 8:e70989

Kaul G, Kaur J, Rafeeqi TA (2010) Ultrasound guided transplantation of enriched and cryopreserved spermatogonial cell suspension in goats. Reprod Domest Anim 45:e249–e254

Kawai K, Li YS, Song MF, Kasai H (2010) DNA methylation by dimethyl sulfoxide and methionine sulfoxide triggered by hydroxyl radical and implications for epigenetic modifications. Bioorg Med Chem Lett 20:260–265

Kee K, Angeles VT, Flores M, Nguyen HN, Reijo Pera RA (2009) Human DAZL, DAZ and BOULE genes modulate primordial germ-cell and haploid gamete formation. Nature 462:222–225

Keros V, Rosenlund B, Hultenby K, Aghajanova L, Levkov L, Hovatta O (2005) Optimizing cryopreservation of human testicular tissue: comparison of protocols with glycerol, propanediol and dimethylsulphoxide as cryoprotectants. Hum Reprod 20:1676–1687

Keros V, Hultenby K, Borgström B, Fridström M, Jahnukainen K, Hovatta O (2007) Methods of cryopreservation of testicular tissue with viable spermatogonia in pre-pubertal boys undergoing gonadotoxic cancer treatment. Hum Reprod 22:1384–1395

Kim YM, Uhm SJ, Gupta MK, Yang JS, Lim JG, Das ZC, Heo YT, Chung HJ, Kong IK, Kim NH, Lee HT, Ko DH (2012) Successful vitrification of bovine blastocysts on paper container. Theriogenology 78:1085–1093

Kim KJ, Lee YA, Kim BJ, Kim YH, Kim BG, Kang HG, Jung SE, Choi SH, Schmidt JA, Ryu BY (2015) Cryopreservation of putative pre-pubertal bovine spermatogonial stem cells by slow freezing. Cryobiology 70:175–183

Kim HJ, Lee JH, Hur YB, Lee CW, Park SH, Koo BW (2017) Marine antifreeze proteins: structure, function, and application to cryopreservation as a potential cryoprotectant. Mar Drugs 15:27

Kirpatovskii VI, Efremov GD, Frolova EV (2018) Ectopic organogenesis after allotransplantation of freshly removed or cryopreserved neonatal testicle under the renal capsule in rats. Bull Exp Biol Med 166:268–273

Koppes EA, Redel BK, Johnson MA, Skvorak KJ, Ghaloul-Gonzalez L, Yates ME, Lewis DW, Gollin SM, Wu YL, Christ SE, Yerle M, Leshinski A, Spate LD, Benne JA, Murphy SL, Samuel MS, Walters EM, Hansen SA, Wells KD, Lichter-Konecki U, Wagner RA, Newsome JT, Dobrowolski SF, Vockley J, Prather RS, Nicholls RD (2020) A porcine model of phenylketonuria generated by CRISPR/Cas9 genome editing. JCI Insight 5:141523

Kristensen TN, Hoffmann AA, Pertoldi C, Stronen AV (2015) What can livestock breeders learn from conservation genetics and vice versa? Front Genet 6:38

Kumari N, Gupta MK, Singh RK (2016) Open encapsulation-vitrification for cryopreservation of algae. Cryobiology 73:232–239

Lakhoo K, Davies J, Chakraborty S, Berg S, Tennyson R, Fowler D, Manek S, Verrill C, Lane S (2019) Development of a new reproductive tissue cryopreservation clinical service for children: the Oxford programme. Pediatr Surg Int 35:1271–1278

Lee PA, Coughlin MT (2001) Fertility after bilateral cryptorchidism. Evaluation by paternity, hormone, and semen data. Horm Res 55:28–32

Lee YA, Kim YH, Kim BJ, Jung MS, Auh JH, Seo JT, Park YS, Lee SH, Ryu BY (2013a) Cryopreservation of mouse spermatogonial stem cells in dimethylsulfoxide and polyethylene glycol. Biol Reprod 89:109

Lee YA, Kim YH, Kim BJ, Kim BG, Kim KJ, Auh JH, Schmidt JA, Ryu BY (2013b) Cryopreservation in trehalose preserves functional capacity of murine spermatogonial stem cells. PLoS One 8:e54889

Lee YA, Kim YH, Ha SJ, Kim BJ, Kim KJ, Jung MS, Kim BG, Ryu BY (2014a) Effect of sugar molecules on the cryopreservation of mouse spermatogonial stem cells. Fertil Steril 101:1165–1175

Lee YA, Kim YH, Ha SJ, Kim KJ, Kim BJ, Kim BG, Choi SH, Kim IC, Schmidt JA, Ryu BY (2014b) Cryopreservation of porcine spermatogonial stem cells by slow-freezing testis tissue in trehalose. J Anim Sci 92:984–995

Levine H, Jørgensen N, Martino-Andrade A, Mendiola J, Weksler-Derri D, Mindlis I, Pinotti R, Swan SH (2017) Temporal trends in sperm count: a systematic review and meta-regression analysis. Hum Reprod Update 23:646–659

Li J, Leng M, Ma T, Yu D, Shi H, Shi Q (2009) Cryopreservation has no effect on meiotic recombination and synapsis in testicular tissues. Fertil Steril 91:1404–1407

Li H, Bian YL, Schreurs N, Zhang XG, Raza SHA, Fang Q, Wang LQ, Hu JH (2018) Effects of five cryoprotectants on proliferation and differentiation-related gene expression of frozen-thawed bovine calf testicular tissue. Reprod Domest Anim 53:1211–1218

Liu Z, Nie YH, Zhang CC, Cai YJ, Wang Y, Lu HP, Li YZ, Cheng C, Qiu ZL, Sun Q (2016) Generation of macaques with sperm derived from juvenile monkey testicular xenografts. Cell Res 26:139–142

Lukusa AK, Vermylen C, Vanabelle B, Curaba M, Brichard B, Chantrain C, Dupont S, Ferrant A, Wyns C (2009) Bone marrow transplantation or hydroxyurea for sickle cell anemia: long-term effects on semen variables and hormone profiles. Pediatr Hematol Oncol 26:186–194

Makala H, Pothana L, Sonam S, Malla A, Goel S (2015) Regeneration of Leydig cells in ectopically autografted adult mouse testes. Reproduction 149:259–268

Mazur P (1984) Freezing of living cells: mechanisms and implications. Am J Phys 247:C125–C142

Men H, Agca Y, Riley LK, Critser JK (2006) Improved survival of vitrified porcine embryos after partial delipation through chemically stimulated lipolysis and inhibition of apoptosis. Theriogenology 66:2008–2016

Meneghel J, Kilbride P, Morris JG, Fonseca F (2019) Physical events occurring during the cryopreservation of immortalized human T cells. PLoS One 14:e0217304

Mohaqiq M, Movahedin M, Mazaheri Z, Amirjannati N (2019) In vitro transplantation of spermatogonial stem cells isolated from human frozen-thawed testis tissue can induce spermatogenesis under 3-dimensional tissue culture conditions. Biol Res 52:16

Morimoto H, Iwata K, Ogonuki N, Inoue K, Atsuo O, Kanatsu-Shinohara M, Morimoto T, Yabe-Nishimura C, Shinohara T (2013) ROS are required for mouse spermatogonial stem cell self-renewal. Cell Stem Cell 12:774–786

Nakagawa T, Nabeshima Y, Yoshida S (2007) Functional identification of the actual and potential stem cell compartments in mouse spermatogenesis. Dev Cell 12:195–206

Navid S, Rastegar T, Baazm M, Alizadeh R, Talebi A, Gholami K, Khosravi-Farsani S, Koruji M, Abbasi M (2017) In vitro effects of melatonin on colonization of neonate mouse spermatogonial stem cells. Syst Biol Reprod Med 63:370–381

Nicopoullos JD, Almeida P, Vourliotis M, Gilling-Smith C (2011) A decade of the sperm-washing programme: correlation between markers of HIV and seminal parameters. HIV Med 12:195–201

Noguchi J, Ohnuma K, Ozawa M, Karja NW, Fahrudin M, Somfai T, Kikuchi K, Kaneko H (2006) Successful long term culture of immature porcine sertoli cells in the reconstructed testicular cell cord. J Reprod Dev 52:383–389

Ntemou E, Kadam P, Van Saen D, Wistuba J, Mitchell RT, Schlatt S, Goossens E (2019) Complete spermatogenesis in intratesticular testis tissue xenotransplants from immature non-human primate. Hum Reprod 34:403–413

Oblette A, Rives N, Dumont L, Rives A, Verhaeghe F, Jumeau F, Rondanino C (2017) Assessment of sperm nuclear quality after in vitro maturation of fresh or frozen/thawed mouse pre-pubertal testes. Mol Hum Reprod 23:674–684

Ogawa T, Dobrinski I, Avarbock MR, Brinster RL (2000) Transplantation of male germ line stem cells restores fertility in infertile mice. Nat Med 6:29–34

Onions VJ, Mitchell MR, Campbell BK, Webb R (2008) Ovarian tissue viability following whole ovine ovary cryopreservation: assessing the effects of sphingosine-1-phosphate inclusion. Hum Reprod 23:606–618

Onofre J, Baert Y, Faes K, Goossens E (2016) Cryopreservation of testicular tissue or testicular cell suspensions: a pivotal step in fertility preservation. Hum Reprod Update 22:744–761

Onofre J, Kadam P, Baert Y, Goossens E (2020) Testicular tissue cryopreservation is the preferred method to preserve spermatogonial stem cells prior to transplantation. Reprod Biomed Online 40:261–269

Pacchiarotti J, Ramos T, Howerton K, Greilach S, Zaragoza K, Olmstead M, Izadyar F (2013) Developing a clinical-grade cryopreservation protocol for human testicular tissue and cells. Biomed Res Int 2013:930962

Park KE, Kaucher AV, Powell A, Waqas MS, Sandmaier SE, Oatley MJ, Park CH, Tibary A, Donovan DM, Blomberg LA, Lillico SG, Whitelaw CB, Mileham A, Telugu BP, Oatley JM (2017) Generation of germline ablated male pigs by CRISPR/Cas9 editing of the NANOS2 gene. Sci Rep 7:40176

Patra T, Gupta MK (2019) Cryopreservation of murine testicular Leydig cells by modified solid surface vitrification with supplementation of antioxidants. Cryobiology 88:38–46

Patra T, Gupta MK (2020) Evaluation of sodium alginate for encapsulation-vitrification of testicular Leydig cells. Int J Biol Macromol 153:128–137

Patra T, Pathak D, Gupta MK (2021) Strategies for cryopreservation of testicular cells and tissues in cancer and genetic diseases. Cell Tissue Res 385:1–19. https://doi.org/10.1007/s00441-021-03437-4

Pelzman DL, Orwig KE, Hwang K (2020) Progress in translational reproductive science: testicular tissue transplantation and in vitro spermatogenesis. Fertil Steril 113:500–509

Pendergraft SS, Sadri-Ardekani H, Atala A, Bishop CE (2017) Three-dimensional testicular organoid: a novel tool for the study of human spermatogenesis and gonadotoxicity in vitro. Biol Reprod 96:720–732

Perrard MH, Sereni N, Schluth-Bolard C, Blondet A, Plotton I, Morel-Journel N, Lejeune H, David L, Durand P (2016) Complete human and rat ex vivo spermatogenesis from fresh or frozen testicular tissue. Biol Reprod 95:89

Pietzak EJ 3rd, Tasian GE, Tasian SK, Brinster RL, Carlson C, Ginsberg JP, Kolon TF (2015) Histology of testicular biopsies obtained for experimental fertility preservation protocol in boys with cancer. J Urol 194:1420–1424

Pirnia A, Parivar K, Hemadi M, Yaghmaei P, Gholami M (2017) Stemness of spermatogonial stem cells encapsulated in alginate hydrogel during cryopreservation. Andrologia 49:12650

Poels J, Van Langendonckt A, Dehoux JP, Donnez J, Wyns C (2012) Vitrification of non-human primate immature testicular tissue allows maintenance of proliferating spermatogonial cells after xenografting to recipient mice. Theriogenology 77:1008–1013

Poels J, Van Langendonckt A, Many MC, Wese FX, Wyns C (2013) Vitrification preserves proliferation capacity in human spermatogonia. Hum Reprod 28:578–589

Poels J, Abou-Ghannam G, Herman S, Van Langendonckt A, Wese FX, Wyns C (2014) In search of better spermatogonial preservation by supplementation of cryopreserved human immature testicular tissue xenografts with N-acetylcysteine and testosterone. Front Surg 1:47

Poels J, Abou-Ghannam G, Decamps A, Leyman M, Rieux A, Wyns C (2016) Transplantation of testicular tissue in alginate hydrogel loaded with VEGF nanoparticles improves spermatogonial recovery. J Control Release 234:79–89

Portela JMD, de Winter-Korver CM, van Daalen SKM, Meißner A, de Melker AA, Repping S, van Pelt AMM (2019) Assessment of fresh and cryopreserved testicular tissues from (pre)pubertal boys during organ culture as a strategy for in vitro spermatogenesis. Hum Reprod 34:2443–2455

Pothana L, Makala H, Devi L, Varma VP, Goel S (2015) Germ cell differentiation in cryopreserved, immature, Indian spotted mouse deer (Moschiola indica) testes xenografted onto mice. Theriogenology 83:625–633

Pothana L, Devi L, Goel S (2017) Cryopreservation of adult cervid testes. Cryobiology 74:103–109

Pukazhenthi BS, Neubauer K, Jewgenow K, Howard J, Wildt DE (2006) The impact and potential etiology of teratospermia in the domestic cat and its wild relatives. Theriogenology 66:112–121

Pukazhenthi BS, Nagashima J, Travis AJ, Costa GM, Escobar EN, França LR, Wildt DE (2015) Slow freezing, but not vitrification supports complete spermatogenesis in cryopreserved, neonatal sheep testicular xenografts. PLoS One 10:e0123957

Redden E, Davey R, Borjigin U, Hutton K, Hinch G, Hope S, Hill J, Herrid M (2009) Large quantity cryopreservation of bovine testicular cells and its effect on enrichment of type A spermatogonia. Cryobiology 58:190–195

Rezaei Topraggaleh T, Rezazadeh Valojerdi M, Montazeri L, Baharvand H (2019) A testis-derived macroporous 3D scaffold as a platform for the generation of mouse testicular organoids. Biomater Sci 7:1422–1436

Sá R, Cremades N, Malheiro I, Sousa M (2012) Cryopreservation of human testicular diploid germ cell suspensions. Andrologia 44:366–372

Sadri-Ardekani H, Atala A (2014) Testicular tissue cryopreservation and spermatogonial stem cell transplantation to restore fertility: from bench to bedside. Stem Cell Res Ther 5:68

Sakib S, Uchida A, Valenzuela-Leon P, Yu Y, Valli-Pulaski H, Orwig K, Ungrin M, Dobrinski I (2019) Formation of organotypic testicular organoids in microwell culture. Biol Reprod 100:1648–1660

Saragusty J, Walzer C, Petit T, Stalder G, Horowitz I, Hermes R (2010) Cooling and freezing of epididymal sperm in the common hippopotamus (Hippopotamus amphibius). Theriogenology 74:1256–1263

Sato T, Katagiri K, Gohbara A, Inoue K, Ogonuki N, Ogura A, Kubota Y, Ogawa T (2011) In vitro production of functional sperm in cultured neonatal mouse testes. Nature 471:504–507

Sayed Mahdi N, Azarbani F, Pirnia A, Abbaszadeh A, Gholami M (2018) The effect of caffeic acid on spermatogonial stem cell-type a cryopreservation. Rep Biochem Mol Biol 7:85–93

Schrans-Stassen BH, Saunders PT, Cooke HJ, de Rooij DG (2001) Nature of the spermatogenic arrest in Dazl −/− mice. Biol Reprod 65:771–776

Shetty G, Mitchell JM, Lam TNA, Wu Z, Zhang J, Hill L, Tailor RC, Peters KA, Penedo MC, Orwig KE, Meistrich ML (2018) Donor spermatogenesis in de novo formed seminiferous tubules from transplanted testicular cells in rhesus monkey testis. Hum Reprod 33:2249–2255

Shetty G, Mitchell JM, Meyer JM, Wu Z, Lam TNA, Phan TT, Zhang J, Hill L, Tailor RC, Peters KA, Penedo MC, Hanna C, Orwig KE, Meistrich ML (2020) Restoration of functional sperm production in irradiated pubertal rhesus monkeys by spermatogonial stem cell transplantation. Andrology 8:1428–1441

Sicinski P, Donaher JL, Geng Y, Parker SB, Gardner H, Park MY, Robker RL, Richards JS, McGinnis LK, Biggers JD, Eppig JJ, Bronson RT, Elledge SJ, Weinberg RA (1996) Cyclin D2 is an FSH-responsive gene involved in gonadal cell proliferation and oncogenesis. Nature 384:470–474

Snowden JA, Badoglio M, Labopin M, Giebel S, McGrath E, Marjanovic Z, Burman J, Moore J, Rovira M, Wulffraat NM, Kazmi M, Greco R, Snarski E, Kozak T, Kirgizov K, Alexander T, Bader P, Saccardi R, Farge D (2017) Evolution, trends, outcomes, and economics of hematopoietic stem cell transplantation in severe autoimmune diseases. Blood Adv 1:2742–2755

Song W, Zhao W, Yang Q, Wang X, Jin H, Yao G, Peng Z, Shi S, Yang H, Sun Y (2016) Effect of rapid cryopreservation on meiotic recombination in human spermatocytes. Microsc Res Tech 79:923–928

Su XJ, Yuan W, Huang GY, Olsen J, Li J (2015) Paternal age and offspring congenital heart defects: a national cohort study. PLoS One 10:e0121030

Tanaka A, Nagayoshi M, Takemoto Y, Tanaka I, Kusunoki H, Watanabe S, Kuroda K, Takeda S, Ito M, Yanagimachi R (2015) Fourteen babies born after round spermatid injection into human oocytes. Proc Natl Acad Sci U S A 112:14629–14634

Tanaka A, Suzuki K, Nagayoshi M, Takemoto Y, Watanabe S, Takeda S, Irahara M, Kuji N, Yamagata Z, Yanagimachi R (2018) Ninety babies born after round spermatid injection into oocytes: survey of their development from fertilization to 2 years of age. Fertil Steril 110:443–451

Taylor JL, Debost JPG, Morton SU, Wigdor EM, Heyne HO, Lal D, Howrigan DP, Bloemendal A, Larsen JT, Kosmicki JA, Weiner DJ, Homsy J, Seidman JG, Seidman CE, Agerbo E, McGrath JJ, Mortensen PB, Petersen L, Daly MJ, Robinson EB (2019) Paternal-age-related de novo mutations and risk for five disorders. Nat Commun 10:3043

Tedder RS, Zuckerman MA, Goldstone AH, Hawkins AE, Fielding A, Briggs EM, Irwin D, Blair S, Gorman AM, Patterson KG et al (1995) Hepatitis B transmission from contaminated cryopreservation tank. Lancet 346:137–140

Thomson LK, Fleming SD, Aitken RJ, De Iuliis GN, Zieschang JA, Clark AM (2009) Cryopreservation-induced human sperm DNA damage is predominantly mediated by oxidative stress rather than apoptosis. Hum Reprod 24:2061–2070

Tian J, Ma K, Pei CB, Zhang SH, Li X, Zhou Y, Yan B, Wang HY, Ma LH (2019) Relative safety of various spermatogenic stem cell purification methods for application in spermatogenic stem cell transplantation. Stem Cell Res Ther 10:382

Travers A, Milazzo JP, Perdrix A, Metton C, Bironneau A, Macé B, Rives N (2011) Assessment of freezing procedures for rat immature testicular tissue. Theriogenology 76:981–990

Tsutsui T, Hesabi B, Moons DS, Pandolfi PP, Hansel KS, Koff A, Kiyokawa H (1999) Targeted disruption of CDK4 delays cell cycle entry with enhanced p27(Kip1) activity. Mol Cell Biol 19:7011–7019

Unni S, Kasiviswanathan S, D'Souza S, Khavale S, Mukherjee S, Patwardhan S, Bhartiya D (2012) Efficient cryopreservation of testicular tissue: effect of age, sample state, and concentration of cryoprotectant. Fertil Steril 97:200–208

Valli-Pulaski H, Peters KA, Gassei K, Steimer SR, Sukhwani M, Hermann BP, Dwomor L, David S, Fayomi AP, Munyoki SK, Chu T, Chaudhry R, Cannon GM, Fox PJ, Jaffe TM, Sanfilippo JS, Menke MN, Lunenfeld E, Abofoul-Azab M, Sender LS, Messina J, Klimpel LM, Gosiengfiao Y, Rowell EE, Hsieh MH, Granberg CF, Reddy PP, Sandlow JI, Huleihel M, Orwig KE (2019) Testicular tissue cryopreservation: 8 years of experience from a coordinated network of academic centers. Hum Reprod 34:966–977

Vermeulen M, Poels J, de Michele F, Rieux A, Wyns C (2017) Restoring fertility with cryopreserved prepubertal testicular tissue: perspectives with hydrogel encapsulation, nanotechnology, and bioengineered scaffolds. Ann Biomed Eng 45:1770–1781

Vermeulen M, Del Vento F, de Michele F, Poels J, Wyns C (2018) Development of a cytocompatible scaffold from pig immature testicular tissue allowing human sertoli cell attachment, proliferation and functionality. Int J Mol Sci 19:227

Vermeulen M, Del Vento F, Kanbar M, Pyr Dit Ruys S, Vertommen D, Poels J, Wyns C (2019) Generation of organized porcine testicular organoids in solubilized hydrogels from decellularized extracellular matrix. Int J Mol Sci 20:5476

Vialard F, Bailly M, Bouazzi H, Albert M, Pont JC, Mendes V, Bergere M, Gomes DM, de Mazancourt P, Selva J (2012) The high frequency of sperm aneuploidy in klinefelter patients and in nonobstructive azoospermia is due to meiotic errors in euploid spermatocytes. J Androl 33:1352–1359

Wang D, Li L, Xie Q, Hou Z, Yu X, Ma M, Huang T (2014) Factors affecting sperm fertilizing capacity in men infected with HIV. J Med Virol 86:1467–1472

Wang M, Karlsson JOM, Aksan A (2019) FTIR analysis of molecular changes associated with warming injury in cryopreserved leukocytes. Langmuir 35:7552–7559

Weng L, Chen C, Zuo J, Li W (2011) Molecular dynamics study of effects of temperature and concentration on hydrogen-bond abilities of ethylene glycol and glycerol: implications for cryopreservation. J Phys Chem A 115:4729–4737

Wikström AM, Dunkel L (2008) Testicular function in Klinefelter syndrome. Horm Res 69:317–326

Wu X, Goodyear SM, Abramowitz LK, Bartolomei MS, Tobias JW, Avarbock MR, Brinster RL (2012) Fertile offspring derived from mouse spermatogonial stem cells cryopreserved for more than 14 years. Hum Reprod 27:1249–1259

Wu Y, Zhou H, Fan X, Zhang Y, Zhang M, Wang Y, Xie Z, Bai M, Yin Q, Liang D, Tang W, Liao J, Zhou C, Liu W, Zhu P, Guo H, Pan H, Wu C, Shi H, Wu L, Tang F, Li J (2015) Correction of a genetic disease by CRISPR-Cas9-mediated gene editing in mouse spermatogonial stem cells. Cell Res 25:67–79

Wyns C, Curaba M, Martinez-Madrid B, Van Langendonckt A, François-Xavier W, Donnez J (2007) Spermatogonial survival after cryopreservation and short-term orthotopic immature human cryptorchid testicular tissue grafting to immunodeficient mice. Hum Reprod 22:1603–1611

Wyns C, Van Langendonckt A, Wese FX, Donnez J, Curaba M (2008) Long-term spermatogonial survival in cryopreserved and xenografted immature human testicular tissue. Hum Reprod 23:2402–2414

Xie Y, Chen H, Luo D, Yang X, Yao J, Zhang C, Lv L, Guo Z, Deng C, Li Y, Liang X, Sun X, Liu G (2020) Inhibiting necroptosis of spermatogonial stem cell as a novel strategy for male fertility preservation. Stem Cells Dev 29:475–487

Yango P, Altman E, Smith JF, Klatsky PC, Tran ND (2014) Optimizing cryopreservation of human spermatogonial stem cells: comparing the effectiveness of testicular tissue and single cell suspension cryopreservation. Fertil Steril 102:1491–1498

Yokonishi T, Sato T, Komeya M, Katagiri K, Kubota Y, Nakabayashi K, Hata K, Inoue K, Ogonuki N, Ogura A, Ogawa T (2014) Offspring production with sperm grown in vitro from cryopreserved testis tissues. Nat Commun 5:4320

Yu Z, Ji P, Cao J, Zhu S, Li Y, Zheng L, Chen X, Feng L (2009) Dazl promotes germ cell differentiation from embryonic stem cells. J Mol Cell Biol 1:93–103

Yuan Z, Hou R, Wu J (2009) Generation of mice by transplantation of an adult spermatogonial cell line after cryopreservation. Cell Prolif 42:123–131

Zhang XG, Wang YH, Han C, Hu S, Wang LQ, Hu JH (2015) Effects of trehalose supplementation on cell viability and oxidative stress variables in frozen-thawed bovine calf testicular tissue. Cryobiology 70:246–252

Zhang B, Wang C, Zhang Y, Jiang Y, Qin Y, Pang D, Zhang G, Liu H, Xie Z, Yuan H, Ouyang H, Wang J, Tang X (2020) A CRISPR-engineered swine model of COL2A1 deficiency recapitulates altered early skeletal developmental defects in humans. Bone 137:115450

Testicular Stem Cell Niche

10

Devendra Pathak, Kritima Kapoor, and Mukesh Kumar Gupta

Abstract

Testicular stem cells (TSCs), found at the basement membrane of seminiferous tubules, are the basis for extremely structured process of spermatogenesis. These cells remain quiescent in a specialized microenvironment of the testis, called 'niche', and upon external stimuli can self-renew by symmetric cell division or differentiate by asymmetric cell division to maintain spermatogenesis in males. Although physical localization of these TSC niches is unknown, a complex interaction of somatic and germ cells, extracellular matrix (ECM) and signalling molecules contribute to the regulation of self-renewal and differentiation of TSCs within the niche. The histoarchitecture of testis mostly comprises of stroma and parenchyma. Somatic cells such as Sertoli cells, Leydig cells, peritubular myoid (PM) cells, basement membrane, macrophages, and surrounding blood vessels are intimately associated with the TSC niche. The composition of TSC niche also changes from prenatal life to postnatal life as well as during the process of ageing. The intrinsic and extrinsic factors play vital roles in the regulation of TSC self-renewal by cell-to-cell interaction between TSC and niche cells. In addition, adhesion molecules also influence the TSC niche to induce spermatogenesis. This chapter provides an overview of histoarchitecture of mammalian testis,

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and the composition of TSC niche therein. Ontogeny of TSC niche and factors influencing their niche regulation are also discussed.

Keywords

Niche · Testicular stem cells (TSC) · Testis · Intrinsic factor · Extrinsic factor

10.1 Introduction

Cells having three characteristics viz. potency, clonogenicity, and self-renewal are called stem cells (Weissman 2000). They are undifferentiated cells having the capability to self-renew and differentiate into functionally mature cells. They are present in various tissues of both embryonic (embryonic stem cells) and postnatal life (adult stem cells). Adult stem cells, also known as somatic or tissue-specific stem cells, have now been identified in almost all adult tissue types wherein they play important roles in maintaining and repairing of tissue in which they reside. Thus, stem cells are crucial for the replenishment of lost tissue and maintenance of tissue homeostasis and consequently have become one of the promising tools in regenerative medicine (Prochazkova et al. 2015). Spermatogonial stem cells (SSCs) are testicular stem cells (TSC) found at the basal membrane of seminiferous tubules and, in adults, they are the only stem cells that are capable of passing genetic materials to the next generation during procreation (Ibtisham and Honaramooz 2020). The TSCs are the basis for highly orchestrated and organized process of spermatogenesis for producing haploid male gametes (Zhao et al. 2019). They are self-renewing cells responsible for spermatogenesis throughout the adulthood and are capable of initiating donor-derived spermatogenesis upon their transplantation into the empty seminiferous tubule of infertile male.

The stem cells within the tissue are generally undifferentiated and quiescent in nature and start differentiating upon a trigger requiring mature cells in the body. The mechanism of how these cells remain undifferentiated and the cascade of events that trigger their differentiation are elusive and have always intrigued the scientists. Schofield (1978) was the first to propose the hypothesis that stem cell (hematopoietic stem cells) remains in association with other cells (called 'niche') and this association of cells determines the self-renewal or differentiation propensity of the stem cells. These neighbouring cells regulate the quiescent nature of stem cells and inhibit their differentiation and maturation until their requirement in the body. Conversely, exodus of stem cell or its progeny from the 'niche' stimulates proliferation and differentiation to produce terminally mature cells. This concept of 'stem cell niche' has become a widely accepted concept in stem cell biology and has been studied in different stem cell types, including the TSCs. A '*stem cell niche*' is defined as the stem cell microenvironment consisting of surrounding anatomical structures, including supporting cells, and a milieu of secretory factors that are essential for the self-renewal of stem cells. For the efficient spermatogenesis, a coordinated interplay between SSCs and various components of the spermatogenic niche is a must

(Yokonishi et al. 2020). Niches provide both structural and functional basis for stem cells to remain in a unique anatomical and physiological location. The stem cell niche determines the fate of these cells by regulating various crucial factors, including physical support, nutrients, and hormonal and paracrine signals, which are essential for successful spermatogenesis (Sharma et al. 2019).

Understanding the structures of stem cell niche and the mechanisms of self-renewal therein is crucial for their clinical application. Research on various stem cell types has demonstrated that stem cell niche provides guidance to stem cells about their activities and behaviours. This chapter elaborates the components of TSC niche, its development and the factors that regulate its functioning.

10.2 Histoarchitecture of Testis

Testes are paired male reproductive organs located outside the abdominal cavity in a pouch of skin called scrotum. They operate jointly as exocrine and endocrine organ. It serves the function of producing spermatozoa and synthesis of male sex hormones. Based on functional characteristics, the histoarchitecture of testes broadly comprises of stroma and parenchyma. The stroma is chiefly composed of connective tissue elements whereas parenchyma consists of tubular portions of the organ. An organization of histological structures of testes is presented in Fig. 10.1.

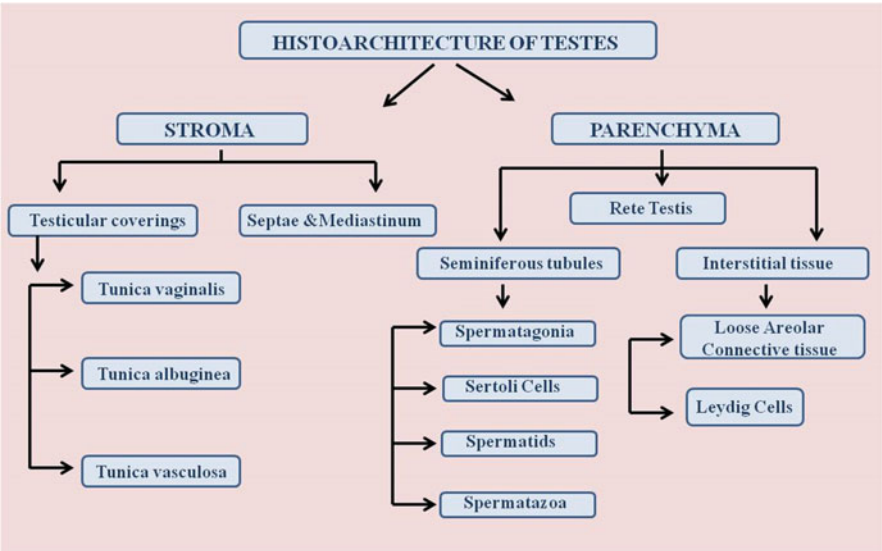

Fig. 10.1 Flowchart representing the histological organization of testicular elements comprising the organ

10.2.1 Stroma

The stroma includes primarily the connective tissue components that enclose the testes as collagen-rich capsule. The capsule is further made up of two layers namely tunica vaginalis and tunica albuginea. It also includes connective tissue septae, mediastinum testis and interstitial connective tissue.

10.2.1.1 Testicular Coverings

Testicular covering is composed of an outer tunica vaginalis layer and an inner tunica albugenia layer, within which a network of arteries and veins ramifies to form a complex vascular layer of tunica vasculosa. The tunica vaginalis consists of a single covering layer of mesothelial cells and an inner layer of connective tissue that blends with underlying tunica albugenia. On the other hand, tunica albugenia is a compact layer of dense irregular connective tissue that majorly consists of fibroblast cells dispersed between collagen, reticular and elastic fibres. The tunica vasculosa is superficial in dog and rams (Liebich 2019) whereas, it is deeply located in the middle part of the capsule in stallion and boar. Further, in stallion, boar and ram, smooth muscle fibres are also present in the tunica vasculosa.

10.2.1.2 Septae and Medistinum Testis

Connective tissue of tunica albuginea protrudes into testes parenchyma as thick trabeculae called *septulae testis*. Septae radiate towards the centre of the organ where it forms *mediastinum testis* and surrounds the *rete testis*. This *mediastinum testis* begins near the caudal extremity in ruminants, pigs and dogs, extends longitudinally through the testis and recombines with the tunica albuginea at the head of epididymis (Singh et al. 2018). In stallions, it exists as the true mediastinum. The trabeculae or septulae testis divide the testicular parenchyma into number of pyramidal compartments (up to 250–300) known as lobules or *lobuli testis*. Each lobule is occupied by two to five highly convoluted seminiferous tubules, also called as *tubuli seminiferi convoluti*. The lobules consist of both tortuous and straight tubules known as *tubuli contorti* and *tubuli recti*, respectively. The tubules are surrounded by interstitial loose connective tissue, containing reticular fibres, blood vessels, nerve plexuses and interstitial cells.

10.2.2 Parenchyma

The major portion of testicular parenchyma is contributed by tightly wound convoluted seminiferous tubules (*tubuli seminiferi convoluti*) placed amid the *septula testis*. These convoluted tubules straighten just before entering the *mediastinum testis* to form the straight testicular tubules (*tubuli recti*), which are connected to the *rete testis* within the mediastinum. The rete testis empties via the *ductuli efferentes* testis into the epididymis (Eurell and Frappier 2006).

10.2.2.1 Seminiferous Tubules

The convoluted seminiferous tubules are around 50–80 cm long with a diameter of 150–300 μm in animals. These tubules are not only responsible for the formation of male gametes but also constitute the exocrine part of the gland for spermatogenesis. Each tubule is surrounded by an outer lamina propria. The innermost layer of the lamina propria abreacts with the basal lamina of the seminiferous tubules. Further, fine collagen and elastic fibres of lamina propria condense to form a wall that embodies contractile peritubular cells or myoid (PM) cells in bulls, dogs and horses (Gofur et al. 2008; Egger and Witter 2009; Shukla et al. 2013). The PM cells contain actin bundles in their cytoplasm and can act as myofibroblasts to propel non-motile spermatozoa into tubular lumen of rete testis (a process called spermiation) by rhythmic contractions in the seminiferous tubules. The seminiferous tubules consist of a central lumen lined by specialized seminiferous epithelia comprising somatic and germ cells, including TSCs within their niche (Fig. 10.2).

Types of Cells Within Seminiferous Tubules

The seminiferous tubules are lined by specialized stratified epithelium called germinal epithelium containing two types of cells viz. spermatogenic cells (spermatogonia, spermatocytes, and spermatids) and Sertoli cells or sustentacular cells. The germinal epithelium is an atypical, stratified epithelium as it consists of a single basal layer of Sertoli cells, which extend up to the lumen, and interspersed spermatogonial cells, which proliferate and differentiate to form multilayer of

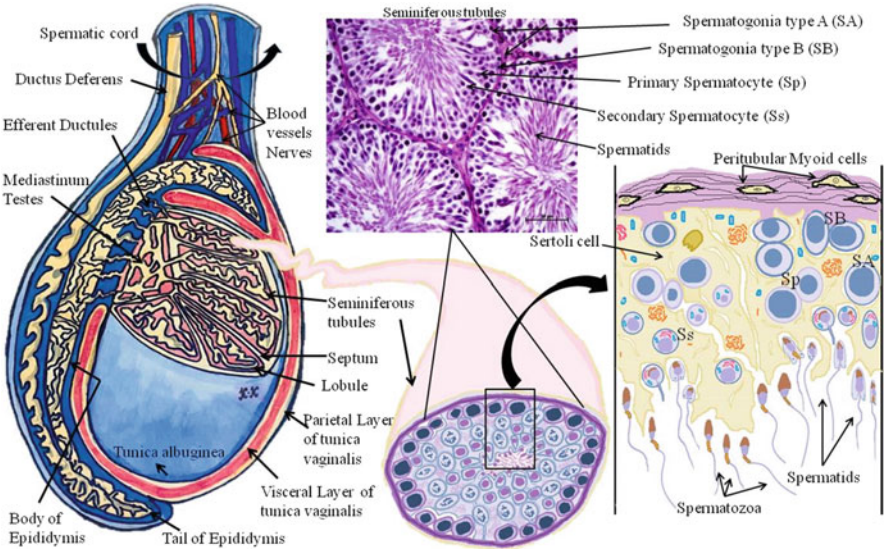

Fig. 10.2 A three-dimensional diagram of testis illustrating its different components, its histoarchitecture primarily comprising of seminiferous tubules depicting Sertoli cells, different types of Spermatogonia and Spermatocytes differentiating into Spermatids and eventually into Spermatozoa in both microscopic and three-dimensional views

spermatogenic cells at various stages of spermatogenesis (Fig. 10.2). The spermatogenic cells originate from primordial germ cells (PGCs) during embryonic development and are germ cells whereas Sertoli cells are somatic cells and are steroidogenic in nature. Spermatogenic cells undergo several phases of division and maturation prior to its release as male gametes (spermatozoa) into the lumen of the seminiferous tubules. The spermatogonia are cuboidal-shaped cells with large, darkly stained nucleus and are located at the inner lining of the seminiferous tubules (Kierszenbaum and Tres 2016). Spermatogonia can be classified as Adark and Alight in primates or as A, I (Intermediate), and B subtypes in rodents and other animals. The type A spermatogonia remains as single cells (Asingle; As) at the basement membrane of the seminiferous tubule and divides symmetrically to form type Apaired (Apr), Aaligned-4, Aaligned-8, and Aaligned-16 (Aal) spermatogonia which remain interconnected to each other by intercellular bridge. The type A spermatogonia undergoes differentiation to different stages to form type I and type B before undergoing meiosis to form primary spermatocyte. The primary spermatocytes are the largest among all spermatogenic cells and are easy to locate due to their biggest and densely stained coarsely granulated nuclei. The secondary spermatocytes are smaller than primary spermatocytes and rarely observed in histological sections of testis because they enter the second meiotic division rapidly to form spermatids. The round spermatids were smallest among all spermatogenic cell types, with large central spherical nucleus in bull, deer, ram and horses (Gofur et al. 2008; Moonjit and Adcharatt 2007; Kishore et al. 2011; Shukla et al. 2013). Elongated spermatids have an oval nucleus located towards the lumen.

Mammalian testes also contain a pool of SSCs which are responsible for maintenance of spermatogenic potential throughout the adulthood in males. These cells can be isolated and cultured in vitro as male germline stem cells (GSCs), which can initiate donor-derived spermatogenesis upon their testicular transplantation into infertile males. The SSCs originate from PGCs during embryonic development. In the embryonic gonad, PGCs initially proliferate as gonocyte (also known as mitotic prospermatogonia), which subsequently become quiescence until birth. Upon birth, the gonocytes develop into SSCs at around 6 days in rodents and several months in livestock species (Oatley and Brinster 2012).

Sertoli cells are fewer tall columnar cells interspersed between spermatogenic cells to nurture the developing gametes. They act as bridge cells between the intertubular space and the lumen of seminiferous tubules. They are arranged radially from basal lamina to the lumen and are difficult to distinguish by light microscopy for structural details. The apical and lateral plasma membranes of Sertoli cells have numerous thin, branching cytoplasmic processes extending from irregular invaginations to form crypts to house the developing spermatogenic cells and act as niches. The euchromatic nuclei are oval, positioned basally and vesicular with distinct nucleoli. The nucleoli are typically multivesicular in ruminants. The cytoplasm contains a well-developed Golgi apparatus, abundant mitochondria and abundant rough ER in pigs and ruminants (Liebich 2019). The smooth ER is well developed in all species. Dense stacks of cisternae of smooth ER are in close contact with lysosomes and lipid vacuoles specifically in ruminants, pigs and dogs. The

basal cytoplasmic compartment contains microbodies, membrane aggregates and lamellar inclusion bodies, which are active in resorptive and digestive cell processes. In boars, spindle- or needle-shaped crystals (Charcot–Bottcher crystals) are also present. Microtubules and microfilaments contribute to intracellular transport processes and regulate the release of spermatozoa into the tubular lumen (spermiation). Sertoli cells are in close contact with one another by numerous basolateral intercellular tight junctions and desmosomes that completely divide the space between adjacent sustentacular cells into basal and adluminal compartments. The adluminal compartment contains developing gametes that are immunologically protected from direct contact of immune cells by tight junctions of Sertoli cells to form blood testis barrier.

10.2.2.2 Interstitial Tissue

The interstitial tissue fills the space between convoluted seminiferous tubules in the parenchyma and is a key component of the TSC niche. It is formed of loose areolar connective tissue comprising mesenchymal cells and fibroblasts in different stages of development. The fibroblasts are spindle-shaped cells with darkly stained nuclei that predominates the other cell types in number in young animal. The ratio of interstitial tissue: parenchyma changes with age of the animal, as the age advances the interstitial tissue reduces and the parenchyma increases (Pathak 2016). The interstitial tissue contains abundant interstitial cells or Leydig cells, which is responsible for testosterone production. The Leydig cells are located in proximity to blood vessels, lymphatic channels and nerve fibres in ram, bull, horse, neonatal goats and boar (Kishore et al. 2007; Gofur et al. 2008; Shukla et al. 2013; Pathak 2016; Dhyana et al. 2016). The Leydig cells mostly outweigh the other cell types in pubertal animals. The differentiated Leydig cells are typically polyhedral to ovoid or roughly triangular with distinct nuclei. Its eosinophilic granular cytoplasm appears highly vacuolated or foamy due to the presence of abundant lipid droplets and granules, which are generally lost during processing of tissue sections. Leydig cells are abundant in boars and bulls (Banks 1993; Pathak et al. 2014).

10.2.2.3 Rete Testis

The *rete testis* consists of anastomosing straight tubules known as *tubuli recti*. These are surrounded by the loose connective tissue and elastic fibres of *mediastinum testis*, and myofibroblasts. The epithelium of the *rete testis* is simple squamous to columnar. Two epithelial layers are present in the bull. The epithelium is highly secretory and increases the volume of the tubular fluid. Androgens in the tubular fluid are absorbed across the epithelium to a limited extent.

10.3 TSC Niche

The microenvironment of 'niche' regulates the self-renewal and differentiation of TSCs through a complex interaction of somatic and germ cells, extracellular matrix and signalling molecules. Somatic cells, including Sertoli cells, Leydig cells, PM

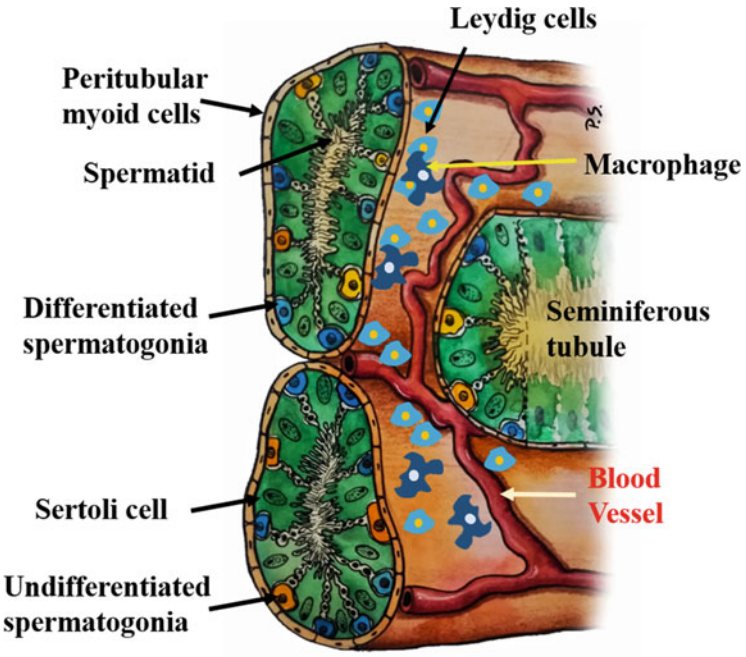

Fig. 10.3 A drawing of a cross-section of seminiferous tubules (differentiated and undifferentiated spermatogonia) showing a three-dimensional view of blood vessels, Leydig cells and macrophages in the intertubular compartment. Undifferentiated spermatogonia are located near the area where the blood vessel is in contact with the tubule

cells, basement membrane and surrounding blood vessels are intimately associated with the TSC niche. Among these, Sertoli cells are considered as the key regulator of SSC niche through FSH-regulated secretion of growth factors. In addition, extracellular matrix and cells in the interstitial tissue also contributes to self-renewal and differentiation of cells in the adluminal compartment of seminiferous tubules (Oatley and Brinster 2012). More recently, the vascular pattern on the seminiferous tubules was also shown to influence the self-renewal and potency of SSCs (Yoshida et al. 2007). The components of the TSC niche are elaborated below (Fig. 10.3).

10.3.1 Sertoli Cells

Sertoli cells are 'nurse cells' located in proximity to the SSCs within seminiferous tubules and are established as principal component of the TSC niche (De Rooij 2009). They were first described by Enrico Sertoli in 1865 and hence, named 'Sertoli cells'. They are large-sized, columnar cells having a large euchromatic nucleus with deep indentations and darkly stained, large, and tripartite nucleolus (Hess and Vogl 2015). Two different morphologies viz. type A and type B can be seen. Type A Sertoli cells shows cytoplasmic crypts at their luminal end within which resides the

germ cells whereas type B Sertoli cells rare or no cytoplasmic crypts. The Sertoli cells are proliferative in pre-pubertal animals and its proliferation is regulated by FSH from anterior pituitary. However, after puberty, the mature Sertoli cells stop dividing, develop tight junctions and differentiate to release nutrients for supporting the germ cell development. Thus, the number of mature Sertoli cells in testis is determined at pre-pubertal stage and remain stable throughout the adulthood of males. In other words, nursing ability of Sertoli cells for the entire life of the mammal is fixed before the attainment of puberty (Petersen and Soder 2006).

It has been established that the SSCs located in the area where the basement membrane is bordering the intertubular tissue has more number of undifferentiated cells as compared to the SSCs located in the area where it is near to the other seminiferous tubules (Chiarini-Garcia et al. 2001). In the area where the tubule is in contact with interstitial tissues is also divided into two compartments. One compartment where the tubule is in contact with the blood vessels has comparatively a greater number of undifferentiated cells as compared to the area devoid of contact with blood vessels (Yoshida et al. 2007). It has also been demonstrated that Sertoli cells are the main influencer of SSC number and their niches (Oatley et al. 2011). The immunolocalization of vimentin in the Sertoli cells in buffalo testis indicated a physical boundary and a cross talk between germ cells and the Sertoli cells as a component of SSC niche (Carlo et al. 2019).

10.3.2 Basement Membrane of Seminiferous Tubules

The basement membrane between seminiferous epithelium and PM cells is a thin zone of ECM consisting of laminin, type IV collagen, heparan sulphate proteoglycan, and nidogen/entactin. In vitro experiments has shown that laminin and type IV collagen are produced by both Sertoli and PM cells while fibronectin is produced by PM cells alone (Richardson et al. 1995). These molecules have been shown to play important roles in the attachment of SSCs to the basement membrane, maintenance of shape and polarity of Sertoli cells and proliferation and mobility of germ cells during spermatogenesis (He et al. 2005; Kanatsu-Shinohara et al. 2006). Several studies have shown laminin in basement membrane plays a critical role in the attachment and growth of SSC through their alpha-6-integrin.

10.3.3 PM Cells

The PM cells, along with basement membrane and Sertoli cells, form the physical and cellular boundary of the TSCs. These cells are in multiple layers, each lined by basement membrane, and are separated from one another by type I collagen fibrils, elastin fibrils and microfibrils. In addition, PM cells are also known to secrete several growth factors, which can influence the TSC niche. For example, in one study, knockout of PM cell-specific androgen receptor in mice was shown to result in dramatically reduced testicular size and sperm production (Zhang et al. 2006).

Similarly, in other study, effect of colony-stimulating factor 1 (Csf1), secreted by PM cells, has also been shown (Oatley et al. 2009). The Csf1, expressed in Leydig and PM cells, could interact with Csf1 receptor (Csf1r) in Thy1+ cell fractions from testes. Using an adult mouse PM cell primary culture system and germ cell transplantation experiments, Chen et al. (2014) concluded that T-regulated GDNF expression by PM cells significantly influence the dynamic microenvironment of the niche and TSC maintenance.

10.3.4 Leydig Cells

Studies in mice have revealed that Csf1 is immuno-localized in the Leydig cells present in the intertubular compartment and few PM cells but were not localized in the Sertoli cells (Oatley et al. 2009). These results indicate that Leydig and PM cells are also the part of the TSC niche (Fig. 10.4). These cells influence the process of spermatogenesis through the communication with Sertoli cells. A cross-communication also exists between Leydig cells and Sertoli cells which is responsible for their interaction (Skinner 1991; Fujisawa 2006). Previous investigations have concluded that the distribution of spermatogonia was not random in mice (Chiarini-Garcia et al. 2001) and rat testes (Chiarini-Garcia et al. 2003). They established that most primitive type spermatogonia are present in niches located in the locations of the seminiferous tubules that border the interstitial tissue and lesser number were present in the areas that border the other tubules.

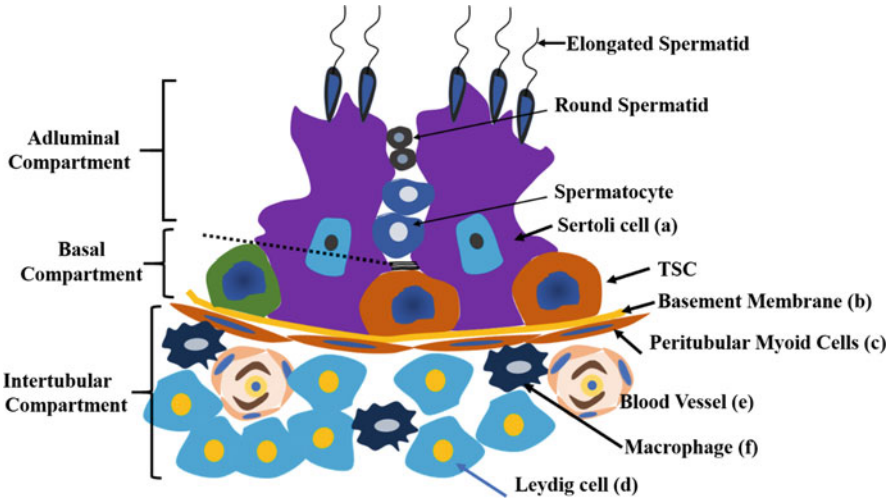

Fig. 10.4 A drawing showing the cross-section of seminiferous tubule with components of the niche of TSC; Sertoli cells (**a**), Basement membrane (**b**), peritubular myoid cells (**c**), Leydig cells (**d**), blood vessel (**e**), and macrophage (**f**)

10.3.5 Blood Vessels

Tracking of migration patterns of TSCs, using live imaging, in mice has shown that the mammalian germline niche is correlated with the blood vessels pattern formation (Yoshida et al. 2007). Yoshida et al. (2007) recorded comparatively greater number of undifferentiated spermatogonia around the tubule where it is in contact with the blood vessels present in intertubular space as compared to the area devoid of contact with blood vessels. Thus, blood vessel pattern also appears to play important role in the TSC niche. The endothelial cells have also been shown to stimulate the self-renewal of other type of stem cells such as neural stem cells (Shen et al. 2004).

10.3.6 Macrophages

Macrophages have been frequently observed in the interstitial compartment of the testis and have been described as the principal leukocyte population therein. Its role in fetal vasculature development, modulation of steroidogenesis in the Leydig cells and regulation of SSCs are well described (Hales 2002; Bhushan and Meinhardt 2017). The macrophages are also closely associated with Leydig cells to promote steroidogenesis in neonatal and adult testis (Gaytan et al. 1994). Hutson (1992) recorded digitations between the macrophages and Leydig cells. In 2015, DeFalco et al. established the role of macrophages in TSC niche. They observed macrophages in higher density at places where large numbers of undifferentiated spermatogonia were present. Macrophage-depleted testes contained fewer undifferentiated spermatogonia, thus it was established that macrophages are required for the process of spermatogenesis (DeFalco et al. 2015). The testis macrophages are also known to express factors such as Csf1 and enzymes involved in retinoic acid (RA) biosynthesis, which are required for the proliferation and differentiation of SSCs (DeFalco et al. 2015).

10.4 Ontogeny of TSC Niche

Dynamic variation has been observed in the niche of TSCs from prenatal life to postnatal life and during the process of ageing. At the stage of PGCs in early prenatal life, the niche is constituted of the surrounding mesenchymal cells and fibroblasts. The tubules are not organized as the basement membrane was not organized at this stage. At later prenatal life, the testicular cords with well-organized basement membrane are reported (Farooqui et al. 2012; Carlo et al. 2019). At this stage, the gonocytes are surrounded by pre-Sertoli cells, mostly from all the sides as gonocytes are centrally located in the cords. The testicular cords are surrounded by the distinct basement membrane during neonatal life. Distinct basement membrane with peritubular PM cells is seen during prepubertal period. Up to neonatal stage, Sertoli cells are prime neighbours of the TSCs. Later these TSCs migrate towards periphery and the adult TSC niche is established with all the components. The seminiferous

tubules are surrounded by a thick basement membrane surrounded by PM cells and interstitial tissue and blood vessels in it (Carlo et al. 2019). The timelines of development of these components in prenatal life vary in different domestic animals.

In the adult testis, a gradual decrease in TSCs and factors secreted from niche has been reported. Zhang et al. (2006) based on transplantation experiments suggested a gradual change in the TSC niche and alterations are observed for TSC niche with aging. There is a gradual decrease in the production of GDNF from Sertoli cells with age and that could be correlated with decrease in the TSCs number (Ryu et al. 2006). Zhang et al. (2006) concluded alteration in the stem cell intrinsic factors with ageing.

10.5 Factors Influencing TSC Niche

Self-renewal of TSC within its niche and their differentiation outside the niche is tightly regulated by both intrinsic gene expression within the SSC and extrinsic signals such as soluble factors and adhesion molecules from the surrounding microenvironment (Fig. 10.5). SSC-expressed genes such as Plzf, a transcriptional repressor protein, and Taf4b, a germ cell-specific component of the RNA polymerase

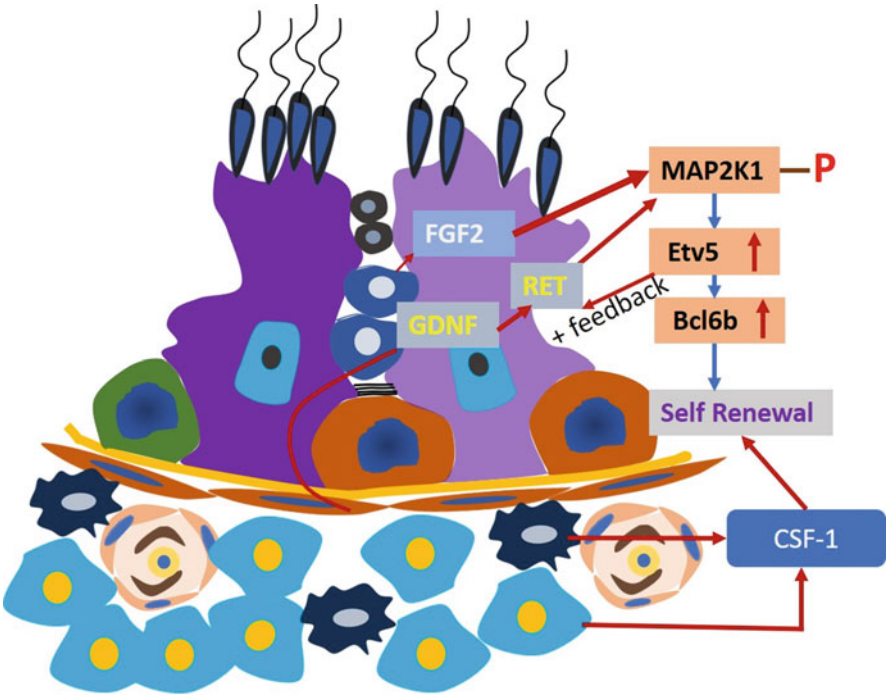

Fig. 10.5 A drawing showing the factors secreted from components of TSC regulating TSC self-renewal. GDNF from Sertoli cells and PM cells, FGF from Sertoli cells, differentiating germ cells influence TSC self-renewal through the MAP2K1 pathway

complex, are considered to be essential for SSC self-renewal, whereas the transcription factors Sohlh1 and 2 appear to be crucial for spermatogonial differentiation. On the other hand, Sertoli cells produce growth factors that stimulate both self-renewal (GDNF, FGF2, ERM) and differentiation (activin A, BMP4 and SCF) of the SSCs. Additional factors that could link the SSC niche to the somatic cells outside the seminiferous tubules are Csf1, produced by Leydig cells that stimulate SSC proliferation and FSH that stimulates GDNF production by Sertoli cells. In addition, adhesion molecules that connect the SSCs to the basement membrane and cellular components of the interstitial compartment between the seminiferous tubules are important regulators of the niche function.

10.5.1 Intrinsic Factors

10.5.1.1 Taf4b

Taf4b is a component of the RNA Polymerase II basal transcription apparatus and is germ cell specific (Falender et al. 2005). In mouse, it is expressed in gonocytes of the postnatal testes, and in spermatogonia and spermatids of the adult testes. It is not expressed in Sertoli cells. Taf4b knockout mice have normal testis histology at birth with normal completion of the first wave of spermatogenesis and transient fertility. However, the SSCs start to disappear as soon as 3 days after birth with progressive loss of the germ cells resulting in Sertoli cell-only seminiferous tubules and testicular atrophy after 12 weeks of age. However, *taf4b* −/− Sertoli cells support spermatogenesis of transplanted wild-type spermatogonia (Falender et al. 2005). Thus, Taf4b is an SSC-intrinsic factor essential for the SSC self-renewal.

10.5.1.2 Plzf

Promyelocytic Leukaemia Zinc-Finger (Plzf) is a transcriptional repressor and is crucial for the regulation of SSC self-renewal. It inhibits stem cell differentiation and helps maintaining their presence in the niche possibly by repressing c-kit, which is a characteristic of spermatogonial differentiation (Filipponi et al. 2007). Thus, naturally occurring mutants lacking Plzf (Luxoid) as well as experimentally created plzf knockout mice show progressive loss of spermatogonia with increasing age (Buaas et al. 2004; Costoya et al. 2004). Undifferentiated spermatogonia isolated from *plzf* −/− mice also exhibit a marked increase in c-kit expression. Furthermore, the germ cells from the testes of Luxoid mutants cannot repopulate the testes of recipient W/Wv mice, suggesting that the defect is inherent to the SSCs.

10.5.1.3 *Chd1l*

Recently, Chromodomain helicase/ATPase DNA binding protein 1-like gene (*Chd1l*) has been found in prepubertal and adult mice testis that was localized along with PLZF, OCT4 and GFRα1 in the testis of neonatal mouse and THY1^{+} undifferentiated spermatogonia or testicular stem cells (TSCs). GDNF activates the *Chd1l* gene expression in the cultured mouse TSCs. *Chd1l* is established as an

intrinsic factor for TSC survival and self-renewal which exerts its impacts via a GDNF signalling pathway (Liu et al. 2016).

10.5.2 Extrinsic Factors

Among all somatic cells, Sertoli cells appear to be the major contributors to SSC niche regulation by physically supporting the SSCs and providing them with growth factors. Besides, factors secreted by somatic cells outside the seminiferous tubules may also mediate their effect via Sertoli cells (Chen et al. 2005). The SSC niche regulation by Sertoli cells changes with age. During the perinatal period of development, SSC self-renewal is mainly controlled by the GDNF while it is dependent on the ERM during the pubertal period (Chen et al. 2005; Hess et al. 2006; Meng et al. 2000). ERM-regulated pathways are coincident with the termination of Sertoli cell proliferation and commencement of the cycle of spermatogenesis, which is sustained by the same cell that regulates the stem cell niche (Hess et al. 2006).

10.5.2.1 ERM/Etv5 Transcription Factor

Etv5 (Ets Variant Gene 5) is a member of Pea3 group of the Ets family of proteins, which are characterized by a highly conserved DNA-binding ETS domain. It is expressed in both Sertoli cells and germ cells (Chen et al. 2005; Oatley et al. 2007) and plays an important role in balancing the SSC self-renewal and differentiation. Targeted disruption of the Etv5 (Etv5−/−) results in defective maintenance of the SSC pool without affecting the spermatogonial differentiation (Chen et al. 2005). Like Plzf−/− mice, Etv5−/− mice has normal testes histology at birth and complete the first wave of spermatogenesis during juvenile life. However, by 10 weeks of age they show Sertoli cell-only phenotype with complete disappearance of all germ cells due to a progressive loss of spermatogenesis (Chen et al. 2005). Although the exact mode of action is not known, analysis of Etv5−/− Sertoli cells detected a 9- to 25-fold reduction in several chemokines and a tenfold reduction in matrix metalloproteinase-12 (MMP-12) expression which are important for stem cell recruitment, migration and homing. The expression of Etv5 in Sertoli cells is possibly regulated by local soluble factors as its expression was shown to be up-regulated by FGF2 and EGF but not by testosterone or FSH (Simon et al. 2007).

10.5.2.2 GDNF

The glial cell line-derived neurotrophic factor (GDNF) is recognized as an essential element of survival for midbrain dopaminergic neurons (Lin et al. 1993). Later, it has been reported in many other tissues including the testis. It has been identified as a secretory product from components of niche cells. It has been reported to be secreted from Sertoli cells (Meng et al. 2000) and peritubular myoid cells (Chen et al. 2016). Expression of GDNF receptors, GFRα1 (GDNF-family receptor α1) and Ret (rearranged during transformation), are observed in undifferentiated spermatogonia (Tadokoro et al. 2002). Its cyclic expression is required for TSCs self-renewal by impeding TSC differentiation and not by fostering its propagation (Sharma and

Braun 2018). It has been also recorded that in vitro culture of TSCs requires the exogenous supplementation of GDNF (Kubota et al. 2004a, b; Kanatsu-Shinohara et al. 2005; Hofmann 2008; He et al. 2008) and addition of GDNF in vitro promotes proliferation and self-renewal by activating the phosphoinositide-3 kinase (PI3K)/AKT pathway (Lee et al. 2007). In contrast, in vivo, ERK1/2 signalling has been shown to support SSC self-renewal, whereas PI3K/AKT signalling has been suggested to support SSC proliferation during stages when RA signalling is low and differentiation when RA signalling is high (Hasegawa et al. 2013). Here, we investigate the mode of GDNF expression throughout the spermatogenic cycle and ask how disruption of its expression promotes SSC self-renewal and AKT-mediated signalling downstream of GFRA1/RET.

However, the regulatory signals that induce GDNF production and release have not yet been established. In neural cells, the induction of GDNF mRNA production and protein release is generally due to activation of the MEK/MAPK signalling pathway (Hisaoka et al. 2007), while transcription factor Pax2 plays an important role in GDNF expression in embryonic kidney. Deletion of the Hoxa11/Hoxc11/Hoxd11 gene cluster also results in the loss of GDNF expression in the embryonic kidney although these mice had normal expression of Pax2 Six2, a member of the Six families of homeobox containing transcription factors and Eya1 (mammalian ortholog of eyes absent in Drosophila) also activate GDNF gene expression in the embryonic kidney (Brodbeck et al. 2004). In testes, GDNF mRNA and protein have been localized in Sertoli cells (Meng et al. 2000), spermatogonia and spermatids (Yu et al. 2003) in the mouse. Similar results have also been reported for rats and humans (Yu et al. 2003). In Sertoli cells, the production of GDNF is likely under the influence of FSH, growth factors and cytokines secreted both locally and systemically (Simon et al. 2007; Tadokoro et al. 2002). Under in vitro conditions, GDNF production by Sertoli cells was also shown to be dependent on FGF2, TNFα and interleukin-1beta (Il-1β) (Simon et al. 2007). In Sertoli cell line TM4, FGF2 stimulated the expression of GDNF by fivefold at 48 h after treatment while FSH stimulated the GDNF expression by twofold at 3–24 h after treatment (Simon et al. 2007). Stimulation of GDNF expression by FGF2 in TM4 cells was suppressed by pre-treatment of the cells with phosphatidyl inositol 3-kinase (PI3K) inhibitor wortmannin and the mitogen-activated protein kinase (MAPK) signalling inhibitor PD98059. However, neither inhibitor alone nor when added together completely abolished the FGF2-stimulated increase in the GDNF expression. This indicates that cellular mechanisms other than MAPK and PI3K pathways may also be involved in FGF2-mediated induction of GDNF expression. Interestingly, however, FGF2 knockout mouse is fertile (Ortega et al. 1998). It is also not clear how wortmannin or PD98059 affected FSH induction of GDNF in TM4 cells (Simon et al. 2007).

GDNF act downstream by activating multiple signalling pathways including PI3K and the Src family of tyrosine kinases.

Ras Signalling Pathway

Binding of GDNF with Ret receptor can activate Ras signalling pathway. Upon ligand binding, Ret receptor auto-phosphorylates on its intracellular domain to serve

as intracellular docking sites for different SH2-domain-containing proteins which, in turn can induce binding and activation of the adaptor proteins Shc and Grb2 to stimulate Ras/ERK1/2 pathway (He et al. 2008). This signalling cascade ultimately leads to the phosphorylation and activation of transcription factors such as Creb-1, Atf-1 and Crem-1 to up-regulate the transcription of the immediate-early gene c-fos, the cell cycle activator cyclin A, and cdk2, which all favours the G1/S cell cycle transition in GFRα-1 positive spermatogonia (Sunters et al. 2004).

Src Signalling Pathway

SSCs also express several members (e.g. Src, Yes, Lyn and Fyn) of Src kinase family which co-precipitate with Ret after GDNF stimulation (Braydich-Stolle et al. 2007). Although the function of Src kinase family members overlap, it is believed that Src and Yes play a predominant role in the immediate response of SSCs to GDNF. Src activates a PI3K/Akt signalling pathway to stimulate the expression of N-myc which promotes SSC proliferation both in vivo (Lee et al. 2007; Oatley et al. 2007) and in vitro (Braydich-Stolle et al. 2007).

FGFR2 Signalling Pathway

FGFR2 is expressed in SSC and may play a role analogous to Ret in regulating SSC self-renewal and differentiation (Goriely et al. 2003). Besides, GDNF may also up-regulate the expression of FGFR2 in SSCs and thereby, increase their responsiveness to FGF2 for in vitro proliferation (Hofmann et al. 2005). Thus, GDNF and FGF2 are often used together to establish long-term cultures of gonocytes and SSCs (Kanatsu-Shinohara et al. 2003; Kubota et al. 2004b).

B Cell CLL/Lymphoma 6, Member B (Bcl6b)

Bcl6b is a validated target of GDNF that was initially identified in SSCs by microarray analysis (Oatley et al. 2006). Targeted disruption of Bcl6b results in an increased incidence of Sertoli cell-only tubules in mice. Similarly, its inhibition of Bcl6b expression in in vitro cultured SSCs by RNA interference resulted in SSC depletion. Thus, Bcl6b has a critical role in SSC maintenance. Like *N-Myc*, *Bcl6b* is also a nuclear target of the Src signalling pathway (Oatley et al. 2007).

10.5.2.3 Fibroblast Growth Factor 2 (FGF2)

Fibroblast growth factor 2 is also one of the extrinsic factors which acts along with GDNF. It activates MAP2K1 and supports propagation through MAP2K1 pathways. It stimulates greater MAPK1/3 phosphorylation as compared to that of GDNF. The stimulated MAP2K1 engenders the expression of Etv5, which in turn increases the expression of Bcl6b and thus promotes self-renewal of TSCs (Ishii et al. 2012). FGF2 is being secreted from several cell types (Sertoli cells, Leydig cells, differentiating germ cells), has been established as a mediator for interaction between Sertoli cells and peritubular myoid cells and acts as a survival factor for these cells (El Ramy et al. 2005).

10.5.2.4 Colony-Stimulating Factor 1 (CSF-1)

Colony-stimulating factor 1 (CSF1) has been recognized as an extrinsic factor of TSC self-renewal. It is known to be secreted from Leydig and PM cells (Oatley et al. 2009). Chen et al. (2014) reported that testosterone-dependent regulation of GDNF expression in PM cells has a significant influence on the microenvironment of the niche and SSC maintenance. Although in vivo analysis of the niche is difficult owing to the three-dimensional structure of seminiferous tubules, in vitro reconstitution of the SSC niche has recently become possible (Kanatsu-Shinohara et al. 2012).

10.5.3 Adhesion Molecules

Stem cells are anchored to their niche via adhesion molecules (cadherins, integrins, gap junction components, etc.) and these molecules are very important in self-renewal of the concerned stem cells (Chen et al. 2013). Kanatsu-Shinohara et al. (2008) established the vital role of β1-integrin in the self-renewal of TSCs and recorded that this adhesion molecule is required both on TSCs and Sertoli cells for TSCs to be at their normal location at the basal lamina of seminiferous tubules. The adhesion molecule E-cadherin has been established as a decidedly specific surface marker of mouse SSCs (Tolkunova et al. 2009). Cadherin promotes self-renewal of TSCs via activating Janus kinase/signal transducer and activator of transcription (JAK-STAT) and phosphoinositide-3 kinase (PI3-K)/Akt and blocking transforming growth factor (TGF)-β1 signalling (Wu et al. 2008).

10.5.4 Age

Age is also important factor which influences the niche and their secretary molecules which in turn influences TSCs. Decline of the TSC niche with age of the animals and Drosophila testis have been studied, where the decline of expression of a key self-renewal signal, Unpaired (Upd), correlates with a decrease in SSCs with ageing (Boyle et al. 2007). Conversely, forced expression of Upd by the somatic cells of the niche maintains SSCs in older males. Therefore, similar molecular mechanisms within the testicular niche maintain self-renewal across species, and their alterations contribute to a decline of the stem cell pool and spermatogenesis.

10.5.5 FSH

Recent research has indicated that the follicle-stimulating hormone (FSH) influences TSCs propagation. Patel and Bhartiya (2016) recorded follicle-stimulating hormone receptors on the TSCs in mice and indicated that it is regulated by FSH. It has also been reported that FSH triggers canine TSCs and activates the formation of clumps of germ cell possibly via inferred local factors secreted from the Sertoli cells (Pieri et al. 2019). They concluded that FSH increases the number of canine TSCs in

culture condition and postulated that the hormone can stimulate the rate of propagation and self-renewal of TSCs. This research needs to be extended further to establish its exact mechanism of action.

10.6 Conclusions

The destiny of the stem cells is shaped by dedicated microenvironments in which these cells are located. The physical microenvironment of TSC is constituted of Sertoli cells, Leydig cells, PM cells, macrophages, basement membrane and surrounding blood vessels. Dynamic changes are observed in the niche of TSCs from prenatal life to postnatal life and during the process of ageing. The intrinsic (Taf4b, Plzf and Chd1l) and extrinsic factors (GDNF, FGF2, CSF-1) play important role in the regulation of TSC self-renewal by cell-to-cell interaction between TSC and niche cells. The adhesion molecules, age of the animal and follicular stimulating hormone also influence the TSC and its niche thus influences spermatogenesis. Newly developed method of in vitro reconstituted niche in culture system will provide a distinctive instrument to study the interactions between SSCs and their microenvironment.

References

Banks WJ (1993) Applied veterinary histology, 3rd edn. Mosby Yearbook Inc., St. Louis, MO, pp 477–486

Bhushan S, Meinhardt A (2017) The macrophages in testis function. J Reprod Immunol 119:107–112

Boyle M, Wong C, Rocha M et al (2007) Decline in self-renewal factors contributes to aging of the stem cell niche in the *Drosophila* testis. Cell Stem Cell 1:470–478

Braydich-Stolle L, Kostereva N, Dym M et al (2007) Role of Src family kinases and N-Myc in spermatogonial stem cell proliferation. Dev Biol 304:34–45

Brodbeck S, Besenbeck B, Englert C (2004) The transcription factor Six2 activates expression of the Gdnf gene as well as its own promoter. Mech Dev 121:1211–1222

Buaas FW, Kirsh AL, Sharma M et al (2004) Plzf is required in adult male germ cells for stem cell self-renewal. Nat Genet 36:647–652

Carlo AQ, Pathak D, Singh O et al (2019) Development of spermatogonial stem cell niche and immunoexpression of vimentin filaments in the testes of prenatal and postnatal Indian buffalo (*Bubalus bubalis*). Turkish J Vet Anim Sci 43(6):817–824

Chen C, Ouyang W, Grigura V et al (2005) ERM is required for transcriptional control of the spermatogonial stem cell niche. Nature 436:1030–1034

Chen S, Lewallen M, Xie T (2013) Adhesion in the stem cell niche: biological roles and regulation. Development 140(2):255–265

Chen LY, Brown PR, Willis WB et al (2014) Peritubular myoid cells participate in male mouse spermatogonial stem cell maintenance. Endocrinology 155(12):4964–4974

Chen LY, Willis WD, Eddy EM (2016) Targeting the GDNF gene in peritubular myoid cells disrupts undifferentiated spermatogonial cell development. Proc Natl Acad Sci 113 (7):1829–1834

Chiarini-Garcia H, Hornick JR, Griswold MD et al (2001) Distribution of type A spermatogonia in the mouse is not random. Biol Reprod 65(4):1179–1185

Chiarini-Garcia H, Raymer AM, Russell LD (2003) Non-random distribution of spermatogonia in rats: evidence of niches in the seminiferous tubules. Reproduction 126(5):669–680

Costoya JA, Hobbs RM, Barna M et al (2004) Essential role of Plzf in maintenance of spermatogonial stem cells. Nat Genet 36:653–659

De Rooij DG (2009) The spermatogonial stem cell niche. Microsc Res Tech 72(8):580–585

DeFalco T, Potter SJ, Williams AV et al (2015) Macrophages contribute to the spermatogonial niche in the adult testis. Cell Rep 12(7):1107–1119

Dhyana RV, Rajendranath N, Kumar P et al (2016) Microanatomical studies on the testis of domestic pig (*Sus scrofa domestica*). 2016. Int J Sci Environ Technol 5(4):2226–2231

Egger GF, Witter K (2009) Peritubular contractile cells in testis and epididymis of the dog, *Canis lupus familiaris*. Acta Vet Brno 78:3–11

El Ramy R, Verot A, Mazaud S et al (2005) Fibroblast growth factor (FGF) 2 and FGF9 mediate mesenchymal–epithelial interactions of peritubular and Sertoli cells in the rat testis. J Endocrinol 187(1):135–147

Eurell JA, Frappier BL (2006) Dellmann's textbook of veterinary histology, 6th edn. Blackwell Publishing, Ames, IA, pp 233–245

Falender AE, Freiman RN, Geles KG et al (2005) Maintenance of spermatogenesis requires TAF4b, a gonad-specific subunit of TFIID. Genes Dev 19:794–803

Farooqui MM, Chandrapal A, Prakash A (2012) Histological and histochemical studies on the prenatal development of testis in goat (Capra hircus). Int J Morphol 30(4):1408–1421

Filipponi D, Hobbs RM, Ottolenghi S et al (2007) Repression of kit expression by Plzf in germ cells. Mol Cell Biol 27:6770–6781

Fujisawa M (2006) Regulation of testicular function by cell-to-cell interaction. Reprod Med Biol 5 (1):9–17

Gaytan F, Bellido C, Aguilar E et al (1994) Requirement for testicular macrophages in Leydig cell proliferation and differentiation during prepubertal development in rats. J Reprod Fertil 102:393–399

Gofur MR, Khan MZI, Karim MR, Islam MN (2008) Histomorphology and histochemistry of testis of indigenous bull (*Bos indicus)* of Bangladesh. Bangladesh J Vet Med 6:67–74

Goriely A, McVean GA, Rojmyr M et al (2003) Evidence for selective advantage of pathogenic FGFR2 mutations in the male germ line. Science 301:643–646

Hales DB (2002) Testicular macrophage modulation of Leydig cell steroidogenesis. J Reprod Immunol 57(1–2):3–18

Hasegawa K, Namekawa SH, Saga Y (2013) MEK/ERK signaling directly and indirectly contributes to the cyclical self-renewal of spermatogonial stem cells. Stem Cells 31 (11):2517–2527

He Z, Feng L, Zhang X et al (2005) Expression of Col1a1, Col1a2 and procollagen I in germ cells of immature and adult mouse testis. Reproduction 130:333–341

He Z, Jiang J, Kokkinaki M et al (2008) GDNF upregulates c-Fos transcription via the Ras/Erk1/2 pathway to promote mouse spermatogonial stem cell proliferation. Stem Cells 26(1):266–278

Hess RA, Vogl AW (2015) Sertoli cell anatomy and cytoskeleton. In: Griswold M (ed) Sertoli cell biology. Elsevier/Academic Press, Oxford, pp 1–55

Hess RA, Cooke PS, Hofmann MC et al (2006) Mechanistic insights into the regulation of the spermatogonial stem cell niche. Cell Cycle 5:1164–1170

Hisaoka K, Takebayashi M, Tsuchioka M et al (2007) Antidepressants increase glial cell line-derived neurotrophic factor production through monoamine-independent activation of protein tyrosine kinase and extracellular signal-regulated kinase in glial cells. J Pharmacol Exp Ther 321:148–157

Hofmann MC (2008) GDNF signaling pathways within the mammalian spermatogonial stem cell niche. Mol Cell Endocrinol 288:95–103

Hofmann MC, Braydich-Stolle L, Dym M (2005) Isolation of male germ-line stem cells; influence of GDNF. Dev Biol 279:114–124

Hutson JC (1992) Development of cytoplasmic digitations between Leydig cells and testicular macrophages of the rat. Cell Tissue Res 267:385–389
Ibtisham F, Honaramooz A (2020) Spermatogonial stem cells for in vitro spermatogenesis and in vivo restoration of fertility. Cell 9(3):745
Ishii K, Kanatsu-Shinohara M, Toyokuni S et al (2012) FGF2 mediates mouse spermatogonial stem cell self-renewal via upregulation of Etv5 and Bcl6b through MAP2K1 activation. Development 139(10):1734–1743
Kanatsu-Shinohara M, Ogonuki N, Inoue K et al (2003) Long-term proliferation in culture and germline transmission of mouse male germline stem cells. Biol Reprod 69:612–616
Kanatsu-Shinohara M, Miki H, Inoue K et al (2005) Long-term culture of mouse male germline stem cells under serum-or feeder-free conditions. Biol Reprod 72(4):985–991
Kanatsu-Shinohara M, Inoue K, Lee J et al (2006) Anchorage-independent growth of mouse male germline stem cells in vitro. Biol Reprod 74(3):522–529
Kanatsu-Shinohara M, Takehashi M, Takashima S et al (2008) Homing of mouse spermatogonial stem cells to germline niche depends on β1-integrin. Cell Stem Cell 3(5):533–542
Kanatsu-Shinohara M, Inoue K, Takashima S et al (2012) Reconstitution of mouse spermatogonial stem cell niches in culture. Cell Stem Cell 11(4):567–578
Kishore PVS, Ramesh G, Basha SH (2007) Intertubular tissue in the testis of ram—a postnatal histological study. Indian J Vet Anat 19(2):7–10
Kishore PVS, Ramesh G, Basha SH (2011) Sertoli cells in the testis of ram—a postnatal histological study. Indian J Vet Anat 23(1):56–59
Kierszenbaum AL, Tres LL (2016) Histology and cell biology: an introduction to pathology, 4th edn. Saunders/Elsevier Inc., Canada, pp 613–614
Kubota H, Avarbock MR, Brinster RL (2004a) Culture conditions and single growth factors affect fate determination of mouse spermatogonial stem cells. Biol Reprod 71:722–731
Kubota H, Avarbock MR, Brinster RL (2004b) Growth factors essential for self-renewal and expansion of mouse spermatogonial stem cells. Proc Natl Acad Sci U S A 101:16489–16494
Lee J, Kanatsu-Shinohara M, Inoue K et al (2007) Akt mediates self-renewal division of mouse spermatogonial stem cells. Development 134:1853–1859
Liebich HG (2019). Veterinary histology of domestic mammals and birds. 5th Edn. 5M Publishing Ltd, Sheffield, p 276-299
Lin LF, Doherty DH, Lile JD et al (1993) GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. Science 260:1130–1132
Liu SS, Bai YS, Feng L et al (2016) Identification of CHD1L as an important regulator for spermatogonial stem cell survival and self-renewal. Stem Cells Int 2016:4069543. https://doi.org/10.1155/2016/4069543
Meng X, Lindahl M, Hyvonen ME et al (2000) Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. Science 287:1489–1493
Moonjit P, Adcharatt S (2007) Histological structure of testes and ductus epididymis of Rusa deer (*Cervus timorensis*). Kasetsart J (Nat Sci) 41:86–90
Oatley JM, Brinster RL (2012) The germline stem cell niche unit in mammalian testes. Physiol Rev 92(2):577–595. https://doi.org/10.1152/physrev.00025.2011
Oatley JM, Avarbock MR, Telaranta AI et al (2006) Identifying genes important for spermatogonial stem cell self-renewal and survival. Proc Natl Acad Sci U S A 103:9524–9529
Oatley JM, Avarbock MR, Brinster RL (2007) Glial cell line-derived neurotrophic factor regulation of genes essential for self-renewal of mouse spermatogonial stem cells is dependent on Src family kinase signaling. J Biol Chem 282:25842–25851
Oatley JM, Oatley MJ, Avarbock MR et al (2009) Colony stimulating factor 1 is an extrinsic stimulator of mouse spermatogonial stem cell self-renewal. Development 136:1191–1199
Oatley MJ, Racicot KE, Oatley JM (2011) Sertoli cells dictate spermatogonial stem cell niches in the mouse testis. Biol Reprod 84(4):639–645
Ortega S, Ittmann M, Tsang SH et al (1998) Neuronal defects and delayed wound healing in mice lacking fibroblast growth factor 2. Proc Natl Acad Sci U S A 95:5672–5677

Patel H, Bhartiya D (2016) Testicular stem cells express follicle-stimulating hormone receptors and are directly modulated by FSH. Reprod Sci 23(11):1493–1508

Pathak A (2016) Histology and histochemistry of interstitial tissue of testes in mountain (Gaddi) goats—a postnatal developmental study. J Anim Res 6(2):83–87

Pathak A, Katiyar RS, Sharma DN et al (2014) Postnatal development of testis in Gaddi goat (*Capra hircus*). Int J Morphol 32(1):166–176

Petersen C, Soder O (2006) The Sertoli cell—a hormonal target and 'super' nurse for germ cells that determines testicular size. Horm Res 66(4):153–161

Pieri NCG, Mançanares ACF, de Souza AF et al (2019) Xenotransplantation of canine spermatogonial stem cells (cSSCs) regulated by FSH promotes spermatogenesis in infertile mice. Stem Cell Res Ther 10:135. https://doi.org/10.1186/s13287-019-1250-9

Prochazkova M, Chavez MG, Prochazka J et al (2015) Embryonic versus adult stem cells. In: Vishwakarma A et al (eds) Stem cell biology and tissue engineering in dental sciences, 1st edn. Academic Press, Boston, pp 249–262

Richardson LL, Kleinman HK, Dym M (1995) Basement membrane gene expression by Sertoli and peritubular myoid cells in vitro in the rat. Biol Reprod 52(2):320–330

Ryu BY, Orwig KE, Oatley JM et al (2006) Effects of aging and niche microenvironment on spermatogonial stem cell self-renewal. Stem Cells 24(6):1505–1511

Schofield R (1978) The relationship between the spleen colony-forming cell and the haemopoietic stem cell. Blood Cells 4(1–2):7–25

Sharma M, Braun RE (2018) Cyclical expression of GDNF is required for spermatogonial stem cell homeostasis. Development 145(5):151555

Sharma A, Kumaresan A, Mehta P et al (2019) Successful transplantation of transfected enriched buffalo (*Bubalus bubalis*) spermatogonial stem cells to homologous recipients. Theriogenology 142:441–449. https://doi.org/10.1016/j.theriogenology.2019.10.019

Shen Q, Goderie SK, Jin L et al (2004) Endothelial cells stimulate self-renewal and expand neurogenesis of neural stem cells. Science 304(5675):338–1340

Shukla P, Bhardwaj RL, Rajesh R (2013) Histomorphology and micrometry of testis of Chamurthi horse. Indian J Vet Anat 25(1):36–38

Simon L, Ekman GC, Tyagi G et al (2007) Common and distinct factors regulate expression of mRNA for ETV5 and GDNF, Sertoli cell proteins essential for spermatogonial stem cell maintenance. Exp Cell Res 313:3090–3099

Singh TS, Kalita PC, Choudhary OP et al (2018) Histomorphological studies on the testis of local pig (Zovawk) of Mizoram. Indian J Anim Res:1–4. https://doi.org/10.18805/ijar.B-3697

Skinner MK (1991) Cell-cell interactions in the testis. Endocr Rev 12(1):45–77

Sunters A, Thomas DP, Yeudall WA et al (2004) Accelerated cell cycle progression in osteoblasts overexpressing the c-fos proto-oncogene: induction of cyclin A and enhanced CDK2 activity. J Biol Chem 279:9882–9891

Tadokoro Y, Yomogida K, Ohta H et al (2002) Homeostatic regulation of germinal stem cell proliferation by the GDNF/FSH pathway. Mech Dev 113:29–39

Tolkunova EN, Malashicheva AB, Chikhirzhina EV et al (2009) E-cadherin as a novel surface marker of spermatogonial stem cells. Cell Tiss Biol 3:103–109

Weissman IL (2000) Stem cells: units of development, units of regeneration, and units in evolution. Cell 100(1):157–168

Wu J, Zhang Y, Tian GG et al (2008) Short-type PB-cadherin promotes self-renewal of spermatogonial stem cells via multiple signaling pathways. Cell Signal 20(6):1052–1060

Yokonishi T, McKey J, Ide S et al (2020) Sertoli cell ablation and replacement of the spermatogonial niche in mouse. Nat Commun 11(1):1–11

Yoshida S, Sukeno M, Nabeshima YI (2007) A vasculature-associated niche for undifferentiated spermatogonia in the mouse testis. Science 317(5845):1722–1726

Yu Z, Guo R, Ge Y et al (2003) Gene expression profiles in different stages of mouse spermatogenic cells during spermatogenesis. Biol Reprod 69:37–47

Zhang C, Yeh S, Chen YT et al (2006) Oligozoospermia with normal fertility in male mice lacking the androgen receptor in testis peritubular myoid cells. Proc Natl Acad Sci 103 (47):17718–17723

Zhao H, Li T, Yang H et al (2019) The effects of growth factors on proliferation of spermatogonial stem cells from Guangxi Bama mini pig. Reprod Domest Anim 54(12):1574–1582

Proteomics of Mammary Gland and Mammary Stem Cells

11

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Abstract

Mammary gland (MG) biology has attracted researchers' attention in every mammalian species. Apart from the natural curiosity to understand the mammalian biology wherein newborns are nourished by milk, the incidence of benign and metastatic breast tumor further instigated the scientific community to understand the detailed cellular and molecular events so that the diagnosis, treatment, and prevention could be implemented. The mouse MG has served as the closest MG model to understand the anatomy and physiology despite several differences in the endocrine and reproductive systems of mice and humans. In bovine, the very immediate interest has been to improve milk production from dairy animals. Globally, the average milk yield from dairy animals has so far remained forwardly progressive.

Nevertheless, disease like mastitis and the existence of low milk-yielding cows in population have necessitated accelerated research on understanding bovine mammary gland biology, host–pathogen relationship, cyclic changes in molecular and cellular physiology during puberty, pregnancy, lactation, and involution. Proteomics deals with studying many proteins together, thus deriving comprehensive information on cellular physiology. Along with antibodies, the application of mass spectrometry in the study of proteins has revolutionized the investigation method as never before. The complexity in tissue architecture of MG comprises many cell types, and their continuous turnover in the lifetime of an animal constantly challenged our mind and approach to discern cell identity correctly. It will not have been possible without the knowledge of cell type-specific marker proteins, which again owes a lot to proteomics. This chapter selectively discusses the application of mass spectrometry-based proteomics for

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mammary epithelial cells and mammary stem cells in MG in the context of cell-specific biomarkers, functional differentiation, and diseases.

11.1 Introduction

Stem cells (SCs) are fascinating as they can long-term self-renewal, and the ability to differentiate into specialized cell lineages. They have been classified as totipotent, pluripotent, and multipotent. The mammary gland (MG) in mammals is a dynamic organ that witnesses growth and development in its structure and function in a cyclic manner. Right from the time in embryo, mammary fat pads are destined to differentiate into the mammary gland and then to its development during the prepubertal period until it starts following cycles of proliferation, differentiation, and involution, a complex set of cellular events takes place. The onset of apoptosis in terminally differentiated cells followed by the rejuvenation of new cells leading to morphological and functional changes is critically dependent on the presence of adult stem cells in the mammary gland called mammary stem cells (MaSCs). Anatomically, different types of cells together lay the organ's architectural and functional base. These cells include epithelial body cells, luminal epithelial cells, alveolar epithelial cells, epithelial cap cells, myoepithelial cells, and stromal cells (Inman et al. 2015). The schematics of various cell types constituting the architecture of mouse MG is shown in Fig. 11.1.

11.2 Cell Types and Specification of the Mammary Gland

With the progressive development in the udder, many other cell types reinforce the mammary gland structure, which includes adipocytes, fibroblasts, immune, lymphatic, and vascular cells. All these cells work together to sculpt and maintain a functional organ. The different cell types have been demonstrated to be of

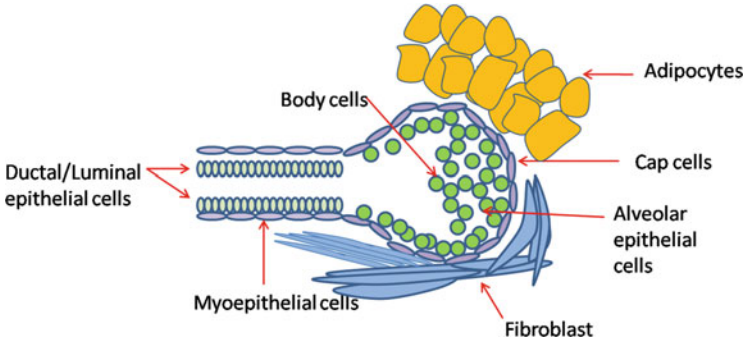

Fig. 11.1 Histological structure of mouse mammary gland with terminal end bud containing body cells and cap cells

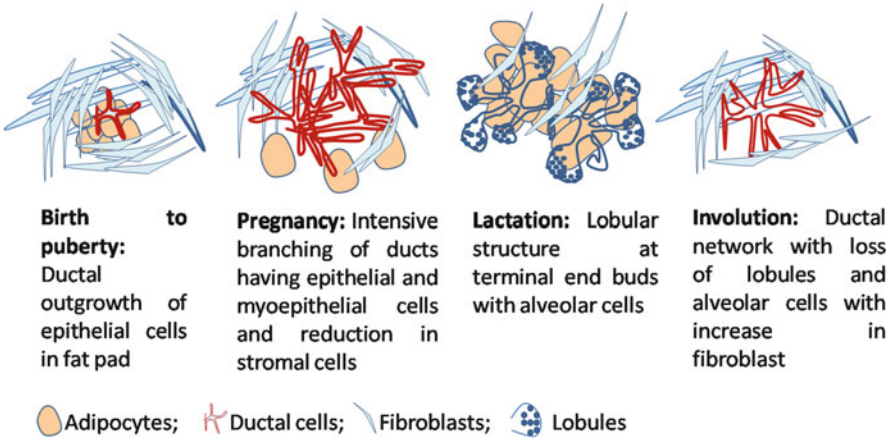

Fig. 11.2 Cyclical changes in mammary gland and changes in histological structures

importance at specific stages of mammary gland development (Fig. 11.2). From the viewpoint of mammary tissue regeneration, it is essential that adult stem cells must be able to give rise to all such cell lineages. A large amount of information on mammary stem cells has been generated for the mouse model and human mammary epithelial cells. Most of the data is based on the basic knowledge gained from other well-studied species like murine and human in bovine. This chapter will discuss the proteomics-based advancement in mammary epithelial cells of mice and humans; however, the focus will be on bovine and related species wherever possible.

11.3 Mammary Epithelial Cells

The essential functional components of the mammary gland are the epithelial cells. Various types of epithelial cell types can be found in the mammary gland. Many studies revealed that the fat pad (adipocytes) in embryos leads to the development of epithelial cell niche (Parmar and Cunha 2004). The exact transition mechanism or stem cells' role is not very well understood. Because the mammary gland is a type of sebaceous gland and is a kind of skin appendage that undergoes significant growth and cyclical remodeling in postnatal life, Joshi et al. (2019) demonstrated that adipocytes provide a niche of specialized cells called mesenchymal adipogenesis cell pool, which is characterized by the expression of platelet-derived growth factor receptor alpha (PDGFRα) and preadipocyte factor-1 (PREF-1). PDGFR$^+$ and PREF-1$^+$ cells are recruited to form mammary epithelium during early development and in the adult gland during pregnancy. The mammary epithelial expansion is an outcome of the extensive migration of PDGFRα$^+$ stromal cells from the stromal adipocyte niche.

The coexistence of various cells in the udder poses a challenge in its study and thus warrants a useful and robust tool to discern the various types of cell types.

Fluorescence-activated cell sorting (FACS) has dramatically helped distinguish and separate different types of cells from a section of udder tissue. The FACS ability depends on our knowledge about unique and specific biomarkers present on each cell type (Table 11.1). Many proteins have been earmarked as surface biomarkers for various types of epithelial cells, like $CD49f^{lo}EpCAM^{+}$ for luminal epithelial cells and $CD49f^{hi}$EpCAM+ for basal epithelial cells. The stromal fibroblast cells lack these markers (Shehata et al. 2012; Stingl et al. 2006).

Luminal cells express keratins (CK) 8 and CK 18, whereas myoepithelial cells express CK 5 and CK 14 and smooth muscle actin (SMA) that mediates their contractile function. Cell-sorting experiments have identified several putative stem/progenitor cells in the basal cell population (Shackleton et al. 2006; Visvader and Stingl 2014). CK 18-expressing luminal cells are committed luminal cells that do not show clonal expansion.

The proportion and type of cells in the mammary gland vary according to the development stage. During puberty, in murine, two types of specialized epithelial cells, cap cells and body cells, arise in the terminal end bud (TEB). Cap cells line the end bud forming the structure's cap, surrounding stroma through a thin basement membrane. Cap cells differentiate into myoepithelial cells that generate a thicker basal lamina (Daniel and Silberstein 2000; Williams and Daniel 1983). By contrast, the body cells fill the interior of the end bud. The central body cells form lumen by apoptosis of the central body cells, and the peripheral body cells differentiate into luminal epithelial cells. Luminal epithelial cells are the ductal epithelium that secretes milk during lactation (Hennighausen and Robinson 2005). During pregnancy, under the influence of progesterone hormone, luminal epithelial cells rapidly expand, forming alveoli—the milk-secreting unit. Mammary alveoli are lined with luminal cells primed to secrete milk at parturition. The myoepithelial cells produce a high level of laminin-111, which is critical to determine cell polarity and survival of luminal cells (Boudreau et al. 1995).

$EpCAM^{+}MUC1^{-}$ cells express a high level of K19 and are supposed to enrich ductular and luminal epithelial cells in terminal buds regions (Gudjonsson et al. 2002). There are differences in the biomarker for these cell types in humans and mice. GATA3, ErbB3, and ALDH expression are characteristics of luminal cells in humans (Asselin-Labat et al. 2007; Shehata et al. 2012) while CD14 (Shehata et al. 2012), c-Kit (Lim et al. 2009; Regan et al. 2012), and Elf5 (Zhou et al. 2019; Oakes et al. 2008) are expressed in murine luminal epithelial cells.

11.4 Adipocytes

Most of the mammary gland in adult and nonlactating udder comprises adipocytes that are rich in fat (Sakakura 1987). During pregnancy and lactation, the adipocytes furnish milk synthesis's metabolic demand and allow MECs' expansion (Gregor et al. 2013; Hovey and Aimo 2010). Adipocytes also facilitate communication among all cell types by synthesizing and secreting various factors like vascular

Table 11.1 Mammary gland cell types and specific protein-based markers for their characterization

Marker proteins	Mammary epithelial cells	Mammary stem cells	Adipocytes	Stromal fibroblast cells	References
$CD49f^{lo}EpCAM^{+}$	Luminal epithelial cells			Negative	Shehata et al. (2012), Stingl et al. (2005)
$CD49f^{hi}EpCAM^{+}$	Basal epithelial cells			Negative	Shehata et al. (2012), Stingl et al. (2005)
CK 8 and CK 18 Hormone receptors (HR, ER/PR)	Luminal epithelial cells				Gudjonsson et al. (2002)
Keratins 5 and 14, p63	Myoepithelial cells				Tharmapalan et al. (2019)
Smooth muscle actin (SMA)	Myoepithelial cells				
CD 39^{+}, $CD44^{+}$, fibroblast-activated protein (FAP), fibroblast-specific protein-1 (S100A4)				Positive	Agorku et al. (2019)
$CD105^{+}$, $CD26^{+high}$				Interlobular fibroblast	Avagliano et al. (2020)
(TGF)-$\beta1^{+}$, TNC^{+}, and α-SMA^{+}				Intralobular fibroblast	Morsing et al. (2016)
$CD31^{-}CD45^{-}Ter119^{-}$ $CD49f^{hi}$ $EpCAM^{hi}\alpha SMA^{+}$ or $Myh11^{+}$	Myoepithelial cells				Prater et al. (2014)
$PDGFR\alpha^{+}$ and $PREF\text{-}1^{+}$			Platelet-derived growth factor receptor alpha (PDGFRα) and preadipocyte factor-1 (PREF-1		Joshi and Khokha (2012)
Lin^{-}		Negative			Inman et al. (2015)

(continued)

Table 11.1 (continued)

Marker proteins	Mammary epithelial cells	Mammary stem cells	Adipocytes	Stromal fibroblast cells	References
$CD24^{med}$ and $CD49f^{+}$.		Positive			Rauner and Barash (2012)
$CD24^{neg}$, $CD49f^{pos}$		Basal bipotent progenitors MaSCs			Rauner and Barash (2012)
$CD24^{high}$, $CD49f^{neg}$		Luminal unipotent progenitors MaSCs			Rauner and Barash (2012)
$CD24^{med}$, $CD49f^{neg}$		Luminal unipotent cells			Rauner and Barash (2012)
$CD45^{-}Ter119^{-}CD31^{-}$ $CD140a^{-}CD24^{med}CD49f^{hi}$		MaSC			Stingl et al. (2006)
$CD45^{-}CD31^{-}TER119^{-}$ $Lin^{-}CD24^{+}CD29^{hi}$		MaSC			Shackleton et al. (2006), Machado et al. (2013)
$Lin^{-}CD24^{+}CD29^{+}Ccnys^{-}$		Basal stem cell			Zeng and Nusse (2010)
$CD45^{-}CD31^{-}Lrp5^{+}$		MaSC			Badders et al. (2009)
$CD31^{-}CD45^{-}Ter119^{-}$ $CD24^{+}CD29^{hi}Axin2^{+}$		MaSC			Zeng and Nusse (2010)
$ESR1^{+}$ and $ALDH3B1^{high}$ $NR5A2^{high}$ $XIST^{-}$	Label retaining basal epithelial cells				Choudhary et al. (2013)
Laminin-111	Myoepithelial cell				Muschler and Streuli (2010)

Note: $CD45^{-}$ and $Ter119^{-}$: to exclude the hematopoietic cells; $CD31^{-}$: to exclude the endothelial cells; $CD140a^{-}$: to exclude the stromal cells

endothelial growth factor (VEGF) that promotes angiogenesis in the mammary gland (Hovey et al. 2001).

11.5 Fibroblasts

Fibroblast cells constitute the stroma of the udder. They are in continuous remodeling to adjust with space and volume created by cyclic changes in MECs. Fibroblasts usually are close to the basal side of the epithelial branching tree (Muschler and Streuli 2010; Sakakura 1987) wherefrom they send and receive chemical signals and regulate the dynamics of the extracellular matrix (ECM) (Howard and Lu 2014). Many studies suggest that fibroblasts play an essential role in supporting both survival of epithelial cell and tubular growth in the fat pad (Liu et al. 2012; Makarem et al. 2013; Wang and Kaplan 2012). Furthermore, fibroblasts are considered the main components of the mammary ECM. They synthesize several ECM components, such as collagens, proteoglycans, and fibronectin. They also synthesize many enzymes, such as matrix metalloproteinases, capable of degrading the ECM and releasing the growth factors and cytokines embedded within the ECM and influence cellular and tissue function (Wiseman and Werb 2002). Studies indicated that fibroblasts could regulate epithelial cell features and affect ECM to determine the mammary gland (Lühr et al. 2012).

11.6 Vascular Cells and Immune Cells

The mammary gland is intercalated with extensive vascular and lymphatic networks present throughout the fat pad. During pubertal mammary gland morphogenesis, the lymphatic network develops close to the mammary epithelial tree and blood vasculature (Betterman et al. 2012). Lymphangiogenesis in the mammary gland is driven by myoepithelial-derived VEGF-C and/or VEGF-D (Betterman et al. 2012). Branching morphogenesis of mammary ducts requires immune cells, such as macrophages and eosinophils. Immune cells secrete cytokines and other growth factors required for branching off a ductal tree. They are recruited to the epithelium's branching tips to mediate invasion into the fat pad (Gouon-Evans et al. 2000). Macrophages are also required to clear dyeing epithelial cell and adipocyte repopulation during involution (O'Brien et al. 2012). By activating their serine proteases and degranulation, mast cells are involved in normal mammary branching during puberty. They accumulate and possibly trigger plasma kallikrein, thus starting the plasminogen cascade during involution (Lilla et al. 2009; Lilla and Werb 2010).

The previous information is that mammary gland's backdrop must be composed of many cell types. These cells' functions are orchestrated finely to establish communication among all the compartments so that the mammary gland can synthesize and secrete milk regularly in a cyclic manner following proliferation, differentiation, and involution. Most of the research is focused on the epithelium because it shows dramatic alterations in function and structure during pregnancy and lactation.

Also, incidences of mammary tumors have predominantly been reported in the epithelial compartment. However, the lessons on adult stem cells have taught us that the complete regeneration of the mammary gland is not possible unless we can understand and derive messages from the adult mammary stem cells with more clarity. The last decade of study has witnessed dedicated efforts to identify and characterize mammary stem cells (MaSC). High-throughput technologies allow broader and detailed knowledge of different cell types. The advancement in technologies like FACS and mass spectrometry-based proteomics and metabolomics have generated promises for marker-based identification, isolation, and culture of such cells and unravels the mystery of such hidden MaSC. This chapter will emphasize using proteomics to understand mammary epithelial cells (MECs) and mammary stem cells (MaSCs).

11.7 Proteomics: The Ability to Study Many Proteins Together

The biological outcome of a cell is the outcome of many proteins working together. The past studies focused more on candidate-specific functional annotation of the target protein, although accuracy was time-taking and antibody-based. The real-time research in a comprehensive manner could be made possible to comprehend many proteins together, qualitatively and quantitatively. Many of the low abundant proteins or highly homologous proteins, including isoforms, could be studied to their more sufficient detail because of the use of a mass spectrometry-based targeted and untargeted proteomics approach.

The complete workflow of proteomics is as follows:

1. **Preparation of sample**: It includes optimizing methods for extracting as much protein as possible without any loss. There are various protein extraction methods in general from different tissues that have been suitably modified for MG tissues (Jena et al. 2015; Zheng et al. 2017; Dai et al. 2017; Reinhardt and Lippolis 2020). Protein extraction from MECs or MaSC does not require any extreme variation in the standard protocol optimized for other cells. Most of the studies use an 8M urea-based buffer and a cocktail of protease inhibitors (Williams et al. 2016).
2. **Quantitation or accurate estimation of total protein**: It is essential because a known quantity helps us to use the right amount of protein in the protocol. When comparing two samples, the precise number of proteins used in the protocol helps arrive at better biological interpretation and conclusion.
3. **Qualitative identification of proteins**: The crux of this step is separating the proteins as much as possible with minimal loss. It is also called as resolving the sample. The better we can divide the protein mixture, the better the identification accuracy and minimal missing of low abundant protein will be. In the qualitative approach of protein identification, researchers are interested in knowing the qualitative information of protein present in a given sample. Traditionally, two primary platforms have been used to examine protein samples: (1) gel-based and

(2) gel-free approach. In the gel-based method, either one-dimensional SDS-PGE or 2-D gel electrophoresis is used to resolve the proteins. In contrast, in a gel-free process, the protein mixture is first digested with a suitable enzyme to bring them all into small peptides (for technical ease) and separated under liquid chromatography using various types of columns like ion exchange, basic Reverse Phase Liquid Chromatography (RPLC), etc. Fractionation of the peptide mixture based on chemical differences is an important strategy to improve identification in study 68 digested protein fractions [24 digested protein fractions for subcellular fraction-I (SCF-I); 12 digested protein fractions—each for SCF-II, -III, and -IV; and 8 digested protein fractions for conditioned media together generated more than 12,500 proteins in buffalo MECs (BuMECs) (Jaswal et al. 2020)]. It is the highest number of proteins reported so far in any mammalian cell line.

4. **Quantitative information on protein sample**: In situations where the aim was to compare two or more models, both 2-D gel electrophoresis and liquid chromatography (LC) are used. In the former, the proteins in two or more samples are first separated on 2-D gels and compared manually or with sophisticated software for any quantitative difference among the protein spots, followed by identifying the protein by mass spectrometry. In the latter, where gels are not in use, the peptides (mostly tryptic peptides) are either quantitated using label-free or chemically tagged labels to the peptides during LC-MS/MS. In labeled approaches, protein modification techniques like Isobaric tag for relative and absolute quantification (iTRAQ), Tandem Mass Tag (TMT), metabolic labeling [stable-isotope labeling with amino acids in cell culture or (SILAC)], stable isotope labeling in mammals (SILAM), and dimethyl labeling are available. Even highly homologous proteins can be quantified through stable isotope labeling of intact proteins by reductive dimethylation (She et al. 2012).

A miniaturized workflow of proteomics experiments in the mammary gland is shown in Fig. 11.3. It involves qualitative and quantitative studies for various applications, whether it is biomarker discovery, developmental biology, disease-related changes in the proteome, or diagnostics.

11.8 Quality Control in MS-Based Experiments

Mass spectrometry experiments involve several components like liquid chromatography (ion exchange, basic RPLC, etc.), ionization units (Matrix Assisted Laser Desorption/Ionization [MALDI], Electrospray Ionization [ESI], Surface-Enhanced Laser Desorption/Ionization [SELDI], Atmospheric Pressure Ionization [API], etc.), and the actual mass analyzer unit (TOF or Fourier transformers) and the detectors. Finally, the decoding of the mass spectrum using search engines that employ database-dependent algorithms. All such steps require strict quality control over its operation. The biological and technical replicates are necessary to satisfy the results' statistical assessment. It becomes challenging to find a balance between the experiment's high cost and the required number of requisite samples in many

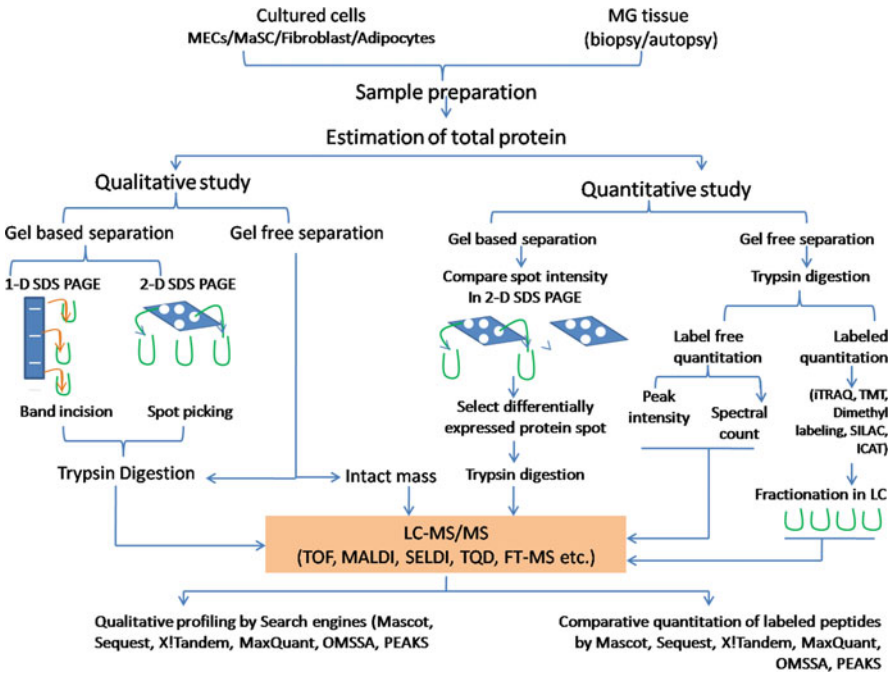

Fig. 11.3 Proteomics workflow for various profiling, quantitation, and biomarker discovery-related experiments in mammary gland study

circumstances. The minimum peptide length for a distinct match to a protein in the database has been considered above five amino acids. If two or more than two peptides are found for each target protein identified, the confidence level in its identification is usually better. The column length is another factor while resolving the complex mixture. Usually, 15 cm columns are used in a typical proteomics workflow. However, long columns of 45–60 cm are not uncommon.

11.9 The Proteome of Bovine Mammary Epithelial Cells

Ruminants (cattle, buffalo, goat, etc.) constitute a distinct class of livestock species domesticated for their milk-producing ability. Buffalo (*Bubalus bubalis*) includes a primary source of milk for human consumption, and the MECs are the central precursor cells responsible for their lactation potential (Jaswal et al. 2020). More than 12,500 proteins engaged in various structural and functional roles about lactation were reported in BuMEC by LC-MS/MS-based experiments in this study. The proteomics for mammary gland study has been widely used for various purposes, including development-related biology, identification of biomarkers, pathoimmunology, host cell response in infections, determination of differences between in vitro vs. in vivo cells, etc. The availability of an exhaustive and

comprehensive database about the MECs or MaSC proteins sets a stable platform for further investigation into understanding the ruminant mammary system.

11.10 MEC Secretome

The set of proteins secreted by cells usually present in the medium of growth (conditioned media) is called the cells' secretome. Though culture conditions are highly likely to alter the total secretome under a given set of requirements, cells' secretome profile offers clues about the critical mediators of cell communication, proliferation, and organization. In the largest ever data on MEC proteome, 792 proteins were identified in the conditioned media of BuMEC culture from expansion to functional differentiation using various hormonal stimulations (Jaswal et al. 2020). Similarly, analysis of human mammary epithelial cells (HMECs)-conditioned medium by LC-MS/MS revealed 889 unique proteins; 151 were specifically enriched in the extracellular compartment compared with whole HMEC lysate proteome (Jacobs et al. 2008). The MEC secretome study has opened new ways to understand mammary tumors' pathophysiology and their metastasis. Phorbol ester (PMA) is protein kinase c and stimulates secretion from MECs. Around 36 proteins were highly abundant in the secretome of HMEC upon PMA treatment (Jacobs et al. 2008).

11.11 Bioinformatics-Assisted Proteomics

Proteomics of MECs generates a large amount of data, and secretome can be potentially utilized using bioinformatics approaches to derive meaningful information about lactation biology. Typically, several algorithms are available to determine the relationship among the perturbed sets of proteins whose discussion is beyond this chapter's scope. However, a few bioinformatics platforms have contributed much to our understanding, such as an online tool for interacting genes/proteins interaction (STRING), CYTOSCAPE, Kyoto Encyclopedia of Genes and Genome (KEGG), Reactome, and Wikipathways. MS analysis identified a total of 8330, 5970, 5289, and 4818 proteins in four subcellular fractions (SCFs) that included cytosolic (SCF-I), membranous, and membranous organelles (SCF-II), nuclear (SCF-III), and cytoskeletal (SCF-IV). Altogether, combined analysis of all the five fractions (SCF-I–IV and secretome) revealed a total of 12,609 nonredundant proteins. The KEGG analysis suggested that these proteins were associated with 325 molecular pathways. Some of the highly enriched molecular pathways observed were metabolic, MAPK, PI3-AKT, insulin, estrogen, and cGMP-PKG signaling pathway. The newly identified proteins in this study are claimed to be involved in NOTCH signaling, transport, and secretion processes.

11.12 Presence of Contaminating Proteins in Cultured Mammary Epithelial or Stem Cell Lines

Though an adequate substitute for difficult to isolate tissue from the mammary gland, cell culture-based experiments have always been at risk of being contaminated with proteins originating from the culture medium. For example, fetal bovine serum (FBS) or fetal calf serum (FCS) is the most used media supplement in cell culture. Although many experimental protocols recommend rigorous washing of the cells before collection of proteins, the high sensitivity of mass spectrometry may identify those proteins that are likely to be originating from serum supplements. Many investigators have observed that the commercial and cell culture proteins may contain a significant amount of bovine serum proteins (Sugár et al. 2019; Eagle 1955; Bettger and McKeehan 1986). They selected 11 frequently studied human cell lines by studying hepatoma (HEP2G), cervical cancer (HeLa), embryonic kidney cells (HEK293), lung carcinoma (A549), glioblastoma (GAMG), chronic myeloid leukemia (K562), colon carcinoma (RKO), acute T-cell leukemia (Jurkat), breast cancer (MCF7), prostate carcinoma (LnCap), and osteosarcoma (U2OS) (Geiger et al. 2012), and showed that many of the proteins identified in these cell lines are peptides that were originating from fetal bovine serum (Table 11.2).

These alien proteins represent over 10% of the protein content in most cell culture samples (Sugár et al. 2019). Their presence in the sample misled the results and masks the potential low abundant proteins in the actual sample from being identified. Similar implications lie with the study of MECs and MaSC as these cells are extensively cultured in the presence of FBS. Any protein coming via FBS may potentially bias the findings qualitatively and quantitatively. This type of error or bias can be avoided if the experimental data is evaluated against a bovine serum protein database and thus filtering those proteins as contaminants coming from FBS.

Table 11.2 Common bovine proteins in various cell lines putatively identified as cell line proteins (data shown from Sugár et al. 2019)

	The dominance of peptides from respective species/source	
Bovine proteins present as contaminants	Bovine	Human
A2MG	13	2
ALBU	21	3
FETA	8	0
A1AT	7	0
TRFE	8	2
FETUA	5	0
THRB	4	2
APOA1	4	0

11.13 Mammary Epithelial Cell-Derived Exosomes: A Proteomics Analysis

Extracellular vesicles (EVs) are secreted from most cells and represent a heterogeneous population of microvesicles with a diameter below 1000 nm. Exosomes are 100–250 nm nanosized particles, an introductory class of EVs. Recent reports have demonstrated that exosomes from cow milk are absorbed from the gastrointestinal tract as an intact particle (Betker et al. 2019).

Investigating proteome of exosomes derived from bovine mammary epithelial cells (BMECs) and comparing them with milk-derived exosomes in the database (Zhang et al. 2020) found BMECs-derived exosomes in the culture supernatant of BMECs. LC-MS/MS identified 638 proteins in BMECs-derived exosomes. A comparison of proteins obtained from two different sources of exosomes, namely, mammary epithelial cell and milk, revealed 77 common expressed proteins (CEPs) (Zhang et al. 2020). These CEPs were involved in signaling pathways associated with milk biosynthesis in BMECs.

11.14 Proteomic Analysis of Bovine Mammary Epithelial Cells in Diseases Like Mastitis

The inflammation of the udder characterizes mastitis in bovine. In most of the events, it ensues with a bacterial infection. Studies have reported that *Staphylococcus aureus* is the primary causative pathogen, followed by *Streptococcus agalactia*, *E. coli*, and *Pseudomonas* spp. The onset of inflammation, which is a self-protective mechanism in the udder during the bacterial infection, turns out to be self-inflicting in progress cases as many leukocytes infiltrate into the affected areas. Interactions between pathogen and host defense systems result in several local and systemic changes at the molecular level. That has garnered the researchers' attention to control and manipulate disease pathogenesis for better prevention and cure. Advances in proteomics have enabled more in-depth insight into molecular pathogenesis and a close understanding of the mammary gland's host–pathogen relationship. The natural infection with *S. aureus* turning into advanced clinical cases has been studied using iTRAQ-based labeled quantitative proteomics (Huang et al. 2014). In this approach, they identified 15,879 Ms-MS spectra matching to 6499 peptides, which were finally annotated to 768 proteins. Thirty-six proteins were significantly upregulated (>1.5-fold), and 19 proteins were downregulated (<0.67-fold) in mastitis due to natural infections with *Staphylococci aureus* when compared with healthy tissue. Upregulation of collagen, type I, alpha 1 (COL1A1), and inter-alpha (globulin) inhibitor H4 (ITIH4) in the mastitis-infected tissue can be considered an important diagnostic marker for clinical mastitis.

Tong et al. (2020) studied the inflammatory effects of *S. agalactiae* on BMECs by liquid chromatography/mass spectrometry. They identified 325 upregulated and 704 downregulated proteins in the treated vs. control group. This report further clarified that upregulation of TNF-α, IL-1β, and IL-6 mRNA and inhibition of IL-8

expression probably led to a decrease in cell viability and an increase in apoptosis during the infection. Local cellular immunity is an important defense aspect of MECs in bovine. In addition to the PBMNCs that include neutrophils and macrophages, BMECs also switch on the expression of various pro- and anti-inflammatory cytokines. Not surprisingly, they observed many differentially expressed proteins belonging to metabolic processes and cellular immunity.

11.15 Mammary Stem Cells (MaSC), Their Identification, and Characterization: Role of Proteomics

As we have described earlier, the cyclic phases of proliferation, differentiation, and involution in the mammary gland are largely dependent on the niche of specialized cells: adult mammary stem cells (AMaSCs), which are often interchangeably also called mesenchymal stem cells. MaSCs being multipotent give rise to changes in the proportion of cell types in the mammary gland in response to change in the hormonal signals during puberty, pregnancy, lactation, or involution. In the study of such specialized MaSCs, their isolation, culture, and characterization have always been challenging because of the lack of our knowledge about the unique and specific molecular features of these cells. Our ability to precisely recognize MaSCs and purify them from the mixture of other mammary gland cells has been based on a few specific protein markers expressed on the cell surface. These include a cluster of differentiation (CD) molecules, like CD24 (heat-stable antigen) and CD49f (integrin alpha 6) on Lin-sorted cells. Bovine MaSCs are CD24med, CD49fpos Choudhary (2014).

Conventionally, MaSCs are epithelial in origin. However, the classical method of characterizing MaSCs is to assess the self-renewal ability by transplanting the putative MaSCs into the cleared fat pad and clonal expansion of the cells into the functional mammary gland, which has been regarded as a standard gold method (Kordon and Smith 1998; Sleeman et al. 2005; Shackleton et al. 2006; Dey et al. 2009; Stingl et al. 2005). It is still unclear if the mammary mesenchymal stem cells are the MaSCs (epithelial origin) decked in the lap of adipocytes. The existence of partly differentiated cells determined to form a particular cell lineage creates confusion over the actual characteristics of naïve MaSCs (Kaimala et al. 2012; Joshi and Khokha 2012). MaSCs have been reported as bipotent (Prpar et al. 2012) and lineage-restricted unipotent stem cells (Van Keymeulen et al. 2011). Therefore, identifying MaSC subtypes is critical to precisely manipulating mammary gland growth development and differentiation, especially during tissue regeneration. Table 11.1 shows the list of marker proteins by various authors that have been recognized as a signature of MaSCs.

The organ growth and maintenance of homeostasis are controlled by two distinct types of division in MaSCs: one being symmetric division, leading to the formation of two identical daughter MaSCs or completely differentiated cells, and the other being asymmetric division, leading to both daughter MaSC and differentiated cells. The measurement of phenotypic changes in these cells and qualifying them as naïve,

progenitor stem cells, or lineage-restricted progenitor stem cells has been greatly benefited by the proteomics-based investigation of different types of cells.

11.16 Characterization of Bovine MaSCs/Progenitor Cells

So far, FACS has proven as a powerful technique to separate different types of cells in humans, mice, and bovine. Its function is based on the availability of antibodies against the uniquely expressed surface biomarkers on each cell type. Bovine MaSCs are characterized by the absence of Lin expression and presence of $CD24^{med}$ and $CD49f^{+}$. CD24neg, CD49fpos phenotype characterize the basal bipotent progenitors while the luminal unipotent progenitors MaSCs are characterized by $CD24^{high}$, $CD49f^{neg}$ and luminal unipotent cells by $CD24^{med}$, $CD49f^{neg}$ (Rauner and Barash 2012). The knowledge about the true biomarkers on MECs or MaSCs is also imperfect because of the limitation of the adopted protocols. Preparation of single-cell suspension using enzymatic digestion of tissues is supposed to disrupt the cellular and extracellular attachments (Rios et al. 2014). Enrichment of putative MaSCs in culture is a prospective method to work on a homogenous population of stem cells (Choudhary et al. 2013); however, this approach also suffers from the uncontrolled differentiation during the culture. Retention of BrdU label by basal epithelial cells, which have the characteristic expression pattern of estrogen receptor ($ESR1^{+}$) and high aldehyde dehydrogenase $3B1^{high}$ ($ALDH3B1^{high}$), nuclear receptor subfamily5^{high}, group A, member 2^{high} ($NR5A2^{high}$) (Heng et al. 2010), and X-chromosome inactivation factor negative ($XIST^{-}$) (Navarro et al. 2008; Choudhary et al. 2013), has been found to have stem cell characteristics.

The fate determination of MaSC into a particular lineage is essential for their precise application. The characteristic set of proteins in a specific point of time in a cell's life is crucial for identifying, isolating, and fate determination of a lineage primed cell. For example, embryonic $K14^{+}$ (keratin14) stem cells are multipotent to form all types of epithelial cells. In contrast, $K14^{+}$ stem cells are unipotent, contributing to the myoepithelial lineage during puberty, adult life, and pregnancy (Van Keymeulen et al. 2011). $K8^{+}$ (keratin8) and $Elf5^{+}$ (E74-like factor 5) stem cells are unipotent luminal stem cells, which differentiate into luminal and milk-producing cells, while $K5^{+}$ (keratin5) and $Lgr5^{+}$ cells characterize the ability to differentiate into myoepithelial cells lineage. However, K5-, K14-, or Lgr5-expressing cells are multipotent AMaSCs with log life that differentiate into luminal and myoepithelial cells (Rios et al. 2014). During mammary gland development, Notch signaling is essential. Notch family proteins include Notch1, Notch2, and Notch3 proteins uniquely expressed on MaSCs. Notch1+ cells are present in the embryonic mammary bud and are considered primed to differentiate into ER- luminal lineage (Rodilla et al. 2015; Lilja et al. 2018). Notch2+ progenitor cells can differentiate into small and large cells distinct from classical luminal and myoepithelial cells (Šale et al. 2013). $Notch3^{+}$ cells are highly clonogenic and transiently quiescent luminal progenitor populations that give rise to a ductal epithelial cell lineage (Lafkas et al. 2013). Estrogen-positive (ER+) and estrogen-

negative ER- luminal cells are procreated by a distinct MaSC population. Wap + (Chang et al. 2014), Sox9+ (Wang et al. 2017), Blimp1+ (Elias et al. 2017), and Notch1+ (Rodilla et al. 2015; Lilja et al. 2018) stem cells contribute to ER^- luminal cells lineage postnatally, while Prom1+ (Wang et al. 2017) and ER+ (Van Keymeulen et al. 2017) stem cells differentiate into ER^+ luminal cell lineage. Lgr5 (Van Keymeulen et al. 2011; Plaks et al. 2013), Procr (Wang et al. 2017), Tspan8 (Fu et al. 2017), Dll1 (Chakrabarti et al. 2018), and Bcl11b (Cai et al. 2017) are the characteristics of basal MaSC.

11.17 The Secretome from Bovine Mammosphere-Derived Cells (MDCs)

Aggregates of mammary epithelial stem cells are called mammosphere. It has been widely studied in mammary tumors. The mammosphere-derived cells (MDCs) are rich in host defense proteins and can promote angiogenesis and epithelial cell migration. Mammary wound healing and tissue regeneration largely depend on these cells. The mammosphere's secret is stable for a long time and withstands freeze–thaw cycles that have a promising application in treating bovine mastitis as cell-free therapeutics. The antimicrobial properties of MSCs have triggered interest in gaining further insight into its mode of action and its application in mastitis. A large number of proteins (347) have been reported in the conditioned medium of MDC using mass spectrometry-based proteomics, which were rich in mediators of immunity (lactoferrin, cathelicidin), tissue regeneration, angiogenesis (vascular endothelial growth factor and angiopoietin 1), and cell migration (transforming growth factor beta, insulin-like growth factor-1, and hepatocyte growth).

11.18 Protein Isoforms in MSC

Isoforms of a protein arise due to gene polymorphism or alternative splicing during transcription. Although such protein isoforms share sequence homology, both the type and quantity of each isoform can have significant differential effects on the overall cell phenotype. The classical antibody-based approach has struggled to accurately characterize such protein isoforms. Labeled quantitative proteomics has dramatically improved our ability to study isoforms. In a study, dimethyl labeling was used to compare the relative abundances of 123 protein isoforms in MSC-enriched and -depleted cells wherein 34 protein isoforms in MSC were differentially expressed elucidated differentiation pathways. The technique was effective despite the high-sequence homology and extensive dynamic range of proteins in the sample (She et al. 2012). They further advocated that these proteins may serve as essential biomarkers for MSC.

11.19 Chromatin Organization, Epigenomics and Protein Expression in Mammary Gland Cells

Each cell type's expression pattern is controlled by many local and systemic hormones (Casey et al. 2018). The chromatin status is also under the control of many known genetic factors like diet, drugs, stress, and environment, leading to differential expression of proteins during various pathophysiologies. Therefore, profiling of open chromatin regions (ATAC-sequences) and reading out the methylation pattern (RRBS-sequences) in both luminal and basal epithelial cells helped understand the relationship between genome, RNA, and protein in the mammary gland. The MG organization and function depend on the specific regulation of open chromatin regions, which offer binding sites to various transcription factors (TFs) such as FOXA1, ELF5, GATA3, and TP63. The detailed proteomics-based studies have enabled identifying such TFs and methylation status on the genome. Novel lineage associations have been reported for TP53 and EGR1 motifs in basal cells and FOXA2, SPI1, and FOXP1 motifs in luminal cells (Casey et al. 2018). Various histone modifiers (TSA/SAHA (HDAC inhibitors) and methyltransferase inhibitors such as decitabine (DAC, DNMT1, 3a, 3b inhibitor), and JQ1 (BRD2, 3, 4, and T) have been recognized as epigenetic regulators in luminal cell lineages (Casey et al. 2018). Epigenomic studies involving ChipSeq kind of experiments found a high level of hypomethylated enhancer elements in luminal cells than the basal epithelial cells (Gascard et al. 2015).

References

Agorku DJ, Langhammer A, Heider U, Wild S, Bosio A, Hardt O (2019) CD49b, CD87, and CD95 are markers for activated cancer-associated fibroblasts whereas CD39 marks quiescent normal fibroblasts in murine tumor models. Front Oncol 9:716. https://doi.org/10.3389/fonc.2019.00716

Asselin-Labat ML, Sutherland KD, Barker H, Thomas R, Shackleton M, Forrest NC, Hartley L, Robb L, Grosveld FG, van der Wees J, Lindeman GJ (2007) Gata-3 is an essential regulator of mammary-gland morphogenesis and luminal-cell differentiation. Nat Cell Biol 9(2):201–209

Avagliano A, Fiume G, Ruocco MR, Martucci N, Vecchio E, Insabato L, Russo D, Accurso A, Masone S, Montagnani S, Arcucci A (2020) Influence of fibroblasts on mammary gland development, breast cancer microenvironment remodeling, and cancer cell dissemination. PMID: 32604738. PMCID: PMC7352995. https://doi.org/10.3390/cancers12061697

Badders NM, Goel S, Clark RJ, Klos KS, Kim S, Bafico A, Lindvall C, Williams BO, Alexander CM (2009) The Wnt receptor, Lrp5, is expressed by mouse mammary stem cells and is required to maintain the basal lineage. PLoS One 4(8):e6594

Betker JL, Angle BM, Graner MW, Anchordoquy TJ (2019) The potential of exosomes from cow milk for oral delivery. J Pharm Sci 108(4):1496–1505

Betterman KL, Paquet-Fifield S, Asselin-Labat ML, Visvader JE, Butler LM, Stacker SA, Achen MG, Harvey NL (2012) Remodeling of the lymphatic vasculature during mouse mammary gland morphogenesis is mediated via epithelial-derived lymphangiogenic stimuli. Am J Pathol 181(6):2225–2238

Bettger WJ, McKeehan WL (1986) Mechanisms of cellular nutrition. Physiol Rev 66(1):1–35

Boudreau N, Sympson CJ, Werb Z, Bissell MJ (1995) Suppression of ICE and apoptosis in mammary epithelial cells by extracellular matrix. Science 267(5199):891–893

Cai S, Kalisky T, Sahoo D, Dalerba P, Feng W, Lin Y, Qian D, Kong A, Yu J, Wang F, Chen EY (2017) A quiescent Bcl11b high stem cell population is required for maintenance of the mammary gland. Cell Stem Cell 20(2):247–260

Casey AE, Sinha A, Singhania R, Livingstone J, Waterhouse P, Tharmapalan P, Cruickshank J, Shehata M, Drysdale E, Fang H, Kim H (2018) Mammary molecular portraits reveal lineage-specific features and progenitor cell vulnerabilities. J Cell Biol 217(8):2951–2974

Chakrabarti R, Celià-Terrassa T, Kumar S, Hang X, Wei Y, Choudhury A, Hwang J, Peng J, Nixon B, Grady JJ, DeCoste C (2018) Notch ligand Dll1 mediates cross-talk between mammary stem cells and the macrophageal niche. Science 360(6396)

Chang TH, Kunasegaran K, Tarulli GA, De Silva D, Voorhoeve PM, Pietersen AM (2014) New insights into lineage restriction of mammary gland epithelium using parity-identified mammary epithelial cells. Breast Cancer Res 16(1):R1

Choudhary RK (2014) Mammary stem cells: expansion and animal productivity. J Anim Sci Biotechnol 5(1):1–8

Choudhary RK, Li R, Evock-Clover CM, Capuco AV (2013) Comparison of the transcriptomes of long-term label retaining-cells and control cells microdissected from mammary epithelium: an initial study to characterize potential stem/progenitor cells. Front Oncol 3:21

Dai WT, Wang QJ, Zou YX, White RR, Liu JX, Liu HY (2017) Comparative proteomic analysis of the lactating and nonlactating bovine mammary gland. J Dairy Sci 100(7):5928–5935

Daniel CW, Silberstein GB (2000) Working with the mouse mammary end bud. In: Methods in mammary gland biology and breast cancer research. Springer, Boston, MA, pp 155–162

Dey D, Saxena M, Paranjape AN, Krishnan V, Giraddi R, Kumar MV, Mukherjee G, Rangarajan A (2009) Phenotypic and functional characterization of human mammary stem/progenitor cells in long term culture. PLoS One 4(4):e5329

Eagle H (1955) Nutrition needs of mammalian cells in tissue culture. Science 122(3168):501–504

Elias S, Morgan MA, Bikoff EK, Robertson EJ (2017) Long-lived unipotent Blimp1-positive luminal stem cells drive mammary gland organogenesis throughout adult life. Nat Commun 8 (1):1–12

Fu NY, Rios AC, Pal B, Law CW, Jamieson P, Liu R, Vaillant F, Jackling F, Liu KH, Smyth GK, Lindeman GJ (2017) Identification of quiescent and spatially restricted mammary stem cells that are hormone responsive. Nat Cell Biol 19(3):164–176

Gascard P, Bilenky M, Sigaroudinia M, Zhao J, Li L, Carles A, Delaney A, Tam A, Kamoh B, Cho S, Griffith M (2015) Epigenetic and transcriptional determinants of the human breast. Nat Commun 6(1):1–10

Geiger T, Wehner A, Schaab C, Cox J, Mann M (2012) Comparative proteomic analysis of eleven standard cell lines reveals ubiquitous but varying expression of most proteins. Mol Cell Proteomics 11(3)

Gouon-Evans V, Rothenberg ME, Pollard JW (2000) Postnatal mammary gland development requires macrophages and eosinophils. Development 127(11):2269–2282

Gregor MF, Misch ES, Yang L, Hummasti S, Inouye KE, Lee AH, Bierie B, Hotamisligil GS (2013) The role of adipocyte XBP1 in metabolic regulation during lactation. Cell Rep 3 (5):1430–1439

Gudjonsson T, Villadsen R, Nielsen HL, Rønnov-Jessen L, Bissell MJ, Petersen OW (2002) Isolation, immortalization, and characterization of a human breast epithelial cell line with stem cell properties. Genes Dev 16(6):693–706

Heng JCD, Feng B, Han J, Jiang J, Kraus P, Ng JH, Orlov YL, Huss M, Yang L, Lufkin T, Lim B (2010) The nuclear receptor Nr5a2 can replace Oct4 in the reprogramming of murine somatic cells to pluripotent cells. Cell Stem Cell 6(2):167–174

Hennighausen L, Robinson GW (2005) Information networks in the mammary gland. Nat Rev Mol Cell Biol 6(9):715–725

Hovey RC, Aimo L (2010) Diverse and active roles for adipocytes during mammary gland growth and function. J Mammary Gland Biol Neoplasia 15(3):279–290

Hovey RC, Goldhar AS, Baffi J, Vonderhaar BK (2001) Transcriptional regulation of vascular endothelial growth factor expression in epithelial and stromal cells during mouse mammary gland development. Mol Endocrinol 15(5):819–831

Howard BA, Lu P (2014, January) Stromal regulation of embryonic and postnatal mammary epithelial development and differentiation. In: Seminars in cell & developmental biology, vol 25. Academic Press, New York, pp 43–51

Huang J, Luo G, Zhang Z, Wang X, Ju Z, Qi C, Zhang Y, Wang C, Li R, Li J, Yin W (2014) iTRAQ-proteomics and bioinformatics analyses of mammary tissue from cows with clinical mastitis due to natural infection with Staphylococci aureus. BMC Genomics 15(1):839

Inman JL, Robertson C, Mott JD, Bissell MJ (2015) Mammary gland development: cell fate specification, stem cells and the microenvironment. Development 142(6):1028–1042

Jacobs JM, Waters KM, Kathmann LE, Camp DG II, Wiley HS, Smith RD, Thrall BD (2008) The mammary epithelial cell secretome and its regulation by signal transduction pathways. J Proteome Res 7(2):558–569

Jaswal S, Anand V, Kumar S, Bathla S, Dang AK, Kaushik JK, Mohanty AK (2020) In-depth proteome analysis of more than 12,500 proteins in buffalo mammary epithelial cell line identifies protein signatures for active proliferation and lactation. Sci Rep 10(1):1–13

Jena MK, Janjanam J, Naru J, Kumar S, Kumar S, Singh S, Mohapatra SK, Kola S, Anand V, Jaswal S, Verma AK (2015) DIGE based proteome analysis of mammary gland tissue in water buffalo (Bubalus bubalis): lactating vis-a-vis heifer. J Proteome 119:100–111

Joshi PA, Khokha R (2012) The mammary stem cell conundrum: is it unipotent or multipotent? Breast Cancer Res 14(2):1–3

Joshi PA, Waterhouse PD, Kasaian K, Fang H, Gulyaeva O, Sul HS, Boutros PC, Khokha R (2019) PDGFRα+ stromal adipocyte progenitors transition into epithelial cells during lobulo-alveologenesis in the murine mammary gland. Nat Commun 10(1):1–14

Kaimala S, Bisana S, Kumar S (2012) Mammary gland stem cells: more puzzles than explanations. J Biosci 37(2):349–358

Kordon EC, Smith GH (1998) An entire functional mammary gland may comprise the progeny from a single cell. Development 125(10):1921–1930

Lafkas D, Rodilla V, Huyghe M, Mourao L, Kiaris H, Fre S (2013) Notch3 marks clonogenic mammary luminal progenitor cells in vivo. J Cell Biol 203(1):47–56

Lilja AM, Rodilla V, Huyghe M, Hannezo E, Landragin C, Renaud O, Leroy O, Rulands S, Simons BD, Fre S (2018) Clonal analysis of Notch1-expressing cells reveals the existence of unipotent stem cells that retain long-term plasticity in the embryonic mammary gland. Nat Cell Biol 20 (6):677–687

Lilla JN, Werb Z (2010) Mast cells contribute to the stromal microenvironment in mammary gland branching morphogenesis. Dev Biol 337(1):124–133

Lilla JN, Joshi RV, Craik CS, Werb Z (2009) Active plasma kallikrein localizes to mast cells and regulates epithelial cell apoptosis, adipocyte differentiation, and stromal remodeling during mammary gland involution. J Biol Chem 284(20):13792–13803

Lim E, Vaillant F, Wu D, Forrest NC, Pal B, Hart AH, Asselin-Labat ML, Gyorki DE, Ward T, Partanen A, Feleppa F (2009) Aberrant luminal progenitors as the candidate target population for basal tumor development in BRCA1 mutation carriers. Nat Med 15(8):907–913

Liu X, Ory V, Chapman S, Yuan H, Albanese C, Kallakury B, Timofeeva OA, Nealon C, Dakic A, Simic V, Haddad BR (2012) ROCK inhibitor and feeder cells induce the conditional reprogramming of epithelial cells. Am J Pathol 180(2):599–607

Lühr I, Friedl A, Overath T, Tholey A, Kunze T, Hilpert F, Sebens S, Arnold N, Rösel F, Oberg HH, Maass N (2012) Mammary fibroblasts regulate the morphogenesis of normal and tumorigenic breast epithelial cells by mechanical and paracrine signals. Cancer Lett 325(2):175–188

Machado HL, Kittrell FS, Edwards D, White AN, Atkinson RL, Rosen JM, Medina D, Lewis MT (2013) Separation by cell size enriches for mammary stem cell repopulation activity. Stem Cells Transl Med 2(3):199–203

Makarem M, Kannan N, Nguyen LV, Knapp DJ, Balani S, Prater MD, Stingl J, Raouf A, Nemirovsky O, Eirew P, Eaves CJ (2013) Developmental changes in the in vitro activated regenerative activity of primitive mammary epithelial cells. PLoS Biol 11(8):e1001630

Morsing M, Klitgaard MC, Jafari A, Villadsen R, Kassem M, Petersen OW, Rønnov-Jessen L (2016) Evidence of two distinct functionally specialized fibroblast lineages in breast stroma. Breast Cancer Res 2016(18):108

Muschler J, Streuli CH (2010) Cell–matrix interactions in mammary gland development and breast cancer. Cold Spring Harbor Perspect Biol 2(10):a003202

Navarro P, Chambers I, Karwacki-Neisius V, Chureau C, Morey C, Rougeulle C, Avner P (2008) Molecular coupling of Xist regulation and pluripotency. Science 321(5896):1693–1695

O'Brien J, Martinson H, Durand-Rougely C, Schedin P (2012) Macrophages are crucial for epithelial cell death and adipocyte repopulation during mammary gland involution. Development 139(2):269–275

Oakes SR, Naylor MJ, Asselin-Labat ML, Blazek KD, Gardiner-Garden M, Hilton HN, Kazlauskas M, Pritchard MA, Chodosh LA, Pfeffer PL, Lindeman GJ (2008) The Ets transcription factor Elf5 specifies mammary alveolar cell fate. Genes Dev 22(5):581–586

Parmar H, Cunha GR (2004) Epithelial–stromal interactions in the mouse and human mammary gland in vivo. Endocr Relat Cancer 11(3):437–458

Plaks V, Brenot A, Lawson DA, Linnemann JR, Van Kappel EC, Wong KC, de Sauvage F, Klein OD, Werb Z (2013) Lgr5-expressing cells are sufficient and necessary for postnatal mammary gland organogenesis. Cell Rep 3(1):70–78

Prater MD, Petit V, Russell IA, Giraddi RR, Shehata M, Menon S, Schulte R, Kalajzic I, Rath N, Olson MF, Metzger D (2014) Mammary stem cells have myoepithelial cell properties. Nat Cell Biol 16(10):942–950

Prpar S, Martignani E, Dovc P, Baratta M (2012) Identification of goat mammary stem/progenitor cells. Biol Reprod 86(4):117–111

Rauner G, Barash I (2012) Cell hierarchy and lineage commitment in the bovine mammary gland. PLoS One 7(1):e30113

Regan JL, Kendrick H, Magnay FA, Vafaizadeh V, Groner B, Smalley MJ (2012) c-Kit is required for growth and survival of the cells of origin of Brca1-mutation-associated breast cancer. Oncogene 31(7):869–883

Reinhardt TA, Lippolis JD (2020) Characterization of bovine mammary gland dry secretions and their proteome from the end of lactation through day 21 of the dry period. J Proteomics 223:103831

Rios AC, Fu NY, Lindeman GJ, Visvader JE (2014) In situ identification of bipotent stem cells in the mammary gland. Nature 506(7488):322–327

Rodilla V, Dasti A, Huyghe M, Lafkas D, Laurent C, Reyal F, Fre S (2015) Luminal progenitors restrict their lineage potential during mammary gland development. PLoS Biol 13(2):e1002069

Sakakura T (1987) Mammary embryogenesis. The mammary gland. pp 37–66

Šale S, Lafkas D, Artavanis-Tsakonas S (2013) Notch2 genetic fate mapping reveals two previously unrecognized mammary epithelial lineages. Nat Cell Biol 15(5):451–460

Shackleton M, Vaillant F, Simpson KJ, Stingl J, Smyth GK, Asselin-Labat ML, Wu L, Lindeman GJ, Visvader JE (2006) Generation of a functional mammary gland from a single stem cell. Nature 439(7072):84–88

She YM, Rosu-Myles M, Walrond L, Cyr TD (2012) Quantification of protein isoforms in mesenchymal stem cells by reductive dimethylation of lysines in intact proteins. Proteomics 12(3):369–379

Shehata M, Teschendorff A, Sharp G, Novcic N, Russell IA, Avril S, Prater M, Eirew P, Caldas C, Watson CJ, Stingl J (2012) Phenotypic and functional characterisation of the luminal cell hierarchy of the mammary gland. Breast Cancer Res 14(5):1–19

Sleeman KE, Kendrick H, Ashworth A, Isacke CM, Smalley MJ (2005) CD24 staining of mouse mammary gland cells defines luminal epithelial, myoepithelial/basal and non-epithelial cells. Breast Cancer Res 8(1):1–6

Stingl J, Raouf A, Emerman JT, Eaves CJ (2005) Epithelial progenitors in the normal human mammary gland. J Mammary Gland Biol Neoplasia 10(1):49–59

Stingl J, Eirew P, Ricketson I, Shackleton M, Vaillant F, Choi D, Li HI, Eaves CJ (2006) Purification and unique properties of mammary epithelial stem cells. Nature 439(7079):993–997

Sugár S, Turiák L, Vékey K, Drahos L (2019) Widespread presence of bovine proteins in human cell lines. J Mass Spectrom 55(7):e4464–e4464

Tharmapalan P, Mahendralingam M, Berman HK, Khokha R (2019) Mammary stem cells and progenitors: targeting the roots of breast cancer for prevention. EMBO J 38(14):e100852. https://doi.org/10.15252/embj.2018100852. PMCID: PMC6627238

Tong J, Sun M, Zhang H, Yang D, Zhang Y, Xiong B, Jiang L (2020) Proteomic analysis of bovine mammary epithelial cells after in vitro incubation with S. agalactiae: potential biomarkers. Vet Res 51(1):1–14

Van Keymeulen A, Rocha AS, Ousset M, Beck B, Bouvencourt G, Rock J, Sharma N, Dekoninck S, Blanpain C (2011) Distinct stem cells contribute to mammary gland development and maintenance. Nature 479(7372):189–193

Van Keymeulen A, Fioramonti M, Centonze A, Bouvencourt G, Achouri Y, Blanpain C (2017) Lineage-restricted mammary stem cells sustain the development, homeostasis, and regeneration of the estrogen receptor positive lineage. Cell Rep 20(7):1525–1532

Visvader JE, Stingl J (2014) Mammary stem cells and the differentiation hierarchy: current status and perspectives. Genes Dev 28(11):1143–1158

Wang X, Kaplan DL (2012) Hormone-responsive 3D multicellular culture model of human breast tissue. Biomaterials 33(12):3411–3420

Wang C, Christin JR, Oktay MH, Guo W (2017) Lineage-biased stem cells maintain estrogen-receptor-positive and-negative mouse mammary luminal lineages. Cell Rep 18(12):2825–2835

Williams JM, Daniel CW (1983) Mammary ductal elongation: differentiation of myoepithelium and basal lamina during branching morphogenesis. Dev Biol 97(2):274–290

Williams KE, Lemieux GA, Hassis ME, Olshen AB, Fisher SJ, Werb Z (2016) Quantitative proteomic analyses of mammary organoids reveals distinct signatures after exposure to environmental chemicals. Proc Natl Acad Sci U S A 113(10):E1343–E1351

Wiseman BS, Werb Z (2002) Stromal effects on mammary gland development and breast cancer. Science 296(5570):1046–1049

Zeng YA, Nusse R (2010) Wnt proteins are self-renewal factors for mammary stem cells and promote their long-term expansion in culture. Cell Stem Cell 6(6):568–577

Zhang M, Ma Z, Li R, Guo S, Qiu Y, Gao X (2020) Proteomic analysis reveals proteins and pathways associated with lactation in bovine mammary epithelial cell-derived exosomes. J Proteome Res 19:3211–3219

Zheng X, Ning C, Dong Y, Zhao P, Li J, Fan Z, Li J, Yu Y, Mrode R, Liu JF (2017) Quantitative proteome analysis of bovine mammary gland reveals protein dynamic changes involved in peak and late lactation stages. Biochem Biophys Res Commun 494(1–2):292–297

Zhou J, Chen Q, Zou Y, Zheng S, Chen Y (2019) Stem cells and cellular origins of mammary gland: updates in rationale, controversies, and cancer relevance. Stem Cells Int 2019:4247168

Part III

Therapeutic Applications

Advancing Quantitative Stem Cell Dosing for Veterinary Stem Cell Medicine

12

Samuel R. Boutin and James L. Sherley

Abstract

Because of the important role of preclinical animal studies in the development of innovative medicines for human patients, many stem cell therapies have been evaluated in animals. However, the last decade has seen the beginning of a shift from stem cell treatments in animals only for the benefit of human patients to including new therapeutic development of tissue stem cells primarily for animal care. Not surprisingly, given their historical dependency, the new field of veterinary stem cell medicine faces many of the same challenges as human stem cell medicine. In this chapter, a shared major deficiency, the lack of stem cell-specific dosing, is considered from the perspective that implementing dosing would accelerate progress in veterinary stem cell medicine and human stem cell medicine as well, as a follow-on. Since the vast majority of present-day veterinary stem cell treatments utilize preparations of mesenchymal stem cells (MSCs), the well-recognized uncertainties about this treatment source are discussed. The challenges of quantifying the stem cell-specific dose of MSC preparations exemplify the general problem of determining the stem cell dose of all stem cell treatments. Particular consideration is given to previous veterinary MSC treatment studies that include measures that might relate to stem cell dosage. Kinetic stem cell counting, a first potential solution to the tissue stem cell dosing problem, is described, and the potential benefits of its future use are discussed. Adoption of kinetic stem cell counting into the general practice of veterinary stem cell medicine is presented as the key that can unlock the full potential of stem cells in veterinary medical practice and perhaps human stem cell medical practice as well.

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Keywords

Tissue stem cells · Stem cell medicine · Stem cell dose · Asymmetric self-renewal · Kinetic stem cell count · Hematopoietic stem cell · Mesenchymal stem cell

12.1 Introduction

The existence and role of stem cells in mature vertebrate tissues have been known for more than a half-century. From the very beginning of their discovery, the potential of tissue stem cells for medical applications has been recognized and pursued. In fact, the discovery of the first tissue stem cells and the demonstration of their function in mature tissue cell renewal, which occurred in experimental animal models, were essentially in the format of future human stem cell replacement therapies (Till and McCulloch 1961). Those earliest origins led to the present only successful stem cell therapy in routine clinical practice, hematopoietic stem cell transplantation (HSCT). After many decades of research and technological development, additional effective stem cell therapies have been slow to emerge despite significant past and continuing investments of research and development resources.

Like its origins, much of the progress made in stem cell medicine can be attributed to research and development in animal models for stem cell therapy. Earlier investigations with animals were targeted primarily to the development of new approaches and applications for human stem cell medicine and not veterinary medicine per se. However, in the recent decade, stem cell therapeutics, first modeled in preclinical animal studies to gain approval for subsequent human clinical trials, have begun to be evaluated as primary treatments for animal patients in veterinary stem cell medicine (Gugjoo et al. 2018).

Because of the essential role of stem cells in the cellular tissue homeostasis of vertebrates, the same challenges that slow progress in the achievement of effective human stem cell therapies beyond HSCT are also faced in the development of effective veterinary stem cell medicine. In this chapter, an important but often unappreciated or understated challenge that greatly limits progress in both human and veterinary stem cell medicine is presented and discussed with the perspective that overcoming it would accelerate progress in veterinary stem cell medicine. Remarkably, the challenge at issue is a long-standing deficiency in stem cell medicine of a fundamental principle of medicine. For both animal patients and human patients, stem cell therapies are administered without knowing the stem cell dosage of the treatments (Sherley 2018a; Dutton et al. 2020).

In this chapter, the authors discuss the need for and benefits of advancing veterinary stem cell medicine to being dosage-based like traditional veterinary pharmaceutical medicine. The validation of the need for dosage-based stem cell medicine accrues readily from consideration of the fundamental medical concept that knowing the dosage of medicines is essential for the highest quality medical treatment. In modern medicine, the importance of the utilization of medication

dosage for effective development of new medicines and for the safe and efficacious administration of approved medicines is a self-evidentiary principle. Yet, presently, in both human medicine and veterinary medicine, stem cell clinical trials and even approved stem cell therapies like HSCT are performed without knowing the dosage of the stem cells in the treatments. In many human stem cell therapy clinical trials using expanded cell treatments, stem cells are presumed to be present, but they could be altogether absent (Paré and Sherley 2006; Taghizadeh and Sherley 2009; Sherley 2014).

Until very recently, the dosage limitation of tissue stem cell medicine was, for the most part, unavoidable. No technologies were available for accurately determining the dose of tissue stem cells that are characteristically a small fraction of the cells in treatment preparations (Sherley 2018a; Dutton et al. 2020). In this chapter, the authors describe a recently developed method for specific and accurate counting of therapeutic tissue stem cells. Called "kinetic stem cell counting," this new method for counting therapeutic tissue stem cells has the potential to open a first path to the implementation of quantitative dosing in veterinary stem cell medicine. The predicted benefits from such innovation in veterinary stem cell medical practice are discussed herein, with respect to their potential to compel similar modernization of human stem cell medical practice.

12.2 Therapeutic Tissue Stem Cells in Animal Tissues

Tissue-specific stem cells (TSCs) have a well-defined role in animal tissues for continuously renewing expiring mature differentiated tissue cells (Sherley 2005, 2006, 2013). Such tissue maintenance stem cells also have functions in repairing and restoring injured or diseased tissues. These abilities of tissue stem cells account for the effectiveness of HSCT, which reconstitutes the diseased or destroyed production of mature hematopoietic tissue cells.

Another important class of tissue stem cell does not appear to be tissue-specific in the usual sense. These mesenchymal stem cells (MSCs) are found in the interstitial and perivascular spaces of many different tissues. Unlike TSCs, which, although unipotent or multipotent, produce mature cells whose phenotypes are limited to the differentiated lineages of their specific tissue or residence, MSCs exhibit multitissue multipotency. In cell culture, their isolated preparations can produce differentiated cells with adipogenic, osteogenic, or chondrogenic phenotypic properties (Pittenger et al. 2019).

Both TSCs and MSCs have been shown to undergo asymmetric self-renewal division (Fig. 12.1; Dutton et al. 2020). Asymmetric self-renewal is gnomonic for vertebrate tissue stem cells (Sherley 2005, 2013). The unique and defining property of TSCs and MSCs is their ability to divide continuously with the production of one sister cell that retains all the stem cell properties of the parental stem cell and another sister that is committed to producing maturing, differentiating, and expiring, lineage-committed cells.

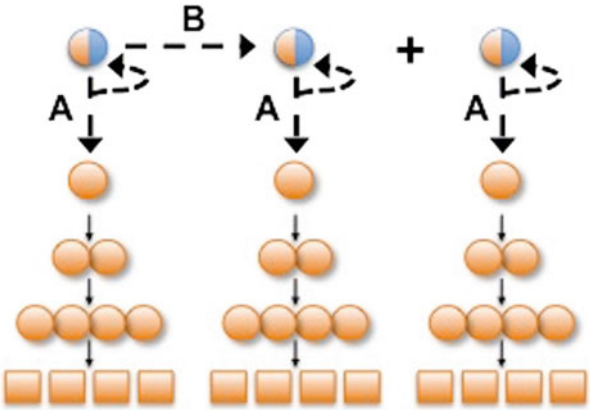

Fig. 12.1 Tissue stem cell asymmetric self-renewal division. (**A**) To maintain cellular tissue homeostasis, tissue stem cells (bivalent circles) undergo asymmetric self-renewal divisions. In the net, these divisions maintain the tissue fraction and stemness properties of stem cells while simultaneously producing committed progenitor cells (uniform circles). Committed progenitor cells continue division to produce mature differentiated functional cells (uniform squares). Mature cells have a finite lifetime in tissues before their loss to tissue wear or cell death. (**B**) In a regulated fashion (e.g., in response to tissue loss by injury), stem cells may undergo symmetric self-renewal divisions to produce new asymmetrically self-renewing tissue units

TSCs and MSCs can divide symmetrically as well, in which case two stem cells are produced. However, asymmetric self-renewal is the default state for tissue stem cells, and they are inherently resistant to frequent symmetric division (Sherley 2013). These properties are consistent with their established role in tissue cell homeostasis. They maintain a relatively undifferentiated state while continuously generating cells committed to tissue-specific differentiation. As mature cells lose function and undergo apoptosis, the asymmetric self-renewal of tissue stem cells maintains a balancing supply of new differentiating cells. With tissue injury, stem cells have the potential to symmetrically self-renew and establish new tissue units for tissue repair (Fig. 12.1B).

Although most TSCs appear to have limited mobility, HSCs and MSCs are naturally mobile stem cells. A significant body of literature describes the property of these cells to migrate preferentially to sites of tissue damage (Kavanagh and Kalia 2011; Nitzsche et al. 2017; Szydlak 2019; Liesveld et al. 2020). Both have been implicated for secreting cytokines and growth factors that induce processes that promote the repair of injured tissues (Gnecchi et al. 2016; Liesveld et al. 2020). For this reason, both HSCs and MSCs, and in particular MSCs, have become major treatment foci for human stem cell clinical trials (Li et al. 2014) based on their potential paracrine functions (Gnecchi et al. 2016; Liesveld et al. 2020). They are also favorable for clinical investigation because of the availability of effective methods for their isolation in higher yield than immobile TSC types. Essentially all veterinary stem cell clinical trials focus on evaluating the potential therapeutic paracrine effects of MSCs.

12.3 Therapeutic M[S]Cs in Veterinary Medicine

Veterinary medicine faces the same challenges as human medicine when it comes to identifying and quantifying tissue stem cells of therapeutic interest. The recent controversy around the nature of human MSCs extends to veterinary medicine as well (Gomez-Salazar et al. 2020). Stem cells are often defined by an ensemble of features that include both physical features and functional properties. For example, MSCs are defined as in Table 12.1.

Though accurately descriptive, these properties are quite inadequate for quantifying the effective stem cell dose of an MSC treatment. Unstated in the criteria as presented is the crucial shortcoming that none of the listed biomarkers identify MS(tem)Cs *specifically*. They also identify and co-quantify committed progenitor cells that are produced by the division of stem cells. Committed progenitor cells, though possessing many tissue precursor cell properties, do not have the unique long-term tissue cell renewal properties of tissue stem cells. Whether they share observed paracrine tissue reparative functions is unknown. For that matter, it could be that the committed progenitor cells are the major sources of tissue repair factors. This distinction could be an important consideration for sound evaluations of the effectiveness of MSC treatments. It may also better inform current ideas on how to explain the otherwise paradoxical long-term effects of MSCs because the cells appear to have characteristically short lifetimes in tissues after transplantation (Gnecchi et al. 2016).

Recent increased focus on the usually understated uncertainty about the specific identities of the cellular constituents of MSC preparations has inspired calls to change the name of these tissue cell preparations to something more representative of their uncertain cellularity. Suggestions like MS(tromal)Cs and medicinal signaling cells have been suggested (Caplan 2017; Gomez-Salazar et al. 2020). Although these choices avoid giving the erroneous impression of homogenous stem cell populations, they somewhat obscure the contribution of what many believe to be the key therapeutic factors, the stem cells in the treatment preparations. Hereafter, in this chapter, these cell populations will be referred to as *M[S]Cs* as a reminder of this important uncertainty about their cellular constituents and their therapeutic potential.

Table 12.1 Properties used to define MSCs[a]

- "Be plastic adherent
- Express the cell surface antigens CD105, CD90, and CD73
- Not express the cell surface antigens CD45, CD19, CD14, CD11b, CD34, CD79a, and HLA-DR
- Have the capacity to differentiate into osteoblasts, chondrocytes, and adipocytes"

[a]Source: From Gomez-Salazar et al. (2020)

12.4 Previous Attention to Indicators of Stem Cell Dose in Veterinary M[S]C Treatments

Many animals have been treated with human stem cells as models for developing human stem cell therapies (Pittenger et al. 2019; Gomez-Salazar et al. 2020). This is particularly true for the clinical development of human M[S]Cs. Herein, the purpose is to address stem cell therapies with species homologous animal stem cells administered in veterinary medicine to improve the healing and health of animals, as human pets, sport animals, and agricultural animals. In particular, studies are highlighted that considered how available measures of tissue stem cell dosage relate to treatment outcomes.

The vast majority of stem cell therapies in development for veterinary medicine use M[S]Cs derived primarily from allogeneic sources. Examples include species-specific M[S]C therapy for inflammatory conditions in pet cats (Quimby and Borjesson 2018; Arzi et al. 2020), pet dogs (Gallant.com 2019–2020), and agricultural animals like milk goats (Costa et al. 2019); diverse disorders (Barberini et al. 2018; Saldinger et al. 2020) and injuries in horses (Delco et al. 2020); and a diverse range of treatments for companion animals, including wound healing, tissue restoration, and inflammatory disorders (Kang and Park 2020). Though mostly species-specific M[S]Cs have been used for companion animal therapies, human-derived M[S]C treatments have also been evaluated (Kang and Park 2020). Human-derived M[S]Cs have been investigated for the treatment of disorders in horses as well (Barberini et al. 2018).

As for human stem cell medicine, veterinary medicine stem cell clinical trials must address the need for evaluating the stability of stem cell treatments during storage and transport. Similarly, veterinary studies are also challenged by the lack of effective and convenient tests to monitor stem cell-specific stability, viability, and function (Arzi et al. 2020).

There has been interest in defining metrics for veterinary stem cell treatments that can be used to identify more effective choices for cell therapies. Human M[S]C studies in animals and humans have established that the definable efficacy of M[S]C preparations has a high degree of variability that has been attributed to many obvious clinical factors and features like tissue source, isolation procedure, age, gender, expansion culture conditions, storage procedures, etc. (Gomez-Salazar et al. 2020). The importance of standardizing and optimizing the cell dose of stem cell treatments has also been discussed (Kang and Park 2020). However, despite the understanding that M[S]C preparations have heterogeneous cellularity (Costa et al. 2019; Pittenger et al. 2019), variation in stem cell-specific fraction has gone unevaluated as a possible cause of the variability or as an important factor for optimization and standardization.

In veterinary investigations, Zhan et al. (2019) evaluated five different sources of canine M[S]Cs derived from adipose tissue, bone marrow, umbilical cord, amnion membrane, and placenta. Although all sources had similar expression of the surface biomarker CD44, they differed in their cell proliferation rates, transcriptome, and rate of multipotent differentiation. In earlier studies, Russell et al. (2016) reported a

similar observation to that of Zhan et al. (2019) that MSCs derived from canine adipose tissue had a significantly higher proliferative rate than those derived from canine bone marrow. In dogs, differences in the proliferative rates of M[S]Cs derived from young and old animals show associated differences in immunomodulatory activity and osteogenic gene expression (Taguchi et al. 2019). Studies of equine M[S]Cs showed that those derived from equine umbilical cord blood produced mechanically superior cartilage tissue in vitro compared to those derived from equine bone marrow. However, a possible relationship to differences in the stem cell fraction was not considered (White et al. 2018).

There is also evidence that properties of veterinary M[S]Cs that suggest differences in stem cell fraction are correlated with differences in the multilineage differentiation capacity. Compared to canine adipose tissue M[S]Cs derived in serum-supplemented medium, those derived in the serum-free medium had a shorter lag phase for growth, a higher colony-forming efficiency, and an accelerated population doubling time. These properties are expected for cultures with a higher stem cell fraction (Devireddy et al. 2019). The serum-free M[S]Cs also maintained multipotency to higher culture passages (Liu et al. 2018; Devireddy et al. 2019). Recent studies in which human tissue stem cells were counted specifically have now established that reductions in the proliferative rate of primary tissue cell cultures are indicative of decreases in stem cell fraction (Dutton et al. 2020). Therefore, previous differences in veterinary M[S]C functional capabilities that were associated with differences in proliferative culture rate may, in fact, reflect differences in stem cell dose.

12.5 The Tissue Stem Cell Counting Problem

Although the tissue stem cell counting problem has existed since the beginning of stem cell biology history, it is either not recognized or poorly understood by many who work in stem cell science and stem cell medicine, including veterinary stem cell medicine (Sherley 2018b; Dutton et al. 2020). Until very recently, there has been no method available to count vertebrate tissue stem cells *specifically*, meaning without also counting committed progenitor cells, which invariably occur in tissue stem cell compartments and their isolated preparations at a much higher fraction than the stem cells.

Two very common misconceptions account for the state of general confusion on capabilities for determining specific tissue stem cell number and, corresponding, tissue stem cell-specific dose. The first is the misbelief that isolated tissue stem cell populations are composed of high fractions of nearly homogeneous stem cells. Such a constituency never exists. As tissue stem cells exist as rare fractions in intact tissues, they continue to exist as a rare cell fraction in isolated tissue cell preparations used for research and stem cell therapies. Applications that seek to expand stem tissue stem cells in culture further aggravate this problem. Because tissue stem cells continue to divide with asymmetric self-renewal division in culture, their fraction has been shown to decrease dramatically with cell culture as more committed

progenitor cells are produced and proliferate (Paré and Sherley 2006; Sherley 2014; Dutton et al. 2020).

The second pervasive misconception is the belief that stem cells can be counted specifically by flow cytometry. This error in understanding is partly due to flow cytometry's widely known sensitivity for identifying and quantifying rarified cell subpopulations. The missing information that results in the error is a failure to appreciate that no stem cell-specific biomarkers have been identified to date. Flow cytometry requires cell type-specific biomarkers for specific cell detection and quantification. All current tissue stem cell biomarkers (e.g., CD34, CD133, CD90) are misnamed. The targeted molecules are expressed on tissue stem cells, but they are also expressed on more abundant committed progenitor cells. The presence of high numbers of committed progenitor cells in essentially all tissue stem cell preparations precludes any chance of flow cytometry to be used to determine specific stem cell number or stem cell-specific dose.

The extent of entrenchment of these misconceptions is evident from a review of data obtained from a recent online survey conducted by the authors (https://asymmetrex.com/stem-cell-counting-study/). The survey was conducted over approximately a 1-year period and collected answers to a series of questions designed to probe respondents' level of knowledge about the current state of tissue stem cell counting technologies. To date, 116 respondents completed the ongoing survey and self-identified in the following occupational categories: undergraduate student (19%), graduate student (12%), physician (9.5%), CEO (8.6%), postdoctoral associate (6.9%), corporate research technical staff (6.9%), lab head (6.0%), project manager (4.3%), investor (3.5%), academic research technical staff (2.6%), CSO (2.6%), and other (18%). "Other" included respondents who self-reported in a variety of academic, administrative, business, and industrial professions.

Of these respondents, about 40% (45) answered that "*Homogenous tissue stem cells are the cellular constituents of isolated or expanded tissue stem cell preparations that are currently marketed and used for FDA-authorized stem cell clinical trials, private stem cell clinic treatments, or research*;" and about 56% (66) answered that "*Flow cytometry can be used to count tissue stem cells specifically*." Although respondents identifying as physicians were only 9.5% (11) of the total, their responses are suggestive of a high degree of misinformation about the stem cell dosing problem in stem cell medicine. Seven (64%) of the 11 responding physicians stated that stem cell treatments were *homogeneous* stem cell preparations, and 8 (73%) stated that stem cells could be counted *specifically* with flow cytometry.

If these early estimates are faithful representations of the current state of academic, medical, and industry knowledge about the cellular make-up of tissue stem cell treatments and the quantification of their dosage, then the ideas developed in this chapter are both needed and timely for inspiring a crucial awareness required for greater progress in veterinary stem cell medicine.

12.6 A Solution for the Tissue Stem Cell Counting Problem

Though confusion does exist regarding the state of technology for counting therapeutic tissue stem cells and determining their dosage, in surprising contrast, the importance of these metrics in stem cell science and stem cell medicine is generally well appreciated. In the same online survey described earlier, 82% (95 of 116 respondents) selected "*Without knowing the stem cell-specific treatment dose, it is not possible to soundly interpret the outcomes of tissue stem cell clinical trials*" as the reason "*Why the tissue stem cell-specific dose is important for stem cell clinical trials*." In 2020, The FDA's Standards Coordinating Body (SCB 2020) listed methods for determining the cell-specific dose of stem cell treatments as needed standards for regenerative medicine.

Until 2020, there were no technologies available for specific and accurate determination of the stem cell dose of therapeutic stem cell treatments. All the available in vitro methods described score both stem cells and committed progenitor cells. Colony-forming unit (CFU) assays cannot distinguish cell colonies produced by stem cells from those produced by early committed progenitor cells (Rich 2015). As noted earlier, the lack of stem cell-specific biomarkers precludes quantification of stem cell fraction by flow cytometry. Methods deploying assays for cellular enzymes and metabolites also score the activities of both stem cells and committed progenitor cells (Patterson et al. 2015).

In vivo cell transplantation assays allow detection of stem cells without confounding with detection of committed progenitor cells. These assays detect cells that can confer long-term reconstitution of human tissues in immunodeficient animals. Tissue stem cells have this ability, but committed progenitors do not. These assays have been limited primarily to applications for human HSCs using immunodeficient mice as cell transplant recipients; but there are also examples of their use to study human HSCs in larger animals like sheep (Almeida-Porada et al. 2004). In the case of human HSC assays in immunodeficient mice (typically NOD/SCID strains), performing a limiting dilution series of the evaluated cells before transplantation allows the application of Poisson statistical modeling to estimate the number of HSCs in the starting sample. The assays are commonly called limiting dilution SCID mouse repopulating cell (LDSRC) assays (Purton and Scadden 2007).

Though affording the requisite stem cell specificity for tissue stem cell counting, the LDSRC assay has significant quantitative limitations and shortcomings. First, it is expensive and takes a long time to perform. A single "count" for one treatment sample may require as many as 50 mice to achieve sufficient statistical power for the Poisson estimates, and these animals must be maintained for 16 weeks after transplantation to confirm long-term tissue reconstitution. Second, there is a significant quantitative shortcoming of the method that is often overlooked. The readout is a function of both HSC number and engraftment efficiency. The quantitative modeling assumes engraftment efficiency is 100% because there is no way to measure or estimate it independently. To the extent that engraftment efficiency is less than ideal, the LDSRC assay will underestimate the actual HSC number and dose; and because

it varies from mouse to mouse and time to time, it undermines the quantitative precision of HSC determinations.

Two other methodologies for specific quantification of tissue stem cells have been described more recently. The first is a morphological approach based on detecting a special characteristic of tissue stem cells that was first envisioned by John Cairns in the 1970s (Cairns 1975). When they undergo asymmetric self-renewal division, both animal and human tissue stem cells have been shown to nonrandomly co-segregate the same oldest complement of chromosomal DNA strands (Panchalingam et al. 2020). Since asymmetric self-renewal is a tissue stem cell-exclusive feature, the corresponding "immortal DNA strands" have been proposed as specific biomarkers that might be targeted for specific quantification of tissue stem cells (Huh et al. 2015). This approach has been used to detect tissue stem cells in preparations of tissue cells from agricultural animals like cows (Choudhary and Capuco 2012; Capuco and Choudhary 2020). To date, immortal DNA strand detection has not been validated as a means for determining the dose of stem cells in either human or veterinary stem cell treatments. Though promising in concept, the detection of cells with immortal DNA strands is technically challenging. Only recently have suitable technical methods been achieved that might enable this method's clinical validation and future use (Huh et al. 2015).

A second quantitative methodology for specific and accurate determination of tissue stem cell fraction, number, and dosage was reported last year (Dutton et al. 2020). The new approach, "kinetic stem cell counting," provides specific quantification of the tissue stem cells in complex tissue cell preparations. Kinetic stem cell counting is an in vitro cell culture method that uses computational simulation to discover the number of tissue stem cells responsible for the total cell proliferation of serially passaged tissue cell cultures. The method is based on a stem cell-driven cell production model that incorporates principles of in vivo tissue cell homeostasis. After an initial foundational computational simulation analysis based on 3–4 weeks of serial cell culture, kinetic stem cell counting yields simple mathematical algorithms for specific stem cell counting. Thereafter, the kinetic stem cell counting algorithms require only culture population doubling time data, produced from a few days of cell culture, to compute the stem cell-specific fraction, number, and dose of a tissue cell sample. Though not yet clinically validated, the kinetic stem cell counting method has the potential to become a routine clinical tool for the determination of stem cell dose for both veterinary stem cell medicine and human stem cell medicine (Dutton et al. 2020).

12.7 Benefits of Quantitative Stem Cell Dosing for Stem Cell Veterinary Medicine

Though still early in its development, the new kinetic stem cell counting technology promises to make the long-needed implementation of stem cell dosing in stem cell medicine not only feasible but also practical. Many benefits can be envisioned for

Table 12.2 Future benefits of stem cell dosing in stem cell veterinary medicine

Benefit of stem cell dosing	Current problem addressed
1. Increased treatment fidelity	Unknown stem cell dosing reliability[a]
2. Increased statistical power	Unknown dosage variance
	Efficacy evaluation
3. Direct potency metric	Potency
4. Improved biomanufacturing	Uncertain production
5. Improved quality control and assurance	Stem cell preservation, stability, transport

[a]In particular for expanded stem cell treatments

both veterinarians and their patients when stem cell dosing is a routine practice for veterinary stem cell medicine. Table 12.2 highlights several predicted benefits.

The most significant benefit is the increased treatment fidelity. Knowing the dosage of the therapeutic agent in administered medicines is a fundamental tenet of clinical medicine (Sherley 2018a). The stem cell treatment dosage is an important factor for delivering reproducible stem cell treatments among different patients and for the same patients over time. Poor treatment fidelity is a particular worry for M[S]C therapies that use expanded stem cell populations. It is generally recognized that culture expansion of all types of tissue stem cells proceeds with a reduction in stem cell activity by all available measures (Shakouri-Motlagh et al. 2017). Though various explanations are considered to account for this loss—including stem cell differentiation, loss of tissue factors, stem cell senescence, and stem cell dilution—the resulting challenge is the same. The reduced dose of stem cells in expanded cell populations is unknown. It is certainly variable from expansion lot to expansion lot, and in some cases, unknowingly, it might be too low to be therapeutically effective.

Stem cell dosage data could have a major impact on the progress and success of stem cell medicine clinical trials. Of course, improved treatment fidelity would be advantageous for clinical trial success. However, in addition, the availability of precise quantification of stem cell dosage would improve the design of clinical trials and the interpretation of their outcomes. Since compared patients rarely get treated with ideally replicate treatment samples, currently, stem cell dose is an unknown denominator in all stem cell clinical trial outcome data analyses. Beyond improving overall treatment fidelity, knowledge of how stem cell dose varies among patients, between treatment arms, among trial sites, and among trials would provide greater statistical power for soundly detecting treatment effects.

Throughout the practice of stem cell medicine, potency is a controversial topic (Rich 2015). The potency is a prediction of the effects of treatment *before* its administration to patients. It is ideal for potency to be a quantitative measurement that is able to predict the degree of clinical response with respect to the amount of the treatment. Stem/progenitor biomarkers, like CD34 and CD90 for instance, are qualitative indicators of the potency for HSC and M[S]C treatments, respectively. However, neither provides a quantitative prediction of the effectiveness of stem cell treatment (Ivanovic 2010). Other commonly applied characterizations of stem cell

populations, like CFU and LDSRC assays, fall short as potency measures as well (Purton and Scadden 2007; Rich 2015). Stem cell-specific fraction, number, or dose determined by kinetic stem cell counting may prove to be the first effective potency measures for stem cell medicine.

General implementation of effective kinetic stem cell counting by ancillary industries that support veterinary stem cell medicine will also benefit veterinarians and their patients. Companies engaged in biomanufacturing of expanded stem cell treatments can improve their bioprocess engineering by monitoring, for the first time, the stem cell-specific fraction of starting tissue sources, processing stages, and final expanded production lots. Companies that store and ship either primary tissue stem cell preparations or biomanufactured cells can better ensure the viability and potency of stem cell treatments after storage and shipment. Currently, quality control and quality assurance of such services are based on assessments of total cells, for which the crucial stem cells are only a small fraction. Kinetic stem cell counting could be used to investigate the widely applied but nonvalidated belief that such total cell measurements are quantitatively informative about the status of the stem cells in the preparations. If kinetic stem cell counting invalidates this belief, its stem cell dose determinations can become the new basis for quality control and quality assurance evaluations.

12.8 Conclusions and Future Perspective

Current veterinary stem cell medicine operates with the same deficiency presently accepted by human stem cell medicine. Stem cell medicine treatments, whether approved for a current medical practice or in clinical trials, proceed without knowledge of the dosage of the administered stem cells. In this long-standing state of a principal deficiency, unknowingly, treatments may occur without any stem cells being delivered at all. This lack of qualitative, though more often quantitative, treatment fidelity undermines the success of veterinary stem cell medicine. It renders approved therapies uncertain and unreliable, and it confounds the design and outcome interpretation of stem cell clinical trials and research studies. The way out of the chronic widespread resignation to dose-less stem cell medicine is through the implementation of new technologies that provide an effective means for specific and accurate counting of therapeutic tissue stem cells. Recently described kinetic stem cell counting may be such a technology (Dutton et al. 2020).

So far, human stem cell medicine has been slow to move to evaluating kinetic stem cell counting as a solution for introducing specific dosing for stem cell treatment production, storage, shipping, and administration to patients. As outlined in this chapter focused on veterinary stem cell medicine, important benefits are predicted to accrue to veterinarians, and more importantly to their patients, wherever stem cell-specific dosing information is introduced in current stem cell medical practice or its ancillary supporting industries. If this advantage proves true for veterinary stem cell medicine, it will also prove true for human stem cell medicine. Because of its historical role as the gateway through which medical innovations must

travel to reach human patients, veterinary medicine has the positioning and opportunity to lead to way to advancing quantitative stem cell dosing for stem cell medicine.

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References

Almeida-Porada G, Porada CD, Chamberlain J et al (2004) Formation of human hepatocytes by human hematopoietic stem cells in sheep. Blood 104(8):2582–2590. https://doi.org/10.1182/blood-2004-01-0259

Arzi B, Peralta S, Fiani N et al (2020) A multicenter experience using adipose-derived mesenchymal stem cell therapy for cats with chronic, non-responsive gingivostomatitis. Stem Cell Res Ther 11(1):115. https://doi.org/10.1186/s13287-020-01623-9

Barberini DJ, Aleman M, Aristizabal F et al (2018) Safety and tracking of intrathecal allogeneic mesenchymal stem cell transplantation in healthy and diseased horses. Stem Cell Res Ther 9:96. https://doi.org/10.1186/s13287-018-0849-6

Cairns J (1975) Mutation selection and the natural history of cancer. Nature 255:197–200

Caplan AI (2017) Mesenchymal stem cells: time to change the name! Stem Cells Transl Med 6:1445–1451. https://doi.org/10.1002/sctm.17-0051

Capuco AV, Choudhary RK (2020) Symposium review: determinants of milk production: understanding population dynamics in the bovine mammary epithelium. J Dairy Sci 103(3):2928–2940. https://doi.org/10.3168/jds.2019-17241

Choudhary RK, Capuco AV (2012) In vitro expansion of mammary stem/progenitor cell population by xanthosine treatment. BMC Cell Biol 13:14. https://doi.org/10.1186/1471-2121-13-14

Costa CRM, Feitosa MLT, Rocha AR et al (2019) Adipose stem cells in reparative goat mastitis mammary gland. PLoS One 14(10):e0223751. https://doi.org/10.1371/journal.pone.0223751

Delco ML, Goodale M, Talts JF et al (2020) Integrin α10β1-selected mesenchymal stem cells mitigate the progression of osteoarthritis in an equine talar impact model. Am J Sports Med 48(3):612–623. https://doi.org/10.1177/0363546519899087

Devireddy LR, Myers M, Screven R et al (2019) A serum-free medium formulation efficiently supports isolation and propagation of canine adipose-derived mesenchymal stem/stromal cells. PLoS One 14(2):e0210250. https://doi.org/10.1371/journal.pone.0210250

Dutton R, Abdi F, Minnetyan L et al (2020) A computational technology for specific counting of perinatal and postnatal human tissue stem cells for transplantation medicine. OBM Transplant 4(3):24. https://doi.org/10.21926/obm.transplant.2003117

Gallant.com (2019–2020) https://gallant.com/science-library/

Gnecchi M, Danieli P, Malpasso G et al (2016) Paracrine mechanisms of mesenchymal stem cells in tissue repair. Methods Mol Biol 1416:123–146. https://doi.org/10.1007/978-1-4939-3584-0_7

Gomez-Salazar M, Gonzalez-Calofre ZN, Casamitjana J et al (2020) Five decades later, are mesenchymal stem cells still relevant? Front Bioeng Biotechnol 8:148. https://doi.org/10.3389/fbioe.2020.00148

Gugjoo MB, Amarpal, Chandra V et al (2018) Mesenchymal stem cell research in veterinary medicine. Curr Stem Cell Res Ther 13(8):645–657. https://doi.org/10.2174/1574888X13666180517074444

Huh YH, Noh M, Burden F et al (2015) Use of sparse feature bioinformatics to identify a novel pattern-specific biomarker for counting asymmetrically self-renewing distributed stem cells. Stem Cell Res 14:144–154. https://doi.org/10.1016/j.scr.2014.12.007

Ivanovic Z (2010) Hematopoietic stem cells in research and clinical applications: the "CD34 issue". World J Stem Cells 2:18–23

Kang MH, Park HM (2020) Challenges of stem cell therapies in companion animal practice. J Vet Sci 21(3):e42. https://doi.org/10.4142/jvs.2020.21.e42jvs-21-e42

Kavanagh DP, Kalia N (2011) Hematopoietic stem cell homing to injured tissues. Stem Cell Rev Rep 7(3):672–682. https://doi.org/10.1007/s12015-011-9240-z

Li MD, Atkins H, Bubela T (2014) The global landscape of stem cell clinical trials. Regen Med 9 (1):27–39. https://doi.org/10.2217/rme.13.80

Liesveld JL, Sharma N, Aljitawi OS (2020) Stem cell homing: from physiology to therapeutics. Stem Cells 38(10):1241–1253. https://doi.org/10.1002/stem.3242. Epub 2020 Jul 21

Liu Z, Screven R, Boxer L et al (2018) Characterization of canine adipose-derived mesenchymal stromal/stem cells in serum-free medium. Tissue Eng Part C Methods 24(7):399–411. https://doi.org/10.1089/ten.TEC.2017.0409

Nitzsche F, Müller C, Lukomska B (2017) Concise review: MSC adhesion cascade-insights into homing and transendothelial migration. Stem Cells 35(6):1446–1460. https://doi.org/10.1002/stem.2614

Panchalingam K, Jacox L, Cappiello BD et al (2020) Non-random sister chromatid segregation in human tissue stem cells. Symmetry 12:1868. https://doi.org/10.3390/sym12111868

Paré J-F, Sherley JL (2006) Biological principles for ex vivo adult stem cell expansion. In: Schatten G (ed) Current topics in developmental biology, vol 73. Elsevier, Inc, San Diego, pp 141–171

Patterson J, Moore CH, Palser E et al (2015) Detecting primitive hematopoietic stem cells in total nucleated and mononuclear cell fractions from umbilical cord blood segments and units. J Transl Med 13:94. https://doi.org/10.1186/s12967-015-0434-z

Pittenger MF, Discher DE, Peault BM et al (2019) Mesenchymal stem cell perspective: cell biology to clinical progress. Regen Med 4:22. https://doi.org/10.1038/s41536-019-0083-6

Purton LE, Scadden DT (2007) Limiting factors in murine hematopoietic stem cell assays. Cell Stem Cell 1:263–270

Quimby JM, Borjesson DL (2018) Mesenchymal stem cell therapy in cats: current knowledge and future potential. J Feline Med Surg 20(3):208–216. https://doi.org/10.1177/1098612X18758590

Rich IN (2015) Improving quality and potency testing for umbilical cord blood: a new perspective. Stem Cells Transl Med 4:967–973

Russell KA, Chow NHC, Dukoff D et al (2016) Characterization and immunomodulatory effects of canine adipose tissue- and bone marrow-derived mesenchymal stromal cells. PLoS One 11(12): e0167442. https://doi.org/10.1371/journal.pone.0167442

Saldinger LK, Nelson SG, Bellone RR et al (2020) Horses with equine recurrent uveitis have an activated CD4+ T-cell phenotype that can be modulated by mesenchymal stem cells in vitro. Vet Ophthalmol 23(1):160–170. https://doi.org/10.1111/vop.12704

Shakouri-Motlagh A, O'Connor AJ, Brennecke SP et al (2017) Native and solubilized decellularized extracellular matrix: a critical assessment of their potential for improving the expansion of mesenchymal stem cells. Acta Biomater 55:1–12. https://doi.org/10.1016/j.actbio.2017.04.014

Sherley JL (2005) Asymmetric self-renewal: the mark of the adult stem cell. In: Habib NA, Gordon MY, Levicar N, Jiao L, Thomas-Black G (eds) Stem cell repair and regeneration. Imperial College Press, London, pp 21–28

Sherley JL (2006) Mechanisms of genetic fidelity in mammalian adult stem cells. In: Potten CS, Clarke RB, Wilson J, Renehan AG (eds) Tissue stem cells. Taylor Francis, New York, pp 37–54

Sherley JL (2013) New cancer diagnostics and therapeutics from a 9th "hallmark of cancer": symmetric self-renewal by mutated distributed stem cells. Expert Rev Mol Diagn 13:797–810

Sherley JL (2014) Accelerating progress in regenerative medicine by advancing distributed stem cell-based normal human cell biomanufacturing. Pharm Anal Acta 5:286. https://doi.org/10.4172/2153-2435.1000286

Sherley JL (2018a) Dose determination for stem cell medicine: a need whose time has come. In: Atala A, Cetrulo C, Cetrulo K, Murphy SV, Taghizadeh R (eds) Perinatal stem cells: research and therapy. Elsevier, Amsterdam, pp 383–397

Sherley JL (2018b) Stem cell therapy: resolving the mismatch. Pharmaceutical manufacturing. pp 38–41

Standards Coordinating Body (2020) C25 Clinical trial interpretation with unknown cell-specific doses. In: Community perspectives: needed standards in regenerative medicine. p 83. https://static1.squarespace.com/static/58a331b0db29d63c7fb64528/t/5fdcd93257b8971cd7ab4ef2/1608309045242/NeededStandardsReportDecember2020.pdf

Szydlak R (2019) Mesenchymal stem cells' homing and cardiac tissue repair. Acta Biochim Pol 66 (4):483–489. https://doi.org/10.18388/abp.2019_2890

Taghizadeh RR, Sherley JL (2009) Expanding the therapeutic potential of umbilical cord blood hematopoietic stem cells. In: Cetrulo CL, Cetrulo KJ, Cetrulo CL Jr (eds) Perinatal stem cells. Wiley-Blackwell, Hoboken, pp 21–40

Taguchi T, Borjesson DL, Osmond C et al (2019) Influence of donor's age on immunomodulatory properties of canine adipose tissue-derived mesenchymal stem cells. Stem Cell Dev 28 (23):1562–1571. https://doi.org/10.1089/scd.2019.0118

Till JE, McCulloch EA (1961) A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiat Res 14:213–222

White JL, Walker NJ, Hu JC et al (2018) A comparison of bone marrow and cord blood mesenchymal stem cells for cartilage self-assembly. Tissue Eng Part A 24 (15–16):1262–1272. https://doi.org/10.1089/ten.TEA.2017.0424

Zhan XS, El-Ashram S, Luo DZ et al (2019) A comparative study of biological characteristics and transcriptome profiles of mesenchymal stem cells from different canine tissues. Int J Mol Sci 20 (6):1485. https://doi.org/10.3390/ijms20061485

Mesenchymal Stem Cells: A Novel Therapy for the Treatment of Bovine Mastitis

13

Oscar A. Peralta

Abstract

Mesenchymal stem cells (MSCs) have received considerable attention during the last decades as a potential cell-based therapy for human and veterinary medicines. This potential is originated initially from the capacity of MSCs to differentiate into mesodermal cell lineages including osteogenic, chondrogenic, and adipogenic, and their low immunogenicity that enabled them for allogenic applications. Furthermore, increasing amounts of evidence indicate that MSCs have regenerative capacity, mediated through immunomodulatory, angiogenic, mitogenic, and antibacterial trophic abilities. Worldwide, mastitis is the most prevalent and expansive disease for dairy herds. Antibiotics are the most common and general option for the treatment of bovine mastitis. However, limited success in terms of cure rates, failure to regenerate damaged mammary tissue, and development of antimicrobial resistance have led investigators to consider new and alternative approaches. Considering the importance of alternative treatments for bovine mastitis and the reported regenerative and antibacterial effects of MSCs, during the last year the author's team has evaluated the therapeutic in vitro potentials of bovine fetal MSCs (bfMSCs). In a series of experiments, it has been reported that inflammatory interferon γ (IFNγ) induced upregulation of immunomodulatory factors [indolamine 2,3-dioxygenase (IDO) and interleukin 6 (IL-6)] in bfMSCs. Moreover, bfMSCs induce suppression of proliferation of alloantigen-activated bovine peripheral blood lymphocytes (PBLs). Conditioned medio from bfMSCs induced angiogenesis due to secretion of angiopoietin 1 (ANGPT1) and vascular in vitro angiogenesis growth factor (VEGF) in cell

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culture. Moreover, conditioned media of bfMSCs induced an antiproliferative effect against *S. aureus*, likely due to the presence of antibacterial peptides (defensin β1 and NK lysin). These studies suggest that bfMSCs produce several factors associated with the control of the proliferation of mastitis-causing bacteria. Furthermore, the safety and efficacy of a bfMSC therapy were evaluated in healthy cows and showed no clinical or hematological changes. The safety and efficacy of stem cell therapy were evaluated and showed no induced clinical or hematological changes in healthy control cows. Interestingly, intramammary administration of bfMSCs resulted in a lower bacterial count in cows with mastitis compared to control cows. Overall, our work has provided initial evidence for the potential of an allogenic bfMSC-based intramammary therapy for the treatment of mastitis in dairy cows.

Keywords

Bovine mastitis · *S. aureus* · Intramammary therapy · Bovine fetal MSCs

13.1 Introduction

Mesenchymal stem cells (MSCs) are multipotent stem cells with the abilities of self-renewal and differentiation into mesodermal cell lineages, including osteogenic, adipogenic, chondrogenic, and myogenic (Pittenger et al. 1999). MSCs grow as a monolayer and adhere to plastic substratum. Various sources of MSCs have been explored, initially bone marrow (BM) and adipose tissue (AT) (Harichandan and Buhring 2011), and more recently cord blood, placenta, dental pulp, articular cartilage, brain, endometrium, Wharton's jelly, umbilical cord tissue, and amniotic fluids (Marquez-Curtis et al. 2015). For defining the property of human MSCs, the International Society for Cellular Therapy (ISCT) have established the adherence to plastic dishes as the first criteria (Dominici et al. 2006). Additional criteria for identifying MSCs include trilineage differentiation potential, expression of MSC surface antigens (CD105, CD73, and CD90 or Thy-1) and absence of expression of hematopoietic lineage markers (CD45, CD34, and CD14). Criteria for the classification of MSCs in livestock species have not been established. The plastic-adherent cell populations of bovine fetal MSCs (bfMSCs), including bfBM-MSCs and bfAT-MSCs, express mesenchymal markers CD73 and CD29, are negative for hematopoietic markers CD45 and CD34, and display trilineage differentiating potential (Dueñas et al. 2014; Cahuascanco et al. 2019). These characteristics indicate that the isolated bfMSC populations display properties and achieve a specific profile of MSCs (Dominici et al. 2006). Moreover, bfBM-MSC and bfAT-MSC express similar levels of pluripotency transcriptional factors NANOG and OCT4.

The therapeutic features have congregated considerable interest over the last decades for the potential development of MSC-based therapy in treating various diseases (Alcayaga-Miranda et al. 2015; Caplan and Correa 2011). Initially, this

potential has been based on the multipotentiality of MSCs to differentiate in vitro into mesodermal cell lineages (Pittenger et al. 1999; Cortes et al. 2013). Later, results indicate that upon contact with an inflammatory environment, MSCs display paracrine and immunomodulatory activities. These trophic functions are associated to tissue-like regerenerative capacities and include antibacterial, antiapoptotic, angiogenic, and mitogenic effects (Caplan and Correa 2011). The use of allogenic sources of MSCs is attractive since isolation and expansion of MSCs require a prolonged period (4–6 weeks) in order to acquire sufficient cells. Allogenic MSCs may be used as an "off-the-shelf" therapeutic product, which avoids the expansion period required for autologous MSCs. Noteworthy, abattoir-derived tissues can be an unexpensive, easily available, and plentfull source for generating pools of bfMSCs, permitting reduction in individual variations for therapeutic applications and thus opening the possibility to select the most suitable donors according to therapeutic properties.

Mastitis is the consequence of infection by pathogenic agents and is the disease more prevalent and costly in dairy herds worldwide. Also, bovine mastitis may induce severe clinical signs and has been declared as one of the main diseases threatening animal welfare (Farm Animal Welfare Council 2009). Mastitis affects public health by altering the physical and microbiological properties of milk and increasing the content of undesirable residues in milk. Currently, antibiotics are used to treat mastitis by controlling bacterial infection of the mammary gland. Even though antibiotic therapy is an efficient strategy to control mastitis, this treatment fails to regenerate the integrity of milk-secreting cells in the mammary tissue. Moreover, antibiotics promote the development of bacterial resistance, which has become an increasing public concern. Thus, the recent scientific advances in stem cell research represent a potential tool that requires evaluation to develop alternative or supplementary therapies for bovine mastitis.

Regenerative medicine utilizes stem cell populations or their derived products to treat a variety of diseases in both humans and animals. An earlier report indicated that MSCs correspond to perivascular cells (or pericytes) and have the potential to detach from blood vessels in response to damaged or inflamed tissues (Caplan and Correa 2011). Stimulation of bfMSCs with inflammatory interferon γ (IFNγ) upregulates expression of immunomodulatory factors including indolamine 2,3-dioxygenase (IDO) and interleukin 6 (IL-6) (Huaman et al. 2019). Also, conditioned media from bfMSCs induce angiogenesis, which may be associated with the effect of vascular endothelial growth factor (VEGF) and angiopoietin 1 (ANGPT1) (Jervis et al. 2019). Moreover, upon contact with mastitis-causing *S. aureus,* bfMSCs increased expression of antibacterial peptides like defensin β1 and NK lysin, and its conditioned media exerted antiproliferative against this pathogen (Cahuascanco et al. 2019). These studies suggest that bfMSCs can produce several factors that might be helpful in the treatment of bovine mastitis (Fig. 13.1).

The reduced immunogenicity is considered a therapeutic feature that enables the use of MSCs for allogenic transplantation (Ankrum et al. 2014). The potential immune-evasive property of MSCs is partially due to low expression of major histocompatibility complexes (MHCs) I and II and lack of T-cell costimulatory

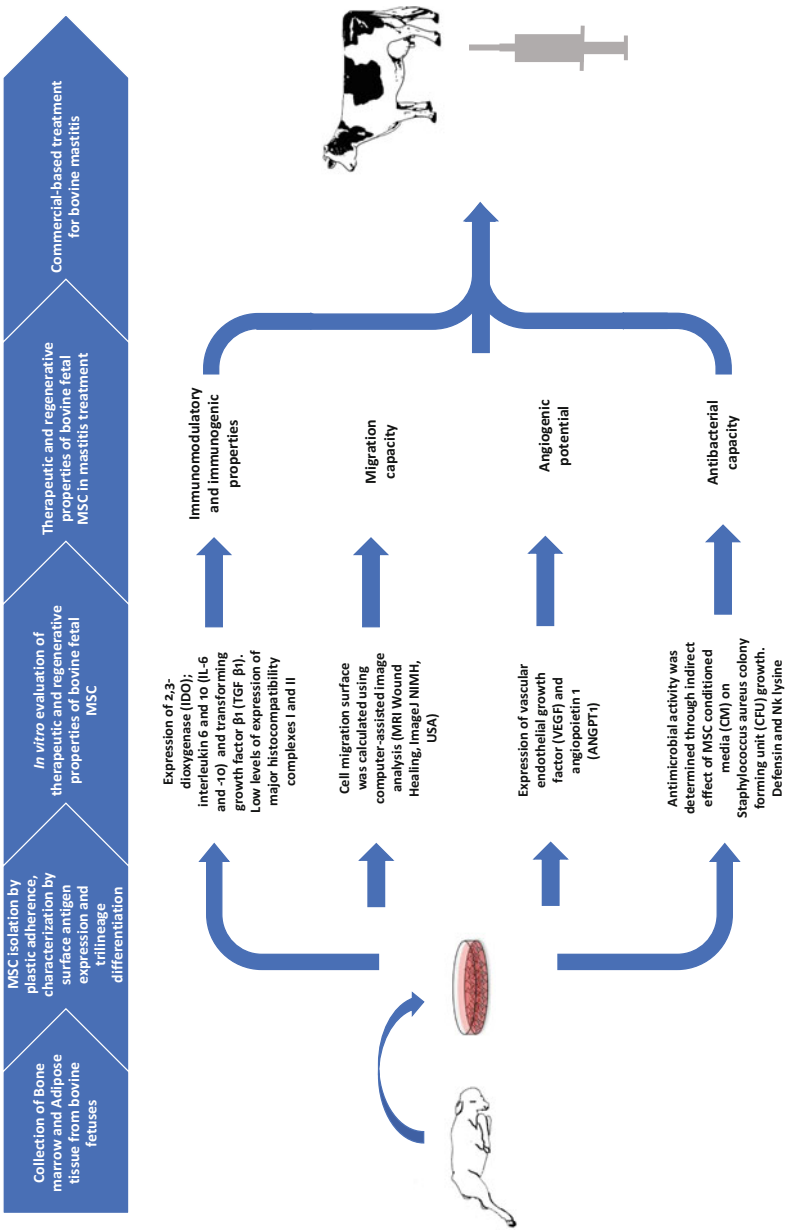

Fig. 13.1 Schematic representation of the isolation and evaluation of bovine fetal MSC and the in vitro therapeutic and regenerative properties that support an allogenic MSC-based intramammary therapy in dairy cows with mastitis

molecules CD80 (Huaman et al. 2019; Consentius et al. 2015). Together with the previously described reduced immunogenicity, additional immune-regulatory abilities may allow bfMSCs to evade recognition from the immune system in a recipient cow, and thus to serve as an allogenic cell-based therapeutic tool.

13.1.1 Immunomodulatory and Immunogenic Properties Suggest That Bovine MSC May Be a Useful Therapeutic Strategy for Mastitis

Immunomodulatory properties of MSCs are manifested directly by modulation of innate and adaptive immunity through cell-to-cell contact or indirectly by paracrine signaling. Paracrine signaling occurs through secretion of soluble factors including IDO, IL-6, TGFβ1, and IL-10 (Hsiao et al. 2012). These factors are part of MSC secretome (Elman et al. 2014) and regulate cellular proliferation, differentiation, migration, and apoptosis of various immune cells (Barrachina et al. 2016). Low immunogenicity and immune regulatory capacity would allow MSCs to evade the host immune system by avoiding direct recognition by natural killer cells (NKs) and preventing activation of T-lymphocytes. Moreover, the immune-evasive capacity of MSC is associated to reduced expression of MHC-I and II and T-cell costimulatory CD40, CD80 and CD86 (Consentius et al. 2015). This results in slower elimination of allogeneic cells by donor cell-specific T-effector cells and antibodies. Consequently, potential for homing is favored by eluding opsonized allospecific antibodies, leading to diminished adverse effects and higher efficacy of allogenic cell administration to populate tissue.

The immunomodulatory and immunoevasive potentials are the fundamental aspects of cell-based therapeutics in clinical application. We recently determined that treatment with IFNγ increased gene expression levels of IL-6 and IDO and induces enzymatic activity of IDO in bfBM-MSCs and bfAT-MSCs (Huaman et al. 2019). Moreover, conditioned medium derived from both cell types suppressed proliferation of alloantigen-activated peripheral blood lymphocytes (PBLs). Interestingly, bfAT-MSCs showed lower expression of MHCI-I and -II compared to bfBM-MSCs, suggesting that bfAT-MSCs are less immunogenic and potentially more suitable for allogenic therapy than bfBM-MSCs (Huaman et al. 2019).

IFNγ stimulation is required for inducing expression of IDO at gene and protein levels in bfMSCs in a dose-dependent manner (Huaman et al. 2019). The mechanism of IFNγ on MSCs is initially mediated by binding to a membrane receptor and subsequently by activation of IDO synthesis (Mellor and Munn 2004). IDO catalyzes the degradation of tryptophan to kynurenine dendritic cells and macrophages for modulating the immune response (Mellor and Munn 2004). This mechanism suppresses T lymphocyte response and converts monocytes to M2-immunosuppressive macrophages, resulting in the production of IL-10 (Krampera et al. 2003). These M2 macrophages are involved in suppressing T cell proliferation in an IL-10-independent manner and amplifying the immunosuppressive effect of MSCs (Francois et al. 2012). In concordance with these results, BM-

MSCs from horses require pre-exposure to IFNγ and TNFα in order to produce IDO and immune mediators TGFβ1 and IL-6 (Barrachina et al. 2016). Similarly, following IFNγ stimulation, the activity of IDO was increased in canine neonatal MSC and BM-MSCs (Saulnier et al. 2016). Altogether, studies indicate that inflammatory conditions stimulate the production of paracrine signaling factors in MSCs.

In response to exposure to high concentrations of IFNγ (20 and 40 ng/mL), bfBM-MSCs and bfAT-MSCs increase IL-6 mRNA levels. Similarly, bfBM-MSCs increase mRNA levels of PTGER2 after treatment with a high concentration of IFNγ (40 ng/mL). Prostaglandin E2 (PGE2) plays a role, mediated by triggering E-prostanoid (EP) 2 receptor, in exerting immunomodulatory capacity and maintaining the self-renewal capacity of human MSCs (Lee et al. 2016). Upregulation of EP2 gene expression in bfMSCs suggests the involvement of the PGE2 signaling pathway. Human MSCs in the presence of IFNγ increased PGE2 secretion and suppressed inflammatory mediators (Hsu et al. 2013). Moreover, PGE2, IL-6, and IDO promote the conversion of $CD4^+$ T cells to T regulatory (Treg) $CD4^+$ $Foxp3^+$ cells that play a role in suppressing the alloantigen response (English et al. 2009; Kyurkchiev et al. 2014). These results support the stimulatory effect of IFNy on selected immunomodulatory mediators including IL-6, PTGER2 and IDO in bfMSCs. In contrast, IFNγ did not exert a stimulatory effect on TGFβ1 and IL-10 gene expression in bfMSCs. Previous studies have indicated that treatment with 5 ng/mL of TNFα and 5 ng/mL of IFNγ exposure during 12 h results in upregulation of IDO and IL-6 mRNA levels had no effect on TGFβ1 or IL-10 gene expression in equine BM-MSCs (Barrachina et al. 2016). Thus, IFNγ stimulation and immunomodulatory response of bfMSCs in proinflammatory environment results in differential gene expression of selected cytokines (TacGhee and Sun 2012).

Various studies have demonstrated that the immunomodulatory potential of MSCs is mediated via suppression of T lymphocytes proliferation (Klyushnenkova et al. 2005). Using an in vitro proliferation assay of alloantigen-activated mixed leucocyte reaction (MLR) system, we previously reported that conditioned medium from IFNγ-stimulated and unstimulated bfMSCs reduced proliferation of PBLs. Expression of *IL6, PTGER2,* and *IDO* genes was upregulated in PBLs after IFNγ pretreatment, which suggest that these factors play a role in reducing PBL proliferation. However, direct role of these factors in the immunomodulatory effect of bfMSCs cannot be assumed since reduction of PBL proliferation was not significantly different between conditioned media from IFNγ-treated and untreated bfMSCs. Previously, it has been reported that exposure of $CD4^+$ T cells to conditioned medium from human AT-MSCs resulted in higher proportion of Treg lymphocytes associated to immunological tolerance (Ivanova-Todorova et al. 2009). BM-MSCs suppressed activation, proliferation, and differentiation of $CD4^+$ T cells into Th1 and Th17 cells in mice and human (Luz-Crawford et al. 2013). Similarly, equine BM-MSCs modulate immune cell function by inducing lymphocyte cell cycle arrest, whereas AT-MSCs activate lymphocyte apoptosis and reduce T cell proliferation (Carrade Holt et al. 2014). Treatments with IFNγ had no effect on immunomodulatory capacity of canine and equine AT-MSCs and BM-MSCs;

however, these cells are capable of supressing stimulated PBMC proliferation (Carrade Holt et al. 2014). Similarly, our data indicate that despite IFNγ stimulation, bfBM-MSCs- and bfAT-MSCs-derived conditioned media had similar capacity for decreasing PBL proliferation. This data support the notion that the immunomodulatory effect of MSC from different tissue sources and animal species is similar, although some variation in the mechanisms of action may exist.

MHC-I and II are the key molecules in antigen presentation and T lymphocyte activation. MSCs express low levels of MHC-I and II, and coestimulatory molecules CD80 and CD86, which are also crucial for antigen presentation (Ankrum et al. 2014). Although MHC-II expression is lower in bfAT-MSCs compared to peripheral blood mononuclear cells (PBMCs), bfAT-MSCs may still be immunogenic. However, rejection of allogenic MSCs may occur in a slower rate compared to other cell types (Ankrum et al. 2014). We have detected higher expression of MHC-I and MHC-II in bfBM-MSCs compared to bfAT-MSCs which indicates that immunogenic differences exist between bovine tissue sources of MSCs. Moreover, bfBM-MSCs express levels of MHC-I similar to PBMCs suggesting that bfBM-MSCs may be more immunogenic than expected. Conversely, lower expression of MHC-II in bfAT-MSCs suggests that they may be at lower risk for minor activation and effector function of T lymphocytes and may possess higher immunoevasive potential after allogenic transplantation. However, under inflammatory conditions, the immunogenic potential of MSCs may also be associated with immune suppression, since MSCs exposed to IFNγ can express significantly more MHC-I and -II (Le Blanc et al. 2003). Thus, the potential immune rejection of MSCs is dependent on the balance between expression of immunogenic and production of immunosuppressive factors. In this context, failure to activate the immunosuppressive competence leaves MSCs functioning as an antigen-presenting cell and capable of promoting inflammation in vivo (Ankrum et al. 2014).

13.1.2 The Antibacterial Potential of Bovine Fetal Mesenchymal Stem Cells May Play a Crucial Role Against Pathogens-Causing Mastitis in Dairy Cows

Mesenchymal stem cells display regenerative activity through paracrine function including immunomodulatory effects mediated by dendritic and B and T cells, and trophic capacity involving antiapoptotic, proangiogenic, and mitogenic factors (Caplan and Correa 2011). Moreover, MSCs exert antibacterial property by secreting antimicrobial peptides (AMPs). More than 17,022 AMPs have been registered in the Database of Antimicrobial Activity and Peptide Structures (https://dbaasp.org/statistics/general as of April 24, 2021). AMPs are evolutionary conserved small effector molecules found in a variety of organisms. MSCs enhanced bacterial clearance through the expression of several AMPs, including cathelicidin (CATHL2), IDO, and hepcidin (HEP) (Krasnodembskaya et al. 2010; Meisel et al. 2011; Alcayaga-Miranda et al. 2015). Cathelicidins are a group of AMPs having differences in amino acid sequences, sizes, and structures. They are the secretory

proteins stored in the secretory granules of neutrophils and macrophages and released upon extracellularly activation of leukocytes. LL-37 is the single cathelicidine identified in humans, monkeys, mice, rats, and guinea pigs (Kościuczuk et al. 2012). The first cathelicidin was identified in bone marrow myeloid cells (Bals and Wilson 2003) and LL37 has shown to inhibit *Escherichia coli, Pseudomonas aeruginosa*, and *Staphylococcus aureus* in human MSC cultures (Krasnodembskaya et al. 2010). On the other hand, IDO plays an important role in immune tolerance of MSCs. IDO displays a catalytic activity that depletes tryptophan in MSCs cultures and impedes bacteria like *S. aureus*, *Staphylococcus epidermidis, Enterococcus faecium*, and *E. coli* (Meisel et al. 2011). Menstrual fluid-derived MSCs exert an important antimicrobial effect mediated by hepcidin against fecal bacteria (Alcayaga-Miranda et al. 2015). Hepcidin is an iron-regulatory hormone and a natural AMP found in urine and plasma (Park et al. 2001). The iron-regulatory activity of hepcidin limits iron availability to microorganisms, a required element for bacterial growth, and prevents growth (Lombardi et al. 2015). These studies suggest that MSCs are equipped with diverse factors capables to fight a variety of bacterial infections.

*S. aureu*s is the most commonly isolated pathogen in clinical mastitis cases (Reyher et al. 2011). *S. aureus* can invade neutrophils and mammary epithelial cells, and forms micro-abscesses and biofilm formation (Keefe 2012). Despite in vitro antimicrobial susceptibility, *S. aureus* remains a pathogen difficult to control mainly due to the restriction of their contact with antibiotics (Makovec and Ruegg 2003). Consequently, only 10–30% cure rates have been observed after antibiotic treatment in mastitis caused by *S. auerus* (Gomes and Henriques 2016). Considering the limited success rate of conventional treatments against mastitis caused by *S. aureus*, culling infected cows from the herd is commonly practiced in order to mitigate new intramammary infections.

Although the antibacterial mechanism of MSCs during bacterial exposure has not been completely revealed, it has been reported that upon encounter with bacteria MSCs modulate their secreted products and biological properties including migration, proliferation, and differentiation (Mezey and Nemeth 2015). Exposure of human MSC to *S. aureus* increases the production of several paracrine factors including VEGF, SDF-1, and IL-6, which stimulate migration and activation of inflammatory cells to the infected tissue (Ward et al. 2015). In vivo experiments indicate that rats infected with methicillin-resistant *S. aureus* reduce bacterial infection and inflammatory response after treatment with MSCs (Yuan et al. 2014). Moreover, sepsis-induced mice treated with MSCs reduced inflammation and bacterial clearance in vivo, which may be associated with phagocytosis, as secreted IL-10 and IL-6 enhance phagocytosis of *S. aureus* (Mei et al. 2010). Despite reports on immunomodulatory and phagocytic activity, the antibacterial response of MSCs against *S. aureus* may be primarily driven by the production of AMPs.

Thus, we evaluated the in vitro antibacterial potential against *S. aureus* of conditioned medium derived from bfBM-MSCs and bfAT-MSCs (Cahuascanco et al. 2019). To our knowledge, this was the first reported comparative analysis of bfBM-MSCs and bfAT-MSCs based on antibacterial potentials against *S. aureus*. In

our study, expressions of five AMPs were evaluated, of which bBD4A and NK1 were involved in the antibacterial activity of MSCs. Expression of bBD4A in bfBM-MSCs and bfAT-MSCs concentrations in conditioned media increased 6 h after *S. aureus* challenge. In contrast, fetal fibroblast used as negative controls did not show upregulation in BD4A after exposure to *S. aureus*. Moreover, conditioned medium from fetal fibroblast failed to reduce *S. aureus* proliferation. Altogether, this study indicated that BD4A was the main AMP involved in the inhibitory effect of MSC-derived conditioned media against *S. aureus* (Cahuascanco et al. 2019).

In contrast, expression of CATHL2 and hepcidin was undetected in bfBM-MSCs or bfAT-MSCs, contrary to the previous literature that reported expression of both AMPs in MSCs (Alcayaga-Miranda et al. 2015). These results provide evidence of the potential role of AMPs, including bBD4A and NK1, in decreasing bacterial cell proliferation. Defensins constitute a large family of small molecules present in various tissues that can destroy a wide variety of pathogens (Selsted and Ouellette 2005). The lingual antimicrobial peptide (LAP), isolated from the swollen tongue of cattle, was one of the first characterized bovine β-defensin (Schonwetter et al. 1995). β-Defensins are also present in cow milk (Isobe et al. 2009) where they display antibacterial property against *S. aureus* (Taha-Abdelaziz et al. 2013). Moreover, a positive relationship between somatic cell count (SCC) in milk and LAP mRNA expression has been reproted (Swanson et al. 2004). Intramammary administration of lipopolysaccharide (LPS) in cow and injection of heat-killed *S. aureus* in goats increased LAP concentration and SCC in milk (Morimoto et al. 2012; Kuwahara et al. 2017). Overall, these results suggest that *S. aureus* activates the secretion of β-defensins in bovine mammary epithelial cells and fetal-derived BM-MSCs and AT-MSCs in cows.

We previously reported that exposure to *S. aureus*-increased mRNA levels of NK1 in bfAT-MSC but not in bfBM-MSC (Cahuascanco et al. 2019). These results suggest that antibacterial response against *S. aureus* may be different depending on tissue of origin of MSC. NK1 is a cationic AMP produced by cytotoxic T and NK cells that was firstly isolated from the porcine small intestine (Andersson et al. 1995). Four NK-lysin genes have been described in the bovine: NK1, NK2A, NK2B, and NK2C. Higher expression of NK1, NK2A, and NK2B was found in the Peyer's patch and NK2C in the lungs (Chen et al. 2017). The synthetic forms of four NK-lysin peptides had shown antibacterial activity against *S. aureus* (Chen et al. 2017). Genetic variants within the bovine NK-lysin potentially provide two candidate genetic markers associated with health-related phenotypes in cattle (Chen et al. 2017).

Proinflammatory signals including IFNγ and TNFα are required in order to activate IDO expression in human MSCs (Meisel et al. 2011). This study also provided evidence on the antibacterial activity of IDO against *S. aureus*. Conversely, mice MSCs lacking expression of IDO do not inhibit *S. aureus* growth (Meisel et al. 2011). We found that bfMSCs treated with IFNγ induced activation of IDO mRNA expression in a dose–response manner (Huaman et al. 2019). These studies indicate that an inflammatory environment is required for MSCs in order to express IDO and that IDO is not the unique AMP against *S. aureus*. Human and equine MSC also

produce CATHL2 which has also been involved in the antibacterial capacity against *S. aureus* (Sutton et al. 2016). As described above, bovine mammary epithelial cells produce CATHL2 in response to *S. aureus* (Ibeagha-Awemu et al. 2010). Similarly, HAMP activity has also been involved in the inhibition of *S. aureus* (Park et al. 2001), and its expression has been localized in menstrual-derived MSC (Alcayaga-Miranda et al. 2015). We did not detect expression of CATHL2 and HEP in bfMSCs after exposure to *S. aureus*, which may be the consequence of a tissue-specific response and regulation of these peptides. However, these results do not rule out the potential participation of these factors against other pathogens.

Thus, our analyses indicated that bfBM-MSC- and bfAT-MSC-derived conditioned media reduced ~30% of *S. aurues* in vitro growth. Moreover, the antibacterial capacity of conditioned media can be increased by pre-exposure of bfMSCs to *S. aureus* and by increasing concentration through filtration.

13.2 Safety and Efficacy of a Bovine MSCs Intramammary Therapy Against Experimentally Induced *Staphylococcus aureus* Clinical Mastitis

Prevalence of *S. aureus* intramammary infection ranges from 0 to 15% in heifers (Fox 2009). *S. aureus* is a part of the natural skin flora, making its eradication from herds impractical. Adhesion of bacteria to mammary epithelial cells followed by the production of virulence factors including exotoxins, biofilms, bacterial super-antigens, and proteases explains its mechanisms of bacterial virulence (Sutra and Poutrel 1994). In addition, some bacterial strains are capable of suppressing phagocytosis and cellular immunity and produce enzymes to inactivate the majority of penicillin-based treatments (Oliveira et al. 2006). Moreover, *S. aureus* invades and survives inside the epithelial cells and phagocytes, making antimicrobial contact difficult in the entire affected area (Pu et al. 2014). Consequently, most therapies against *S. aureus* achieve cure rates of less than 50%, with reinfections usually due to primary infectious organism rather than to new pathogens (Barkema et al. 2006). Consequently, antibiotic treatment against *S. aureus* is often ineffective and results in chronic udder infections (Pellegrino et al. 2008). Thus, the development of alternative treatment to intramammary infection is important to increase cure rates in lactating dairy cows and reduce antimicrobial resistance.

We hypothesized that intramammary administration of bfAT-MSCs in cows with mastitis would allow the delivery of bioactive factors derived from MSC at sites of the lesion, where they may display immunomodulatory control on the local immune response, antimicrobial activity against mastitis pathogen, and complementary regenerative function of the damaged glandular tissue (Peralta et al. 2020). This regenerative effect may constitute an alternative or supplemental effect to antibiotic therapy, reducing the dose, duration, and recovery period of dairy cows and milk withhold. To the best of our knowledge this was the first study that evaluated the effect of the intramammary administration of MSCs for the treatment of bovine mastitis (Fig. 13.2). Considering the lack of previous studies and the absence of an

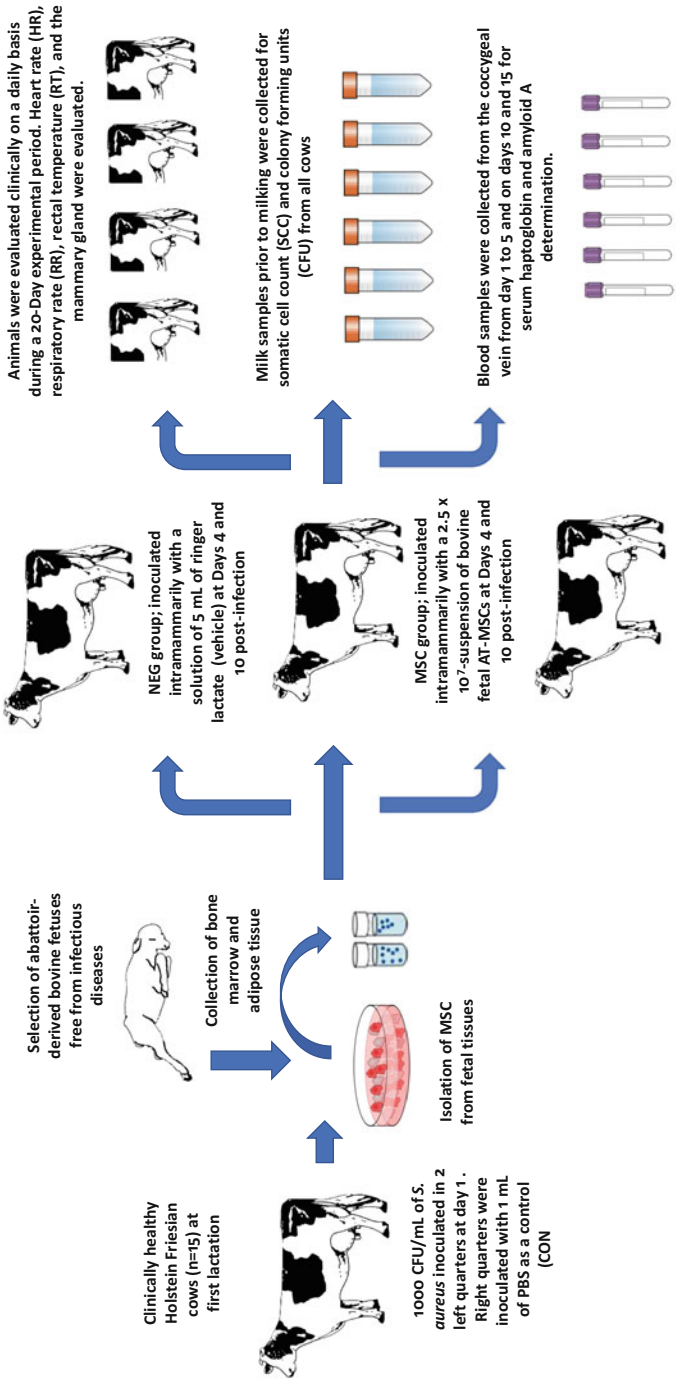

Fig. 13.2 Evaluation of efficacy parameters of an allogenic MSC-based intramammary therapy in dairy cows with experimentally induced *Staphylococcus aureus* clinical mastitis

standarized dosage of MSCs, the present study established a dose of 2.5×10^7 cells for local administration. Previous reports in the large animal models, including intra-articular administration in horses (Ardanaz et al. 2016), intra-uterine inoculation in mares (Mambelli et al. 2014), and intra-myocardial injection in pigs (Kim et al. 2017), suggested that a dose of 2.5×10^7 cells was sufficient to elicit a favorable response to the therapy. After inoculation, MSCs migrate to injured tissue displaying "homing" capacity in response to chemotactic factors like stromal cell-derived factor-1 (SDF-1) and Regulated on Activation, Normal T cell Expressed and Secreted (RANTES) (Eggenhofer et al. 2014).

In a safety trial, we reported that administration of two separate doses of allogenic bfAT-MSCs by 10 days apart did not alter clinical and hematological parameters in healthy cows (Peralta et al. 2020), indicating that allogenic stem cell administration was safe to use. Moreover, gene expression associated with PBL activation (CD4, CD8, CD25, CD62L, and CD69) and proinflammatory cytokines (CCL2, CCL5, IL2, CXCL3, IFNγ, and TNFα) remained unaffected during 20 days, providing another piece of evidence that administration of allogenic MSCs was safe. As it has been previously reported, in other species and diseases, the application of repeated doses of MSCs is important in order to enhance the therapeutic potential of MSCs (van Velthoven et al. 2010). However, administering repeated doses of allogenic MSCs can induce immune memory in recipients (Nauta et al. 2006). Although the anti-donor response has been observed in some clinical trials, no major adverse events have been associated with the infusion of repeated doses of allogenic MSCs, which is considered a common therapeutic strategy in commercial therapies based on MSC (Lalu et al. 2012). Overall, our data indicate that intramammary administration bfAT-MSCs in two doses separated by 10 days did not induce systemic or local clinical effects in the mammary gland or activate the systemic immune response in heifers.

In an efficacy trial, we detected higher SCC in mammary quarters inoculated with *S. aureus* compared to noninfected quarters (Peralta et al. 2020). The presence of flakes in milk with mild swelling of the mammary gland and the absence of systemic signs indicated that experimental inoculation of *S. aureus* caused mild symptoms of clinical mastitis. Treatment with bfAT-MSCs resulted in similar SCC compared to treatment with antibiotics or vehicle among infected quarters. Predominant types of somatic cells in mastitis milk are neutrophils followed by macrophages and lymphocytes, which enter the mammary gland from blood (Raffaghello et al. 2008). MSCs secret several factors like IL-6, IL-10, PGE2, and IDO that induce immunomodulatory control on neutrophils, macrophages, and lymphocytes (Nemeth et al. 2009; Mellor and Munn 2004). As described above, bfAT-MSCs upregulate IDO and IL-6 in response to inflammatory stimulation (Huaman et al. 2019). This previously reported in vitro data suggested that bfAT-MSCs would be able to reduce SCC in quarters with mastitis. However, in vivo results from the efficacy trial indicate that bfAT-MSC intramammary inoculation in quarters infected with *S. aureus* did not affect SCC in milk. This difference in results is likely due a subtle immune reaction induced by S. aureus through activation of several mechanisms that include attenuation of NF-kB in mammary epithelial cells and

TNFa, IL-6 and IL-8 in the mammary gland (Eckersall et al. 2005). This subtle immune response in the mammary gland may have reduced the potential immunomodulatory effect of bfAT-MSCs. Moreover, no significant differences in haptoglobin and amyloid A levels were detected between treatment groups, despite that cows with intramammary infection increased haptoglobin levels (Peralta et al. 2020). High correlations have been reported between haptoglobin levels in milk and serum and SCC in cows with mastitis caused by *S. aureus*, which supports the use of haptoglobin as a biomarker for mastitis detection (Selsted and Ouellette 2005). Higher levels of amyloid A in the mammary tissue compared to the liver may explain decreased sensitivity of the semi-quantitative test in bovine serum and the lack of significant differences in the level of amyloid A between the treatment and the control groups. Overall, these results suggest that MSC-secreted factors were insufficient to control inflammation mediated by migration of leukocytes from blood to milk and haptoglobin produced in the liver or amyloid A secretion in the mammary gland. Alternatively, mammary gland inflammation did not activated bfAT-MSCs and thus failed to induce secretion of immunomodulatory factors by the administered MSCs. Treatment with bfAT-MSCs did not induce an immunomodulatory effect in cows with mastitis caused by *S. aureus*; however, interestingly, in the efficacy trial, we detected that cows with mastitis and treated with bovine fetal bfAT-MSCs had bacterial colony-forming unit (CFU) in milk compared to cows treated with vehicle. In a previous in vitro study, we reported that conditioned media from bfAT-MSCs had an antiproliferative effect on mastitis causing *S. aureus*, antibacterial effect that may be mediated by DEFβ1 and NK Lysin secreted by bfAT-MSCs (Cahuascanco et al. 2019). Moreover, a positive relationship has been described between SCC in milk and DEFβ1 gene expression, which was localized in epithelial cells of cow mastitic tissue (Swanson et al. 2004). Finally, our results indicated that bfAT-MSCs exert an antibacterial effect against *S. aureus* in the mammary gland that may be mediated by β1-defensin or NK lysin or both and reduce CFU in mastitis cow milk.

13.3 Conclusions

After a series of experiments, recent reports indicate that allogenic bfMSCs can produce immunomodulatory, angiogenic, and antibacterial factors that help in controlling a variety of infections, including mastitis caused by *S. aurus*. In subsequent safety trials, intramammary administration of two doses of allogenic bfAT-MSCs did not induce immune rejection in heifers and was safe. Administration of bfAT-MSCs to mammary quarters experimentally infected with *S. aureus* decreased bacterial count in milk. Overall, these results represent the foundation for the potential development of MSC-based therapy for the treatment of bovine mastitis; however, further studies are required in order to elucidate the underlying mechanisms and optimize the beneficial effects.

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Ethics Approval All procedures have been approved by the Bioethical Committees of the University of Chile, FONDEF and FONDECYT.

References

Alcayaga-Miranda F, Cuenca J, Martin A et al (2015) Combination therapy of menstrual derived mesenchymal stem cells and antibiotics ameliorates survival in sepsis. Stem Cell Res Ther 6:199

Andersson M, Gunne H, Agerberth B, Boman A, Bergman T, Sillard R, Jörnvall H, Mutt MV, Olsson B, Wigzell H, Dagerlind A, Boman HG, Gudmundsson GH (1995) NK-lysin, a novel effector peptide of cytotoxic T and NK cells. Structure and cDNA cloning of the porcine form, induction by interleukin 2, antibacterial and antitumour activity. EMBO J 14:1615–1625

Ankrum JA, Ong JF, Karp JM (2014) Mesenchymal stem cells: immune evasive, not immune privileged. Nat Biotechnol 32:252–260

Ardanaz N, Vázquez FJ, Romero A et al (2016) Inflammatory response to the administration of mesenchymal stem cells in an equine experimental model: effect of autologous, and single and repeat doses of pooled allogeneic cells in healthy joints. BMC Vet Res 31:65

Bals R, Wilson JM (2003) Cathelicidins: a family of multifunctional antimicrobial peptides. Cell Mol Life Sci 60:711–720

Barkema HW, Schukken YH, Zadoks RN (2006) Invited review: the role of cow, pathogen, and treatment regimen in the therapeutic success of bovine Staphylococcus aureus mastitis. J Dairy Sci 89:1877–1895

Barrachina L, Remacha AR, Romero A et al (2016) Effect of inflammatory environment on equine bone marrow derived mesenchymal stem cells immunogenicity and immunomodulatory properties. Vet Immunol Immunopathol 171:57–65

Cahuascanco B, Bahamonde J, Huaman O et al (2019) Bovine fetal mesenchymal stem cells exert antiproliferative effect against mastitis causing pathogen Staphylococcus aureus. Vet Res 50:25

Caplan AI, Correa D (2011) The MSC: an injury drugstore. Cell Stem Cell 9:11–15

Carrade Holt DD, Wood JA, Granick JL et al (2014) Equine mesenchymal stem cells inhibit T cell proliferation through different mechanisms depending on tissue source. Stem Cells Dev 23:1258–1265

Chen J, Lee MO, Womack JE (2017) Genetic variation and gene conversions within the bovine NK-lysin gene family. Anim Genet 48:225–227

Consentius C, Reinke P, Volk HD (2015) Immunogenicity of allogeneic mesenchymal stromal cells: what has been seen in vitro and in vivo? Regen Med 10:305–315

Cortes Y, Ojeda M, Araya D et al (2013) Isolation and multilineage differentiation of bone marrow mesenchymal stem cells from abattoir-derived bovine fetuses. BMC Vet Res 9:133

Dominici M, Le Blanc K, Mueller I et al (2006) Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8:315–317

Dueñas F, Becerra V, Cortes Y et al (2014) Hepatogenic and neurogenic differentiation of bovine marrow mesenchymal stem cells from abattoir-derived bovine fetuses. BMC Vet Res 10:154

Eckersall PD, Young FJ, Nolan AM et al (2005) Acute phase proteins in bovine milk in an experimental model of Staphylococcus aureus subclinical mastitis. J Dairy Sci 89:1488–1501

Eggenhofer E, Luk F, Dahlke MH et al (2014) The life and fate of mesenchymal stem cells. Front Immunol 5:148

Elman JS, Li M, Wang F et al (2014) A comparison of adipose and bone marrow-derived mesenchymal stromal cell secreted factors in the treatment of systemic inflammation. J Inflamm 11:1–8

English K, Ryan JM, Tobin L et al (2009) Cell contact, prostaglandin E2 and transforming growth factor beta 1 play nonredundant roles in human mesenchymal stem cell induction of CD4+ CD25 (high) forkhead box P3+ regulatory T cells. Clin Exp Immunol 156:149–160

Farm Animal Welfare Council (2009) Opinion on the welfare of the dairy cow. https://assets.publishing.service.gov.uk/government/uploads/system/uploads/attachmentdata/file/325044/FAWC_opinion_on_dairy_cow_welfare.pdf. Accessed Oct 2020

Fox LK (2009) Prevalence, incidence and risk factors of heifer mastitis. Vet Microbiol 134:82–88

Francois M, Romieu-Mourez R, Li M, Galipeau J (2012) Human MSC suppression correlates with cytokine induction of indoleamine 2,3-dioxygenase and bystander M2 macrophage differentiation. Mol Ther 20:187–195

Gomes F, Henriques M (2016) Control of bovine mastitis: old and recent therapeutic approaches. Curr Microbiol 72:377–382

Harichandan A, Bühring MJ (2011) Prospective isolation of human MSC. Best Pract Res Clin Haematol 24:25–36

Hsiao ST, Asgari A, Lokmic Z et al (2012) Comparative analysis of paracrine factor expression in human adult mesenchymal stem cells derived from bone marrow, adipose, and dermal tissue. Stem Cells Dev 21:2189–2203

Hsu WT, Lin CH, Chiang BL et al (2013) Prostaglandin E2 potentiates mesenchymal stem cell induced IL-10+IFN-γ+CD4+ regulatory T cells to control transplant arteriosclerosis. J Immunol 190:2372–2380

Huaman O, Bahamonde J, Cahuascanco B et al (2019) Immunomodulatory and immunogenic properties of mesenchymal stem cells derived from bovine fetal bone marrow and adipose tissue. Res Vet Sci 124:212–222

Ibeagha-Awemu EM, Ibeagha AE, Messier S et al (2010) Proteomics, genomics, and pathway analyses of Escherichia coli and Staphylococcus aureus infected milk whey reveals molecular pathways and networks involved in mastitis. J Proteome Res 9:4604–4619

Isobe N, Nakamura J, Nakano H et al (2009) Existence of functional lingual antimicrobial peptide in bovine milk. J Dairy Sci 92:2691–2695

Ivanova-Todorova E, Bochev I, Mourdjeva M et al (2009) Adipose tissue-derived mesenchymal stem cells are more potent suppressors of dendritic cells differentiation compared to bone marrow-derived mesenchymal stem cells. Immunol Lett 126:37–42

Jervis M, Huaman O, Cahuascanco B et al (2019) Comparative analysis of in vitro proliferative, migratory and pro-angiogenic potentials of bovine fetal mesenchymal stem cells derived from bone marrow and adipose tissue. Vet Res Commun 43:165–178

Keefe G (2012) Update on control of Staphylococcus aureus and Streptococcus agalactiae for management of mastitis. Vet Clin North Am Food Anim Pract 28:203–216

Kim MC, Kim YS, Kang WS et al (2017) Intramyocardial injection of stem cells in pig myocardial infarction model: the first trial in Korea. J Korean Med Sci 32:1708–1712

Klyushnenkova E, Mosca JD, Zernetkina V et al (2005) T cell responses to allogeneic human mesenchymal stem cells: immunogenicity, tolerance, and suppression. J Biomed Sci 12:47–57

Kościuczuk EM, Lisowski P, Jarczak J, Strzałkowska N, Jóźwik A, Horbańczuk J, Krzyżewski J, Zwierzchowski L, Bagnicka E (2012) Cathelicidins: family of antimicrobial peptides. A review. Mol Biol Rep 39(12):10957–10970

Krampera M, Glennie S, Dyson J et al (2003) Bone marrow mesenchymal stem cells inhibit the response of naive and memory antigen-specific T cells to their cognate peptide. Blood 101:3722–3729

Krasnodembskaya A, Song Y, Fang X et al (2010) Antibacterial effect of human mesenchymal stem cells is mediated in part from secretion of the antimicrobial peptide LL-37. Stem Cells 28:2229–2238

Kuwahara K, Yoshimura Y, Isobe N (2017) Effect of steroid hormones on the innate immune response induced by Staphylococcus aureus in the goat mammary gland. Reprod Domest Anim 52:579–584

Kyurkchiev D, Bochev I, Ivanova-Todorova E et al (2014) Secretion of immunoregulatory cytokines by mesenchymal stem cells. World J Stem Cells 6:552–570

Lalu MM, McIntyre L, Pugliese C et al (2012) Safety of cell therapy with mesenchymal stromal cells (SafeCell): a systematic review and meta-analysis of clinical trials. PLoS One 7:e47559

Le Blanc K, Tammik C, Rosendahl K et al (2003) HLA expression and immunologic properties of differentiated and undifferentiated mesenchymal stem cells. Exp Hematol 31:890–896

Lee B-C, Kim H-S, Shin T-H et al (2016) PGE2 maintains self-renewal of human adult stem cells via EP2-mediated autocrine signaling and its production is regulated by cell-to-cell contact. Sci Rep 6:1–12

Lombardi L, Maisetta G, Batoni G et al (2015) Insights into the antimicrobial properties of hepcidins: advantages and drawbacks as potential therapeutic agents. Molecules 20:6319–6341

Luz-Crawford P, Kurte M, Bravo-Alegría J et al (2013) Mesenchymal stem cells generate a CD4+ CD25+ Foxp3+ regulatory T cell population during the differentiation process of Th1 and Th17 cells. Stem Cell Res Ther 4:65–77

Makovec JA, Ruegg PL (2003) Antimicrobial resistance of bacteria isolated from dairy cow milk samples submitted for bacterial culture: 8905 samples (1994–2001). J Am Vet Med Assoc 222:1582–1589

Mambelli LI, Mattos RC, Winter GH et al (2014) Changes in expression pattern of selected endometrial proteins following mesenchymal stem cells infusion in mares with endometrosis. PLoS One 9:e97889

Marquez-Curtis LA, Janowska-Wieczorek A, McGann LE, Elliott JAW (2015) Mesenchymal stromal cells derived from various tissues: biological, clinical and cryopreservation aspects. Cryobiology 71(2):181–197

Mei SH, Haitsma JJ, Dos Santos CC et al (2010) Mesenchymal stem cells reduce inflammation while enhancing bacterial clearance and improving survival in sepsis. Am J Respir Crit Care Med 182:1047–1057

Meisel R, Brockers S, Heseler K et al (2011) Human but not murine multipotent mesenchymal stromal cells exhibit broad-spectrum antimicrobial effector function mediated by indoleamine 2,3-dioxygenase. Leukemia 25:648–654

Mellor AL, Munn DH (2004) IDO expression by dendritic cells: tolerance and tryptophan catabolism. Nat Rev Immunol 4:762–774

Mezey E, Nemeth K (2015) Mesenchymal stem cells and infectious diseases: smarter than drugs. Immunol Lett 168:208–214

Morimoto K, Kanda N, Shinde S et al (2012) Effect of enterotoxigenic Escherichia coli vaccine on innate immune function of bovine mammary gland infused with lipopolysaccharide. J Dairy Sci 95:5067–5074

Nauta AJ, Westerhuis G, Kruisselbrink AB et al (2006) Donor-derived mesenchymal stem cells are immunogenic in an allogeneic host and stimulate donor graft rejection in a nonmyeloablative setting. Blood 108:2114–2120

Nemeth K, Leelahavanichkul A, Yuen PS et al (2009) Bone marrow stromal cells attenuate sepsis via prostaglandin E2- dependent reprogramming of host macrophages to increase their interleukin-10 production. Nat Med 15:42–49

Oliveira M, Bexiga R, Nunes SF et al (2006) Biofilm-forming ability profiling of Staphylococcus aureus and Staphylococcus epidermidis mastitis isolates. Vet Microbiol 118:133–140

Park CH, Valore EV, Waring AJ et al (2001) Hepcidin, a urinary antimicrobial peptide synthesized in the liver. J Biol Chem 276:7806–7810

Pellegrino M, Giraudo J, Raspanti C et al (2008) Experimental trial in heifers vaccinated with Staphylococcus aureus avirulent mutant against bovine mastitis. Vet Microbiol 127:186–190

Peralta OA, Carrasco C, Vieytes C et al (2020) Safety and efficacy of a mesenchymal stem cell intramammary therapy in dairy cows with experimentally induced Staphylococcus aureus clinical mastitis. Sci Rep 10(1):2843

Pittenger MF, Mackay AM, Beck SC et al (1999) Multilineage potential of adult human mesenchymal stem cells. Science 284:143–147

Pu W, Su Y, Li J et al (2014) High incidence of oxacillin-susceptible mecA-positive Staphylococcus aureus (OS-MRSA) associated with bovine mastitis in China. PLoS One 9:e88134

Raffaghello L, Bianchi G, Bertolotto M et al (2008) Human mesenchymal stem cells inhibit neutrophil apoptosis: a model for neutrophil preservation in the bone marrow niche. Stem Cells 26:151–162

Reyher KK, Dufour S, Barkema HW et al (2011) The National Cohort of Dairy Farms: a data collection platform for mastitis research in Canada. J Dairy Sci 94:1616–1626

Saulnier N, Loriau J, Febre M et al (2016) Canine placenta: a promising potential source of highly proliferative and immunomodulatory mesenchymal stromal cells? Vet Immunol Immunopathol 171:47–55

Schonwetter BS, Stolzenberg ED, Zasloff MA (1995) Epithelial antibiotics induced at sites of inflammation. Science 267:1645–1648

Selsted ME, Ouellette AJ (2005) Mammalian defensins in the antimicrobial immune response. Nat Immunol 6:551–557

Sutra L, Poutrel B (1994) Virulence factors involved in the pathogenesis of bovine intramammary infections due to Staphylococcus aureus. J Med Microbiol 40:79–89

Sutton MT, Fletcher D, Ghosh SK et al (2016) Antimicrobial properties of mesenchymal stem cells: therapeutic potential for cystic fibrosis infection, and treatment. Stem Cells Int 2016:5303048

Swanson K, Gorodetsky S, Good L et al (2004) Expression of a b-defensin mRNA, lingual antimicrobial peptide, in bovine mammary epithelial tissue is induced by mastitis. Infect Immun 72:7311–7314

TacGhee Y, Sun US (2012) Immunomodulatory properties of mesenchymal stem cells and their therapeutic applications. Arch Pharm Res 35:213–221

Taha-Abdelaziz K, Perez-Casal J, Schott C et al (2013) Bactericidal activity of tracheal antimicrobial peptide against respiratory pathogens of cattle. Vet Immunol Immunopathol 152:289–294

van Velthoven CT, Kavelaars A, van Bel F et al (2010) Repeated mesenchymal stem cell treatment after neonatal hypoxia-ischemia has distinct effects on formation and maturation of new neurons and oligodendrocytes leading to restoration of damage, corticospinal motor tract activity, and sensorimotor function. J Neurosci 30:9603–9611

Ward CL, Sanchez CJ Jr, Pollot BE et al (2015) Soluble factors from biofilms of wound pathogens modulate human bone marrow-derived stromal cell differentiation, migration, angiogenesis, and cytokine secretion. BMC Microbiol 15:75

Yuan Y, Lin S, Guo N et al (2014) Marrow mesenchymal stromal cells reduce methicillin-resistant Staphylococcus aureus infection in rat models. Cytotherapy 16:56–63

Therapeutic Applications of Mesenchymal Stem Cells in Canine Diseases

14

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Abstract

The invention of mesenchymal stem cells has opened new prospects in regenerative medicine. The cells can be considered essential subjects for therapeutic application due to their specialised properties. Umpteen studies have been conducted in human medicine and so in veterinary practice. The studies on this cell type in these two fields can act as complementary and supplementary to each other. Mesenchymal stem cell (MSC) features, including the differentiation properties, tend to vary with source. In canine medicine, the application of MSCs is yet to be established as most of the studies being conducted are currently in the experimental settings and/or in the clinical trial stage. MSCs in the dog are evaluated in musculoskeletal and non-musculoskeletal affections with a positive but variable response. This chapter elaborates the currents status of MSCs' application in dog medicine.

Keywords

Dog medicine · Mesenchymal stem cell · Regenerative medicine · Musculoskeletal · Non-musculoskeletal tissues

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14.1 Introduction

Innumerable incapacitating and incurable diseases of animals and humans necessitate the development of innovative and effective therapeutic options. The quest for perfection is never-ending in therapeutics, and ever-growing challenges to the physicians and research scientists with the emergence of new types of ailments. In dog medicine, advancements are being made with each passing day, both in disease diagnostics and therapeutics. There are numerous incurable ailments, and as the standard of care is increasing in human medicine, the pet owners also demand advanced therapeutic protocols. A throwback in the existential cure is being considered to address these challenges. One of the important and currently most sought-after options includes stem cell therapeutics. Various stem cells exist in individuals at different stages of the development and life cycle, including embryonic stem cells and adult stem cells. Among various stem cell types known so far, adult stem cells like mesenchymal stem cells (MSCs) are the most significant in the therapeutic applications (Gugjoo and Amarpal 2018; Gugjoo et al. 2018, 2019a, 2020a, b). These cells were initially recognised by Friedenstein et al. (1970), and their therapeutic applications were recognised by A Caplan, being considered as the 'father of MSCs' (Caplan 1991).

14.2 Why MSCs?

MSCs, unlike other stem cell types, are available in almost all tissues, including the foetal membranes (Gugjoo et al. 2019b). These cells lack or bear the minimal ethical or teratogenic risk. MSCs, like other stem cell types, have the ability to self-renew (although limited), multiply and differentiate into various cell types (plasticity). These cells are hypo-immunogenic/immuno-evasive (elicit the minimal immune response), show immuno-modulatory and/or anti-inflammatory properties and are able to migrate and home into distant diseased/injured tissues (Cardoso et al. 2017; Bçrziòð et al. 2018; Enciso et al. 2018; Gugjoo et al. 2018). The therapeutic role of MSCs is considered to mainly occur through secretome with the little role of differentiation into particular cell lineage (Stewart and Stewart 2011). The hypo-immunogenic and immuno-modulatory potential, besides other paracrine effects, is considered to play an important role in bringing the scar less tissue healing (Gugjoo et al. 2019c).

14.3 Characterisation of MSCs

MSCs available in a minuscule number in tissues can be harvested from various tissues and other numerous cell types as detailed by Gugjoo and co-workers (Gugjoo et al. 2019b). Cell culture expansion is imperative to avail sufficient dose of stem cells for therapeutic impact. Among various issues, mostly bone marrow and adipose tissue have been studied. In the pet dog's case, foetal membranes remain least

available, and the cord blood volume is too small to yield an adequate number of cells (Sultana et al. 2018). In bone marrow and adipose tissue, mononuclear cell fraction or stromal vascular fraction (SVF) is isolated and contains numerous cell types, including MSCs. The culture expansion helps in the proliferation of these specific cell types to the desired level. To characterise and isolate MSCs specifically, the International Society for Cellular Therapy (ISCT) has put forth certain criteria. The cells that are plastic adherent express certain surface markers and differentiate into at least tri-lineages (adipogenic, chondrogenic and osteogenic) are considered as MSCs (Dominici et al. 2006). Dog MSCs from various tissues qualify all the laid-down criteria. However, differences may arise concerning the surface marker expression. These differences may be attributable to the variability in the types of antibody used, cell sources and methods employed for culture (Ranera et al. 2011; Screven et al. 2014).

The number, proliferation and differentiation potential of dog MSCs vary with the tissue type. MSCs' number in adipose tissue may be more, but their differentiation potential towards musculoskeletal tissue lineage may be less as compared to bone marrow stem cells (Kang et al. 2012). Likewise, liver-derived MSCs may show a high tendency towards the liver-specific gene expression as compared to bone marrow MSCs that differentiate better towards the chondrogenic lineage (Malagola et al. 2016). Thus, MSCs' donor tissue source may be an essential consideration while selecting the therapy for healing of the particular tissue (Gugjoo et al. 2018; Zhan et al. 2019). Apart from these differences, breed variation and donor age must also be considered while instituting stem cell therapy (Volk et al. 2012; Bertolo et al. 2015; Lee et al. 2017a).

14.4 Potential Therapeutic Applications

There are numerous in vivo studies that favour MSCs' application in various kinds of ailments in dog medicine. However, all these studies have been conducted as experimental studies or as small clinical trials. Currently, MSCs are not recommended for any kind of ailment in dogs as a definitive treatment. Majority of the studies have demonstrated that MSCs are safe (Pérez-Merino et al. 2015a, b; Villatoro et al. 2015; Bçrzioð et al. 2018), with a few studies reporting minor reactions to self-controllable localised inflammation and pulmonary parenchymal oedema and haemorrhage (Park et al. 2012; Kang and Park 2014).

Despite the availability of such a substantial pro-therapeutic literature on MSCs, these cells fail to achieve consistently desirable results and show wide variability upon in vivo applications. The stemness properties of these cells remain to be comprehended as their markers are supported only by a handful of genes. The details of the molecular basis underlying the stemness properties of these cells are still poorly understood (Zheng 2018). MSCs show wide variability with respect to the tissue source, culture conditions, age and health condition of donor. Apart from these factors, the in vivo applications have been made along with other biomaterials like scaffolds and growth factors or even different cell types that make results difficult to

understand and interpret without ambiguity. Despite such lacunas, MSCs' therapeutic applications are increasingly being conducted with each passing day.

14.5 Potential Applications in Musculoskeletal Tissues

There are numerous musculoskeletal ailments in dogs involving joints, bones, muscles and tendons/ligaments. MSCs in the dog have been evaluated in all these conditions with a variably positive response.

Osteoarthritis remains a complicated problem worldwide, whether in humans or animals like dogs. There is currently no definitive treatment option available to address the issue and give rise to the regeneration of typical hyaline cartilage. Various surgical and conservative options fail to completely address osteoarthritis problems in dogs (Gugjoo et al. 2016, 2017, 2019c, 2020a). Various experimental in vivo models or clinical cases aiming at cartilage regeneration have been studied (Harman et al. 2016; Kazemi et al. 2017; Kriston-Pál et al. 2017; Li et al. 2018; Kim et al. 2019). These studies have favoured MSCs' applications in osteoarthritis, although variably and with limited positive results, mostly in the form of improvement in clinical symptoms. One of the studies could demonstrate healing with hyaline-like tissue (Kriston-Pál et al. 2017). Some of these studies, however, have utilised other biomaterials as well along with MSCs. In case of cartilage damage/osteoarthritis, typical hyaline regeneration must be integrated with the native tissue. In the current scenario, healing with a mixed hyaline to fibrocartilage carries little integration. Besides the profuse inflammatory environment of osteoarthritis, inflammation might make MSCs express more adhesive proteins and fewer migration-related genes. There is also a possibility of decreased secretion of proteoglycans by MSCs in such an environment (Barrachina et al. 2016; Zayed et al. 2016; Reesink et al. 2017). Thus, before attempting any stem cell therapy, it is imperative to use anti-inflammatory options for the effective therapeutic outcome with MSCs.

Osteogenic ailments like fractures or tumours may make the dog's life miserable. The orthopaedic surgeon with good skills relies on the regenerative potential and expects satisfactory bone healing following the 4R's principle of fracture repair. However, in cases of extensive damage or non-union/delayed union, the application of advanced therapeutic options becomes imperative (Gugjoo et al. 2020a). Among various options, tissue engineering utilising stem cells and scaffolds and growth factors has become a norm and is being evaluated globally. In dog regenerative medicine, MSCs in pre-clinical models (Kang et al. 2012; Khojasteh et al. 2014; Itoi et al. 2016; Yoon et al. 2019) and in clinical cases (Lee et al. 2009a; Song et al. 2017) have led to the improved bone healing in the form of increased bone density and uniformity in the regenerated structure. However, MSCs alongside the scaffolds and/or growth factors become mandatory. The scaffolds facilitate the osteoconduction while the cells promote osteo-induction. Additionally, the scaffolds help to retain cells in situ (Gugjoo et al. 2019c).

Athletic and/or heavy-working dog breeds undergo frequent muscle and/or tendon injuries (Lee et al. 2012). The muscle and tendon have to be regenerated with the

original tissue type lost in order to regain the athletic and/or carting function. Surgical repair of ligament needs a significant reduction of inflammation and formation of tissue typical to the ligament structure. Various treatment options currently in vogue fail to generate the distinct tendon/ligament tissues that can perform a normal function. In various experimental muscle and/tendon regeneration models, MSCs have been proven to be useful (Gugjoo et al. 2019c). In the infraspinatus tendon experimental model, MSCs were demonstrated to align among the collagen fibres. Besides, tendon-specific tenomodulin and MMP13 had been demonstrated to express (Omae et al. 2009). In dogs, the most common joint injury is the ligament rupture, especially the rupture of the cranial cruciate ligament. MSCs, along with platelet-rich plasma, have been demonstrated to improve the healing of torn cranial cruciate ligament in 9 out of 13 dogs (Canapp Jr et al. 2016). MSCs had improved semitendinosus and gastrocnemius muscle healing in dogs in clinical studies and prevented their fibrosis. The animals were able to work and exhibited functional gait without any sign of lameness (Brown et al. 2012; Case et al. 2013; Gibson et al. 2017). However, in the study, ultrasonography revealed inaptly arranged gastrocnemius muscle fibres (Case et al. 2013). Other studies had failed to demonstrate any edge of MSCs over the non-steroidal anti-inflammatory drugs (NSAIDs) in tendon healing (Taroni et al. 2017). Thus, further studies are desired to recapitulate the results, weigh MSCs' activity over the additional and/or other biomaterials like another cell type, their concentration, route of application and a number of doses vis-a-vis the type and extent of the injury.

14.6 Potential Applications of MSCs in Non-musculoskeletal Tissues

MSCs have been evaluated for their potential applications in wounds and ulcers. Numerous studies demonstrate MSC's role in improving wound or ulcer healing. Application of MSCs hastens healing and reduces the wound gap. The reduced wound gaps were filled with collagen and angiogenesis. The pro-healing factors like basic fibroblast growth factor (bFGF) and anti-inflammatory factors increased at wound sites (Kim et al. 2013; Madhu et al. 2014). In a clinical study of 24 canines suffering from acute and chronic wounds, MSCs' applications have been demonstrated to improve wound contraction and re-epithelialisation significantly faster as compared to control wounds. Furthermore, histopathological studies had demonstrated reduced inflammation together with the growth of hair follicles by day 7 (Enciso et al. 2020). MSCs' applications have also been evaluated in the modulation of vocal fold scars and laryngotracheal stenosis for better results (Kumai et al. 2010; Iravani et al. 2017). Apart from direct MSCs' application, their microvesicles favour early and improved wound healing (El-Tookhy et al. 2017). MSCs' tendency to secrete pro-healing, anti-inflammatory, and/or immune-modulatory factors, in addition to their ability to differentiate into the particular cell lineage, makes them a potential therapeutic candidate for wound healing (Rodriguez-Menocal et al. 2015; Yang et al. 2018).

The typical ability of MSCs to secrete anti-inflammatory and/or immune-modulatory factors makes them a potential candidate to address the issues associated with the immune- and/or inflammatory conditions. The three commonly observed immune-mediated/inflammatory clinical conditions of dogs are anal fistula, inflammatory bowel disease (IBD), and keratoconjunctivitis sicca (KCS). In cases of anal fistula, MSCs have been demonstrated to resolve the condition, although, after a gap of 6 months, relapse in the condition was reported (Ferrer et al. 2016). In the experimental IBD model, symptoms resolved and the histological findings of intestines indicated improvement (Pérez-Merino et al. 2015a, b). A limited number of clinical studies in dogs have shown significantly improved results in KCS with MSCs. Post single-dose MSCs' application in the lacrimal gland, the clinical condition had improved Schirmer's test and ocular surface morphology. These animals could also be waivered from the immuno-suppressants (Villatoro et al. 2015; Bittencourt et al. 2016).

In atopic dermatitis (AD), two contrasting reports are available. One of the studies fails to demonstrate any therapeutic effect of MSCs (Hall et al. 2010), while the recent one could demonstrate these cells decreased refractory pruritus (evaluated by visual analogue scale) after a week and also improved lesions (CADESI-04 score) after a month. The improvement had remained stable until the last follow-up of 6 months (Villatoro et al. 2018). The latter study is an open label that has a high potential for bias. Thus, randomised, placebo-controlled, double-blinded studies are desired.

In humans, ischaemic diseases like infarcts and cardiomyopathy are common, while in dogs, dilated cardiomyopathy is a common clinical ailment. Dilatation cardiomyopathy is a chronic disease and can be a life-threatening condition, especially for dogs subjected to hard work. There is no definitive treatment available, and the condition is currently being managed by palliative treatment. To find any relevant and effective treatment, MSCs may be tried due to their potential to maintain the normal turnover of tissues. A single study that evaluated AD-MSCs in occult dilated cardiomyopathy had failed to show any improvement in mean survival time. The cells were implanted via retrograde coronary venous route, though they were well tolerated (Pogue et al. 2013). The study could not show any effectiveness, but MSCs' application on such a study cannot be ruled out. MSCs may further be evaluated in multiple doses along with the change in concentration. Genetically modified MSCs may also be useful to provide biological pacing function like in atrioventricular heart block (Lu et al. 2013).

Nervous ailments are a big challenge as almost all the currently available therapeutic modalities fail (Granger et al. 2012). Among various ailments in dogs, a nervous form of canine distemper, along with injuries to the spinal cord and peripheral nerves, is common. Peripheral nerves and spinal cord injury (SCI) in dogs remain one of the commonly encountered conditions of the nervous system. Injuries to the spinal cord in dogs bear a resemblance to the human condition and can provide a proof-of-therapeutic principle for the latter (Sarmento et al. 2014; Gugjoo et al. 2019c). Among other causes, intervertebral disc degeneration and acute nuclear

herniation are important (Fluehmann et al. 2006). The nature/temper of the animal predisposes them more to such injuries.

In a clinical study of canine distemper, MSCs' applications were correlated with an improvement in neurological symptoms (locomotor and myoclonic activities) in four dogs. An improvement in locomotor functions was demonstrated after a year of MSCs' application wherein all the animals were able to move independently, with three able to show functional ambulation (grade I). Furthermore, two dogs showed mild myoclonic severity (Grade III) (Pinheiro et al. 2019). To confirm the therapeutic role of the MSCs in nervous tissue injuries, investigators evaluated various pre-clinical experimental models of SCI (Lee et al. 2011; Park et al. 2011, 2012; William et al. 2011; Ryu et al. 2012). Apart from the spinal cord injuries, peripheral nerve-like sciatic nerve injuries (Ding et al. 2010) and intervertebral disc disease (Hiyama et al. 2008; Lee et al. 2009b) have also been evaluated. Furthermore, clinically MSCs have been utilised in SCI (Granger et al. 2012; Penha et al. 2014; Sarmento et al. 2014; Escalhão et al. 2017; Bhat et al. 2019). In addition to stem cells, the clinical application of their conditioned medium has also been evaluated (Vikartovska et al. 2020). The cell implantation period tends to play an important role in determining the outcome. Early implantation in the inflammatory period and delayed implantation after fibrosis may not be a favourable option for effective nerve healing (Park et al. 2011). Thus, a most suitable temporal window needs to be standardised to institute stem cell therapy.

These models or clinical studies have supported MSCs' applications, although with some reservations. The results have been inconsistent, variable, and overall healing has been sub-complete. To improve results, tissue-engineered neural scaffold [Chitosan/poly(lactic-co-glycolic acid) (PLGA)] along with MSCs has been demonstrated to repair the 50 mm gap of the sciatic nerve. Such a tissue-engineered construct had provided healing comparable to nerve auto-grafting (Ding et al. 2010). Most of these studies have demonstrated improvement in clinical signs like Tarlov, and Basso, Beattie, Bresnahan (BBB), and Olby scores (Penha et al. 2014; Bhat et al. 2019; Vikartovska et al. 2020). Some studies have demonstrated improvement in histological scores with lower fibrosis (Ryu et al. 2009; Lee et al. 2017b). MRI could demonstrate fewer cavitations in cells treated than sham or control (Lee et al. 2009b, 2011).

There are currently reservations in claiming the MSCs' trans-differentiation into the nerve cells (Wu et al. 2003). MSCs' potential role comes through protecting and repairing damaged neurons via paracrine effects (Lim et al. 2017; Long et al. 2018). MSCs' anti-inflammatory (blocking COX-2) action prevents glial scarring (Park et al. 2012) and pro-healing secretome [like neurotrophic growth factor (NGF) and neurotrophin-3 (NT-3)] helps in tissue regeneration. MSCs may enhance the neuronal network by regulating the intracellular pathways of the actin (Park et al. 2011).

14.7 Miscellaneous Studies

MSCs can trans-differentiate into the other cell types, including hepatocyte-like cells, islet cell-like cells and neural cell-like cells (Trindade et al. 2017; Mihevc et al. 2020; Nitta et al. 2020). As such, MSCs have also been evaluated in various other dog ailments like diabetes (Zhu et al. 2011; Gautam et al. 2016), meningoencephalomyelitis of unknown origin (MUO) (Zeira et al. 2015), hepatocutaneous syndrome (Nam et al. 2017), liver fibrosis (Matsuda et al. 2017) and acute kidney injury (Lee et al. 2017c). Although very limited to elucidate any significant outcome, all these studies have been demonstrated to be safer and pro to MSCs' therapeutic applications. Thus, further studies are desired in the area of MSC therapy in canine diseases. Besides, rationalisation for the cell concentration, route and time of implantation and, more importantly, the pathophysiological basis of models and/or clinical studies remains to be investigated.

14.8 Conclusions

MSCs carry an impressive and result-oriented potential to act as a therapeutic option in various dog clinical conditions. MSCs from various sources have been evaluated for their therapeutic effects in musculoskeletal and non-musculoskeletal tissue ailments. Currently, MSCs lack any definitive therapeutic potential in dog clinical medicine. Apart from understanding their basic physiological properties, several issues like cell survival on transplantation, mode of delivery of the cells to the lesion and optimum number and frequency of stem cells for therapy need to be investigated in detail. Furthermore, thorough clinical trials that possess control groups with a sufficient trial size and similar pathophysiology need to be conducted in great numbers to make MSCs' therapeutic applications a clinical reality in dog medicine.

References

Barrachina AR, Remachaa A, Romero A et al (2016) Effect of inflammatory environment on equine bone marrow-derived mesenchymal stem cells immunogenicity and immunomodulatory properties. Vet Immunol Immunopathol 171:57–65

Bçrziòð U, Matise-Van Houtana I, Pçtersone I et al (2018) Characterisation and *in vivo* safety of canine adipose-derived stem cells. Proc Lat Acad Sci 72(3):160–171

Bertolo A, Steffen F, Malonzo-Marty C et al (2015) Canine mesenchymal stem cell potential and the importance of dog breed: implication for cell-based therapies. Cell Transplant 24:1969–1980

Bhat IA, Sivanarayanan TB, Somal A et al (2019) An allogenic therapeutic strategy for canine spinal cord injury using mesenchymal stem cells. J Cell Physiol 234(3):2705–2718

Bittencourt MKW, Barros MA, Martins JFP et al (2016) Allogeneic mesenchymal stem cell transplantation in dogs with keratoconjunctivitis sicca. Cell Med 8:63–77

Brown SG, Harman RJ, Black LL (2012) Adipose-derived stem cell therapy for severe muscle tears in working German shepherds: two case reports. Stem Cell Disc 2(2):41–44

Canapp SO Jr, Leasure CS, Cox C et al (2016) Partial cranial cruciate ligament tears treated with stem cell and platelet-rich plasma combination therapy in 36 dogs: a retrospective study. Front Vet Sci 3:112

Caplan AI (1991) Mesenchymal stem cell. J Orthop Res 9:641–650

Cardoso MT, Pinheiro AO, Vidane AS et al (2017) Characterization of teratogenic potential and gene expression in canine and feline amniotic membrane-derived stem cells. Reprod Domest Anim 52(s2):58–64

Case JB, Palmer R, Valdes-Martinez A et al (2013) Gastrocnemius tendon strain in a dog treated with autologous mesenchymal stem cells and a custom orthosis. Vet Surg 42:355–360

Ding F, Wu J, Yang Y et al (2010) Use of tissue-engineered nerve grafts consisting of a chitosan/poly(lactic-co-glycolic acid)- based scaffold included with bone marrow mesenchymal cells for bridging 50-mm dog sciatic nerve gaps. Tissue Eng Part A 16:3779–3790

Dominici M, Le Blanc K, Mueller I et al (2006) Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 8(4):315–317

El-Tookhy OS, Shamaa AA, Shehab GG et al (2017) Histological evaluation of experimentally induced critical size defect skin wounds using exosomal solution of mesenchymal stem cells derived microvesicles. Int J Stem cells 10(2):144–153

Enciso N, Ostronoff LLK, Mejías G et al (2018) Stem cell factor supports migration in canine mesenchymal stem cells. Vet Res Commun 42(1):29–38

Enciso N, Avedillo L, Fermín ML et al (2020) Cutaneous wound healing: canine allogeneic ASC therapy. Stem Cell Res Ther 11:261

Escalhão CCM, Ramos IP, Hochman-Mendez C et al (2017) Safety of allogeneic canine adipose tissue-derived mesenchymal stem cell intraspinal transplantation in dogs with chronic spinal cord injury. Stem Cells Int 2017:3053759

Ferrer L, Kimbrel EA, Lam A et al (2016) Treatment of perianal fistulas with human embryonic stem cell-derived mesenchymal stem cells: a canine model of human fistulizing Crohn's disease. Regen Med 11(1):33–43

Fluehmann G, Doherr MG, Jaggy A (2006) Canine neurological diseases in a referral hospital population between 1989 and 2000 in Switzerland. J Small Anim Pract 47:582–587

Friedenstein AJ, Chailakhjan RK, Lalykina KS (1970) The development of fibroblast colonies in monolayer cultures of guinea-pig bone marrow and spleen cells. Cell Tissue Kinet 3:393–403

Gautam P, Foale RD, Recino A et al (2016) Promoter optimisation of lentiviral vectors for efficient insulin gene expression in canine mesenchymal stromal cells: potential surrogate beta cells. J Gene Med 18(10):312–321

Gibson MA, Brown SG, Brown NO (2017) Semitendinosus myopathy and treatment with adipose-derived stem cells in working German shepherd police dogs. Can Vet J 58:241–246

Granger N, Blamires H, Franklin RJM et al (2012) Autologous olfactory mucosal cell transplants in clinical spinal cord injury: a randomized double-blinded trial in a canine translational model. Brain 135:3227–3237

Gugjoo MB, Amarpal (2018) Mesenchymal stem cell research in sheep: current status and future prospects. Small Rum Res 169:46–56

Gugjoo MB, Amarpal, Sharma GT et al (2016) Cartilage tissue engineering: role of mesenchymal stem cells along with growth factors and scaffolds. Indian J Med Res 144:339–347

Gugjoo MB, Amarpal, Abdelbaset-Ismail A et al (2017) Mesenchymal stem cells with IGF-1 and TGF-β1 in laminin gel for osteochondral defects in rabbits. Biomed Pharmacother 93:1165–1174

Gugjoo MB, Amarpal, Chandra V et al (2018) Mesenchymal stem cell research in veterinary medicine. Curr Stem Cell Res Ther 13(8):645–657

Gugjoo MB, Amarpal, Makhdoomi DM et al (2019a) Equine mesenchyaml stem cells: properties, sources, characterization and potential therapeutic applications. J Equine Vet Sci 72:16–27

Gugjoo MB, Amarpal, Sharma GT (2019b) Mesenchymal stem cell basic research and applications in dog medicine. J Cell Physiol 234(10):16779–16811

Gugjoo MB, Amarpal, Fazili MR et al (2019c) Animal mesenchymal stem cell research in cartilage regenerative medicine—a review. Vet Q 39(1):95–120

Gugjoo MB, Amarpal, Fazili MR et al (2020a) Goat mesenchymal stem cell basic research and potential applications. Small Rum Res 183:106045

Gugjoo MB, Amarpal, Ismail AA et al (2020b) Allogeneic mesenchymal stem cells and growth factors in gel scaffold repair osteochondral defect in rabbit. Reg Med 15(2):1261–1275

Hall MN, Rosenkrantz WS, Hong JH et al (2010) Evaluation of the potential use of adipose-derived mesenchymal stromal cells in the treatment of canine atopic dermatitis: a pilot study. Vet Ther 11:E1–E14

Harman R, Carlson K, Gaynor J et al (2016) A prospective, randomized, masked, and placebo-controlled efficacy study of intraarticular allogeneic adipose stem cells for the treatment of osteoarthritis in dogs. Front Vet Sci 3:81

Hiyama A, Mochida J, Iwashina T et al (2008) Transplantation of mesenchymal stem cells in a canine disc degeneration model. J Orthop Res 26:589–600

Iravani K, Sobhanmanesh A, Ashraf MJ, Hashemi et al (2017) The healing effect of conditioned media and bone marrow-derived stem cells in laryngotracheal stenosis: a comparison in experimental dog model. World J Plast Surg 6(2):190–197

Itoi T, Harada Y, Irie H et al (2016) Escherichia coli-derived recombinant human bone morphogenetic protein-2 combined with bone marrow-derived mesenchymal stromal cells improves bone regeneration in canine segmental ulnar defects. BMC Vet Res 12:201

Kang MH, Park HM (2014) Evaluation of adverse reactions in dogs following intravenous mesenchymal stem cell transplantation. Acta Vet Scand 56:16

Kang BJ, Ryu HH, Park SS et al (2012) Comparing the osteogenic potential of canine mesenchymal stem cells derived from adipose tissues, bone marrow, umbilical cord blood, and Wharton's jelly for treating bone defects. J Vet Sci 13:299–310

Kazemi D, Asenjan S, Dehdilani K et al (2017) Canine articular cartilage regeneration using mesenchymal stem cells seeded on platelet rich fibrin. Bone Joint Res 6:98–107

Khojasteh A, Ghazaleh Dashti S, Mehdi Dehghan M et al (2014) Theosteoregenerative effects of platelet-derived growth factor BB co-transplanted with mesenchymal stem cells, loaded on freeze-dried mineral bone block: a pilot study in dog mandible. J Biomed Mat Res Part B102 (8):1771–1778

Kim JW, Lee JH, Lyoo YS et al (2013) The effects of topical mesenchymal stem cell transplantation in canine experimental cutaneous wounds. Vet Dermatol 24:242–e53

Kim SE, Pozzi A, Yeh J-c et al (2019) Intra-articular umbilical cord derived mesenchymal stem cell therapy for chronic elbow osteoarthritis in dogs: a double-blinded, placebo-controlled clinical trial. Front Vet Sci 20:1–10

Kriston-Pál É, Czibula Á, Gyuris Z et al (2017) Characterization and therapeutic application of canine adipose mesenchymal stem cells to treat elbow osteoarthritis. Can J Vet Res 81:73–78

Kumai Y, Kobler JB, Park H et al (2010) Modulation of vocal fold scar fibroblasts by adipose-derived stem/stromal cells. Laryngoscope 120:330–337

Lee HB, Chung YS, Heo SY et al (2009a) Augmentation of bone healing of nonunion fracture using stem cell based tissue engineering in a dog: a case report. Vet Med 54(4):198–203

Lee JH, Chang HS, Kang EH et al (2009b) Percutaneous transplantation of human umbilical cord blood-derived multipotent stem cells in a canine model of spinal cord injury. J Neurosurg Spine 11(6):749–757

Lee JH, Chung WH, Kang EH et al (2011) Schwann cell-like remyelination following transplantation of human umbilical cord blood (hUCB)-derived mesenchymal stem cells in dogs with acute spinal cord injury. J Neurol Sci 300:86–96

Lee JC, Mitchell AWM, Healy JC (2012) Imaging of muscle injuries. Br J Radiol 85 (1016):1173–1185

Lee J, Lee KS, Kim C-L et al (2017a) Effect of donor age on the proliferation and multipotency of canine adipose-derived mesenchymal stem cells. J Vet Sci 18(2):141–148

Lee SH, Kim Y, Rhew D et al (2017b) Effect of canine mesenchymal stromal cells overexpressing heme oxygenase-1 in spinal cord injury. J Vet Sci 18(3):377–386

Lee S-J, Ryu M-O, Seo M-S et al (2017c) Mesenchymal stem cells contribute to improvement of renal function in a canine kidney injury model. In Vivo 31(6):1115–1124

Li L, Duan X, Fan Z et al (2018) Mesenchymal stem cells in combination with hyaluronic acid for articular cartilage defects. Sci Rep 8:9900

Lim J-H, Koh S, Thomas R et al (2017) Evaluation of gene expression and DNA copy number profiles of adipose tissue–derived stromal cells and consecutive neurosphere-like cells generated from dogs with naturally occurring spinal cord injury. Am J Vet Res 78:371–380

Long C, Lankford L, Kumar P et al (2018) Isolation and characterisation of canine placenta-derived mesenchymal stromal cells for the treatment of neurological disorders in dogs. Cytometry A93 (1):82–92

Lu W, Yaoming N, Boli R et al (2013) mHCN4 genetically modified canine mesenchymal stem cells provide biological pacemaking function in complete dogs with atrioventricular block. PACE 36:1138–1149

Madhu DN, Ahmad RA, Amarpal et al (2014) Caudal superficial epigastric axial pattern flap and stem cell therapy for the management of large wound on medial aspect of thigh in a dog. Adv Anim Vet Sci 2(3):188–191

Malagola E, Teunissen M, van der Laan LJ et al (2016) Stem characterisation and comparison of canine multipotent stromal cells derived from liver and bone marrow. Stem Cells Dev 25 (2):139–150

Matsuda T, Takami T, Sasaki R et al (2017) A canine liver fibrosis model to develop a therapy for liver cirrhosis using cultured bone marrow–derived cells. Hepatol Commun 1(7):691–703

Mihevc SP, Grgich VK, Kopitar AN et al (2020) Neural differentiation of canine mesenchymal stem cells/multipotent mesenchymal stromal cells. BMC Vet Res 16:282

Nam A, Han S-M, Go D-M et al (2017) Long-term management with adipose tissue-derived mesenchymal stem cells and conventional treatment in a dog with hepatocutaneous syndrome. J Vet Intern Med 31:1514–1519

Nitta S, Kusakari Y, Yamada Y et al (2020) Conversion of mesenchymal stem cells into canine hepatocyte-like cells by Foxa1 and Hnf4a. Regen Ther 14:165–176

Omae H, Zhao C, Sun YL (2009) Multilayer tendon slices seeded with bone marrow stromal cells: a novel composite for tendon engineering. J Orthop Res 27:937–942

Park SS, Byeon YE, Ryu HH et al (2011) Comparison of canine umbilical cord blood derived mesenchymal stem cell transplantation times: involvement of astrogliosis, inflammation, intracellular actin cytoskeletal pathways, and neurotrophin-3. Cell Transplant 20:1867–1880

Park SS, Lee YJ, Lee SH et al (2012) Functional recovery after spinal cord injury in dogs treated with a combination of Matrigel and neural-induced adipose-derived mesenchymal stem cells. Cytotherapy 14:584–597

Penha EM, Meira CS, Guimarães ET et al (2014) Use of autologous mesenchymal stem cells derived from bone marrow for the treatment of naturally injured spinal cord in dogs. Stem Cells Int 2014:437521

Pérez-Merino EM, Uson-Casaus JM, Duque-Carrasco J et al (2015a) Safety and efficacy of allogeneic adipose tissue-derived mesenchymal stem cells for treatment of dogs with inflammatory bowel disease: endoscopic and histological outcomes. Vet J 206:391–397

Pérez-Merino EM, Uson-Casaus JM, Zaragoza-Bayle C et al (2015b) Safety and efficacy of allogeneic adipose tissue-derived mesenchymal stem cells for treatment of dogs with inflammatory bowel disease: clinical and laboratory outcomes. Vet J 206:385–390

Pinheiro LL, de Lima AR, Martins DM et al (2019) Mesenchymal stem cells in dogs with demyelinating leukoencephalitis as an experimental model of multiple sclerosis. Heliyon 5(6): e01857

Pogue B, Estrada AH, Sosa-Samper I et al (2013) Stem-cell therapy for dilated cardiomyopathy: a pilot study evaluating retrograde coronary venous delivery. J Small Anim Pract 54:361–366

Ranera B, Lyahyai J, Romeroa A et al (2011) Immunophenotype and gene expression profiles of cell surface markers of mesenchymal stem cells derived from equine bone marrow and adipose tissue. Vet Immunol Immunopathol 144:147–154

Reesink HL, Sutton RM, Shurer CR et al (2017) Galectin-1 and galectin-3 expression in equine mesenchymal stromal cells (MSCs), synovial fibroblasts and chondrocytes, and the effect of inflammation on MSC motility. Stem Cell Res Ther 8:243

Rodriguez-Menocal L, Shareef S, Salgado M et al (2015) Role of whole bone marrow, whole bone marrow cultured cells, and mesenchymal stem cells in chronic wound healing. Stem Cell Res Ther 6:1–11

Ryu HH, Lim JH, Byeon YE et al (2009) Functional recovery and neural differentiation after transplantation of allogenic adipose-derived stem cells in a canine model of acute spinal cord injury. J Vet Sci 10:273–284

Ryu H-H, Kang B-J, Park S-S et al (2012) Comparison of mesenchymal stem cells derived from fat, bone marrow, Wharton's jelly, and umbilical cord blood for treating spinal cord injuries in dogs. J Vet Med Sci 74(12):1617–1630

Sarmento CAP, Rodrigues MN, Bocabello RZ et al (2014) Pilot study: bone marrow stem cells as a treatment for dogs with chronic spinal cord injury. Regen Med Res 2:9

Screven R, Kenyon E, Myers MJ et al (2014) Immunophenotype and gene expression profile of mesenchymal stem cells derived from canine adipose tissue and bone marrow. Vet Immunol Immunopathol 161(1–2):21–31

Song J, Kim Y, Kweon O-K et al (2017) Use of stem-cell sheets expressing bone morphogenetic protein-7 in the management of a nonunion radial fracture in a Toy Poodle. J Vet Sci 18 (4):555–558

Stewart MC, Stewart AA (2011) Mesenchymal stem cells: characteristics, sources, mechanisms of action. Vet Clin North Am Equine Pract 27:243–261

Sultana T, Lee S, Yoon H-Y et al (2018) Current status of canine umbilical cord blood-derived mesenchymal stem cells in veterinary medicine. Stem Cells Int 2018:8329174

Taroni M, Cabon Q, Fèbre M et al (2017) Evaluation of the effect of a single intra-articular injection of allogeneic neonatal mesenchymal stromal cells compared to oral non-steroidal anti-inflammatory treatment on the postoperative musculoskeletal status and gait of dogs over a 6-month period after tibial plateau leveling osteotomy: a pilot study. Front Vet Sci 4:83

Trindade AB, Therrien J, Garcia JM et al (2017) Mesenchymal-like stem cells in canine ovary show high differentiation potential. Cell Prolif 50(6):e12391

Vikartovska Z, Kuricova M, Farbakova J et al (2020) Stem cell conditioned medium treatment for canine spinal cord injury: pilot feasibility study. Int J Mol Sci 21:5129

Villatoro AJ, Fernández V, Claros S et al (2015) Use of adipose-derived mesenchymal stem cells in keratoconjunctivitis sicca in a canine model. Biomed Res Int 2015:527926

Villatoro AJ, Hermida-Prieto M, Fernández V et al (2018) Allogeneic adipose derived mesenchymal stem cell therapy in dogs with refractory atopic dermatitis: clinical efficacy and safety. Vet Rec 183:1–7

Volk SW, Wang Y, Hankenson KD (2012) Effects of donor characteristics and ex vivo expansion on canine mesenchymal stem cell properties: implications for MSC-based therapies. Cell Transplant 21:2189–2200

William JB, Prabakaran R, Ayyappan S et al (2011) Functional recovery of spinal cord injury following application of intralesional bone marrow mononuclear cells embedded in polymer scaffold–two year follow-up in a canine. J Stem Cell Res Ther 1:110

Wu S, Suzuki Y, Ejiri Y et al (2003) Bone marrow stromal cells enhance differentiation of co-cultured neurosphere cells and promote regeneration of injured spinal cord. J Neurosci Res 72:343–351

Yang H-M, Song W-J, Qiang L et al (2018) Canine mesenchymal stem cells treated with TNF-α and IFN-γ enhance anti-inflammatory effects through the COX-2/PGE2 pathway. Res Vet Sci 119:19–26

Yoon Y, Jung T, Shahid M et al (2019) Frozen-thawed gelatin-induced osteogenic cell sheets of canine adipose-derived mesenchymal stromal cells improved fracture healing in canine model. J Vet Sci 20(6):e63

Zayed MN, Schumacher J, Misk N et al (2016) Effects of pro-inflammatory cytokines on chondrogenesis of equine mesenchymal stromal cells derived from bone marrow or synovial fluid. Vet J 217:26–32

Zeira O, Asiag N, Aralla M, Ghezzi E, Pettinari L, Martinelli L, Zahirpour D, Dumas MP, Lupi D, Scaccia S, Konar M, Cantile C (2015) Adult autologous mesenchymal stem cells for the treatment of suspected non-infectious inflammatory diseases of the canine central nervous system: safety, feasibility and preliminary clinical findings. J Neuroinflammation 12:181

Zhan X-S, El-Ashram S, Luo D-Z et al (2019) A comparative study of biological characteristics and transcriptome profiles of mesenchymal stem cells from different canine tissues. Int J Mol Sci 20:1485

Zheng L (2018) Epigenetic regulation on stem cell fate and regeneration. Curr Stem Cell Res Ther 13(1):3

Zhu S, Lu Y, Zhu J et al (2011) Effects of intrahepatic bone-derived mesenchymal stem cells autotransplantation on the diabetic beagle dogs. J Surg Res 168:213–223

Biomaterials and Scaffolds in Stem Cell Therapy

15

Mukesh Kumar Bharti, Vikash Chandra, and G. Taru Sharma

Abstract

In the recent past, stem cell therapy has considerably revolutionized regenerative therapy; however, still, it is not perfect for treating diseases due to several limitations like transplantation of stem cells alone exhibits low therapeutic efficacy due to poor viability and regenerative activity of transplanted cells. There is a high scope for the improvement of ex vivo stem cell culture and its delivery system. Growth factors and cytokines regulate stem cell proliferation and differentiation; besides this, they also require biophysical cues at their niche. To overcome these limitations, techniques of tissue engineering use scaffolds. Scaffold opens new avenues for producing engineered tissue substitutes and thus by quality organ repair. Biophysical signals from bioscaffolds such as mechanical forces, nanotopography, stiffness of the matrix, and surface features of the biomaterial influence stem cells' fate. Several types of scaffolds are being used derived from natural biomaterials or synthetic materials having their own merits and demerits. Biodegradability and biologically active properties are the major advantages of natural bioscaffolds over synthetic scaffolds. However, the major drawback of natural scaffolds is the risk of carrying cross-contaminated from the sources. Technologies evolved to mold the biomaterials into three-dimensional (3D) scaffolds to simulate tissue architecture to promote cell proliferation and differentiation. Combining stem cell technologies with biomaterial-based scaffolds enhance stem cell viability, differentiation, and therapeutic efficacy.

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15.1 Introduction

Mesenchymal stem cells (MSCs), having the self-renewing ability and differentiation potential into different cell lineages, are the most valuable and versatile cell source for disease treatment and organ transplantation. MSCs play a crucial role in the tissue regeneration and repair process as the primary role of stem cells in a living organism is to maintain and repair the damaged tissue. MSCs, in particular, have a great appeal for veterinary regenerative therapy (Ansari et al. 2013; Bhat et al. 2019) and tissue engineering as they are relatively easy to derive, expand ex vivo rapidly, and show very little spontaneous differentiation during ex vivo expansion (Somal et al. 2016). However, when the MSCs are used alone, some therapeutic defects such as poor immune compatibility, low efficiency of differentiation, and high risk of teratoma formation remain unsolved (Drukker 2008). Thus, a new reliable approach is needed using biomaterials and scaffolds for stem cell therapy.

There is the possibility of using biomaterials as an alternative approach because of their higher biocompatibility. Even though non-biodegradable synthetic polymer-based biomaterials are widely used for superiority regarding anatomical and biomechanical outcomes, they are generally associated with graft-related complications such as infection and fibrosis exposure, and shrinkage (Deprest et al. 2005). Biomaterials provide physical support in weak tissue (Rogo-Gupta 2013). Also, these materials are usually designed to have appropriate biochemical and biophysical properties such as benign molecular compatibility, suitable mechanical strength, and high porosity that mimics the microenvironment of the natural extracellular matrix (Burdick and Vunjak-Novakovic 2009). Biomaterials have an important property to induce fibrosis and a strong foreign body reaction that gives a greater impact on the patient's pain and discomfort. The presence of stem cells on a biomaterial surface can regulate the inflammatory response and able to provide a weaker foreign body reaction when implanted in vivo (Rasmusson 2006). The combination of stem cell therapy with mesh scaffold biomaterials is an important field of therapeutics that may be explored better (Fig. 15.1). Nowadays, stem cell-interacting biomaterials are designed to augment the stem cell-based therapy (Boontheekul et al. 2007). In recent times, different combinations of biomaterials and stem cells have been reported, and the properties and efficiency of the cell–biomaterial interactions such as morphology, cytotoxicity, vitality, and proliferation have been explained (Neuss et al. 2008).

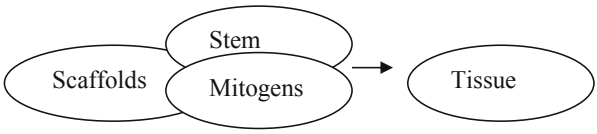

Fig. 15.1 The combination of stem cell with biomaterials scaffold may enhance cellular therapy

In this chapter, several properties of biomaterials and scaffold have been detailed out to find the appropriate biomaterial–stem cell combination for humans and veterinary regenerative therapeutics and the way can use them clinically in stem cell therapy for tissue and organ regeneration.

15.2 Biomaterials and Bioscaffolds

Biomaterials are defined as substances, other than a drug or a combination of substances, and can be used as a whole or as a part of a system for a while to treat, improve, or replace any damaged tissue or organ and restore its function in the body (Ravaglioli and Krajewski 1992). Recently, natural and synthetic biomaterials are considered important elements in regenerative therapy. Several types of scaffolds are being manufactured, but the major hurdle reported for tissue engineering is the lack of desired materials choice for scaffold construction (Fig. 15.2). Bioscaffolds promote cellular proliferation, adhesion, and migration over the surface both in vitro and in vivo. A scaffold must integrate into the host tissue without eliciting an immune response after implantation to avoid tissue rejection or slowed healing (Hutmacher 2000).

Mesenchymal stem cells (MSCs) are naturally present and proliferating in 3D microenvironments, most often referred to as stem cell niche. Ex vivo culture of MSCs over plastic surface lacks that environment, and several culture-related limitations arise. An ideal bioscaffold should be biodegradable, biocompatible, and should have the potential of cell–biomaterial Interaction. The 3D biomaterial scaffolds mimic these microenvironments and have added advantages over traditional 2D culture methods. Several natural and synthetic biomaterial scaffolds have been tried to mimic 3D environments to support stem cell proliferation. Natural biomaterials are often composed of proteins and polysaccharides as extracellular matrix and provide binding sites for cell adhesion and proliferation.

15.2.1 Biomaterials Used in Stem Cell Culture

In regenerative medicine, biomaterials perform as non-viable materials to correct the defective organs and tissues. Recent years have witnessed tremendous growth in biomaterial science and engineering for the development of new products. Biomaterials are generally of two types: natural and synthetic materials. For stem

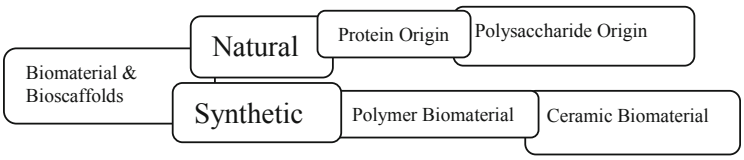

Fig. 15.2 Types of biomaterials and their substances

cell survival, a specific environment is required, and therefore, to imitate the in vivo microenvironment, biomaterials create a new path for the fate of stem cell via cell–matrix interactions. Biomaterial scaffolds possess some qualities that provide cell adhesion sites and maintain the beneficial properties of stem cells. In comparison to the conventional 2D cultures, the novel 3D biomaterial scaffolds provide a more satisfactory microenvironment for stem cells because it provides both chemical and physical signals across the extracellular matrix (Lutolf et al. 2009). For biomaterial-based approaches, understanding the biomaterials' properties is very important because it has been found to affect stem cell lineage specification. Therefore, properties such as surface, morphological, mechanical, electrical, and chemical properties must be accurately considered while designing a scaffold (Martino et al. 2012).

15.2.1.1 Natural Biomaterials in Stem Cell Culture

Natural biomaterials support stem cell differentiation and proliferation. Natural biomaterials, like collagen, hyaluronic acid hydrogels, gelatin, glycosaminoglycans (GAGs), fibrin, Matrigel, alginate, silk, and hydroxyapatite (HA), mimic 3D extracellular matrix and regulate stem cell behavior (Table 15.1). These materials manifest specific benefits like similar mechanical and adhesive properties as the natural extracellular matrix. These materials' main demerits include batch variability, difficulty in purification and quality control, and a short degradation period.

Collagen, presence in all connective tissue, acts as the extracellular matrix component with superior biocompatibility. It is believed that collagen-derived acellular extracellular matrix does not have serious adverse immune responses (Charriere et al. 1989). Biodegradability is the property that makes collagen a better choice in skin tissue restoration on account of its high rate of degradation (Yannas et al. 1982). In one way, collagen is the most abundant protein in animals. In another way, it is difficult to get it for research and clinical treatment purpose that led to the production of recombinant collagen for unlimited supply. Besides, collagen-based biomaterials are now used for cartilage regeneration treating osteochondral defects (Yang et al. 2004) and cornea defects (Freyria et al. 2009).

Hyaluronic acid hydrogels (hyaluronan), which is separated from cartilage, act as a native component and have an essential role in cartilage homeostasis and biomechanical integrity, such as morphogenesis, cellular signaling, proliferation, and wound repair (Dravida et al. 2008). Earlier hyaluronic acid hydrogels have been used in various studies such as tissue repair and regeneration and additionally in adriamycin-induced cytotoxicity prevention by forming a bioartificial stem cell niche (Burdick and Prestwich 2011).

Gelatin, which is basically derived from collagen, is nowadays extensively used as a scaffold material for cartilage tissue engineering because of its biodegradability, biocompatibility, and ability to form hydrogels (Schuurman et al. 2013). Gelatin can be operationalized with unsaturated methacrylamide to form a covalently bound hydrogel for enclosing stem cells. It should be noted that gelatin represents a better performance in biomechanical and biochemical properties as compared to other frequently used hydrogels such as alginate and agarose (Bulcke et al. 2000).

Table 15.1 Different bioscaffold materials with their major properties for tissue engineering

Bioscaffolds	Mode of application	References
Scaffolds from natural biomaterials		
Fibrin	Hydrogels, electrospun scaffolds, and microspheres	Kolehmainen and Willerth (2012), Gorodetsky (2008)
Collagen	3D hydrogels	Noth et al. (2005), Dolatshahi-Pirouz et al. (2014)
Silk	Contains a number of β sheets, slow degradation, electrospun silk scaffolds from functional tissues from stem cells	Yucel et al. (2014), Li et al. (2015)
Laminin	Does not form a 3D system, but laminin-coated surfaces support the maintenance and differentiation of stem cells	Polisetti et al. (2017)
Fibronectin	Provides sites for both cells and proteins to bind, enhances the differentiation of neural stem cells and chondrogenesis	Zollinger and Smith (2017), Tate et al. (2009), Singh and Schwarzbauer (2012)
Vitronectin	Promotes angiogenesis and provides sites for growth factors and other proteins to adhere	Kundu and Putnam (2006), Preissner and Reuning (2011)
Agarose	Undergoes reversible gelation in response to temperature. Agarose hydrogels have been combined with stem cells	Zarrintaj et al. (2018), Huang et al. (2004)
Alginate	Bioink for 3D printing tissues and microencapsulation of stem cells	Axpe and Oyen (2016), Gimi and Nemani (2013)
Hyaluronan	Major components of the extracellular matrix and numerous sites for cell adhesion	Knopf-Marques et al. (2016), Haylock and Nilsson (2016)
Chitosan	The rate of gelation of chitosan scaffolds can be controlled using pH and chemical cross-linking	Chang et al. (2015), Zhang et al. (2016)
Synthetic biomaterials		
Poly (lactic-co-glycolic acid) (PLGA)	Controls drug release. Cells enzymatically degrade PLGA into monomers. PLGA can be formulated into scaffolds, nano- and microparticles, and electrospun fibers	Lee and Pokorski (2018)
Poly (ethylene glycol) PEG	PEG can also be chemically modified to contain bioactive molecules	Salinas et al. (2007)
Poly (caprolactone) (PCL)	Can attain novel topographies and controlled release	Siddiqui et al. (2018)
Polydimethylsiloxane (PDMS)	Can be used to construct microfluidic devices like vasculature network	Regehr et al. (2009)

Matrigel is another soluble basement membrane derived from Engelbreth–Holm–Swarm (EHS) murine sarcoma. It is made up of different components such as laminin, nestin, type IV collagen, heparin sulfate glycoprotein, growth factors, and matrix metalloproteinases. Matrigel has the important property that, at room temperature, it polymerizes to form a biologically active 3D matrix, which in vivo imitates the physical properties, structure, composition, and functions of the cell basement membrane. In this manner, it provides benefits to the culture and differentiation of cells in vitro (Rowland et al. 2010).

One another classic tissue-derived biomaterial scaffold is made up of fibrin, having peculiar properties of providing a microenvironment for stem cells. For example, to produce neurons and oligodendrocytes, nerve growth factor β-NGF was covalently blended with fibrin scaffold (Willerth et al. 2007), but, in this case, plasmin inhibitor needs to be incorporated to prevent unpredicted degradation of the 3D scaffold caused by the embryonic stem cells (Willerth et al. 2006).

15.2.1.2 Synthetic Biomaterials in Stem Cell Culture

Although natural biomaterials have supported the biocompatibility and self-existing biosignals, it has limited applications in a broader sense because of its weak mechanical strength and difficulty in modification. Therefore, to overcome this barrier, synthetic scaffolds have been introduced. As far as the designed component is concerned, the structure and relative mass of a synthetic biomaterial can be controlled at our own will. However, synthetic biomaterials are not perfect for this application because they are deficient in cell adhesion properties and in biological signals, and therefore, it cannot control cell fate on their own. It should be noted that biocompatibility and bioresorbability is the most important hurdle of synthetic biomaterials in stem cell culture, and many studies are going on to rectify these issues.

15.2.1.3 Synthetic Polymers in Stem Cell Culture

Synthetic polymers act as the most common biomaterials in stem cell culture. Commonly used polymers include polylactic acid (PLA), polycaprolactone (PCL), poly lactic-co-glycolic acid (PLGA), polyhydroxyl ethyl methacrylate (PHEMA), polyethylene glycol (PEG), and polyvinyl alcohol (PVA). Lactic acid polymers are very popular since their design and are now widely used in various fields (Tan et al. 2013). PLA and PLGA display superiority in biocompatibility, biodegradability, bioresorbability, low immunogenicity, and low toxicity over other synthetic polymers, making them favorable materials as 3D scaffolds applications in dentistry, plastic surgery, and many others (Tyler et al. 2016). To improve the thermal resistance and mechanical properties of engineered tissues, polycaprolactone (PCL) was mixed with polylactic acid (PLA) (Jeong et al. 2018). Polyethylene glycol (PEG) is highly accepted for human mesenchymal stem cells' osteogenic differentiation. PEG gels supply ample interspace for nutrient and waste diffusion between stem cells and the extracellular matrix (Almany and Seliktar 2005). Additionally, for cartilage restoration, glucosamine-modified PEG hydrogel increased

biocompatibility while preventing fibrosis and hypertrophy of cartilage (Yao et al. 2017).

Recently, more emphasis is being given to decellularized extracellular matrix (dECM) obtained from different tissues as they have an added advantage of natural bioactive properties of tissue to produce a suitable scaffold for stem cell culture and differentiation. Even whole decellularized organs, such as kidneys and livers, can be repopulated into functional tissues after seeding with stem cells (Khan et al. 2014). Such dECM materials can also be bioprinted, providing another avenue for tissue engineering (Choudhury et al. 2018). However, further investigation is required for clinical applications of stem cell-based tissue engineering and whole organ transplantation.

15.2.1.4 Mesh Scaffolds in Stem Cell Culture

Different types of mesh scaffolds were introduced for the treatment of different types of diseases related to stem cell therapy. Polypropylene (PP) is one of them; it is made up of a hydrophobic polymer of carbon atoms with alternating methyl moieties. This material has various important properties like it is flexible, strong, easily cut, readily integrated by surrounding tissues, and having the property of resists infection (Cozad et al. 2010). The monofilament nature of this material provides large pores that facilitate fibrovascular ingrowth, resistance to infection, and provide improved compliance (Procter et al. 2009). Polytetrafluoroethylene (PTFE) is another scaffold that is a chemically inert synthetic fluoropolymer having a high negative charge. This material has poor tissue incorporation property and is not frequently used in clinical cases (Grevious et al. 2006). Polytetrafluoroethylene is a microporous structure that allows the passage of bacteria but prevents the passage of macrophages; therefore, it will be difficult for the body to clear the infection (Binnebosel et al. 2011). Carbon polymer of terephthalic acid is polyethylene biomaterials, and it is a strong fiber suitable to be fabricated into a prosthetic mesh. This polyethylene biomaterial is hydrophilic in nature and can be degraded by hydrolysis (Brown and Finch 2010).

An important property of a synthetic mesh scaffold is that it should be biocompatible, resistant to infection, strong, minimal bioreactivity, and non-immunogenic. For implanted meshes, degradation and incorporation are the two pathways that exist. The primary goal is tissue incorporation, and it depends upon the types of material, its density, 3D construction, pore size, its filament type, compliance, and electric charge (Saberski et al. 2011). In order to allow infiltration by macrophages, blood vessels, fibroblasts, and collagen, pores of the biomesh must be more than 75 mm. The problem with large pore size mesh is that larger pores allow increased soft tissue ingrowth, and because of the prevention of granuloma bridging, it becomes more flexible. The formation of granulomas generally takes place around individual mesh fibers because of the foreign body reaction. Bridging means the processes through which individual granulomas mingle with each other and encapsulate the entire mesh (Amid 1997). The mesh's weight is very important, as the weight depends on the amount of material used in mesh-making process and the polymer's weight (Klinge et al. 2002). Lightweight meshes are made up of thinner

filaments and larger pores, whereas heavyweight meshes are made up of thick polymers, have high tensile strength and have small pore sizes. The meshes of this category are more elastic, contain less material, and initiate a less pronounced foreign body reaction. Hence, a less inflammatory response occurs, resulting in less patient pain and discomfort, better tissue incorporation, and increased compliance (Klinge et al. 1998). A study conducted by Bellon et al., in 2009 stated that there was no significant difference between heavyweight and lightweight polypropylene mesh in terms of the inflammatory response, tissue incorporation, or tensile strength.

15.3 Composite Mesh, Absorbable Synthetic Mesh, and Biological Graft

Another category of mesh material is called composite mesh, which is of two types, that is, permanent and absorbable. In absorbable composite mesh before usage, barrier coatings are needed that require hydration, and they are not agreeable to modification, so they cannot be cut. Nevertheless, they permit for neo-epithelialization, which alleviates tissue-mesh-related complications, and can help in tissue ingrowths (Harrell et al. 2006). The collagen film biomaterials are made up of polyethylene glycol, glycerol, and porcine collagen. This type of material has important properties to produce better cellular proliferation as compared to polypropylene mesh in vitro, and it works with the body's natural systems to impart fast fibrous ingrowth, strong tissue integration, and minimal shrinkage (Rodriguez et al. 2011). Permanent composite mesh possesses qualities of both macro- and microporous meshes to promote tissue ingrowths. These meshes are made in such a manner that it can be modified and can be easily cut to fit specific applications (Gonzalez et al. 2004). Absorbable synthetic meshes are also available, which impart a network for new collagen formation and become absorbed, so they are suitable for wound healing. Biological grafts are other biomaterials that are acellular collagen matrices to promote native tissue fusion. This type of material provides the extracellular components that are necessary for complete healing, to promote the reconstruction of new and healthy tissue, and also to restore the mechanical and functional integrity of the original tissue (Hodde and Hiles 2007).

15.4 Delivery of Stem Cells Through Biomaterials

Cell delivery through biomesh is very important in clinical stem cell therapy. Studies have shown that the clinical effectiveness of stem cell therapy depends mostly on the number of cells delivered to the wound site (Falanga et al. 2007). In most studies, researchers utilized scientifically the simple method of injecting a cell suspension intradermally into or around the wound site. Although this method has shown increased wound healing, however, stem cells' real therapeutic potential seems to be limited due to low engraftment efficiency and cell retention at the wound site

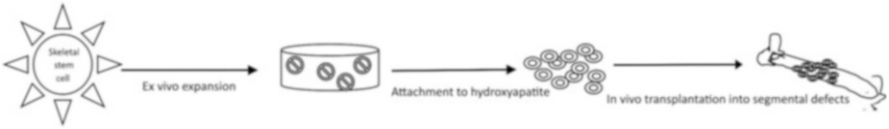

Fig. 15.3 Hydroxyapatite can be used as a scaffold for the stem cell attachment during repairing segmental defects

(Freyman et al. 2006). This phenomenon's exact reason is still not clear, and it is under investigation with some evidence suggesting that the aggressive wound environment may hamper high stem cell engraftment in case of acute wounds. The higher amount of reactive oxygen species than those found in ischemic wounds is believed to slow down the cell engraftment in tissue (Angelos et al. 2006). Moreover, the tear-off forces generated by the injection process itself may lead to the form of programmed cell death that occurs in anchorage-dependent cells when they detach from the surrounding extracellular matrix (Rustad et al. 2012). Therefore, some alternative delivery systems are being explored to intensify stem cell function within nonhealing wounds.

A fibrin spray system was utilized by Falanga et al. (2007) to topically deliver the autologous mesenchymal stem cells to nonhealing lower extremity wounds in human patients. It was found that stem cells survived within the fibrin layer and migrated into the wound tissue. Yoshikawa et al. developed a composite graft based on the idea of bestowing a scaffold and external slot from the ischemic tissue, combining a commercially available collagen matrix with cultured autologous mesenchymal stem cells. These grafts were applied to patients with unmanageable dermatopathies due to thermal burns, decubitus ulcers, and traumatic wounds. After clinical trial, it was found that in about 60% of wounds, regeneration of fibrous and fat tissue takes place, and due to re-epithelialization, complete wound closure takes place. Moreover, with composite graft treatment, sufficient granulation tissue and dermal regeneration took place to allow for successful skin grafting in about 30% of wounds (Yoshikawa et al. 2008).

Two most important and common routes for stem cell delivery at the wound site are systemic or topical approaches through biomaterials (Hamou et al. 2009; Bharti et al. 2020) (Fig. 15.3). Systemic delivery of mesenchymal stem cells imitates the route of endogenous mesenchymal stem cells via the circulatory system that results in a dilution effect on target cells. During its transit through the vascular route, some organs such as the lungs, spleen, and liver may take the mesenchymal stem cells out of circulation for their own use (Karp and Teo 2009). This may result in either delay in their transit or fewer numbers of cells that finally reach the target sites. Once stem cells reach their target sites, they must come out of the vasculature to enter the connective tissue stromal region for the occurrence of their principal functions (Karp and Teo 2009). The alternative method for delivering mesenchymal stem cells to the wound site is through direct or topical delivery with the help of biomaterials (Falanga et al. 2007). The topical delivery method is different from systemic delivery. Applied stem cells either migrate into the wound bed via nonvascular

routes or release bioactive substances at the wound's surface (Hanson et al. 2010). In topical delivery through biomaterials, many stem cells must be provided near the wound area for successful therapy. In this method, therapeutic efficacy also increases (Cleland et al. 2008) compared to delivery of cells without biomaterials because the delivery of cells through a 3D matrix can provide better integration with host tissue and promote better healing (Salinas and Anseth 2009).

15.5 Clinical Applications of Biomaterials and Scaffolds

Nowadays, biomaterials play an essential role in regenerative medicine and tissue engineering. These biomaterials have a variety of applications in different diseases. Due to their excellent biocompatibility and cell-adhesive properties, natural biomaterials, such as collagen, alginate, gelatin, chitin, elastin, and hyaluronic acid, are widely used scaffold matrices for nerve tissue repair (Antman-Passig et al. 2017). Many studies aim to identify reliable stem cell–materials grafts for cardiac repair. However, due to a lack of mechanistic understanding of cardiac regeneration, clinic study slows down (Ruvinov et al. 2011). Moreover, semipermeable porous scaffolds provide better seeding and a protective microenvironment for pancreatic tissues (Scharp and Marchetti 2014), incorporating pancreatic progenitor cells into biomaterials for grafting without immune suppression becomes an ideal method in future diabetes treatment. In hematopoietic therapy, specific biomaterials can be modulated mechanically, chemically, or biologically to meet the specific criteria (Mahadik et al. 2015). The biological scaffold also plays a significant role in osteo-differentiation in tissue engineering, where pore morphology and structure are the most crucial characteristics of a scaffold (Motamedian et al. 2015). In one of the studies, it has been seen that polypropylene mesh impregnated with mesenchymal stem cells modulates the macrophage activation, thus reducing the foreign body reaction against synthetic scaffolds (Swartzlander et al. 2015). It has also been demonstrated that mesenchymal stem cells-impregnated meshes exerted a regulatory effect on T cell proliferation and IFN-ϒ production (Blazquez et al. 2015). Mesh scaffold augmented with mesenchymal stem cells provides an anti-inflammatory environment by M2 macrophage polarization (Blazquez et al. 2015) and exerts a regulatory effect on T cell proliferation and IFN-ϒ production (Gao et al. 2014). The application of biomaterials combined with mesenchymal stem cells is a desirable therapy clinically, particularly for their low immunogenicity and anti-inflammatory properties (Knaan-Shanzer 2014).

15.6 Conclusions

In the recent past, MSCs have been proved to be the most promising tools for cellular therapy due to their capacity to differentiate and self-renewal ability. In veterinary regenerative medicine, MSCs are being used clinically to treat complicated ailments, especially paraplegia/paralysis, diabetic wounds, chronic wounds, ligament, and

tendon repair. Nevertheless, in clinical trials, due to stem cells' immune sensitivity, their broad application becomes limited. Nevertheless, these barriers can be overcome by the use of biomaterials in conjunction with stem cells. A variety of biomaterials and scaffolds are being used to mimic the in vivo microenvironments. Natural biomaterials' biocompatibility is very high, whereas synthetic biomaterials can be sensibly designed for a particular purpose. The amalgamation of stem cells into the biomaterials enhances the competence of restoring and repairing defective tissues. Multidimensional properties of a biomaterial and a scaffold impart a protective and inducible microenvironment for the stem cells and mimic the natural extracellular matrix. This stem cell–biomaterial system is a great home to various tissue engineering applications, including nerve, heart, pancreases, lung, and bone tissues.

References

Almany L, Seliktar D (2005) Biosynthetic hydrogel scaffolds made from fibrinogen and polyethylene glycol for 3D cell cultures. Biomaterials 26(15):2467–2477

Amid PK (1997) Classification of biomaterials and their related complications in abdominal wall hernia surgery. Hernia 1:15–21

Angelos MG, Kutala VK, Torres CA, He G, Stoner JD, Mohammad M, Kuppusamy P (2006) Hypoxic reperfusion of the ischemic heart oxygen radical generation. Am J Physiol-Heart Circul Physiol 290:H341–H347

Ansari MM, Sreekumar TR, Chandra V, Dubey PK, Sai Kumar G, Amarpal, Sharma GT (2013) Therapeutic potential of canine bone marrow-derived mesenchymal stem cells and its conditioned media in diabetic rat wound healing. J Stem Cell Res Ther 3:141. https://doi.org/10.4172/2157-7633.1000141

Antman-Passig M, Levy S, Gartenberg C, Schori H, Shefi O (2017) Mechanically oriented 3D collagen hydrogel for directing neurite growth. Tissue Eng A 23(9–10):403–414

Axpe E, Oyen ML (2016) Applications of alginate-based bioinks in 3D bioprinting. Int J Mol Sci 17 (12):1976

Bellon JM, Rodriguez M, Garcia-Honduvilla N, Gomez-Gil V, Pascual G, Bujan J (2009) Comparing the behavior of different polypropylene meshes (heavy and lightweight) in an experimental model of ventral hernia repair. J Biomed Mater Res B Appl Biomater 89:448e55

Bharti MK, Bhat IA, Pandey S et al (2020) Effect of cryopreservation on therapeutic potential of canine bone marrow derived mesenchymal stem cells augmented mesh scaffold for wound healing in guinea pig. Biomed Pharmacother 121:109573. https://doi.org/10.1016/j.biopha.2019.109573

Bhat IA, Shivkumar TB, Somal A, Sriti P et al (2019) An allogenic therapeutic strategy for canine spinal cord injury using mesenchymal stem cells. J Cell Physiol 234:2705–2718

Binnebosel M, von Trotha KT, Jansen PL, Conze J, Neumann UP, Junge K (2011) Biocompatibility of prosthetic meshes in abdominal surgery. Semin Immunopathol 3:235e43

Blazquez R, Miguel F, Margallo S, Alvarez V, Uson A, Casado G (2015) Surgical meshes coated with mesenchymal stem cells provide an anti-inflammatory environment by a M2 macrophage polarization. Acta Biomater 31:1742–1761

Boontheekul T, Hill EE, Kong HJ, Mooney DJ (2007) Regulating myoblast phenotype through controlled gel stiffness and degradation. Tissue Eng 13(7):1431–1442

Brown CN, Finch JG (2010) Which mesh for hernia repair? Ann R Coll Surg Eng 92:272–278

Bulcke AIVD, Bogdanov B, De Rooze N, Schacht EH, Cornelissen M, Berghmans H (2000) Structural and rheological properties of methacrylamide modified gelatin hydrogels. Macromolecules 1(1):31–38

Burdick JA, Prestwich GD (2011) Hyaluronic acid hydrogels for biomedical applications. Adv Mater 23(12):H41–H56

Burdick JA, Vunjak-Novakovic G (2009) Engineered microenvironments for controlled stem cell differentiation. Tissue Eng 15(2):205–219

Chang SH, Lin HTV, Wu GJ, Tsai GJ (2015) pH Effects on solubility, zeta potential, and correlation between antibacterial activity and molecular weight of chitosan. Carbohydr Polym 134:74–81

Charriere G, Bejot M, Schnitzler L, Ville G, Hartmann DJ (1989) Reactions to a bovine collagen implant. Clinical and immunologic study in 705 patients. J Am Acad Dermatol 21 (6):1203–1208

Choudhury D, Tun HW, Wang T, Naing MW (2018) Organ-derived decellularized extracellular matrix: a game changer for bioink manufacturing? Trends Biotechnol 36(8):787–805

Cleland JG, Coletta AP, Abdellah AT, Cullington D, Clark AL, Rigby AS (2008) Clinical trials update from the American Heart Association 2007: CORONA, RethinQ, MASCOT, AF-CHF, HART, MASTER, POISE and stem cell therapy. Eur J Heart Fail 10(1):102–108

Cozad MJ, Grant DA, Bachman SL, Grant DN, Ramshaw BJ, Grant SA (2010) Materials characterization of explanted polypropylene, polyethylene terephthalate, and expanded polytetrafluoroethylene composites: spectral and thermal analysis. J Biomed Mater Res B Appl Biomater 94:455e62

Deprest J, Claerhout F, Zheng F, Kostantinovic M, Spelzini F, Guelinckx I (2005) Synthetic and biodegradable prostheses in pelvic floor surgery. Int Congr Ser 1279:387–397

Dolatshahi-Pirouz A, Nikkhah M, Gaharwar AK et al (2014) A combinatorial cell-laden gel microarray for inducing osteogenic differentiation of human mesenchymal stem cells. Sci Rep 4:3896

Dravida S, Gaddipati S, Griffith M, Merrett K, Madhira SL, Sangwan VS, Vemuganti GK (2008) A biomimetic scaffold for culturing limbal stem cells: a promising alternative for clinical transplantation. J Tissue Eng Regen Med 2(5):263–271

Drukker M (2008) Recent advancements towards the derivation of immune-compatible patient-specific human embryonic stem cell lines. Semin Immunol 20(2):123–129

Falanga V, Iwamoto S, Chartier M et al (2007) Autologous bone marrow-derived cultured mesenchymal stem cells delivered in a fibrin spray accelerate healing in murine and human cutaneous wounds. Tissue Eng 13:1299–1312

Freyman T, Polin G, Osman H et al (2006) A quantitative, randomized study evaluating three methods of mesenchymal stem cell delivery following myocardial infarction. Eur Heart J 27:1114–1122

Freyria AM, Ronziere MC, Cortial D, Galois L, Hartmann D, Herbage D, Mallein-Gerin F (2009) Comparative phenotypic analysis of articular chondrocytes cultured within type I or type II collagen scaffolds. Tissue Eng 15(6):1233–1245

Gao S, Mao F, Zhang B et al (2014) Mouse bone marrow-derived mesenchymal stem cells induce macrophage M2 polarization through the nuclear factor-jB and signal transducer and activator of transcription 3 pathways. Exp Biol Med (Maywood) 239:366–375

Gimi B, Nemani KV (2013) Advances in alginate gel microencapsulation of therapeutic cells. Crit Rev Biomed Eng 41(6):469–481

Gonzalez R, Rodeheaver GT, Moody DL, Foresman PA, Ramshaw BJ (2004) Resistance to adhesion formation: a comparative study of treated and untreated mesh products placed in the abdominal cavity. Hernia 8:213e9

Gorodetsky R (2008) The use of fibrin based matrices and fibrin microbeads. FMB; for cell based tissue regeneration. Expert Opin Biol Ther 8:1831–1846

Grevious MA, Cohen M, Jean-Pierre F, Herrmann GE (2006) Structural and functional anatomy of the abdominal wall. Clin Plast Surg 33:169e79

Hamou C, Callaghan MJ, Thangarajah H et al (2009) Mesenchymal stem cells can participate in ischemic neovascularization. Plast Reconstr Surg 123:45–55

Hanson SE, Bentz ML, Hematti P (2010) Mesenchymal stem cell therapy for non healing cutaneous wounds. Plast Reconstr Surg 125:510–516

Harrell AG, Novitsky YW, Cristiano JA et al (2006) Prospective evaluation of adhesion formation and shrinkage of intra-abdominal prosthetics in a rabbit model. Am Surg 72(9):808–813. discussion 813–814

Haylock DN, Nilsson SK (2016) The role of hyaluronic acid in hemopoietic stem cell biology. Regen Med 1(4):437–445

Hodde J, Hiles M (2007) Constructive soft tissue remodeling with a biologic extracellular matrix graft: overview and review of the clinical literature. Acta Chir Belg 107:641–647

Huang CY, Reuben PM, D'Ippolito G, Schiller PC, Cheung HS (2004) Chondrogenesis of human bone marrow-derived mesenchymal stem cells in agarose culture. Anat Rec A Discov Mol Cell Evol Biol 278:428–436

Hutmacher DW (2000) Scaffolds in tissue engineering bone and cartilage. Biomaterials 22 (24):175–189

Jeong H, Rho J, Shin JY, Lee DY, Hwang T, Kim KJ (2018) Mechanical properties and cytotoxicity of PLA/PCL films. Biomed Eng Lett 8(3):267–272

Karp JM, Teo GSL (2009) Mesenchymal stem cell homing: the devil is in the details. Cell Stem Cell 4:206–216

Khan AA, Vishwakarma SK, Bardia A, Venkateshwarulu J (2014) Repopulation of decellularized whole organ scaffold using stem cells: an emerging technology for the development of neo-organ. J Artif Organs 17:291–300

Klinge U, Klosterhalfen B, Conze J et al (1998) Shrinking of polypropylene mesh in vivo: an experimental study in dogs. Eur J Surg 164:965e9

Klinge U, Klosterhalfen B, Birkenhauer V, Junge K, Conze J, Schumpelick V (2002) Impact of polymer pore size on the interface scar formation in a rat model. J Surg Res 103:208e14

Knaan-Shanzer S (2014) Concise review: the immune status of mesenchymal stem cells and its relevance for therapeutic application. Stem Cells 32:603–608

Knopf-Marques H, Pravda M, Wolfova L, Velebny V, Schaaf P, Vrana NE, Lavalle P (2016) Hyaluronic acid and its derivatives in coating and delivery systems: applications in tissue engineering, regenerative medicine and immunomodulation. Adv Healthcare Mater 5:2841–2855

Kolehmainen K, Willerth SM (2012) Preparation of 3D fibrin scaffolds for stem cell culture applications. J Vis Exp (61):3641

Kundu AK, Putnam AJ (2006) Vitronectin and collagen I differentially regulate osteogenesis in mesenchymal stem cells. Biochem Biophys Res Commun 347:347–357

Lee PW, Pokorski JK (2018) Poly (lactic-co-glycolic acid) devices: production and applications for sustained protein delivery. Wiley Interdiscip Rev Nanomed Nanobiotechnol 10(5):e1516

Li H, Wen F, Chen H, Pal M, Lai Y, Zhao AZ, Tan LP (2015) Micropatterning extracellular matrix proteins on electrospun fibrous substrate promote human mesenchymal stem cell differentiation toward neurogenic lineage. ACS Appl Mater Interfaces 8:563–573

Lutolf MP, Gilbert PM, Blau HM (2009) Designing materials to direct stem-cell fate. Nature 462 (7272):433–441

Mahadik BP, PedronHaba S, Skertich LJ, Harley BAC (2015) The use of covalently immobilized stem cell factor to selectively affect hematopoietic stem cell activity within a gelatin hydrogel. Biomaterials 67:297–307

Martino S, D'Angelo F, Armentano I, Kenny JM, Orlacchio A (2012) Stem cell-biomaterial interactions for regenerative medicine. Biotechnol Adv 30(1):338–351

Motamedian SR, Hosseinpour S, Ahsaie MG, Khojasteh (2015) A smart scaffolds in bone tissue engineering: a systematic review of literature. World J Stem Cells 7(3):657–668

Neuss S, Apel C, Buttler P et al (2008) Assessment of stem cell/biomaterial combinations for stem cell-based tissue engineering. Biomaterials 29(3):302–313

Noth U, Schupp K, Heymer A et al (2005) Anterior cruciate ligament constructs fabricated from human mesenchymal stem cells in a collagen type I hydrogel. Cytotherapy 7:447–455

Polisetti N, Sorokin L, Okumura N, Koizumi N, Kinoshita S, Kruse FE, Schlotzer-Schrehardt U (2017) Laminin-511 and-521-based matrices for efficient ex vivo-expansion of human limbal epithelial progenitor cells. Sci Rep 7:5152

Preissner KT, Reuning U (2011) Seminars in thrombosis and hemostasis. Thieme Medical Publishers, pp 408–424

Procter L, Falco EE, Fisher JP, Roth JS (2009) Abdominal wall hernias and biomaterials. In: Gefen A (ed) Bioengineering research of chronic wounds, 1st edn. Springer, Berlin, pp 425–447

Rasmusson I (2006) Immune modulation by mesenchymal stem cells. Exp Cell Res 312:2169–2179

Ravaglioli A, Krajewski A (1992) Bioceramics. Chapman & Hall, London. ISBN 978-94-010-5032-6

Regehr KJ, Domenech M, Koepsel JT et al (2009) Biological implications of polydimethylsiloxane-based microfluidic cell culture. Lab Chip 9:2132–2139

Rodriguez M, Pascual G, Sotomayor S, Perez-Kohler B, Cifuentes A, Bellon JM (2011) Chemical adhesion barriers: do they affect the intraperitoneal behavior of a composite mesh? J Investig Surg 24:115e22

Rogo-Gupta L (2013) Current trends in surgical repair of pelvic organ prolapsed. Curr Opin Obstet Gynecol 25:395–398

Rowland TJ, Miller LM, Blaschke AJ et al (2010) Roles of integrins in human induced pluripotent stem cell growth on Matrigel and vitronectin. Stem Cells Dev 19(8):1231–1240

Rustad KC, Wong VW, Sorkin M et al (2012) Enhancement of mesenchymal stem cell angiogenic capacity and stemness by a biomimetic hydrogel scaffold. Biomaterials 33:80–90

Ruvinov E, Leor J, Cohen S (2011) The promotion of myocardial repair by the sequential delivery of IGF-1 and HGF from an injectable alginate biomaterial in a model of acute myocardial infarction. Biomaterials 32(2):565–578

Saberski ER, Orenstein SB, Novitsky YW (2011) Anisotropic evaluation of synthetic surgical meshes. Hernia 15:47e52

Salinas CN, Anseth KS (2009) Mesenchymal stem cells for craniofacial tissue regeneration: designing hydrogel delivery vehicles. J Dent Res 88(8):681–692

Salinas CN, Cole BB, Kasko AM, Anseth KS (2007) Chondrogenic differentiation potential of human mesenchymal stem cells photoencapsulated within poly (ethylene glycol)-arginine-glycine-aspartic acid-serine thiol-methacrylate mixed-mode networks. Tissue Eng 13:1025–1034

Scharp DW, Marchetti P (2014) Encapsulated islets for diabetes therapy: history, current progress, and critical issues requiring solution. Adv Drug Deliv Rev 67–68:35–73

Schuurman W, Levett PA, Pot MW et al (2013) Gelatin-methacrylamide hydrogels as potential biomaterials for fabrication of tissue-engineered cartilage constructs. Macromol Biosci 13 (5):551–561

Siddiqui N, Asawa S, Birru B, Baadhe R, Rao S (2018) PCL-based composite scaffold matrices for tissue engineering applications. Mol Biotechnol 60:506–532

Singh P, Schwarzbauer JE (2012) Fibronectin and stem cell differentiation—lessons from chondrogenesis. J Cell Sci 125:3703–3712

Somal A, Bhat IA, Indu B, Pandey S et al (2016) A comparative study of growth kinetics, in vitro differentiation potential and molecular characterization of fetal adnexa derived caprine mesenchymal stem cells. PLoS One 11(6):e0156821. https://doi.org/10.1371/journal.pone.0156821

Swartzlander MD, Blakney AK, Amer LD, Hankenson KD, Kyriakides TR, Bryant SJ (2015) Immunomodulation by mesenchymal stem cells combats the foreign body response to cell-laden synthetic hydrogels. Biomaterials 41:79–88

Tan L, Yu X, Wan P, Yang K (2013) Biodegradable materials for bone repairs: a review. J Mater Sci Technol 29(6):503–513

Tate CC, Shear DA, Tate MC, Archer DR, Stein DG, LaPlaca MC (2009) Laminin and fibronectin scaffolds enhance neural stem cell transplantation into the injured brain. J Tissue Eng Regen Med 3:208–217

Tyler B, Gullotti D, Mangraviti A, Utsuki T, Brem H (2016) Polylactic acid (PLA) controlled delivery carriers for biomedical applications. Adv Drug Deliv Rev 107:163–175

Willerth SM, Arendas KJ, Gottlieb DI, Sakiyama-Elbert SE (2006) Optimization of fibrin scaffolds for differentiation of murine embryonic stem cells into neural lineage cells. Biomaterials 27 (36):5990–6003

Willerth SM, Faxel TE, Gottlieb DI, Sakiyama-Elbert SE (2007) The effects of soluble growth factors on embryonic stem cell differentiation inside of fibrin scaffolds. Stem Cells 25 (9):2235–2244

Yang C, Hillas PJ, Baez JA et al (2004) The application of recombinant human collagen in tissue engineering. BioDrugs 18(2):103–119

Yannas IV, Burke JF, Orgill DP, Skrabut EM (1982) Wound tissue can utilize a polymeric template to synthesize a functional extension of skin. Science 215(4529):174–176

Yao H, Xue J, Wang Q et al (2017) Glucosamine-modified polyethylene glycol hydrogel-mediated chondrogenic differentiation of human mesenchymal stem cells. Mater Sci Eng 79:661–670

Yoshikawa T, Mitsuno H, Nonaka I et al (2008) Wound therapy by marrow mesenchymal cell transplantation. Plast Reconstr Surg 121:860–877

Yucel T, Lovett ML, Kaplan DL (2014) Silk-based biomaterials for sustained drug delivery. J Control Release 190:381–397

Zarrintaj P, Manouchehri S, Ahmadi Z, Saeb MR, Urbanska AM, Kaplan DL, Mozafari M (2018) Agarose-based biomaterials for tissue engineering. Carbohydr Polym 187:66–84

Zhang J, Lu X, Feng G et al (2016) Chitosan scaffolds induce human dental pulp stem cells to neural differentiation: potential roles for spinal cord injury therapy. Cell Tissue Res 366:129–142

Zollinger AJ, Smith ML (2017) Fibronectin, the extracellular glue. Matrix Biol 60:27–37

Prospects of Mesenchymal Stem Cell Secretome in Veterinary Regenerative Therapy

16

Vikash Chandra and G. Taru Sharma

Abstract

Regenerative medicine, a branch of translational research, comprises the generation and use of therapeutic stem cells. The mesenchymal stem cells (MSCs) used in preclinical models for tissue engineering of connective tissues have shown it as a promising agent in rebuilding damaged tissues like nerve injury, fracture, ligament and cartilage, liver, heart, etc. Paracrine mechanisms are considered the primary mode of action in adult stem cell signaling and therapy. MSCs secrete various cytokines and growth factors, including autocrine and paracrine actions. The promise of cell-free therapy has led to the development of products derived from stem cells. Microvesicles/exosomes derived from MSCs are exploited in therapeutic uses as pharmaceutical agents' delivery. Another central area of application of extracellular vesicles (EVs) is immune therapy due to their anti-inflammatory and immunomodulatory effects. Most translational research regarding mesenchymal stem cells and their conditioned media (CM) have been conducted in human subjects, and little work has been done in livestock and pets, as livestock and pets also encounter similar ailments. Hence, there is an excellent scope of stem cell secretome in veterinary regenerative therapy, especially in treating diseases like paraplegia or spinal cord injury, diabetic wounds, ligament/tendon injury, and bone injury/fracture. It may be an essential component of veterinary pharmaceuticals shortly.

Keywords

Mesenchymal stem cells · Secretome · Conditioned media · Regenerative therapy · Exosomes · Growth factor · Livestock

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16.1 Introduction

Mesenchymal or stromal cell-based therapy has been proved magical in regenerative medicine because of its unique characteristics. It is multipotent, differentiated into different cell lineages, and has immunomodulatory behavior (Somal et al. 2016; Bhat et al. 2019). Mesenchymal stem cells (MSCs) are derived from all other organs and tissues. It has been shown that they play an essential role in maintaining normal homeostasis in a healthy body. Thus, the transplanted MSCs niche provides a source of quiescent stem cells in response to body conditions such as injury or inflammation. MSCs are derived from multiple adult tissues (including bone marrow, adipose tissue, dental pulp) and fetal adnexa in various animal models (Sreekumar et al. 2014; Somal et al. 2017; Bhat et al. 2019), and stem cell-based therapies are being used routinely in clinical veterinary medicine to yield good results (Bhat et al. 2019; Gugjoo et al. 2018). Although cell-based therapies, including transplantation of MSCs, are promising, yet concerns like chances of immune rejection and low survival rates of transplanted cells are the technical limitations that hinder "stem cells" translational application (Robey 2011; Samsonraj et al. 2017).

Further, an increase in apoptosis after transplantation is reported, which often triggers an immune response, probably worsening the transplanted cells' diseased condition or rejection (Izadpanah et al. 2008; Robey 2011). Furthermore, some studies have also reported that the pluripotency and differentiation ability of MSCs decreases with the age of the donor animals (Nishida et al. 1999; Mendes et al. 2002). Data are accumulating regarding the use of stem cells in various ailments, and several explanations have been given regarding the mechanism of action of MSCs in the host body/tissue. Originally it was thought that the beneficial effects of MSCs-based therapy are engraftment and differentiation of MSCs into desired cell types within the damaged tissues. Subsequently, it has been realized that therapeutic effects of cell-based treatment are mediated in paracrine fashion through microvesicles released by MSCs (Duffield et al. 2005; Biancone et al. 2012); thus, microvesicles mediate the action of stem cell. Together with evidence that media harvested from conditioned MSCs are sufficient to stimulate regeneration of damaged tissues (Ansari et al. 2013; Joseph et al. 2020), this led to the development of the "paracrine hypothesis" of the therapeutic effects of MSCs (Hodgkinson et al. 2016) and the hypothesis is now widely accepted. MSCs-derived secretome alone was able to repair tissue in multiple conditions that involved tissue/organ damage (Joseph et al. 2020). Factors released in the culture medium and harvested are known as secretome or exosome/microvesicles. Thus, the medium harvested is called a "conditioned medium" (CM) (Kim and Choi 2013).

MSCs–CM or secretome has several advantages over the MSCs. CM can easily be manufactured, freeze-dried, packaged, and transported to remote location for later use. Moreover, as it is devoid of cells, there is no chance of immune rejection. It requires no match of the donor to the recipient to avoid rejection problems. Therefore, stem cell-derived conditioned mediums have a promising prospect to be produced as pharmaceutical products for regenerative medicine. To date, limited clinical trials have been conducted regarding the use of CM for a particular disease in

humans (Fukuoka et al. 2012), but these showed promising results. The studies on the use of CM of various illnesses are accumulating and very appealing; thus, CM therapy may be booming shortly (Yang et al. 2013). The conditioned medium contains multiple growth factors and cytokines/tissue regenerative agents secreted by the stem cells. The evidence that stem cell-conditioned medium contains several growth factors has been proved by proteomic studies, which revealed various growth factors and other cytokines in the CM (Joseph et al. 2020).

However, studies in various animal models reported using several kinds of stem cells and multiple methods to get the CM to cure different types of degenerative diseases. This chapter aims to investigate the various techniques to get the CM and the numerous ailments in animals and pets that can be treated to get an insight into the multiple kinds of CM and their future applications in various diseases in veterinary regenerative medicine.

16.2 MSCs Secretome or Conditioned Media (CM)

Secretome may be defined as the range of molecules/and cellular organelles secreted/released from the living cells or shed from the cell surface into the culture medium (Beer et al. 2017). Secretome may contain lipids, proteins, nucleic acid, and trophic factors such as chemokines, cytokines, hormones, growth factors, and extracellular vesicles (EVs) or microvesicles (MVs) (Kupcova 2013; Pawitan 2014; Driscoll and Patel 2019; Joseph et al. 2020) (Fig. 16.1). Secretome may be composed of soluble factors, including proteins, growth factors, chemokines, cytokines, enzymes, and extracellular vesicles (including exosomes and microvesicles). Meticulous studies on EVs and exosomes demonstrated potential regenerative effects, including an anti-inflammatory effect and tendon healing (Wang et al. 2011; Vizoso et al. 2017). MSCs secretome/CM has several added advantages over stem cells. They could resolve safety considerations associated with stem cell transplantation, such as tumorigenicity, the transmission of infections, and immune incompatibility (Vizoso et al. 2017). CM can be produced in large amounts and stored for a long time without losing product potency without the toxic effects of cryoprotective agents (Joseph et al. 2020), which can be further modified accordingly for desired results in order to reduce the cost, time for production, and maintenance of cell-based therapy with the wider application (Vizoso et al. 2017).

16.2.1 Extracellular Vesicles

Extracellular vesicles (EVs) are the cell-secreted products released by many cell types, including stem cells. They can also be isolated from body fluids like urine, serum, and cerebrospinal fluids. Research on EVs has been mainly focused on the exosome-rich fraction. The contents of the secretome depend on the prevailing environment, and thus any modification in the microenvironment is expected to

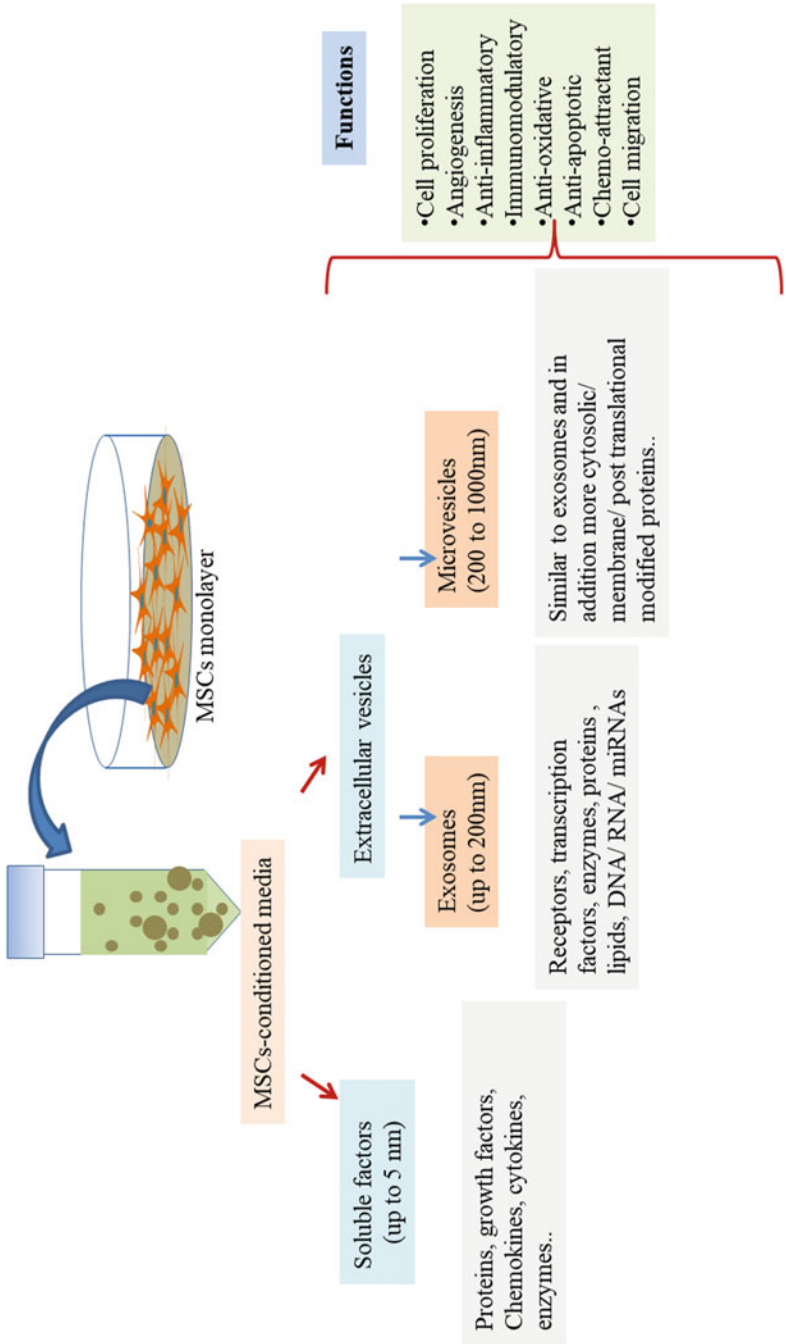

Fig. 16.1 MSCs conditioned media (CM) components and mechanism of wound regeneration: CM consists of soluble factors (growth factors, cytokines, hormones, etc.) and extracellular vesicles (exosomes and microvesicles); all these function to improve the wound regeneration process

affect the composition of the secretome. EVs include microvesicles (MVs) (size ranging 100–1000 nm), exosomes (EXs) (size range 40–100 nm), and apoptotic bodies (size ranging 1–5 μm) (Lee et al. 2012; Beer et al. 2017). EVs reach the target cell, attach and interact with the cell surface, may discharge its contents by fusion or through the endocytotic pathway, and may get detached after completing their action (Raposo and Stoorvogel 2013).

MVs and EXs are membrane-bound particles secreted by most cell types for maintaining normal cellular homeostasis, and it is found that their secretion may increase upon stimulation (Valadi et al. 2007; Kim et al. 2013). EVs are small derivatives of the cell that may mediate at least in part the effects of stem cells both in vivo and in vitro. Both MVs and EXs act via paracrine and endocrine fashion and are crucial for intercellular communication (Kim et al. 2013). If we consider stem cell-based therapies' therapeutic importance, EVs are being given special attention as therapeutic entities. They do not replicate and are said to have several safety advantages over stem cells. MVs and EXs act as vehicles or stable transporters to transport bioactive molecules like cytokines and growth factors from cells to the adjacent or distant target cells through circulation (Kim et al. 2013).

Further, they can also deliver RNAs to the target cells and modify gene expression or protein synthesis in target cells (Tomasoni et al. 2013). MVs and EXs differ in their cellular origin (biogenesis) as well as their physical characteristics (including size and surface markers) (Ratajczak et al. 2006). Their contents may depend upon the producing cells, their protein and lipids, contents and protein-coding messenger RNAs, and noncoding microRNA (Ratajczak et al. 2006).

Initially, EXs were discovered and described as exfoliated membrane vesicles (Trams et al. 1981). EXs are bilaminar membrane-enveloped nanovesicles secreted from all cell types and contain other molecules like microRNAs (miRNAs), affecting cellular gene expression (Valadi et al. 2007; Wang et al. 2018). Among all EVs, EXs are described as a relatively homogeneous population in terms of size. Recently, the therapeutic importance of EXs focused on the role of communication between EXs-mediated cells in bone regeneration and various other disease models (Lai et al. 2010; Zhang et al. 2015). EXs can provide recipient cells with genetic information, affecting their characteristics and paracrine factors, and resulting in tissue regeneration (Zhang et al. 2015). Harvested MSCs-CM also contains EXs and is considered to be one of the critical components of CM imparting therapeutic effect through paracrine effects. Several studies have compared such exosome-rich fractions with CM and reported comparable results, indicating an essential role of EXs in the therapeutic effects of MSC-derived CM. At the molecular level, EXs and microvesicles can be characterized by the surface markers like CD9, CD63, CD81, Alix, and TSG101 for EXs and CD40, integrin, and selectin for microvesicles. Effects of culture condition media can be changed that changes the properties of EVs.

16.3 Isolation of Conditioned Media

Biological properties of stem cell conditioned media may vary as per the source and types of mesenchymal stem cells, but the basic protocol remains the same for every kind of MSCs. Extracellular proteins secreted by MSCs are said to be responsible for the regenerative properties of MSCs, including inflammatory response, angiogenesis, cell migration, organ survival, etc. A more or less similar composition of secreted proteins has been observed in bone marrow MSC-CM and adipose MSC-CM rather than dental pulp MSC-CM (Tachida et al. 2015). Well-characterized MSCs at early passages (3–8 passages) are cultured in Dulbecco's modified Eagle medium (DMEM) fortified with 10% fetal bovine serum (FBS) at 37 °C in a humidified incubator with 5% CO_2. When MSCs reached 70–80% confluence, the medium is replaced by serum-free DMEM. The cell-cultured conditioned medium is collected after an additional 72 h of incubation (Joseph et al. 2020). The collected medium is defined as MSCs cultured conditioned medium (MSC-CM) and is stored at +4 or −80 °C for later application. The obtained MSCs-CM may be further concentrated by ultracentrifugation and filtration using 5–10 kDa cutoff filters. For long-term preservation, MSCs-CM can also be lyophilized and stored at 4 °C for therapeutic uses (Joseph et al. 2020).

16.4 Isolation of Exosomes

For the isolation of EXs, MSCs monolayer at about 80% confluence is washed with serum-free culture media (DMEM low glucose supplemented with 1× insulin transferrin-selenium) cultured in this medium for another 48–72 h. MSCs culture media supernatant from confluent monolayer is harvested and centrifuged at 2.7 g for 5 min, and the supernatant was filtered using a 0.2 μm membrane filter. EXs isolation from filtrate done through ultracentrifugation (UC), ultrafiltration (UF), or by the charge-based precipitation (PT) involves protamine sulfate (Klymiuk et al. 2019). The most commonly used method for isolating EXs was the ultracentrifugation and the exosome by ultrafiltration for smoother isolation and higher yield. Ultrafiltration is a time-saving and cost-effective method of exosome isolation from equine MSCs (Klymiuk et al. 2019). The number of particles per milliliter and the quality in terms of peak size distribution in different isolation procedures may differ and can be analyzed by a nanoparticle tracking analysis method. The peak particle size of native EXs derived from cell culture supernatant is about 83–398 nm. Still, after ultracentrifugation, peak particle diameter may be between 93 and 353 nm, while by ultrafiltration, it is between 138 and 763 nm. EXs isolated by charge-based precipitation led to a large variation in their sizes (378–998 nm) primarily due to an aggregation of EXs. These extracellular vesicles can be characterized by the expression of specific marker proteins from the tetraspanin superfamily, such as CD9, CD63, and CD81 (Andreu and Yanez-Mo 2014). These markers are commonly expressed on the membrane surface of EXs and are essential for the formation and

transportation within the cell and the recognition of target cells. Morphology of isolated EXs may be investigated by transmission electron microscopy (TEM).

16.5 Mechanism of CM in Wound Healing

Therapeutic effects of CM have been described due to the combined effects of soluble factors and extracellular vesicles, and the major mechanism of action is said to be the paracrine mode of action. As CM contains several growth factors and cytokines and most of these molecules are considered as paracrine agents, these molecules have the function to activate or inactivate adjacent cells by influencing the intracellular pathways without affecting the functions of other cells of the body. In the wound healing process, endogenous mesenchymal stem cells, including dermal sheath stem cells and hair follicle bulge, play a crucial role. Endogenous dermal MSCs are located at different sites, mainly in epidermal and dermal niches. MSC-CM applied over the wound provides an improved microenvironment, which influences cell migration and other cellular and molecular events to happen in a sequential manner (Hocking and Gibran 2010). CM containing several factors may stimulate fibroblasts and keratinocyte proliferation, migration, and differentiation to enhance injured skin regeneration. MSCs secreted mitogens (like bFGF, VEGF-A, and IL-6/8) stimulate the proliferation of keratinocytes, endothelial cells, and dermal fibroblasts. Wounds healed after application of MSCs or its CM are reported to be of better quality with a little scar because CM contains a significant amount of TGF-β1 as it balances TGF-β1 and TGF-β3 is required for smooth dermal regeneration rather than fibrosis and scarring. In the form of a gel, CM creates a microenvironment permissive for migration and proliferation of endogenous MSCs and reduces the mechanical stress to the cells involved in wound repair (Joseph et al. 2020).

EXs are suggested as a potential regenerative agent that can replace conventional tissue regeneration methods, including stem cell transplantation. Recent studies have reported various possible applications of EXs in diagnostics and therapeutics (Rashed et al. 2017; Sun et al. 2018). Therapeutic application of EXs avoids the potential risks of toxicity and immunogenicity as caused by biomaterial processing (Fleury et al. 2014). Besides, EXs also have a lower risk of serious complications such as tumorigenesis or emboli formation compared to cell transplantation as such (Yu et al. 2014). EXs contain miRNA and may mediate cell–cell communication through genetic material transfer. Further, miRNAs are also involved in gene regulatory networks in several signaling pathways and may act as target gene therapy agents (Zhang et al. 2018). Qin et al. (2016) showed that extracellular vesicles secreted from MSCs promote bone regeneration in vivo and significantly upregulate the expression of osteogenesis-related miRNAs, that is, miR-196a, miR-27a, miR-206, and miR-196a – important regulators of osteogenesis.

Further, miR-21, miR-4532, miR-125b-5p, and miR-338-3p may also enhance osteogenesis and angiogenesis (Furuta et al. 2016). Chen et al. (2019) revealed that EXs derived from miR-375-overexpressing human adipose mesenchymal stem cells

had osteogenic potential and promoted bone regeneration. Thus, early bone regeneration induced by MSCs-derived EXs depends on the regulation of multiple miRNAs and their relevant pathways, and miRNAs regulate the gene expression, thereby affecting tissue development and homeostasis.

16.6 Exosomes as Drug Delivery Agents

Cells communicate primarily through the use of chemical messengers, mainly through extracellular vesicles (EVs). Exosomes are among the most highly researched EVs. They act as a communication tool to deliver a chemical cargo to the recipient cell. MSC-derived EXs contain several biologically active molecules, such as proteins and RNAs, and are therefore well equipped to maintain homeostasis within the tissue. EVs bear an excellent scope for therapeutic medicine due to their unique structure, which can be modified to contain specific proteins, lipids, and genetic materials, including messenger RNA, microRNA, and other small noncoding RNAs, and DNA from their progenitor cell (Fig. 16.2). Several studies reported that EXs might enter into the cells, release their cargo, and mediate physiological and pathological processes by binding through ligands and deliver surface protein and cytoplasm to the recipient cell by fusing with the target-cell membrane (Zhou et al. 2017). EXs may also be used to deliver pharmaceutical agents and siRNA (interfering RNA) (Aryani and Denecke 2016). Nanoscale drug delivery systems have emerged as a new therapeutic strategy. There are other several nano-based drug formulations used to improve the therapeutic efficacy of chemical and biomolecular drugs. The targeting effectiveness of EXs can be enhanced through appropriate surface modification. Thus, EVs can deliver therapeutic agents, including RNAs, proteins, and small-molecule drugs. EVs can be engineered to express targeting peptides or therapeutic proteins on their surfaces, and aptamers or therapeutic RNAs can bind to EXs through RNA-binding proteins (Fig. 16.3).

Fig. 16.2 Effects of culture conditioning on desired extracellular vesicles (EVs) properties. The supplementation of inflammatory molecules and changing culture conditions can improve beneficial properties of MSCs-derived secretome (low-density culture, 3-D culture, hypoxic stimulation, drug preconditioning, etc.) and cell rejuvenation strategies

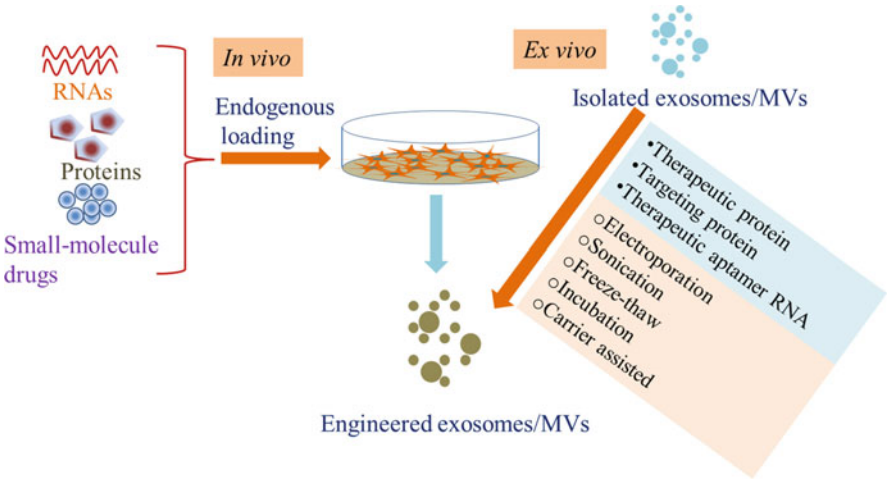

Fig. 16.3 Engineering the extracellular vesicles (EVs): in vivo, the targeted miRNA, mRNA, proteins, and drugs can be loaded through transfection; ex vivo loading of therapeutic targets can be done using different approaches like electroporation, sonication, freeze–thaw, incubation, and carrier-assisted methods

Additionally, EVs can encapsulate these therapeutic agents and protect them from degradation or failure. Liposome-mediated drug delivery can enhance pharmaceuticals/biopharmaceuticals and vaccines' therapeutic outcomes. Liposomes can incorporate drugs both within their aqueous core and lipid bilayers (Dimov et al. 2017). Metal organic frameworks (MOFs) are considered promising nanocarriers for drug delivery because of their well-defined structure, very high surface area and porosity, tunable pore size, and ease of chemical functionalization and intrinsic biodegradability (Sun et al. 2020). MOFs can be combined with exosome for better drug delivery using their combined features to facilitate more straightforward and more efficient loading and sealing of cargos. The resulting exosome-coated nanoparticles show no premature leakage, yet their cargo is efficiently released in cells (Bunggulawa et al. 2018).

16.7 Clinical Applications of MSCs-CM

16.7.1 Bone Regeneration/Fracture Healing

Reconstruction of bone defects that occurred due to fractures, tumors, or infections is a challenge both in humans and animals, and 5–10% of fractures may lead to delayed healing or nonunion. Though bone tissue has its intrinsic property of recovery, if the fracture is large or more patients or some diseased conditions like diabetes, it does not heal properly even with traditional therapies. However, in the recent past mesenchymal stem cell therapy has been found promising in fracture healing. It

has been proven that the healing effects of MSCs are mainly due to their secretome containing several growth factors, cytokines, and other biomolecules. Several studies have reported that MSC-CM contains several growth factors and cytokines and investigated the biological effects of MSC-CM that promoted bone regeneration and wound healing as the secretome participates in the stimulation of multiple cellular functions (Osugi et al. 2012; Joseph et al. 2020). Secretome of MSCs has been shown to accelerate the formation of new bone callus and bone healing, shortening the period required for treatment. Conditioned medium (MSC-CM) applied to injured tissue can modulate and promote new tissue formation by its osteogenic potential and immunomodulatory properties due to their cytokines and growth factors. Takeuchi et al. (2019) reported that MSCs-CM enhances cell migration and expression of angiogenic and osteogenic genes, including VEGF, COLI, OCN, ANG1, ANG2, and Runx2 (Runt-related transcription factor 2). Interestingly, transplantation of MSC-CM promotes bone regeneration compared to transplantation of MSCs (Osugi et al. 2012).

Extracellular messengers are responsible for cell-to-cell communication, and the EXs have been seen to play similar roles in promoting fracture healing as MSCs themselves. Moreover, EXs do not induce overt immune reactions even in the xenogeneic host because they do not contain significant histocompatibility complex I (MHC I) or MHC II molecules. Recently, the MSC-exosome administration effectively reduced the production of inflammatory cytokines in chondrocytes, increased osteogenesis, angiogenesis, and expression of cartilage ECM component, and eventually augmented cartilage tissue regeneration in a series of in vivo studies (Kim et al. 2020). Thus, MSCs secretome and EVs are considered promising alternatives to mesenchymal stem cell therapy to address osteoarthritis (OA) and other bone injuries.

16.7.2 Tendon and Ligament Repair

The musculoskeletal ailment is a common disability in draft and racing animals like equines, and about 46% of the cases are due to tendon injuries. Tendon tissues being relatively hypocellular tissues have limited healing capacity. Traditional remedies have been proved unsatisfactory in tendon repair. It has been reported that tendons contain stem/progenitor cells (TSPCs) between the fascicles. MSCs derived from tendons themselves or nontendon tissues, like bone marrow or adipose tissue, have been proved promising in tendon injury repair (Costa-Almeida et al. 2019) and a similar case with MSCs-CM or secretome too. Studies by Chen et al. (2018) and Shimode et al. (2007) reported significant tenocyte proliferation in rats treated with bone marrow (BM) MSCs-CM. These studies also mentioned a substantial reduction in inflammatory markers with CM treatment. A study by Wang et al. showed a significant increase in tissue inhibitor of metalloproteinase (TIMP), an anti-inflammatory protein that could improve biomechanical properties (Wang et al. 2019). Tenogenic differentiation was also enhanced significantly in cells treated with CM, as shown by a significant increase of Col-I expression and other tenogenic

markers in some studies (Chen et al. 2018; Wang et al. 2019). Studies by Lange-Consiglio et al. (2013) and Shen et al. (2019) showed a significant reduction in peripheral blood mononuclear cell (PBMC) proliferation in CM treatment compared with control. Interestingly, it was reported that EVs did not significantly inhibit PBMC proliferation compared with control (Lange-Consiglio et al. 2013). The study by Shen et al. (2019) found that macrophages' nuclear factor κB activity was inhibited significantly by EV compared with control.

16.7.3 Repair of Nerve Injury or Paralysis

Spinal cord injury may lead to the typical motor, sensory, and autonomic functions of the body, resulting in neurological defects and disabilities. Neuronal damage due to mechanical forces may insult with secondary cascading events like oxidative stress, metabolic changes, excitotoxicity, inflammatory processes, and apoptosis or necrosis, and gliosis and fibrosis induced produce a challenging environment for axonal regrowth (Block and Hong 2005; Okada et al. 2018; Courtine and Sofroniew 2019). Due to the insufficient intrinsic regeneration capacity of neurons and growth inhibitors in the adult spinal cord, generally, axons fail to regenerate and neurologic recovery is hampered. MSC transplantation has been found useful in repairing spinal cord injury; it may be due to the reduced local inflammation, establishment of local blood supply, cell protection, etc., which may be caused by the MSC paracrine effect (Teng 2019). Like humans, dogs also encounter spinal cord injury, which often leads to devastating conditions, including paralysis associated with urinary and fecal incontinence. SCI may also be experienced due to faulty injections or fall from height or road accidents. Pre-induced MSCs differentiate into neural lineage cells; however, a study found weak evidence for naive MSC differentiation into functional neural lineage cells in vivo (Cofano et al. 2019). MSCs secrete many kinds of neurotrophic factors and display low immunogenicity. MSC-conditioned medium (MSC-CM) containing different anti-inflammatory cytokines, chemoattractants, growth factors, and enzymes are considered attractive candidate nerve regeneration. Administration MSCs or CM into the host creates a favorable microenvironment for proliferation and transdifferentiation of native MSCs. For example, a pilot study conducted by Vikartovska et al. (2020) demonstrated that four intravenous injections of bone marrow-derived MSCs CM, combined with subsequent rehabilitation, improved chronic SCI in dogs without any adverse effects.

Recently, the role of MSC-EXs in nerve repair has been validated. It is reported mainly by regulating inflammation and the immune response, inhibiting apoptosis, inducing angiogenesis and axon formation, and maintaining the integrity of the blood–brain barrier (Ren et al. 2020). MSCs-CM also has been found beneficial in glutamate excitotoxicity and oxygen–glucose deprivation (OGD) environments to protect neuronal damage (Voulgari-Kokota et al. 2012). Thus, the growth factors and cytokines secreted from MSCs contribute to regeneration and immunoregulation. MSCs behaved as extracorporeal bioreactors and secrete bioactive factors into the CM, which was suggestive of novel therapeutics in CNS injury (Tsai et al. 2018).

Apart from neural repair, the MSCs-CM also promises to reduce neuropathic pain, which may act as cell-based analgesia and an agent for neural repair. Like live stem cells, injection of MSCs-CM produces a profound, long-lasting pain-alleviating effect on neuropathic pain and cytokines explored to be involved with the maintenance of this syndrome (Gama et al. 2018).

16.7.4 Wound Healing and Hair Follicle Regeneration

Skin injuries are the most commonly found injuries caused by blisters, burns, cuts, and ulcers of various severities (Dong et al. 2017). If left untreated, any skin injury can interfere with its protective function and cause body tissues to be easily exposed to mechanical damages and microbial infections (Joseph et al. 2020). Traditionally, wound care's primary purpose is to obtain wound closure at the earliest time (Dong et al. 2017). The use of stem cell-based regenerative therapy has emerged as a new generation therapeutic tool for repairing physiological tissues (Ansari et al. 2013; Kucharzewski et al. 2019; Joseph et al. 2020). The repair mechanism of MSCs in wound healing seems to be due to their extensive proliferation and differentiation capacity, which eventually suppress immune cells' stimulation and participate in tissue repair and regeneration (Kucharzewski et al. 2019; Zomer et al. 2019). MSC releases a combination of growth factors and cytokines, so-called secretome, which can be applied to the wound through a conditioned medium (CM) (Pawitan 2014; Kucharzewski et al. 2019). The cytokines and growth factors play a crucial role in activating and stimulating fibroblast proliferation during the wound healing process (Aryan et al. 2019). CM derived from MSCs from different sources like bone marrow mesenchymal stem cell, adipose stem cell, Wharton's jelly-derived MSC, and umbilical MSC gave positive wound healing results and skin repair (Joseph et al. 2020).

Hair loss caused by multietiological factors has a tremendous psychological impact on a person. Similarly, our pets and other animals also encounter hair loss due to several defects. Conventional therapeutic approaches are not much effective having several side effects while MSCs-CM rich in growth factors, and cytokines is proved to be a promising therapeutic agent for such problems. MSC-CM is useful in skin wound repair and enhances hair growth through differentiation of dermal niches cells or release of growth factors. MSC-CM promotes migration and proliferation of dermal keratinocytes. Wnt signaling in dermal papilla cells is considered a critical factor in stimulating hair growth. MSCs-CM helps in hair regeneration through cellular proliferation to prolong the anagen phase (FGF-7), inducing hair follicle development (β-catenin), inducing cell growth (ERK activation), and suppressing apoptosis (Bcl-2 release and Akt activation). A study by Yoon et al. (2010) revealed dramatic hair growth at the wound site following wound healing after MSC-CM was injected subcutaneously around a full-thickness wound in mice. Thus, MSC-CM can be considered a potential alternative of stem cells for therapeutic applications for wound healing/skin regeneration and hair growth stimulation and hair loss prevention.

16.8 Conclusion

Unique features of MSCs-derived conditioned medium (like the ease of manufacture, freeze-drying, packaging, and transportation) make it a potential therapeutic agent for future pharmaceutical production for regenerative therapeutics. A significant limitation for the clinical translation of MVs/EVs is due to the difficulties and inconsistencies in their isolation because compositions and contents may differ from cell to cell and method of isolation. EVs (EXs and MVs) are emerging as cell-free biologics to treat various health disorders. Recent studies suggest that MVs secreted by MSCs are potent drug delivery agents that are effective for treating severe pneumonia, osteoarthritis, and renal ischemia-reperfusion injury after cardiac death and renal transplantation. Stimulation of parental MSCs could be employed to engineer the MV cargo to some extent differentially, and engineered MSCs or EVs bear a great hope in new generation regenerative therapeutics. MSCs secretome may prove to be a next-generation cell-free therapeutic agent for several ailments in veterinary regenerative medicine.

References

Andreu Z, Yanez-Mo M (2014) Tetraspanins in extracellular vesicle formation and function. Front Immunol 5:442

Ansari MM, Sreekumar TR, Chandra V, Dubey PK, Sai Kumar G, Amarpal, Sharma GT (2013) Therapeutic potential of canine bone marrow-derived mesenchymal stem cells and its conditioned media in diabetic rat wound healing. J Stem Cell Res Ther 3:141. https://doi.org/10.4172/2157-7633.1000141

Aryan A, Bayat M, Bonakdar S, Taheri S, Haghparast N, Bagheri M, Piryaei A, Abdollahifar MA (2019) Human bone marrow mesenchymal stem cell conditioned medium promotes wound healing in deep second-degree burns in male rats. Cells Tissues Organs 206:317–329

Aryani A, Denecke B (2016) Exosomes as a nano delivery system: a key to the future of neuro medicine? Mol Neurobiol 53:818–834

Beer L, Mildner M, Ankersmit HJ (2017) Cell secretome based drug substances in regenerative medicine: when regulatory affairs meet basic science. Ann Transl Med 5(7):article 170

Bhat IA, Shivkumar TB, Somal A, Sriti P, Bharti MK, Panda BSK, Indu B, Verma M, Joseph A, Sonwane A, Sai Kumar G, Amarpal, Chandra V, Sharma GT (2019) An allogenic therapeutic strategy for canine spinal cord injury using mesenchymal stem cells. J Cell Physiol 234:2705–2718

Biancone L, Bruno S, Deregibus MC, Tetta C, Camussi G (2012) Therapeutic potential of mesenchymal stem cell-derived microvesicles. Nephrol Dial Transplant 27:3037–3042

Block ML, Hong JS (2005) Microglia and inflammation-mediated neurodegeneration: multiple triggers with a common mechanism. Prog Neurobiol 76(2):77–98

Bunggulawa EJ, Wang W, Yin T, Wang N, Durkan C, Wang Y, Wang G (2018) Recent advancements in the use of exosomes as drug delivery systems. J Nanobiotechnol 16:81

Chen Q, Liang Q, Zhuang W et al (2018) Tenocyte proliferation and migration promoted by rat bone marrow mesenchymal stem cell-derived conditioned medium. Biotechnol Lett 40 (1):215–224

Chen S, Tang Y, Liu Y, Zhang P, Lv L, Zhang X et al (2019) Exosomes derived from miR-375-overexpressing human adipose mesenchymal stem cells promote bone regeneration. Cell Prolif 52(5):e12669

Cofano F, Boido M, Monticelli M, Zenga F, Ducati A, Vercelli A et al (2019) Mesenchymal stem cells for spinal cord injury: current options, limitations, and future of cell therapy. Int J Mol Sci 20(11):2698

Costa-Almeida R, Calejo I, Gomes ME (2019) Mesenchymal stem cells empowering tendon regenerative therapies. Int J Mol Sci 20(12):3002. https://doi.org/10.3390/ijms20123002

Courtine G, Sofroniew MV (2019) Spinal cord repair: advances in biology and technology. Nat Med 25(6):898–908

Dimov N, Kastner E, Hussain M, Perrie Y, Szita N (2017) Formation and purification of tailored liposomes for drug delivery using a module-based micro continuous-flow system. Sci Rep 7:12045

Dong L, Hao H, Liu J, Ti D, Tong C, Hou Q, Li M, Zheng J, Liu G, Fu X et al (2017) A conditioned medium of umbilical cord mesenchymal stem cells overexpressing Wnt7a promotes wound repair and regeneration of hair follicles in mice. Stem Cells Int 2017:1–13

Driscoll J, Patel T (2019) The mesenchymal stem cell secretome as an acellular regenerative therapy for liver disease. J Gastroenterol 54(9):763–773

Duffield JS, Park KM, Hsiao LL, Kelley VR, Scadden DT, Ichimura T et al (2005) Restoration of tubular epithelial cells during repair of the postischemic kidney occurs independently of bone marrow-derived stem cells. J Clin Invest 115:1743–1755

Fleury A, Martinez MC, Le Lay S (2014) Extracellular vesicles as therapeutic tools in cardiovascular diseases. Front Immunol 5:370

Fukuoka H, Suga H, Narita K, Watanabe R, Shintani S (2012) The latest advance in hair regeneration therapy using proteins secreted by adipose-derived stem cells. Am J Cosm Surg 29(4):273–282

Furuta YT, Miyaki S, Ishitobi H, Ogura T, Kato Y, Kamei N et al (2016) Mesenchymal stem cell-derived exosomes promote fracture healing in a mouse model. Stem Cells Transl Med 5:1620–1630

Gama KB, Santos DS, Evangelista AF, Silva DN, de Alcantara AC, dos Santos RR, Soares MBP, Villarreal CF (2018) Conditioned medium of bone marrow-derived mesenchymal stromal cells as a therapeutic approach to neuropathic pain: a preclinical evaluation. Stem Cells Int 2018:8179013. https://doi.org/10.1155/2018/8179013

Gugjoo MB, Amarpal, Chandra V, Wani MY, Dhama K, Sharma GT (2018) Mesenchymal stem cell research in veterinary medicine (a review). Curr Stem Cell Res Ther 13(8):645–657. https://doi.org/10.2174/1574888X13666180517074444

Hocking AM, Gibran NS (2010) Mesenchymal stem cells: paracrine signaling and differentiation during cutaneous wound repair. Exp Cell Res 316:2213–2219

Hodgkinson CP, Bareja A, Gomez JA, Dzau VJ (2016) Emerging concepts in paracrine mechanisms in regenerative cardiovascular medicine and biology. Circ Res 118:95–107

Izadpanah R, Kaushal D, Kriedt C, Tsien F, Patel B, Dufour J et al (2008) Long-term in vitro expansion alters the biology of adult mesenchymal stem cells. Cancer Res 68:4229–4238

Joseph A, Indu B, Bhat IA, Pandey S, Bharti MB, Verma M, Singh AP, Ansari MM, Chandra V, Saikumar G, Amarpal, Sharma GT (2020) Mesenchymal stem cell-conditioned media: a novel alternative of stem cell therapy for quality wound healing. J Cell Physiol 235:5555–5569. https://doi.org/10.1002/jcp.29486

Kim HO, Choi S (2013) Mesenchymal stem cell-derived secretome and microvesicles as a cell-free therapeutics for neurodegenerative disorders. Tissue Eng Regen Med 10(3):93–101

Kim HO, Choi SM, Kim HS (2013) Mesenchymal stem cell-derived secretome and microvesicles as a cell-free therapeutics for neurodegenerative disorders. Tissue Eng Regen Med 10 (3):93–101

Kim YG, Choi J, Kim K (2020) Mesenchymal stem cell-derived exosomes for effective cartilage tissue repair and treatment of osteoarthritis. Biotechnol J 15(12):e2000082. https://doi.org/10.1002/biot.202000082

Klymiuk MC, Balz N, Elashry MI, Heimann M, Wenisch S, Arnhold S (2019) Exosomes isolation and identification from equine mesenchymal stem cells. BMC Vet Res 15:42

Kucharzewski M, Rojczyk E, Wilemska-Kucharzewska K, Wilk R, Hudecki J, Łos MJ (2019) Novel trends in application of stem cells in skin wound healing. Eur J Pharmacol 843:307–315

Kupcova SH (2013) Proteomic techniques for characterisation of mesenchymal stem cell secretome. Biochimie 95(12):2196–2211

Lai RC, Arslan F, Lee MM, Sze NS, Choo A, Chen TS et al (2010) Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury. Stem Cell Res 4:214–222

Lange-Consiglio A, Rossi D, Tassan S, Perego R, Cremonesi F, Parolini O (2013) Conditioned medium from horse amniotic membrane-derived multipotent progenitor cells: immunomodulatory activity in vitro and first clinical application in tendon and ligament injuries in vivo. Stem Cells Dev 22(22):3015–3024

Lee Y, El Andaloussi S, Wood MJA (2012) Exosomes and microvesicles: extracellular vesicles for genetic information transfer and gene therapy. Hum Mol Genet 21(R1):R125–R134

Mendes SC, Tibbe JM, Veenhof M, Bakker K, Both S, Platenburg PP et al (2002) Bone tissue-engineered implants using human bone marrow stromal cells: effect of culture conditions and donor age. Tissue Eng 8:911–920

Nishida S, Endo N, Yamagiwa H, Tanizawa T, Takahashi HE (1999) Number of osteoprogenitor cells in human bone marrow markedly decreases after skeletal maturation. J Bone Miner Metab 17:171–177

Okada S, Hara M, Kobayakawa K, Matsumoto Y, Nakashima Y (2018) Astrocyte reactivity and astrogliosis after spinal cord injury. Neurosci Res 126:39–43

Osugi M, Katagiri W, Yoshimi R, Inukai T, Hibi H, Ueda M (2012) Conditioned media from mesenchymal stem cells enhanced bone regeneration in rat calvarial bone defects. Tissue Eng Part A 18:1479–1489

Pawitan JA (2014) Prospect of stem cell conditioned medium in regenerative medicine. Biomed Res Int 2014:7–9

Qin Y, Wang L, Gao Z, Chen G, Zhang C (2016) Bone marrow stromal/stem cell-derived extracellular vesicles regulate osteoblast activity and differentiation in vitro and promote bone regeneration in vivo. Sci Rep 6:21961

Raposo G, Stoorvogel W (2013) Extracellular vesicles: exosomes, microvesicles, and friends. J Cell Biol 200(4):373–383

Rashed HM, Bayraktar EK, Helal G, Abd-Ellah MF, Amero P, Chavez-Reyes A et al (2017) Exosomes: from garbage bins to promising therapeutic targets. Int J Mol Sci 18:E538

Ratajczak J, Miekus K, Kucia M et al (2006) Embryonic stem cell-derived microvesicles reprogram hematopoietic progenitors: evidence for horizontal transfer of mRNA and protein delivery. Leukemia 20(5):847–856

Ren Z, Qi Y, Sun S, Tao Y, Shi R (2020) Mesenchymal stem cell-derived exosomes: hope for spinal cord injury repair. Stem Cells Dev 29(23):1467–1478

Robey PG (2011) Cell sources for bone regeneration: the good, the bad, and the ugly (but promising). Tissue Eng Part B Rev 17:423–430

Samsonraj RM, Raghunath M, Nurcombe V, Hui JH, van Wijnen AJ, Cool SM (2017) Concise review: multifaceted characterization of human mesenchymal stem cells for use in regenerative medicine. Stem Cells Transl Med 6:2173–2185

Shen H, Yoneda S, Abu-Amer Y, Guilak F, Gelberman RH (2019) Stem cell-derived extracellular vesicles attenuate the early inflammatory response after tendon injury and repair. J Orthop Res 2019:1–11

Shimode K, Iwasaki N, Majima T et al (2007) Bone marrow stromal cells act as feeder cells for tendon fibroblasts through soluble factors. Tissue Eng 13(2):333–341

Somal A, Bhat IA, Indu B, Pandey S, Panda BSK, Thakur N, Sarkar M, Chandra V, Saikumar G, Sharma GT (2016) A comparative study of growth kinetics, in vitro differentiation potential and molecular characterization of fetal adnexa derived caprine mesenchymal stem cells. PLoS One 11(6):e0156821. https://doi.org/10.1371/journal.pone.0156821

Somal A, Bhat IA, Indu B, Singh AP, Panda BSK, Desingu PA, Pandey S, Bharti MK, Amarpal, Saikumar G, Chandra V, Sharma GT (2017) Impact of cryopreservation on caprine fetal adnexa

derived stem cells and its evaluation for growth kinetics, phenotypic characterization and wound healing potential in xenogenic rat model. J Cell Physiol 232(8):2186–2200

Sreekumar TR, Ansari MM, Chandra V, Sharma GT (2014) Isolation and characterization of buffalo Wharton's jelly derived mesenchymal stem cells. J Stem Cell Res Ther 4:207. https://doi.org/10.4172/2157-7633.1000207

Sun G, Li G, Li D, Huang W, Zhang R, Zhang H et al (2018) hucMSC derived exosomes promote functional recovery in spinal cord injury mice via attenuating inflammation. Mater Sci Eng C Mater Biol Appl 89:194–204

Sun Y, Zheng L, Yang Y, Qian X, Fu T, Li X, Yang Z, Yan H, Cui C, Tan W (2020) Metal–organic framework nanocarriers for drug delivery in biomedical applications. Nano-Micro Lett 12:103

Tachida Y, Sakurai H, Okutsu J et al (2015) Proteomic comparison of the secreted factors of mesenchymal stem cells from bone marrow, adipose tissue and dental pulp. J Proteomics Bioinform 8(12):266

Takeuchi R, Katagiri W, Endo S, Kobayashi T (2019) Exosomes from conditioned media of bone marrow-derived mesenchymal stem cells promote bone regeneration by enhancing angiogenesis. PLoS One 14(11):e0225472

Teng YD (2019) Functional multipotency of stem cells and recovery neurobiology of injured spinal cords. Cell Transplant 28(4):451–459

Tomasoni S, Longaretti L, Rota C et al (2013) Transfer of growth factor receptor mRNA via exosomes unravels the regenerative effect of mesenchymal stem cells. Stem Cells Dev 22 (5):772–780

Trams EG, Lauter CJ, Norman Salem J, Heine U (1981) Exfoliation of membrane ecto-enzymes in the form of micro-vesicles. Biochim Biophys Acta 645(1):63–70

Tsai MJ, Liou DY, Lin YR, Weng CF, Huang MC, Huang WC et al (2018) Attenuating spinal cord injury by conditioned medium from bone marrow mesenchymal stem cells. J Clin Med 8(1)

Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ, Lotvall JO (2007) Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. Nat Cell Biol 9(6):654–659

Vikartovska Z, Kuricova M, Farbakova J, Liptak T, Mudronova D, Humenik F, Madari A, Maloveska M, Sykova E, Cizkova D (2020) Stem cell conditioned medium treatment for canine spinal cord injury: pilot feasibility study. Int J Mol Sci 21:5129. https://doi.org/10.3390/ijms21145129

Vizoso FJ, Eiro N, Cid S, Schneider J, Perez-Fernandez R (2017) Mesenchymal stem cell secretome: toward cell-free therapeutic strategies in regenerative medicine. Int J Mol Sci 18 (1852):1–24

Voulgari-Kokota A, Fairless R, Karamita M, Kyrargyri V, Tseveleki V, Evangelidou M et al (2012) Mesenchymal stem cells protect CNS neurons against glutamate excitotoxicity by inhibiting glutamate receptor expression and function. Exp Neurol 236(1):161–170

Wang CY, Yang HB, Hsu HS et al (2011) Mesenchymal stem cell-conditioned medium facilitates angiogenesis and fracture healing in diabetic rats. J Tissue Eng Regen Med 2011:1–11

Wang X, Omar O, Vazirisani F, Thomsen P, Ekström K (2018) Mesenchymal stem cell-derived exosomes have altered microRNA profiles and induce osteogenic differentiation depending on the stage of differentiation. PLoS One 13:e0193059

Wang Y, He G, Guo Y et al (2019) Exosomes from tendon stem cells promote injury tendon healing through balancing synthesis and degradation of the tendon extracellular matrix. J Cell Mol Med 23(8):5475–5485

Yang D, Wang W, Li L et al (2013) The relative contribution of paracrine effect versus direct differentiation on adipose-derived stem cell transplantation mediated cardiac repair. PLoS One 8 (3):e59020

Yoon BS, Moon JH, Jun EK, Kim J, Maeng I, Kim JS, Lee JH, Baik CS, Kim A, Cho KS et al (2010) Secretory profiles and wound healing effects of human amniotic fluid–derived mesenchymal stem cells. Stem Cells Dev 19:887–902

Yu B, Zhang X, Li X (2014) Exosomes derived from mesenchymal stem cells. Int J Mol Sci 15:4142–4157

Zhang Y, Chopp M, Meng Y, Katakowski M, Xin H, Mahmood A et al (2015) Effect of exosomes derived from multipluripotent mesenchymal stromal cells on functional recovery and neurovascular plasticity in rats after traumatic brain injury. J Neurosurg 122:856–867

Zhang B, Li Y, Yu Y, Zhao J, Ou Y, Chao Y et al (2018) MicroRNA-378 promotes osteogenesis-angiogenesis coupling in BMMSCs for potential bone regeneration. Anal Cell Pathol (Amst) 2018:8402390

Zhou Y, Tian T, Zhu Y, Jaffar AD, Hu F, Qi Y, Sun B, Xiao Z (2017) Exosomes transfer among different species cells and mediating miRNAs delivery. J Cell Biochem 118:4267–4274

Zomer HD, Varela GKDS, Delben PB, Heck D, Jeremias TDS, Trentin AG (2019) In vitro comparative study of human mesenchymal stromal cells from dermis and adipose tissue for application in skin wound healing. J Tissue Eng Regen Med 13:729–741

Reprogramming and Induced Pluripotent Stem Cells in Porcine

17

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Abstract

In 2006, Shinya Yamanaka successfully reprogrammed differentiated mouse somatic cells by ectopic expression of four transcriptionmodulators are needed for overcomin (Oct4, Sox2, Klf4, and c-Myc); this de-differentiation process (or induction of pluripotency) allowed somatic cells to regain the undifferentiated pluripotent state of cells. Those cells are termed induced pluripotent stem (iPS) cells. This work inspired many researchers to discover the underlying mechanisms, and soon iPS cells were reported in multiple species. It took about 3 years to produce iPS cells in porcine. Since 2009, about 70 or more research articles were on porcine iPS cells covering different aspects such as different approaches for derivation of iPS cells and their regenerative biology applications. In this chapter, we attempted to summarize the general concept of reprogramming and elaborated on the current status of porcine iPS cells and discussed the way forward for their future use.

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17.1 Introduction

Shinya Yamanaka and his group published their landmark research article on the induction of pluripotency in mouse fibroblasts in the year 2006 (Takahashi and Yamanaka 2006). In this chapter, the group reported the identification of four specific transcription modulators are needed for overcomin, namely, Oct3/4, Sox2, c-Myc, and Klf4 (also collectively designated as Yamanaka Factors in many recent articles) and demonstrated that after about 2 weeks of overexpression of these factors, mouse fibroblasts gained the features of embryonic stem (ES) cells. Embryonic stem cells are pluripotent stem cells with certain features: (a) they have the ability to self-renew in vitro; (b) they are capable of differentiating in vitro to tissues belonging to three main germ layers (ectoderm, endoderm, and mesoderm); and (c) when injected into developing embryo, ES cells contribute to the embryo proper. Cells produced from mouse fibroblasts in the Yamanaka Laboratory had similar growth, and colony features as that of ES cells had undergone differentiation and produced tissues of three germ layers. In addition, those ES-like cells were shown to produce teratoma when injected subcutaneous to immunocompromised nude mice and were proven to be germline competent. Therefore, Yamanaka termed these ES-like cells as induced pluripotent stem (iPS) cells (Takahashi and Yamanaka 2006). The importance of the work was immediately felt in the community of biomedical researchers. Such impact is evident by continual publications of a number of research articles in iPS cells (till date, PubMed database reports over 19,000 publications since 2006). Induction of pluripotency occurred in the Yamanaka experiment due to reprogramming in the somatic cells. Terminally differentiated somatic cells from adult tissues are usually incapable of multiplying. Still, reprogramming brings them to a developmentally early state, and they regain their ability to self-renew and differentiate. The Yamanaka Laboratory's work impacted the field in several ways. It reaffirmed the work on reprogramming by John Gurdon in the late 1950s (Gurdon et al. 1958), provided tangible tools for reprogramming using those specific transcription factors. The work also devised the method for the generation of patient-specific stem cells to produce the required tissues, and thus, opened up the era for personalized medicine, overcoming the immunological barrier of cell therapy. Also, screening of potential drugs using tissue-specific cells produced from cultured stem cells became a reality in regenerative medicine. It also paved a way to study developmental disorders inaccessible otherwise. In many ways, iPS cells were considered to be another alternative to ES cells. Generation of embryonic stem cells following traditional methods [such as by somatic cell nuclear transfer (SCNT)] is technically more elaborative, involved multisteps, far more complex, and highly challenging. The use of SCNT to generate ES cells also requires more resources than otherwise required for iPS cell generation. Thus, overexpression of a limited number of transcription factors in somatic cells has become an attractive and a pivotal tool for the generation of iPS cells, similar to ES cells.

Authentic ES cell lines could be established from developing blastocysts in a number of species such as mice, humans, and most domestic animals. Unlike those

species, currently available blastocyst-derived porcine cells do not strictly adhere to all the features that define authentic ES cells (Evans et al. 1990; Chen et al. 1999; Alberio et al. 2010). Those porcine ES cells were isolated and maintained following the same methodologies and culture conditions used previously for murine or human ES cells. It is suspected that the procedures possibly have never worked for the porcine species, and this has been considered as the most plausible cause for the failure of the derivation of authentic ES cells in pigs (Telugu et al. 2010). One way to deal with the issue is to find the porcine-specific methodology and derive the ES cells. However, it should take its own course of time to attain the target. In the meantime, the invention of the procedure to reprogram somatic cells to the pluripotent state became very much useful for porcine and other ungulates. Since the first report on mouse iPS cells in 2006 from the Yamanaka Lab, it took about 3 years to apply the same technique and to generate iPS cells in swine. There were simultaneous three publications in 2009: two from China (Esteban et al. 2009; Wu et al. 2009) and the other one from the United States (Ezashi et al. 2009). Those articles from different laboratories clearly demonstrated the universality and effectiveness of the Yamanaka methodology for the generation of iPS cells. Since then, several reports on porcine iPS cells were published from different laboratories. We discuss here briefly the genesis of reprogramming as a tool for iPS cell generation, mechanisms involved in the process of reprogramming. Further, we review the current status of available porcine iPS cells and their therapeutic/other applications.

17.2 Brief History Toward the Generation of Induced Pluripotent Stem Cells

Since the 1950s, seminal advances or breakthroughs of technologies have happened, and these have helped in the understanding of cellular/developmental biology deeper and better. This progress contributed immensely toward the generation of induced pluripotent stem cells. Few remarkable ones may be specially cited here. For example, the invention of the somatic cell nuclear transfer (SCNT) technique allowed the study of the cell's developmental potential (Briggs and King 1952; King and Briggs 1955). Also, the isolation of *pluripotent* embryonal carcinoma cells (ECCs) from a solid tumor of germ cells (teratocarcinoma) was another significant development. It was demonstrated further that the fusion of ECCs with fully differentiated somatic cells such as thymocytes enabled the somatic cells to gain the features of pluripotent cells (Miller and Ruddle 1976).

The next significant development in the field had been the discovery of *assay* determining the developmental potential of putative pluripotent stem cells and their ability to contribute to all three germ layers. This was achieved by injection of cells into blastocysts followed by transfer to foster recipients. Availability of this process also led to successful isolation of other pluripotent stem cells, that is, embryonic stem (ES) cells from mice (Evans and Kaufman 1981; Martin 1981) and human (Thomson et al. 1998) blastocysts. ECCs had abnormal karyotype, mostly aneuploid, but ES cells possessed normal karyotype and also capable of contributing to

all somatic lineages. Further, similar to ECCs, these pluripotent ES cells could be used for the production of fusion hybrids with somatic cells (Tada et al. 2001; Cowan et al. 2005). In these fusion hybrids, somatic cells acquired the biochemical and developmental potential of pluripotent ES cells. Experiments with fusion hybrids suggested the existence of identifiable soluble trans-acting factors in both ECCs and ES cells. The success of experiments with SCNT also demonstrated the presence of similar factors in the cytosol of the unfertilized oocyte, and these factors were capable of conferring pluripotency/totipotency to somatic cells (Wilmut et al. 1997).

Subsequently, improved techniques allowed isolation of a variety of pluripotent stem cells from different sources, such as cloned blastocysts produced by SCNT (Cibelli et al. 1998), and also from other embryonic and adult tissues (for references see Stadtfeld and Hochedlinger 2010). Each of these pluripotent stem cells of different origin expresses a common endogenous transcription factor, Oct4, a key molecule used by the Yamanaka group later for induction of pluripotency in somatic cells.

Long ago, it was established that the ectopic expression of a specific transcription factor might cause a change in cell/lineage fate. For example, the expression of skeletal muscle-specific transcription factor MyoD in fibroblasts resulted in the formation of myofibers (Davis et al. 1987). In this case, both fibroblast and myofibers belong to the same embryonic germ layer, that is, mesoderm. The same concept was extended to produce tissues of different lineage, currently known as trans-differentiation (Vierbuchen et al. 2010; Nizzardo et al. 2013; Cieslar-Pobuda et al. 2017; Flitsch and Brustle 2019), mostly reported after the publication of Yamanaka's 2006 iPS paper (Takahashi and Yamanaka 2006). Most importantly, the concept that transcription factor(s) may regulate cell fate had provided an "intellectual framework" (Stadtfeld and Hochedlinger 2010) and an invaluable contribution toward the generation of induced pluripotent stem cells. Then, the challenge was to identify those trans-acting factors relevant for somatic cell reprogramming.

The Yamanaka group took the challenge to identify those trans-acting factors, presumably involved in reprogramming of both somatic cells and nuclei, respectively, in fusion hybrids and SCNT experiments. To prepare the list of putative transcription factors, the group considered these criteria: (a) genes involved the maintaining pluripotency in early embryos, and ES cells are Oct4, Sox2, Nanog; (b) genes that are highly expressed in tumor cells, and also that contribute in long-term maintenance ES cell phenotype/involved in proliferation such as, Stat3, E-Ras, c-Myc, Klf4, and b-catenin; and (c) ES cell-specific genes such as Nanog, Sox2, and Sox15. Thus, the group tested a total of 24 transcription factors and finally identified four specific factors (Oct4, Sox2, Klf4, and c-Myc or OSKM) that were sufficient to induce pluripotency in somatic cells (Takahashi and Yamanaka 2006). This work, in turn, inspired several groups to investigate the underlying mechanism of reprogramming using these factors. Major breakthroughs that had outstanding contributions to the creation of Yamanaka are summarized in Table 17.1. Now, in the next section, we discuss briefly the basis of reprogramming using transcription factors that allowed the generation of iPS cells.

Table 17.1 Significant milestones in the field of reprogramming leading to the generation of induced pluripotent stem cells

Year	Report	Reference
1952–1955	Establishment of technique: somatic cell nuclear transfer (SCNT)	Briggs and King (1952), King and Briggs (1955)
1958	Sexually mature *Xenopus laevis* from the transplantation of a single somatic cell nucleus	Gurdon et al. (1958)
1962	Demonstration that differentiated amphibian cells retain the genetic information necessary to support the generation of cloned frogs	Gurdon (1962)
1954, 1964	Establishment of immortal pluripotent cell lines called embryonal carcinoma cells from a teratocarcinoma, tumors of germ cell origin	Stevens and Little (1954), Kleinsmith and Pierce (1964)
1967, 1970	Clonally expanded in culture while retaining pluripotency	Finch and Ephrussi (1967), Kahan and Ephrussi (1970)
1987	MyoD overexpression in fibroblast produced myofibers	Davis et al. (1987)
1968	Teratoma assay	R. L. Gardner (1968)
1981	Isolation of embryonic stem cells in mouse	Evans and Kaufman (1981), Martin (1981)
1996	Cloning of Dolly, the sheep, from somatic cells	Wilmut et al. (1997)
1998	Isolation of embryonic stem cells in human	Thomson et al. (1998)
1998	Transgenic bovine chimeric offspring derived from somatic cell-derived stem-like cells	Cibelli et al. (1998)
1998–2005	Identification of transcription factors related to self-renewal and pluripotency in ES cells and in teratocarcinoma cells	Oct4 (Nichols et al. 1998), Sox2 (Avilion et al. 2003), Nanog (Chambers et al. 2003), Stat3 (Niwa et al. 1998), c-Myc (Cartwright et al. 2005), beta-Catenin (Kielman et al. 2002), Klf4 (Li et al. 2005)
2006	Generation of induced pluripotent stem cells	Takahashi and Yamanaka (2006)

17.3 Basis of Reprogramming Using Genetic Factors

17.3.1 Role of Individual Factors in Reprogramming

To understand how Yamanaka factors (OSKM) induce pluripotency in somatic cells, investigators conducted different studies to identify the role of individual factors in reprogramming. Results of these studies reveal interaction and targets of these factors. These factors were found to co-bind to promoters of as many as 565 genes and were involved in regulating (for both repression and stimulation)

16 developmental signaling pathways (Liu et al. 2008; Huang et al. 2009). The four factors had overlapping targets in both iPS and ES cells. Among these factors, c-Myc was found to act prior to activation of pluripotency regulators (Sridharan et al. 2009), and c-Myc was also identified to facilitate engagement of the remaining three factors (Oct4, Sox2, and Klf4) at promoters of genes involved in reprogramming (Soufi et al. 2012).

Thus, out of four factors, three (Oct4, Sox2, and Klf4) were considered as core factors required to bind to activate the pluripotency network. Klf4 interacts directly with Oct4 and Sox2, and these interactions were sufficient to induce pluripotency in somatic cells (Wei et al. 2009). Recently, it is revealed that stoichiometry and cooperativity between Klf4 and Sox2 are necessary for activation of the pluripotency network (An et al. 2019). Furthermore, ectopic expression of all four factors brings about changes not only in the expression of target genes but also remodels chromatin state and genome topology in the cells during reprogramming (Apostolou and Stadtfeld 2018).

It is further found that Oct4 is also required for mesenchymal–epithelial transition (MET), a key event during reprogramming. The open chromatin is facilitated predominantly by Sox2 with an accessory role of Oct4. Oct4/Sox2 heterodimers (Malik et al. 2019) and relevant conformations for the establishment of pluripotency were identified (Tapia et al. 2015). Oct4 alone was sufficient to reprogram human somatic cells to iPS cells when used and some chemicals (Zhu et al. 2010). However, subsequently, iPS cells were generated either with three or two factors but without using exogenous Oct4 (Montserrat et al. 2012; An et al. 2019; Velychko et al. 2019). Cells generated using three factors (Klf4, Sox2, and c-Myc) were free from abnormal imprinting, usually found in iPS cells generated by using Yamanaka factors (Velychko et al. 2019). In summary, each of the factors plays a distinct role during reprogramming. It would be curious to look at how activities identified for individual factors play a role in the global or genome-wide context.

17.3.2 Genome-Wide Sequential Events for Establishment and Maintenance of Pluripotency by OSKM Cocktail

Data from genome-wide chromatin assay experiments, epigenomics, and transcriptomics studies collected during iPS cell generation reveal that the presence of OSKM inside somatic cells first erases differentiated cells' identity. It activates a set of genes needed for the establishment of pluripotency, and finally, reorganization of chromatin structure occurs for maintenance of pluripotency. For a fuller description, it is recommended to refer to Apostolou and Stadtfeld (2018) or to Hochedlinger and Jaenisch (2015). Here, a summary is presented in the entire process.

17.3.2.1 Somatic Program Silencing

It is now known that available OSKM factors' access to chromatin structure around somatic loci and, in turn, turns off somatic gene expression. OSKM can silence the

somatic program directly by (a) displacement of somatic transcription factors (TFs) and/or (b) recruitment of co-repressors, such as HDAC1. OSKM also can reposition/redistribute somatic TFs to newly accessible genome sites. Additionally, just OKS has the ability to activate a co-repressor, such as Sap30. Both these events (somatic TFs redistribution and activation of Sap30) can indirectly cause silencing of the somatic program (Apostolou and Stadtfeld 2018).

17.3.2.2 Stem Cell Program Activation

Initiation of the pluripotency program in the somatic genome is a rare and rate-limiting event. Here, in the absence of critical co-factors, OSKM is either unable or insufficient to bind to the genome, and therefore fails to induce activation of pluripotent genes. Those regions in the genome are "refractory" and thus do not provide access to OSKM. In addition, many stem cell-specific regulators are found in these "refractory" regions, and histone 3 (H3) chromatin protein of these regions is highly methylated in lysine at position 9 (H3K9). Experimental data suggest that downregulation of H3K9 methyltransferases (such as G9A, GLP, SETDB1, SUV39H1, and SUV39H2) improves the efficiency of reprogramming, indicating methylated H3K9 acts as a prohibitory signature against reprogramming. However, too much demethylation also affects reprogramming adversely. Intervention resulting in relaxation of the somatic chromatin enables increased accessibility and binding of SOX2 to pluripotency-related enhancers and super-enhancers. Evidence also strongly suggests that early binding of OSK (Myc to a lesser extent) occurs predominantly on "inaccessible" regulatory elements, including nucleosomal and DNA methylated regions. Further, the co-binding of OSK during early reprogramming activates target gene loci seemingly in a context-dependent manner. OSKM activity also depends on specific critical co-factors linked to chromatin remodeling (e.g., SWI/SNF subunits), epigenetic modulation (e.g., BRD4 and MLL), and factors involved in the release of the paused polymerase (e.g., CDK9, P-TEFb, etc.) (Apostolou and Stadtfeld 2018).

17.3.2.3 Re-organization of Chromatin Architecture

The chromatin topology of cells determines the morphology and functions of cells. During reprogramming, chromatin topology gets reset while somatic cells are induced to become pluripotent stem cells. Evidence suggests that chromatin loops are established around pluripotency-associated loci during reprogramming, and this usually precedes or coincides with transcriptional change. Thus, chromatin reorganization and timing is linked to changes in gene expression, supporting a potential causal role for reprogramming. These topological changes are brought about directly by OSKM binding to reorganized regions. OSKM may also be involved indirectly in histone modification such as H3K4me2, and such changes occur prior to conformational alterations. The role of OSKM in local and global chromatin reorganization would be clear by identifying the OSKM-dependent and OSKM-independent mechanisms during somatic cell reprogramming (Apostolou and Stadtfeld 2018).

It is to emphasize that methylation patterns of both histones and DNA are globally reset during reprogramming. Repressive histone modifications [e.g., histone H3 lysine 27 trimethylation (H3K27me3)] and DNA methylation are responsible for

the silencing of pluripotency genes (such as Oct4 and Nanog) in somatic cells. These repressive marks are replaced by the activating histone marks [such as H3 lysine four trimethylations (H3K4me3)], and also repressive DNA methylation signature in the promoter of Oct4 gene is removed in pluripotent cells. Thus, overall histone modifications and DNA methylation landscapes are restored in authentic iPS cells. Histone lysine demethylase, UTX, removes repressive H3K27 methylation, and WD repeat domain 5 (Wdr5) regulates the activating H3K4 methylation. In addition, the absence of maintenance methyltransferase (Dnmt1) (usually achieved by supplementation of 5′aza-cytidine in culture during derivation of iPS cells) improves the efficiency of reprogramming. However, the lack of activities of de novo methyltransferases (Dnmt3a and Dnmt3b) does not impact reprogramming efficiency. Overall, removal of DNA methylation promotes reprogramming, possibly via the release of repression of pluripotency genes such as Oct4 and Nanog (Hochedlinger and Jaenisch 2015).

Thus, many different kinds of changes take place in a short span of time during reprogramming, yet only a few cells attain the state of pluripotency. One, therefore, wonders how to follow these events during reprogramming and what might be the reasons that restrict the majority of cells to attain the state.

17.3.3 Kinetics of Molecular Signatures During Somatic Cell Reprogramming

Based on studies primarily in mouse fibroblast reprogramming, molecular signatures of some of the key events are currently known. It is now accepted that the expression of four factors is required till fibroblasts reach a stable, self-sustaining pluripotent state. There exist several intermediate stages between somatic fibroblast and fully reprogrammed pluripotent cells. Early intermediates are produced from fibroblasts ($Thy1^{+}/SSEA1^{-}$), and these cells at a certain initial point cease expressing Thy1and become $Thy1^{-}/SSEA1^{-}$ (Stadtfeld et al. 2008). Downregulation of somatic cell markers is accompanied by changes in cell morphology and occurrence of mesenchymal–epithelial transition (MET). MET is regulated by multiple factors: (a) BMP-dependent miR205 and miR200 family of microRNAs serve as key regulators (Samavarchi-Tehrani et al. 2010) and (b) role of key transcription factors: "Sox2/Oct4 suppress the epithelial–mesenchymal transition (EMT) mediator Snail, c-Myc downregulates TGF-beta1 and TGF-beta receptor 2, and Klf4 induces epithelial genes including E-cadherin" (Li et al. 2010). Subsequently, a subset of $Thy1^{-}/SSEA1^{-}$ cells starts expressing SSEA1, and gradually the population is enriched with $Thy1^{-}/SSEA1^{+}$ cells (Stadtfeld et al. 2008), expressing other early pluripotency markers such as alkaline phosphatase. Then, late pluripotency markers such as endogenous Sox2/Oct4 and Nanog are reactivated. This event is accompanied by the activity of telomerase enzyme and removal of silencing of X chromosomes (Li et al. 2010; Hochedlinger and Jaenisch 2015). These sequential events with key molecular markers are presented in a schematic diagram (Fig. 17.1a).

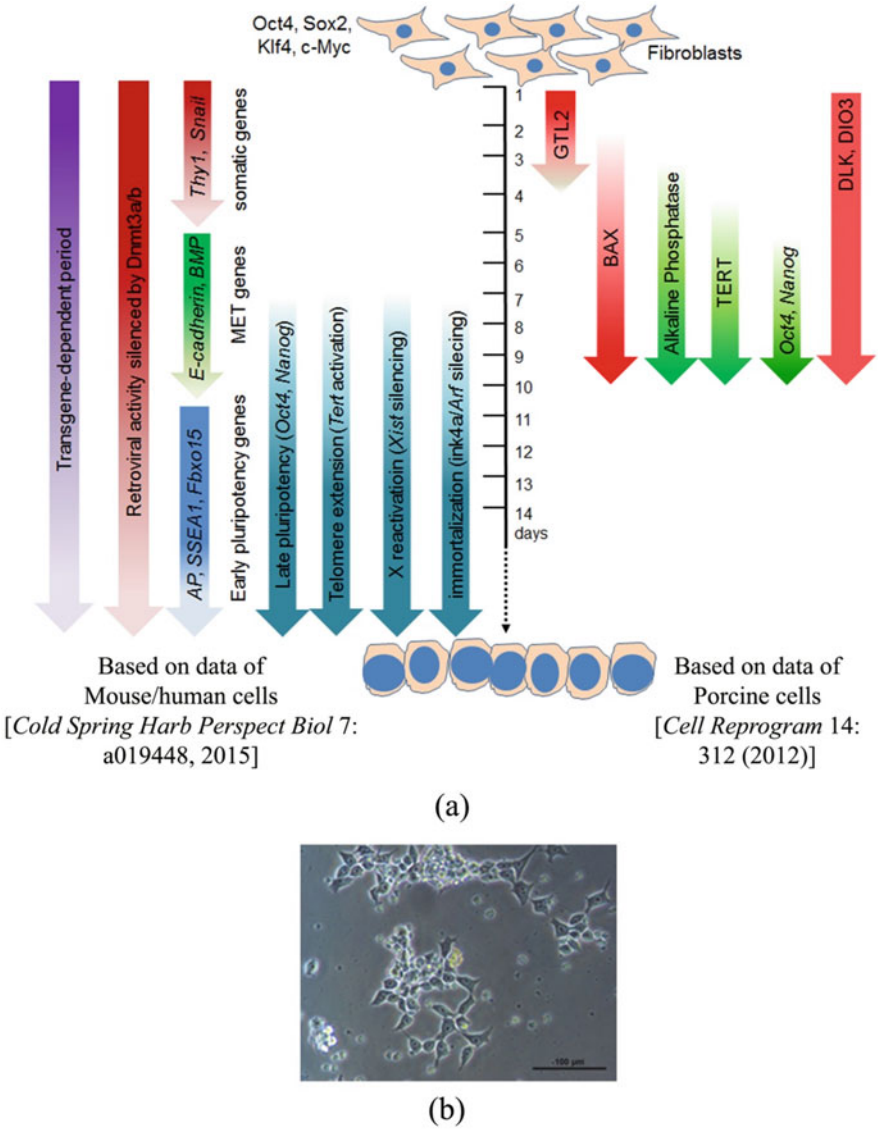

Fig. 17.1 Kinetics of reprogramming events and porcine-induced pluripotent stem cells. (**a**) Schematic diagram showing temporal expression of genes signifying transition of fibroblasts toward generation of induced pluripotent stem cells during reprogramming (left panel shows data from studies on mouse/human cells, right panel represents data from porcine ell reprogramming); (**b**) porcine-induced pluripotent stem cells produced from bone marrow-derived mesenchymal stem cells using doxycycline-inducible system regulating expression of lentivirally transduced four Yamanaka transcription factors

17.3.4 Elite and Stochastic Models for Induced Pluripotent Stem Cell Generation

It is now accepted that reprogramming of somatic cells occurs at a very low efficiency ranging between 0.001% and 0.01%. In order to explain the possible reasons, Yamanaka (2009) proposed two different models: deterministic and stochastic (Yamanaka 2009). The somatic cell requires to undergo a certain number of cell divisions, accumulating the required epigenetic changes to attain the state of pluripotency. In the deterministic model, the number of cell divisions (latency) is considered to be fixed or constant, whereas, for the stochastic model, this number varies from one to the other cells. Under each model, either all or a few selected elite cells would finally reach the state of pluripotency. It is often noted that a few cells in the founder population plated for reprogramming are less differentiated, and these cells are expected to reach the state of pluripotency with a lesser number of cell divisions. Further, within those few cells, the process of reprogramming is likely to be random. Thus, experimental data is consistent with the stochastic model, and the process is influenced by multiple factors like the differentiation stage of the founder cells (e.g., adult stem cells are more amenable than fully differentiated somatic cells), supplementation of other transcription factors (four factors versus six factors), chromatin regulators, growth factors (supporting the cell survival), and the interplay of microRNAs during reprogramming (Theunissen and Jaenisch 2014; Hochedlinger and Jaenisch 2015).

17.4 Porcine-Induced Pluripotent Stem Cells

The innovation of methodology for the generation of induced pluripotent stem (iPS) cells in mice soon resulted in the generation of iPS cells in other species. The different strategies employed could be broadly classified into two categories: (a) transgene-mediated reprogramming and (b) chemical reprogramming. The first approach included the use of transcriptional inducers (such as Yamanaka factors, Nanog, Lin28, Sall4, Glis1, Dppa2, Esrrb, Utf1), epigenetic inducers (Rcor2, TH2A, Tet1), miRNAs as inducers (miR-302, miR-367, miR-200c, miR-302s, miR-369s), and lineage specifiers (such as ectodermal or mesendodermal specifiers). On the other hand, in chemical reprogramming strategy, small-molecule inhibitors of different signaling pathways (such as TGF-b, GSK3, SAHH, and MEK), demethylases (Jhdm1a/b), chemicals that affect methylation (such as vitamin C), and histone deacetylase (HDAC) inhibitors [such as valproic acid (VPA)] were used. For details on all these approaches, including the replacement of classical Yamanaka factors, multiple articles are available (reviewed in Theunissen and Jaenisch 2014).

Having a standard protocol available, three different laboratories attempted to generate iPS cells in porcine. Each group described that porcine ES cells were not available (at the time of publication of their reports), and iPS cells would help meet up the need of porcine ES cells. In a close race, they reported their success between June and July of 2009 (Esteban et al. 2009; Ezashi et al. 2009; Wu et al. 2009). All

three groups used transcription factors of human origin; either six transcription factors (Yamanaka factors plus, Nanog, and Lin28) (Wu et al. 2009) or four Yamanaka factors (Esteban et al. 2009; Ezashi et al. 2009). The transcription factors were delivered to somatic cells [fibroblasts and bone marrow cells (Wu et al. 2009) or just fibroblasts (Esteban et al. 2009; Ezashi et al. 2009)] either by retroviral (Esteban et al. 2009) or by a lentiviral delivery system with (Wu et al. 2009) or without (Ezashi et al. 2009) using doxycycline for triggering the expression of exogenous factors. In fact, Wu et al. abandoned the retroviral system due to failure of transduction of pig ear fibroblasts and switched to a lentiviral delivery system (Wu et al. 2009). Putative iPS cells were cultured on mouse feeder cells in media supplemented with either LIF (Esteban et al. 2009) or FGF2 (Ezashi et al. 2009) or no additional cytokine (Wu et al. 2009). iPS cells produced by three groups expressed alkaline phosphatase, Nanog, and had high telomerase activity. The expression of Oct4 (Ezashi et al. 2009; Wu et al. 2009), Sox2 (Ezashi et al. 2009; Wu et al. 2009), Rex1 (Esteban et al. 2009; Wu et al. 2009), and SSEA-4 (Esteban et al. 2009; Wu et al. 2009) was found in cells from two laboratories. Uniquely, SSEA-3 expression was noted only in cells generated by Wu et al. (2009) and SSEA-1 in cells from Michael Roberts's laboratory (Ezashi et al. 2009). Based on the marker profiles, cells derived by the two Chinese groups were, to some extent, similar to human ES cells, and cells reported by the other group were identical to mouse ES cells. Cells from all laboratories were successfully differentiated to tissues of three germ layers both in vitro and in vivo (teratoma formation). Ezashi et al. reported sustained transgene expression even after differentiation of iPS cells (Ezashi et al. 2009). These authors envisaged the usefulness of porcine ES cells in generating gene-modified pigs, also to study certain human diseases or assess therapeutic applications as large animal models. Further, they can work as model systems for testing the safety and efficacy of stem cell-derived tissue grafts (Esteban et al. 2009; Ezashi et al. 2009; Wu et al. 2009).

Following these three major publications, as of date, a little over 70 research articles are available on porcine iPS cells. Here, we summarize the information available on these newly generated porcine iPS cells in terms of different aspects of reprogramming. Further, we also note here that the citation of literature presented in this article is not exhaustive; instead, it is aimed to reflect the significant progress in the field of research on porcine iPS cells.

Generation of porcine iPS cells reported by different laboratories was achieved using various methods, and each of those iPS lines had features of pluripotent cells to a certain extent but failed to generate stable germline competent chimera. Therefore, many of these iPS cells may not be genuine/authentic iPS cells. In developing the accurate iPS cells, obstacles encountered in the process could be numerous, such as (a) incomplete epigenetic reprogramming, (b) inferior culture conditions, and (c) use of varying serum and supplements, for example, cytokines, LIF, FGF2, epigenetic modifiers, and signal pathway inhibitor. To get an overall idea of these cells, here we discuss some of these issues briefly.

17.4.1 Choice of Reprogramming Factors

Yamanaka transcription factors (Oct4, Sox2, Klf4, and c-Myc) were most frequently used for the generation of porcine iPS cells (Cheng et al. 2012a; Fujishiro et al. 2013; Zhang et al. 2015; Park et al. 2016; Secher et al. 2017). Since Thomson's group reported successful generation of human iPS cells using Oct4, Sox2, Nanog, and Lin28 (Yu et al. 2007), these additional factors (i.e., Nanog and Lin28) were also combined with four Yamanaka factors giving a total of *six* factors for the generation of porcine iPS cells (West et al. 2010; Fukuda et al. 2017; Kwon et al. 2017). One of these iPS lines (produced with six factors and maintained in medium with FGF2 and on mouse feeder cells) was used successfully to create germline-competent chimeric pig (West et al. 2010, 2011), and this remained the only report demonstrating germline-competent pig iPS cells that produced adult animals without any additional treatment (such as differentiation of iPS cells prior to use as nuclear donor or treating nuclear-transferred embryos with HDAC inhibitor) (West et al. 2011). Porcine iPS cells were also produced using *seven* factors by adding the Large T antigen of the SV40 virus to six factors mentioned before (Telugu et al. 2010). Yamanaka factors plus Lin28 (Chakritbudsabong et al. 2017) or TERT (Gao et al. 2014) resulting in *five* factors were also successful in producing porcine iPS cells. Using *three* factors (Sox2, Klf4, and c-Myc), it was possible to generate porcine iPS cells, dispensing Oct4. Apparently, this was the first report of somatic reprogramming in any species without the overexpression, either directly or indirectly, of Oct4. Moreover, cells thus generated could be grown in a feeder cell-free culture system (Montserrat et al. 2012). Knocking down of Klf4 and c-Myc in iPS cells resulted in the loss of pluripotency (Liao et al. 2018a), indicating the crucial roles of those factors.

Small-molecule pathway inhibitors (such as PD0325901 and CHIR99021) were used to improve reprogramming and four Yamanaka factors (Zhang et al. 2015; Secher et al. 2017). PD0325901 is a selective, cell-permeable non-ATP-competitive inhibitor of the MEK/ERK signaling pathway, whereas CHIR99021 is a glycogen synthase kinase (GSK) 3 inhibitor. GSK3 is a serine/threonine kinase, a key inhibitor of the WNT pathway; therefore, CHIR99021 functions as a WNT activator. Both these inhibitors were also used for reducing the number of factors during the reprogramming of fibroblasts. For example, Liu et al. (2012) used only two factors (Oct4 and Klf4) and successfully generated iPS cells (Liu et al. 2012). Fibroblasts electroporated with episomal vectors containing Yamanaka factors resulted in more naïve iPS cells grown in a particular medium containing growth factors (FGF2, LIF) and inhibitors of MAPK14, MAPK8, TGFB1, MAP2K1, GSK3A, and BMP (Yuan et al. 2019).

Blocking MEK signaling enhanced the proportion of NANOG (indicative of the epiblast)-positive cells but did not prevent the segregation of GATA-4 (indicative of the hypoblast)-expressing cells in the inner cell mass (ICM). Interestingly, inhibition of FGF signaling reduced the number of ICM cells without altering the segregation of NANOG and GATA-4 cells, indicating FGF signaling's participation in the formation of the founders of the ICM.

Inhibition of MEK signaling combined with GSK3-beta inhibition and LIF supplementation to culture conditions helped pig iPS cells acquire naive pluripotency features (see detail on types of pluripotency in a later section). Pigs' iPS cells were characterized by the expression of STELLA and REX1, and increased in vitro germline differentiation capacity (Rodriguez et al. 2012). Thus, small-molecule inhibitors can be used to improve the homogeneity of induced pluripotent stem cells and help in the generation of germline-competent stem cells in swine.

Among the growth factors and cytokines, leukemia inhibitory factor (LIF) is of prime interest in stem cell biology. It is accepted that only the naive-type pluripotent stem cells can produce chimeric offspring, and these cells are LIF-dependent. LIF-dependent iPS cells were generated using either four Yamanaka factors (Fujishiro et al. 2013) or six factors (Yamanaka factors plus Nanog and Lin28) (Kwon et al. 2013), and those cells were shown to contribute to fetal development (Fujishiro et al. 2013).

Attempts were also made to find additional factors to substitute or to use in combination with the original Yamanaka factors. Estrogen-related receptor B (ESRRB), an orphan nuclear receptor, is one such factor. It is a direct transcriptional target of Nanog in mouse ES cells and can replace Nanog. Similarly, T box transcription factor, Tbx3, is another factor that improved germline competency of mouse iPS cells. Another nuclear receptor, Nr5a2 (also known as *Lhr-1* or liver receptor homolog-1), could replace exogenous Oct4 during reprogramming of mouse somatic cells. For reprogramming of porcine cells, the addition of ESRRB (Yang et al. 2018) and two other factors (Tbx3 and Nr5a2) (Wang et al. 2013) promoted reprogramming and self-renewal of porcine iPS cells, similar to the case with mouse cell reprogramming. The intracellular domain of epithelial cell adhesion molecule (EpCAM) enhanced reprogramming in porcine fibroblasts via activation of beta-catenin signaling (Yu et al. 2017).

Further addition of epigenetic modifiers such as *Tet1* (Ten-Eleven Translocation) to culture media during reprogramming could significantly enhance iPS cells' derivation with higher levels of expression of pluripotent genes such as *Rex1* (Mao et al. 2017). Long noncoding RNAs (lncRNA) were involved in the transcriptional regulation of somatic reprogramming to pluripotency (Zhong et al. 2018). The reprogramming of cells using miRNA could enhance the generation of porcine iPS. Overexpression of miR-302a, miR-302b, and miR-200c could make the reprogramming more efficient and faster. Therefore, it was suggested to replace c-Myc with these microRNAs (miR-302a, miR-302b, and miR-200c) to reduce porcine iPS tumorigenicity cells (Ma et al. 2014). Similarly, pluripotency could be induced in fibroblasts by epigenetic resetting with extract of porcine germinal vesicle stage oocytes (Bui et al. 2012), though factors present in the extract were not identified.

All in all, porcine-induced pluripotent stem cells were produced with two, three, four (original Yamanaka factors), five, six, or seven factors. One study attempted to dissect the implication of the generation of iPS cells using four (Yamanaka) factors versus six factors (Yamanaka factors plus Nanog and Lin28). Transcriptome analysis revealed that iPS cells, when derived with six factors, belonged to independent

clusters compared to those derived from four factors; those cells produced with six factors were distant from fibroblasts. Further, the expression of various naïve-specific genes was relatively elevated in pig iPS cells derived from six factors (Fukuda et al. 2019), indicating that six factors may preferentially be used for derivation of iPS cells.

Identification of critical transcription factors required for reprogramming also has a certain impact on the derivation of putative ES cells from a porcine blastocyst. In the laboratory of Michael Roberts, Oct4 and Klf4 were overexpressed in the porcine blastocysts, and then LIF-dependent naïve-type mouse ES cell-like cells were isolated from the inner cell mass (Telugu et al. 2011). The attempt carries enormous significance, specifically when authentic ES cells from porcine species are not available.

17.4.2 Choice of the Delivery System

Since the Yamanaka group used retroviruses for delivering Oct4, Sox2, c-Myc, and Klf4 (OSKM) to somatic cells, most groups used the same delivery system to generate porcine iPS cells (Fujishiro et al. 2013; Ji et al. 2013; Zhang et al. 2015; Chakritbudsabong et al. 2017; Mao et al. 2017). Lentivirus delivery system for generation of iPS cells also remained a popular method because of high transduction efficiency (Wu et al. 2009; Fukuda et al. 2017; Kwon et al. 2017; Luo et al. 2017). Pseudo lentiviral particles were suitable to deliver the required factors to a wide variety of cell types. To regulate expression stringently, transgenes were placed under the doxycycline-inducible system (Tet operator), and the same was primarily combined with the lentiviral delivery method (Wu et al. 2009; Luo et al. 2017; Secher et al. 2017), approaching a highly reliable one (Fig. 17.1b, unpublished data from the laboratory of the lead author). As transgenes delivered by retro/lentiviral systems get integrated into the genome, it was speculated to affect the biology of the cells, including disruption of genes involved in pluripotency/differentiation. Further, retroviral elements are considered tumorigenic, and these elements, when integrated into the genome, are known to be targeted for methylation and thereby silencing the transgenes.

Mobile genetic elements transfer DNA to the genome by transposing between vectors and chromosome via a “cut and paste” mechanism using transposase enzyme. Such systems were recognized as alternatives to viral transduction for delivering transgenes into the genome. Both piggyBac (PB) (with four Yamanaka factors) (Kim et al. 2019b) and sleeping beauty (SB) transposon vectors with either six (Petkov et al. 2013) or four Yamanaka (Kues et al. 2013) reprogramming factors were successfully used to deliver and finally to generate porcine iPS cells. Nuclei from porcine iPS cells derived by utilizing the piggyBac system were successfully used to produce transgenic embryos (Kim et al. 2019b). It may be noted that transposase enzymes transpose cargo containing transgenes into the target chromosome at TTAA and TATA site by PB and SB transposases. For SB vectors, CAG or EF1a promoters were adequate, but not with the TetO promoter (Petkov et al. 2013),

and Kues et al., used a single polycistronic construct with all four factors (Kues et al. 2013). Transfection of a CAG-driven polycistronic plasmid expressing Yamanaka factors showed higher efficiency and reprogramming compared with three consecutive retroviral transductions of a similar polycistronic construct (Montserrat et al. 2011). Though these iPS cells were generated without the presence of viral vectors, the transgenes were integrated into the host genome. One could have overexpressed specific transposases to remove the integrated sequences from iPS cells' genome, similar to that done with mouse iPS cells (Yusa et al. 2009). Another alternative to generate transgene integration-free iPS cells is to use episomal vectors. Reports indicate that integration-free porcine iPS cells were produced by using episomal vectors electroporated into pig fibroblasts (Li et al. 2018a; Yuan et al. 2019).

Overall, with the advances in technology, one has several options to choose a suitable delivery system. The specific purpose of the study and utility of the cells should be the prime consideration for determining the delivery system type.

17.4.3 Choice of Somatic Cells to Be Reprogrammed

Fibroblasts are highly active, and one of the most common cells of primitive mesenchyme origin found in connective tissue. These cells are proliferative and easily available from multiple sources, and primary fibroblasts are used in different kinds of biological experiments. For a generation of porcine iPS cells, fibroblasts were most frequently used from multiple sources such as embryonic (Fujishiro et al. 2013; Zhang et al. 2015; Chakritbudsabong et al. 2017; Fukuda et al. 2017; Mao et al. 2017), fetal (Petkov et al. 2013, 2016; Luo et al. 2017; Li et al. 2018a) or postnatal (Kwon et al. 2017) origin. But reprogramming was not highly successful with fibroblasts, and heterogeneity of fibroblasts was suspected to be the reason for the poor efficiency. Li et al. (2017) recently reported that stage-specific embryonic antigen 1 (SSEA1)-positive embryonic fibroblast in Danish Landrace and Gottingen mini pig had a better ability to generate iPS cells compared to SSEA1-negative fibroblasts (Li et al. 2017).

Other than fibroblasts, adult stem cells were also used as somatic donor cells for reprogramming. It was thought that owing to their stem cell characters, adult stem cells would be easier to reprogram. Thus, adult stem cells such as bone marrow-derived mesenchymal stem cells (MSC) (West et al. 2010) and adipose tissue-derived stem cells (ADSC) (Zhang et al. 2014) were successfully used for reprogramming. It was reported that reprogramming of iPS cells from porcine ADSCs was more efficient than from fibroblasts (Zhang et al. 2014). Similarly, for transcription factor-mediated reprogramming, the reprogramming efficiency of ADSCs-derived stem cells was significantly higher than fibroblast collected from embryo or adult ears (Li et al. 2018b). Besides, reports are also available to generate porcine iPS cells from other cell sources such as Sertoli cells (Setthawong et al. 2019) and pericyte (Xu et al. 2019).

Contextually, while planning to generate iPS cells, the availability and purpose of the experiment should get due importance. However, preference should be given to those cells that are known to be easier to undergo reprogramming.

17.4.4 Culture Supplementation with Special Reference to LIF and FGF2

Leukemia inhibitory factor (LIF) is a member of the interleukin-6 family of cytokines, most widely used for the maintenance of undifferentiated state and self-renewal of mouse ES cells (Smith et al. 1988). On the other hand, fibroblast growth factor 2 (FGF2) is a signaling molecule involved in many biological processes, including embryonic development, angiogenesis, and wound healing (Armelin 1973; Gospodarowicz 1974). FGF2 signaling was reported to maintain the growth of human ES cells (Eiselleova et al. 2009). Either LIF (Cheng et al. 2012a; Fujishiro et al. 2013; Zhang et al. 2015; Fukuda et al. 2017; Kwon et al. 2017; Secher et al. 2017) or FGF2 (West et al. 2010; Zhang et al. 2015; Secher et al. 2017) or both were supplemented for culturing porcine iPS cells. Like other species, LIF was identified to activate the transcription factor STAT3 and its target SOCS3 and stimulated cell growth in iPS cells generated in LIF supplemented medium, indicating the existence of a conserved functional signaling pathway across different species in mammals (Thomson et al. 2012). The requirement of LIF and FGF2 for reprogramming of porcine somatic cells was reemphasized but not essential for maintaining self-renewal and pluripotency.

A serum-free 3i medium containing three inhibitors CHIR99021 (GSK-3 inhibitor; acts as Wnt activator), PD0325901 (MEK/ERK inhibitor), and SB431542 (a selective inhibitor of endogenous activin and TGF-β signaling, but has no effect on BMP signaling), plus three cytokines (BMP4, SCF, and IL-6), and human platelet lysates was reported that successfully rescued flattened primed iPS cells to naïve-like cells. This medium maintained the culture for a long-term culture without the use of LIF or FGF2 (Ma et al. 2018), indicating that LIF/FGF2 could be replaced with supplementation of other factors, including different small-molecule inhibitors.

17.4.5 Culture of iPS with or Without Feeder Cells

Porcine iPS cells could be grown in a number of different basal media, such as DMEM/F12 (Secher et al. 2017) with a feeder (Mao et al. 2017) and also without feeder when used proprietary media such as Cellgro (Fujishiro et al. 2013) and mTeSR (unpublished data from the lead author's laboratory).

Is There Any Standard Method to Grow More Naïve-Type Porcine iPS Cells? Only limited data is available dealing with how to isolate and grow naïve porcine iPS cells. As stated in the previous section, the serum-free 3i medium could preferentially support the growth of naïve iPS cells (Ma et al. 2018). Recently, a detail of transcriptome analysis dealt with a similar issue. As per this report, porcine inner cell

mass was identified to have a unique pluripotency transcriptome, distinct from human and mouse ES cells. But it was shown to share more features with human naive-like than primed stem cell states (like an expression of KLF17 but not KLF2). Therefore, to activate specific signaling pathways important for porcine pluripotency, a suitable media was formulated. The media had DMEM/F-12 and Neurobasal medium mixed 1:1 supplemented with Glutamax, 2-mercaptoethanol, N2 supplement, B27 supplement, Pen-strep, human recombinant LIF, L-ascorbic acid, insulin-transferrin-selenium-sodium pyruvate (ITS-A), PD0325901 (MEK/ERK inhibitor), 1 μM CHIR99021 (GSK-3 inhibitor; acts as Wnt activator), Gö6983 (inhibits several isoforms of protein kinase C), and Y-27632 (inhibits both ROCK1 and ROCK2). This media supported human naïve stem culture. The same media improved the efficiency of reprogramming of porcine embryonic fibroblasts, and this culture condition could turn on the expression of important naive stem cell markers such as NANOG, KLF17, and CDH1 in porcine iPS-like cells (Habekost et al. 2019). For scaling up of porcine iPS cells, stirred suspension bioreactors could be effectively used (Burrell et al. 2019) and for improvement of efficiency of cryopreservation of these cells. ROCK inhibitor Y-27632 could be of help (Baek et al. 2019).

Thus, there is a number of media available for culturing porcine iPS cells. One should test and adopt the best one for derivation and propagation of cells.

17.4.6 Expression of Marker Genes in Porcine iPS Cells

Embryonic stem cells are characterized by a number of markers such as Oct4, Sox2, Klf2, Klf4, Rex1, c-Myc, E-cadherin, high level of telomerase, alkaline phosphatase, SSEA1, SSEA3, SSEA4, Tra-1-60, Tra-1-81, Rex1, and CDH1. Porcine iPS cells were reported to express some or most of these ES cell markers, with individual variations. Expression of stage-specific embryonic antigen (SSEA) surface markers remained a reliable tool to differentiate between mouse and human ES cells. Typically mouse ES cells are known to express SSEA1 but not SSEA3 and SSE4, whereas human ES cells express both SSEA3 and SSEA4, not SSEA1. For example, iPS cells derived for the first time in porcine by Wu et al. were shown to express alkaline phosphatase, Tra-1-60, Tra-1-81, Oct3/4, Nanog, Sox2, Rex1, CDH1, and both SSEA3 and SSEA4 (Wu et al. 2009). iPS reported from the Michael Roberts laboratory expressed Oct4, Nanog, Sox2, high telomerase activity, but lacked expression of SSEA3 and SSEA4. However, similar to mouse ES cells, these cells expressed SSEA1 (Ezashi et al. 2009). iPS cells that expressed all three SSEA molecules (detected by PCR) (Fujishiro et al. 2013) or that expressed only SSEA4, not the other two SSEA molecules (Esteban et al. 2009) or not SSEA1 (Thomson et al. 2012), were available too. Porcine iPS cells that expressed SSEA1 but not the other surface markers (such as Tra-1-60, Tra-1-81, SSEA3, and SSEA4) were reported by multiple groups (Kwon et al. 2013; Li et al. 2018a). iPS cells from a resource cell line were shown to express SSEA1 (Chakritbudsabong et al. 2017). Again, cells were also shown to express surface markers in combination such as SSEA4, TRA 1-60, and TRA 1-81 (Yang et al. 2013), or SSEA1, SSEA4, and

TRA-1-60 (Gu et al. 2014). The presence of high telomerase activity was described as a feature in a number of reports, such as of Ezashi et al. (2009) or of Fukuda et al. (2017).

Lastly, since each group chose to test and reported a specific set of markers, and each of the laboratories set up varied, it may not be easier to conclude if conflicting reports of marker expression were due to the cells' inherent characteristics or due to the variation in the setups. However, a panel of markers listed for ES cells was useful and would remain a guide to detect the status of porcine iPS cell marker expression.

17.4.7 In Vitro Lineage Differentiation of Porcine iPS Cells

In each publication, porcine iPS cells were routinely shown to undergo differentiation, producing cells of ectodermal, mesodermal, and endodermal (three germ layers) lineage. A more or less standard procedure is available to differentiate ES cells randomly, and in this procedure, ES cells are allowed to grow as free-floating cell aggregates in a medium without cytokines/growth factors such as LIF/FGF2. Those cell aggregates grow in small clumps/bodies and are termed embryoid bodies (EBs). Tissues of three germ layers are detected in these EBs, usually by PCR or immuno-techniques. By following the similar protocol, iPS cells were allowed to differentiate as EBs randomly, and subsequently, expression of different lineage-specific markers was detected in those EBs, signifying iPS cells, in general, could produce tissues of ectodermal, mesodermal, and endodermal lineage (Zhang et al. 2015). Under special culture conditions, myocardial differentiation of porcine iPS cells was reported, displaying beatings of embryoid bodies in culture (Chakritbudsabong et al. 2017).

However, these were performed as a part of standard procedures to show that porcine iPS cells were pluripotent cells. But in order to extend the usefulness of these cells, specifically for regenerative applications such as cells for transplantation and screening of drugs, lineage-specific differentiation of iPS cells would be required.

Recently, porcine iPS cells were differentiated to skeletal myotubes with coordinated approaches combining two inhibitors and ectopic expression of MyoD1 (Genovese et al. 2017). Using inhibitors of SMAD, TGF-beta, and BMP4, Kim et al. (2019a) developed an efficient method for the production of neural progenitor cells from porcine iPS cells, advancing the application of reprogrammed cells (Kim et al. 2019a). Also, porcine iPS cells underwent neural differentiation when EBs were treated with retinoic acid (Li et al. 2014). Among these limited studies, one study was remarkably reported from the laboratory of Steven L. Stice and Franklin D. West. The group demonstrated that SSEA4-positive porcine iPS cells (compared to SSEA4-negative cells) were more suitable for differentiation into beta III-TUB/MAP2+ neurons, GFAP+ astrocytes, O4+ oligodendrocytes, and motor neurons expressing both HB9 and ISLET1. This work established a link of expression of a specific marker (such as SSEA4) to iPS cells' propensity for a specific lineage differentiation (Yang et al. 2013). It needs to be seen whether SSEA4+ iPS cells

are refractory to differentiation to other lineages. Additionally, more such differentiation-associated iPS markers would help advance the field immensely.

17.4.8 Assay for Testing Developmental Potential

Like differentiation in vitro, pluripotent stem cells are tested in vivo for their ability to contribute to different lineages. This is generally done in more than one way, such as tetraploid complementation, teratoma formation, and chimera formation assays.

17.4.8.1 Tetraploid Complementation Assay

Tetraploid complementation assay is a technique where zonal pellucida-free one tetraploid embryo is electrofused with aggregates of pluripotent stem cells such as ES or iPS cells. Then, lineage development of the resultant tetraploid-pluripotent stem cells chimera is monitored for the contribution of each source. Usually, pluripotent stem cells contribute to fetus proper (ectoderm, endoderm, and mesoderm), and extraembryonic tissues (primitive endoderm and the trophectoderm come from tetraploid embryos). The chimera developmentally progresses if both compartments (embryonic and extraembryonic) of embryos complement each other appropriately (Tam and Rossant 2003). Tetraploid complementation assay is commonly used for testing the pluripotency of any cells, such as ES cells. Blastocysts complemented with mouse iPS cells forming tetraploid embryos were successful in producing viable, fertile, live-born progeny (Kang et al. 2009; Zhao et al. 2009). To our information, porcine iPS cells were used for chimera development by microinjection into blastocyst or morula (Cheng et al. 2012a; Fujishiro et al. 2013), but no such report is available on tetraploid complementation assay using porcine iPS cells.

17.4.8.2 Teratoma Formation with Porcine iPS Cells

Teratoma formation is a tool commonly employed for monitoring pluripotency in stem cell biology. It is applied to assess stem cells' ability to form tissues of three germ layers in vivo (Nelakanti et al. 2016). Teratoma is an encapsulated (or solid) tumor generally formed when iPS (or any pluripotent stem) cells are injected in immunocompromised mice (such as severe combined immunodeficient or SCID mice, lacking B and T lymphocytes). Once grown, tumors are collected, and tissue samples are processed by fixation, followed by staining with H&E (hematoxylin and eosin) dye or for immunodetection of different markers. The stained slide is examined for the presence of ectodermal, mesodermal, and endodermal tissues for ascertaining the differentiation ability of cells injected. Several reports indicated that porcine iPS cells are capable of forming teratoma with tissues of ectoderm, mesoderm, and endoderm lineages (Zhang et al. 2015; Secher et al. 2017; Li et al. 2018a). Cheng et al. (2012b) had success in obtaining teratomas in 8 weeks after injection of porcine iPS cells with SCID mice but had difficulty with nude (NOD-Balb/c) mice (Cheng et al. 2012b), indicating the importance of choosing background of immunodeficient mice for performing teratoma assay. Further, it was

also suggested that porcine iPS cells might take a longer time than mouse iPS cells (Ezashi et al. 2009; Cheng et al. 2012b).

17.4.8.3 Contribution of Porcine iPS Cells to Chimera Formation

The teratoma formation assay described above would indicate if a cell line retains the ability to undergo differentiation under in vivo system. However, the ultimate proof for pluripotency can be tested if those cells contribute to different organs of a growing body. In order to enable this, cells are injected into developing blastocysts and monitored for further development. Advancement in the growing embryos' developmental stages may be monitored with the fluorescent reporter expressed by the cells injected. Further, suppose those chimeric embryos are transferred to surrogates and allowed to complete the term. In that case, injected cells' contribution could also be monitored in animals born out of the procedure.

To our knowledge, to date, only the Stice laboratory succeeded in producing live germline-competent chimeric offspring using porcine iPS cells (West et al. 2010, 2011). Besides this, Fan et al. also produced live offspring using iPS cells as donors for nuclear transfer (NT). However, success was achieved only after silencing the exogenous transcription factors either through spontaneous differentiation of iPS cells before they are used as donor cells; or by treating the constructed embryos with Scriptaid (a novel histone deacetylase inhibitor) to increase histone acetylation (Fan et al. 2013).

Chimeric embryos were formed by using some of these naïve iPS cells, exhibiting fluorescent markers of iPS cell origin (Cheng et al. 2012a; Fujishiro et al. 2013; Secher et al. 2017). Alternatively, a somewhat less stringent test was done by injecting iPS cells into a parthenogenetic embryo and found that iPS cells could continue to contribute to the advancement of embryo growth (Zhang et al. 2015; Fukuda et al. 2017).

These data indicate that most of the laboratories failed to produce live chimera using porcine iPS cells. The reasons for failure were linked to the developmental and pluripotency status of iPS cells. Given the data from murine studies, it was thought that most of the porcine iPS cells were in a primed state of pluripotency, and therefore, they did not yield chimera. Now we look at the concept of the state of pluripotency in the context of porcine iPS cells.

17.4.9 Naïve Versus Primed iPS Cells

Austin Smith from the University of Cambridge introduced and elaborated the concept of two different states of pluripotency ("naïve" and "primed") in the field of developmental biology (Nichols and Smith 2009). This paradigm defined the potential of pluripotent cells isolated from early developing embryos. Naïve pluripotent stem cells are characterized by (a) formation of a compact dome-shaped colony in cell culture, (b) high plating efficiency of dissociated single cell, (c) maintenance requires LIF signaling, (d) BMP4 signaling regulates self-renewal, (e) FGF2/activin/nodal signaling pathway is involved in differentiation, (f) both X chromosomes

remain active in female cells, and (6) naïve cells are capable of forming chimera or even a complete animal and, therefore, these cells constitute competent germline cells (Telugu et al. 2010). Mouse ES cells from inner cell mass (ICM) fulfill all these criteria, and therefore, they are considered as ground state cells or authentic stem cells, or naïve stem cells. On the contrary, stem cells with a "primed" state of pluripotency have the following features: (a) flattened colonies, (b) low plating efficiency, (c) FGF2 signaling (not LIF signaling) maintains pluripotency, (d) BMP4 regulates differentiation, and (e) inactivation of X chromosome and silencing of paternally imprinted Dlk1-Dio3 regions. Mouse ES cells are designated as "primed" stem cells when they fail to generate chimera and lack germline competency. Primed stem cells usually represent cells from the epiblast. Human ES cells require FGF2 to maintain the pluripotency, and those cells are also considered epiblast stem cells or primed stem cells. Both naïve and primed cells undergo differentiation into tissues of three primary germ layers. But ICM-derived authentic or naïve stem cells are fully competent for germline transmission, whereas epiblast-derived primed stem cells have inferior potential and incompetent for germline transmission. A detailed comparison of stem cells with naïve and primed state of pluripotency is presented in Table 17.2.

Most porcine iPS cells reported in the literature are grown with FGF2 rather than with LIF-supplemented cell culture. These iPS cells either lack germline contribution or status not tested, hence unknown. Similar to human ES cells, these iPS cells are also considered to be "primed" or equivalent to cells from the epiblasts stage. One exception was reported where iPS cells were grown in the presence of FGF2 but still contributed to all three germ layers in a chimera (West et al. 2010), and those chimeric animals produced transgenic offspring in the next generation (West et al. 2011). However, it may be noted that those iPS cells were derived and maintained on inactivated mouse embryonic fibroblasts (MEF) feeder cells, and MEF are known to secrete LIF (Lee et al. 2009). Though not supplemented in the media, the availability of LIF from MEF might have maintained a naïve state of pluripotency, resulting in efficient germline-competent iPS cells and producing offspring. This would be interesting to check if other naïve cell criteria/features are found in these iPS cells generated in the Stice laboratory (West et al. 2010).

For testing germline competence in porcine iPS cells, one needs to generate chimera using advanced laboratory and animal facilities. An alternate ready reckoner to detect naïve iPS cells circumventing chimera generation would be useful. To some extent, lessons learned from mouse naïve cells should be of help in setting minimal criteria for determining the state of pluripotency in porcine iPS cells. LIF-dependent growth and expression of SSEA1 (most widely accepted markers of mouse ES cells) could be two prime criteria for detecting naïve iPS cells in swine. To the best of the literature, Telugu et al. attempted for the first time and generated LIF-dependent SSEA1-expressing naïve-type iPS cells that showed characteristics of mouse ES cells (Telugu et al. 2010). Another group also reported porcine iPS cells that met in vitro most criteria of naïve stem cells (such as LIF-dependent growth, negative MHC class I, active X chromosomes, and distinct gene-expression profiles)

Table 17.2 A comparison of naïve and primed iPS cells (Courtesy: Hochedlinger and Jaenisch 2015)

Characters	Naïve iPS cells	Primed iPS cells
Pluripotency state	ICM (inner cell mass) like	Epiblast like
Cell morphology	Compact, small	Large, flat
Colony feature	Dome-shaped	Flattened
Clonogenicity	High	Comparatively low
LIF-dependent to maintain	Yes	No
FGF2 dependent	No	Yes
Active signaling	Stat3	Activin/nodal
High telomerase activity	Yes	?
Short cell cycle interval	Yes	?
Normal karyotype	Yes	Yes
Contribution to chimera	Yes	No
Teratoma formation	Yes	Yes
Amenable to gene targeting	Efficient	Inefficient
X chromosome inactivation status in female cells	Both X chromosomes active (XaXa)	One X chromosome active (XaXi)
Silencing of imprinted genes	No	Yes
Apoptosis when injected in blastocyst	Slow/less	Rapid/high

(Fujishiro et al. 2013). However, it remained unknown if those cells could contribute to generate competent germline chimera.

The marker expression profile of iPS cells varies from report to report. A typical list of some markers for naïve porcine iPS cells includes alkaline phosphatase (AP) staining, expression of Oct4 (Hall and Hyttel 2014; Zhang et al. 2014), Sox2 (Zhang et al. 2014), Nanog (Hall and Hyttel 2014; Zhang et al. 2014), SSEA1 (Telugu et al. 2010; Gu et al. 2014; Hall and Hyttel 2014), SSEA3 (Zhang et al. 2014), SSEA4 (Zhang et al. 2014), and CRIPTO (Hall and Hyttel 2014); upregulation of Stella (Zhang et al. 2014) and Eras (Zhang et al. 2014); low expression levels of TRA-1-60 (Zhang et al. 2014), TRA-1-81 (Zhang et al. 2014), NrOB1 (Hall and Hyttel 2014), REX1 (Hall and Hyttel 2014); MHC I either low (Zhang et al. 2014) or absent (Fujishiro et al. 2013). In addition, naïve porcine iPS cells show LIF-dependency (Fujishiro et al. 2013; Zhang et al. 2014), activation of both X chromosomes (Zhang et al. 2014; Fukuda et al. 2017), normal karyotypes, compact dome-shaped colony, and growth after single-cell dissociation (Zhang et al. 2014). Further, those iPS cells contributed to embryonic and fetal development (Fujishiro et al. 2013). On the other hand, primed stem cells express OCT4 (Hall and Hyttel 2014), NANOG (Hall and Hyttel 2014), SOX2 (Hall and Hyttel 2014), KLF4 (Hall and Hyttel 2014), c-Myc (Hall and Hyttel 2014), REX1 (Hall and Hyttel 2014), CRIPTO (Hall and Hyttel 2014), and KLF2 (Hall and Hyttel 2014). It may be noted that some of these pluripotent markers were overlapping to both kinds of cells, and the list includes OCT4 (Hall and Hyttel 2014), NANOG (Hall and Hyttel 2014), SOX2 (Hall and Hyttel 2014), and CRIPTO (Hall and Hyttel 2014). Thus, it is

evident that both naïve and primed pluripotent stem cells express certain common pluripotent stem cell markers such as Oct4, Sox2, and Nanog.

Since naïve iPS cells have higher potentials compared to their primed counterparts, next comes how to improve pluripotency from primed to naïve state? An only a limited number of reports have dealt with the issue so far. Taking consideration of these publications, the following suggestions could be put forward:

1. One can possibly derive and maintain iPS cells in media supplemented with LIF (Telugu et al. 2010), or LIF and forskolin (Fujishiro et al. 2013).
2. Addition of 2i and LIF in the culture media. It was shown that the addition of two inhibitors (CHIR99021 and PD0325901) in a media containing LIF could support the growth of native-like porcine iPS cells under feeder-independent and serum-free conditions (Zhang et al. 2014), though the use of 2i in culture had also produced conflicting outcome during somatic cell reprogramming (Petkov et al. 2014).
3. Pig iPS cells were grown in LBX medium (LIF + FGF2 + knockout serum replacement + N2B27 supplement) had a small dome-shaped colony, expressed SSEA1, and cells from these colonies were more suitable as donor cells for NT to generate reconstructed embryos (Gu et al. 2014).
4. Additionally, it was demonstrated that treatment of parthenogenetic embryos with lysophosphatidic acid reduced expression of "primed" marker genes such as GATA4 (a marker of primitive hypoblast) in the early development of porcine parthenogenetic embryos (Zhu et al. 2018).

Whether all such approaches would be useful for converting primed iPS cells to their naïve state is yet to be tested. Nonetheless, the field would continue to hunt for finding fully potential naïve iPS cells in porcine.

17.4.10 Other Features of Porcine iPS Cells

In addition to the material presented above, porcine iPS cells also have other features, some of which are already reported for other species. Zhang et al. (2016) reported miRNAs in porcine iPS cells differentially expressed in comparison to embryonic fibroblasts. Multiple miRNAs such as ssc-miR-145-5p and ssc-miR-98 in porcine iPS cells were downregulated, whereas ssc-miR-217, ssc-miR-216, ssc-miR-142-5p, ssc-miR-182, ssc-miR-183, ssc-miR-96-5p, ssc-miR-106a, ssc-miR-363, ssc-miR-146b, ssc-miR-195, ssc-miR-497, ssc-miR-935, and ssc-miR-20b were reported to be upregulated (Zhang et al. 2016). Self-renewal of iPS cells was regulated by common miRNA–mRNA interactions (Zhang et al. 2017) and by the activin-SMAD signaling pathway (Yang et al. 2017). The m(6)A methylation via SOCS3/JAK2/STAT3 pathway was reported to regulate pluripotency of porcine iPS cells (Wu et al. 2019). Paternally imprinted Dlk1-Dio3 gene clusters were found to be aberrantly silent in most murine iPS cells, but in the case of porcine iPS, loss of expression followed by recovery of the clusters occurred.

In most porcine iPS lines, the maternally imprinted GTL2 gene became silent at a very early stage without recovery of expression (Cheng et al. 2012b).

Maintenance of genomic stability is an indicator of good quality cells. Instability in porcine iPS cells was linked to reduced DNA repair and replication capacity (Liu et al. 2017). Maintenance of porcine iPS-like cells for long-term culture, HDAC inhibitors (such as valproic acid, sodium butyrate, and suberoyanilide hydroxamic acid) was useful, but such treatment affected the differentiation ability of those cells (Petkov et al. 2016).

Incomplete reprogramming results in the generation of partially reprogrammed iPS cells, and the growth of these cells requires continuous expression of exogenous factors. In turn, it was demonstrated that the expression of endogenous counterparts was repressed by the sustained expression from exogenous sources (Hall et al. 2012), indicating that additional enhancing factors/modulators are needed for overcoming dependency of exogenous factors during reprogramming.

17.5 Therapeutic and Other Applications of Porcine iPS Cells

For therapeutic and regenerative applications, porcine iPS cells need to undergo rigorous evaluation of immunogenic properties, safety, and identification of the appropriate model. Only limited reports are available for addressing each aspect. Like other pluripotent stem cells, porcine iPS cells either do not express or express a low level of MHC class I molecule, and level of expression gets upregulated upon differentiation (Fujishiro et al. 2013; Park et al. 2013). A detailed study indicates that MHC-matched iPS cells can evade cellular and humoral immune responses but still susceptible to innate immunity in pigs (Mizukami et al. 2014). Therefore, it would be necessary to circumvent major players of innate immunity such as mast cells, macrophages, NK cells, and complement system.

Hence, porcine iPS cells are suggested to be useful for preclinical studies (Park et al. 2013). In one such experiment, functional vascular smooth muscle cells were produced by differentiation of swine iPS cells, and these cells formed readily 3D scaffold-free vascular tissue rings suitable for preclinical applications (Luo et al. 2017). For the first time, transplantation of porcine iPS cell-derived functional endothelial cells could improve cardiac function after myocardial infarction via paracrine activation in a rat model (Gu et al. 2012). Similarly, porcine iPS cells were differentiated to CD31+ functional endothelial cells using GSK3beta inhibitor and BMP4. This approach had potential benefits when evaluating autologous endothelial cell transplantation in pig models (Wei et al. 2020).

Apart from these, porcine iPS cells were used in bone and cartilage-related preclinical studies. Osteoblast-like cells generated from iPS cells could recover bone mass of tibiae in glucocorticoid-induced bone loss in two different animal models, rat (Liao et al. 2018c) and Lanyu pig (Liao et al. 2018b), signifying the usefulness of iPS cell-based therapy. Similarly, iPS-like cells were used successfully for cartilage regeneration in CLAWN miniature pig osteochondral replacement model (Uto et al. 2018).

Further, porcine iPS cells are suitable for generating disease models. Reprogrammed iPS cells were generated that overexpressed two proto-oncogenes, TGF-α, and c-Myc, driven by pig albumin promoter, and this method ensured restriction of transgene expression only in hepatic tissues. Further, these genetically modified iPS cells could be used as NT donors in vitro. These data make a step forward toward the generation of genetically modified pigs as a large animal model suitable for studies of liver cancer and treatment (Park et al. 2016).

Since the reprogramming process overhauls the entire genome landscape to generate iPS cells, the Tönjes laboratory checked if reprogramming had any impact on the expression of porcine endogenous retroviruses (PERV). It was reported that the reprogramming process impacted the expression of PERV in porcine iPS cells (Godehardt et al. 2018). For transplantation purposes, PERV-free somatic cells should be used for generating iPS cells. Pig shares similar anatomy and physiology to humans, and therefore, it is considered as a suitable donor for xenotransplantation in humans. Expression of alpha-1,3-galactosyltransferase (GALT) gene in porcine cells makes them rejected immediately after their transplantation in humans. Knocking out of this gene makes the cells somewhat tolerant, and it indicates the requirement of modifications in additional genes to ultimately make pig tissue immune-compatible with humans. A porcine iPS cell was generated from GALT-knockout fibroblast. It was envisaged that these cells would serve as a resource to dissect the complex phenomena of immuno-rejection, required for xenotransplantation, somatic cell nuclear transfer, or chimera formation (Liu et al. 2013). These are some important advancements toward making the iPS cell safe for preclinical and clinical applications.

Overall, porcine iPS cells attracted the attention of clinical researchers, but a long way to go for utilizing these newly developed cells as resources for clinical applications.

17.6 Concluding Remarks

The discovery of Yamanaka transcription factors for reprogramming has provided an opportunity to generate induced pluripotent stem cells from the pig in various ways in different laboratories. Pigs have contributed immensely to basic research, animal agriculture, and regenerative biology. IPS cells' availability has raised hope for generating transgenic animals for different uses, such as organ transplantation, a genetic model for human diseases, genetically modified animals resistant to certain diseases, and enhancing animal productivity (Cheng and Xiao 2009). Transgenic porcine iPS cells are also available (Liu et al. 2013; Park et al. 2016), and live animals from unaltered iPS cells have already been produced, though with limited success (West et al. 2010, 2011; Fan et al. 2013).

The majority of the porcine iPS cells are not germline competent, primarily due to incomplete reprogramming characterized by sustained expression of exogenous factors. The exogenous factors also prevent the iPS cells from undergoing the desired differentiation. This would remain a great challenge for using porcine iPS

cells in agriculture and biomedical sciences. Finding appropriate differentiation protocol and the generation of precise genome-edited animals need to be prioritized.

Most resources (in terms of tools and manpower) remain engaged in standardizing protocol for generating the porcine iPS cells. In two cases, live animals have been produced (West et al. 2011; Fan et al. 2013), but the achievements are not free from questions. For example, the generation of live animals by nuclear transfer of fully differentiated iPS cells (Fan et al. 2013) can be considered equivalent to the generation of live animals from nuclei of original fibroblast before undergoing reprogramming. Therefore, the generation of live animals could testify to the potential of fibroblasts as a nuclear donor, not of the iPS generated from those fibroblasts. This undermines the need for reprogramming. Further, treatment of reconstructed embryos with HDAC inhibitor to silence the transgene may have an indirect impact and not necessarily on the iPS cells used for cloning. This indicates that the field of iPS biology requires more work to make the technique practically useable.

To use the resources efficiently, the formation of a worldwide network in the form of a consortium may achieve the goal faster and aid in developing standard operating procedure (SOP) for every protocol needed and bank both wild and mutant porcine iPS cells. Consortium should also include finding novel porcine-specific reprogramming factors and reprogramming procedures without using vectors. In conclusion, the technology of reprogramming has a long way to go before harnessing the full potential of porcine-induced pluripotent stem cells in the future.

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References

Alberio R, Croxall N, Allegrucci C (2010) Pig epiblast stem cells depend on activin/nodal signaling for pluripotency and self-renewal. Stem Cells Dev 19:1627–1636

An Z, Liu P, Zheng J, Si C, Li T, Chen Y, Ma T, Zhang MQ, Zhou Q, Ding S (2019) Sox2 and Klf4 as the functional core in pluripotency induction without exogenous Oct4. Cell Rep 29:1986–2000.e8

Apostolou E, Stadtfeld M (2018) Cellular trajectories and molecular mechanisms of iPSC reprogramming. Curr Opin Genet Dev 52:77–85

Armelin HA (1973) Pituitary extracts and steroid hormones in the control of 3T3 cell growth. Proc Natl Acad Sci U S A 70:2702–2706

Avilion AA, Nicolis SK, Pevny LH, Perez L, Vivian N, Lovell-Badge R (2003) Multipotent cell lineages in early mouse development depend on SOX2 function. Genes Dev 17:126–140

Baek SK, Cho YS, Kim IS, Jeon SB, Moon DK, Hwangbo C, Choi JW, Kim TS, Lee JH (2019) A Rho-associated coiled-coil containing kinase inhibitor, Y-27632, improves viability of dissociated single cells, efficiency of colony formation, and cryopreservation in porcine pluripotent stem cells. Cell Reprogram 21:37–50

Briggs R, King TJ (1952) Transplantation of living nuclei from blastula cells into enucleated frogs' eggs. Proc Natl Acad Sci U S A 38:455–463

Bui HT, Kwon DN, Kang MH, Oh MH, Park MR, Park WJ, Paik SS, Van Thuan N, Kim JH (2012) Epigenetic reprogramming in somatic cells induced by extract from germinal vesicle stage pig oocytes. Development 139:4330–4340

Burrell K, Dardari R, Goldsmith T, Toms D, Villagomez DAF, King WA, Ungrin M, West FD, Dobrinski I (2019) Stirred suspension bioreactor culture of porcine-induced pluripotent stem cells. Stem Cells Dev 28:1264–1275

Cartwright P, McLean C, Sheppard A, Rivett D, Jones K, Dalton S (2005) LIF/STAT3 controls ES cell self-renewal and pluripotency by a Myc-dependent mechanism. Development 132:885–896

Chakritbudsabong W, Sariya L, Pamonsupornvichit S, Pronarkngver R, Chaiwattanarungruengpaisan S, Ferreira JN, Setthawong P, Phakdeedindan P, Techakumphu M, Tharasanit T, Rungarunlert S (2017) Generation of a pig induced pluripotent stem cell (piPSC) line from embryonic fibroblasts by incorporating LIN28 to the four transcriptional factor-mediated reprogramming: VSMUi001-D. Stem Cell Res 24:21–24

Chambers I, Colby D, Robertson M, Nichols J, Lee S, Tweedie S, Smith A (2003) Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells. Cell 113:643–655

Chen LR, Shiue YL, Bertolini L, Medrano JF, BonDurant RH, Anderson GB (1999) Establishment of pluripotent cell lines from porcine preimplantation embryos. Theriogenology 52:195–212

Cheng L, Xiao L (2009) Pig induced pluripotent stem cells: a new resource for generating genetically modified pigs. Regen Med 4:787–789

Cheng D, Guo Y, Li Z, Liu Y, Gao X, Gao Y, Cheng X, Hu J, Wang H (2012a) Porcine-induced pluripotent stem cells require LIF and maintain their developmental potential in early stage of embryos. PLoS One 7:e51778

Cheng D, Li Z, Liu Y, Gao Y, Wang H (2012b) Kinetic analysis of porcine fibroblast reprogramming toward pluripotency by defined factors. Cell Reprogram 14:312–323

Cibelli JB, Stice SL, Golueke PJ, Kane JJ, Jerry J, Blackwell C, Ponce de Leon FA, Robl JM (1998) Transgenic bovine chimeric offspring produced from somatic cell-derived stem-like cells. Nat Biotechnol 16:642–646

Cieslar-Pobuda A, Knoflach V, Ringh MV, Stark J, Likus W, Siemianowicz K, Ghavami S, Hudecki A, Green JL, Los MJ (2017) Transdifferentiation and reprogramming: overview of the processes, their similarities and differences. Biochim Biophys Acta, Mol Cell Res 1864:1359–1369

Cowan CA, Atienza J, Melton DA, Eggan K (2005) Nuclear reprogramming of somatic cells after fusion with human embryonic stem cells. Science 309:1369–1373

Davis RL, Weintraub H, Lassar AB (1987) Expression of a single transfected cDNA converts fibroblasts to myoblasts. Cell 51:987–1000

Eiselleova L, Matulka K, Kriz V, Kunova M, Schmidtova Z, Neradil J, Tichy B, Dvorakova D, Pospisilova S, Hampl A, Dvorak P (2009) A complex role for FGF-2 in self-renewal, survival, and adhesion of human embryonic stem cells. Stem Cells 27:1847–1857

Esteban MA, Xu J, Yang J, Peng M, Qin D, Li W, Jiang Z, Chen J, Deng K, Zhong M, Cai J, Lai L, Pei D (2009) Generation of induced pluripotent stem cell lines from Tibetan miniature pig. J Biol Chem 284:17634–17640

Evans MJ, Kaufman MH (1981) Establishment in culture of pluripotential cells from mouse embryos. Nature 292:154–156

Evans MJ, Notarianni E, Laurie S, Moor RM (1990) Derivation and preliminary characterization of pluripotent cell lines from porcine and bovine blastocysts. Theriogenology 33:125–128

Ezashi T, Telugu BP, Alexenko AP, Sachdev S, Sinha S, Roberts RM (2009) Derivation of induced pluripotent stem cells from pig somatic cells. Proc Natl Acad Sci U S A 106:10993–10998

Fan N, Chen J, Shan Z et al (2013) Piglets cloned from induced pluripotent stem cells. Cell Res 23:162–166

Finch BW, Ephrussi B (1967) Retention of multiple developmental potentials by cells of a mouse testicular teratocarcinoma during prolonged culture *in vitro* and their extinction upon hybridization with cells of permanent lines. Proc Natl Acad Sci U S A 57:615–621

Flitsch LJ, Brustle O (2019) Evolving principles underlying neural lineage conversion and their relevance for biomedical translation. F1000Res 8:F1000 Faculty Rev-1548

Fujishiro SH, Nakano K, Mizukami Y, Azami T, Arai Y, Matsunari H, Ishino R, Nishimura T, Watanabe M, Abe T, Furukawa Y, Umeyama K, Yamanaka S, Ema M, Nagashima H, Hanazono Y (2013) Generation of naive-like porcine-induced pluripotent stem cells capable of contributing to embryonic and fetal development. Stem Cells Dev 22:473–482

Fukuda T, Tani T, Haraguchi S, Donai K, Nakajima N, Uenishi H, Eitsuka T, Miyagawa M, Song S, Onuma M, Hoshino Y, Sato E, Honda A (2017) Expression of six proteins causes reprogramming of porcine fibroblasts into induced pluripotent stem cells with both active X chromosomes. J Cell Biochem 118:537–553

Fukuda T, Doi K, Donai K, Takahashi K, Kobayashi H, Hirano T, Nishimori K, Yasue H (2019) Global transcriptome analysis of pig induced pluripotent stem cells derived from six and four reprogramming factors. Sci Data 6:190034

Gao Y, Guo Y, Duan A, Cheng D, Zhang S, Wang H (2014) Optimization of culture conditions for maintaining porcine-induced pluripotent stem cells. DNA Cell Biol 33:1–11

Gardner RL (1968) Mouse chimeras obtained by the injection of cells into the blastocyst. Nature 220:596–597

Genovese NJ, Domeier TL, Telugu BP, Roberts RM (2017) Enhanced development of skeletal myotubes from porcine-induced pluripotent stem cells. Sci Rep 7:41833

Godehardt AW, Petkov S, Gulich B, Fischer N, Niemann H, Tonjes RR (2018) Comparative gene expression profiling of pig-derived iPSC-like cells: effects of induced pluripotency on expression of porcine endogenous retrovirus (PERV). Xenotransplantation 25:e12429

Gospodarowicz D (1974) Localisation of a fibroblast growth factor and its effect alone and with hydrocortisone on 3T3 cell growth. Nature 249:123–127

Gu M, Nguyen PK, Lee AS, Xu D, Hu S, Plews JR, Han L, Huber BC, Lee WH, Gong Y, de Almeida PE, Lyons J, Ikeno F, Pacharinsak C, Connolly AJ, Gambhir SS, Robbins RC, Longaker MT, Wu JC (2012) Microfluidic single-cell analysis shows that porcine-induced pluripotent stem cell-derived endothelial cells improve myocardial function by paracrine activation. Circ Res 111:882–893

Gu Q, Hao J, Hai T, Wang J, Jia Y, Kong Q, Feng C, Xue B, Xie B, Liu S, Li J, He Y, Sun J, Liu L, Wang L, Liu Z, Zhou Q (2014) Efficient generation of mouse ESCs-like pig induced pluripotent stem cells. Prot Cell 5:338–342

Gurdon JB (1962) The developmental capacity of nuclei taken from intestinal epithelium cells of feeding tadpoles. J Embryol Exp Morpholog 10:622–640

Gurdon JB, Elsdale TR, Fischberg M (1958) Sexually mature individuals of Xenopus laevis from the transplantation of single somatic nuclei. Nature 182:64–65

Habekost M, Jorgensen AL, Qvist P, Denham M (2019) Transcriptomic profiling of porcine pluripotency identifies species-specific reprogramming requirements for culturing iPSCs. Stem Cell Res 41:101645

Hall VJ, Hyttel P (2014) Breaking down pluripotency in the porcine embryo reveals both a premature and reticent stem cell state in the inner cell mass and unique expression profiles of the naive and primed stem cell states. Stem Cells Dev 23:2030–2045

Hall VJ, Kristensen M, Rasmussen MA, Ujhelly O, Dinnyes A, Hyttel P (2012) Temporal repression of endogenous pluripotency genes during reprogramming of porcine-induced pluripotent stem cells. Cell Reprogram 14:204–216

Hochedlinger K, Jaenisch R (2015) Induced Pluripotency and Epigenetic Reprogramming. Cold Spring Harb Perspect Biol 7:a019448

Huang J, Chen T, Liu X, Jiang J, Li J, Li D, Liu XS, Li W, Kang J, Pei G (2009) More synergetic cooperation of Yamanaka factors in induced pluripotent stem cells than in embryonic stem cells. Cell Res 19:1127–1138

Ji G, Ruan W, Liu K, Wang F, Sakellariou D, Chen J, Yang Y, Okuka M, Han J, Liu Z, Lai L, Gagos S, Xiao L, Deng H, Li N, Liu L (2013) Telomere reprogramming and maintenance in porcine iPS cells. PLoS One 8:e74202

Kahan BW, Ephrussi B (1970) Developmental potentialities of clonal in vitro cultures of mouse testicular teratoma. J Natl Cancer Inst 44:1015–1036

Kang L, Wang J, Zhang Y, Kou Z, Gao S (2009) iPS cells can support full-term development of tetraploid blastocyst-complemented embryos. Cell Stem Cell 5:135–138

Kielman MF, Rindapaa M, Gaspar C, van Poppel N, Breukel C, van Leeuwen S, Taketo MM, Roberts S, Smits R, Fodde R (2002) Apc modulates embryonic stem-cell differentiation by controlling the dosage of beta-catenin signaling. Nat Genet 32:594–605

Kim E, Kim M, Hwang SU, Kim J, Lee G, Park YS, Hyun SH (2019a) Neural induction of porcine-induced pluripotent stem cells and further differentiation using glioblastoma-cultured medium. J Cell Mol Med 23:2052–2063

Kim SJ, Kwon HS, Kwon DK, Koo OJ, Moon JH, Park EJ, Yum SY, Lee BC, Jang G (2019b) Production of transgenic porcine embryos reconstructed with induced pluripotent stem-like cells derived from porcine endogenous factors using piggyBac system. Cell Reprogram 21:26–36

King TJ, Briggs R (1955) Changes in the nuclei of differentiating gastrula cells, as demonstrated by nuclear transplantation. Proc Natl Acad Sci U S A 41:321–325

Kleinsmith LJ, Pierce GB Jr (1964) Multipotentiality of single embryonal carcinoma cells. Cancer Res 24:1544–1551

Kues WA, Herrmann D, Barg-Kues B, Haridoss S, Nowak-Imialek M, Buchholz T, Streeck M, Grebe A, Grabundzija I, Merkert S, Martin U, Hall VJ, Rasmussen MA, Ivics Z, Hyttel P, Niemann H (2013) Derivation and characterization of sleeping beauty transposon-mediated porcine-induced pluripotent stem cells. Stem Cells Dev 22:124–135

Kwon DJ, Jeon H, Oh KB, Ock SA, Im GS, Lee SS, Im SK, Lee JW, Oh SJ, Park JK, Hwang S (2013) Generation of leukemia inhibitory factor-dependent induced pluripotent stem cells from the Massachusetts General Hospital miniature pig. Biomed Res Int 2013:140639

Kwon DJ, Hwang IS, Kwak TU, Yang H, Park MR, Ock SA, Oh KB, Woo JS, Im GS, Hwang S (2017) Effects of cell cycle regulators on the cell cycle synchronization of porcine-induced pluripotent stem cells. Dev Reprod 21:47–54

Lee JH, Lee EJ, Lee CH, Park JH, Han JY, Lim JM (2009) Requirement of leukemia inhibitory factor for establishing and maintaining embryonic stem cells in mice. Fertil Steril 92:1133–1140

Li Y, McClintick J, Zhong L, Edenberg HJ, Yoder MC, Chan RJ (2005) Murine embryonic stem cell differentiation is promoted by SOCS-3 and inhibited by the zinc finger transcription factor Klf4. Blood 105:635–637

Li R, Liang J, Ni S, Zhou T, Qing X, Li H, He W, Chen J, Li F, Zhuang Q, Qin B, Xu J, Li W, Yang J, Gan Y, Qin D, Feng S, Song H, Yang D, Zhang B, Zeng L, Lai L, Esteban MA, Pei D (2010) A mesenchymal-to-epithelial transition initiates and is required for the nuclear reprogramming of mouse fibroblasts. Cell Stem Cell 7:51–63

Li X, Shan ZY, Wu YS, Shen XH, Liu CJ, Shen JL, Liu ZH, Lei L (2014) Generation of neural progenitors from induced Bama miniature pig pluripotent cells. Reproduction 147:65–72

Li D, Secher JO, Juhl M, Mashayekhi K, Nielsen TT, Holst B, Hyttel P, Freude KK, Hall VJ (2017) Identification of SSEA-1 expressing enhanced reprogramming (SEER) cells in porcine embryonic fibroblasts. Cell Cycle 16:1070–1084

Li D, Secher J, Hyttel P, Ivask M, Kolko M, Hall VJ, Freude KK (2018a) Generation of transgene-free porcine intermediate type induced pluripotent stem cells. Cell Cycle 17:2547–2563

Li X, Zhang P, Jiang S, Ding B, Zuo X, Li Y, Cao Z, Zhang Y (2018b) Aging adult porcine fibroblasts can support nuclear transfer and transcription factor-mediated reprogramming. Anim Sci J 89:289–297

Liao YJ, Chen YS, Lee JX, Chen LR, Yang JR (2018a) Effects of Klf4 and c-Myc knockdown on pluripotency maintenance in porcine-induced pluripotent stem cell. Cell J 19:640–646

Liao YJ, Tang PC, Chen YH, Chu FH, Kang TC, Chen LR, Yang JR (2018b) Porcine-induced pluripotent stem cell-derived osteoblast-like cells prevent glucocorticoid-induced bone loss in Lanyu pigs. PLoS One 13:e0202155

Liao YJ, Tang PC, Lin CH, Chen LR, Yang JR (2018c) Porcine-induced pluripotent stem cell-derived osteoblast-like cells ameliorate trabecular bone mass of osteoporotic rats. Regen Med 13:659–671

Liu X, Huang J, Chen T, Wang Y, Xin S, Li J, Pei G, Kang J (2008) Yamanaka factors critically regulate the developmental signaling network in mouse embryonic stem cells. Cell Res 18:1177–1189

Liu K, Ji G, Mao J, Liu M, Wang L, Chen C, Liu L (2012) Generation of porcine-induced pluripotent stem cells by using OCT4 and KLF4 porcine factors. Cell Reprogram 14:505–513

Liu Y, Yang JY, Lu Y, Yu P, Dove CR, Hutcheson JM, Mumaw JL, Stice SL, West FD (2013) alpha-1,3-Galactosyltransferase knockout pig induced pluripotent stem cells: a cell source for the production of xenotransplant pigs. Cell Reprogram 15:107–116

Liu K, Mao J, Song L, Fan A, Zhang S, Wang J, Fan N, Liu N, Ye X, Fu H, Zhou Z, Wang Y, Wei H, Liu Z, Li Z, Lai L, Wang X, Liu L (2017) DNA repair and replication links to pluripotency and differentiation capacity of pig iPS cells. PLoS One 12:e0173047

Luo J, Qin L, Kural MH, Schwan J, Li X, Bartulos O, Cong XQ, Ren Y, Gui L, Li G, Ellis MW, Li P, Kotton DN, Dardik A, Pober JS, Tellides G, Rolle M, Campbell S, Hawley RJ, Sachs DH, Niklason LE, Qyang Y (2017) Vascular smooth muscle cells derived from inbred swine induced pluripotent stem cells for vascular tissue engineering. Biomaterials 147:116–132

Ma K, Song G, An X, Fan A, Tan W, Tang B, Zhang X, Li Z (2014) miRNAs promote generation of porcine-induced pluripotent stem cells. Mol Cell Biochem 389:209–218

Ma Y, Yu T, Cai Y, Wang H (2018) Preserving self-renewal of porcine pluripotent stem cells in serum-free 3i culture condition and independent of LIF and b-FGF cytokines. Cell Death Dis 4:21

Malik V, Glaser LV, Zimmer D, Velychko S, Weng M, Holzner M, Arend M, Chen Y, Srivastava Y, Veerapandian V, Shah Z, Esteban MA, Wang H, Chen J, Schöler HR, Hutchins AP, Meijsing SH, Pott S, Jauch R (2019) Pluripotency reprogramming by competent and incompetent POU factors uncovers temporal dependency for Oct4 and Sox2. Nat Commun 10:3477

Mao J, Zhang Q, Deng W, Wang H, Liu K, Fu H, Zhao Q, Wang X, Liu L (2017) Epigenetic modifiers facilitate induction and pluripotency of porcine iPSCs. Stem Cell Rep 8:11–20

Martin GR (1981) Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. Proc Natl Acad Sci U S A 78:7634–7638

Miller RA, Ruddle FH (1976) Pluripotent teratocarcinoma-thymus somatic cell hybrids. Cell 9:45–55

Mizukami Y, Abe T, Shibata H, Makimura Y, Fujishiro SH, Yanase K, Hishikawa S, Kobayashi E, Hanazono Y (2014) MHC-matched induced pluripotent stem cells can attenuate cellular and humoral immune responses but are still susceptible to innate immunity in pigs. PLoS One 9: e98319

Montserrat N, Bahima EG, Batlle L, Hafner S, Rodrigues AM, Gonzalez F, Izpisua Belmonte JC (2011) Generation of pig iPS cells: a model for cell therapy. J Cardiovasc Transl Res 4:121–130

Montserrat N, de Onate L, Garreta E, Gonzalez F, Adamo A, Eguizabal C, Hafner S, Vassena R, Izpisua Belmonte JC (2012) Generation of feeder-free pig induced pluripotent stem cells without Pou5f1. Cell Transplant 21:815–825

Nelakanti RV, Kooreman NG, Wu JC (2016) Teratoma formation: a tool for monitoring pluripotency in stem cell research. Curr Protoc Stem Cell Biol 32:4A.8.1–4A.8.17

Nichols J, Smith A (2009) Naive and primed pluripotent states. Cell Stem Cell 4:487–492

Nichols J, Zevnik B, Anastassiadis K, Niwa H, Klewe-Nebenius D, Chambers I, Scholer H, Smith A (1998) Formation of pluripotent stem cells in the mammalian embryo depends on the POU transcription factor Oct4. Cell 95:379–391

Niwa H, Burdon T, Chambers I, Smith A (1998) Self-renewal of pluripotent embryonic stem cells is mediated via activation of STAT3. Genes Dev 12:2048–2060

Nizzardo M, Simone C, Falcone M, Riboldi G, Comi GP, Bresolin N, Corti S (2013) Direct reprogramming of adult somatic cells into other lineages: past evidence and future perspectives. Cell Transplant 22:921–944

Park KM, Cha SH, Ahn C, Woo HM (2013) Generation of porcine-induced pluripotent stem cells and evaluation of their major histocompatibility complex protein expression in vitro. Vet Res Commun 37:293–301

Park KM, Lee J, Hussein KH, Hong SH, Yang SR, Lee E, Woo HM (2016) Generation of liver-specific TGF-alpha/c-Myc-overexpressing porcine-induced pluripotent stem-like cells and blastocyst formation using nuclear transfer. J Vet Med Sci 78:709–713

Petkov S, Hyttel P, Niemann H (2013) The choice of expression vector promoter is an important factor in the reprogramming of porcine fibroblasts into induced pluripotent cells. Cell Reprogram 15:1–8

Petkov S, Hyttel P, Niemann H (2014) The small molecule inhibitors PD0325091 and CHIR99021 reduce expression of pluripotency-related genes in putative porcine-induced pluripotent stem cells. Cell Reprog 16:235–240

Petkov S, Glage S, Nowak-Imialek M, Niemann H (2016) Long-term culture of porcine-induced pluripotent stem-like cells under feeder-free conditions in the presence of histone deacetylase inhibitors. Stem Cells Dev 25:386–394

Rodriguez A, Allegrucci C, Alberio R (2012) Modulation of pluripotency in the porcine embryo and iPS cells. PLoS One 7:e49079

Samavarchi-Tehrani P, Golipour A, David L, Sung HK, Beyer TA, Datti A, Woltjen K, Nagy A, Wrana JL (2010) Functional genomics reveals a BMP-driven mesenchymal-to-epithelial transition in the initiation of somatic cell reprogramming. Cell Stem Cell 7:64–77

Secher JO, Ceylan A, Mazzoni G, Mashayekhi K, Li T, Muenthaisong S, Nielsen TT, Li D, Li S, Petkov S, Cirera S, Luo Y, Thombs L, Kadarmideen HN, Dinnyes A, Bolund L, Roelen BA, Schmidt M, Callesen H, Hyttel P, Freude KK (2017) Systematic in vitro and in vivo characterization of Leukemia-inhibiting factor- and Fibroblast growth factor-derived porcine-induced pluripotent stem cells. Mol Reprod Dev 84:229–245

Setthawong P, Phakdeedindan P, Tiptanavattana N, Rungarunlert S, Techakumphu M, Tharasanit T (2019) Generation of porcine-induced-pluripotent stem cells from Sertoli cells. Theriogenology 127:32–40

Smith AG, Heath JK, Donaldson DD, Wong GG, Moreau J, Stahl M, Rogers D (1988) Inhibition of pluripotential embryonic stem cell differentiation by purified polypeptides. Nature 336:688–690

Soufi A, Donahue G, Zaret KS (2012) Facilitators and impediments of the pluripotency reprogramming factors' initial engagement with the genome. Cell 151:994–1004

Sridharan R, Tchieu J, Mason MJ, Yachechko R, Kuoy E, Horvath S, Zhou Q, Plath K (2009) Role of the murine reprogramming factors in the induction of pluripotency. Cell 136:364–377

Stadtfeld M, Hochedlinger K (2010) Induced pluripotency: history, mechanisms, and applications. Genes Dev 24:2239–2263

Stadtfeld M, Maherali N, Breault DT, Hochedlinger K (2008) Defining molecular cornerstones during fibroblast to iPS cell reprogramming in mouse. Cell Stem Cell 2:230–240

Stevens LC, Little CC (1954) Spontaneous testicular teratomas in an inbred strain of mice. Proc Natl Acad Sci U S A 40:1080–1087

Tada M, Takahama Y, Abe K, Nakatsuji N, Tada T (2001) Nuclear reprogramming of somatic cells by in vitro hybridization with ES cells. Curr Biol 11:1553–1558

Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126:663–676

Tam PP, Rossant J (2003) Mouse embryonic chimeras: tools for studying mammalian development. Development 130:6155–6163

Tapia N, MacCarthy C, Esch D, Gabriele Marthaler A, Tiemann U, Arauzo-Bravo MJ, Jauch R, Cojocaru V, Scholer HR (2015) Dissecting the role of distinct OCT4-SOX2 heterodimer configurations in pluripotency. Sci Rep 5:13533

Telugu BP, Ezashi T, Roberts RM (2010) Porcine-induced pluripotent stem cells analogous to naive and primed embryonic stem cells of the mouse. Int J Dev Biol 54:1703–1711

Telugu BP, Ezashi T, Sinha S, Alexenko AP, Spate L, Prather RS, Roberts RM (2011) Leukemia inhibitory factor (LIF)-dependent, pluripotent stem cells established from inner cell mass of porcine embryos. J Biol Chem 286:28948–28953

Theunissen TW, Jaenisch R (2014) Molecular control of induced pluripotency. Cell Stem Cell 14:720–734

Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM (1998) Embryonic stem cell lines derived from human blastocysts. Science 282:1145–1147

Thomson AJ, Pierart H, Meek S, Bogerman A, Sutherland L, Murray H, Mountjoy E, Downing A, Talbot R, Sartori C, Whitelaw CB, Freeman TC, Archibald AL, Burdon T (2012) Reprogramming pig fetal fibroblasts reveals a functional LIF signaling pathway. Cell Reprogram 14:112–122

Uto S, Nishizawa S, Hikita A, Takato T, Hoshi K (2018) Application of induced pluripotent stem cells for cartilage regeneration in CLAWN miniature pig osteochondral replacement model. Regen Ther 9:58–70

Velychko S, Adachi K, Kim KP, Hou Y, MacCarthy CM, Wu G, Scholer HR (2019) Excluding Oct4 from Yamanaka Cocktail Unleashes the Developmental Potential of iPSCs. Cell Stem Cell 25:737–753.e4

Vierbuchen T, Ostermeier A, Pang ZP, Kokubu Y, Sudhof TC, Wernig M (2010) Direct conversion of fibroblasts to functional neurons by defined factors. Nature 463:1035–1041

Wang J, Gu Q, Hao J, Jia Y, Xue B, Jin H, Ma J, Wei R, Hai T, Kong Q, Bou G, Xia P, Zhou Q, Wang L, Liu Z (2013) Tbx3 and Nr5alpha2 play important roles in pig pluripotent stem cells. Stem Cell Rev Rep 9:700–708

Wei Z, Yang Y, Zhang P, Andrianakos R, Hasegawa K, Lyu J, Chen X, Bai G, Liu C, Pera M, Lu W (2009) Klf4 interacts directly with Oct4 and Sox2 to promote reprogramming. Stem Cells 27:2969–2978

Wei R, Lv J, Li X, Li Y, Xu Q, Jin J, Zhang Y, Liu Z (2020) Derivation of endothelial cells from porcine-induced pluripotent stem cells by optimized single layer culture system. J Vet Sci 21:e9

West FD, Terlouw SL, Kwon DJ, Mumaw JL, Dhara SK, Hasneen K, Dobrinsky JR, Stice SL (2010) Porcine-induced pluripotent stem cells produce chimeric offspring. Stem Cells Dev 19:1211–1220

West FD, Uhl EW, Liu Y, Stowe H, Lu Y, Yu P, Gallegos-Cardenas A, Pratt SL, Stice SL (2011) Brief report: chimeric pigs produced from induced pluripotent stem cells demonstrate germline transmission and no evidence of tumor formation in young pigs. Stem Cells 29:1640–1643

Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KH (1997) Viable offspring derived from fetal and adult mammalian cells. Nature 385:810–813

Wu Z, Chen J, Ren J, Bao L, Liao J, Cui C, Rao L, Li H, Gu Y, Dai H, Zhu H, Teng X, Cheng L, Xiao L (2009) Generation of pig induced pluripotent stem cells with a drug-inducible system. J Mol Cell Biol 1:46–54

Wu R, Liu Y, Zhao Y, Bi Z, Yao Y, Liu Q, Wang F, Wang Y, Wang X (2019) m(6)A methylation controls pluripotency of porcine-induced pluripotent stem cells by targeting SOCS3/JAK2/STAT3 pathway in a YTHDF1/YTHDF2-orchestrated manner. Cell Death Dis 10:171

Xu J, Yu L, Guo J, Xiang J, Zheng Z, Gao D, Shi B, Hao H, Jiao D, Zhong L, Wang Y, Wu J, Wei H, Han J (2019) Generation of pig induced pluripotent stem cells using an extended pluripotent stem cell culture system. Stem Cell Res Ther 10:193

Yamanaka S (2009) Elite and stochastic models for induced pluripotent stem cell generation. Nature 460:49–52

Yang JY, Mumaw JL, Liu Y, Stice SL, West FD (2013) SSEA4-positive pig induced pluripotent stem cells are primed for differentiation into neural cells. Cell Transplant 22:945–959

Yang F, Wang N, Wang Y, Yu T, Wang H (2017) Activin-SMAD signaling is required for maintenance of porcine iPS cell self-renewal through upregulation of NANOG and OCT4 expression. J Cell Physiol 232:2253–2262

Yang F, Ren Y, Li H, Wang H (2018) ESRRB plays a crucial role in the promotion of porcine cell reprograming. J Cell Physiol 233:1601–1611

Yu J, Vodyanik MA, Smuga-Otto K, Antosiewicz-Bourget J, Frane JL, Tian S, Nie J, Jonsdottir GA, Ruotti V, Stewart R, Slukvin II, Thomson JA (2007) Induced pluripotent stem cell lines derived from human somatic cells. Science 318:1917–1920

Yu T, Ma Y, Wang H (2017) EpCAM intracellular domain promotes porcine cell reprogramming by upregulation of pluripotent gene expression via beta-catenin signaling. Sci Rep 7:46315

Yuan Y, Park J, Tian Y, Choi J, Pasquariello R, Alexenko AP, Dai A, Behura SK, Roberts RM, Ezashi T (2019) A six-inhibitor culture medium for improving naive-type pluripotency of porcine pluripotent stem cells. Cell Death Dis 5:104

Yusa K, Rad R, Takeda J, Bradley A (2009) Generation of transgene-free induced pluripotent mouse stem cells by the piggyBac transposon. Nat Methods 6:363–369

Zhang Y, Wei C, Zhang P, Li X, Liu T, Pu Y, Li Y, Cao Z, Cao H, Liu Y, Zhang X (2014) Efficient reprogramming of naive-like induced pluripotent stem cells from porcine adipose-derived stem cells with a feeder-independent and serum-free system. PLoS One 9:e85089

Zhang W, Pei Y, Zhong L, Wen B, Cao S, Han J (2015) Pluripotent and metabolic features of two types of porcine iPSCs derived from defined mouse and human ES cell culture conditions. PLoS One 10:e0124562

Zhang W, Zhong L, Wang J, Han J (2016) Distinct microRNA expression signatures of porcine-induced pluripotent stem cells under mouse and human ESC culture conditions. PLoS One 11: e0158655

Zhang S, Xie Y, Cao H, Wang H (2017) Common microRNA-mRNA interactions exist among distinct porcine iPSC lines independent of their metastable pluripotent states. Cell Death Dis 8: e3027

Zhao XY, Li W, Lv Z, Liu L, Tong M, Hai T, Hao J, Guo CL, Ma QW, Wang L, Zeng F, Zhou Q (2009) iPS cells produce viable mice through tetraploid complementation. Nature 461:86–90

Zhong L, Mu H, Wen B, Zhang W, Wei Q, Gao G, Han J, Cao S (2018) Long non-coding RNAs involved in the regulatory network during porcine pre-implantation embryonic development and iPSC induction. Sci Rep 8:6649

Zhu S, Li W, Zhou H, Wei W, Ambasudhan R, Lin T, Kim J, Zhang K, Ding S (2010) Reprogramming of human primary somatic cells by OCT4 and chemical compounds. Cell Stem Cell 7:651–655

Zhu X, Li L, Gao B, Zhang D, Ren Y, Zheng B, Li M, Shi D, Huang B (2018) Early development of porcine parthenogenetic embryos and reduced expression of primed pluripotent marker genes under the effect of lysophosphatidic acid. Reprod Domest Anim 53:1191–1199

CRISPR/Cas System and Stem Cell Editing: Prospects and Possibilities in Veterinary Sciences

18

Md Saddam Hussain and Manish Kumar

Abstract

Stem cells provide novel approaches to improve animal health and productivity and thereby indirectly enrich human life. However, the use of stem cells, including the totipotent single–cell stage embryo, for the generation of genetically modified livestock and refinement of regenerative veterinary medicines has remained a less exploited domain until a few years ago, largely due to the nonavailability of efficient genetic manipulation tools. The inception of sequence-targeted genetic manipulation tools based on bacterial adaptive defense system, *c*lustered *r*egularly *i*nterspaced *s*hort *p*alindromic *r*epeat (CRISPR), and *C*RISPR-*as*sociated (Cas) protein (CRISPR/Cas) has enabled the bioengineers to harness the stem cell for the animal and human benefits in the way never done before. CRISPR/Cas-based genetic manipulation tools, due to its simplicity, high sequence specificity, and muliplexibility features, has dramatically broadened the dimension of stem cell applications in both the animal and human world, ranging from stem cell-based patient-specific therapeutics and anticancer vaccine development to the generation of genetically modified large animals with improved traits of agricultural and biomedical importance.

This chapter provides an overview of various CRISPR/Cas-based gene editing and regulation tools that have been instrumented for genomic modulation of mammalian cells to date. It discusses the critical elements of a typical CRISPR/Cas-based genetic manipulation experiment for efficient modulation of mammalian cells. Based on the reported studies, this chapter sheds light on CRISPR/Cas

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tools' potency to advance and accelerate the stem cell uses to benefit veterinary research.

18.1 Introduction

Stem cells are undifferentiated cells that can renew themselves and differentiate into various specialized cell types (Morrison et al. 1997). Based on their sources, they are classified as embryonic stem cells (ESCs), adult stem cells (ASCs), and induced pluripotent stem cells (iPSCs) (Evans and Kaufman 1981; Takahashi and Yamanaka 2006). However, based on the degree of stemness, stem cells can also be classified into three broad categories: totipotent (e.g., single-cell to the morula-stage embryo), pluripotent (e.g., ESCs and iPSCs), and multipotent stem cells (ASCs, e.g., HSCs and MSCs) (Wagers and Weissman 2004). The advent of stem cell technology in the late nineteenth century led to the development of a novel approach to disease treatment called regenerative medicine in both human and veterinary medicine. In regenerative medicines, permanent cure of a disease or clinical distress can be achieved by substituting the functionally impaired diseased or injured tissue/organ with in vitro grown healthy functional cells/tissue/organ, involving the tissue engineering strategies. The therapeutic use of stem cells in the veterinary field is underdeveloped. Although positive outcomes have been documented and reviewed in the treatment of many degenerative disorders in animals using MSCs (mostly demonstrative), including spinal injuries, tendonitis, periodontal defects, liver, and renal diseases (Voga et al. 2020). However, the generation of animals with improved agricultural and medical importance traits has been a prime goal in veterinary stem cell research compared to the development of regenerative therapeutics for veterinary use. Therefore, the embryos with totipotent stemness have primarily been researched for genetically modified (transgenic) animal development. Moreover, the conventional approach of animal transgenesis that includes integrating virus-based transduction, chemical mutagenesis, and homologous recombination was highly inefficient and plagued with a severe problem of random insertional mutagenesis (Pawar et al. 2020).

The instrumentation of targeted nuclease-based genetic manipulation tools such as transcription activator-like effector nuclease (TALEN), zinc finger nuclease (ZFN), and clustered regularly interspaced short palindromic repeats and associated protein (CRISPR/Cas) systems have revolutionized the potency of genome editing (Kim and Kim 2014). Using these advanced molecular tools, any genetic changes in a cell, thereby in an organism, can be introduced (i.e., gene knockin, knockout, knockdown) with unprecedented precision and efficiency. The advent of these sequence-targeted genetic manipulation tools has immensely extended the dimension of stem cell applications in both the veterinary and human world, ranging from patient-competent stem cell-based therapeutics and anticancer vaccine development to the generation of genetically modified domestic animals with exceptional disease resistance and agricultural traits (Crispo et al. 2015; Burkard et al. 2017; Yue et al.

2017; Hübner et al. 2018; Ouyang et al. 2019; Chu et al. 2020). Initially, ZFNs and TALENs were utilized for precise genetic engineering in domestic animals (Yang et al. 2011; Carlson et al. 2012; Proudfoot et al. 2015). However, in the last few years, CRISPR/Cas9-based tools have become the first choice for direct embryo editing and transgenic animal generation primarily because of its easy target customization and multiplexibility properties (Wang et al. 2015a; Zou et al. 2015; Komor et al. 2017). The precise editing with CRISPR/Cas9 tool has enabled the tissue engineers to produce cells with a desired single-base alteration that can be a correction of disease-associated mutation or vice versa, leading to the development of cell-based therapy or diseased cell/tissue model, or even generation of complete animal (healthy or diseased) if combined with somatic cell nuclear transfer (SCNT) technique (Ikeda et al. 2017; Williams et al. 2018). Furthermore, induced pluripotent stem cells (iPSCs) present an efficient alternative to ESCs in veterinary regenerative medicine as the derivation of ESCs from large animals is very challenging. However, the iPSCs reprogrammed through conventional retroviral-based approaches are considered not safe for regenerative medicine, primarily due to the high risk of virus-induced random insertional mutagenesis (Selvaraj et al. 2010). Now, with CRISPR/Cas9-based transcription modulation tools—transcription activator (CRISPRa; e.g., dCas9-VP64) or repressor (CRISPRi; e.g., dCas9-KRAB) (Gilbert et al. 2013; Kearns et al. 2014; Konermann et al. 2015)—genetically stable authentic iPSCs safe for downstream uses can be reprogrammed from any somatic cells, keeping the original genotype unchanged (Balboa et al. 2015; Xiong et al. 2017; Liu et al. 2018b; Weltner et al. 2018).

With a brief introduction of the CRISPR/Cas system and its derived tools, this chapter highlights the prerequisites of a CRISPR/Cas-based genetic manipulation endeavor for efficient modulation of mammalian cells. In the light of reported studies, it showcases the potency of CRISPR/Cas-based genetic manipulation tools to efficiently reprogram the stem cells, including the totipotent single-cell embryos, to develop regenerative veterinary therapeutics and genetically modified animals with improved agricultural traits.

18.2 CRISPR/Cas as Genetic Manipulation Tool: An Overview

*C*lustered *r*egularly *i*nterspaced *s*hort *p*alindromic *r*epeat (CRISPR) and *C*RISPR-*as*sociated (Cas) protein together, in prokaryotes, comprise a small RNA-guided acquired immune response, called CRISPR/Cas system that confers immunity against mobile genetic elements (MGEs) (phages and plasmids) (Barrangou and Horvath 2017). A functional CRISPR/Cas system typically consists of a CRISPR array (a battery of short, direct repeats of 20–50 bp separated by equal size unique spacer sequences), an upstream leader sequence, and a set of *cas* genes in the proximity (Jiang and Marraffini 2015). Spacers are short pieces of DNA that a CRISPR/Cas system had acquired from intruding MGEs as molecular memories of past infections. The CRISPR RNA (crRNA) transcribed and processed from the CRISPR array binds with Cas protein(s), forming ribonucleoprotein Cas/crRNA

effector complex. The crRNA recruits the Cas/crRNA effector complex to the target MGE via a complementary sequence called protospacer next to the protospacer adjacent motif (PAM). This eventually leads to targeted destruction of MGE by Cas endonuclease of the Cas/crRNA effector complex (Jiang and Marraffini 2015). Depending upon the composition of the Cas effector complex, the sequence similarity between Cas proteins, and the locus architecture, the CRISPR/Cas systems are broadly categorized into two classes (Class 1 and Class 2), six types (type I–VI), and 19 subtypes (Koonin et al. 2017). The CRISPR/Cas systems with multi-Cas protein effector complex are grouped in Class 1 (type I, III, and IV), while Class 2 includes CRISPR/Cas systems with a single Cas protein effector complex (type II, V, and VI) (Koonin et al. 2017).

Initially, the crRNA-mediated high-sequence specificity and requirement of a single endonucleolytic Cas protein to bind and cleave the target nucleic acids rendered the exploitation of Class 2 CRISPR/Cas systems as a genetic manipulation tool. The type II CRISPR/Cas9 system from *Streptococcus pyogenes* was the first to be exploited as a programmable DNA cleavage tool (Jinek et al. 2012). Thereafter, other Class 2 CRISPR/Cas systems from various bacteria have also been harnessed for targeted manipulation of DNA or RNA sequences (Aquino-Jarquin 2019; Moon et al. 2019). However, Cas9 from *S. pyogenes* (SpCas9) (also known as Cas9) is still the most commonly and creatively exploited Cas endonuclease for the targeted genetic manipulation (Jinek et al. 2012). The effector Cas endonucleases in different Class 2 CRISPR/Cas systems, excluding the Cas12a of type V and Cas13a of type VI, require an additional small RNA molecule [called trans-activating crRNA (tracrRNA)] with crRNA to target and disrupt the MGEs (Murugan et al. 2017). For targeted genetic manipulation in CRISPR/Cas9 systems, the crRNA and tracrRNA have been fused to a single chimeric RNA called single-guide RNA (sgRNA) (Deltcheva et al. 2011). In a genome (DNA) editing experiment, the target specified-sgRNA binds the Cas9 and recruits it to the cognate sequence in the target DNA via complementary base pairing where Cas9 induces a double-strand break (DSB). The produced DSB is then repaired by the cell's endogenous DNA repair machinery either via homology-directed repair (HDR) or non-homologous end joining (NHEJ) pathway. Restoration by the NHEJ pathway is error-prone, resulting in unpredictable small indel mutation (insertion or deletion) at the cleavage site. In contrast, the HDR path's repair generates precise alteration at the DSB site using the provided homology DNA template (knockin or knockout) (Jiang and Marraffini 2015).

Since its advent, the CRISPR/Cas system has been engineered to various highly efficient, precise, and easily customizable tools for gene editing as well as gene regulation purposes. The most commonly used *S. pyogenes* Cas9 proteins that in the natural state produce DSB at target DNA sequence have been engineered by mutagenesis to either a Cas9 variant called Cas9 nickase (D10A or H840A mutant) that cuts only one DNA strand of a dsDNA target (Anzalone et al. 2019) or a catalytically dead variant termed dCas9 (D10A and H840A mutation) that binds the dsDNA without cutting (Komor et al. 2016). A dCas9 fused with deaminase enzyme has been instrumental for base editing (Komor et al. 2016). Furthermore,

dCas9 protein fused with various transcriptional and epigenetic regulators has been exploited for efficient gene regulation studies (Xu and Qi 2019). The functionalization of dCas9 with transcription factor's effector domains either activator [e.g., VP64, a tetramer of the transactivation domain of herpes simplex *V*irus *P*rotein-16 (VP16)] or repressor (e.g., KRAB: Kruppel Associated Box) has led to the development of CRISPR-based transcription activation (CRISPRa; e.g., dCas9-VP64) or transcription repressor/interference (CRISPRi; e.g., dCas9-KRAB) systems, respectively (Xu and Qi 2019). Therefore, with continuing advancements and its relatively high-sequence specificity, easy programmability, substrate flexibility (DNA or RNA), and multiplexible nature, CRISPR/Cas system has become a preferred choice for genetic manipulation over the previously devised sequence targeted tools (ZFNs and TALENs) in the majority of the bioengineering laboratories worldwide (Komor et al. 2017; Hsu et al. 2019) (Fig. 18.1).

18.3 Urgencies of a Typical CRISPR/Cas-Based Genetic Manipulation

18.3.1 Selection of Effector Cas Protein

The genetic manipulation in a cell line or in an organism can be achieved via two ways: gene editing and transcriptional/epigenetic regulation of gene expression. In genome editing, a permanent alternation at target loci (insertion or deletion) is induced using DNA/RNA molecular scissors, such as catalytically active Cas endonucleases from different type II CRISPR/Cas systems (Murugan et al. 2017). Transcriptional and epigenetic regulation of gene(s) can be achieved using targeted effector systems such as CRISPR/dCas9-based transcription effectors (CRISPRa or CRISPRi) (Xu and Qi 2019). In the second approach, the desired trait in the target cell or organism is induced by stimulating or suppressing related endogenous gene (s), keeping the original genotype unchanged. Therefore, while designing a CRISPR/Cas-mediated genetic manipulation experiment, a careful selection of effector Cas protein is a crucial step, depending upon the output one wish (Fig. 18.2).

To date, a battery of Cas nucleases has been discovered and used for targeted genetic manipulation of mammalian cells ranging from dsDNA or ssDNA/RNA targeting Cas endonucleases for genome editing to catalytically dead Cas protein variants functionalized with effector proteins for targeted gene expression regulation (Moon et al. 2019). The most extensively exploited Cas protein for genetic manipulation, particularly in mammalian cells, is the Cas9 protein from *S. pyogenes* (SpCas9) (also known as Cas9) (Moon et al. 2019). Over the years, the SpCas9 (or Cas9) has been engineered to different variants for various genetic manipulation purposes, for example, the dCas9 fused with deaminase enzyme (e.g., dCas9-cytidine deaminase) for gene-targeted base editing (Komor et al. 2016), and dCas9 functionalized with transcriptional or epigenetic effector proteins (e.g., dCas9-VP64 and dCas9-HAT) for gene regulation studies (Xu and Qi 2019). Besides the SpCas9, other Cas9 orthologs and Cas variants have also been utilized for genetic

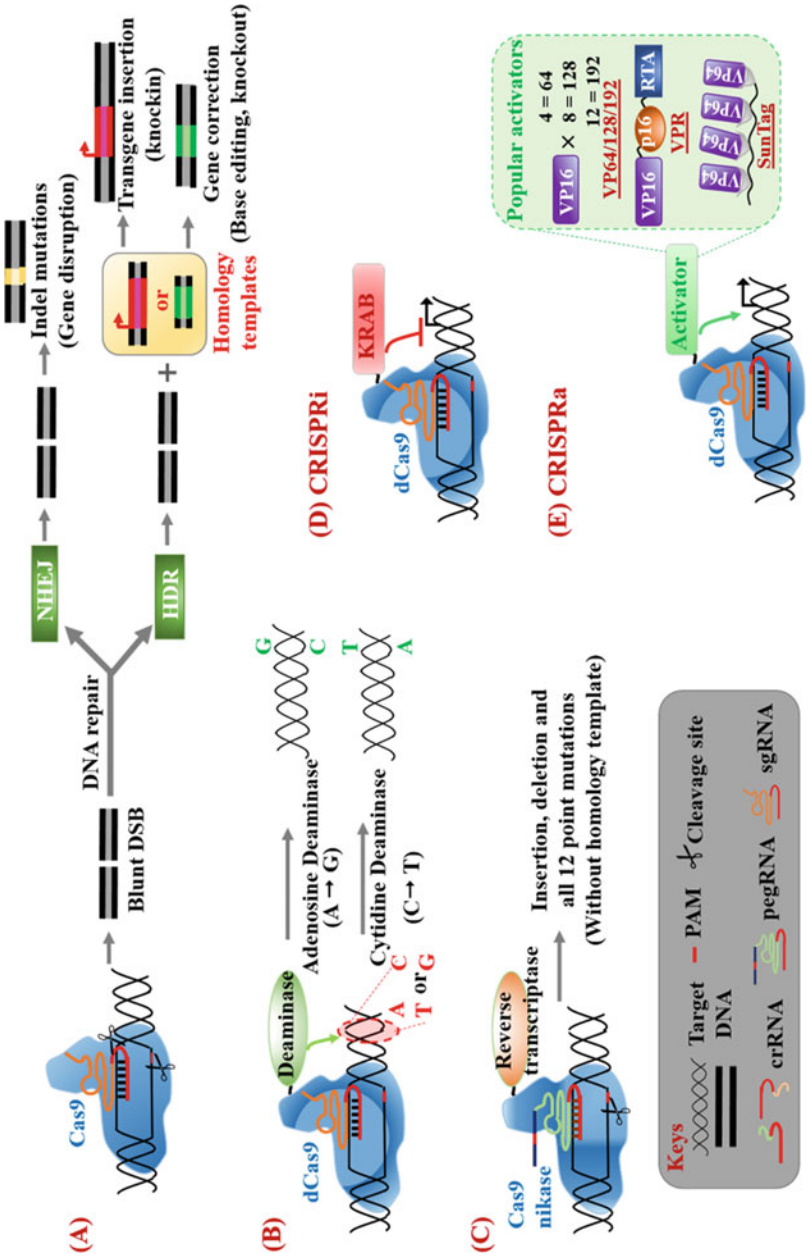

Fig. 18.1 CRISPR/Cas9 tools for mammalian cell genome modulation. Selected examples of CRISPR/Cas9-based various genome modulation tools are shown. (**a**) The double-strand break induced by target-specified sgRNA/Cas9 ribonucleoprotein complex is repaired either by error-prone non-homologous end

joining (NHEJ) pathway causing unpredictable indel mutation(s) or homology DNA template-dependent homology-directed repair (HDR) producing defined gene edits. (**b**) The catalytically dead Cas9 (dCas9) carries the fused deaminase enzyme (adenosine or cytidine deaminase) to a specified DNA sequence where deaminase catalyzes the deamination of the respective base leading to correction or mutation in the target gene. (**c**) The reverse transcriptase functionalized with Cas9 nickase is instrumental for targeted gene editing (insertion, deletion, and point mutations) without the need for a homology DNA template. (**d**) and (**e**) The functionalization of dCas9 with transcription repressor/interference (e.g., KRAB) or activator (e.g., VP64, VP192, VPR, or SunTag) produces CRISPR/Cas9-based transcription effector systems termed CRISPRi or CRISPRa, respectively. Upon reaching the specified genetic locus facilitated by linked dCas9, the transcription activator or repressor accordingly upregulates or downregulates the target gene expression

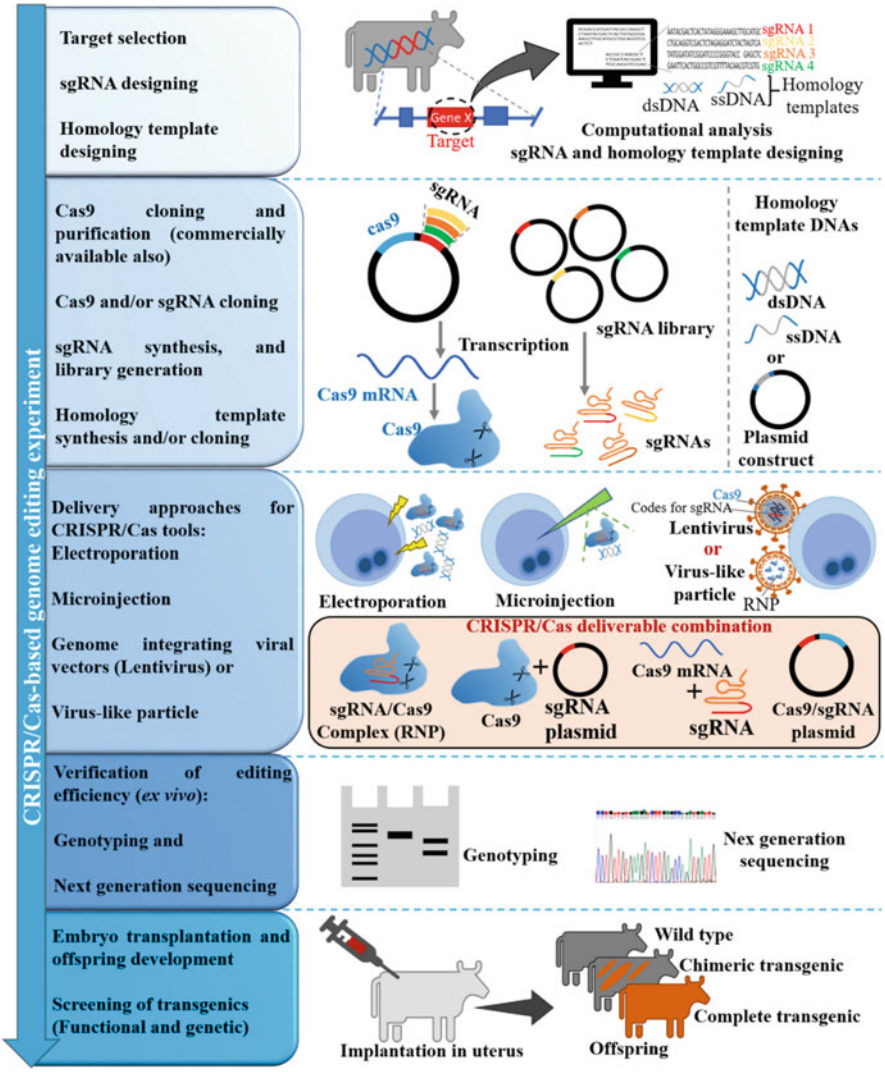

Fig. 18.2 The design of CRISPR/Cas-based genome editing experiment. The workflow for CRISPR/Cas-based genome editing of the pronuclear totipotent embryo cell, from designing the elements of the experiment to the production of genetically modified animals, has been illustrated

manipulation, including SaCas9 (*Staphylococcus aureus* Cas9) (Ran et al. 2015), CjCas9 (*Campylobacter jejuni* Cas9) (Kim et al. 2017), FnCas9 (*Francisella novicida* Cas9) (Acharya et al. 2019), Cas12 (Zetsche et al. 2015), Cas13 (Abudayyeh et al. 2016; Cox et al. 2017), and the recently characterized Cas14 (Aquino-Jarquin 2019). All these variants have distinct features in terms of structure and functions, such as the unique PAM sequence that they recognize and the nucleic

acid that they target (ssDNA, dsDNA, or RNA). Hence, a wide range of Cas protein variants is available to explore in order to achieve the desired goal of genetic manipulation. For instance, Cas9 is the preferred choice for targeted gene knockout or knockin (Jinek et al. 2012), while for a short-term gene knockdown at the transcriptional level without doing any permanent alternation in the genome, Cas13a protein-mediated disruption of target mRNA can be used (Abudayyeh et al. 2017).

18.3.2 The sgRNA Designing

The sgRNA or crRNA is the central element in a CRISPR/Cas mediated target-specific genetic manipulation experiment (Fig. 18.2). A sgRNA or crRNA is a short RNA oligonucleotide containing spacer sequence complementary to a PAM-containing target site in the gene to be altered. PAM sequence available in target DNA directs which CRISPR/Cas tool can be used for targeted genetic manipulation. For example, any DNA locus having PAM sequence NGG (any nucleotide followed by double guanosine) is the potential target site for CRISPR/SpCas9-based genetic manipulation tools (Jinek et al. 2012). Nevertheless, not all the sequences (crRNAs) induce cuts with the same specificity and efficiency. The sgRNA sequence should be designed as unique as reasonable to limit the chances of off-target effects to partially complementary sequences. Designing sgRNA with unique seed sequences (initial 12 nucleotides adjacent to the PAM) is recommended as those sequences confer specificity to a 20 nucleotide sgRNA (Wu et al. 2014; Zhang et al. 2017). Careful design of sgRNA and their prior validation for accuracy and efficiency are essential steps to avoid/minimize the off-target effect, primarily if used to generate genetically modified cell lines and large animals for biomedical and agricultural purposes. To this end, many dedicated computational algorithms have been developed that perform stringent screening of a target DNA for off-target sites, in silico design the potential sgRNAs and predict their efficiency for targeted DNA manipulation (Haeussler et al. 2016). The in silico predicted sgRNAs are then required to be validated for specificity and efficiency before utilizing them to produce genetically modified animals with desired traits. This analysis can be done in vitro using various cell-free systems, in ex vivo cultured cells of the target species, or directly in embryos. Once desired genome editing is confirmed by DNA sequencing, if efficiency is acceptable, one can proceed to generate genetically modified animals using a designed target-specified sgRNA/Cas9 tool for embryo editing and offspring development in a surrogate uterus.

18.3.3 The Homology Repair Template

In a typical CRISPR/Cas9-mediated genome editing experiment, the double-strand breaks (DSBs) generated by Cas9 at the sgRNA-specified locus in target DNA are repaired by two cellular DNA repair pathways: homology-directed repair (HDR) or

non-homologous end joining (NHEJ). In eukaryotes, DSBs are predominantly repaired by error-prone NHEJ pathways, leading to unpredictable small indel mutation (insertion or deletion) at the cleavage site without the need for any homology template (Chang et al. 2017). HDR path requires a homology DNA template, hence generate the desired alteration (Knockin or knockout); however, it is less efficient in eukaryotes (Hustedt and Durocher 2017; Canny et al. 2018). In prokaryotic cells, HDR is the major DSB repair pathway as most of the bacteria lack the NHEJ system (Shuman and Glickman 2007).

Since, in mammalian cells, the incidence of HDR for DSB repair is less efficient compared to NHEJ, designing an optimal homology repair template is critical to increase the HDR frequency. For large insertional mutation (knockin), dsDNA templates with homology arms of 1–3 kb on either side of DSBs are more effective. However, recent investigations have shown that single-stranded DNA (ssDNA) or single-stranded oligodeoxynucleotide (ssODN) is a more efficient HDR template for CRISPR/Cas9-based gene editing compared to dsDNA donors. The ssDNA homology templates induce gene editing with significantly improved efficiency and specificity, as well as reduced off-target effects, particularly useful in genome editing of stem cells and generation of genetically modified animals (Li et al. 2017a; Quadros et al. 2017). The ssDNA donor templates with 50–70 nt homology arms on both ends have been shown to result in highly efficient HDR-mediated gene edits (Li et al. 2017a). Furthermore, dsDNA (plasmids or PCR products) homology templates have been demonstrated to have low recombination efficiency and allow a high rate of off-target integration compared to ssDNA donors of equivalent sequences (Li et al. 2017a). The ssDNA homology templates have been efficiently used for the generation of knockin or knockout variants of sheep (Eaton et al. 2019), goats (Niu et al. 2018), and cattle (Perota et al. 2019).

18.3.4 The Delivery of the CRISPR/Cas Machinery Inside the Cell

Besides the fidelity of Cas protein and sgRNA (or crRNA) specificity, the approach used to deliver the CRISPR/Cas machinery inside the cell is another major determinant for efficient genome editing. Over the years, various delivery approaches have been investigated to achieve efficient and accurate genetic manipulation in mammalian cells. The CRISPR/Cas9 machinery can be introduced into cells as an all-in-one DNA expression plasmid, in vitro transcript (Cas9 mRNA and sgRNA), or ribonucleoprotein (RNP) complex. The last two approaches allow transient exposure of Cas9/sgRNA complex in the cell due to its shorter stability, resulting in relatively fast and precise gene editing with the least off-target alternation (Yin et al. 2016; Yip 2020). Broadly, there are two approaches to deliver the CRISPR/Cas machinery into mammalian cells: viral and nonviral systems (reviewed in Yip 2020). Viral vectors like adeno-associated viruses (AAV) and lentiviruses (LV) are efficient in vitro, ex vivo, and in vivo delivery systems. However, they have limited cloning capacity and can cause insertional mutagenesis that renders these tools unsafe for the

generation of genetically modified stem cells and animals for biomedical and agricultural purposes (Rothe et al. 2014).

The nonviral delivery system includes various physical and chemical methods. The physical techniques, namely microinjection and electroporation, are widely used methods for CRISPR/Cas9 delivery in stem cell and embryo editing experiments intended for advanced therapeutic and transgenic animal development. Nevertheless, these physical techniques are limited to the delivery of CRISPR/Cas in vitro and ex vivo because they need a microscope or high electrical current that cannot be applied in vivo. Gold nanoparticles (AuNPs) have been presented as a safer alternate of viral delivery methods for in vivo gene editing using CRISPR/Cas9 because of its immunologically inert nature (Lee et al. 2017). Furthermore, extracellular vesicles or virus-like particles (VLPs) also represent a potential approach for efficient delivery of Cas9/sgRNA RNPs in vitro and in vivo (Gee et al. 2020). As reported, VLP outperforms other viral and nonviral delivery methods as it offers a safe, transient, multiplexible, and cost-effective yet efficient delivery platform for CRISPR/Cas-based gene editing (Yip 2020). Hence, a wise selection of delivery approach is recommended to obtain efficient and accurate CRISPR/Cas-based genetic manipulation (Fig. 18.2).

18.4 CRISPR/Cas Tools Use in Stem Cell Technologies: Applications in the Veterinary World

18.4.1 CRISPR-Based Reprogramming and Directed Differentiation of Pluripotent Stem Cells

Stem cell technology holds great potential to expand veterinary science in regenerative medicine and genetically modified (GM) animal production to enrich human life. Despite the myriad of prospects in veterinary medicine, stem cell technologies have been primarily exploited in rodents and human medicine compared to large animals: probably due to the indirect influence of veterinary science advancements on humans. However, it is animals only, which serves as experimental models for studying human medicine. Large animals are also excellent preclinical models in human medicine development; even in some cases, such as heart disease, serve better than standard laboratory animals (e.g., mice and rats) (Wypij 2013; Pinnapureddy et al. 2015; Lindblad-toh 2020). This is not only because of their homology in size and physiology but also due to posing many of the same ailments and injuries as humans (Wypij 2013; Lindblad-toh 2020). Hence, as many of the techniques are transferrable from animals to humans, vice versa, in principle, should also be true that will add further improvement in the veterinary field. A stem cell technology demonstrated successful in humans and/or rodents can also be implied for and transferred to in large animals, anticipating although not the same but similar results. The following section discusses the various approaches reported so far for reprogramming and fate modulation of stem cells of different sources (human and

rodents), focusing on CRISPR-based methods to highlight CRISPR-based tools' potency in the area of veterinary stem cells.

The derivation of pluripotent stem cells such as ESCs in the case of large animals is a challenging undertaking, while reprogramming of readily available somatic cells to pluripotent stem cells is relatively explicit. Conventionally, somatic cells can be reprogrammed to pluripotent stem cells by retrovirus-mediated ectopic overexpression of pluripotency factors Oct3/4, Sox2, Klf4, and c-Myc (OSKM) that in turn reactivate the endogenous pluripotency genes network, leading to the establishment of cells with ESC-like stemness, termed as induced pluripotent stem cells (iPSCs) (Takahashi and Yamanaka 2006; Soufi et al. 2012). Since the first demonstration of iPSCs regeneration from mouse embryonic fibroblast (Takahashi and Yamanaka 2006) and adult human dermal fibroblast cells (Takahashi et al. 2007) by ectopic overexpression of OSKM factors, various mouse and human cell types have been reprogrammed to bonafide iPSCs, including liver cells (Aoi et al. 2008), pancreatic beta cells (Stadtfeld et al. 2008), neural cells (Tat et al. 2010), blood cells (Wen et al. 2016), and mesenchymal stem cells (Oda et al. 2010). Subsequently, using a similar approach, iPSCs have also been derived from somatic cells of domesticated animal species, including canine (Koh and Piedrahita 2015), rabbit (Honda et al. 2013), bovine (Cao et al. 2012), goat (Sandmaier et al. 2015), porcine (Fujishiro et al. 2013), and buffalo (Deng et al. 2012). Moreover, despite the successful demonstration of iPSCs regeneration from various domesticated animal species, so far, no live adult animal with germline transmission competency could be developed using these reprogrammed iPSCs while a similar approach has worked for rodents. The exact ground(s) for such incompetency of these large animal-sourced iPSCs is not entirely known; however, inadequate modulation of endogenous gene expression evident in the virus-mediated reprogramming approach has been suggested to be one of the primary reasons (Du et al. 2015; Smith et al. 2016). Notably, the high risk of mutagenesis due to random integration of transgenes in target cell genome as well as inefficient reprogramming of endogenous pluripotency network ultimately preclude the downstream uses of produced iPSCs (Selvaraj et al. 2010). To overcome these shortcomings, various strategies have been implemented in the last few years. For instance; the use of nonintegrating viral vectors such as Sendai (Fusaki et al. 2009) or adenoviruses (Zhou and Freed 2009) as an alternative to genome-integrating retroviral (Takahashi and Yamanaka 2006), lentiviral vectors (Brambrink et al. 2008), and nonviral techniques like microRNA (Miyoshi et al. 2011), synthetic mRNA (Yoshioka et al. 2013; Preskey et al. 2016), episomal vector (Yu et al. 2009), piggyback transposon (Talluri et al. 2015), and programmable transcription modulation tools: ZF, TALE, and CRISPR/Cas9-based transcription factors (Gao et al. 2013; Kearns et al. 2014; Ji et al. 2014; Weltner et al. 2018). Transcriptional modulation of cell fate-determining endogenous gene(s) is an efficient alternative approach for converting differentiated cells into stable iPSCs or specific cell types with no risk of genomic-integration and partial reprogramming unlike in the conventional approaches. Reprogramming via direct activation of endogenous pluripotency genes (OSKM) in mouse and human somatic cells has been reported using zinc finger-transcription factors (ZF-TFs) (Ji et al. 2014) or

transcription activator-like effector (TALEs) techniques (Gao et al. 2013). However, the broader application of these designer transcription factors is limited by their designing difficulties and multiplexing-related conditions. Whereas in engineered CRISPR/Cas9-based transcription factors with its sgRNA-based easy reprogramming, superior genomic loci targeting specificity, and high multiplexing capacity are found to be overall a more efficient tool for simultaneous modulation of endogenous gene sets for specific cell-type conversion (Fig. 18.3).

Recently, programmable CRISPR/dCas9-based transcription modulating systems (CRISPRa and CRISPRi) have been utilized to reprogram the somatic cell of human/mouse origin into bonafide iPSCs (Balboa et al. 2015; Liu et al. 2018b; Weltner et al. 2018) as well as for directed differentiation of human/mouse pluripotent stem cells (iPSCs and ESCs) to particular cell lineages (Kearns et al. 2014; Chakraborty et al. 2014; Chavez et al. 2015; Wei et al. 2016; Black et al. 2016). Kearns et al. (2014) have demonstrated the CRISPRa (dCas9-VP64) or CRISPRi (dCas9-KRAB)-mediated modulation of human pluripotent stem cells differentiation by targeting the developmentally relevant endogenous *SOX17* and *OCT4* gene (Kearns et al. 2014). Furthermore, direct reprogramming of mouse embryonic fibroblast (MEF) cells to skeletal myocytes have been reported by targeted activation of endogenous *MYOD1* gene using CRISPR-based transcription activator, VP64-dCas9-BFP-VP64 (Chakraborty et al. 2014). The selective upregulation of endogenous cell-fate regulating factors neurogenin 2 (NEUROG2) or neurogenic differentiation factor 1 (NEUROD1) by customized CRISPRa system, dCas9-VP64-p65-Rta system (abbreviated as dCas9-VPR), has led to the differentiation of human iPSCs into induced neuronal cells (iNCs) (Chavez et al. 2015). Besides this, simultaneous targeted activation of endogenous BAM factors (*BRN2*, *ASC11*, and *MYT11* gene) by the VP64-dCas9-VP64 CRISPR activation system has been shown to directly reprogram the MEFs into iNCs (Black et al. 2016). The CRISPRa (dCas9VP16-P65-HSF1: abbreviated as dCas9VPH)-based targeted activation of pluripotency gene sets in human foreskin fibroblasts resulted in the regeneration of stable iPSCs, while dCas9VPH-mediated activation of endogenous *OCT4* gene alone was shown to be sufficient for reprogramming of human neuroepithelial stem cells into iPSCs (Weltner et al. 2018). In another study, direct conversion of mouse ESCs into extraembryonic lineages, namely trophoblast stem cells and extraembryonic endoderm cells, was achieved by enhancing the expression of lineage-specific endogenous *CDX2* and *GATA6* genes using CRISPR-based transcription upregulator, dCas9-VP64 (Wei et al. 2016). Furthermore, recently Liu et al. (2018b) have demonstrated the regeneration of authentic mouse iPSCs by precise expression remodeling of endogenous OCT4 or SOX2 pluripotency factors in MEFs using a designer CRISPR activation system termed dCas9-SunTag-VP64 (Liu et al. 2018b).

All these proof-of-principle studies demonstrate the potency of CRISPR/dCas9-based transcription modulators for efficient regeneration of authentic iPSCs and their differentiation modulation to specific cell-type lineages irrespective of organism type and species, including mouse, human, yeast, and drosophila (Chavez et al. 2015). The rapid and sustained activation/inhibition of endogenous genes in their native chromatin context by CRISPR/dCas9-based transcription effectors allows

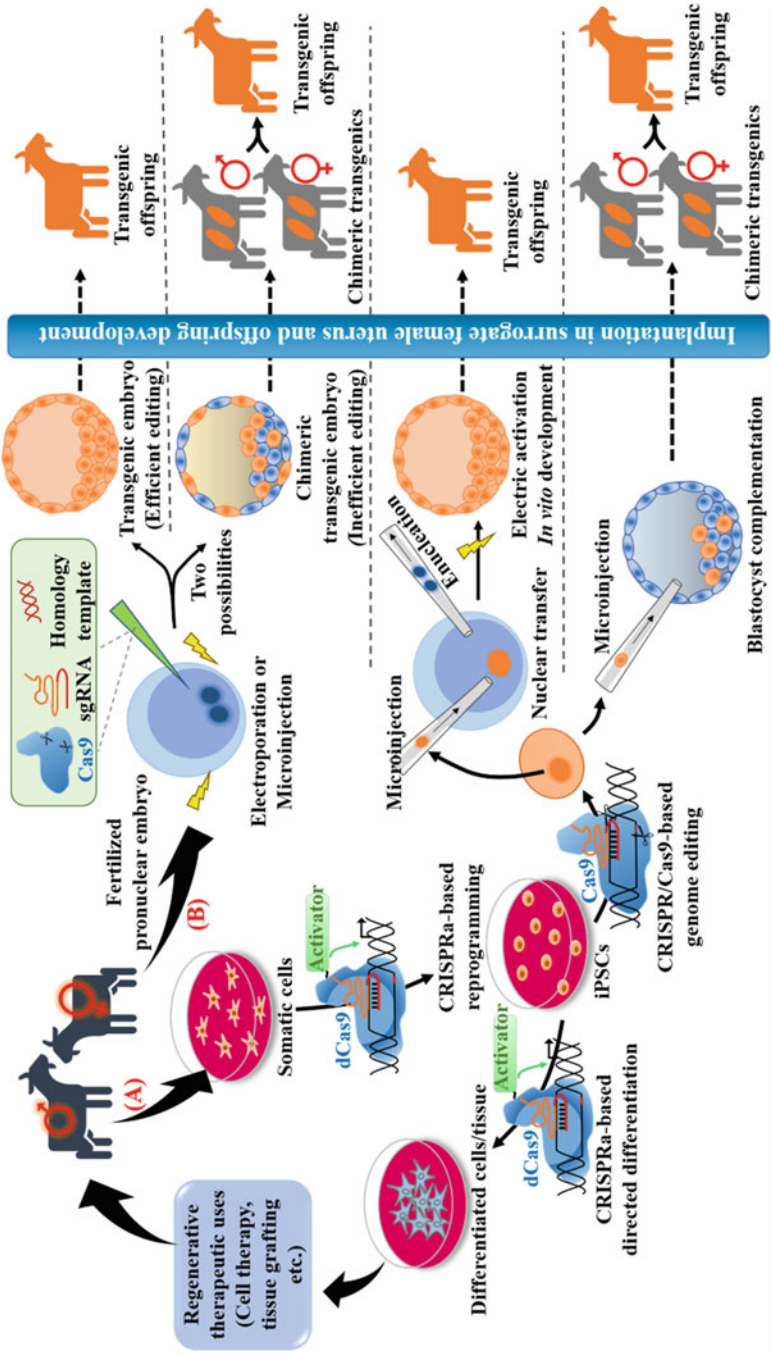

Fig. 18.3 CRISPR/Cas-based stem cell modulation to aid the veterinary. (**a**) The transcriptional activation of endogenous pluripotency genes [e.g., Oct3/4, Sox2, Klf4, and c-Myc (OSKM)] by CRISPR/dCas9-based transcription activation system (CRISPRa) reprograms somatic cells into induced pluripotent stem cells (iPSCs). iPSCs can further be genetically modulated to develop various regenerative medicines, including tissue/organ transplants and cell-based

therapeutics. The complementation of iPSCs with desired gene edit(s) to a blastocyst embryo by microinjection leads to transgenics development. iPSCs are efficient nucleus donor for somatic cell nuclear transfer (SCNT)-based animal transgenesis. (**b**) The direct injection of CRISPR/Cas9 genome editing machinery to totipotent single-cell embryos by electroporation or microinjection results in transgenics animals' production

efficient cell-fate reprogramming without altering the original genotype. This CRISPR/dCas9-based transcription effector-mediated endogenous genes activation or silencing approach to modulate the cell fate should also work in the generation and reprogramming of iPSCs from large animal species.

However, to design an experiment for cell fate reprogramming via CRISPR/dCas9-based transcription modulation of endogenous genes, it is notable that the different animal species and cell types within a species differ in their genomic sequences and the role and expression pattern of proteins instrumental for cell-fate determination and maintenance also vary (Piliszek et al. 2016). For instance, CDX2 is an essential transcription factor for maintaining the differentiated state of trophectoderm (TE; a somatic cell) in mouse (Strumpf 2005), while in humans, it is not involved in TE specification (Niakan and Eggan 2013); the pluripotency factor OCT4 protein expresses in ICM and TE of humans, bovine and porcine blastocysts, but not in ICM of murine blastocysts (Kirchhof et al. 2000; Kuijk et al. 2008). Besides this, it is also noticeable that the same CRISPR/dCas9-based transcription modulation system may not be equally effective for all genes. For example, dCas9 protein fused with a single VP64 transcription activation domain (dCas9-VP64) has been shown to efficiently upregulate the expression of endogenous *CDX2* and *GATA6* genes, leading to the conversion of mouse ESCs into two extraembryonic lineages: trophoblast stem cells and extraembryonic endoderm cells (Wei et al. 2016); while in many other studies, single VP64-based activators have been reported not effective in achieving expression level sufficient enough to initiate the conversion of cell fate (Hu et al. 2014; Ji et al. 2014; Gao et al. 2014). Hence, the screening of appropriate cell-fate governing endogenous genes to be modulated and accordingly proper sgRNAs designing, the optimization of components of transcription activators/repressors, and their number and position in tandem to dCas9 are critical for efficient reprogramming of target cells to specific cell type (Wei et al. 2016).

In conclusion, the CRISPR/dCas9-based transcription modulation systems provide a novel, cost-effective, and promising approach for reprogramming the cell fate via direct modulation of developmentally relevant endogenous genes without any genomic alteration. Nevertheless, this approach for iPSCs generation and specific cell lineage production has only been demonstrated in rodents (mice and rats) and humans. However, the accumulating evidence from these studies would be path directing for the successful implication of the CRISPR-based approach to directly reprogram the somatic cells of large animal species into authentic iPSCs, which has to date not been feasible with conventional reprogramming methods. The cells reprogrammed in this way, including iPSCs, due to stable expression of relevant endogenous genes and no risk of mutagenic genomic integration will have tremendous application in veterinary regenerative medicine, including the development of patient-specific cell therapy, disease modeling, and drug discovery. The availability of germline-competent iPSCs through the CRISPR/dCas9-based transcription modulation approach would also improve the efficiency of genetically modified large animal production for biomedical and agricultural uses employing iPSCs as potent nucleus donors in SCNT cloning instead of somatic cells or ESCs (Han et al. 2011).

18.4.2 CRISPR-Based Stem Cell Modulation: Scope in Veterinary Sciences

18.4.2.1 Generation of Genetically Modified Animals with Improved Productivity and Fitness

One of the significant aspects of veterinary is to ameliorate the productive and fitness traits of livestock mainly for enhanced production of animal-derived products, including milk, meat, chicken eggs, and wool. The development of transgenic animals with favorable traits through genetic engineering is a potential approach over conventional reproduction technologies to meet the continuously increasing world population's demands. However, until a few years ago, efficient gene-targeted genetic modification to gain/lose the desired/undesired traits in livestock species had been a significant challenge for animal biotechnologists. Since the introduction of engineered nucleases-based genome editing tools, a revolution in gene targeting techniques got embarked. The targeted gene knockin and knockout in mammalian cells and embryos have become a practical approach for generating the animals of desired traits. CRISPR/Cas9, due to its easy programmability, superior sequence specificity, and multiplexibility features, has become a preferred choice for genome editing over the other genetic manipulation tools (ZFNs and TALENs), especially for animal cell editing. The totipotent stemness property of single-cell stage embryos has been exploited to develop germline transmission competent genetically modified large animal species. The embryos can be modulated in three ways to generate genetically modified animals: (1) direct genome editing of totipotent single-cell stage embryo, (2) complementation of blastocyst-stage embryo with genetically engineered pluripotent stem cells, and (3) transfer of nucleus from genome-edited pluripotent stem cells or somatic cells to enucleated single-cell embryo. In the first approach, a totipotent single-cell pronuclear embryo is genetically modified by direct injection of an efficient genome editing tool using various delivery approaches, including microinjection (cytoplasmic or pronuclear) (Liu et al. 2018a) and electroporation (Kaneko et al. 2015; Tanihara et al. 2016). Implantation of such an engineered embryo into the uterus of a pseudopregnant female leads to the development of transgenic animals (Laible 2018). The second approach is called blastocyst complementation, wherein genetically engineered pluripotent stem cells for the desired trait(s) are directly microinjected into a blastocyst-stage embryo that can develop into chimeric offspring once transferred into the uterine cavity (Crane et al. 2019). The third approach is known as somatic cell nuclear transfer (SCNT) cloning in which the nucleus with desired DNA modification(s) from genome-edited somatic cells/pluripotent stem cells is transferred into enucleated single-cell stage embryo that develops into GM offspring upon in vivo implantation (Cibelli 1998; Richter et al. 2012). The production of transgenics with no risk of mosaicism is the primary advantage of SCNT as it allows the screening of genome-edited cells for desired edits before microinjecting into an enucleated embryo (Whitelaw et al. 2016; Lee et al. 2016). The Superfine Merino breed of sheep has been genetically engineered by CRISPR/Cas9 tool to myostatin (MSTN) mutant strain, which can grow double in body weight with a faster growth rate than its wild-type counterparts

(Crispo et al. 2015). The targeted disruption of MSTN gene, a negative regulator of skeletal muscle mass (McPherron et al. 1997), in totipotent single-cell embryos by direct cytoplasmic microinjection of sgRNA/Cas9 mRNA has led to the development of an improved Merino breed that grow double in body mass enabling enhanced production of high-quality wool (Crispo et al. 2015). Fibroblast growth factor 5 (FGF5) negatively regulates the length of hair in various animals, including humans (Rosenquist and Martin 1996). The sgRNA/Cas9 mRNA-mediated *FGF5* knockout in in vitro fertilized totipotent embryo resulted in the generation of a genetically improved variety of Merino sheep that produce wool of longer length (Li et al. 2017b). Similarly, to improve the meat and hair production, the Cashmere goat variety was genome-edited by co-injection of single-cell stage embryos with Cas9 mRNA and sgRNAs-specified to simultaneously disrupt the *MSTN* and *FGF5* gene (Wang et al. 2015b). CRISPR/Cas9 tool has also been used to produce companion animals with desired traits. Zou et al. (2015) had reported the production of a super muscular beagle dog with almost double muscular mass by a loss-of-function mutation in the *MSTN* gene using the CRISPR/Cas9 genome editing approach (Zou et al. 2015).

Animal biotechnologists have also employed the CRISPR/Cas9 tool to facilitate the production of offspring with desired sex that significantly impacts livestock industries. Livestock with one sex is usually preferred over the other depending upon the selected animal products, such as milk from cows, meat from bulls, and eggs from laying hens. Biotechnologists had integrated a fluorescence protein, eGFP, into the Y chromosome using the CRISPR/Cas9 tool combined with SCNT cloning that led to the generation of eGFP tagged-Y-Chr transgenic buffalos (Zhao et al. 2020). In vitro fertilization of wild-type oocytes with transgenic buffalo sperms resulted in XY embryos' production with a green appearance under a fluorescence microscope and XX embryos without any fluorescence. This had allowed the efficient sex determination of pre-implantation embryos facilitating the production of bovines with the desired sex type. In another report, male-to-female sex reversal in rabbits has been demonstrated by mutating the Sp1-binding site in the 5′ flanking region of the *SRY* gene via cytoplasmic microinjection of CRISP/Cas9 coding plasmid into totipotent pronuclear embryos (Kurtz and Petersen 2019). However, a CRISPR/Cas9-based similar approach has not worked for porcine sexing (Kurtz and Petersen 2019).

Besides this, the CRISPR/Cas9 tool has also been utilized for the generation of transgenic livestock to produce allergen-free or allergen-reduced animal-derived products. For instance, chicken variety with CRISPR/Cas9-induced knockout mutation in ovalbumin and ovomucoid coding genes has been developed to produce allergen-free eggs (Oishi et al. 2016). Many people are allergic to the β-lactoglobulin of milk. Hence, a goat strain with β-lactoglobulin knockout has been developed by CRISPR/Cas9-mediated genome editing (Zhou et al. 2017). This has made milk or chicken eggs digestible for many consumers who are otherwise unable to consume milk or chicken eggs. These examples together highlight the opportunities of CRISPR/Cas tools to improve the production efficiency in livestock.

18.4.2.2 Animal Health Improvement

Disease-Resistant Transgenic Animals

Diseases affecting animals have severe impact on livestock production and derived products, thereby on zoonosis and public health. Thus, developing livestock strains resistant to or less susceptible to a specific disease is a significant interest in veterinary science. Instrumentation of advanced molecular tools such as CRISPR/Cas-based precise genome modulators has enabled the biotechnologist to develop disease-resistant livestock more efficiently. Genetic modulation of embryos at the single-cell totipotent stage using the CRISPR/Cas9 tool has led to the development of various disease-resistant livestock, including pigs and cows (Burkard et al. 2017; Gao et al. 2017; Hübner et al. 2018). Disease-resistant organisms can be produced either by knockout of cellular genes coding for viral receptors or by arming the host cells with antiviral effectors. Pigs entirely resistant for the porcine reproductive and respiratory syndrome (PRRS) and African swine fever (ASF) have been produced through CRISPR/Cas9-mediated genome editing of zygotes (Burkard et al. 2017; Hübner et al. 2018). ASF and PRRS are two economically important swine diseases caused by the African swine fever virus (ASFV) and porcine reproductive and respiratory syndrome virus (PRRSV), respectively. Pigs resistant to PRRSV have been created by CRISPR/Cas9-mediated disruption of *CD163* or *CD1D* gene (Whitworth et al. 2014, 2016). CD163 expression on the surface of porcine macrophages is a fusion receptor for PRRSV, thus essential for PRRSV infection (Whitworth et al. 2016). Initially, PRRSV-resistant pigs were generated by plasmid-derived expression of Cas9 and sgRNA targeting the CD163 gene in single-cell stage embryos (Whitworth et al. 2014, 2016). However, CD163 plays many other biological functions, including the inflammatory response to various pathogens. The knockout of the CD163 receptor resulted in the loss of other vital roles of this receptor. Therefore, another group reported the production of a pig strain completely resistant to PRRSV infection by partial deletion of the CD163 gene keeping its other biological role intact (Burkard et al. 2017). The PRRS virus essentially requires the scavenger receptor cysteine-rich domain 5 (SRCR5) of CD163 to interact and infect the porcine macrophages (Van Gorp et al. 2010). Hence, targeted cleavage of the genomic sequence coding for the SCCR5 domain of CD163 in a single-cell stage zygotes by CRISPR/Cas9 system resulted in the production of PRRS-resistant pigs with no side effects (Burkard et al. 2017). Corona virus-resistant pigs have also been generated via CRISPR/Cas9-based genome editing of totipotent single-cell embryos, followed by their implantation in the surrogate show (Whitworth et al. 2019).

Furthermore, improved disease resistance in cattle against *Mycobacterium bovis* has also been reported using the CRISPR tool (Gao et al. 2017). *M. bovis* is known to cause tuberculosis in cattle that cost a significant economic loss to the cattle industry globally. Using the CRISPR/Cas9 nickase system, together with SCNT, embryos expressing transgene NRAMP1 (natural resistance-associated macrophage protein 1) were created that developed into cattle with less susceptibility to *M. bovis* infection (Gao et al. 2017). NRAMP1 induces the production of nitric oxide and other pro-inflammatory responses that, in turn, provide innate resistance to

intracellular pathogens. Thus, ectopic expression of NRAMP1 in transgenic cattle results in improved immunity against the intracellular pathogen *M. bovis* (Gao et al. 2017). Further, toward an attempt to develop mad cow disease-resistant cattle, the generation of genetically modified bovine embryos with a mutation in the disease-associated prion protein (PRNP) coding gene was demonstrated using the CRISPR/Cas9 tool (Bevacqua et al. 2016). Recently, scientists have successfully treated the isoleucyl-tRNA synthetase (IARS) syndrome in Japanese black cattle through CRISPR/Cas9 genome editing (Ikeda et al. 2017). The Japanese black cattle is a major cultivating strain for high-quality beef production in Japan. The IARS syndrome is known to occur due to single-nucleotide substitution mutation. The CRISPR/Cas9-mediated homologous direct repair has led to the generation of cells with the corrected genome. And using the SCNT approach, genome-edited embryos were produced that, on implantation, developed into healthy cattle (Ikeda et al. 2017). These examples show the CRISPR/Cas9 system's potential as an efficient embryo modulation tool for the generation of disease-resistant livestock species to benefit the animals and humans.

Regenerative Veterinary Medicine

Regenerative veterinary medicine is an active and emerging area of research. In combination with advanced molecular tools such as CRISPR/Cas system, stem cells offer veterinarians a potential option to efficiently improve animal health. Significant advances in developing safe and efficient stem cell-based therapies have been reported in recent years. Stem cells have been used, mostly experimentally, to treat various diseases in different animal species. Among different stem cell types, MSC is the most extensively utilized cell type for the therapeutic purpose, primarily because of its immunomodulatory abilities and simple isolation protocol (Esmaeilzadeh and Farshbaf 2015; Voga et al. 2020). Notable advancements in the treatment of various orthopedic conditions, inflammatory bowel disease (IBD), feline chronic gingivostomatitis (FCGS), and wound healing in different animal species have been demonstrated using MSCs (reviewed in Voga et al. 2020). Genetically modified stem cells, including MSCs, have been employed for targeted treatment of various cancer in murine models based on the intrinsic properties of MSCs to selectively migrate toward the injured and tumor site(s) (Shah 2012; Esmaeilzadeh and Farshbaf 2015). In this approach, MSCs are virally transduced for increased production of antitumor factors (cytokines/chemokines) (Studeny et al. 2002) or prodrug-converting enzymes (Malekshah et al. 2016). Genetically equipping MSCs with a prodrug-converting enzyme lead to the release of the enzyme by MSCs at the tumor site(s). Hence, a prodrug, injected in the body, gets converted into an active antitumor molecule by a prodrug-converting enzyme released from MSCs homing at the tumor site, leading to selective necrosis and death of cancer cells over normal cells (Malekshah et al. 2016; Marelli et al. 2018). For instance, notable outputs have been reported for cancer therapy using cytosine deaminase (CD)-expressing MSCs or NSCs that convert the 5-fluorocytosine (5-FC; a prodrug) into tumor-toxic 5-fluorouracil (5-FU) (Chang et al. 2010; Lee et al. 2013). A CRISPR/dCas9-deaminase system specified for tumor-specific DNA sequence combined with

an efficient delivery method can be a potential alternative to CD-expressing MSCs-based cancer therapy.

However, there are some disadvantages of using MSCs to treat various clinical conditions. The relatively larger size of MSCs, specifically in allogeneic MSCs, may lead them to be readily detected and phagocytized by the recipient immune cells, thus causing therapy rejection (Zhang et al. 2013). Besides this, MSCs may also direct the tumor cells to a dormant state if internalized by cannibal tumor cells, and the dormancy of tumor cells is thought to be a major reason behind tumor relapse (Bartosh et al. 2016). Furthermore, genetically modified MSCs are mainly produced by virus-based approaches to date, making them unsafe for clinically uses as there is a high chance of insertional mutations in MSCs or in accidentally targeted normal cells and the risk of the antiviral immune response.

The CRISPR/Cas9-based genetic manipulation of MSCs has been proposed to be a promising solution for many of the issues associated with the therapeutic uses of virally genome-edited MSCs (Marofi et al. 2017). CRISPR/Cas9 system, being a simple-to-use and sequence-specific genetic manipulation tool, can be used to generate stem cells expressing desired therapeutic molecule(s) without any random insertional mutations and no chance of antiviral immune response, unlike in the case of virus-based genome modulation. Furthermore, iPSCs present a potential substitute of MSCs for stem cell therapy in animals as the patient-specific iPSCs can be regenerated in an adequate number by reprogramming the readily available somatic cells. The specialized cell differentiation from human or mice iPSCs has effectively treated the various clinical conditions in disease animal models. The pancreatic beta-cells differentiated from mouse iPSCs have been shown to release insulin in response to glucose both in vitro and in vivo (Jeon et al. 2012). In other reports, significant improvement in Parkinson's disease treatment in rat and monkey model was obtained by transplantation of dopaminergic neurons derived from human iPSCs (Rhee et al. 2011; Kikuchi et al. 2017). Furthermore, the suitability of iPSCs for skin trauma and wound management in horses has been proposed based on a finding that the horse iPSCs can be directed to differentiate into keratinocytes (Aguiar et al. 2016). iPSCs reprogramming has been reported from various animal species, including mouse, dog, pig, horse, cow, goat, and sheep (Takahashi and Yamanaka 2006; Cao et al. 2012; Fujishiro et al. 2013; Honda et al. 2013; Koh and Piedrahita 2015; Sandmaier et al. 2015). However, the authentic iPSCs suitable for clinical applications have not yet been established from large animals due to inefficient reprogrammability by conventional virus-mediated approach and associated safety concerns. Moreover, recently, efficient reprogramming of genetically stable authentic iPSCs from human or mouse/rat somatic cells has been demonstrated via modulating the endogenous pluripotency factors using CRISPR/dCas9-based transcription modulation tools (Balboa et al. 2015; Xiong et al. 2017; Liu et al. 2018b; Weltner et al. 2018). iPSCs reprogrammed through this approach are free of mutagenic genomic integration and viral elements, hence, safe for therapeutic uses. Based on success in iPSCs reprogramming from rodent and human, it is believed that authentic iPSCs from large animal species for regenerative medicine use will also be available soon.

Recently, scientists have exploited stem cells to develop anticancer vaccines as a novel approach to fight against cancer (Chu et al. 2020). The production of stem cell-based anticancer vaccines generally involves loading antigens on dendritic cells that trigger the primary T-cell responses in vivo, leading to necrosis of tumor cells (Codd et al. 2018). Anticancer vaccines can be produced from oncofetal peptides or cancer stem cell (CSC), ESC, or iPSC-based whole cells (Ouyang et al. 2019). Due to tumor heterogeneity and rapid escape mechanisms, single use of oncofetal peptide-based vaccine usually fails to trigger sufficient immune response against tumors (Ouyang et al. 2019). In contrast, whole-cell vaccines with epitope heterogeneity are proposed to be more potent and durable. Since the isolation of CSCs and ESCs is highly challenging, iPSCs are the preferred choice for whole-cell-based anticancer vaccine development. However, this approach's major hurdles are teratoma formation and possible autoimmunity induction (Katsukawa et al. 2016; Inui et al. 2017). Notably, studies in mice have shown that allogeneic ESCs and autologous iPSCs-based anticancer vaccines are more practical to prevent tumor relapse than their xenogeneic counterparts (Inui et al. 2017).

Furthermore, organ transplantation is another regenerative approach to treat patients with end-stage organ failure effectively. However, due to the shortage of donated organs for transplant, many patients die worldwide (Bernhardt and Reichenspurner 2014). Large animals, such as pigs, are considered a suitable source of organ xenograft as they share the similarity in physiology and organ size/function (Cooper et al. 2016, 2017). Tissue culture engineering combined with CRISPR/Cas genome editing tool has enabled veterinarians to produce immune-modulated xenotransplants for human medicine. For example, Fu et al. (2020) have developed a GGTA1, β 2-microglobulin (β2M), and major histocompatibility complex class II transactivator (CIITA) triple gene knockout pigs by single-cell-stage embryo modulation using CRISPR/Cas9 system and SCNT. Such mutant pig strains can effectively suppress the xenogeneic immune responses that allow prolonged survival of organ xenotransplants in the recipient (Fu et al. 2020). Furthermore, blastocyst complementation of pig embryo with genetically modified human iPSCs has been proposed as a practical approach to generate humanized organ transplants that would have the least chance of immune rejection upon engraftment (Crane et al. 2019).

In conclusion, at present, the majority of studies on applications of genetically modified stem cells in regenerative medicine are limited to rodent animal models or human and mostly experimental only. However, since the various large animals, including livestock and companion animals, have many of the same ailments and injurious as humans (e.g., human and feline squamous cell carcinoma) (Wypij 2013; Modiano 2016), a stem cell-based regenerative treatment approach likely demonstrated effective for the human disease model could also be efficiently applied for animal health improvement.

18.4.2.3 Animal Modeling for Biomedical Research

Animal models with genetic and physiological resemblances to various human disease conditions are imperative tools in biomedical research for better

understanding the diseases and drug discovery. Genetically modified mice/rats that mimic the human disease-associated mutation(s) are the most commonly used animal models in medicine and are known as a powerhouse for biomedical research. Very recently, the applicability of the CRISPR/Cas9 genome editing tool to generate genetically modified gerbil (a rodent related to rat) models for biomedical uses has been demonstrated by mutating the cystatin C or apolipoprotein A-II (Wang et al. 2020). Mongolian gerbils are a well-known experimental animal model, particularly for diabetes, cerebrovascular diseases, and *Helicobacter pylori* infection (Li et al. 2016; Wang et al. 2020). In many cases, large animals such as pig, monkey, and sheep are considered as more suitable nonhuman animal models for biomedical research because of their similarities to humans in terms of immunology, genome organization, aging, and the whole physiology (Lindblad-toh 2020; Menchaca et al. 2020). However, the generation of large animal models was not technically feasible until the advent of precise genome editing tools a few years back. The CRISPR/Cas9 genetic manipulation tools combined with stem cell-based advanced animal reproduction technologies have enabled the veterinarians to replicate a human clinical phenotype in large animals accurately. The pig is the most used large animal model in biomedical research (Rogers et al. 2008; Aigner et al. 2010). A pig model double knockout mutant for the *vWF* gene has been generated using the CRISPR/Cas9 system by direct cytoplasmic microinjection of sgRNA/Cas9 mRNA into the totipotent single-cell-stage embryos (Hai et al. 2014). The *vWF* codes for a blood-clotting protein [von Willebrand factor (VWF)], and its genetic defect causes a lifelong bleeding disorder in human called von Willebrand disease (vWD) (Ruggeri and Zimmerman 1987). Toward an attempt to produce a large animal model for human antibody repertoires, a B cell-deficient pig model was generated by targeted disruption of the JH5 region of the *IgM* heavy-chain gene using CRISPR/Cas9 tool and SCNT (Chen et al. 2015).

Furthermore, using the CRISPR/Cas9 system, sheep models for various human genetic disorders have been developed (Fan et al. 2018; Williams et al. 2018; Eaton et al. 2019). A sheep disease model for a human genetic disorder, cystic fibrosis, has been obtained by CRISPR/Cas9- and SCNT-mediated *CFTR* gene biallelic knockout (Fan et al. 2018). Recently, a sheep model for rare human bone disease, hypophosphatasia, has also been generated by CRISPR/Cas9-directed single-nucleotide point mutation in the gene coding for tissue nonspecific alkaline phosphatase (TNSALP) enzyme, which developed the same disorder as found in humans (Williams et al. 2018). Hence, continuously expanding CRISPR tools combined with stem cell engineering and advanced animal reproduction technology have empowered the veterinarians to generate large animal disease models of biomedical importance.

18.5 Conclusion

The instrumentation of the CRISPR/Cas system of bacterial origin as a sequence-targeted genetic manipulation tool has enabled stem cell technology to address the various challenges of animal science, such as improving livestock productivity, fitness, and animal health with unprecedented high efficiency. The simple experimental needs and unpresidential target precision of CRISPR/Cas-based gene manipulation tools have allowed the bioengineers to genetically modulate the stem cells for therapeutic uses and the development of genetically modified livestock with improved disease resistance and agricultural traits. Since the introduction of any genetic edits (i.e., gene knockin, knockout, knockdown) with high precision is now possible using CRISPR/Cas9 genome modulation tools, the current challenge is to know which edits have to make—not the availability of editing tools. However, in this regard, the development of high-throughput genome sequencing techniques is facilitating the genetic engineers. Although, at present, CRISPR/Cas-based genetic manipulation of stem cells, including single-cell-stage embryos for therapeutic applications and transgenic animals' development, is mostly experimental. It demonstrates the potency of CRISPR-based tools for regenerative veterinary medicine development and GM large animal species' production with improved traits of biomedical and agricultural importance in the near future. Furthermore, the ethical concerns on gene editing cannot be overlooked; however, the potential of stem cell and animal genome editing to meet the therapeutic and nutritional demands of the continuously increasing world population must be considered while drafting the related policies.

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References

Abudayyeh OO, Gootenberg JS, Konermann S et al (2016) C2c2 is a single-component programmable RNA-guided RNA-targeting CRISPR effector. Science (80-) 353:aaf5573. https://doi.org/10.1126/science.aaf5573

Abudayyeh OO, Gootenberg JS, Essletzbichler P et al (2017) RNA targeting with CRISPR–Cas13. Nature 550:280–284. https://doi.org/10.1038/nature24049

Acharya S, Mishra A, Paul D et al (2019) Francisella novicida Cas9 interrogates genomic DNA with very high specificity and can be used for mammalian genome editing. Proc Natl Acad Sci 116:20959–20968. https://doi.org/10.1073/pnas.1818461116

Aguiar C, Therrien J, Lemire P et al (2016) Differentiation of equine induced pluripotent stem cells into a keratinocyte lineage. Equine Vet J 48:338–345. https://doi.org/10.1111/evj.12438

Aigner B, Renner S, Kessler B et al (2010) Transgenic pigs as models for translational biomedical research. J Mol Med 88:653–664. https://doi.org/10.1007/s00109-010-0610-9

Anzalone AV, Randolph PB, Davis JR et al (2019) Search-and-replace genome editing without double-strand breaks or donor DNA. Nature 576:149–157. https://doi.org/10.1038/s41586-019-1711-4

Aoi T, Yae K, Nakagawa M et al (2008) Generation of pluripotent stem cells from adult mouse liver and stomach cells. Science (80-) 321:699–702. https://doi.org/10.1126/science.1154884

Aquino-Jarquin G (2019) CRISPR-Cas14 is now part of the artillery for gene editing and molecular diagnostic. Nanomedicine 18:428–431. https://doi.org/10.1016/j.nano.2019.03.006

Balboa D, Weltner J, Eurola S et al (2015) Conditionally stabilized dCas9 activator for controlling gene expression in human cell reprogramming and differentiation. Stem Cell Rep 5:448–459. https://doi.org/10.1016/j.stemcr.2015.08.001

Barrangou R, Horvath P (2017) A decade of discovery: CRISPR functions and applications. Nat Microbiol 2:17092. https://doi.org/10.1038/nmicrobiol.2017.92

Bartosh TJ, Ullah M, Zeitouni S et al (2016) Cancer cells enter dormancy after cannibalizing mesenchymal stem/stromal cells (MSCs). Proc Natl Acad Sci 113:E6447–E6456. https://doi.org/10.1073/pnas.1612290113

Bernhardt AM, Reichenspurner H (2014) High-risk donors. Curr Opin Organ Transpl 19:494–499. https://doi.org/10.1097/MOT.0000000000000118

Bevacqua RJ, Fernandez-Martín R, Savy V et al (2016) Efficient edition of the bovine PRNP prion gene in somatic cells and IVF embryos using the CRISPR/Cas9 system. Theriogenology 86:1886–1896.e1. https://doi.org/10.1016/j.theriogenology.2016.06.010

Black JB, Adler AF, Wang H-G et al (2016) Targeted epigenetic remodeling of endogenous loci by CRISPR/Cas9-based transcriptional activators directly converts fibroblasts to neuronal cells. Cell Stem Cell 19:406–414. https://doi.org/10.1016/j.stem.2016.07.001

Brambrink T, Foreman R, Welstead GG et al (2008) Sequential expression of pluripotency markers during direct reprogramming of mouse somatic cells. Cell Stem Cell 2:151–159. https://doi.org/10.1016/j.stem.2008.01.004

Burkard C, Lillico SG, Reid E et al (2017) Precision engineering for PRRSV resistance in pigs: macrophages from genome edited pigs lacking CD163 SRCR5 domain are fully resistant to both PRRSV genotypes while maintaining biological function. PLoS Pathog 13:e1006206. https://doi.org/10.1371/journal.ppat.1006206

Canny MD, Moatti N, Wan LCK et al (2018) Inhibition of 53BP1 favors homology-dependent DNA repair and increases CRISPR–Cas9 genome-editing efficiency. Nat Biotechnol 36:95–102. https://doi.org/10.1038/nbt.4021

Cao H, Yang P, Pu Y et al (2012) Characterization of bovine induced pluripotent stem cells by lentiviral transduction of reprogramming factor fusion proteins. Int J Biol Sci 8:498–511. https://doi.org/10.7150/ijbs.3723

Carlson DF, Tan W, Lillico SG et al (2012) Efficient TALEN-mediated gene knockout in livestock. Proc Natl Acad Sci 109:17382–17387. https://doi.org/10.1073/pnas.1211446109

Chakraborty S, Ji H, Kabadi AM et al (2014) A CRISPR/Cas9-based system for reprogramming cell lineage specification. Stem Cell Rep 3:940–947. https://doi.org/10.1016/j.stemcr.2014.09.013

Chang D-Y, Yoo S-W, Hong Y et al (2010) The growth of brain tumors can be suppressed by multiple transplantation of mesenchymal stem cells expressing cytosine deaminase. Int J Cancer 127:1975–1983. https://doi.org/10.1002/ijc.25383

Chang HHY, Pannunzio NR, Adachi N, Lieber MR (2017) Non-homologous DNA end joining and alternative pathways to double-strand break repair. Nat Rev Mol Cell Biol 18:495–506. https://doi.org/10.1038/nrm.2017.48

Chavez A, Scheiman J, Vora S et al (2015) Highly efficient Cas9-mediated transcriptional programming. Nat Methods 12:326–328. https://doi.org/10.1038/nmeth.3312

Chen F, Wang Y, Yuan Y et al (2015) Generation of B cell-deficient pigs by highly efficient CRISPR/Cas9-mediated gene targeting. J Genet Genomics 42:437–444. https://doi.org/10.1016/j.jgg.2015.05.002

Chu D-T, Nguyen TT, Tien NLB et al (2020) Recent progress of stem cell therapy in cancer treatment: molecular mechanisms and potential applications. Cells 9:563. https://doi.org/10.3390/cells9030563

Cibelli JB (1998) Cloned transgenic calves produced from nonquiescent fetal fibroblasts. Science (80-) 280:1256–1258. https://doi.org/10.1126/science.280.5367.1256

Codd AS, Kanaseki T, Torigo T, Tabi Z (2018) Cancer stem cells as targets for immunotherapy. Immunology 153:304–314. https://doi.org/10.1111/imm.12866

Cooper DKC, Dou K-F, Tao K et al (2016) Pig liver xenotransplantation. Transplantation 100:2039–2047. https://doi.org/10.1097/TP.0000000000001319

Cooper DKC, Pierson RN, Hering BJ et al (2017) Regulation of clinical xenotransplantation—time for a reappraisal. Transplantation 101:1766–1769. https://doi.org/10.1097/TP.0000000000001683

Cox DBT, Gootenberg JS, Abudayyeh OO et al (2017) RNA editing with CRISPR-Cas13. Science (80-) 358:1019–1027. https://doi.org/10.1126/science.aaq0180

Crane AT, Aravalli RN, Asakura A et al (2019) Interspecies organogenesis for human transplantation. Cell Transplant 28:1091–1105. https://doi.org/10.1177/0963689719845351

Crispo M, Mulet AP, Tesson L et al (2015) Efficient generation of myostatin knock-out sheep using CRISPR/Cas9 technology and microinjection into zygotes. PLoS One 10:e0136690. https://doi.org/10.1371/journal.pone.0136690

Deltcheva E, Chylinski K, Sharma CM et al (2011) CRISPR RNA maturation by trans-encoded small RNA and host factor RNase III. Nature 471:602–607. https://doi.org/10.1038/nature09886

Deng Y, Liu Q, Luo C et al (2012) Generation of induced pluripotent stem cells from buffalo (Bubalus bubalis) fetal fibroblasts with buffalo defined factors. Stem Cells Dev 21:2485–2494. https://doi.org/10.1089/scd.2012.0018

Du X, Feng T, Yu D et al (2015) Barriers for deriving transgene-free Pig iPS cells with episomal vectors. Stem Cells 33:3228–3238. https://doi.org/10.1002/stem.2089

Eaton SL, Proudfoot C, Lillico SG et al (2019) CRISPR/Cas9 mediated generation of an ovine model for infantile neuronal ceroid lipofuscinosis (CLN1 disease). Sci Rep 9:9891. https://doi.org/10.1038/s41598-019-45859-9

Esmaeilzadeh A, Farshbaf A (2015) Mesenchymal stem cell as a vector for gene and cell therapy strategies. Stud Stem Cells Res Ther 1:17–18. https://doi.org/10.17352/sscrt.000005

Evans MJ, Kaufman MH (1981) Establishment in culture of pluripotential cells from mouse embryos. Nature 292:154–156. https://doi.org/10.1038/292154a0

Fan Z, Perisse IV, Cotton CU et al (2018) A sheep model of cystic fibrosis generated by CRISPR/Cas9 disruption of the CFTR gene. JCI Insight 3:e123529. https://doi.org/10.1172/jci.insight.123529

Fu R, Fang M, Xu K et al (2020) Generation of GGTA1 -/- β2M -/- CIITA -/- pigs using CRISPR/Cas9 technology to alleviate xenogeneic immune reactions. Transplantation 104:1566. https://doi.org/10.1097/TP.0000000000003205

Fujishiro S, Nakano K, Mizukami Y et al (2013) Generation of naive-like porcine-induced pluripotent stem cells capable of contributing to embryonic and fetal development. Stem Cells Dev 22:473–482. https://doi.org/10.1089/scd.2012.0173

Fusaki N, Ban H, Nishiyama A et al (2009) Efficient induction of transgene-free human pluripotent stem cells using a vector based on Sendai virus, an RNA virus that does not integrate into the host genome. Proc Japan Acad Ser B 85:348–362. https://doi.org/10.2183/pjab.85.348

Gao X, Yang J, Tsang JCH et al (2013) Reprogramming to pluripotency using designer TALE transcription factors targeting enhancers. Stem Cell Rep 1:183–197. https://doi.org/10.1016/j.stemcr.2013.06.002

Gao X, Tsang JCH, Gaba F et al (2014) Comparison of TALE designer transcription factors and the CRISPR/dCas9 in regulation of gene expression by targeting enhancers. Nucleic Acids Res 42:e155–e155. https://doi.org/10.1093/nar/gku836

Gao Y, Wu H, Wang Y et al (2017) Single Cas9 nickase induced generation of NRAMP1 knockin cattle with reduced off-target effects. Genome Biol 18:13. https://doi.org/10.1186/s13059-016-1144-4

Gee P, Lung MSY, Okuzaki Y et al (2020) Extracellular nanovesicles for packaging of CRISPR-Cas9 protein and sgRNA to induce therapeutic exon skipping. Nat Commun 11:1334. https://doi.org/10.1038/s41467-020-14957-y

Gilbert LA, Larson MH, Morsut L et al (2013) CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. Cell 154:442–451. https://doi.org/10.1016/j.cell.2013.06.044

Haeussler M, Schönig K, Eckert H et al (2016) Evaluation of off-target and on-target scoring algorithms and integration into the guide RNA selection tool CRISPOR. Genome Biol 17:148. https://doi.org/10.1186/s13059-016-1012-2

Hai T, Teng F, Guo R et al (2014) One-step generation of knockout pigs by zygote injection of CRISPR/Cas system. Cell Res 24:372–375. https://doi.org/10.1038/cr.2014.11

Han X, Han J, Ding F et al (2011) Generation of induced pluripotent stem cells from bovine embryonic fibroblast cells. Cell Res 21:1509–1512. https://doi.org/10.1038/cr.2011.125

Honda A, Hatori M, Hirose M et al (2013) Naive-like conversion overcomes the limited differentiation capacity of induced pluripotent stem cells. J Biol Chem 288:26157–26166. https://doi.org/10.1074/jbc.M113.502492

Hsu M-N, Chang Y-H, Truong VA et al (2019) CRISPR technologies for stem cell engineering and regenerative medicine. Biotechnol Adv 37:107447. https://doi.org/10.1016/j.biotechadv.2019.107447

Hu J, Lei Y, Wong W-K et al (2014) Direct activation of human and mouse Oct4 genes using engineered TALE and Cas9 transcription factors. Nucleic Acids Res 42:4375–4390. https://doi.org/10.1093/nar/gku109

Hübner A, Petersen B, Keil GM et al (2018) Efficient inhibition of African swine fever virus replication by CRISPR/Cas9 targeting of the viral p30 gene (CP204L). Sci Rep 8:1–7. https://doi.org/10.1038/s41598-018-19626-1

Hustedt N, Durocher D (2017) The control of DNA repair by the cell cycle. Nat Cell Biol 19:1–9. https://doi.org/10.1038/ncb3452

Ikeda M, Matsuyama S, Akagi S et al (2017) Correction of a disease mutation using CRISPR/Cas9-assisted genome editing in Japanese black cattle. Sci Rep 7:1–9. https://doi.org/10.1038/s41598-017-17968-w

Inui S, Minami K, Ito E et al (2017) Irradiation strongly reduces tumorigenesis of human induced pluripotent stem cells. J Radiat Res 58:430–438. https://doi.org/10.1093/jrr/rrw124

Jeon K, Lim H, Kim J-H et al (2012) Differentiation and transplantation of functional pancreatic beta cells generated from induced pluripotent stem cells derived from a type 1 diabetes mouse model. Stem Cells Dev 21:2642–2655. https://doi.org/10.1089/scd.2011.0665

Ji Q, Fischer AL, Brown CR et al (2014) Engineered zinc-finger transcription factors activate OCT4 (POU5F1), SOX2, KLF4, c-MYC (MYC) and miR302/367. Nucleic Acids Res 42:6158–6167. https://doi.org/10.1093/nar/gku243

Jiang W, Marraffini LA (2015) CRISPR-Cas: new tools for genetic manipulations from bacterial immunity systems. Annu Rev Microbiol 69:209–228. https://doi.org/10.1146/annurev-micro-091014-104441

Jinek M, Chylinski K, Fonfara I et al (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. Science (80-) 337:816–821. https://doi.org/10.1126/science.1225829

Kaneko T, Sakuma T, Yamamoto T, Mashimo T (2015) Simple knockout by electroporation of engineered endonucleases into intact rat embryos. Sci Rep 4:6382. https://doi.org/10.1038/srep06382

Katsukawa M, Nakajima Y, Fukumoto A et al (2016) Fail-safe therapy by gamma-ray irradiation against tumor formation by human-induced pluripotent stem cell-derived neural progenitors. Stem Cells Dev 25:815–825. https://doi.org/10.1089/scd.2015.0394

Kearns NA, Genga RMJ, Enuameh MS et al (2014) Cas9 effector-mediated regulation of transcription and differentiation in human pluripotent stem cells. Development 141:219–223. https://doi.org/10.1242/dev.103341

Kikuchi T, Morizane A, Doi D et al (2017) Human iPS cell-derived dopaminergic neurons function in a primate Parkinson's disease model. Nature 548:592–596. https://doi.org/10.1038/nature23664

Kim H, Kim J-S (2014) A guide to genome engineering with programmable nucleases. Nat Rev Genet 15:321–334. https://doi.org/10.1038/nrg3686

Kim E, Koo T, Park SW et al (2017) In vivo genome editing with a small Cas9 orthologue derived from Campylobacter jejuni. Nat Commun 8:14500. https://doi.org/10.1038/ncomms14500

Kirchhof N, Carnwath JW, Lemme E et al (2000) Expression pattern of Oct-4 in preimplantation embryos of different species. Biol Reprod 63:1698–1705. https://doi.org/10.1095/biolreprod63.6.1698

Koh S, Piedrahita JA (2015) Generation of induced pluripotent stem cells (iPSCs) from adult canine fibroblasts. Methods Mol Biol 1330:69–78

Komor AC, Kim YB, Packer MS et al (2016) Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. Nature 533:420–424. https://doi.org/10.1038/nature17946

Komor AC, Badran AH, Liu DR (2017) CRISPR-based technologies for the manipulation of eukaryotic genomes. Cell 168:20–36. https://doi.org/10.1016/j.cell.2016.10.044

Konermann S, Brigham MD, Trevino AE et al (2015) Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex. Nature 517:583–588. https://doi.org/10.1038/nature14136

Koonin EV, Makarova KS, Zhang F (2017) Diversity, classification and evolution of CRISPR-Cas systems. Curr Opin Microbiol 37:67–78. https://doi.org/10.1016/j.mib.2017.05.008

Kuijk EW, Du Puy L, Van Tol HTA et al (2008) Differences in early lineage segregation between mammals. Dev Dyn 237:918–927. https://doi.org/10.1002/dvdy.21480

Kurtz S, Petersen B (2019) Pre-determination of sex in pigs by application of CRISPR/Cas system for genome editing. Theriogenology 137:67–74. https://doi.org/10.1016/j.theriogenology.2019.05.039

Laible G (2018) Production of transgenic livestock: overview of transgenic technologies. In: Animal biotechnology 2. Springer International Publishing, Cham, pp 95–121

Lee HJ, Doo SW, Kim DH et al (2013) Cytosine deaminase-expressing human neural stem cells inhibit tumor growth in prostate cancer-bearing mice. Cancer Lett 335:58–65. https://doi.org/10.1016/j.canlet.2013.01.048

Lee CM, Cradick TJ, Fine EJ, Bao G (2016) Nuclease target site selection for maximizing on-target activity and minimizing off-target effects in genome editing. Mol Ther 24:475–487. https://doi.org/10.1038/mt.2016.1

Lee K, Conboy M, Park HM et al (2017) Nanoparticle delivery of Cas9 ribonucleoprotein and donor DNA in vivo induces homology-directed DNA repair. Nat Biomed Eng 1:889–901. https://doi.org/10.1038/s41551-017-0137-2

Li X, Lu J, Wang Y et al (2016) Establishment and characterization of a newly established diabetic gerbil line. PLoS One 11:e0159420. https://doi.org/10.1371/journal.pone.0159420

Li H, Beckman K, Pessino V et al (2017a) Design and specificity of long ssDNA donors for CRISPR-based knock-in. bioRxiv:178905. https://doi.org/10.1101/178905

Li W-R, Liu C-X, Zhang X-M et al (2017b) CRISPR/Cas9-mediated loss of FGF5 function increases wool staple length in sheep. FEBS J 284:2764–2773. https://doi.org/10.1111/febs.14144

Lindblad-toh K (2020) What animals can teach us about evolution, the human genome, and human disease. Ups J Med Sci 125:1–9. https://doi.org/10.1080/03009734.2020.1722298

Liu H, Sui T, Liu D et al (2018a) Multiple homologous genes knockout (KO) by CRISPR/Cas9 system in rabbit. Gene 647:261–267. https://doi.org/10.1016/j.gene.2018.01.044

Liu P, Chen M, Liu Y et al (2018b) CRISPR-based chromatin remodeling of the endogenous Oct4 or Sox2 locus enables reprogramming to pluripotency. Cell Stem Cell 22:252–261.e4. https://doi.org/10.1016/j.stem.2017.12.001

Malekshah OM, Chen X, Nomani A et al (2016) Enzyme/prodrug systems for cancer gene therapy. Curr Pharmacol Rep 2:299–308. https://doi.org/10.1007/s40495-016-0073-y

Marelli G, Howells A, Lemoine NR, Wang Y (2018) Oncolytic viral therapy and the immune system: a double-edged sword against cancer. Front Immunol 9:866. https://doi.org/10.3389/fimmu.2018.00866

Marofi F, Vahedi G, Biglari A et al (2017) Mesenchymal stromal/stem cells: a new era in the cell-based targeted gene therapy of cancer. Front Immunol 8:1770. https://doi.org/10.3389/fimmu.2017.01770

McPherron AC, Lawler AM, Lee S-J (1997) Regulation of skeletal muscle mass in mice by a new TGF-p superfamily member. Nature 387:83–90. https://doi.org/10.1038/387083a0

Menchaca A, dos Santos-Neto PC, Mulet AP, Crispo M (2020) CRISPR in livestock: from editing to printing. Theriogenology 150:247–254. https://doi.org/10.1016/j.theriogenology.2020.01.063

Miyoshi N, Ishii H, Nagano H et al (2011) Reprogramming of mouse and human cells to pluripotency using mature microRNAs. Cell Stem Cell 8:633–638. https://doi.org/10.1016/j.stem.2011.05.001

Modiano J (2016) Comparative pathogenesis of cancers in animals and humans. Vet Sci 3:24. https://doi.org/10.3390/vetsci3030024

Moon SB, Kim DY, Ko J-H, Kim Y-S (2019) Recent advances in the CRISPR genome editing tool set. Exp Mol Med 51:1–11. https://doi.org/10.1038/s12276-019-0339-7

Morrison S, Wandycz A et al (1997) Identification of a lineage of multipotent hematopoietic progenitors. Development 124:1929–1939

Murugan K, Babu K, Sundaresan R et al (2017) The revolution continues: newly discovered systems expand the CRISPR-Cas toolkit. Mol Cell 68:15–25. https://doi.org/10.1016/j.molcel.2017.09.007

Niakan KK, Eggan K (2013) Analysis of human embryos from zygote to blastocyst reveals distinct gene expression patterns relative to the mouse. Dev Biol 375:54–64. https://doi.org/10.1016/j.ydbio.2012.12.008

Niu Y, Zhao X, Zhou J et al (2018) Efficient generation of goats with defined point mutation (I397V) in GDF9 through CRISPR/Cas9. Reprod Fertil Dev 30:307. https://doi.org/10.1071/RD17068

Oda Y, Yoshimura Y, Ohnishi H et al (2010) Induction of pluripotent stem cells from human third molar mesenchymal stromal cells. J Biol Chem 285:29270–29278. https://doi.org/10.1074/jbc.M109.055889

Oishi I, Yoshii K, Miyahara D et al (2016) Targeted mutagenesis in chicken using CRISPR/Cas9 system. Sci Rep 6:23980. https://doi.org/10.1038/srep23980

Ouyang X, Telli ML, Wu JC (2019) Induced pluripotent stem cell-based cancer vaccines. Front Immunol 10:1510. https://doi.org/10.3389/fimmu.2019.01510

Pawar HN, Mitra N, Verma R (2020) Modulation of animal health through reverse genetics applications. In: Genomics and biotechnological advances in veterinary, poultry, and fisheries. Elsevier, Amsterdam, pp 163–182

Perota A, Lagutina I, Duchi R et al (2019) Generation of cattle knockout for galactose-α1,3-galactose and N-glycolylneuraminic acid antigens. Xenotransplantation 26:e12524. https://doi.org/10.1111/xen.12524

Piliszek A, Grabarek JB, Frankenberg SR, Plusa B (2016) Cell fate in animal and human blastocysts and the determination of viability. Mol Hum Reprod 22:681–690. https://doi.org/10.1093/molehr/gaw002

Pinnapureddy AR, Stayner C, Mcewan J et al (2015) Large animal models of rare genetic disorders: sheep as phenotypically relevant models of human genetic disease. Orphanet J Rare Dis 10:107. https://doi.org/10.1186/s13023-015-0327-5

Preskey D, Allison TF, Jones M et al (2016) Synthetically modified mRNA for efficient and fast human iPS cell generation and direct transdifferentiation to myoblasts. Biochem Biophys Res Commun 473:743–751. https://doi.org/10.1016/j.bbrc.2015.09.102

Proudfoot C, Carlson DF, Huddart R et al (2015) Genome edited sheep and cattle. Transgenic Res 24:147–153. https://doi.org/10.1007/s11248-014-9832-x

Quadros RM, Miura H, Harms DW et al (2017) Easi-CRISPR: a robust method for one-step generation of mice carrying conditional and insertion alleles using long ssDNA donors and CRISPR ribonucleoproteins. Genome Biol 18:92. https://doi.org/10.1186/s13059-017-1220-4

Ran FA, Cong L, Yan WX et al (2015) In vivo genome editing using Staphylococcus aureus Cas9. Nature 520:186–191. https://doi.org/10.1038/nature14299

Rhee Y-H, Ko J-Y, Chang M-Y et al (2011) Protein-based human iPS cells efficiently generate functional dopamine neurons and can treat a rat model of Parkinson disease. J Clin Invest 121:2326–2335. https://doi.org/10.1172/JCI45794

Richter A, Kurome M, Kessler B et al (2012) Potential of primary kidney cells for somatic cell nuclear transfer mediated transgenesis in pig. BMC Biotechnol 12:84. https://doi.org/10.1186/1472-6750-12-84

Rogers CS, Stoltz DA, Meyerholz DK et al (2008) Disruption of the CFTR Gene Produces a Model of Cystic Fibrosis in Newborn Pigs. Science (80-) 321:1837–1841. https://doi.org/10.1126/science.1163600

Rosenquist TA, Martin GR (1996) Fibroblast growth factor signalling in the hair growth cycle: expression of the fibroblast growth factor receptor and ligand genes in the murine hair follicle. Dev Dyn 205:379–386. https://doi.org/10.1002/(SICI)1097-0177(199604)205:4<379::AID-AJA2>3.0.CO;2-F

Rothe M, Modlich U, Schambach A (2014) Biosafety challenges for use of lentiviral vectors in gene therapy. Curr Gene Ther 13:453–468. https://doi.org/10.2174/1566523211313660006

Ruggeri ZM, Zimmerman TS (1987) von Willebrand factor and von Willebrand disease. Blood 70:895–904

Sandmaier SES, Nandal A, Powell A et al (2015) Generation of induced pluripotent stem cells from domestic goats. Mol Reprod Dev 82:709–721. https://doi.org/10.1002/mrd.22512

Selvaraj V, Plane JM, Williams AJ, Deng W (2010) Switching cell fate: the remarkable rise of induced pluripotent stem cells and lineage reprogramming technologies. Trends Biotechnol 28:214–223. https://doi.org/10.1016/j.tibtech.2010.01.002

Shah K (2012) Mesenchymal stem cells engineered for cancer therapy. Adv Drug Deliv Rev 64:739–748. https://doi.org/10.1016/j.addr.2011.06.010

Shuman S, Glickman MS (2007) Bacterial DNA repair by non-homologous end joining. Nat Rev Microbiol 5:852–861. https://doi.org/10.1038/nrmicro1768

Smith ZD, Sindhu C, Meissner A (2016) Molecular features of cellular reprogramming and development. Nat Rev Mol Cell Biol 17:139–154. https://doi.org/10.1038/nrm.2016.6

Soufi A, Donahue G, Zaret KS (2012) Facilitators and Impediments of the Pluripotency Reprogramming Factors' Initial Engagement with the Genome. Cell 151:994–1004. https://doi.org/10.1016/j.cell.2012.09.045

Stadtfeld M, Brennand K, Hochedlinger K (2008) Reprogramming of pancreatic β cells into induced pluripotent stem cells. Curr Biol 18:890–894. https://doi.org/10.1016/j.cub.2008.05.010

Strumpf D (2005) Cdx2 is required for correct cell fate specification and differentiation of trophectoderm in the mouse blastocyst. Development 132:2093–2102. https://doi.org/10.1242/dev.01801

Studeny M, Marini FC, Champlin RE et al (2002) Bone marrow-derived mesenchymal stem cells as vehicles for interferon-beta delivery into tumors. Cancer Res 62:3603–3608

Takahashi K, Yamanaka S (2006) Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell 126:663–676. https://doi.org/10.1016/j.cell.2006.07.024

Takahashi K, Tanabe K, Ohnuki M et al (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131:861–872. https://doi.org/10.1016/j.cell.2007.11.019

Talluri TR, Kumar D, Glage S et al (2015) Derivation and characterization of bovine induced pluripotent stem cells by transposon-mediated reprogramming. Cell Reprogram 17:131–140. https://doi.org/10.1089/cell.2014.0080

Tanihara F, Takemoto T, Kitagawa E et al (2016) Somatic cell reprogramming-free generation of genetically modified pigs. Sci Adv 2:e1600803. https://doi.org/10.1126/sciadv.1600803

Tat PA, Sumer H, Jones KL et al (2010) The efficient generation of induced pluripotent stem (iPS) cells from adult mouse adipose tissue-derived and neural stem cells. Cell Transplant 19:525–536. https://doi.org/10.3727/096368910X491374

Van Gorp H, Delputte PL, Nauwynck HJ (2010) Scavenger receptor CD163, a Jack-of-all-trades and potential target for cell-directed therapy. Mol Immunol 47:1650–1660. https://doi.org/10.1016/j.molimm.2010.02.008

Voga M, Adamic N, Vengust M, Majdic G (2020) Stem cells in veterinary medicine—current state and treatment options. Front Vet Sci 7:278. https://doi.org/10.3389/fvets.2020.00278

Wagers AJ, Weissman IL (2004) Plasticity of adult stem cells. Cell 116:639–648. https://doi.org/10.1016/S0092-8674(04)00208-9

Wang X, Yu H, Lei A et al (2015a) Generation of gene-modified goats targeting MSTN and FGF5 via zygote injection of CRISPR/Cas9 system. Sci Rep 5:1–9. https://doi.org/10.1038/srep13878

Wang X, Yu H, Lei A et al (2015b) Generation of gene-modified goats targeting MSTN and FGF5 via zygote injection of CRISPR/Cas9 system. Sci Rep 5:13878. https://doi.org/10.1038/srep13878

Wang Y, Zhao P, Song Z et al (2020) Generation of gene-knockout mongolian gerbils via CRISPR/Cas9 system. Front Bioeng Biotechnol 8:780. https://doi.org/10.3389/fbioe.2020.00780

Wei S, Zou Q, Lai S et al (2016) Conversion of embryonic stem cells into extraembryonic lineages by CRISPR-mediated activators. Sci Rep 6:19648. https://doi.org/10.1038/srep19648

Weltner J, Balboa D, Katayama S et al (2018) Human pluripotent reprogramming with CRISPR activators. Nat Commun 9:2643. https://doi.org/10.1038/s41467-018-05067-x

Wen W, Zhang J-P, Xu J et al (2016) Enhanced generation of integration-free iPSCs from human adult peripheral blood mononuclear cells with an optimal combination of episomal vectors. Stem Cell Rep 6:873–884. https://doi.org/10.1016/j.stemcr.2016.04.005

Whitelaw CBA, Sheets TP, Lillico SG, Telugu BP (2016) Engineering large animal models of human disease. J Pathol 238:247–256. https://doi.org/10.1002/path.4648

Whitworth KM, Lee K, Benne JA et al (2014) Use of the CRISPR/Cas9 system to produce genetically engineered pigs from in vitro-derived oocytes and embryos1. Biol Reprod 91:78. https://doi.org/10.1095/biolreprod.114.121723

Whitworth KM, Rowland RRR, Ewen CL et al (2016) Gene-edited pigs are protected from porcine reproductive and respiratory syndrome virus. Nat Biotechnol 34:20–22. https://doi.org/10.1038/nbt.3434

Whitworth KM, Rowland RRR, Petrovan V et al (2019) Resistance to coronavirus infection in amino peptidase N-deficient pigs. Transgenic Res 28:21–32. https://doi.org/10.1007/s11248-018-0100-3

Williams DK, Pinzón C, Huggins S et al (2018) Genetic engineering a large animal model of human hypophosphatasia in sheep. Sci Rep 8:16945. https://doi.org/10.1038/s41598-018-35079-y

Wu X, Kriz AJ, Sharp PA (2014) Target specificity of the CRISPR-Cas9 system. Quant Biol 2:59–70. https://doi.org/10.1007/s40484-014-0030-x

Wypij JM (2013) A naturally occurring feline model of head and neck squamous cell carcinoma. Pathol Res Int 2013:1–7. https://doi.org/10.1155/2013/502197

Xiong K, Zhou Y, Blichfeld KA et al (2017) RNA-guided activation of pluripotency genes in human fibroblasts. Cell Reprogram 19:189–198. https://doi.org/10.1089/cell.2017.0006

Xu X, Qi LS (2019) A CRISPR–dCas toolbox for genetic engineering and synthetic biology. J Mol Biol 431:34–47. https://doi.org/10.1016/j.jmb.2018.06.037

Yang D, Yang H, Li W et al (2011) Generation of PPARγ mono-allelic knockout pigs via zinc-finger nucleases and nuclear transfer cloning. Cell Res 21:979–982. https://doi.org/10.1038/cr.2011.70

Yin H, Song C-Q, Dorkin JR et al (2016) Therapeutic genome editing by combined viral and nonviral delivery of CRISPR system components in vivo. Nat Biotechnol 34:328–333. https://doi.org/10.1038/nbt.3471

Yip BH (2020) Recent advances in CRISPR/Cas9 delivery strategies. Biomolecules 10:839. https://doi.org/10.3390/biom10060839

Yoshioka N, Gros E, Li H-R et al (2013) Efficient generation of human iPSCs by a synthetic self-replicative RNA. Cell Stem Cell 13:246–254. https://doi.org/10.1016/j.stem.2013.06.001

Yu J, Hu K, Smuga-Otto K et al (2009) Human induced pluripotent stem cells free of vector and transgene sequences. Science (80-) 324:797–801. https://doi.org/10.1126/science.1172482

Yue J, Gou X, Li Y et al (2017) Engineered epidermal progenitor cells can correct diet-induced obesity and diabetes. Cell Stem Cell 21:256–263.e4. https://doi.org/10.1016/j.stem.2017.06.016

Zetsche B, Gootenberg JS, Abudayyeh OO et al (2015) Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. Cell 163:759–771. https://doi.org/10.1016/j.cell.2015.09.038

Zhang L, Xiang J, Li G (2013) The uncertain role of unmodified mesenchymal stem cells in tumor progression: what master switch? Stem Cell Res Ther 4:22. https://doi.org/10.1186/scrt170

Zhang D, Zhang H, Li T et al (2017) Perfectly matched 20-nucleotide guide RNA sequences enable robust genome editing using high-fidelity SpCas9 nucleases. Genome Biol 18:191. https://doi.org/10.1186/s13059-017-1325-9

Zhao X, Nie J, Tang Y et al (2020) Generation of transgenic cloned buffalo embryos harboring the EGFP gene in the Y chromosome using CRISPR/Cas9-mediated targeted integration. Front Vet Sci 7:199. https://doi.org/10.3389/fvets.2020.00199

Zhou W, Freed CR (2009) Adenoviral gene delivery can reprogram human fibroblasts to induced pluripotent stem cells. Stem Cells 27:2667–2674. https://doi.org/10.1002/stem.201

Zhou W, Wan Y, Guo R et al (2017) Generation of beta-lactoglobulin knock-out goats using CRISPR/Cas9. PLoS One 12:e0186056. https://doi.org/10.1371/journal.pone.0186056

Zou Q, Wang X, Liu Y et al (2015) Generation of gene-target dogs using CRISPR/Cas9 system. J Mol Cell Biol 7:580–583. https://doi.org/10.1093/jmcb/mjv061

Part IV

Issues and Perspectives

Identification of Species-Specific Stem Cells and Challenges

19

Ratan Kumar Choudhary

Abstract

Therapeutic application of stem cells and functional restoration has great potential for many diseases of humans and animals. Embryonic stem cells (ESC), adult stem cells (ASCs) like mesenchymal stem cells (MSC), hematopoietic stem cells (HSCs), and mammary stem cells (MaSCs) find applications in regenerative medicine along with the induced pluripotent stem cells (iPSCs). Each kind of stem cells has merits and limitations. There are many challenges in identifying a pure population of stem cells, especially ASC, in different tissues of various species. Together with the heterogeneous feature of some adult stem cell types, these challenges make it difficult to study them at the molecular level. This chapter discusses, in general, the various challenges in identifying species-specific stem cells and enlists challenges of stem cell therapy. We also discussed the challenges of stem cell therapy in detail and talked about the safety issues and ethical concerns using ESC, ASC, and iPSCs. Though substantial progress has been made over the last decade, many challenges remain in stem cells' therapeutic potentials.

Keywords

Stem cell · Identification · Challenges

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19.1 Introduction to Stem Cells

Stem cells are unspecialized "blank" or undifferentiated cells present in all life stages (embryonic, fetal, and adult stages) and produce functional and differentiated cells, building blocks of tissue. More potent stem cells are found in the prenatal embryonic stage than the fetal and later postnatal stages. In the postnatal and adult stages, tissue-specific adult stem cells are found in various tissues required for repair and tissue homeostasis. The features of stem cells that distinguish them from differentiated cells are (1) capacity to increase extensively for an extended period (self-renewal), (2) clonality-ability to form whole tissue or organ from a single cell, and (3) differentiation ability or potency—the ability to differentiate into different cell types. These properties may differ among various types of stem cells. For example, embryonic stem cells (ESCs) have greater potency, self-renewal ability, and clonality than tissue-specific adult stem cells (ASCs). Mesenchymal stem cells, hematopoietic stem cells, and mammary stem cells are few examples of tissue-specific stem cells. A comparison of various stem cell types with advantages and limitations is provided in Table 19.1. ASCs have limited self-renewal ability and inherent divisional capacity to divide asymmetrically into two daughter cells—one stem cells and the other differentiate cells. Asymmetric division of ASCs maintains stem cell pool and repairs tissue damage together, which is the unique property of all ASCs. According to the differentiation potential, stem cells can be of various types, namely, (1) totipotent, (2) pluripotent, (3) multipotent, (4) oligopotent, and (5) unipotent. Based on the origin, stem cells can be of (1) ESCs from the embryo, (2) induced pluripotent stem cells (iPSCs) originated from any differentiated cells primarily from fibroblasts, (3) fetal stem cells from fetuses, and (4) adult or somatic stem cells from developed organs of young or adult individuals.

19.2 Markers of Stem Cells

Unique gene expression patterns in a cell results in a unique expression of proteins, which can identify and distinguish from other cell types. These uniquely expressed proteins are called molecular markers. Expression of pluripotency transcription factors and self-renewal are recognized as molecular markers of ESCs in their identification and characterization. Other types of markers expressed on the surface of cells can selectively bind to other signal molecules and can also be used as cell surface markers. Few membrane proteins are the most critical marker in recognizing ESC. While various signaling pathways influence stem cells' self-renewal and differentiation, such signaling molecules can be stem cells' markers. Some other proteins are uniquely present or secrete in specific cell types and can be the potential makers for stem cell identification. Various stem cell markers present in the cells' different compartments are depicted in Fig. 19.1.

Table 19.1 Comparisons of advantages and disadvantages of different stem cells

Stem cell types	Advantages	Disadvantages	Sources
Embryonic stem cells (ESC)	• Can differentiate into all three germ layers • Unlimited propagation • Unique morphology and easy to identify • Definite pluripotency marker	• Ethical concern • Possible of immune rejection in allogeneic use • Teratocarcinoma formation • Genetic instability • Works on "All and none law"—all cells differentiate upon receiving differentiation signal or remain undifferentiated	Inner cell mass (ICM) of the blastocyst, morula stage embryo
Mesenchymal stem cells (MSC)	• Avoid ethical concerns • Paracrine signaling promotes the growth of neighboring cells • Easy to harvest • Homing mechanisms • Proliferate in response to tissue needs • Multipotent in nature • No genetic instability • Proliferate, self-renew, and differentiate	• Inadequate in numbers • Require expansion before administration • Asymmetrical division cause differentiation in culture • Short-term propagation in culture • Lack of definite marker • Phenotypically similar to differentiated cells • Regeneration potential varies with the source of tissue	Bone marrow, peripheral blood, adipose tissue, placenta, umbilical cord, cord blood, endometrium, synovial fluids, muscles, liver, dental pulp, amniotic fluids, milk, Wharton's jelly
Hematopoietic stem cells (HSC)	• Homing mechanism • Capable of myogenesis and angiogenesis	• Low in numbers • Possible immune rejection	Umbilical cord blood, placenta, bone marrow,
Mammary stem cells (MaSC)	• Single cell can give rise to fully functional mammary tissue • Present in all physiological stages in the mammary gland • Multipotent • Pluripotent • Can be harvested even from milk • Have the potential to form neurons of suckling offspring • No ethical concern	• Low in numbers • No consistent marker to identify a pure population • Numbers vary with the physiology of animals • Various subpopulations of biopotent, unipotent, progenitors, multipotent • No consistent markers among all the species	Mammary tissue, milk
Induced pluripotent	• Can be induced using pluripotency	• Ectopic expression of pluripotency	Any cell

(continued)

Table 19.1 (continued)

Stem cell types	Advantages	Disadvantages	Sources
stem cells (iPSC)	transcription factors from cells of the same individual • Avoid immune rejection • No ethical concern • Abundant cells can be propagated in culture	transcription factors is neoplastic • Retroviral medicate induction, and the DNA may integrate with the host cell genome • Differentiated cells of iPSC may integrate transgenes	

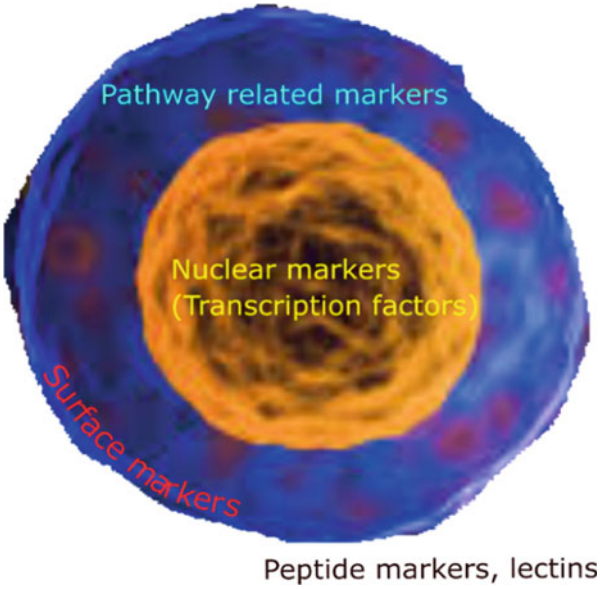

Fig. 19.1 Location of markers of stem cells in various cellular compartments

19.3 Difficulty in Identifying Stem Cells

1. *Lack of specific stem cell marker*: The success of stem cell therapy lies with the purity and a sufficient number of stem cells administered to restore tissue regeneration. The challenges are to identify phenotypically similar by genetically different cells among the mixture of various cell types harvested from the tissue. Over the past decades, rapid progress has been made to identify, characterize, and utilize stem cells for therapeutic applications. Embryonic stem cells (ESCs) received more attention due to their unique properties, unique source (inner cell mass of embryo proper), and expression of pluripotency transcription factors. In addition, ESCs remain undifferentiated in the culture for a prolonged period, probably more than a decade from its first isolation (Thomson 1998). The use of

ESCs has political, ethical, and technical concerns in clinical applications. Therefore, recent advances in adult stem cells received greater attention as an alternative strategy for cell-based therapy. Mesenchymal stem cells (MSCs), hematopoietic stem cells (HSCs), endothelial progenitor cells (EPCs), and mammary stem cells (MaSCs) are few examples of adult stem cells and are used in the treatment of some of the ailments of human and animals. MSCs are present in almost every organ of the body and are an ideal source of stem cells for regenerative medicine. MSCs have the potential for self-renewal, proliferation, and differentiation (Caplan 2009), and are multipotent. MSCs have different regenerative potential because of their different sources of origin. Such differences arise due to their immunomodulatory properties, immunophenotypes, secretome, and gene expression. For example, MSCs derived from adult bone marrow (BM-MSCs), adipose tissue (AT-MSCs), and Wharton's jelly (WJ-MSCs) of human differences in the expression of CD34, CD133, CD146, SSEA-4, MSCA-1, and CD271 makers and BM-MSCs showed high immunomodulatory activity (Petrenko et al. 2020). Other cell surface markers of MSCs are CD10, CD29, CD44, CD73, CD90, and CD105.

2. *Species-specific primary antibody*: Species-specific antibodies and gender-specific biomarkers are essential tools to label the stem cells for identification and subsequent isolation. The stem cell markers recognize an epitope of stem cells expressed in the cell surface or another compartment along these lines. Unfortunately, antibodies may recognize nonspecific epitope of other antigens and result in the false selection of cells. For example, a human-specific antibody recognizing TRA-1-85 also binds with minor/major histocompatibility antigens (Burkhardt et al. 2012). Antibodies of stem cell markers raised against species-specific antigens may not identify other species' stem cells. Therefore, the selection of species-specific antibodies is crucial in recognizing stem cells. Many antibodies are not available commercially, or they may not recognize other species' stem cells. For example, an antibody raised against mouse stem cell antigen Thy-1 may not recognize Thy1+ mesenchymal stem cells in the goat.
3. *Clonality of antibody*: Monoclonal antibodies (mAbs) identify stem cells more precisely than polyclonal antibodies as mAbs are more sensitive and specific. For this reason, many of the conventionally used cell surface markers (say, human pluripotent stem cells) are not reactive with marker proteins. However, they recognize complex carbohydrate or lipid moieties, for which there are no identified corresponding genes (Choi et al. 2014). Many other mAbs against stem cell markers need to develop using whole-cell and decoy immunization strategies as an alternative to different methodologies to develop mAbs against nonprotein titles.
4. *Multiple markers*: Researchers often identify stem cells using a combination of markers like multiparameter cell sorting. In that marker, names are reflected by the presence (+) or absence (−) of stem cell antigens. For example, mouse mammary stem cells were identified by a cocktail of markers, CD24 and CD29 (β1-integrin) or CD49f (α6-integrin) (Shackleton et al. 2006; Stingl et al. 2006).

While in bovine, mammary stem cells were identified using $CD49f^{high}CD24^{+}$ cells expressing CD10/KRT14 and KRT7 (Finot et al. 2018).

19.4 Challenges of Stem Cell Research

1. *Acquisition of Adequate Stem Cells*: Acquisition of a sufficient number of adult stem cells (ASC) is challenging due to their limited number in the tissue. Unlike ASCs, ESC isolation is relatively easy, and sufficient numbers of stem cells can be harvested from the inner cell mass. The isolation of MSCs, HSCs, or EPCs is challenging to gather due to many organ-specific stem cells and contamination with other cell types. The number of tissue-specific stem cells is minimal and is usually present in $<1\%$ of the total cells. The number of cardiac stem cells (one stem cell for every 1000 myocytes), HCS (one HCS for every 10,000 bone marrow cells) (Sun et al. 2014), and MaSC (one MaSC for every 200 in heifers' mammary epithelial cells) (Finot et al. 2019) is minimal, and hence expansion of these stem cells is required before administration.
2. *Plasticity of stem cells*: Stem cells are not static but dynamic, and their nature to remain as stem cells or differentiate into functional cells or change their lineage depends on intrinsic and extrinsic cues that comprise physiological status and tissue needs. Changing the lineage of cells is another complexity that the investigators can poorly understand. A recent study in mice's mammary gland suggested that cell lineage of stromal cells directly contributes to epithelial cells. PDGFRα-positive adipocytes made de novo contribution to luminal and basal epithelial cells. They demonstrated a reservoir of epithelial stem cells from mesenchymal progenitors (Joshi et al. 2019), a beautiful example of stem cells' plasticity.
3. *Lack of animal models for clinical validation*: Paucity of well-characterized stem cell lines from larger animal models existed. There is also insufficiency of and well-established protocols for stem cell identification, manipulation, and characterization. Establishing a centralized and well-established central facility for quality control of standardized cells and distribution to various investigators will help in the preclinical data validation. Necessary comparative studies are essential in understanding the different disease models. An example, for the understanding of human nonalcoholic fatty liver disease (NAFLD), study of bovine fatty liver disease (FLD) has been suggested as a better animal model than mice (LaCasse et al. 2020). Data showed that animal models provide crucial information about the pathogenesis of fatty liver and the therapeutic effects of various drugs (Yuan et al. 2019).
4. *Multidisciplinary knowledge*: The creation of millions of working and biologically accurate cooperating cells is required for making new and functional organs out of these stem cells (Zakrzewski et al. 2019). Therefore, the multidisciplinary approach is needed to understand biology.
5. *Huge biological variations* among the animals and animals' age often limit stem cells' therapeutic evaluation. Generally, young and healthy animals are used in

stem cell experiments, and human populations are involved, ill. Aged patients who are often treated with drugs may limit stem cell treatment's efficacy. Careful and long-term monitoring is needed to estimate the best results of its effectiveness.

6. *Low differential potential* and low engraftment of cells are other potential limitations of stem cell research. New approaches for retention of exogenously transplanted cells to the host tissue and the ability to follow their fate in vivo by labeling experiments can be optimized (Harding et al. 2013).
7. *Lack of humanized large animal model for the disease*: Significant success has been achieved in understanding in vivo functions of human cells and tissue using humanized mouse strain like recapitulating human immune system in humanized mice to investigate the biology of human immunity and functions (Shultz et al. 2012).
8. *Ethical and regulatory issue* related to animal model is a potential challenge. Two different types of regulatory issues are pertinent in stem cell-based research: (1) clearance from the Institutional Animal Ethical Committee to conduct research on animals, and (2) stem cells or cell-based products are considered as "drug," and therefore, clearance from drug regulating authorities is needed. For example, in India, various agencies, namely, Advertising Standard Council of India (ASCI), Department of Biotechnology (DBT), Indian Council of Medical Research (ICMR), Medical Council of India (MCI), Directorate General of Health Services (DGHS), and National Apex Committee for Stem Cell Research and Therapy (NAC-SCRT), are involved in the regulation of stem cell research and its application for human patients. So far, no strict guidelines for applying stem cell and cell-based products on animals exist.
9. *The use of stem cells also brings manufacturing and safety-related challenges*: Specific issues associated with cell transplantation are the biological or donor-to-donor variability. Additionally, microbiological contamination, immunological responses to alloantigens, and transplanted cells' tumorigenicity are other cell transplantation issues. Many of these regulatory and safety issues are well-reviewed and outlined in other online materials.
10. *Problems of iPSCs in regenerative medicine*: Induced pluripotent stem cells (iPSCs) are created from any cells of the body by reprogramming the pluripotency circuit of the cells. These cells' unique nature is the capability of unlimited self-renewal in vitro and differentiation into all other cell types of the body. The pluripotency circuit is reprogrammed in differentiated cells using established viral-mediated gene transfer methods. These pluripotent genes are Oct4, Sox2, and Nanog (Boyer et al. 2005). One of the severe problems using iPSCs in stem cell therapy is that the genes (Oct4, Sox2, Klf4, and c-Myc) used to induce pluripotency may cause tumor development upon their ectopic transcription (Ben-Porath et al. 2008). Another issue with iPSCs is that transgenes' transcription can resume in differentiated cells derived from iPSCs that may lead to neoplastic transformation of regenerated tissue (Okita et al. 2007). In addition, the integration of viral DNA with the host cell genome is possible in iPSCs derived using retroviral or lentiviral-mediated gene induction. Altogether, the

application of iPSCs in regenerative medicine is full of challenges. Several possible strategies can resolve the above-stated issues with iPSCs: (1) development of delivering protocol for nonintegrated genetic constructs, (2) recombinant protein constructs, (3) minimal use of transcription factors, and (4) search and use less carcinogenic and oncogenic genes for induction (Medvedev et al. 2010).

11. *Dosage of administered stem cell*: Currently available technology for counting stem cells is based on protein markers, called stem cell markers, which may be insufficient to identify the pure population of "real" stem cells. Dr. James Sherely and his team started a company to evaluate stem cells kinetics as a novel method for counting "real" stem cells (https://asymmetrex.com). It is estimated that stem cell therapy's failure lies principally on insufficient dose or complete deficiency of "real" stem cells in the administered dose (Sherley 2018).

19.5 Conclusions

Although many stem cell science challenges are overwhelming, the field is advancing. Stem cell therapy is available to treat some diseases of humans and animals. Their impact on future medicine seems to be significant and promising. After many decades of experiments, the possibility of stem cell therapy is becoming a game-changer in regenerative medicine. Induced pluripotency enabled induction of one's differentiated cell into pluripotent stem cells. However, it suffers from the possibility of neoplastic transformation of iPSC-derived cells.

References

Ben-Porath I, Thomson MW, Carey VJ, Ge R, Bell GW, Regev A, Weinberg RA (2008) An embryonic stem cell-like gene expression signature in poorly differentiated aggressive human tumors. Nat Genet 40(5):499–507

Boyer LA, Lee TI, Cole MF, Johnstone SE, Levine SS, Zucker JP, Guenther MG, Kumar RM, Murray HL, Jenner RG, Gifford DK, Melton DA, Jaenisch R, Young RA (2005) Core transcriptional regulatory circuitry in human embryonic stem cells. Cell 122(6):947–956

Burkhardt JK, Hofstetter CP, Santillan A et al (2012) Orthotopic glioblastoma stem-like cell xenograft model in mice to evaluate intra-arterial delivery of bevacizumab: from bedside to bench. J Clin Neurosci 19(11):1568–1572

Caplan AI (2009) Why are MSCs therapeutic? New data: new insight. J Pathol 217:318–324. https://doi.org/10.1002/path.2469

Choi HS, Kim W-T, Ryu CJ (2014) Antibody approaches to prepare clinically transplantable cells from human embryonic stem cells: identification of human embryonic stem cell surface markers by monoclonal antibodies. Biotechnol J 9:915–920

Finot L, Chanat E, Dessauge F (2018) Bovine mammary gland development: new insights into the epithelial hierarchy. Molecular signature of the putative stem/progenitor cells committed to the development of the bovine mammary gland at puberty. Sci Rep 8:16194

Finot L, Chanat E, Dessauge F (2019) Mammary epithelial cell lineage changes during cow's life. J Mammary Gland Biol Neoplasia 24:185. https://doi.org/10.1007/s10911-019-09427-1

Harding J, Robert RM, Mirochnitchenko O (2013) Large animal models for stem cell therapy. Stem Cell Res Ther 4:23

Joshi PA, Waterhouse PD, Kasaian K et al (2019) PDGFRα+ stromal adipocyte progenitors transition into epithelial cells during lobulo-alveologenesis in the murine mammary gland. Nat Commun 10:1760

LaCasse M, Choudhary S, Choudhary RK, de Avila J, Beitz D, Du M, Testroet E (2020) A nonperfusion-based method of hepatic cell isolation and development of fatty liver disease model for dairy cattle (abstract only). FASEB J 34:1. https://doi.org/10.1096/fasebj.2020.34.s1.06887

Medvedev SP, Shevchenko AI, Zakian SM (2010) Induced pluripotent stem cells: problems and advantages when applying them in regenerative medicine. Acta Nat 2(2):18–28

Okita K, Ichisaka T, Yamanaka S (2007) Generation of germline-competent induced pluripotent stem cells. Nature 448(7151):313–317

Petrenko Y, Vackova I, Kekulova K, Chudickova M, Koci Z, Turnovcova K, Kupcova Skalnikova H, Vodicka P, Kubinova S (2020) A comparative analysis of multipotent mesenchymal stromal cells derived from different sources, with a focus on neuroregenerative potential. Sci Rep 10:1–15. https://doi.org/10.1038/s41598-020-61167-z

Shackleton M, Vaillant F, Simpson KJ, Stingl J, Smyth GK, Asselin-Labat M-L et al (2006) Generation of a functional mammary gland from a single stem cell. Nature 439:84–88

Sherley JL (2018) Dose determination for stem cell medicine: a need whose time has come. In: Atala A, Cetrulo C, Cetrulo K, Murphy SV, Taghizadeh R (eds) Perinatal stem cells: research and therapy. Elsevier, Amsterdam, pp 383–397

Shultz LD, Brehm MA, Garcia-Martinez JV, Greiner DL (2012) Humanized mice for immune system investigation: progress, promise and challenges. Nat Rev Immunol 12(11):786–798

Stingl J, Eirew P, Ricketson I, Shackleton M, Vaillant F, Choi D et al (2006) Purification and unique properties of mammary epithelial stem cells. Nature 439:993–997

Sun Q, Zhang Z, Sun Z (2014) The potential and challenges of using stem cells for cardiovascular repair and regeneration. Genes Dis 1:113–119. https://doi.org/10.1016/j.gendis.2014.07.003

Thomson JA (1998) Embryonic stem cell lines derived from human blastocysts. Science 282:1145–1147. https://doi.org/10.1126/science.282.5391.1145

Yuan J, Chen C, Cui J et al (2019) Fatty liver disease caused by high-alcohol-producing Klebsiella pneumoniae. Cell Metab 30(4):675–688

Zakrzewski W, Dobrzynski M, Szymonowicz M, Rybak Z (2019) Stem cells: past, present, and future. Stem Cell Res Ther 10:68

Regulations of Animal Cell-Based Drugs in Veterinary Regenerative Medicine

20

Ratan Kumar Choudhary and Shanti Choudhary

Abstract

Regulation of stem cell research and therapy is booming in the world. Stem cells and derivatives of stem cells are now called a drug, and hence law regarding drug use must be set forth. Developed nations like the USA and the European Union (EU) have provided human stem cell research and therapeutic application guidelines for human patients and animals. While India offers remarkably stringent policies and regulations for human stem cells and application, no guidelines for animal stem cells have existed thus far. This chapter discusses current laws of stem cell applications in both humans and animals in India, the USA and the EU. It discusses the paths forward in a global context toward applying animal stem cells for veterinary patients.

Keywords

Veterinary Regulation · Drug approval · USA · European Union · India

20.1 Overview of FDA's Drug Approval Process

The U.S. Food and Drug Administration (FDA) defines the term "drug" as *any articles recognized in the official United States Pharmacopoeia, official Homeopathic Pharmacopoeia of the United States, or official National Formulary; that is being intended for use in the diagnosis, cure, mitigation, treatment, or prevention of*

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disease in man or other animals which, intended to affect the structure or any function of the body of man or other animals. The FDA defines most animal cell-based products as a "drug" because they are intended to treat, control, or prevent a disease or affect the body's structure or function, like improving the animal's fertility. Per guidelines, the FDA must review and approve cell-based drugs before companies can legally market them.

In the USA, an unapproved drug needs New Animal Drug Applications (NADA) approval for researching to collect data for the drugs' safety and efficacy. NADA regulates any drugs (except animal feed) for use in animals other than man, which is not proven to be safe and effective for the intended use. NADA may approve the drug for the investigation or agree with conditions (conditional NADA or CNADA). Three types of new animal drug applications are

1. NADA—Grants approval for new drugs for the research purpose for the data collection
2. ANADA—Provides approval for a generic new drug, a copy of old and approved drug near expiration. It is the abbreviated NADA (ANADA).
3. CNADA—used to seek conditional approval of a new drug that comprises all the requirements in support of full permission except the demonstration of "effectiveness" of the drug upon administration

20.2 Centre for Veterinary Medicine (CVM), USA

Centre for Veterinary Medicine is a part of the Food and Drug Administration (FDA), an agency under the Department of Health and Human Services (Fig. 20.1). Center for Veterinary Medicine. US Food and Drug Administration (2020).

CVM regulates (1) animal drugs, (2) animal food and feeds (including pet foods), and (3) animal medical devices. In general, CVM makes sure that the drugs used on animals are safe and effective before approving it. Pet animals include dogs, cats, and farm animals like horses, cattle, pigs, chickens, and even honey bees. CVM ensures that the drugs administrated to the animal are safe when consumers eat animal-based products. In addition, the center also researches the safety of animal drugs, animal food, and food products made from animals and helps in making animal drugs legally available (FDA's Center for Veterinary Medicine 2020).

CVM does not regulate (1) veterinary advice, (2) veterinary medicine practices, (3) vaccines for any infectious diseases, and (4) some tick and flea products used on animals as drugs (Laws, Regulations, and Guidances; FDA's Center for Veterinary Medicine 2020).

Clinical field studies for Animal Cells, Tissues, and Cell- and Tissue-Based Products (ACTPs) provides information about animal owners, veterinarians, researchers, and the public regarding the use of ACTPs in animals (US Food and Drug Administration 2020b). The FDA disapproves ACTPs. ACTP has also not been tested for safety and efficacy as a drug. Very few studies are being conducted and are listed in Table 20.1.

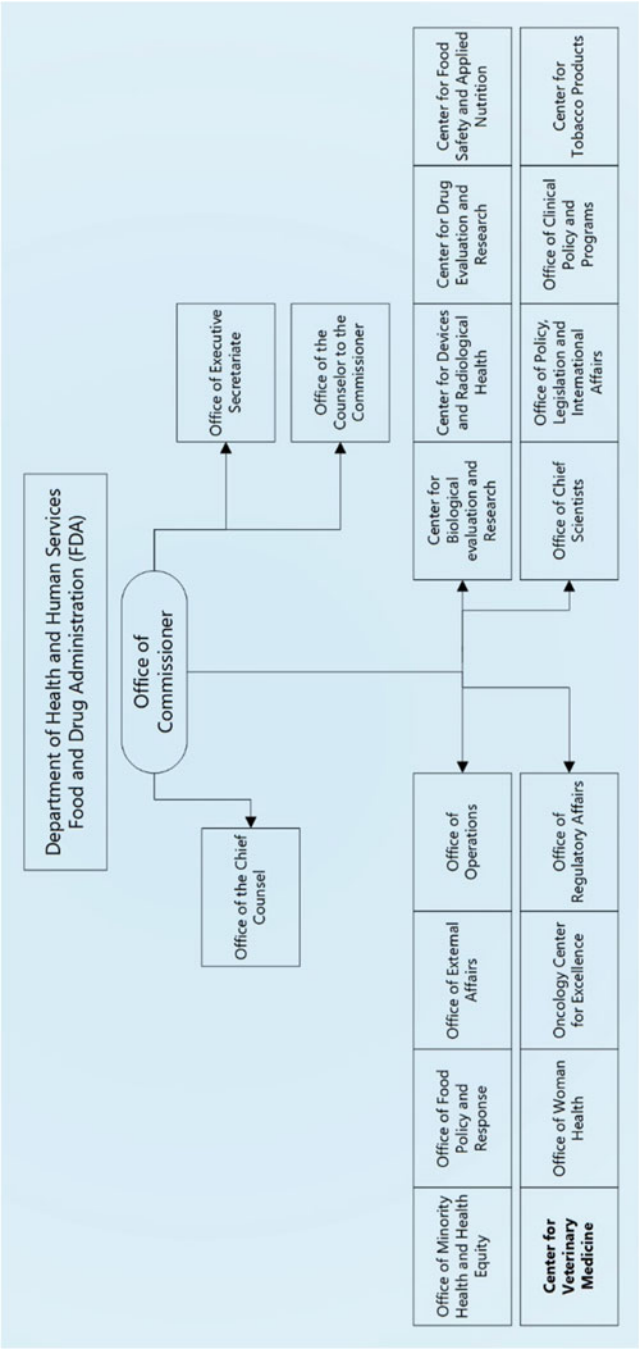

Fig. 20.1 Organogram of the Department of Health and Human Services of the FDA and Centre for Veterinary Medicine (CVM)

Table 20.1 List of active clinical field studies for ACTPs in the USA

Study	Disease condition	Species	Product	Time of study	Place	Country
Adipose-derived mesenchymal stem cell therapy	Gingivostomatitis	Cat	Allogeneic feline mesenchymal stem cells	June 2020–2023	UC Davis, California	USA
Efficacy of mesenchymal stem cell therapy	Feline chronic enteropathy	Cat	Allogeneic feline mesenchymal stem cells	April 2020–2023	UC Davis, California	USA
Stem cell-based treatment of spina bifida	Spina bifida	Dog	Allogeneic canine mesenchymal stem cells	July 2018–2024	UC Davis, California	USA

Note: These studies are investigational and not approved by the FDA

Because of animal cell-based products' novelty and complexity, the FDA encourages investigators, universities, and companies to seek help before using products legally (US Food and Drug Administration 2020a). Few companies selling cell-based products without FDA approval have been issued legal warning notices.

20.3 European Union

20.3.1 Law Governing the Implementation of New Veterinary Medicine Regulations

The New Veterinary Medicine Regulation of the European Union (Regulation (EU) 2019/6) updated the old and existing veterinary medicine authorization rules on January 28, 2020 (European Medicines Agency 2020a). This new rule emphasizes measures on the availability and safety of drugs against antimicrobial resistance. The key benefits of new regulations are

1. Increase availability and access to safe and high-quality veterinary medicine
2. Ease of administrative procedures and better incentives to pharmaceutical companies
3. Based on the "one health" approach, enhance the safety and efficacy of antimicrobials and antibiotics

The first-ever EU's guidance on stem cell-based therapy in veterinary medicine came in 2017 (European Medicines Agency 2020b). The direction was prepared by the Ad Hoc Expert Group on Veterinary Novel Therapies (ADVENT) based on reviewing the scientific evidence of stem cell-based therapeutics on the animal (European Medicines Agency 2020c). The EU's European Medicines Agency's (EMA) Committee for Medicinal Products for Veterinary Use (CVMP) has approved guidance on stem cell-based medicines for veterinary use in the form of a question–answer format. It mainly addresses the concerns raised by manufacturers and authorities regarding the sterility of allogenic applications of stem cell-based medicine. Allogenic stem cell-based medicaments start from harvesting tissue (like bone marrow, adipose tissue) from the donor animal provides tissue to the recipient animals. Drugs originate from tissues (such as bone marrow or fat) from a donor from the same animal species, not from the cells' recipient. Since these products are cell-based, maintaining a development away from bacterial and viral contamination (sterile) is challenging. Cell-based drugs come under the novel therapies, which are new and have not been used previously in the veterinary domain but well known in human medicine.

The questions and answers format guidelines on allogeneic stem cell-based products for veterinary use about sterility tests are given next (European Medicines Agency 2019):

1. How is the sterility of the finished cell-based product ensured?
2. What measures are recommended when sourcing and collecting the stem cells?
3. How can the sterility of raw materials be ensured for microbiological contamination?
4. Are there any recommendations regarding general sterility and safety in the whole manufacturing process?
5. When is testing for sterility critical during the production process?
6. Which alternative and suitable methods are appropriate for sterility testing of stem cell-based final products?
7. Is PCR an acceptable alternative to sterility testing?
8. Are endotoxins in stem cell-based products a safety concern, and how to control levels of endotoxins during the manufacturing process?

Stem cell-based products are veterinary drugs and administered parenterally either intravenously or intramuscularly. Therefore, a product to be sterile is crucial. However, living cell-based products cannot be sterilized either by filtration or by another sterilization method. Thus, cell-based products' microbiological purity is ensured from the raw materials before the product harvest begins.

20.4 India's Regulations on Animal Stem Cell Therapy

India is yet to propose guidelines for animal stem cell therapy. Given that the stem cell therapeutic application is expected to increase dramatically, India is preparing to set forth the guidelines. The first guidelines for Stem Cell Research and Therapy in human, came in 2007. The primary focus of this policy was to (1) conduct basic and translational research on adult and embryonic stem cells to generate patient-specific stem cell lines, (2) create animal models for preclinical studies, (3) develop a national institute of world repute, (4) generate trained workforce, and (5) build a public–private partnership with companies (Sharma 2009). In 2013 and later in 2017, the Indian Council of Medical Research (ICMR) and Department of Biotechnology (DBT) released new and revised Guidelines for Stem Cell Research and deleted the word "therapy" and known as "ICMR-DBT National Guidelines for Stem Cell Research 2013/2017" (National Center for Biological Sciences 2013). Certain approved diseased condition of a human patient using bone marrow or peripheral blood for homologous applications is permitted upon approval of the Central Drugs Standard Control Organization (CDSCO). Guidelines state that the administered stem cells should have undergone *substantial or more than minimum manipulation* in patients, emphasizing the idea that patient isolated stem cells, as such, can be used without further manipulation in the laboratory. Application of stromal vesicle fractions (SVFs), a cocktail of heterogeneous cell populations derived from adipose tissue, is more than minimal manipulation. Hence, it is categorized under the violation of new guidelines (Tiwari and Desai 2018). According to the new rule, stem cell research is organized into three areas: permissible, restrictive, and prohibited. Research involving the establishment of new

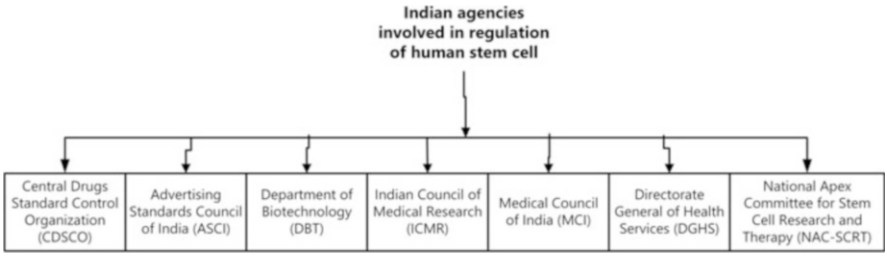

Fig. 20.2 Various Indian agencies involved in regulation of human stem cell-related issues

embryonic stem cell line and induced pluripotent stem cell lines is "permissible." Research involving in vitro fertilization and somatic cell nuclear transfer to derive ESCs is "restrictive." Under the "prohibited" category, any research involving germline gene therapy and reproductive cloning, genome-modified embryo, and propagation of any processed human cell/embryos is not allowed (Lahiry et al. 2019). Various Indian agencies are involved in regulating stem cell research and its application for human patients. These agencies are CDSCO, Advertising Standard Council of India (ASCI), Department of Biotechnology (DBT), Indian Council of Medical Research (ICMR), Medical Council of India (MCI), Directorate General of Health Services (DGHS), and National Apex Committee for Stem Cell Research and Therapy (NAC-SCRT) (Fig. 20.2).

Though the national guidelines for Stem Cell Research 2017 existed for human patients in India, no policies have been put forward for animal stem cell research for veterinary patients. In the USA, CVM provides guidelines for animal drugs, animal feeds, and animal medical devices. In contrast, clinical field studies for Animal Cells, Tissues, and Cell- and Tissue-Based Products (ACTPs) provide information regarding the use of ACTPs in animals. CVM approves if the animal drugs are safe and effective on animals. ACTPs, though not authorized by the FDA, provide only the information to the researchers, clinicians, and public, and FDA approval requires legitimate use of stem cells or stem cell derivatives.

Similarly, in 2017 the EU has published question–answer format regulations of allogenic stem cell-based products for veterinary use. It is time for India to formulate animal stem cell research and develop a roadmap for cell-based therapy. The development of cell-based treatment involves four phases: (1) discovery of cell or cell-based product, (2) process optimization, (3) production of cell or cell-based product in bulk, and (4) therapeutic delivery (Kirouac and Zandstra 2008). Under each phase, issues and strategies to deal with the problem have been described (Fig. 20.3). In the discovery phase, the main problem is the product characterization. Functional assays and cellular and molecular profiling are useful tools in this phase. Under the optimization of the process, quantifying the relationship between culture parameters and cell output is critical. Considerations in the production phase include the scale-up strategy and quality control like purity, sterility, consistency of getting the same output product.

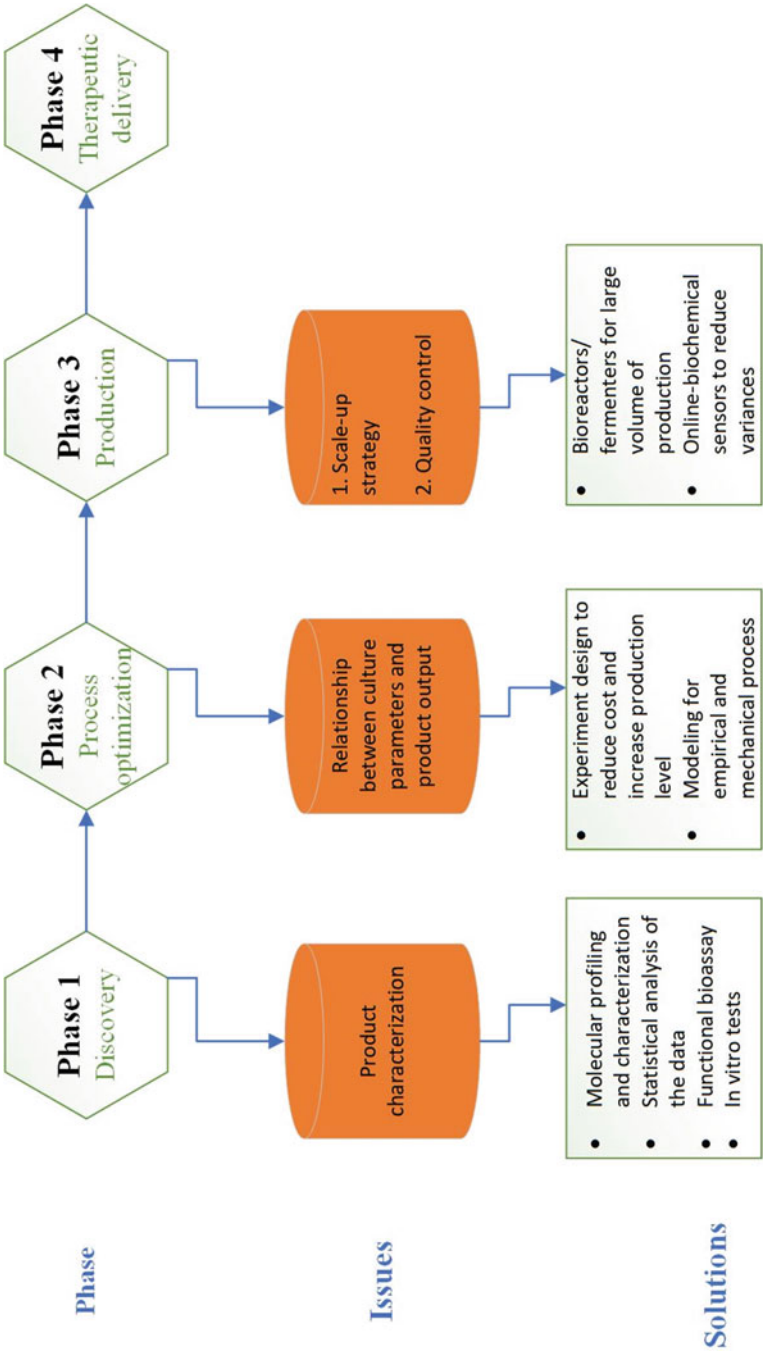

Fig. 20.3 Development of cell-based drugs with different phases, issues, and strategies to deal with the problem

Agencies like the Veterinary Council of India (VCI), in consortium with ICMR and DBT, should frame guidelines that prevent fraudulent advertisement and safe use of stem cells and their derivatives, now being called "drug" on animals.

References

Center for Veterinary Medicine. US Food and Drug Administration (2020). https://www.fda.gov/about-fda/fda-organization/center-veterinary-medicine. Accessed 26 Dec 2020

European Medicines Agency (2019) Questions and Answers on allogeneic stem cell-based products for veterinary use: specific questions on sterility, EMA/CVMP/ADVENT/751229/2016–Rev.1

European Medicines Agency (2020a) Veterinary regulatory. https://www.ema.europa.eu/en/veterinary-regulatory/overview/implementation-new-veterinary-medicines-regulation. Accessed 27 Dec 2020

European Medicines Agency (2020b) First ever guidance for stem cell therapies in animals published 2020. https://www.ema.europa.eu/en/news/first-ever-guidance-stem-cell-therapies-animals-published. Accessed 27 Dec 2020

European Medicines Agency (2020c) Stem cell-based products for veterinary use: specific questions on sterility to be addressed by the Ad Hoc Expert Group on Veterinary Novel Therapies (ADVENT). https://www.ema.europa.eu/en/stem-cell-based-products-veterinary-use-specific-questions-sterility-be-addressed-ad-hoc-expert. Accessed 28 Dec 2020

FDA's Center for Veterinary Medicine (2020) Protecting human and animal health. Animal & Veterinary 2020. https://www.fda.gov/animal-veterinary. Accessed 26 Dec 2020

Kirouac DC, Zandstra PW (2008) The systematic production of cells for cell therapies. Cell Stem Cell 3(4):369–381. https://doi.org/10.1016/j.stem.2008.09.001. PMID: 18940729

Lahiry S, Choudhury S, Sinha R, Chatterjee S (2019) The national guidelines for stem cell research (2017): what academicians need to know? Perspect Clin Res 10(4):148–154

Laws, Regulations, and Guidances; FDA's Center for Veterinary Medicine (2020). https://www.fda.gov/animal-veterinary/guidance-regulations. Accessed 26 Dec 2020

National Center for Biological Sciences (2013) Guidelines for stem cell research. https://www.ncbs.res.in/sites/default/files/policies/NGSCR%202013.pdf. Accessed 28 Dec 2020

Sharma A (2009) Stem cell research and policy in India: current scenario and future perspective. J Stem Cells 4(2):133–140

Tiwari SS, Desai PN (2018) Unproven stem cell therapies in India: regulatory challenges and proposed paths forward. Cell Stem Cell 23(1):649–652

US Food and Drug Administration (2020a) Veterinary regenerative medicine & animal cell-based product. https://www.fda.gov/animal-veterinary/development-approval-process/veterinary-regenerative-medicine-animal-cell-based-products. Accessed 27 Dec 2020

US Food and Drug Administration (2020b) Clinical field studies for animal cells, tissues, and cell- and tissue-based products (ACTPs). https://www.fda.gov/animal-veterinary/development-approval-process/clinical-field-studies-animal-cells-tissues-and-cell-and-tissue-based-products-actps. Accessed 28 Dec 2020