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Mariusz Z. Ratajczak *Editor*

Stem Cells

Therapeutic Applications

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Preface

It is obvious that stem cells and regenerative medicine have become the “hottest” contemporary scientific topics, covered by many scientific journals, textbooks, and public media. Therefore, in order to remain highly competitive and abreast with new developments, this new book should help students and researchers to better reflect and update the broadened scope of the field. There have been proposed different types of stem cells for therapeutic applications. However, with the exception of hematopoietic stem cells (HSCs) for hematological applications, the current clinical results with other types of stem cells are somewhat disappointing.

Hematopoietic stem cells (HSCs) isolated from hematopoietic tissues have been successfully employed for 50 years in hematological transplantations to treat leukemia, lymphomas, bone marrow aplasia, and some cases of immunodeficiency as well as metabolic disorders. The successful clinical application of HSCs has helped to establish the rationale for the development of stem cell therapies and regenerative medicine, and regenerative medicine is still looking for a pluripotent/multipotent stem cell, more primitive in development than HSC, that will be able to differentiate across germ layers into cells belonging to meso-, ecto-, and endoderm and be safely employed without unwanted side effects in therapy.

There have been proposed some solutions to this problem by employing embryonic stem cells (ESCs) derived from fertilized embryos or created by nuclear transfer and induced pluripotent stem cells (iPSCs) obtained by genetic modification of adult postnatal cells. Nevertheless, the potential applications of the more primitive embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) in the clinic have so far been discouraging as both these types of stem cells have exhibited several problems, including genomic instability, a risk of teratoma formation, and the possibility of rejection.

Therefore, taking into consideration safety and ethical issues, the only stem cells at the current point that could be employed in regenerative medicine are monopotent or tissue committed stem cells (TCSCs) isolated from postnatal tissues. However, their monopotency, and therefore limited differentiation potential across germ layers, is a barrier to their broader application in the clinic. Nevertheless, what is important is that there have also been described in adult tissues very rare stem

cells with broader differentiation potential that could be perhaps a solution to this problem. Moreover, there is also solid evidence that stem cells that are employed for therapy secrete a variety of growth factors—cytokines, chemokines, and bioactive components—as well as shed from the cell surface or secrete from the endosomal compartment small circular membrane fragments enriched in mRNA, miRNA, bioactive lipids, nucleotides, and proteins (extracellular vesicles (ExVs)), which seem to play an important and underappreciated role in improving the function and regeneration of damaged organs. Such cell-derived paracrine signals were very well demonstrated in the case of mesenchymal stem cells (MSCs) and may explain why the some final therapeutic benefits are similar when applying different types of adult stem cells for nonhematopoietic applications. Based on this, our book is focused on stem cells isolated from adult tissues and the various potential mechanisms involved in their therapeutic effects.

In Chap. 1 of this book, there are discussed different types of stem cells that could be potentially employed in regenerative medicine, including those isolated from adult tissues, as well as the pros and cons for the application of ESCs and iPSCs. The general point of this chapter is that regenerative medicine is still searching for optimal stem cells that could be employed in the clinic to treat damaged organs (e.g., myocardial infarction, liver damage, ischemic kidney failure, or stroke).

As mentioned above, there are serious concerns with the application of iPSC in the clinic because of the risk of teratoma formation and their genomic instability. In Chap. 2, Dr. Yasuhiro Murakawa is discussing this issue that delays the potential application of these cells in the clinical settings.

HSCs isolated from bone marrow, mobilized peripheral blood, or umbilical cord blood have been successfully employed for more than 50 years in hematological transplantations to treat leukemia, lymphomas, bone marrow aplasia, and some cases of immunodeficiency as well as metabolic disorders. The identification of these cells, as well as available isolation strategies, is reviewed in Chap. 3.

Neural stem cell (NSC) transplantation has provided the basis for the development of potentially powerful new therapeutic cell-based strategies for a broad spectrum of clinical diseases, including stroke, psychiatric illnesses such as fetal alcohol spectrum disorders, and cancer. In Chap. 4, Dr. Borlongan discusses preclinical investigations involving NSCs, including how NSCs can ameliorate these diseases, the current barriers hindering NSC-based treatments, and future directions for NSC research.

MSCs migrating at the sites of tissue injury suggested an innate ability for these cells to be involved in baseline tissue repair. The bone marrow is one of the primary sources of MSCs, though they can be ubiquitous. An attractive property of MSCs for clinical application is their ability to cross allogeneic barrier. However, alone, MSCs are not immune suppressive cells as third party cells suppress the immune response that generally recapitulates graft versus host responses (GvHD). In Chap. 5, Dr. Rameshwar focuses on the potential use of MSC for immunological disorders such as Crohn's disease and GvHD.

Very small embryonic-like stem cells (VSELs) were isolated from postnatal tissues, including gonads. VSELs in the gonads can be targeted to regenerate the gonads of patients with gonadal insufficiency, including cancer survivors, and are excellent candidates to differentiate into gametes *in vitro*. This exciting topic that provides hope for improving fertility treatment is discussed by a pioneer in the field, Dr. Bhartiya, in Chap. 6.

Gastrointestinal stem cell research, including the application of stem cells for therapy, the phenomenon of circulating and cancer stem cells, as well studies on the role of microbiota in various clinical entities, opens up a new area of investigation in gastroenterology. These topics are reviewed by Dr. Marlicz in Chap. 7.

For many years, a role of stem cells in psychiatric disorders was understudied and even neglected. Dr. Kucharska-Mazur presents new exciting data that change this old point of view.

Evidence accumulated that stem cells act in a paracrine manner through the secretion of biologically active cargo that acts on cells locally and systemically. These active molecules include not only soluble factors but also the mentioned above ExVs, which act as vehicles that transfer molecules between originator and recipient cells, therefore modifying the phenotype and function of the latter. As ExVs released from stem cells may successfully activate regenerative processes in injured cells, their application as a form of therapy can be envisaged. One of the world leaders in this field, Dr. Camussi, discusses this very timely topic in Chap. 9.

Ischemic heart disease (IHD), which includes heart failure induced by myocardial infarction, is a significant cause of morbidity and mortality worldwide. In the past decades, stem cell therapy has become a promising strategy for cardiac regeneration. However, stem-cell-based therapy yielded modest success in human clinical trials. Dr. Abdel-Latif in Chap. 10 will examine the types of cells examined in cardiac therapy in the setting of IHD, with a brief introduction to ongoing research aiming at enhancing the therapeutic potential of transplanted cells.

Dr. Smadja in Chap. 11 discusses the reported findings from *in vitro* data and also preclinical studies that aimed to explore stem cells at the origin of vasculogenesis in humans and then explore the potential use of endothelial progenitor cells to promote newly formed vessels or serve as a liquid biopsy to understand vascular pathophysiology.

The skin is the organ with the highest number of stem cells. Dr. Lenkiewicz in Chap. 12 presents a current view on stem cell compartment in human epidermis. In Chap. 13, Dr. Ciechanowicz embraces the difficult topic of lung stem cells, as well as data that show that stem cells could be applied to treat lung disorders.

There are several important signaling molecules involved in regulating stem cell biology and migration. One of the hottest topic areas are studies on the role of extracellular purines and purinergic signaling in these phenomena. Stem-cell-based therapy, mainly through mesenchymal and hematopoietic stem cells, showed promising results in improving symptoms caused by neurodegeneration. World-recognized leader in purinergic signaling Dr. Ulrich, based on his own data, proposes that purinergic receptor activity regulation combined with stem cells could enhance proliferative and differentiation rates, as well as cell engraftment.

Finally, the dream of slowing down the aging process has always inspired mankind. Since stem cells are responsible for tissue and organ rejuvenation, it is logical that we should search for encoded mechanisms affecting life span in these cells. Dr. Kucia elegantly reviews the topic of aging and stem cells in Chap. 15.

Saying this, there are many individuals that I wish to thank, without whose efforts this book would not have been possible. First and foremost, special thanks go to all the authors who wrote the chapters and shared with readers their experience and novel research. These individuals are recognized experts in the various areas of stem cell research. I would also like to thank the Springer Nature team and our acquisition editors for their patience, encouragement, and valuable help. It was an intellectual pleasure to work with them on this book.

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Contents

1 Potential Clinical Applications of Stem Cells in Regenerative Medicine	1
Suman Suman, Alison Domingues, Janina Ratajczak, and Mariusz Z. Ratajczak	
2 Genomic Instability of iPSCs and Challenges in Their Clinical Applications	23
Masahito Yoshihara, Akiko Oguchi, and Yasuhiro Murakawa	
3 Hematopoietic Stem and Progenitor Cells (HSPCs)	49
Kamila Bujko, Magda Kucia, Janina Ratajczak, and Mariusz Z. Ratajczak	
4 Neural Stem Cells	79
Julian P. Tuazon, Vanessa Castelli, Jea-Young Lee, Giovambattista B. Desideri, Liborio Stuppia, Anna Maria Cimini, and Cesar V. Borlongan	
5 Therapeutic Potential of Mesenchymal Stem Cells in Immune-Mediated Diseases	93
Adam Eljarrah, Marina Gergues, Piotr W. Pobiarzyn, Oleta A. Sandiford, and Pranela Rameshwar	
6 Stem Cells in the Mammalian Gonads	109
Deepa Bhartiya, Sandhya Anand, Ankita Kaushik, and Diksha Sharma	
7 Stem and Progenitor Cells in the Pathogenesis and Treatment of Digestive Diseases	125
Wojciech Marlicz, Igor Łoniewski, and Karolina Skonieczna-Żydecka	

8	Stem Cells in Psychiatry	159
	Jolanta Kucharska-Mazur, Mariusz Z. Ratajczak, and Jerzy Samochowiec	
9	The Role of Extracellular Vesicles as Paracrine Effectors in Stem Cell-Based Therapies	175
	Stefania Bruno, Sharad Kholia, Maria Chiara Deregibus, and Giovanni Camussi	
10	Cellular Therapy for Ischemic Heart Disease: An Update	195
	Hsuan Peng and Ahmed Abdel-Latif	
11	Vasculogenic Stem and Progenitor Cells in Human: Future Cell Therapy Product or Liquid Biopsy for Vascular Disease	215
	David M. Smadja	
12	Epidermal Stem Cells	239
	Anna M. Lenkiewicz	
13	Stem Cells in Lungs	261
	Andrzej Ciechanowicz	
14	Targeting Purinergic Signaling and Cell Therapy in Cardiovascular and Neurodegenerative Diseases	275
	Roberta Andrejew, Talita Glaser, Ágatha Oliveira-Giacomelli, Deidiane Ribeiro, Mariana Godoy, Alessandro Granato, and Henning Ulrich	
15	Plausible Links Between Metabolic Networks, Stem Cells, and Longevity	355
	Magdalena Kucia and Mariusz Z. Ratajczak	
	Index	389

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Chapter 1

Potential Clinical Applications of Stem Cells in Regenerative Medicine



Suman Suman, Alison Domingues, Janina Ratajczak,
and Mariusz Z. Ratajczak

Abstract The field of regenerative medicine is looking for a pluripotent/multipotent stem cell able to differentiate across germ layers and be safely employed in therapy. Unfortunately, with the exception of hematopoietic stem/progenitor cells (HSPCs) for hematological applications, the current clinical results with stem cells are somewhat disappointing. The potential clinical applications of the more primitive embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) have so far been discouraging, as both have exhibited several problems, including genomic instability, a risk of teratoma formation, and the possibility of rejection. Therefore, the only safe stem cells that have so far been employed in regenerative medicine are monopotent stem cells, such as the abovementioned HSPCs or mesenchymal stem cells (MSCs) isolated from postnatal tissues. However, their monopotency, and therefore limited differentiation potential, is a barrier to their broader application in the clinic. Interestingly, results have accumulated indicating that adult tissues contain rare, early-development stem cells known as very small embryonic-like stem cells (VSELs), which can differentiate into cells from more than one germ layer. This chapter addresses different sources of stem cells for potential clinical application and their advantages and problems to be solved.

Keywords Monopotent stem cells · Tissue-committed stem cells · Pluripotent stem cells · Embryonic stem cells · Induced pluripotent stem cells · Teratoma

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formation · Genomic instability · Paracrine effects · Extracellular microvesicles · Very small embryonic-like stem cells · Nuclear transfer · Therapeutic cloning

1.1 Introduction

In humans, like other species that reproduce sexually, development starts from a single most primitive stem cell that is the fertilized oocyte called zygote [1]. This single-cell zygote has potential to divide and differentiate to generate all the specialized cell types of the adult human body giving rise to an entirely new organism. This unique property of a cell is referred to as “totipotency” [2, 3]. A totipotent cell can develop into any type of cell in the adult human body. Zygote is a totipotent stem cell (TSC) and has a distinguishing quality to give rise to both the placenta and embryo proper [3, 4]. Totipotency is retained after first few divisions of zygote into blastomeres that initiate growth of morula. In a next step, morula develops into blastocyst, and the inner cell mass of developing blastocyst contains embryonic stem cells (ESCs) and is also called as pluripotent stem cells (PSCs) that are able to differentiate into cells from all three germ layers and gametes [5–8]. PSCs in contrast to TSCs cannot give rise to the placenta. Accumulated evidence suggests that PSCs may also survive in dormant state in postnatal tissues. During the process of embryogenesis, PSCs establish all three germ layers (meso-, ecto-, and endoderm) and within germ layers give rise to monopotent tissue-committed stem cells (TCSCs), which are responsible for organogenesis and in postnatal life secure rejuvenation of organs and tissues [1, 9–15]. PSCs-derived cells that give rise to germ layers are called as multipotent stem cells (MPSCs).

Stem cells are the units of biological organization with unique self-renewal capability as well as differentiation potential to give rise to multiple cell lineages [5, 16, 17]. These two unique properties make them indispensable for the development and regeneration of organ and tissue system. The robustness of the adult stem cell compartment is one of the major factors that directly impacts quality of life as well as life span, and their proper functioning ensures healthy aging [1, 18, 19]. It is well known that stem cells continuously replace differentiated cells in adult tissues that are used up during life, and this replacement occurs at a different pace in the various organs [11]. It is ideal to state that different tissues require and employ distinct strategies to self-renew and repair. The local adult stem cells residing in the stem cell niches in adult tissues are responsible for organ rejuvenation and the replacement of senescent cells in a given tissue. These stem cells can be multipotent or unipotent and may be present in quiescent or actively dividing states. Thus, this process occurs at a varying pace in different organs, like the intestinal epithelium and epidermis, and hematopoietic cells are continuously replaced by new cells, whereas this process of self-renewal is extremely slow in other organs (e.g., the heart, skeletal muscles, liver, or pancreas), and its existence is still questioned for the central nervous system (the brain and spinal cord) [11, 20–22].

There is urgent need to replace or regenerate damaged tissues under various circumstance like tissue injury, trauma, cancers, and age-related and other degenerative diseases. The unique properties of stem cells to self-renew and differentiate are being exploited to regenerate damaged tissues/organs and have laid the foundation for regenerative medicine [23]. It is a new branch of translational research in tissue engineering and molecular biology with the aim to regenerate damaged cells, tissues, or organs in order to restore or establish their normal function [23–26]. It also includes a long-term goal that is the possibility to grow tissues, organ fragments, and even the entire organs in the laboratory and to implant them when the body cannot heal itself. This would potentially solve the problem of shortage of organs available for donation and the problem of organ transplant rejection if the organ is established from patients autologous cells without having the issue of histocompatibility.

For almost half a century, the successful application of hematopoietic stem cells (HSCs) in hematopoietic transplants has encouraged clinicians to employ adult stem cells in treating several other clinical problems, including (i) damaged myocardium after heart infarction, (ii) the brain after stroke, (iii) the spinal cord after mechanical injury, (iv) age-related macular degeneration (AMD) of the retina, (v) diabetes, (vi) extensive skin burns, (vii) damaged liver, and (viii) Parkinson’s disease [27–33]. The stem cells most frequently employed so far for this purpose are adult tissue-derived cells isolated mainly from bone marrow, mobilized peripheral blood (mPB), umbilical cord blood, fat tissue, and even myocardial biopsies [1, 5].

Overall, in this chapter we will discuss the two major goals of regenerative medicine (Fig. 1.1). The first goal is to employ stem cells in emergency situations, and

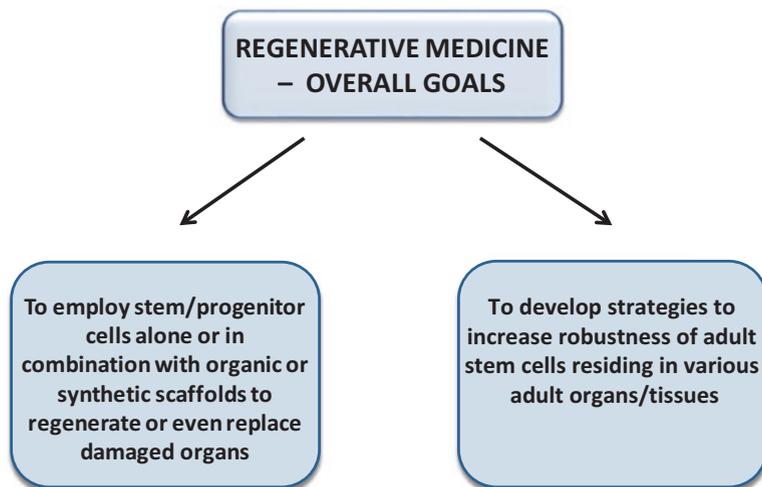


Fig. 1.1 Overall goals of regenerative medicine. There are two major goals of regenerative medicine—(i) to employ stem cells in emergency situations and (ii) to modulate in positive way adult stem cell compartment so that stem cells will retain their robustness during adult life

the second goal is to modulate the adult stem cell compartment in a positive way so that stem cells will retain their robustness during adult life. Recent advances in stem cell biology and technology have fueled the field of regenerative medicine and hold great potential in advanced tissue engineering and cell-based therapies [33].

Regenerative medicine as therapeutic procedure involves injection of stem cells or progenitor cells isolated from adult tissues to damaged organs and may result in tissue/organ structural regeneration as well as functional improvement in the affected tissue/organ [1]. These effects are believed to be a consequence of stem cell differentiation or due to the biologically active molecules administered alone or as a part of “secretome” or secreted extracellular microvesicles (ExMVs) by the cells employed in therapy [34]. There is hope that in the future, advances in regenerative therapy may allow the transplantation of laboratory-grown organ fragments and tissues employing appropriate synthetic or nature-derived scaffolds [1, 35]. The latter is however still a remote goal due to the fact that true organs grow in a three-dimensional structures and contain cells belonging to different germ layers such as the nerves, blood vessels, lymphatics, etc. In other words, to make this goal achievable, the implanted stem cells should recapitulate the exact organogenesis giving rise to a three-dimensional functional tissue/organ composed of cells from different germ layers and hence simulating the embryonic development in a given organ, but this requirement appears to be far from feasible both technically and practically.

It is important to note that so far, adult stem cells have given promising data for their safe usage in regenerative medicine [36]. Adult stem cells that are scattered throughout various tissues and organs have the capability to produce at least a differentiated functional progeny. These cells can be isolated from various sources like bone marrow, umbilical cord blood, mobilized peripheral blood, skin epithelium, myocardium, adipose tissues, and skeletal muscle biopsies [37]. Unfortunately, except hematopoietic stem cell transplants, the clinical results for stem cell therapies in other conditions have been rather disappointing, and several encouraging results initially reported in laboratory animals have not been reproduced in humans. In clinical settings, if any improvement eliciting functional tissue repair of the damaged solid organs has been noted, it is mainly due to the paracrine effects of stem cells employed for therapy. These stem cells are a source of soluble trophic factors, cytokines, growth factors, and extracellular matrix (ECM) molecules that modulate the molecular composition in the target organ and evoke responses from the resident cells, improving survival of the cells in damaged organs and promoting vasculogenesis of hypoxic tissues [33, 38]. Thus, adult stem cells employed in regenerative medicine may have some beneficial effects because of their immunomodulatory properties or because they secrete membrane-derived extracellular microvesicles that cargo different mRNA, miRNA, proteins, and bioactive lipids and may promote regeneration of damaged tissues [39, 40].

At the same time, attempts to employ embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) in the clinic have failed, due to problems with their differentiation into fully functional cells, risk of tumor formation by these cells, and their significant genomic instability [41–43]. In addition, the use of human embryos for embryonic stem cells faces lot of ethical controversies and makes their use even

more problematic [44, 45]. In this chapter, we will discuss various potential sources of stem cells that are currently employed in regenerative medicine and the mechanisms that explain some of their limitations. We will also discuss some noted potential beneficial effects.

However, the danger in regenerative medicine approaches makes it premature to hope for effectiveness of these therapies. It poses danger of the so-called stem cell tourism that refers to situations when patients travel abroad to get stem cell treatments that would not be available to them in their home countries [46, 47]. These treatments in the foreign countries are expensive, sometimes unproven, ineffective, risky, and many times conducted at unregulated clinics. Since regenerative medicine is a young medical field, different countries are at different places in their development and regulation for potential application of stem cell therapies. There is no doubt that regenerative medicine has future, but more patience and concrete research are necessary to pave the way for these therapies for better results and achievable goals.

1.2 Stem Cells and Their Role in Tissue Development and Regeneration

Because of scientific progress, at the beginning of the third millennium, human beings have reached out to and discovered new technologies and till now are the only “supreme beings.” The development of physics has enabled us to explore nuclear energy, and the development of biology and genetics has explained the mystery of the organism regeneration thus leading this biotechnology research into the fascinating world of stem cells. However, we have to remember that with both of these technologies, there is always a risk of technological abuse. For instance, nuclear energy is not only employed for therapy but also facilitates construction of weapons of mass destruction such as atomic or nuclear bombs. Similarly, biotechnology poses a danger where stem cell technology could be employed for therapeutic reproduction and cloning of human beings.

Stem cell compartment during embryogenesis shows hierarchical organization (Table 1.1). The most primitive stem cell during development as already mentioned above is fertilized oocyte that turns to totipotent stem cells (TSCs) [1–3]. These cells can give rise to both the body of an embryo and tissues of the placenta. Under normal conditions, TSCs are fertilized oocyte (zygote) and initial few blastomeres (up to four divisions) in developing morula [48, 49]. Under artificial conditions as in the case of therapeutic cloning, totipotency is retained by the zygote equivalent to the so-called clonote created as a result of transferring the nucleus of a somatic cell to the enucleated oocyte [50, 51].

As mentioned above, the developing blastocyst contains inside a group of stem cells that are called embryonic stem cells (ESCs) and are equipped with pluripotent differentiation potential and are also known as pluripotent stem cells (PSCs), as they

Table 1.1 Developmental hierarchy of stem cell compartment

Totipotent stem cells (TSCs)	<i>They give rise to both the body of an embryo and tissues of the placenta. Under normal conditions, totipotent cells are fertilized oocyte (zygote) and initial few blastomeres (up to four cells). Under artificial conditions, totipotency is retained by the zygote equivalent to the so-called clonote created as a result of nuclear transfer of the nucleus of somatic cell to the oocyte</i>
Pluripotent stem cells (PSCs) Multipotent stem cells (MPSCs)	<i>They give rise to cells of all three germ layers. Pluripotent cells are cells of internal mass of blastocyst and cells in the epiblast. They do not contribute anymore to the placenta. These stem cells give rise to one of the germ layers (meso-, ecto-, or endoderm)</i>
Monopotent or tissue-committed stem cells (TCSCs)	<i>They include the so-called tissue-specific cells which give onset to one line of cells. Examples include stem cells of the intestinal epithelium, hematopoietic stem cells, epidermal stem cells, neural stem cells, hepatic stem cells, and stem cells of skeletal muscles</i>

Table 1.2 Contribution of germ layers to postnatal tissues

Ectoderm	The brain, sympathetic ganglions, peripheral nerves, eye, epidermis, skin appendices, pigment cells
Mesoderm	Hemato/lymphopoietic cells, the endothelium, skeletal muscles, heart muscle cells, adipocytes, connective tissues (the bone, tendon, cartilage), smooth muscles, tubule cells of the kidney
Endoderm	The lung, gut, liver, pancreas, thyroid gland

give rise to the cells of all three germ layers (meso-, ecto-, and endoderm) [5–8, 52]. PSCs are also present in developing epiblast that gives rise to the embryo proper. Finally, as mentioned in the introduction, there are germ layer specific multipotent stem cells (MPSCs) that give rise to monopotent or tissue-committed stem cells (TCSCs). TCSCs differentiate into one line of adult cells. Examples include stem cells of the intestinal epithelium, hematopoietic stem cells, epidermal stem cells, neural stem cells, hepatic stem cells, and stem cells of skeletal muscles. Table 1.2 shows examples of tissues derived from MPSCs for ecto-, meso-, and endoderm through their descendant stem cell population—TCSCs. Accordingly, ectoderm MPSCs give rise to TCSCs for the brain, sympathetic ganglions, peripheral nerves, eye, epidermis, skin appendices, and pigment cells; mesodermal MPSCs differentiate into TCSCs for hemato-/lymphopoietic cells, the endothelium, skeletal muscles, heart, adipocytes, connective tissues (the bone, tendon, cartilage), smooth muscles, and tubule cells of the kidney; and finally endodermal MPSCs contribute to TCSCs for the liver, pancreas, lung, gut, and thyroid gland [9, 14, 15, 53].

As mentioned above, it has been postulated that in addition to TCSCs, adult tissues may also contain some development of early PSCs/MPSCs in quiescent state [5, 54]. Several potential candidate stem cells have been described that could differentiate across germ layers. The presence of these cells in adult tissue may explain a decade ago proposed concept of stem cell plasticity, based on the wrong assumption that adult monopotent TCSCs (e.g., hematopoietic stem cells) may trans-differentiate into other TCSCs (e.g., for cardiomyocytes).

In support of the presence of early-development stem cells in postnatal life, several types of putative PSCs or MPSCs have been described and isolated, primarily from hematopoietic tissues that are able to give rise to cells from more than one germ layer. Most likely, they represent overlapping populations of early-development stem cells that, depending on isolation strategy, *ex vivo* expansion protocol, and markers employed for their identification and characterization, have been given different names, for example, multipotent adult stem cells (MASCs) [55], multilineage-differentiating stress-enduring (Muse) cells [56], multipotent adult progenitor cells (MAPCs) [57, 58], unrestricted somatic stem cells (USSCs) [59], marrow-isolated adult multilineage inducible (MIAMI) cells [60], multipotent progenitor cells (MPCs) [61], omnicytes [62], spore-like stem cells [63], and elutriation-, lin-, after BM homing-derived stem cells (ELHs) [64–66]. The presence of PSCs/MPSCs in adult tissues can be explained by the possibility that during early embryogenesis, not all of the earliest-development stem cells disappear from the embryo after giving rise to TCSCs, but some may have survived in developing organs as a dormant backup population of more primitive stem cells. Most likely, such population of PSCs corresponds to the recently discovered very small embryonic-like stem cells (VSELs) [13, 67, 68].

The presence of VSELs that can differentiate into cells from more than one germ layer, in adult tissues including BM, have been currently confirmed by at least 25 independent laboratories [13, 67, 69–74]. VSELs are small cells, with their size corresponding to the cells in the inner cell mass of the blastocyst, and, depending on the measurement conditions (in suspension or after adhesion to slides), they measure ~3–5 μm in mice and ~5–7 μm in humans. Thus, they are slightly smaller than red blood cells and require a special gating strategy during fluorescence-activated cell sorting (FACS) [13]. Transmission electron microscopy analysis revealed that VSELs have large nuclei containing euchromatin and a thin rim of the cytoplasm enriched in spherical mitochondria, which are characteristic of early-development cells, e.g., primordial germ cells (PGCs). They also express several genes characteristic for pluripotent/multipotent stem cells such as stage-specific antigen (SSEA), Oct-4, Nanog and Rex-1, and highly expressed Rif-1 telomerase protein. Studies performed on highly purified double-sorted VSELs isolated from murine BM revealed that these cells highly express mRNA and proteins (e.g., Stella, Fragilis, Blimp1, Nanos3, Prdm14, and Dnd1) characteristic for late migratory PGCs (e.g., Dppa2, Dppa4, and Mvh) [13, 68, 75]. VSELs could give rise to monopotent TCSCs and have great potential to be involved in tissue/organ rejuvenation and in organ regeneration following organ injury [73, 76–81].

According to widely accepted stem cell definition, TCSCs possess the ability for self-renewal and may differentiate into progenitors for adult tissue residing cells [5, 9, 10, 82]. Progenitor cells cannot any more self-renew, but they are able to differentiate into functional somatic cells. In order to fulfill this mission, TCSCs can be programmed to undergo asymmetric divisions where one of daughter cells retains stem cell potential and the other becomes progenitor cell. This mechanism of asymmetric cell division is required to keep constant number of HSC in hematopoietic organs and prevent their depletion thus maintaining the homeostatic balance [83, 84]. Another feature of most TCSCs is their quiescent state, except for stem cells

of the intestinal epithelium, epidermis, and hematopoiesis. TCSCs usually show some level of resistance to radio-chemotherapy and cytostatic drugs. They also possess some characteristic morphology having high nuclear/cytoplasmic ratio.

These unique properties of TCSCs make them candidates for two important clinical applications. As mentioned above and shown in Fig. 1.1, they could be directly employed in clinical settings to regenerate damaged tissues and improve the function of the affected organs [1]. These applications would require their isolation and *ex vivo* expansion followed by systemic or local delivery. The main obstacle with TCSCs therapies for damage of solid organs is as mentioned above that in humans, there is no concrete evidence for significant contribution of infused or locally injected TCSCs in improving damaged organ parenchyma. The only beneficial so far well-demonstrated effects are credited to the release of soluble trophic factors and ExMV_s that may indirectly improve the function of damaged organs [39, 40, 85, 86].

Second, an even more important aspect of regenerative medicine is to increase stem cell robustness and regenerative potential directly *in vivo* in adult organisms by therapeutic means, including (i) regular physical activity, (ii) caloric restriction, and finally (iii) stem cell-targeted pharmacological interventions [87–91].

However, this second preventive aspect of clinical regenerative medicine, in contrast to stem cell applications as therapeutics in emergency situations, is still somewhat underappreciated. In particular, this area awaits the development of more specific drugs, in addition to already employed such as metformin, berberine, nicotinamide, or AMP-activated protein kinase (AMPK) activators that would increase robustness of TCSCs. This ambitious task provides a challenge to the development of stem cell-tailored pharmacology.

1.3 Therapeutic Application of Stem Cells Isolated from Adult Tissues

Despite the hype created by the media about ESCs and iPSCs, clinical data shows that TCSCs are the only cells to be employed safely in regenerative medicine so far. Several types of TCSCs have been employed to treat and regenerate organs in cardiology, neurology, dermatology, gastroenterology, ophthalmology, and orthopedics [27–32, 92]. The most commonly used stem cells from adult tissues are those isolated from bone marrow (BM), umbilical cord blood (UCB), mobilized peripheral blood (mPB), adipose tissue, skin epithelium, and rarely from the myocardium and skeletal muscle biopsies. However, despite promising animal data and safety, in hematological applications of BM-, mPB-, or UCB-derived TCSCs, the clinical efficacy of these TCSCs in other areas is still not satisfactory [32].

Unfortunately, in contrast to animal models, there is no solid and reproducible evidence in humans—despite several clinical trials—that these cells (except hematopoietic transplants) contribute to generating functional cells in damaged organs. In fact, the beneficial therapeutic effects of stem cells delivered to various

tissues or organ during therapy are mostly related to their paracrine effects [33]. To explain this finding, it is well known that TCSCs currently employed in therapies, as mentioned before, are rich source of growth factors, cytokines, chemokines, and bioactive lipids that have (i) trophic, (ii) antiapoptotic, and (iii) pro-angiopoietic effects [38, 40, 93, 94]. All of these factors have beneficial effects on damaged tissues. Moreover, in addition to soluble factors, stem cells also release membrane-derived extracellular microvesicles (ExMV), ranging in size from 100 nm to 1 μ m in diameter, which may deliver mRNA, miRNA, and functional proteins to target cells, thereby additionally promoting cell survival and proliferation. Smaller ExMVs are also known as exosomes. Accumulated evidence suggests that all these paracrine effects mediated by (i) soluble factors and/or (ii) by ExMVs are major factors responsible for the positive results observed in patients after systemic or local stem cell therapies [39, 40, 95, 96].

The best examples of the paracrine effects of stem cell therapies involve TCSCs for mesenchyme—mesenchymal stromal cells (MSCs) isolated from bone marrow, adipose tissues, umbilical cord, or umbilical cord Wharton jelly. In the literature these cells for mesenchyme have been wrongly termed “mesenchymal stem cells,” as only a very low percentage of these cells have the properties required for clonal growth and are real progenitors of connective tissue and the bulk of these cells derived from expansion cultures are merely differentiated fibroblasts [97]. MSCs are safe for clinical applications, easy to isolate, and grow *in vitro* [98–101]. However, it is now well known that their beneficial effects are transient and mainly due to the release of soluble paracrine factors and ExMVs [95, 102].

1.4 The Search for Other Alternative Sources of PSCs for Potential Therapeutic Applications in Regenerative Medicine

The field of regenerative medicine is still searching for ethically acceptable and efficient stem cells that could be employed for therapy. The ideal stem cells for application in regenerative medicine are PSCs that, according to their definition, give rise to cells from all three germ layers (meso-, ecto-, and endoderm) [5, 103]. PSCs hold great promise in biomedical fields as they can serve as unlimited cell source and their pluripotent differentiation potential enables to generate any desired cell type *in vitro*; Table 1.3 shows *in vitro* and *in vivo* criteria that are expected from PSCs. *In vitro* PSCs criteria include (i) undifferentiated morphology, euchromatin, and high nuclear/cytoplasm ratio; (ii) expression of PSCs markers (e.g., Oct-4, Nanog, SSEA), the presence of bivalent domains, and female PSCs reactivate X chromosome, and (iii) their ability for multilineage differentiation into cells from all three germ layers (meso-, ecto-, and endoderm). *In vivo* PSCs criteria include (i) complementation of blastocyst development and (ii) teratoma formation. The following are the currently available PSCs that have been proposed to be employed in the clinic:

Table 1.3 In vitro and in vivo criteria for stem cell pluripotency

In vitro PSC criteria
Undifferentiated morphology, high nuclear/cytoplasm ratio, euchromatin
Markers of pluripotency (e.g., Oct-4, Nanog, SSEA), the presence of bivalent domains, female PSCs reactivate X chromosome
Multilineage differentiation into cells from all three germ layers (meso-, ecto-, and endoderm)
In vivo PSC criteria
Ability to complement blastocyst development
Teratoma formation

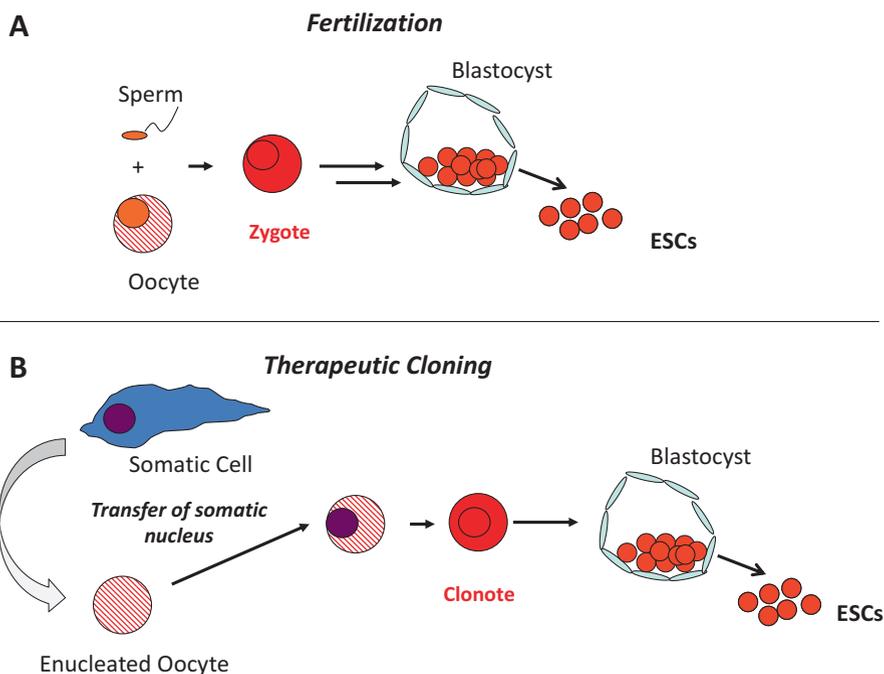


Fig. 1.2 Embryonic stem cells (ESCs) obtained from embryos by fertilization of the oocyte by sperm (a) or after nuclear transfer of a somatic nucleus into an enucleated oocyte (b). *Panel A.* ESCs isolated from blastocysts derived from an oocyte fertilized by sperm (a zygote). *Panel B.* ESCs can also be obtained by means of therapeutic cloning as the result of transfer of the nucleus from an adult somatic cell (e.g., the nucleus of a fibroblast) into an enucleated oocyte. A totipotent stem cell generated by this strategy is called a clonote, which, like a zygote, gives rise to a blastocyst. In both cases, stem cells isolated from inner cell mass of blastocyst are pluripotent

Embryonic stem cells (ESCs) These immortalized cells are derived from early embryos at the blastula stage (Fig. 1.2) [52, 104]. They can be generated from the blastula following the physiological process of fertilization (Fig. 1.2a) or derived by employing a nuclear transfer strategy (Fig. 1.2b). The blastula is an early embryonic stage which contains a group of PSCs that are close to animal pole inside its cavity that have the ability to differentiate into stem cells for all of the germ layers. These

cells can be isolated and under appropriate cell culture conditions become immortalized to proliferate as an established ESCs line. This process, however, is not so easy and to obtain PSCs from inner cell mass of blastocyst still encounters technical problems [52, 105]. Figure 1.2a shows ESCs derived from blastocysts, generated by the physiological process of fertilization. These cells can be obtained from unused embryos that are stored in liquid nitrogen at in vitro fertilization clinics. Unfortunately, generation of immortalized ESCs requires that the blastocyst be destroyed, and this strategy has been questioned from an ethical point of view [106, 107]. Besides ethical considerations, the problem with such ESCs is that they give rise to differentiated cells that have a unique combination of histocompatibility genes inherited from the sperm and egg and would be rejected by a histoincompatible recipient [105, 108, 109]. This leaves the problem of finding a matched donor for the recipient of such cells. On the other hand, ESCs are difficult to control, as they may grow teratomas, and it is still a problem to obtain fully functional differentiated somatic cells from them [110, 111]. So far, for example, fully functional hematopoietic stem cells have not yet been generated from ESCs.

Another strategy is to create immortalized ESCs lines from fertilized oocytes, which is therapeutic cloning. The first step in this strategy requires the insertion of a donor patient-derived nucleus isolated from a somatic cell into an enucleated oocyte derived from an ovulating female (Fig. 1.2b) [50, 51, 112]. The patient-derived “bare” nucleus isolated from a differentiated somatic cell, when inserted into the cytoplasm of an enucleated oocyte, is dedifferentiated by the plethora of enzymes, proteins, mRNAs, and miRNAs present in the oocyte cytoplasm to a state mimicking the nucleus in ESCs [50, 51]. Such artificially created stem cells (called, in contrast to the physiologically fertilized oocyte zygote, a clonote) have the potential for development into a blastocyst and can be employed to obtain ESCs lines from the inner cell mass of the blastocyst. Important to note is that, since the nucleus of the patient donor cell encodes all of the histocompatibility genes, it is possible to create “tailored” ESCs that potentially would not be rejected by the patient’s immune system. Nevertheless, there are serious ethical concerns with this method, as well as the inherent risk of karyotypic abnormalities associated with proliferative advantage or teratoma formation, that are similar to those raised for ESCs isolated from blastocysts obtained by physiological fertilization. As a result of these ethical concerns and technical obstacles, no further progress has been made, and this potential source of pluripotent stem cells has been abandoned [106, 107, 110–113]. This strategy of using embryos created by nuclear transfer implanted into the uterus may also lead to the so-called reproductive cloning, which was employed, for example, in cloning Dolly the sheep. Therapeutic cloning can also be made to work in humans, as recently demonstrated, but it poses a great threat and creates a serious ethical danger surrounding any attempts to perform reproductive cloning in human beings.

Induced pluripotent stem cells (iPSCs) As mentioned above, strategies to obtain PSCs using human oocytes to create embryos have become highly controversial from an ethical point of view. Therefore, an alternative strategy has been developed to obtain cells with multigerm layer differentiation potential by ex vivo induction

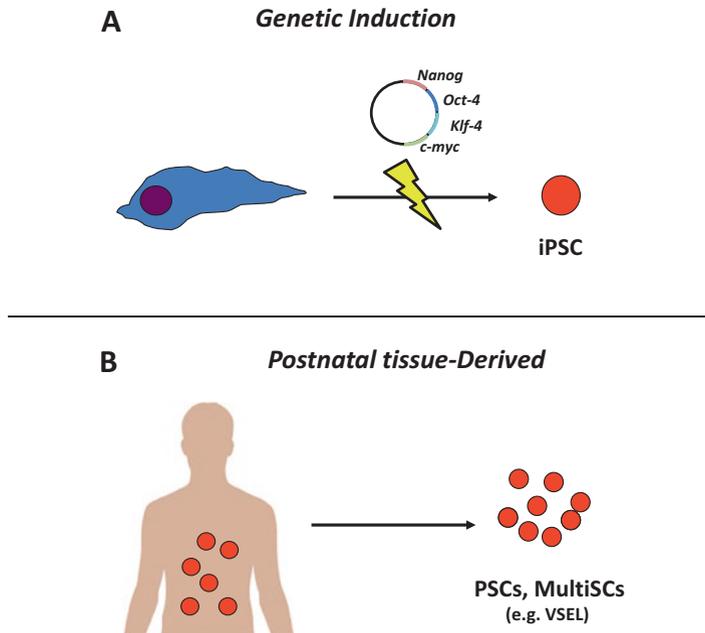


Fig. 1.3 Pluripotent stem cells obtained from postnatal tissues by genetic modification of adult cells (a) or pluripotent/multipotent stem cells isolated from postnatal tissues (b). *Panel A.* PSCs can be obtained by transforming somatic cells (e.g., fibroblasts) using genes that encode embryonic transcription factors (e.g., Oct-4, Nanog, Klf-4, and c-Myc). There are also alternative strategies that replace DNA with mRNA, proteins, or regulatory miRNA. *Panel B.* PSCs can also be obtained from the tissues of mature individuals (e.g., very small embryonic-like stem cells, also known as VSELs). With advances in expansion strategy, these cells what we believe could soon become real game changers in regenerative medicine

(transformation) of postnatal adult somatic cells into the embryonic stem cell state (Fig. 1.3a). The target population of cells used for this reprogramming is isolated from normal adult tissues (e.g., skin fibroblasts). Stem cells generated by genetic reprogramming (transformation) of adult somatic cells are called as induced pluripotent stem cells (iPSCs) and can differentiate into a wide spectrum of tissues [114, 115]. The first iPSCs were discovered in 2006 by Dr. Shinya Yamanaka who was awarded the Nobel Prize in Physiology and Medicine in 2012 [115]. The initial strategy for obtaining iPSCs was based on the transduction of somatic mouse fibroblasts with a set of four genes called as “Yamanaka factors” (Oct3/4, Sox2, c-Myc, Klf4), which encode transcription factors governing pluripotency and the resulting proliferation of embryonic cells. Most importantly this technology allowed PSCs to be obtained that are histocompatible with the initial donor cell used for reprogramming. Unfortunately, several limitations have been identified for these cells, including the risk of teratoma and cancer formation that may be attributed to the two Yamanaka—factors c-Myc and Klf-4—that are potent oncogenes [116–118]. Recent evidence indicates that iPSCs have a significant problem with (i) genomic

dysregulation, transcriptional, and epigenetic instability [117, 119–122]; (ii) the risk of insertional mutagenesis [123, 124]; (iii) the immune response, even to autologous iPSCs [125]; (iv) variability in differentiation capacity [1, 126]; and (v) significant variability among iPSC clones derived from the same donor cells [1, 127]. These limitations explain why the first clinical trials using these cells, which were regarded as “promising” cells, were suspended [43, 119, 128, 129]. The recently published report where iPSC-derived cardiomyocytes were employed in the clinic concluded that some weak beneficial effects were observed due to paracrine effects of these cells.

In order to overcome the abovementioned obstacles, some strategies to mitigate the risk of therapies using iPSCs have been proposed. The risk of mutagenesis in iPSCs is based on the fact that the genes employed for the induction of pluripotency (Oct3/4, Sox2, c-Myc, Klf4) integrate randomly into the chromosomes of manipulated cells and, if incorporated into chromosome “hot spots,” could trigger the activation of oncogenes or inactivate tumor suppressor genes by insertional mutagenesis [123]. To mitigate this possibility, several alternative strategies have been proposed, such as (i) employing non-integrating DNA plasmids [130–133], (ii) replacing DNA sequences with mRNA or miRNA [134–137], and (iii) employing protein products in the form of cell-penetrating Oct3/4, Sox2, c-Myc, and Klf4 proteins instead of the genomic DNA itself and even employing small molecules that modify the DNA structure of target cells and induce the pluripotent state [138–140]. Other strategies to mitigate the potential risks of iPSC therapy include (i) the use of suicide genes to eliminate any remaining undifferentiated and highly proliferative iPSCs from the recipient’s body after therapy [141, 142]; (ii) the selection of a proper source of cells that are free of mutation prior to immortalization by transduction [143]; (iii) employing better gene delivery methods for reprogramming, such as using non-integrating vectors, Sendai virus, or episomal plasmid vectors [144]; and (iv) requiring a lower passage number for iPSCs, since mutations may accumulate in cells during passaging [145]. It will be important to scan the behavior of these reprogrammed cells in cell culture and their performance in clinical studies.

Based on these concerns, until significant progress is achieved in increasing the clinical safety of iPSCs, these cells can serve only as experimental models to study cell differentiation processes or as tools to identify the genes responsible for the origin of certain disorders. However, even in this potential setting, we have to cautiously evaluate the data obtained using iPSCs because of their genomic instability and variability.

Potential PSCs isolated from adult tissues Evidence has accumulated as mentioned above that adult tissues harbor a population of very rare stem cells with pluripotent stem cell characteristics that express early-development embryonic markers. These cells were named as VSELs [13, 146] (Fig. 1.3b). VSELs are small cells, corresponding in size to the cells in the inner cell mass of the blastocyst, and, depending on the measurement conditions (in suspension or after adhesion to slides), they measure ~3–5 μm in mice and ~5–7 μm in humans. Transmission electron microscopy analysis revealed that they have large nuclei containing euchromatin

and a thin rim of the cytoplasm enriched in spherical mitochondria, which are characteristic of early-development cells. Evidence accumulated that VSELs originate from cells related to the germ line, are deposited in developing organs during embryogenesis, and play a role as a backup population for multipotent TCSCs [147]. VSELs are quiescent but are activated during stress situations and mobilized into the circulation [76]. The number of these cells decreases with age. Overall, the presence of these early-development cells in postnatal tissues challenges the accepted hierarchy within the adult stem cell compartment in bone marrow. VSELs express some embryonic stem cell markers, such as stage-specific antigen (SSEA), nuclear Oct-4A, Nanog, and Rex1 [68]. The true expression of these genes has been confirmed by the open structure of chromatin in their respective promoters, by their association with histones promoting transcription, and by the sequencing of RT-PCR products. VSELs also express several markers characteristic of migrating primordial germ cells (PGCs), such as Stella and Fragilis. Our single-cell cDNA libraries revealed that the gene expression profile in murine BM-isolated VSELs, sorted as very small Sca-1⁺lin⁻CD45⁻ cells, varies [71]. VSELs residing in adult tissues are highly quiescent due to the erasure of regulatory sequences for certain paternally imprinted genes (e.g., at the Igf2–H19 locus) and thereby protected from insulin/insulin-like growth factor stimulation [71]. They also express bivalent domains at genes encoding transcription factors in the homeobox family. Recent proteomic data have confirmed that genes involved in proliferation and cell signaling are expressed in VSELs at a low level and become upregulated during their expansion.

Moreover, evidence has accumulated that VSELs are at the top of the stem cell hierarchy in normal bone marrow, giving rise to HSCs, MSCs, and endothelial progenitor cells (EPCs). VSELs expand *in vivo* in response to stimulation by pituitary gonadotropins and gonadal sex hormones, which, from a developmental point of view, further links these cells to migrating PGCs. It has been convincingly demonstrated that VSELs can be isolated from the ovarian surface epithelium of young and postmenopausal women as well as from the testes. Recently, it has been reported that ovary-isolated VSELs differentiate into oocyte-like cells in response to sperm cells and release the zona pellucida, which is the first step in the fertilization process [71]. This differentiation of ovary- or testes-derived VSELs into gametes will be discussed in another chapter in this book.

The number of VSELs correlates with longevity in certain long-living murine strains. Their number can be increased in experimental animals by caloric restriction, regular exercise, and administration of DNA modifiers, such as nicotinamide or valproic acid [71]. By contrast, the exposure of animals to increased insulin/insulin-like growth factor signaling leads to premature aging and depletion of VSELs from the tissues. Several papers have been published showing the contribution of injected purified VSELs in hematopoiesis, osteogenesis, and angiogenesis as well as to the myocardium, liver, and pulmonary alveolar epithelium in appropriate *in vivo* models. The well-demonstrated presence of chimerism in several organs indicates the potential of these cells to differentiate across germ layers. The most important breakthrough in the potential application of VSELs came with

the development of more efficient *ex vivo* expansion strategies for these rare cells. VSELs can now be expanded *ex vivo* in the presence of nicotinamide or valproic acid or in the presence of the small-molecule UM177. These modifications do not require transduction of VSELs by DNA or RNA or employing supportive third-party feeder layer cells. To explain our expansion approach, both of the small molecules employed in our expansion medium, nicotinamide and valproic acid, are inhibitors of the histone deacetylase sirtuin (Sirt-1). It turned out that Sirt-1 inhibits the activity of the *de novo* DNA methyltransferase 3-like (DNMT3L), which is crucial for methylation of the regulatory regions of paternally imprinted genes. These loci as mentioned above are demethylated (erased) during early embryogenesis in VSELs, as they are in PGCs migrating to the genital ridges. These epigenetic changes explain why PGCs and VSELs are so quiescent and cannot complement blastocyst development, and, what is even more important, these cells do not grow teratomas, despite their pluripotency. The fact that Sirt-1 maintains a low intracellular level of DNMT3L explains why it has beneficial effects on longevity by preventing premature depletion of VSELs from adult tissues. By contrast, downregulation of Sirt-1 by nicotinamide or valproic acid in culture promotes *ex vivo* expansion of these cells [71].

Mounting evidence from independent laboratories should encourage other investigators to study this promising population of cells isolated from adult tissues, which is being facilitated recently by *ex vivo* expansion approaches. Importantly, VSELs in our hands during expansion undergo asymmetric divisions, which is a crucial feature of primitive stem cells. As reported we are able to expand VSELs up to 3000 folds in Dulbecco's medium supplemented with artificial "knock-out" serum in the presence of Sirt-1 inhibitors nicotinamide or valproic acid. We facilitate this process by a cocktail of follicle-stimulating hormone (FSH), luteinizing hormone (LH), bone morphogenetic protein (BMP-4), and kit ligand (KL). Expanded *ex vivo* cells contain as result of asymmetric divisions small VSEL-like cells and also more differentiated cells. An open question remains if VSELs-expanded cells will fully differentiate and integrate with other cells in the damaged tissues. It is also important to prove that they can reestablish a three-dimensional fully functional tissue structures, which will be crucial to justify their potential application in the clinic. In addition, since almost all VSELs studies so far have been performed with cells isolated from hematopoietic tissues, one can ask whether VSELs purified from other non-hematopoietic sources have the same properties and can differentiate into cells from all three germ layers.

However, although our preliminary data show that they do not grow teratoma in immunocompromised mice, some further deep sequencing analysis is needed to evaluate the genomic stability of VSELs-derived cells after current expansion strategies employing small molecular DNA-modifying agents [1]. While VSELs isolated from adult tissues and expanded *ex vivo* could be employed to regenerate damaged organs, another experimental approach would be to develop, in parallel, strategies to maintain the pool of VSELs residing in adult tissues. This goal provides a challenge for modern pharmacology: to develop drugs that protect VSELs from insulin/insulin-like

growth factor signaling. Metformin, which is currently employed to modulate insulin signaling and increases longevity, or in particular rapamycin that is m-TOR signaling inhibitor has, unfortunately, several side effects.

1.5 Conclusion

There is no doubt that stem cell therapies are the future of clinical medicine. However, news stories published by nonprofessional media predicting that clinical applications for a variety of medical problems will soon be available to foster premature and often unrealistic expectations in the public. Serious problems hampering progress in the field include patent issues and the financial involvement of biotechnology companies, which are frequently driven by competition, at the expense of cooperation.

The ethical concerns that have emerged around stem cells isolated from human embryos are somewhat muted, as these cells will not be employed in the clinic in the foreseeable future because of the risk of teratoma formation and genomic instability. Similarly, problems have emerged with iPSCs. In this chapter, we have tried to cool overheated expectations for the clinical application of stem cells isolated from the embryos as well as iPSCs. On the other hand, the identification of developmentally primitive VSELs residing in adult tissues and promising evidence that these cells can be isolated and expanded *ex vivo* opens the door to a new chapter in regenerative medicine. However, although there is no evidence so far that these cells form teratomas, it remains crucial to assess genomic stability of VSELs-expanded cells. It is also necessary to perform appropriate cell tracking studies. We propose that pluripotent VSELs isolated from adult tissues should be studied further by independent groups in solid organ injury models, as they may enlighten a path forward that solves several problems with the use of controversial ESCs and iPSCs in regenerative medicine.

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Chapter 2

Genomic Instability of iPSCs and Challenges in Their Clinical Applications



Masahito Yoshihara, Akiko Oguchi, and Yasuhiro Murakawa

Abstract Generation of human-induced pluripotent stem cells (iPSCs) from somatic cells has opened the possibility to design novel therapeutic approaches. In 2014, the first-in-human clinical trial of iPSC-based therapy was conducted. However, the transplantation for the second patient was discontinued at least in part due to genetic aberrations detected in iPSCs. Moreover, many studies have reported genetic aberrations in iPSCs with the rapid progress in genomic technologies. The presence of genomic instability raises serious safety concerns and can hamper the advancement of iPSC-based therapies. Here, we summarize our current knowledge on genomic instability of iPSCs and challenges in their clinical applications. In view of the recent expansion of stem cell therapies, it is crucial to gain deeper mechanistic insights into the genetic aberrations, ranging from chromosomal aberrations, copy number variations to point mutations. On the basis of their origin, these genetic aberrations in iPSCs can be classified as (i) preexisting mutations in parental somatic cells, (ii) reprogramming-induced mutations, and (iii) mutations that arise during in vitro culture. However, it is still unknown whether these genetic aberrations in iPSCs can be an actual risk factor for adverse effects. Intersection of the genomic data on iPSCs with the patients' clinical follow-up data will help to produce evidence-based criteria for clinical application. Furthermore, we discuss novel approaches to generate iPSCs with fewer genetic aberrations. Better understanding of iPSCs from both basic and clinical aspects will pave the way for iPSC-based therapies.

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2.1 Background: Why We Need to Study Genomic Instability

Yamanaka and his colleagues succeeded in reprogramming somatic cells into iPSCs by introducing sets of key transcription factors [1, 2]. The use of iPSCs has solved the ethical issue of using embryonic stem cells (ESCs) because iPSCs are generated without destroying pre-implantation-stage embryos. iPSC technologies have enabled the creation of patient-matched pluripotent stem cells that can be propagated and differentiated into desired cell types. iPSCs and ESCs have enormous promise for disease modeling, pharmacological screening, and regenerative medicine (reviewed in [3, 4]). Cells differentiated from patient-derived iPSCs are unlikely to be rejected by the immune system after transplantation and can be used for autologous stem cell transplantation [5–7]. Banks of human leukocyte antigen (HLA)-homozygous iPSC lines are good cell sources for allogeneic iPSC-based therapies [8–10]. Indeed, iPSC-based therapies are beginning to be used to treat patients with intractable diseases [11–15].

The first-in-human clinical trial of iPSC-based cell therapy was conducted by Takahashi and her colleagues. In September 2014, a patient with exudative age-related macular degeneration (AMD) was transplanted with a retinal pigment epithelial (RPE) cell sheet, which was differentiated from iPSCs generated from her own skin fibroblasts [11–13]. At 1 year after transplantation, the transplanted RPE cell sheet remained intact, without serious adverse effects. The second patient was enrolled in 2014; however, he did not undergo transplantation because genetic alterations were observed in the iPSCs and iPSC-derived RPE cells. Three alterations of DNA copy number were identified: loss of heterozygosity in the *YAF2*-coding region of chromosome 12, loss of heterozygosity with two small homozygous deletions in the *SNRPN*-coding region of chromosome 15, and loss of the *STS*-coding region of chromosome X [13]. Although the iPSC-derived RPE cells passed the *in vivo* tumorigenicity test based on their injection into the subretinal area of five nude rats, the transplantation surgery for the second patient was cancelled because of concerns about the unpredictable effects of the genomic deletions (Fig. 2.1) [13, 16].

This result shows that the presence of genetic aberrations in iPSCs raises serious safety concerns and can hamper the advancement of iPSC-based therapies, although it remains elusive whether these genetic aberrations can be an actual risk factor for adverse effects. To circumvent this problem, it is crucial to gain deeper insights into the genomic aberrations observed in iPSCs (Fig. 2.2) [17–20]. Next, we overview the characteristics of mutations in iPSCs, with a particular focus on their origins and mechanisms of the onset.

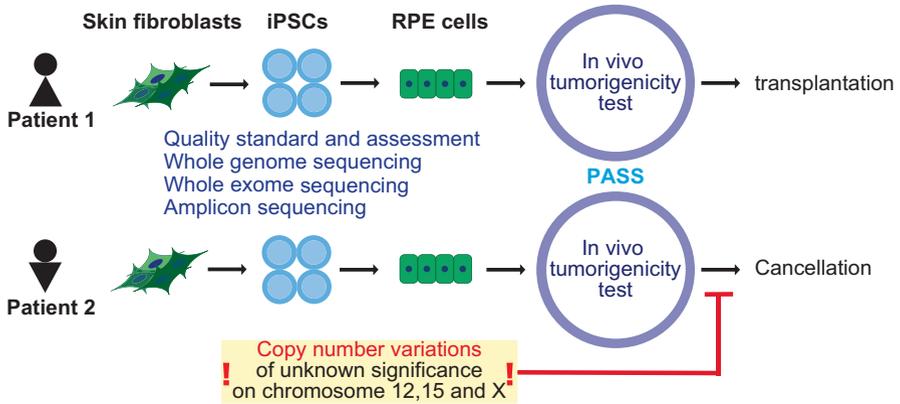


Fig. 2.1 Outline of the first-in-human clinical trial of iPSC-based cell therapy. Transplantation for patient 2 was cancelled because of genetic aberrations detected in the iPSCs

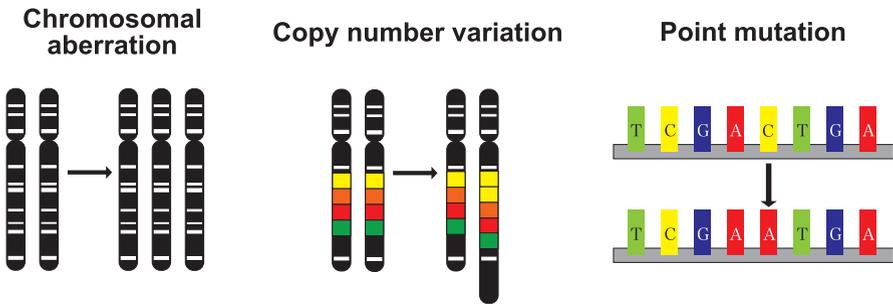


Fig. 2.2 Types of genetic aberrations observed in iPSCs

2.2 Genomic Aberrations in iPSCs

2.2.1 Chromosomal Aberrations

Many different methods are available to detect genetic aberrations. The most common and traditional method is G-banding, which can detect numerical (aneuploidy and polyploidy) or large structural chromosomal changes, including translocations and inversions [21].

Chromosomal instability of human iPSCs was first reported by Mayshar et al. in 2010 [22]. A large-scale study [23] and several individual studies [22, 24, 25] showed that trisomy 12 is the most frequently observed chromosomal aberration in human iPSCs and accounts for about 30% of cases of iPSC aneuploidy [23]. Because chromosome 12 is rich in cell cycle-related genes and carries the pluripotency-related gene *NANOG* [22], trisomy 12 might confer the selective proliferative and reprogramming advantage in pluripotent stem cells. The gain of 12p is a characteristic

of testicular germ cell tumors [26, 27]. Other types of chromosomal aneuploidy frequently observed in human iPSCs are trisomies 8 and 20q [23]. In iPSCs, accumulation of chromosomal aneuploidy is frequently found after prolonged in vitro culture [28].

Trisomy 12 is also the predominant abnormality in human ESCs, accounting for about 40% of total aberrations; other typical chromosomal aneuploidies in human ESCs are found in chromosomes 1, 17, 20, and X [29–31]. This pattern differs from that of the most common aneuploidies seen at live births (autosomes 13, 18, 21, and sex chromosomes) and spontaneous abortions (autosomes 4, 7, 13, 15, 16, 21, and 22) (reviewed in [32]).

2.2.2 Copy Number Variations

Array-based technologies including comparative genomic hybridization [33] and single nucleotide polymorphism (SNP) arrays [34] have been adopted to achieve high resolution. These approaches allow the assessment of copy number variations (CNVs; i.e., duplications and deletions) across the whole genome at kilobase resolution (reviewed in [35]).

Chin et al. performed the first CNV analysis of three human iPSC lines using array comparative genomic hybridization. They found a few CNVs in each iPSC line, yet none of them were shared between iPSC lines [36]. A larger-scale study using 32 human iPSC lines found several recurrent CNVs in human iPSCs [24]. Recurrent CNVs at 1q31.3 and 17q21.1 are uniquely identified in human iPSCs (>25% of human iPSC samples). The loss of 8q24.3 is also unique to human iPSCs (12%). Duplications of 20q11.21 (18%) and 2p11.2 (>25%) are often detected in human iPSCs [24], which are also recurrently acquired in human ESCs [37]. A more recent study described the systematic genotyping and phenotyping of 711 human iPSC lines, identifying 35 regions where CNVs are significantly enriched including trisomy X and duplications of the chromosome 17 and 20 [38]. Amplification of 20q11.21, one of the most recurrent CNVs in human iPSCs [39], is also frequently found in cancer [40, 41]. This region is rich in genes linked to pluripotency and anti-apoptosis, such as those encoding DNA methyltransferase 3B (*DNMT3B*), inhibitor of DNA binding 1 (*ID1*), and BCL2-like1 (*BCL2L1*).

Interestingly, at least half of the CNVs observed in iPSCs were found to be derived from low-frequency somatic alterations in the parental skin fibroblasts using whole genome sequencing (WGS) analysis [42]. Next-generation sequencing (NGS) technologies enabled sensitive detection of such low-frequency variations in the parental cells, providing deeper insights into the origin of genomic instability (reviewed in [43]).

Laurent et al. [25] and Hussein et al. [44] analyzed the dynamic changes of CNVs during reprogramming and time in culture using SNP arrays. Laurent et al. reported that deletions of tumor-suppressor genes tend to occur with reprogramming, whereas duplications of tumor-promoting genes accumulate with passaging [25]. Hussein et al. identified a large number of CNVs in early-passage human

iPSCs, and the number decreased during cell propagation [44]. They also showed that most CNVs are formed de novo and generate genetic mosaicism in early-passage human iPSCs; this mosaicism then gradually disappears during cell passaging as a result of selective pressure [44].

2.2.3 Point Mutations

Point mutations observed in iPSCs have been studied using NGS technology [45]. Unlike conventional methods, NGS can detect low-frequency mutations [46]. Whole exome sequencing (WES) selectively sequences protein-coding regions (~1% of the human genome), whereas WGS interrogates the entire genome. Recent reduction in the cost of sequencing has enabled investigation of the iPSC genome at an affordable cost in a high-throughput manner.

On average, a human iPSC line has ~10 mutations in the protein-coding regions [47–53] and hundreds to thousands of mutations in the whole genome [47, 52, 54–59]. On the basis of their origin, point mutations in iPSCs can be classified as (i) preexisting mutations in parental somatic cells, (ii) reprogramming-induced mutations, and (iii) mutations that arise during in vitro culture (Fig. 2.3).

2.2.3.1 Preexisting Mutations

Many iPSC mutations were already present in parental somatic cells [47, 48, 53, 54, 60–62]. Mutations exist in a minority of parental cells and become detectable in the iPSC genome after cloning during iPSC generation. These mutations are present in

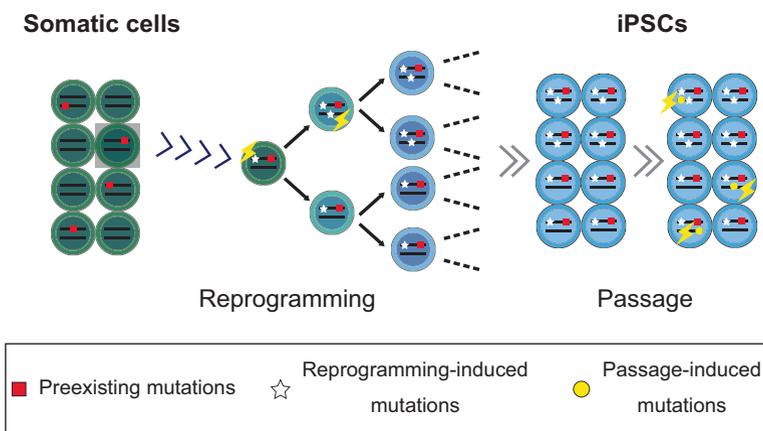


Fig. 2.3 Origin of genetic aberrations in iPSCs. On the basis of their origin, genetic aberrations can be classified as (i) preexisting mutations in parental somatic cells, (ii) reprogramming-induced mutations, and (iii) mutations that occur during in vitro culture

one allele of all the iPSCs (i.e., ~50% allele frequency in the NGS data). However, it is sometimes difficult to conclude that mutations in the iPSC genome were present in the parental cells. Ultra-deep sequencing can be applied to identify preexisting alterations, which exist at low frequencies in parental somatic cells [42, 48, 60]. However, alterations in parental cells may still exist at undetectably low frequencies. In one WGS study of mouse iPSCs, 157 shared point mutations were identified in four iPSC clones established from the same embryonic fibroblasts; this strongly suggests that these point mutations were derived from parental cells [55]. A recent genomic study of human iPSCs derived from skin fibroblasts showed that ~50% of the iPSC clones harbor mutations due to ultraviolet-induced damage, suggesting that they existed in the parental cell [58]. These mutations are characterized by over-representation of C-to-T or CC-to-TT transitions [58] and are often observed in melanoma [63–65].

Preexisting mutations may be just randomly captured and expanded during iPSC generation, or alternatively some of them may facilitate the generation or proliferation of iPSCs and be preferentially propagated because of selective advantage [43].

2.2.3.2 Reprogramming-Induced Mutations

Ji et al. argued that a large fraction of the point mutations is induced during human iPSC reprogramming [49]. More recently, Sugiura et al. provided multiple lines of evidence that hundreds of point mutations occur just after the onset of reprogramming [56]. They generated iPSCs from mouse embryonic fibroblasts to minimize preexisting mutations, which accumulate as the parental cells age and performed a WGS analysis. They established ESCs and iPSCs under nearly identical conditions and demonstrated that iPSCs had many more point mutations than ESCs [56]. WGS on human iPSCs derived from monoclonal somatic cells identified subclonal mutations that were unique to each iPSC line and thus were acquired during reprogramming [52].

Reprogramming-induced mutations occur immediately after the onset of iPSC reprogramming before the first cell division or after the first or second cell division [56]. Accordingly, these mutations are expected to be observed at ~50%, ~25%, and ~12.5% allele frequencies, respectively [56]. Point mutations with allele frequencies of ~50% can be either preexisting or reprogramming induced, but reprogramming-induced mutations show a transversion-dominant pattern, whereas preexisting mutations show a transition-dominant pattern [56]. Yoshihara et al. investigated reprogramming-induced mutations identified by WGS in the context of epigenetic status [66]. They showed that reprogramming-induced mutations exhibit an oxidative stress-induced DNA mutation signature (C-to-A transversions) and preferentially occur in structurally condensed lamina-associated domains (LADs) located at the nuclear periphery. LADs are sensitive to reactive oxygen species released from mitochondria because heterochromatin formation in LADs prevents the access of DNA repair proteins [67–69]. A burst of oxidative phosphorylation at the initial phase of iPSC reprogramming [70] might be the cause of transversion-predominant point mutations introduced during reprogramming.

2.2.3.3 Passage-Induced Mutations

Mutations induced during culture pose a potential risk for the use of iPSCs in regenerative medicine. Gore et al. applied WES to the same human iPSC line at early and late passages and identified four point mutations that occurred during the prolonged culture [48]. Passage-induced mutations occur stochastically and are expected to exhibit much lower allele frequencies than preexisting mutations or reprogramming-induced mutations. Although NGS can detect low-frequency variants, it is sometimes difficult to distinguish them from NGS errors. Recently, Kuijk et al. determined the mutation accumulation rate per population doubling in human iPSCs and adult stem cells during in vitro culture [59]. They also showed that passage-induced mutations are characterized by C-to-A transversions linked to oxidative stress. Physiologic oxygen concentration (2%) reduces chromosomal abnormalities in cultured human ESCs compared with room oxygen (21%) [71]. These observations are valuable for further insights into the optimization of culture conditions.

2.3 Effects of Mutations on the Phenotype of iPSCs

Understanding whether mutations in iPSCs can actually lead to unfavorable outcomes such as malignant outgrowth is crucial for clinical applications. Yet, it is difficult to distinguish “driver” mutations, which confer a proliferative advantage to cancer cells, from “passenger” mutations, which do not have such an effect [72].

Gore et al. first reported many protein-coding point mutations in human iPSCs using WES [48]. The majority of these mutations are nonsynonymous or nonsense or result in splice variants and are enriched in cancer-associated genes listed in the Catalogue of Somatic Mutations in Cancer (COSMIC) database [48, 73]. Contrary to this observation, Cheng et al. as well as other groups have showed that point mutations found in iPSCs are not enriched in cancer-associated genes [47, 50, 57–59, 66]. Ruiz et al. generated iPSC clones that carried point mutations in protein-coding regions, and they showed that these mutations did not provide a selective advantage for reprogramming [50]. However, one cannot exclude that a fraction of them are cell-type dependent (reviewed in [74, 75]) and that the tumorigenic potential depends on the environment [76]. A combination of mutations might lead to tumorigenic potential, as illustrated by the multiple-hit hypothesis [77, 78]. Moreover, donor-derived hematopoietic stem cells that harbored mutations in the cancer-related genes *IDH2* and *DNMT3A* led to leukemia in long-term follow-up after transplantation [79], indicating the importance of long-term monitoring of the functional consequences of point mutations. Therefore, the phenotypic impact of genetic mutations needs further investigation.

Hundreds to thousands of point mutations have been detected throughout the genome by WGS in human iPSCs [47, 52, 54–59]. Functional assessment of the noncoding mutations is also crucial. Noncoding regions constitute ~99% of the genome and contain a large number of *cis*-regulatory elements such as promoters

and enhancers, which are critical for the regulation of gene expression [80]. Interestingly, several studies have identified cancer mutations within *cis*-regulatory regions that can affect gene expression [81–84]. Furthermore, disease-causing SNPs are most overrepresented in enhancer regions [85] (reviewed in [86]). However, in contrast to disease-causing SNPs, point mutations introduced during reprogramming were found mostly in transcriptionally repressed LADs and were significantly underrepresented in protein-coding genes and in *cis*-regulatory elements [66]. This finding indicates that most of the reprogramming-induced mutations are benign [66]. Bhutani et al. also showed that noncoding point mutations are generally benign [57] based on the combined annotation-dependent depletion score [87]. D’Antonio et al. reported that most of the point mutations were found in repressed chromatin regions but that subclonal mutations (that are not present in all iPSCs within a line) are enriched in active promoters and could alter gene expression [58]. The pattern of mutations can vary in different iPSC lines, and further large-scale genomic studies are needed for better characterization.

2.4 Improved Reprogramming Methods to Reduce Genomic Instability

Since the establishment of human iPSCs in 2007 [2, 88], specific and innovative approaches have been used to produce iPSCs more efficiently and safely. Better understanding of the mechanisms underlying mutations in iPSCs would help us to reduce these mutations. Genomic and functional evaluation of iPSCs is important with the advent of newer iPSC generation protocols. Here, we overview the methods to minimize the acquisition of genomic instability in iPSCs (Fig. 2.4).

2.4.1 Starting Cell Source

Many genetic variations detected in clonal iPSCs were variants inherited from the parental fibroblasts [48, 54, 60–62]. To produce iPSCs more efficiently and safely, the choice of starting somatic cells is important.

Since the first human iPSCs were generated from skin fibroblasts [2, 88], skin fibroblasts have been commonly used as starting cell sources. In fact, RPE cell sheet differentiated from skin fibroblasts-derived iPSCs was transplanted into the patient with AMD in the first-in-human clinical trial [13]. A recent study using an integrative multi-omics approach, however, showed that mutations due to ultraviolet-induced damage are present in ~50% of human iPSCs generated from skin fibroblasts [58].

Other types of cells can also be used for reprogramming. Similar frequencies of protein-coding mutations were reported in human iPSCs derived from fibroblasts, keratinocytes, mesenchymal stem cells (MSCs), neural stem cells, and umbilical

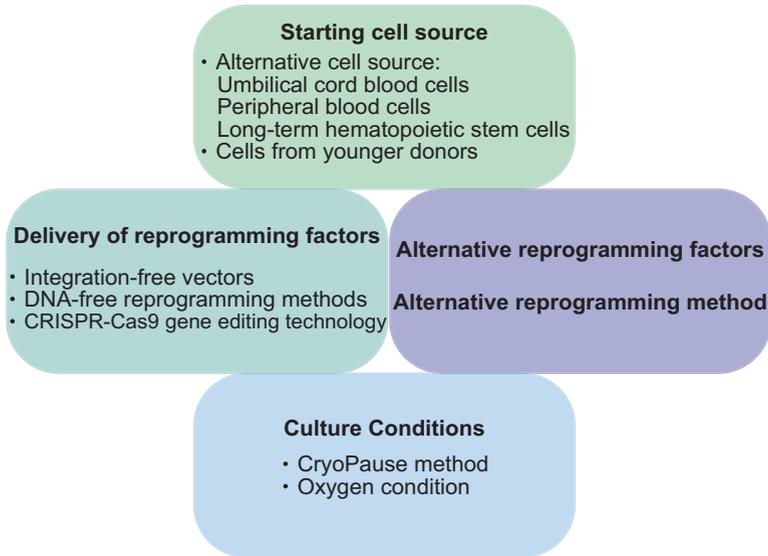


Fig. 2.4 Improved methods to minimize the acquisition of genomic instability in iPSCs

vein endothelial cells [50]. Interestingly, long-term hematopoietic stem cells can be reprogrammed into iPSCs at high efficiency and contain a significantly fewer somatic point mutations and indels than skin fibroblasts [89]. In addition, peripheral blood cells might also be an ideal source for reprogramming, because a large quantity of these cells can be readily harvested by noninvasive methods [90–92].

Multiple lines of evidence suggest that cells from younger donors may be advantageous. Aging is associated with increased DNA damage [93]. Noncancerous skin cells from the elderly, and skin cancer cells, harbor similarly high numbers of somatic mutations, some in cancer-associated genes [94]. In blood cells, somatic mutations, including cancer driver mutations, also accumulate with aging [95, 96]. The frequency of mitochondrial DNA mutations in human iPSCs increases with age, and this can lead to metabolic defects in iPSCs [97]. iPSCs derived from older mice have lower proliferative activity and reprogramming efficiency than those from young mice [98]. A recent work using iPSCs derived from blood cells of 16 donors aged 21–100 demonstrated that mutations in iPSCs increase linearly with age; the study showed that increased donor age is associated with an increased risk of abnormalities in human iPSCs [53]. A study using skin fibroblast-derived iPSCs also demonstrated a positive correlation between the number of mutations and donor age [99].

Umbilical cord blood cells, collected noninvasively at the time of birth, include hematopoietic stem cells and are stored in blood banks together with immunological information for the treatment of hematological malignancies [100]. iPSCs have been successfully generated from umbilical cord blood cells [101, 102]. A WES study revealed that umbilical cord blood-derived iPSCs had a significantly lower

frequency of point mutations than fibroblast-derived iPSCs [103]. Therefore, umbilical cord blood appears to be a particularly promising alternative cell source to generate iPSCs.

iPSC-based cell therapies are switching from autologous transplantation to allogeneic transplantation [104]. In 2017, allogeneic iPSC-derived RPE cell transplantations were carried out for the treatment of AMD [105]. Although autologous transplantation of patient-matched iPSCs could circumvent concerns about immune rejection [5, 6], obtaining patient-derived iPSCs is time-consuming and expensive [8]. Hence, autologous transplantation cannot be readily suitable for acute progressive disorders. A small number of homozygous HLA types could cover a large part of populations [9, 10], and the concept of the banks of HLA-homozygous cell iPSC lines has emerged [8]. Considering the low mutational load in umbilical cord blood cells and the availability of immunological information, HLA-matched umbilical cord blood-derived iPSCs can be useful in allogeneic iPSC-based cell therapies [106]. It would be important to store iPSCs in cell banks together with genomic data [8, 107]. These data could be linked to the clinical outcome of the transplantation of iPSC-derived products, which would help to produce evidence-based criteria for clinical applications.

2.4.2 Delivery of Reprogramming Factors

A number of studies have aimed to improve the efficiency and safety of iPSC reprogramming. Originally, iPSCs were generated by using retrovirus [1, 2, 88]; however, retrovirus transfer could produce insertional mutations and reactivate transgenes [108], which might play a role in tumorigenesis. Indeed, tumor formation by mouse iPSCs was attributed to reactivation of c-Myc transgene [109]. To circumvent this problem, integration-free vectors (such as expression plasmids [110], Sendai virus vectors [111], and episomal plasmid vectors [112, 113]) and several DNA-free reprogramming methods based on direct delivery of proteins [114, 115], mRNA [116], or miRNA [117] have been developed. More recently, Weltner et al. reprogrammed human skin fibroblasts into iPSCs using CRISPR-Cas9-based gene activation [118].

It is important to compare genomic instabilities in iPSCs generated by different methods. An mRNA-based method showed the lowest aneuploidy rate, although its success rate was lower than that of the use of retrovirus, Sendai virus, and episomal plasmid vectors [119]. Gore et al. and Bhutani et al. showed that the numbers of point mutations were comparable between different reprogramming methods [48, 57]. Popp et al. detected no significant differences in the number, size, and gene content of CNVs between iPSCs generated with retrovirus and those generated with Sendai virus [99]. However, Sugiura et al. showed that retrovirally transduced iPSCs harbored about twice as many mutations as integration-free iPSCs [56]. Cheng et al. reported a low incidence of genetic variations in human iPSCs reprogrammed by

using episomal vectors [47]. Effective and integration-free delivery methods should be ideal for clinical applications. In fact, episomal plasmid vectors were used in the first iPSC-based cell therapy [13].

2.4.3 Reprogramming Factors

Reprogramming factors have been explored to generate safer iPSCs more effectively. Rais et al. showed that the nucleosome remodeling and deacetylation repressor complex Mbd3/NuRD is the predominant molecular block preventing iPSC induction, and Mbd3 depletion markedly boosted the efficiency of iPSC reprogramming [120]. Jiang et al. reported that the oocyte factor Zscan4 not only increases reprogramming efficiency but also improves genomic stability during mouse iPSC reprogramming [121]. Increasing the levels of checkpoint kinase 1 (CHK1) reduces reprogramming-induced replication stress and increases reprogramming efficiency and genomic stability in both mouse and human iPSCs [122]. Currently used reprogramming factors have oncogenic potential [123–129], and the use of a chemical reprogramming strategy has potential to reduce the risk of tumorigenesis. Notably, Hou et al. succeeded in generating iPSCs by using a combination of seven small-molecule compounds [130].

2.4.4 Alternative Reprogramming Method

Back in 1962, Gurdon cloned frogs by transferring the nucleus differentiated tadpole's somatic cells into oocytes [131]. This method is referred to as somatic cell nuclear transfer (SCNT). In 2014, Chung et al. generated human ESCs via SCNT using dermal fibroblasts [132]. Human somatic cells can be immediately reprogrammed to pluripotency by SCNT but gradually when using reprogramming factors [133], suggesting that the reprogramming mechanisms are different. Several groups have performed genome-wide analyses of iPSCs and SCNT-ESCs derived from genetically matched somatic cells [51, 134, 135]. Surprisingly, human SCNT-ESCs and iPSCs derived from the same somatic cells contained comparable numbers of CNVs [134] and protein-coding mutations [51]. In addition, the cells differentiated from human iPSCs were found to be comparable to SCNT-ESCs in terms of transcriptome, epigenome, and pharmacological features [135]. A potential advantage of SCNT-ESCs is that the SCNT technology can rescue the mitochondrial DNA mutations by replacing old somatic mitochondria with oocyte mitochondria [97]. However, generation of SCNT-ESCs still faces technical difficulties [132] and the ethical issue of using oocytes and manipulating pre-implantation-stage embryos.

2.4.5 Culture Conditions

As we described previously, deleterious CNVs that occur at early passages could be negatively selected and lost during subsequent passages [44]. However, several studies reported that aneuploidies [22, 136], CNVs [25, 136], and point mutations [48, 59] accumulate at high passages, indicating that prolonged cell culture should be avoided. The CryoPause method, in which iPSCs are dissociated into single cells and stored as ready-to-use aliquots, allows the use of the cells without the need for expansion [137]. This method will reduce the frequency of passage-induced genetic aberrations in iPSCs.

Garitaonandia et al. investigated the effect of passaging method on human ESCs and iPSCs over 100 continuous passages and found that enzymatic passaging on a feeder-free substrate was associated with increased accumulation of genetic aberrations compared with mechanical passaging on feeder layers [138].

Overrepresentation of C-to-A transversions that occur during reprogramming and cell passaging [52, 59, 66] suggests that antioxidants can reduce the occurrence of these mutations. One study reported that antioxidants reduced CNVs in iPSCs [139]. Further studies are required to investigate the effect of antioxidants on mutations in iPSCs.

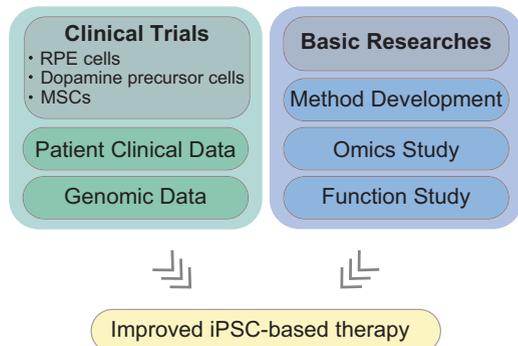
2.5 Challenges in Clinical Applications

Genomic instability can occur at any stage of iPSC processing. Mutations could also arise during differentiation from iPSCs to the final cell products used for transplantation. Careful monitoring is needed to ensure iPSC safety in the clinical settings [48]. Even though low-cost NGS technologies are becoming available [140], routine extensive genome-wide analysis is still prohibitively expensive. Tumor formation assay might be a way of assessing the tumorigenic potential of iPSC-derived products [141]. There are currently no evidence-based guidelines for tumorigenicity testing of such products. It has been reported that human iPSC-derived neurospheres formed tumors in a mouse model after long-term observation [142]; this emphasizes the importance of long-term follow-up. In the case of iPSC-derived RPE cell transplantation, the ocular fundus can be observed noninvasively, and morphological changes of transplanted RPE can be monitored at the cellular level by using optical coherence tomography [13]. In addition to genomic instability, contamination with residual undifferentiated iPSCs or residual exogenous genes could cause undesirable teratomas after transplantation [143]; several strategies have been developed to prevent teratoma formation [143–145].

It should be kept in mind that the presence of mutations does not always lead to serious adverse events. Although a number of genomic aberrations have been

reported in ESCs [29–32, 146], none of the clinical trials using ESCs have reported cancer-associated adverse effects (reviewed in [20]). In the first clinical trial, autologous iPSC-based cell therapy did not cause any major adverse event [13]. Although one of the subsequent patients with an allogeneic iPSC-derived RPE cell transplant developed a preretinal membrane that needed to be surgically removed, this was caused by the surgical method used for transplantation, not by the transplanted iPSC-derived RPE cells themselves [147]. In October 2018, dopamine precursor cells derived from iPSCs were implanted into the brain of a patient with Parkinson’s disease, showing so far no major adverse effect [14]. The first allogeneic clinical trial using iPSC-derived MSCs for the treatment of graft-versus-host disease has started, and no treatment-related serious adverse effects have been reported at primary evaluation after 100 days [15]. A further four clinical studies using iPSCs have been approved: heart muscle cell sheets for heart failure [148, 149], blood transfusion for aplastic anemia [150, 151], neural precursor cells for spinal cord injuries [152, 153], and corneal epithelial cell sheets for corneal epithelial stem cell deficiency [154–156]. These studies will be carried out in the near future. To ensure the safety of these novel iPSC-based therapies, a more comprehensive understanding of variations in the iPSC genome would be important. Several concerns such as malignant outgrowth have been raised for allogeneic iPSC-based therapies requiring an immunosuppressant to prevent rejection [157, 158]. It would be important to obtain and store WGS data on iPSCs used in clinical trials and to intersect the genomic data with the patients’ clinical follow-up data in the forthcoming iPSC-based therapies. Large-scale WGS studies of iPSCs might identify genetic variations relevant to clinical outcome. Better understanding of iPSCs from both basic and clinical aspects will pave the way for iPSC-based therapies (Fig. 2.5).

Fig. 2.5 Better understanding of iPSCs from both basic and clinical aspects will pave the way for iPSC-based therapies



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Chapter 3

Hematopoietic Stem and Progenitor Cells (HSPCs)



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Abstract Hematopoietic stem/progenitor cells (HSPCs) isolated from bone marrow have been successfully employed for 50 years in hematological transplantations. Currently, these cells are more frequently isolated from mobilized peripheral blood or umbilical cord blood. In this chapter, we overview several topics related to these cells including their phenotype, methods for isolation, and *in vitro* and *in vivo* assays to evaluate their proliferative potential. The successful clinical application of HSPCs is widely understood to have helped establish the rationale for the development of stem cell therapies and regenerative medicine.

Keywords Hematopoiesis · Hematopoietic stem cells · Hematopoietic progenitors · Stem cell purification · Hematopoietic stem cell markers · *In vitro* assays · *In vivo* assays · Expansion of hematopoietic stem cells · Very small embryonic-like stem cells · Primordial germ cells

3.1 Introduction

Hematopoiesis is a process that involves the production of all mature blood cells from hematopoietic stem cells (HSCs) by maintaining a fine balance between enormous production and need to supply and regulate the number of erythrocytes, granulocytes, monocytes, lymphocytes, and blood platelets throughout the lifetime of an individual. It has been calculated that the average adult person produces more

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than 500 billion blood cells every day, and it reflects on the highly dynamic feature of HSC compartment. In fact, HSCs and their progeny hematopoietic progenitor cells (HPCs) represent the best characterized stem cells in mammals. Several well-established experimental and diagnostic *in vitro* assays as well as appropriate *in vivo* animal models are available to study these cells [1–10].

HSCs have been identified and can be isolated from embryonic and adult tissues including the yolk sac (YS), aorta-gonad-mesonephros (AGM) region, fetal liver (FL), bone marrow (BM), peripheral blood (PB), and umbilical cord blood (UCB) [9, 11, 12]. This chapter summarizes the following: (i) properties of HSC, (ii) their developmental origin, (iii) hierarchy of the stem cell compartment in hematopoietic organs, (iv) assays to study these cells, and (v) phenotypic markers that allow isolation and identification of HSCs. Recent research has shed new light on better understanding of the embryonic development of hematopoiesis from mesoderm and HSC migration during embryogenesis, which involves multiple anatomical sites in an embryo proper before adult hematopoiesis is established in BM micro-environment [13–16].

Evidence accumulated describes that during early stages of embryogenesis, proximal epiblast-derived pluripotent stem cells (PSCs) give rise to hemangioblasts that are precursors of both HSCs and endothelial progenitor cells (EPCs). Moreover, adult BM most likely still contains a population of development early PSCs that gives rise to the long-term repopulating of HSC (LT-HSC), mesenchymal stromal cells (MSCs), and endothelial progenitors (EPCs) [17–20]. This challenging concept is discussed later on in this chapter. New strategies to isolate/purify HSC have been developed based on a presence of cell surface markers and metabolic properties of these cells [21–23]. It is noteworthy that expression of some of these surface markers depends on the developmental status of HSCs and may change during their activation and isolation. This chapter deals with both murine and human HSCs and discusses their developmental heterogeneity, phenotype, and the experimental strategies that allow to identify these rare cells in embryonic tissues (YS, AGM, FL) as well as in adult BM, PB, and UCB.

According to widely accepted stem cell definition, HSCs possess the ability for self-renewal and may differentiate into HPCs giving rise to any blood cell type of the myeloid and lymphoid lineages. In order to fulfill this mission, HSC must undergo asymmetric divisions where one of the daughter cells retains HSC potential and the other becomes HPCs (Fig. 3.1). HPCs lose the ability to self-renew; however, they are able to expand into clones of functional hematopoietic cells. This mechanism of asymmetric cell division is required to keep constant number of HSCs in hematopoietic organs and prevent their depletion. Therefore, HSCs are endowed with the unique ability to balance self-renewal and differentiation into HPCs in such a way that mature cells necessary for tissue function can be quickly generated and replaced at the same time without exhausting the HSCs pool [5, 7, 16]. Evidence gathered indicates that with age, cells expressing HSC markers increase in the hematopoietic organs, despite the fact that they are less robust as compared to those isolated from the young individuals. Moreover, HSC from old BM are also biased toward myeloid differentiation on expense of other hemato-/lymphopoietic lineages [24–26].

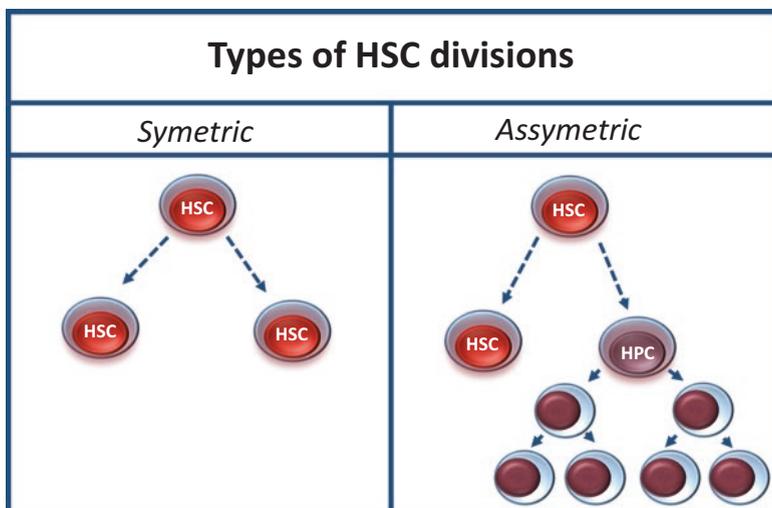


Fig. 3.1 Symmetric and asymmetric division of HSC. The most physiologically relevant is asymmetric cell division that gives rise to one HSC and one HPC. While new HSC secures constant number of HSC in BM, HPC differentiates and gives rise to clone of mature hematopoietic cells

It is well known that HSC are untired travelers and during embryogenesis migrate from one site to another anatomical site where hematopoiesis is active in a well-regulated manner. This developmental journey of the HSCs begins with their first appearance in the YS and subsequently in aorta-gonad-mesonephros (AGM) region or para-aortic splanchnopleure and continues through the FL stage to BM microenvironment. In mammals, FL is a main hematopoietic organ in second trimester of gestation before HSCs could migrate to BM in the third trimester of gestation and also to the spleen in case of rodents [13–16, 27, 28]. In postnatal life, HSCs circulate at low level in the PB, maintaining a balanced number of stem cells located in BM microenvironment in remote locations of skeleton ensuring maintenance of blood homeostasis under normal conditions as well as during state of severe hematopoietic injury. On average, BM constitutes 4% of the total body mass in humans. Based on this calculation, in an adult having 65 kilograms BM typically accounts for approximately 2.6 kilograms. Active hematopoietic BM is called “red” marrow. This color is due to the erythropoietic lineage that is prominent in this organ. In humans with aging, hematopoietic “red” BM is gradually replaced in long bones by “yellow” marrow containing mostly fat tissue also called as marrow adipose tissue (MAT). As a result of this constraint with aging, active hematopoiesis is limited to the “red” marrow present in central skeleton, such as the pelvis, sternum, cranium, ribs, vertebrae, and scapulae, and variably found in the proximal epiphyseal ends of long bones such as the femur and humerus [29, 30]. In contrast in mice, active hematopoiesis in long bones as well as the spleen is maintained during their entire life [31]. In frog, active hematopoiesis occurs in the liver [32], and in fish, the major hematopoietic organ is the kidney [33].

The circulating blood and lymph are highways for trafficking of HSCs and HPCs, and a number of circulating hematopoietic precursor cells in PB follow circadian rhythm where twice as many HSC are detected in early morning hours as compared to late night [34–36]. The number of HSCs increases in PB during strenuous exercise, inflammation, tissue/organ injury (e.g., heart infarct, stroke) [37, 38], and pharmacological mobilization (e.g., after administration of granulocyte-colony stimulating factor; G-CSF [39, 40] or CXCR4 receptor antagonist AMD3100 [41, 42]). Interestingly, these pharmacological agents increase the number of circulating HSCs and HPCs in PB up to 100 times. Pharmacological mobilization is an indispensable mean to harvest HSCs for hematopoietic transplants in clinical setting [39–42].

HSC development has been well characterized in mouse models, which serves as a surrogate model for better understanding of hematopoiesis. As it is shown in Fig. 3.2, HSC gives rise to HPC for myeloid and lymphoid lineage. The HPC for the myeloid lineage is called as the common myeloid progenitor (CMP), and for the lymphoid lineage, it is called as the common lymphoid progenitor (CLP). It has been postulated that HSC, before proceeding into the myeloid vs lymphoid pathway, may form bipotent myelo-erythroid and myelo-lymphoid progenitors. CMP gives rise to the mature cells of myeloid lineages (e.g., erythroblasts, megakaryocytes, monocytes, neutrophilic-, eosinophilic-, and basophilic granulocytes),

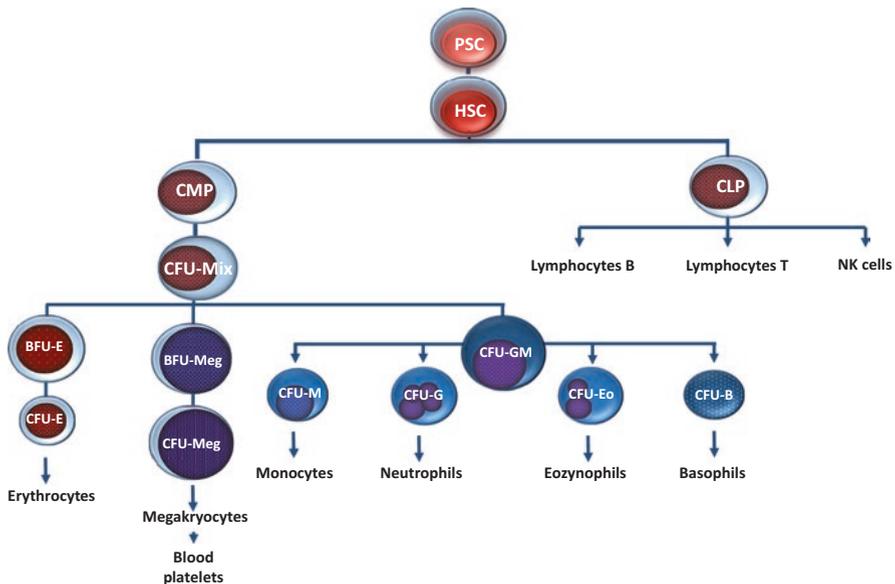


Fig. 3.2 Hierarchy of stem cell compartment in adult BM. The most primitive stem cell in adult BM is proposed as PGC-related PSC (VSEL?). PSC gives rise to HSC that leads to generation of common myeloid progenitor (CMP) and/or common lymphocytic progenitor (CLP). These cells give rise to clonogenic hematopoietic and lymphopoietic HPC

whereas CLP differentiates into lymphoid lineages (e.g., T and B lymphocytes, natural killer (NK) cells) (Fig. 3.2) [9, 10, 43].

However, this chapter discusses features of both murine and human HSCs and HPCs; it is obvious that there are considerable differences in expression of some surface markers between murine and human counterparts. As a consequence, it is sometimes difficult to identify human HSCs based solely on murine phenotype (e.g., the human analog of murine Sca-1 antigen has not been identified yet). Moreover, murine HSCs in contrast to human HSCs loose expression of cell surface marker CD34 antigen very early in development and are predominantly regarded as marker of human HSCs, and in contrast murine HSCs are CD38⁺ [44, 45]. Due to obvious ethical reasons, functional assays to evaluate human HSCs function are very limited, in contrast to the functional assays available to study murine HSCs. Therefore, human HSC is studied *in vivo* by using surrogate heterotransplantation models into immunodeficient mice that do not reject human cells or in intrauterine transplants in sheep [46–50]. Development of hematopoietic system is also widely studied with obvious limitations in surrogate zebra fish model [2, 51, 52].

3.2 Developmental Origin of HSCs

The developmental origin of hematopoietic cells during embryogenesis is biphasic, starting from primitive hematopoiesis and culminating in definitive hematopoiesis, and two separate anatomical sites have been identified where these processes occur. Accordingly, the first primitive hematopoietic precursors are identified outside the embryo proper in the so-called hematopoietic islands located at the bottom of the YS that is a part of the extraembryonic ectoderm. The blood islands where primitive hematopoiesis is initiated are believed to develop from Flk-1 (Kdr)⁺ hemangioblasts, which give rise to both HSCs and endothelial progenitors (EPCs). These cells initiate primitive hematopoiesis in blood islands at 7.0–8.0 days post coitum (dpc) giving rise to endothelial cells and primitive nucleated erythrocytes. Next, at 8.25 dpc. Mixed lineage and myelo-erythroid progenitors as well as primitive pre-HSCs become detectable in this anatomical location [12, 16–20, 53].

The developmental origin of hemangioblasts at day 6.25 dpc in an extraembryonic location at bottom of YS in mice is connected with the migration of a population of primordial germ cells (PGCs) from proximal epiblast of embryo proper (embryonic ectoderm) into extraembryonic ectoderm at the bottom of YS. Interestingly, PGCs are the first stem cells specified in proximal epiblast of developing embryo. These precursors of gametes enter at 6.0 dpc to the extraembryonic ectoderm, and the developmental migration of these cells through this region correlates with appearance of first hemangioblasts at the bottom of YS [54–56]. This may indicate that PGCs or some subpopulation of these cells give rise to hemangioblasts. Moreover, later in embryonic development, definitive hematopoiesis is observed when the first HSC becomes identified in the dorsal aorta wall in the so-called hematogenic endothelium. This area is part of a wider anatomical area of the

embryo known as the aorta-gonad-mesonephros (AGM) region [54–56]. In mice, the origin of definitive HSCs in AGM occurs at the time when PGC after entering through primitive streak of embryo proper migrate on 11 dpc through this region to genital ridges where ultimately, between 12.5 and 14.5 dpc, they will settle down and give rise to gametes [53–56]. Based on above information, initiation of both primitive and definitive hematopoiesis is tightly connected with the migration of primordial germ cells (PGCs), and we cannot exclude the possibility that a sub-population of cells related to migrating PGCs may be responsible for establishing hematopoiesis at both places.

This challenging concept postulates that potential developmental origin of HSCs could be from PGC-related cells. A potential intermediate developmental link between PGCs and HSCs could be a population of very small embryonic-like stem cells (VSELs) identified in embryonic and adult hematopoietic organs (Fig. 3.3) [57–60]. This is somehow supported by expression of functional sex hormone receptors on PGCs, VSELs, and HSCs. Attesting this statement like VSELs, normal human, and murine HSCs expresses several functional pituitary and gonadal sex hormone receptors and responds by proliferation to follicle-stimulating hormone

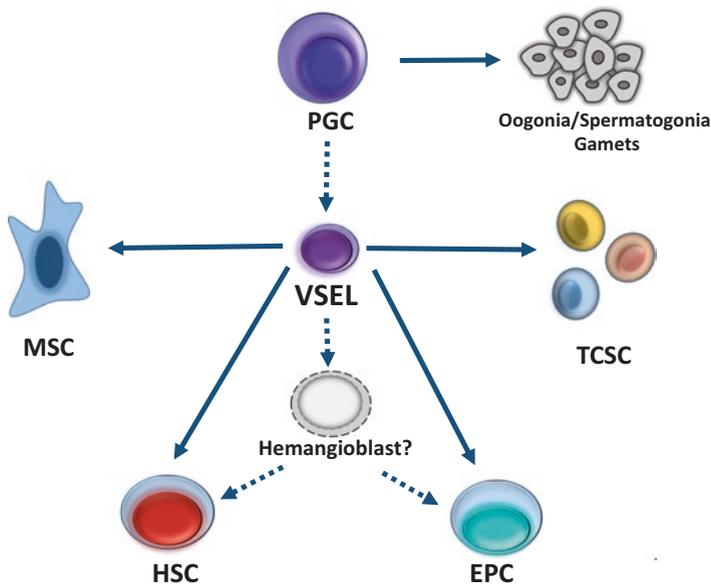


Fig. 3.3 Proposed developmental interrelationship between primordial germ cells (PGCs), very small embryonic-like stem cells (VSELs), hemangioblasts, hematopoietic stem cells (HSCs), and endothelial progenitor cells (EPCs). We propose that migratory PGCs, aside from their major role in establishing gametogenesis, may be a source of certain developmentally primitive stem cells (e.g., VSEL) that in bone marrow give rise to HSCs and EPCs and are a source of mesenchymal stem cells (MSCs) and in other tissues a source of tissue-committed stem cells (TCSCs). Specification of VSEL into HSC and EPC may involve putative hemangioblast as an intermediate precursor cell. Dotted line pathways are still under investigation

(FSH), luteinizing hormone (LH), prolactin, estrogens, and androgens [61, 62]. Since VSELs are also present in adult BM, it is possible that this rare population of PGC descendants (?) residing in adult BM could be developmental precursor of potential long-term repopulating HSCs (LT-HSCs) and perhaps a reserve source of these LT-HSCs. This may change our view on stem cell compartment in adult BM where on the top of stem cell hierarchy are VSELs that can give rise to both HSCs and other stem/progenitor cells including mesenchymal stromal cells (MSCs) and endothelial progenitor cells (EPCs) (Fig. 3.3).

Nevertheless, a current view postulates that hemangioblasts are specified in extraembryonic ectoderm from unknown precursors and definitive hematopoiesis in AGM region occurs due to the blood stream transfer of pre-HSCs from YS blood islands to AGM. This transfer requires initiation of blood circulation in embryo and emergence of a beating heart that pumps blood. In support of this, murine *Ncx1*-KO embryos, which do not develop a beating heart by 8.25 dpc but still continue to develop until 10 dpc, do not initiate definitive hematopoiesis in AGM region. As reported, while there was no observed significant difference in the number of hematopoietic precursors in the YS from wild-type and *Ncx1*-KO embryos, the embryo proper, including the AGM region, was nearly devoid of HSC in mutant embryos [63, 64]. However this concept, which pre-HSC from the YS may specify into definitive HSC and colonize aortic endothelium, does not preclude the possibility that the aortic endothelium could be colonized by other PGC-derived cells (e.g., VSELs) migrating through the AGM region by 11 dpc in mice [53, 56]. Moreover, the initiation of definitive hematopoiesis in the aortic “hemogenic” endothelium as suggested is also dependent on an effect induced by shear forces generated due to active blood flow [63, 64]. Again, we cannot preclude that these shear forces act on migrating PGC-derived VSELs that infiltrate the AGM region. What is not clear in support of hemogenic endothelium as source of HSCs is that since shear forces occur in all large vessels in the embryo, why AGM region still seems to be a preferable anatomic location to initiate definitive hematopoiesis. To support this statement and taking into consideration that at this time PGCs migrate through AGM region, it is pertinent to state that derivatives of these cells could be the origin of definitive HSCs [59, 61, 65].

The proposed developmental link between PGCs, VSELs, and HSCs is supported by the presence of germ line precursor cells in adult BM. It has been demonstrated, for example, that SSEA-1⁺Oct-4⁺Stella⁺Mvh⁺ cells, isolated from murine BM, may differentiate in the presence of BMP-4 (bone morphogenetic factor 4) into gamete precursors [66]. On the other hand, BM-derived Oct-4⁺Mvh⁺Dazl⁺Stella⁺ putative germ cells supported and restored oogenesis in mice sterilized by chemotherapy [67, 68]. Similarly, Oct-4⁺Mvh⁺ Stella⁺ cells isolated from the BM of male Stra8-GFP mice expressed several molecular markers of spermatogonial stem cells and spermatogonia indicating transdifferentiation of BM cells to male germ cells [69]. Interestingly, in direct mutagenesis studies, it has also been demonstrated that BM cells exposed to methylchloranthrene may give rise to germ line tumors [70]. It is most likely that all these cells employed in the abovementioned studies are related to VSELs. Moreover, in support of the intriguing concept that HSCs may be derived

from PGCs, it has been demonstrated that PGCs isolated from murine embryos, murine testes [71], and certain teratocarcinoma cell lines [72–74] can be specified into HSCs. Similarly, there are reports on the clonal origin of both teratomas and germ line leukemia tumors [75, 76]. Interestingly, it has been demonstrated that VSELs isolated from adult BM has potential to differentiate in vitro into HSCs and HPCs. Also, VSEL isolated from ovaries has been demonstrated to differentiate into precursors of oocytes [77]. Nevertheless, despite accumulating evidence, more studies are warranted to strengthen and better understanding of the developmental link between PGCs, VSELs, and HSC (Fig. 3.3).

3.3 Phenotype of HSC: From YS to Adult BM

As mentioned above, first primitive pre-HSCs are identified in murine YS and AGM at 8.5–10.5 dpc. These cells express CD41 and CD34 antigens but lack CD45 and Sca-1 expression (Table 3.1). Similar populations of pre-HSCs can be also detected in the murine placenta. These pre-HSCs finally give rise to definitive HSCs (Table 3.1) that express CD34, CD45, and Sca-1 antigens at 11.0–12.5 dpc. These

Table 3.1 Phenotype of murine and human HSC at different stages of embryonic and postnatal development. These markers are usually used in combination in order to isolate cells highly enriched for HSC

HSC	Mouse	Human
Hemangioblast (<i>yolk sac</i>)	Flk-1 (Kdr) ⁺	?
Pre-HSC (<i>AGM</i>)	CD34 ⁺ CD41 ⁺ Sca-1 ⁻ CD45 ⁻	?
HSC (<i>AGM, placenta</i>)	CD34 ⁺ CD45 ⁺ CD41 ^{+/-} Sca-1 ^{+/-}	CD34 ⁺
HSC (<i>fetal liver</i>)	Sca-1 ⁺ CD34 ⁺ CD45 ⁺ Mac1 ⁺ CXCR4 ⁺	CD34 ⁺ CD133 ⁺ CXCR4 ⁺ Lin ⁻
HSC Long-term engrafting HSC (LT-HSC) Short-term engrafting HSC (ST-HSC) (<i>bone marrow</i>)	CD34 ^{lo/-} Sca-1 ⁺ Thy1.1 ^{+/lo} CD38 ⁺ c-kit ⁺ lin ⁻ c-kit ⁺ Thy1.1(CD90) ^{lo} Lin ⁻ Sca ^{hi} (<i>KTLS</i>) CD150 ⁺ CD48 ⁻ CD244 ⁻ (<i>SLAM</i>) Rh123 ^{low} , Hoe3342 ^{low} , pyroninY ^{low} 5-FU resistant, ALDH ^{hi} Fr25 (small cells) Lin ⁻ Side population (SP) – Cells LT-HSC: CD34 ⁻ CD38 ⁻ Sca-1 ⁺ Thy1.1 ^{+/lo} c-kit ⁺ lin ⁻ CD135 ⁻ Slamf1/ CD150 ⁺ ST-HSC: CD34 ⁺ CD38 ⁺ Sca-1 ⁺ Thy1.1 ^{+/lo} c-kit ⁺ lin ⁻ CD135 ⁻ Slamf1/ CD150 ⁺ Mac-1 (CD11b) ^{lo}	CD34 ⁺ CD59 ⁺ Thy1/CD90 ⁺ CD38 ^{lo/-} c-kit/CD117 ⁺ lin ⁻ CD34 ⁺ CD38 ⁻ Lin ⁻ CD34 ⁺ CD133 ⁺ CXCR4 ⁺ Lin ⁻ Rh123 ^{low} , Hoe3342 ^{low} , pyroninY ^{low} ALDH ^{high} (ALDH ^{hi} CD133 ⁺ Lin ⁻) CD150 ⁺ CD48 ⁻ CD244 ⁻ (<i>SLAM</i>) Side population (SP) – Cells

definitive HSCs begin to colonize FL at 11.5 dpc, and FL becomes a major hematopoietic organ during the second trimester of gestation, and hematopoietic activity remains detectable in murine FL several weeks postnatal [53–56]. As mentioned, FL is also major hematopoietic organ in humans during this period of gestation, and AGM-derived definitive HSCs migrate to FL through the umbilical artery, placenta, and umbilical vein. This migratory route of HSCs may explain why the placenta is also enriched for HSCs [12, 16–20, 53]. HSCs present in second trimester of gestation in murine FL express CD34, CD45, Sca-1, Mac-1, and CD150 antigen (Table 3.1) [78].

It is apparent that HSCs in their developmental migration in embryo must follow gradients of chemoattractants. So far, very few chemoattractants have been identified for murine and human HSCs that include (i) α -chemokine stromal-derived factor-1 (SDF-1) [79, 80]; (ii) two bioactive phosphosphingolipids, namely, sphingosine-1 phosphate (PLc) [81, 82] and ceramide-1 phosphate (C1P) [83, 84]; and (iii) extracellular adenosine triphosphate (ATP) [85, 86].

Interestingly, the migration of embryonic HSCs from AGM to FL does not depend on SDF-1. Evidence to support this notion is that mice with SDF-1 or its corresponding receptor CXCR4 knockout possesses a normal number of HSCs in FL [87, 88]. This suggests the existence of an alternative homing factor/chemoattractant during early embryogenesis that may compensate for SDF-1 deficiency and allows accumulation of embryonic HSCs in FL. This could be S1P, C1P, ATP, or another not yet identified chemoattractant.

In the third trimester of gestation in mice (18.0 dpc.), HSCs begin to seed fetal BM and the spleen [53–56]. Here the colonization of fetal BM by HSCs depends on the expression of SDF-1 that becomes expressed in the developing BM microenvironment [87, 88]. In response to an SDF-1 gradient, HSCs that express the corresponding receptor, CXCR4, move from the FL to BM and establish adult-type hematopoiesis. The HSCs in neonatal murine BM are CXCR4⁺, Sca-1⁺, and CD45⁺, but as mentioned earlier, murine HSC gradually loses the expression of CD34 antigen (Table 3.1). Human HSCs are CD34⁺ CD38⁻, and some of them express CD133 [45].

The colonization of BM by HSCs does not terminate the developmental and postnatal journey of HSCs and HPCs as these cells continuously circulate in PB and lymph. As discussed in introduction, symmetric division results in two niche bound HSCs, and asymmetric division results in one daughter HSC, and one daughter cell enabled with differentiation program has to leave the BM niche and released into the circulation to find a new niche [89]. This mechanism may be responsible for maintaining homeostasis between HSC niches in different areas of BM distributed across various bones. It has been postulated that circulating HSCs and HPCs are also patrolling peripheral tissues for the presence of potential pathogens or damages [90]. This phenomenon allows them to react rapidly to fight infections by providing clones of granulocytes, monocytes, or dendritic cells. Moreover, since not only HSCs and HPCs but also MSCs, EPCs, and VSELs circulate at low level in PB and lymph, all these circulating cells may potentially mend small organ damages.

3.4 Novel View on Possible Hierarchy of Stem Cell Compartment in Adult BM

Stem cells residing in the BM demonstrate developmental hierarchy. It has been proposed that at the top resides the most primitive PGC-derived pluripotent stem cell (VSEL?) that can give rise to LT-HSCs, short-term repopulating HSCs, MSCs, and EPCs (Fig. 3.3).

In fact, the presence of VSELS which can differentiate into germ line cells has been confirmed in adult tissues including BM, by at least 20 independent laboratories. VSELS are small cells, corresponding in size to the cells in the inner cell mass of the blastocyst, and depending on the measurement conditions (in suspension or after adhesion to slides), they measure ~3–5 μm in mice and ~5–7 μm in humans [58–60]. Thus, they are slightly smaller than red blood cells and require a special gating strategy during FACS sorting. Transmission electron microscopy analysis revealed that VSELS have large nuclei containing euchromatin and a thin rim of the cytoplasm enriched in spherical mitochondria, which are characteristic of early-development cells, for example, PGCs. They also express several genes characteristic for pluripotent/multipotent stem cells such as stage-specific embryonic antigen (SSEA), Oct-4, Nanog and Rex-1, and highly expressed Rif-1 telomerase protein. Studies performed on highly purified double-sorted VSELS isolated from murine BM revealed that these cells express high mRNA and/or protein levels of (e.g., *Stella*, *Fragilis*, *Blimp1*, *Nanos3*, *Prdm14*, and *Dnd1*) and late migratory PGCs (e.g., *Dppa2*, *Dppa4*, and *Mvh*) [91].

Murine VSELS are isolated as a population by multiparameter sorting as Sca-1⁺ Lin⁻ CD45⁻ cells and human VSELS as CD34⁺ CD133⁺ Lin⁻ CD45⁻ cells [57, 59]. As mentioned earlier, these cells have potential to differentiate into functional HSCs and HPCs. Accordingly, VSELS that are CD45⁻ if expanded toward hematopoietic lineage give rise to CD45⁺ hematopoietic cells that transplanted into experimental animals and protect them from lethal irradiation by differentiating in vivo into all the major hematopoietic lineages (e.g., as demonstrated Gr-1⁺, B220⁺, and CD3⁺ cells) [92]. In parallel, the hematopoietic specification of murine VSELS is accompanied by the upregulation of mRNA for several genes regulating hematopoiesis (e.g., PU-1, c-myb, LMO2, Ikaros). Additionally, functional VSELS have also been purified from human UCB and BM [59, 60]. Despite this exciting data, the proposed hierarchy of stem cell compartment in adult murine and human BM requires further studies.

Figure 3.2 shows widely accepted hierarchy of HSCs and HPCs in adult BM. HSC gives rise both to stem cell for hematopoiesis (CMP) and lymphopoiesis (CLP). Hematopoietic stem cells differentiate into HPCs for common and separate hematopoietic lineages. At the top of CMP hierarchy is the so-called colony-forming unit of mix lineages (CFU-Mix) – a HPC that is able to grow in vitro colonies containing cells from myeloid, erythroid, and megakaryocytic lineage. Therefore, CFU-Mix is often called colony-forming unit–granulocyte-erythrocyte-monocyte-megakaryocyte (CFU-GEMM). Downstream in this hierarchy, we can distinguish more differentiated HPCs specified to erythroid lineage forming big or small

colonies of red blood cells called burst-forming unit of erythrocytes (BFU-E) and colony-forming unit of erythrocytes (CFU-E), respectively. Common progenitor for myeloid cells, colony-forming unit of granulocyte and monocytes (CFU-GM), gives rise to more restricted progenitors such as colony-forming unit of granulocytes (CFU-G) and colony-forming unit of monocytes (CFU-M). In myeloid lineage, we distinguish also progenitor for eosinophils – colony-forming unit of eosinophils (CFU-Eo) and basophils – colony-forming unit of basophils (CFU-Baso). Megakaryocytes that give rise to blood platelets develop from more primitive burst-forming unit of megakaryocytes (BFU-Meg) and further into more differentiated colony-forming unit of megakaryocytes (CFU-Meg). In contrast, CLP for lymphoid lineage gives rise to T cells, B cells, or natural killer (NK) cells [9, 10].

The specification and differentiation of HPCs of both myeloid lymphoid progenitor cells are regulated by several hematopoietic growth factors and cytokines. Figure 3.4 shows the most important players involved in this process. These factors regulate different levels of hematopoiesis and lymphopoiesis in vivo and could also be employed for in vitro assays to grow colonies composed of cells from different lineages [93, 94]. Figure 3.5 shows some examples of such hematopoietic colonies growing in clonogenic assays in vitro. These assays have both experimental and diagnostic meanings and implications.

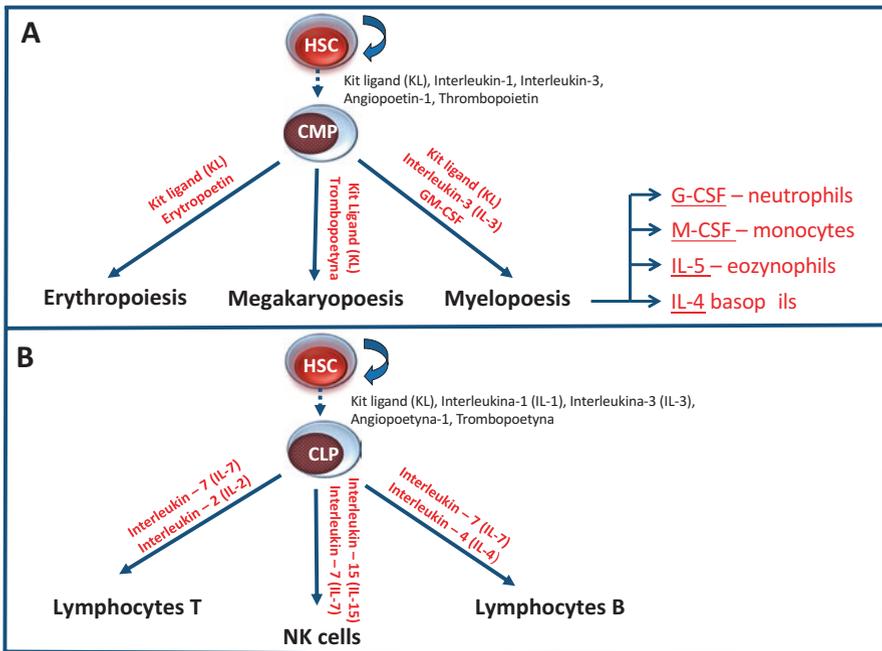


Fig. 3.4 Specification of HSC into CMP and hematopoietic lineages (Panel a) and specification of HSC into CLP and lymphopoiesis (Panel b). On the scheme, there are indicated growth factors and cytokines that regulate the development of various hematopoietic and lymphopoietic lineages

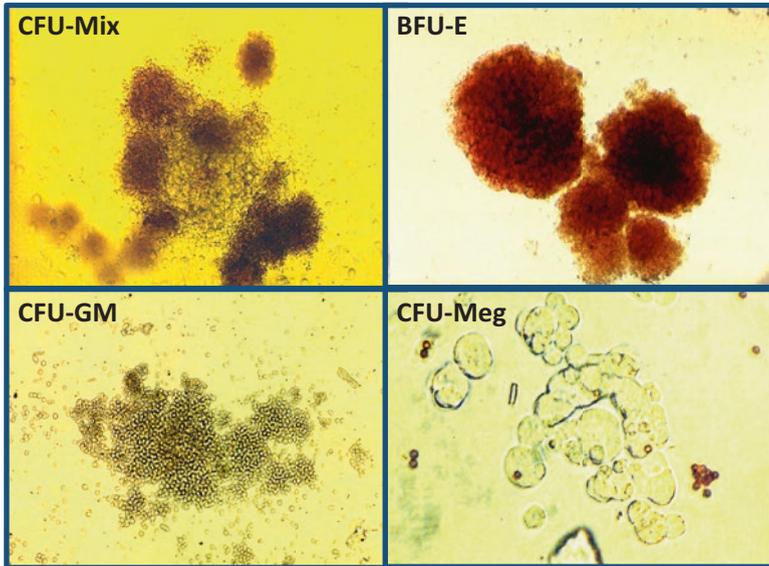


Fig. 3.5 Examples of clonogenic growth of CFU-Mix, BFU-E, CFU-GM, and CFU-Meg. There are shown representative HPC colonies growing in semisolid cloning media

3.5 The Hematopoietic Niche and Retention of HSPCs in BM Microenvironment

HSCs reside in bone marrow niches, where they are anchored due to the interaction between the ligands α -chemokine stromal-derived factor 1 (SDF-1) and vascular cell adhesion molecule-1 (VCAM-1), expressed in cells forming stem cell niches interacting, respectively, with corresponding receptors CXCR4 and $\alpha\beta 1$ integrin, very late antigen-4 (VLA-4) expressed on HSCs [89]. Of note, both CXCR4 and VLA-4 receptors are membrane lipid raft-associated receptors, and their incorporation into cell surface membrane lipid rafts is essential to their optimal biological function. Nevertheless, the BM stem cell niche remains incompletely defined and has been described by competing models. Recent research indicates that this niche is perivascular [SDF-1^+ and kit ligand (KL)^+], created partially by mesenchymal stromal cells and endothelial cells and, often but not always, located near trabecular bone [95]. While HSCs are located around perivascular cells, early lymphoid progenitors are associated with the osteoblastic niche. The existence of distinct niches, including quiescent $\text{nestin}^{\text{bright}} \text{NG2}^+$ arteriolar and proliferative $\text{nestin}^{\text{dim}} \text{Lep}^+$ sinusoidal niches as a source of factors required for the maintenance of distinct subpopulations of HSCs, has been proposed [96]. On the other hand, evidence also indicates the involvement of an osteoblastic niche that is located along trabecular bone in the BM microenvironment, where osteoblasts provide cellular support for HSCs [89]. Within the BM niche, several factors affect the fate of HSCs, including

interactions with adhesion molecules and ligands. In particular, the SDF-1–CXCR4 and VCAM-1–VLA4 axes play important roles in retention of HSCs in BM niches [97]. HSCs are also under the influence of several hematopoietic growth factors, cytokines, and chemokines secreted by niche cells or delivered to the niche via the circulating blood and lymph [98]. In addition, an important role is also played by sympathetic nervous system via innervating HSC niches with neural β -adrenergic fibers and local oxygen tension. Under steady-state conditions, HSPCs are actively retained in BM niches, and these retention mechanisms counteract the continuous sphingosine-1-phosphate (S1P) gradient originating in blood plasma, as recently demonstrated by our research work [99]. This gradient is playing an important role in egress or mobilization of HSCs from BM into PB.

3.6 HSC Egress from BM into PB (Mobilization)

A crucial role in HSC mobilization process is played by the induction of a proteolytic microenvironment in BM due to the release of proteolytic enzymes from granulocytes and monocytes. This, for example, occurs, after administration of pro-mobilizing agent such as G-CSF [39, 40]. It has been demonstrated that several proteolytic enzymes released by cells in BM digest proteins involved in retention of HSCs in the bone marrow microenvironment (e.g., SDF-1-CXCR4 and VCAM-1-VLA-4 axes) [97, 100]. Interestingly, the crucial proteases involved in this process have not yet been identified, since mice with knockout of multiple proteolytic enzymes, such as MMP-9, MMP-2, cathepsin G, and elastase, mobilize HSCs in a similar manner as control wild-type mice [101, 102]. This finding may indicate that their deficiency is compensated by other proteolytic enzymes.

Another previously unrecognized possibility is the involvement of other types of non-proteolytic enzymes. Our recent results indicate the involvement of the lipolytic enzyme hematopoietic cell-specific phospholipase C β 2 (PLC- β 2) [82]. PLC- β 2 is an enzyme that targets VCAM-1 expressed in stem cell niches and the glycolipid glycosylphosphatidylinositol anchor (GPI-A), which is important lipid raft component for optimal function of CXCR4 and VLA-4 receptors to support their role in retention of HSCs in BM niches [82].

3.7 Chemoattractants for HSC and Stem Cell Homing

In contrast to differentiated hematopoietic cells and lymphocytes, the complete list of chemoattractants for HSC is quite short. As previously mentioned, HSC responds robustly to SDF-1 gradients [79, 80] as well as to gradients of two phosphosphingolipids, S1P [81, 99] and ceramide-1 [83, 84]. Importantly, extracellular adenosine triphosphate (ATP) [85, 86] has emerged as an important chemoattractant for

HSPCs in addition to SDF-1, S1P, and C1P. These factors, however, would be crucial for homing and engraftment of HSCs after hematopoietic transplant.

Homing is a process where transplanted HSCs circulating in PB migrate to their niches in BM and is followed by engraftment in the bone marrow microenvironment and is a process opposite to mobilization. HSCs infused into PB after myeloablative conditioning by radio- or chemotherapy respond to the chemotactic gradients originating from BM, attach to the BM endothelium, transmigrate through the basal membrane in a metalloproteinase (MMP)-dependent manner, and finally home to the niche where they subsequently survive, expand, and proliferate. Homing of HSCs to BM niches is the first step in the engraftment process before HSCs self-renew and differentiate into hematopoietic lineages. *Short-term engraftment* can be assessed in mice by evaluating the hematopoietic recovery from lethal irradiation of PB parameters after transplant or the formation of spleen colonies in irradiated recipients (colony-forming units-spleen [CFU-S] assay). *Long-term engraftment*, which is most important for long-term survival after transplantation, is achieved by the most primitive HSCs, which are endowed with long-term repopulating capacity to maintain hematopoiesis for months after transplantation [103].

3.8 Markers and Strategies to Isolate HSC and HPC

Neither a single specific marker nor a biological property exists which, if employed alone, allows for isolation of pure murine or human HSCs. Hence, combinations of several markers must be used to enrich murine BM- or human BM-, PB-, or UCB-derived mononuclear cells for HSCs isolation identification and characterization.

3.8.1 Markers of HSCs

To purify HSCs, several markers/parameters have to be employed in combination. Figure 3.6 shows the most important cell surface expressed antigens (CD34, CD133, CXCR4, and c-kit) and metabolic fluorochromes (Hoechst 33342, pyronin Y, and rhodamine 123) that are useful to identify and potentially isolate HSCs. However, there are some differences between markers for murine and human HSCs [104, 105].

The most relevant cell surface markers associated for murine HSCs include Sca-1, c-kit, and Thy1.1 (CD90) and for human HSCs: CD34, CD133, CXCR4, and CD150 [45]. Both murine and human HSCs lack lineage differentiation markers (Lin⁻). They also show low accumulation/staining with metabolic fluorochromes such as Hoe33342 (DNA marker), Rh123 (efflux pump substrate), and pyronin Y (mRNA marker). HSCs are also in vivo resistance to fluorouracil (5-FU), express high activity of aldehyde dehydrogenase enzyme (ALDH) that conveys resistance to cyclophosphamide, and are characterized by small size (e.g., on FACS cytograms, HSCs are included in the so-called lymph-gate and during elutriation are recovered

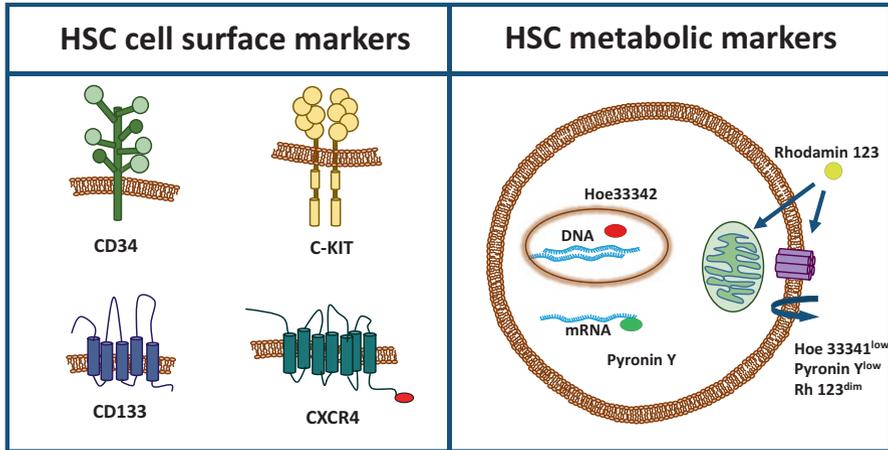


Fig. 3.6 Cell surface antigens (Panel a) and metabolic fluorochromes (Panel b) that are employed for isolation of HSC. These markers are usually employed in combination to purify HSC

with a fraction FR25 containing small lymphocytes). The majority of HSCs are quiescent. Interestingly, a combination of signaling lymphocyte activation molecule (SLAM) markers ($CD150^+CD48^-CD244^-$) has been recently proposed to define and sort LT-HSCs [106–108]. The SLAM is a group of more than ten molecules whose genes are located mostly tandemly in a single locus on chromosome 1 (mouse). SLAM molecules belong to a subset of the immunoglobulin gene superfamily, and originally thought to be involved in T-cell stimulation include CD150, CD48, CD244, and CD150. The marker CD150 is the founding member of SLAM family and is known as *slamF1*, that is, SLAM family member 1 [106, 108].

Unfortunately, cell surface markers employed for isolation of HSC such as CD34 antigen are also expressed on more differentiated hematopoietic cells and EPC [109]. Similarly, CXCR4, a receptor for α -chemokine SDF-1, which as mentioned is a major chemoattractant of HSCs, is also expressed by more differentiated hematopoietic cells, for example, by megakaryocytes, monocytes, platelets, and lymphocytes [110]. This requires multiparameter FACS sorting targeting different cell surface and metabolic markers.

3.8.2 Strategies to Purify and Isolate HSCs

HSC is purified and enriched from BM, PB, or UCB mononuclear cells (MNCs). The MNCs in first step may be depleted of erythrocytes and granulocytes using Ficoll gradient centrifugation or hypotonic erythrocyte lysis. MNCs may be also isolated from PB, BM cell suspension, or UCB by leukapheresis [111].

Different methods are employed for HSC isolation, for example, (i) high speed FACS sorters, (ii) elutriators, (iii) paramagnetic beads, or (iv) avidin columns. Additionally, recent development of microfluidic strategy to develop cell-based assays looks very attractive and have provided promising means to monitor and study cellular phenotype and behavior in real time and in a well-controlled micro-environment simulating the *in vivo* settings [112]. In FACS isolation MNCs are (i) labeled with fluorochrome-conjugated antibodies against above-listed HSC markers [23, 112] or (ii) are exposed to metabolic fluorochromes (Rh123, Hoe33342, pyronin Y) [105] or the fluorescent substrate of ALDH that is Aldefluor. After labeling, HSCs are subsequently sorted from the lymphocyte gate as (i) cells showing the HSC-specific phenotype (e.g., CD34⁺CD38⁻Lin⁻ or CD34⁺CD133⁺Lin⁻); (ii) a population that is characterized by low accumulation of Hoe33342, Rh123, and/or pyronin Y (Rh123^{dim}Hoe33342^{dim} pyronin Y^{low} cells); or (iii) the so-called side population (SP) of cells showing low accumulation of Hoe33342. A convenient tool to pre-enrich for HSC is elutriation where HSCs are enriched in the so-called FR25 fraction containing small cells. These cells could be subsequently employed as sorting material to isolate, for example, Sca-1⁺Lin⁻ cells or as reported recently the CD133⁺Lin⁻ALDH^{hi} cells [113]. Moreover, cell surface markers and metabolic fluorochromes may also be combined together to sort, for example, CD34⁺c-kit⁺Rh123^{dull} cells [114].

For isolation and enrichment of HSCs, magnetic cell separation or biotin columns are used for both experimental and clinical applications. In this approach, HSCs are labeled with antibodies conjugated with paramagnetic beads or biotin. Cells labeled with paramagnetic beads are isolated through columns using magnetic field. In contrast, cells labeled with antibodies conjugated with biotin (biotinylated antibodies) are isolated on streptavidin columns. Paramagnetic beads of biotinylated antibodies may be employed for both positive selection of HSCs (e.g., CD34⁺ or CD133⁺ paramagnetic beads or biotinylated antibodies) or to deplete MNCs of more differentiated lineage-positive cells (e.g., Lin⁺ beads or biotinylated antibodies) [115].

As it is shown in Table 3.1, HSCs are most commonly isolated from the adult mouse BM by FACS as c-kit⁺ Thy1.1^{lo} Lin⁻ Sca^{hi} (KTLS) cells or as Sca-1⁺c-kit⁺Lin⁻ (SKL) cells [116]. The phenotype of murine long-term engrafting HSCs is described as Thy 1.1^{lo} Lin⁻ Sca^{hi} Mac1⁻ CD4⁻ or Thy1.1^{lo} Flk-2⁻ cells [112, 117, 118]. The short-term engrafting HSCs were identified among a KTLS population as Mac-1^{lo} CD4⁻ or Mac-1^{lo} CD4⁺ Thy-1.1⁺ Flk-2⁻ cells [103]. Murine HSCs can be enriched *in vivo* before isolation by depletion of cycling cells *in vivo* by exposing to 5-fluorouracil (5-FU) [119]. Murine HSCs are also isolated according to signaling lymphocyte activation molecule family (SLAM) phenotype as CD150⁺ CD48⁻ CD244⁻ cells. In contrast human HSCs are purified as a population of CD34⁺CD38⁻Lin⁻, CD34⁺ CD133⁺ CXCR4⁺ Lin⁻, or CD150⁺ CD48⁻ CD244⁻ (SLAM) cells [106–108].

3.8.3 Markers of HPCs

In addition to identification of HSCs, SLAM family of markers has been proposed to identify HPCs. Accordingly, the multipotent progenitor cells (MPP) are identified as CD150⁻CD48⁻CD244⁺, the lineage-restricted progenitor cells (LRP) as CD150⁻CD48⁺CD244⁺, the common myeloid progenitor (CMP) as lin⁻Sca-1⁻c-kit⁺CD34⁺CD16/32^{mid}, the granulocyte-macrophage progenitor (GMP) as lin⁻Sca-1⁻c-kit⁺CD34⁺CD16/32^{hi}, and finally the megakaryocyte-erythroid progenitor (MEP) as lin⁻sca-1⁻c-kit⁺CD34⁻CD16/32^{low} [1, 120].

Murine multipotent progenitor (MMP) cells could also be isolated by FACS as early MPP: CD34⁺, SCA-1⁺, Thy1.1⁻, c-kit⁺, lin⁻, CD135⁺, Slamf1/CD150⁻, Mac-1 (CD11b)^{lo}, and CD4^{lo} and as late MPP according to phenotype: CD34⁺, SCA-1⁺, Thy1.1⁻, c-kit⁺, lin⁻, CD135^{high}, Slamf1/CD150⁻, Mac-1 (CD11b)^{lo}, and CD4^{lo} [1, 78].

3.9 Expansion of HSC

A common problem in hematopoietic transplants in clinical settings is lack of sufficient number of HSCs. This occurs when the harvest of HSCs from BM or PB is poor or UCB is used as a source of HSCs for transplant, in particular for patients with bigger body mass that require higher number of graft cells. To eliminate this problem, several ex vivo expansion protocols have been proposed for these cells [120–123]. Nevertheless, almost all available HSC expansion protocols result in an increase in the number of HPCs and more differentiated hematopoietic progenitor cells at the expense and loss of HSCs. To explain the failure of ex vivo expansion procedures of HSCs, it has been postulated that we still have not identified all factors operating in vivo in HSC niches that are crucial for the self-renewal and maintenance of these cells. Another explanation is that HSCs employed for expansion claimed to be the most primitive in hematopoietic lineage and have in fact already entered a pathway of differentiation at the expense of their true self-renewing potential [165].

Recent studies point toward significant progress that has been made in this direction both in experimental and clinical settings by employing prostaglandin E2 or small molecular compounds such as aryl hydrocarbon receptor antagonist – SR-1 [124, 125]. Nevertheless, it is clear that in order to proceed with most optimal expansion, we should begin this process with the most primitive quiescent stem cells likely able to give rise to all LT-HSC. Taking into consideration that VSELs are on the top of hierarchy of stem cell compartment in adult BM, expansion protocols should be initiated using these cells (Fig. 3.7) [65]. In fact the promising strategy to expand HSCs in the presence of some small-molecule inhibitors UM171 or histone deacetylase-3 (HDAC) inhibitor nicotinamide [126–129] most likely initiates expansion of HSCs from population of quiescent VSELs.

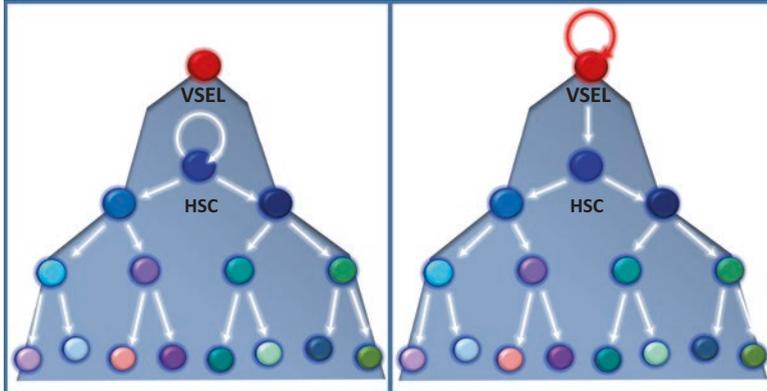


Fig. 3.7 A possible reason for the current poor results for clinical expansion of HSCs. It is most likely that the most of the current clinical expansion procedures employ HSCs that are already “rolling downhill” in following a differentiation pathway (left panel). The expansion strategy should be better initiated at the level of VSELs, which are highly quiescent and positioned at the top of the stem cell compartment hierarchy (right panel)

3.10 Experimental Approaches to Identify and Study HSC

Depending on their developmental stage and hematopoietic organ origin, HSCs are studied using different *in vitro* and *in vivo* assays that are listed in Table 3.2. The most primitive pre-HSCs in YS are evaluated by their ability to grow blast colonies (BL-CFC) [130]. YS-derived cells studied in this particular assay are Flk-1 (Kdr)⁺ hemangioblasts that may give rise *in vitro* under appropriate culture conditions to both hematopoietic and endothelial cells [19, 20, 130]. Molecular analysis of cells isolated from these colonies has revealed that they express genes associated with both hematopoietic and vascular developments. The most important genes are Flk-1, Scl/Tal1, VE-cadherin, GATA1, fetal β H1 globin, and β -major globin [20, 130]. Moreover, cells isolated from solubilized BL-CFC, after plating in methylcellulose, grow into primitive erythroid and macrophage colonies in the presence of appropriate growth factors [131–133].

In contrast to YS-derived murine HSCs, more differentiated HSCs can be assayed for their ability to establish long-term (>4–6 months) hematopoiesis after transplantation into recipient mice. Cells from these mice can be further assayed for their ability to reconstitute secondary recipients [134, 135]. Unfortunately, as reported murine pre-HSCs display defective homing in adult animals [136–138]. There are several possible explanations for this defect including (i) lack of expression of MHC-I antigens which makes these cells susceptible to recipient NK cell attack, (ii) poor responsiveness of pre-HSCs to chemoattractants that are required for colonization of adult BM (e.g., defective SDF-1-CXCR4 signaling), (iii) lack of a proper repertoire of adhesion molecules, or (iv) lack of production/secretion of matrix metalloproteinases crucial to cross blood-BM barrier on the way to hematopoietic microenvironment [139–141].

Table 3.2 Assays to evaluate murine and human pre-HSC and HSC

Source of HSC	Mouse	Human
Hemangioblast (<i>yolk sac</i>)	BL-CFC in vitro assay	Not available
Pre-HSC (<i>AGM, YS</i>)	Expansion on OP9 stroma support Expansion on AGM-derived endothelial cells Transplantation “in utero” into embryos transplantation into NOD/Shi-scid/IL-2R γ^{null} (NOG) mice Transplantation into newborn liver Intra-bone injection	Not available
HSC (<i>fetal liver, BM, UCB, PB</i>)	Transplantation into syngeneic mice followed by transplantation into secondary recipients	Clinical results of hematopoietic transplants Engraftment in NOD/Shi-scid/IL-2R γ^{null} (NOG) mice (<i>SRC assay</i>), humanized SCID mice (hu-SCID), or engraftment in large animals (into sheep or goat fetuses)

Therefore, pre-HSCs isolated from embryonic tissues are assayed in vitro for their hematopoietic potential in special conditions that favor development/maturation of classical embryonic stem cells (ESCs). This includes co-culturing with cell lines derived from murine embryonic tissues such as the AGM-derived endothelial cell line AGM-S3 [142]. Since the stromal cell line OP9 (derived from the skull bones of osteopetrotic op/op mice) may also support the expansion/maturation of pre-HSCs, OP9 cells are employed to stimulate proliferation of pre-HSCs [27, 143, 144]. Important to mention that the hematopoietic cells isolated from cultures of pre-HSCs over AGM-S3 or OP9 cell lines acquire the ability to grow hematopoietic colonies in vitro and most importantly engraft in lethally irradiated mice. Furthermore, pre-HSC derived from the YS or AGM regions could also be transplanted in utero into recipient E.18.0 embryos. In order to facilitate this approach, pregnant mice are exposed to sub-myeloablative doses of busulfan [28].

Murine pre-HSCs that have homing defects and do not express high MHC-I antigens and thus are susceptible to NK attack could also be assayed by employing (i) direct intra-bone injection, (ii) transplantation into newborn livers, or (iii) transplantation into Rag2^{-/-} mice that lack lymphocytes or Rag2^{-/-} γ c^{-/-} mice that lack both lymphocytes and more importantly NK cells [145]. Another popular immunodeficient mouse model employed as recipients of HSCs is NOD/Shi-scid/IL-2R γ^{null} (NOG) animals [47].

Murine FL-derived HSCs are able to engraft long term and establish hematopoiesis in adult mice. Interestingly, similar evidence does not exist for human HSCs isolated from FL. In the past, a number of studies were performed in attempt to employ these cells for hematopoietic transplants, but results were disappointing. It is not clear at this point whether human FL cells have defective engraftment in adult BM as hematopoietic transplants with HLA-matched human FL cells have not yet been performed in the clinic.

The most valuable and conclusive preclinical assay for studying human HSCs is one that demonstrates that they engraft long term in immunodeficient mice. In this surrogate transplant assay, human hematopoietic cells are tested as xenotransplants to evaluate the number of hematopoietic–repopulating cells that differentiate into multilineage mature cells and self-renew in mouse, and these cells are defined as SCID mouse-repopulating cells (SRCs) [145]. SCID mice lack T and B lymphocytes but possess NK cells; therefore it is more ideal to use NOD/SCID mice with some defects of NK cells. To enhance engraftment of human cells in NOD/SCID, these mice could be pretreated with intraperitoneal injections of TM- β 1, a monoclonal antibody against murine IL-2R β , to eliminate any remaining NK cell activity [146]. Alternatively, human HSCs could be tested in Rag2^{-/-} or even Rag2^{-/-} γ c^{-/-} mice that, as mentioned above, are deficient in both lymphoid and NK cells [145]. Recently, another suitable recipients employed for human HSCs are highly immunodeficient NOD/Shi-scid/IL-2R γ ^{null} (NOG) animals [47]. A useful modification of xenotransplant is transplantation of human HSCs into the so-called humanized mice, immunodeficient animals that carry human tissue implants (e.g., fragments of bones) [48, 49]. This provides a more suitable physiological microenvironment for human cells [50]. Xenotransplants in NOD/Shi-scid/IL-2R γ ^{null} (NOG) mice that give rise to all hematopoietic/lymphoid lineages are currently the best surrogate assay for human HSCs. Nevertheless, because of very low posttransplant human-murine chimerism, the question remains how much real engraftment occurs in this model as opposed to a simple “lodging” and survival of HSC due to the immunodeficient state of the recipient mouse. Human HSCs could also be tested after intrauterine injection into developing fetuses in sheep or goat [46]. Nevertheless, it is an expensive procedure.

In clinical settings, evidence of hematopoietic reconstitution with BM, UCB, or mobilized PB-derived cells indicates involvement of transplanted HSCs. Hematopoietic transplants with a more purified population of HSC (CD34⁺ or CD133⁺) are not performed on routine basis in humans owing to the risk of losing engraftment potential. It is speculated that purification of HSCs may deplete transplant from engraftment facilitating cells.

Finally, evidences are accumulating, suggesting human UCB contains more primitive HSCs that do not engraft optimally after intravenous injection (CD34^{fl}-Lin⁻ cells) [147]. These cells show poor responsiveness to SDF-1; in contrast, these cells engraft much better after introducing cells directly to the mouse bone marrow or intra-bone marrow (iBM) and minimize the factors that interfere with homing of HSCs during intravenous injections [147–150].

3.11 Conclusion

The compartment of HSCs and HPCs has been intensively investigated over the past years. The progress in this area is marked by the development of new experimental tools such as omics strategies. These strategies allow better understanding

of the mechanisms that govern self-renewal and differentiation of HSCs. Furthermore, proteomic approach and progress in phage display libraries lead to the discovery of new markers that are helpful for isolating HSCs. In parallel, there is advancement in better developed imaging in vivo systems, microfluidic devices, and new generations of cell sorters. In addition to mammals, there are other low vertebrates such as zebra fish that are employed as models in studying hematopoiesis [97, 98]. Moreover, progress in techniques of cell labeling, for example, using iron oxide nanoparticles, which allows tracking of HSCs in vivo by magnetic resonance imaging (MRI) and application of in vivo imaging strategies, opens new possibilities for studying HSC homing and migration in living animals. Finally, further studies are needed to assess the biological and physiological significance of VSELs' presence in BM and their potential supply as a pool of long-term repopulating HSCs. In particular, hematopoietic specification of these cells will provide a new source of HSC for transplantation.

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Chapter 4

Neural Stem Cells



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Abstract Neural stem cell (NSC) transplantation has provided the basis for the development of potentially powerful new therapeutic cell-based strategies for a broad spectrum of clinical diseases, including stroke, psychiatric illnesses such as fetal alcohol spectrum disorders, and cancer. Here, we discuss pertinent preclinical investigations involving NSCs, including how NSCs can ameliorate these diseases, the current barriers hindering NSC-based treatments, and future directions for NSC research. There are still many translational requirements to overcome before clinical therapeutic applications, such as establishing optimal dosing, route of delivery, and timing regimens and understanding the exact mechanism by which transplanted NSCs lead to enhanced recovery. Such critical lab-to-clinic investigations will be necessary in order to refine NSC-based therapies for debilitating human disorders.

Keywords Stem cell therapy · Graft · Stroke · Fetal alcohol spectrum disorders · Cancer · Neural stem cell · Preclinical research · Clinical research

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4.1 Background

Neural transplantation has become a topic of strong interest in stem cell therapy, as stem cell transplantation is a promising treatment for several diseases and disorders that currently lack a cure. Initially, cell therapy was focused on neurodegenerative disorders such as Parkinson's disease, Huntington's disease, stroke, and traumatic brain injury but is now being evaluated for its potential to ameliorate neuropsychiatric disorders [43]. Neural stem cells (NSCs) are multipotent cells possessing a great capacity for self-renewal and the ability to generate progeny cells that differentiate into neurons, astrocytes, and oligodendrocytes [94]. Compared to embryonic stem cells (ESCs), NSCs are more restricted in their ability to differentiate but lead to all cell types necessary for the central nervous system (CNS) and possess a minimal risk of tumorigenesis [8, 60]. Endogenous stem cells are broadly observed in the subgranular zone of the dentate gyrus, the subventricular area, and the subependymal region in the spinal cord [22]. Following ischemic insult in stroke models, NSCs in these specific regions are sharply upregulated. While NSCs are found in the injury site, they are unable to support functional repair [50]. Recent evidence suggests that injecting exogenous stem cells can stimulate endogenous stem cell production in the infarct site [32, 52]. Interestingly, these exogenous NSCs do not replace injured cells but create a microenvironment [14, 16] that induces endogenous neurogenesis [15, 37, 86]. Thus, NSCs may be useful in overcoming the restrictions associated with current graft materials or gene transfer vehicles. In fact, NSC transplantation represents an important therapeutic advancement that may be useful for treating different neurodegenerative disorders, as well as stroke and other injuries. Current studies indicate that NSCs grafts are capable of integrating into the host tissue and improving behavioral deficits in a variety of *in vivo* models, including Alzheimer's, Parkinson's [13], and Huntington's disease [38, 40].

Numerous logistical difficulties interfere with stem cell transplantation, especially in clinical applications. NSC quantities and conditions require optimization, the ideal transplantation window after injury is small, and the dosage timing (i.e., number of transplantations within a determined period) needs to be regulated. Most preclinical experiments for neurological conditions, such as traumatic brain injury and stroke, have used a single-dose treatment [5, 29, 76–78]. Recent findings indicate that the minority of these neurological disorders are consequences of acute brain injury. On the contrary, a greater number are caused by chronic neurodegenerative disorders [4, 10, 56], indicating that more than a single dosage is necessary to ameliorate clinical outcomes. With multiple dosages of stem cells, it is possible to optimize clinical outcomes for brain disorders [20, 23, 64, 82]. NSC transplantation depends on the quantities of the delivered cells, but it is evident that more cells do not always guarantee better results. In fact, transplanted FGF-2-responsive human precursor cells (PNPCs) are not able to survive with a density below 1 million cells [51]. However, low-density PNPC transplants show the same ability to proliferate and expand their fibers to the CNS compared to higher density transplants and yet have the added advantage of avoiding an adverse immune response,

which typically occurs with larger grafts. Moreover, transplantation at very high densities (around 2 million cells) does not induce enhanced differentiation or fiber growth into the host tissue [51]. This information will assist in translating stem cell therapy to the clinic and highlights the necessity of additional research in order to better understand the optimal dose, route, and timing for transplanting cell grafts.

Another obstacle involves finding a suitable source of NSCs for transplantation treatments. Autologous transplantation may be less ideal, as harvesting these cells from patients necessitates major surgical procedures. Instead, NSCs can be retrieved from the CNS tissue of humans postmortem, from ESCs, or from allogeneic fetal tissues. Utilizing these cells for CNS regions lacking neurogenic activity will first require the cells to be expanded *in vitro* and differentiated. Moreover, to facilitate differentiation of the NSCs to the desired neuronal types, regulatory signals from the exogenous stem cells or from further interventional treatments may be necessary. Alternatively, partial differentiation mediated by stem cells such as ESCs or induced pluripotent stem cells can be used to create NSCs and other stem cell lineages [11, 73].

Moreover, as stem cells have the ability to form tumors, precautions are necessary to avoid this outcome. Adult stem cells are less prone to tumorigenesis, likely due to their limited proliferation. In contrast, ESCs grow without differentiation and, thus, are more sensitive to tumor formation. For instance, transplantation of NSCs collected from fetal sources into a child with ataxia telangiectasia resulted in the development of tumors, as the transplanted cell population was heterogeneous and not all cells were characterized as NSCs [7]. Using purified populations of stem cells for transplant therapies will be crucial to circumvent tumorigenesis [30, 63].

Furthermore, collecting sufficient quantities of cells for transplantation is another problem preventing successful cell therapy in the clinic. While ESC or fetal stem cells have high capacities for proliferation, there are several techniques to counter the low proliferation rates of stem cells taken from adult sources. For example, an England-based stem cell therapeutics company, ReNeuron Ltd., has added oncogenes such as a c-Myc regulator gene and a mutated estrogen receptor transgene [55]. Additionally, with the possible side effect of generating undesired non-NSCs, proliferation can be increased by long-term cell culture [83]. As an alternative, cells can be immortalized, as demonstrated by the human teratocarcinoma immortalized neural cell lines [73].

4.2 Neural Stem Cell (NSC) Applications

4.2.1 *Stroke*

As mentioned above, NSCs are multipotent cells able to differentiate into neurons, astrocytes, and oligodendrocytes, making them valid candidates for stroke treatment [94]. As a consequence of stroke insult, activity in the subgranular and subventricular zone is enhanced, and NSCs are able to dynamically migrate to the injured tissues [91]. This recruitment is of considerable relevance in stroke therapy.

Nonetheless, the neuroprotective mechanism triggered by NSC therapy is not clearly understood.

Various *in vitro* and *in vivo* NSCs investigations reveal fundamental concepts regarding the neurogenic, angiogenic, and neuroprotective characteristics triggered by these stem cells. For instance, it is possible to culture NSCs *in vitro* by only adding EGF (epidermal growth factor) and bFGF (basic fibroblast growth factor) [12, 65], and these NSCs are able to differentiate into all three primary neural cells [71]. In addition, endogenous NSCs enhance angiogenesis after stroke injury [90, 91]. Interestingly, the hypoxic environment (that occurs during stroke) triggers the increased differentiation of NSCs into neural cell types, thus, supporting neurogenesis. In fact, NSCs respond to hypoxic environments, differentiating faster compared to control conditions [12]. Moreover, a sharp increase in PI3k/Akt phosphorylated proteins occurs, suggesting that NSCs differentiation is strictly dependent on this signaling pathway [12]. NSCs in hypoxic conditions experience a quiescent state, and low oxygen levels impede differentiation, indicating the existence of a minimum and maximum oxygen levels that are necessary for NSCs to exert therapeutic effects [65]. Furthermore, it has been demonstrated that NSCs react positively to miR-381 overexpression, NFkB (nuclear factor kappa B), as well as Nox4 (NADPH oxidase 4)-oxidative stress [71, 80, 92]. In contrast, direct contact with endothelial cells induces NSCs to quiescence, inhibiting the differentiation process [18]. Additionally, elevated glucose concentrations in NSCs in oxygen and glucose deprivation (OGD) conditions induce apoptosis and decrease proliferation via JNK/p38 MAPK pathway activation [14–16]. A key part in counteracting inflammatory pathway activity is exerted by NSCs exosomes, including VEGF, BDNF, NGF, and other neurotrophins [48], but the mechanism needs to be elucidated.

It is well known that in *in vivo* models, NSCs are able to migrate into the injured tissue after stroke [25, 91]. In fact, after ischemic stroke, NSCs are directed toward the infarct core through chemokine signals released from the injured tissue, such as SDF-1 (stromal cell-derived factor 1), VEGF (vascular endothelial growth factor), and angiopoietin [25]. Exogenous implantation of NSCs represents a potential therapy *in vivo* with neuroprotective effects and the ability to restore neural circuits. In the MCAo rat model, human NSCs transplanted intravenously ameliorate motor and cognitive conditions as well as decrease the core infarct volume relative to controls [70]. Moreover, exogenous NSC transplants increase endogenous proliferation and differentiation and induce angiogenesis [90]. Comparable results occur in a photothrombotic stroke model, in which rats transplanted with NSCs show enhanced behavioral recovery compared to controls [27]. This finding also supports the notion that NSCs differentiate into astrocytes and neurons *in vivo* [27]. The survival and differentiation process of NSCs depends on inflammatory pathways and on pro-inflammatory T-cell activity. Indeed, the quantity of endogenous NSCs decreases significantly with glucocorticoid-induced TNF receptor expression [79]. Despite this, it has been reported that NSCs exert anti-inflammatory effects *in vivo*. For instance, rodent stroke models, following intravenous transplantation of NSCs, show decreased OX-42+ microglia and MPO+ infiltration into injured tissue, as well as

significant reduction in TNF- α , IL-6 and NF- κ B expression in the brain and spleen [39]. Furthermore, NSCs transplanted in MCAo rats induce a significant reduction in inflammatory activity, decrease pro-inflammatory cytokine expression, reduce adhesion molecule numbers and microglial activity, and restore the blood-brain barrier following injury [28]. In order to better understand NSCs' anti-inflammatory potential, the decreased survival level of NSCs due to neuroinflammation needs to be considered, with respect to its pro-proliferative activities.

In conclusion, most studies indicate that NSC transplantation is able to ameliorate brain functions, and a recent meta-analysis supports NSCs' preclinical efficacy for ischemic stroke therapy in vivo [17]. It is noteworthy, as explained in the meta-analysis, that NSC efficacy depends on the donor, which should be the same species as the recipient and on the treatment time window (within 72 hours to achieve a better outcome) [17]. However, the mechanism underlying NSCs' neuroprotective effects needs to be elucidated. It likely includes, as mentioned above, the overall combined effects of NSCs' exosomes, cell/neuron replacement, and enhanced endogenous NSCs proliferation [28, 44–46, 48, 71, 90].

4.2.2 Fetal Alcohol Spectrum Disorders (FASD)

Neurological disorders are characterized by a loss of neurons or loss of neuronal function which cannot be recovered due to the adult brain's limited ability to generate new functional nerve cells post-injury from mature neurons and endogenous NSCs. Indeed, traumatic brain injury and stroke result in a loss of an array of neurons, and Parkinson's disease is typically marked by a reduction of the substantia nigra's dopaminergic neurons. Moreover, lateral sclerosis leads to the loss of spinal cord motor neurons, resulting in an inhibition of muscle activation, and multiple sclerosis causes a loss of the oligodendrocytes that protect neurons. Given the nervous system's minimal regenerative capacity after injury, stem cell transplantation has gained traction as a potential therapy to replace lost or damaged neurons, and grafted stem cells have successfully survived and integrated into the brain in recent studies involving diseased animal models [75].

Clinical psychiatric conditions may be attributed to cerebral structural modifications, as indicated by recent studies. In particular, symptoms of psychiatric disorders may be mitigated by disrupting neural systems with ineffective neurogenesis. Thus, NSCs may be a viable treatment for fetal alcohol spectrum disorders (FASD) such as fetal alcohol syndrome, given the association of FASD with neurodegeneration. Indeed, NSC transplantation in rat FASD models recovers the levels of post-synaptic density protein 95 in the anterior cingulate cortex, amygdala, and hippocampus and amends the decrease in GABAergic interneuron PV-positive phenotypes caused by alcohol. In addition, anomalous social behaviors and cognitive deficits are remedied after intravenously transplanting NSCs in rats exposed to alcohol in utero [74]. Moreover, injecting NSCs intravenously in rat models for fetal alcohol effects results in NSC migration to various brain regions and improves

behavioral deficits [89]. While it is uncertain how NSCs exert their therapeutic effects in FASD, it is speculated that they may promote endogenous neurogenesis or provide neuroprotection via increased levels of trophic factors [6, 54, 87]. Furthermore, there may be another mechanism underlying how transplanted NSCs replace lost neurons and integrate into host tissues. To fully evaluate NSCs' potential benefits in neuropsychiatric disorders such as FASD, further preclinical research is warranted.

Stem cell therapies may be effective during early embryonic development given the developing brain's considerable plasticity and may amplify already active endogenous repair mechanisms that can ameliorate the outcomes of congenital abnormalities [62], which affect around 5% of all live births annually [53, 61]. Of note, delivering stem cells in the uterus may boost the treatment's efficacy as the fetus's incomplete immune system enables a lower chance of host rejection of the transplanted cells, the cells are administered during an earlier time window, and the fetus's swift growth can potentially multiply the grafted cells [49]. Indeed, injecting stem cells into the uterus during the period of accelerated brain growth is crucial, as evidenced by the ability of transplanted mesenchymal stem cells to correct sensory and motor anomalies associated with spina bifida aperta [41].

Additional information and evidence need to be provided before NSCs can be utilized clinically for FASD. For instance, NSCs cannot differentiate into the ideal cell type to ameliorate behavioral deficits and brain damage with complete certainty. Furthermore, the mechanism in which neural progenitor cells migrate after transplantation is not clear, though antigen, cell surface receptor, and cytokine signaling or breakdown of the blood-brain barriers enable human NSCs administered intravenously to reach the injured brain tissue in stroke model studies [9, 47].

Creating an ideal method for stem cell delivery is another dilemma impeding the successful execution of stem cell therapies for neuropsychiatric disorders such as FASD. Additional injury to the host can be generated from intracerebral injections, which are less precise than the accurate stereotaxic surgeries involving specific CNS coordinates. Of note, stem cells have needed intraparenchymal injections to be effective in several studies involving neurodegenerative disease model animals. In these cases, the size and extent of the main lesion determine the efficacy of neural transplantation. However, intravenous administration of NSCs may be more suitable for psychiatric disorders which involve more complex, unknown pathologies and may not be directly associated with the magnitude of the neural lesion. It will be imperative to evaluate the effectiveness and safety of injecting stem cells systemically for psychiatric disorders such as FASD in future investigations.

4.2.3 Cancer

Brain metastasis is a significant cause of mortality and morbidity associated with cancer as it may swiftly disrupt CNS function. It is the most prominent intracranial neoplasm, occurring in 10–40% of cancer patients [24]. Additionally, brain metastasis

eventually manifests in around 40% of all individuals afflicted with lung cancer and expresses the highest incidence in primary lung cancer [67].

While radiation and surgery are effective for treating an isolated metastasis, these are ineffective therapies for the multiple metastases present in many patients [69]. Radiation therapy for the entire brain and system-wide chemotherapeutics are potential treatments for multiple metastases, but the efficacy of chemotherapy is limited by the development of drug resistance in the body and the blood-brain barrier, which prevents proper delivery of the chemotherapeutic to the brain; moreover, radiation is accompanied by radiation necrosis, reduced cognition, and other unfavorable side effects [1]. Thus, a new treatment regimen for brain metastases that circumvents these issues is warranted.

NSCs may bolster chemotherapy treatments with their innate tumor-tropic characteristics and provide a novel technique for delivering therapeutic genes to tumors in the brain [66]. In this case, NSCs, which migrate selectively to brain tumors, can administer beneficial gene products specifically to invasive tumors present in multiple regions, acting as a vehicle for treatment distribution [36]. Indeed, human NSCs expressing the cytosine deaminase-herpes simplex thymidine kinase fusion gene or yeast cytosine deaminase gene increase survival rates and decrease tumor sizes in animals with brain metastases from lung cancer [81, 88]. Treating brain tumors with human NSCs possessing therapeutic suicide genes such as carboxylesterase (CE), which hydrolyzes the prodrug CPT-11 (irinotecan) to a topoisomerase I inhibitor and selective killer of cells undergoing cell division, SN-38 (7-ethyl-10-hydroxy-camptothecin) [42, 68, 93], and cytosine deaminase, which converts the prodrug 5-fluorocytosine into the tumor-killing 5-fluorouracil [2, 19, 31, 33–35, 72, 88], shows promising results. In both in vivo and in vitro settings, A549 human non-small cell lung adenocarcinoma cell growth is suppressed by treatment with both CPT-11 and human NSCs expressing rabbit CE. SN-38 is produced after activating CPT-11 with the CE enzyme, enabling the NSCs with the CE gene to selectively deliver chemotherapeutics to brain tumors. This dual treatment regimen enhances survival and reduces tumor volumes in mice, advocating this paradigm for metastatic brain tumors derived from lung cancer [26].

Evidently, both primary and distant metastatic lung cancer can be treated by NSCs, and this NSC transplantation therapy is currently being evaluated for recurrent glioblastoma in a phase I clinical trial (clinical trial ID NCT01172964; <http://clinicaltrials.gov/ct2/show/NCT01172964>). NSCs may bypass disadvantages present in current treatment regimens with their revolutionary ability to specifically administer high levels of chemotherapy drugs to tumors. Additionally, gene therapy for brain tumors may take advantage of the beneficial properties of NSCs that have been modified genetically or immortalized [3, 36].

CPT-11 has potential for treating a variety of tumors in the brain and is a chemotherapeutic that can successfully cross the blood-brain barrier [3, 42, 68, 93]. Of note, CPT-11 is participating in phase II clinical trials for malignant gliomas and is currently approved for their treatment [58, 59]. However, the primary issue in utilizing CPT-11 for metastatic brain tumor therapy is finding an ideal dosage to compromise between efficacy and CPT-11-associated toxicity, which includes

hepatotoxicity, leukopenia, severe gastrointestinal problems, and immune system disruption [21, 57, 59]. Adding bevacizumab and other chemotherapy drugs as a combination therapy is a potential solution that limits the necessary concentration of CPT-11 while increasing the overall efficacy against tumors by inhibiting tumor growth through other routes [21]. Moreover, local administration of CE may limit overall toxicity while maintaining potency against tumors and may even augment the effects of combination therapy.

4.3 Future Directions

Ultimately, NSCs can be derived from various sources such as human fetal tissues, induced pluripotent stem cells, and postmortem human CNS tissues, and accumulating evidence has supported the therapeutic potential of transplantation of NSCs in a myriad of human disorders (Fig. 4.1). However, translational studies are necessary to improve NSC purification and culturing procedures in order to stimulate homologous cell proliferation and to reduce tumorigenicity. Furthermore, graft dosage, route of administration, and poststroke timing need to be optimized.

As mentioned above, NSCs' neuroprotective mechanism needs to be elucidated and is fundamental in order to translate NSCs to clinical therapies. Future research should also examine NSC transplantation combined with other cell types or drugs to evaluate if therapeutic activity is amplified. For instance, in a stroke model, combined treatment with kallikrein gene counteracts inflammation and induces neurogenesis, generating a more tolerable environment for NSCs [84, 85]. With these promising results, additional studies should analyze other possible combination treatments.

NSCs have potential for treating neuropsychiatric disorders such as FASD, as evidenced by successful preclinical studies utilizing FASD animal models. NSC transplantation therapies for FASD can be refined by conducting additional studies that fully entail the genetic, molecular, and cellular profiles of FASD. Highly clinically relevant FASD models will be necessary to produce an NSC transplant regimen that is safe and effective and probe how neuropsychiatric disorders such as FASD are improved by NSCs. Moreover, NSC injections should ideally be performed in the uterus, while the brain is developing at an accelerated rate, and there is a higher possibility of an improved outcome [41, 49, 62]. It may also be worthwhile to test if systemic administration of NSCs is more effective for treating neuropsychiatric disorders such as FASD, which may have more complex, unknown pathologies. Furthermore, actions must be taken to avoid tumor formation, such as by ensuring that the transplanted cells are derived from a purified, homogenous population [30, 63].

Administering F3 human NSCs transduced with the CE gene and accompanied by CPT-11 appears to be an effective treatment for lung cancer–derived brain metastases [26]. Thus, it will be imperative to evaluate if this model of utilizing NSCs to

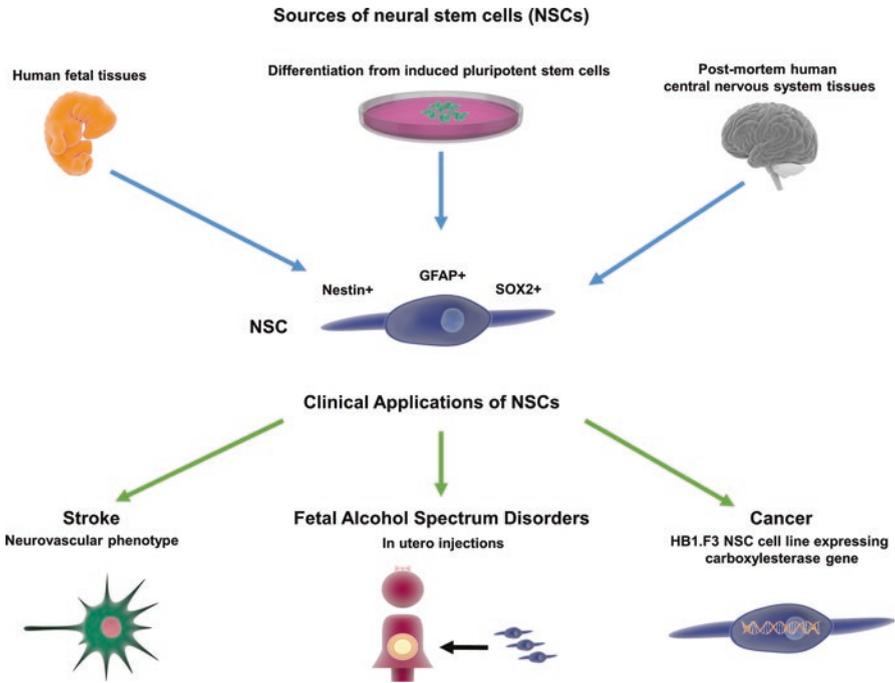


Fig. 4.1 Sources of neural stem cells (NSCs) and its clinical applications. NSCs can be derived from human fetal tissues, from postmortem human central nervous system tissues, and from differentiation from induced pluripotent stem cells. NSCs express markers such as nestin, GFAP, and SOX2. NSC transplantation has potential as a clinical therapeutic. In stroke, NSCs can differentiate into cells of the neurovascular phenotype. Additionally, in utero injections of NSCs may be effective for treating fetal alcohol spectrum disorders. Moreover, HB1.FE NSC cell lines transduced with the carboxylesterase gene can be administered with CPT-11 to selectively target metastatic tumors

deliver chemotherapeutics specifically to tumors can also be used to treat metastatic tumors in other tissues besides the brain or metastatic tumors that are derived from other forms of cancer. Additionally, positive outcomes from clinical trials such as the phase I clinical trial involving NSCs for glioblastoma and the phase II clinical trial for CPT-11 and malignant gliomas will provide further evidence for this NSC-mediated therapy's clinical effectiveness. For the treatment paradigm of NSCs and CPT-11, exploring ways to reduce the accompanying toxicity of CPT-11, replacing CPT-11 with a less toxic but highly potent chemotherapeutic, or adding an additional synergistic compound to increase antitumor efficacy while lowering toxicity will be a welcome achievement. The concept of utilizing NSCs' selective migration to tumors and a gene present in NSCs to convert an inert compound to an active chemotherapeutic can advance the development of cancer therapies that minimize damage to healthy tissues and maximize the inhibition of tumor growth.

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Chapter 5

Therapeutic Potential of Mesenchymal Stem Cells in Immune-Mediated Diseases



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Abstract Mesenchymal stem cells (MSCs) are multipotent cells that can self-renew and differentiate into cells of all germ layers. MSCs can be easily attracted to the site of tissue insult with high levels of inflammatory mediators. The general ability of MSCs to migrate at the sites of tissue injury suggested an innate ability for these cells to be involved in baseline tissue repair. The bone marrow is one of the primary sources of MSCs, though they can be ubiquitous. An attractive property of MSCs for clinical application is their ability to cross allogeneic barrier. However, alone, MSCs are not immune suppressive cells. Rather, they can be licensed by the tissue microenvironment to become immune suppressor cells. Immune suppressor functions of MSCs include those that blunt cytotoxicity of natural killer cells, suppression of T-cell proliferation, and “veto” function. MSCs, as third-party cells, suppress the immune response that generally recapitulates graft-versus-host disease (GvHD) responses. Based on the plastic functions of MSCs, these cells have dominated the field of cell-based therapies, such as anti-inflammatory and drug delivery. Here, we focus on the potential use of MSC for immunological disorders such as Crohn’s disease and GvHD.

Keywords Mesenchymal stem cells; Cytokines · Cell therapy · Drug delivery · Immune response · Graft-versus-host response

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5.1 Introduction

MSCs are multipotent stromal cells with multi-lineage differentiation potential. MSCs differentiate into various cell types, depending on the location and associated microenvironment [1, 2]. Among these cell types are mesodermal cells (chondrocytes, myocytes, osteocytes, and adipocytes), ectodermal cells (neurons), and cells of endodermal lineage (hepatocytes) [3]. MSCs represent a small percentage of stem cells in the bone marrow (BM), comprising 0.001–0.1% of total cells [4, 5]. In the BM, MSCs play a crucial role in the maintenance of the hematopoietic stem cell (HSC) niche [6]. MSCs are present in different regions of the BM, including the vascular regions [7]. Since other cells entering and leaving the BM must bypass the vascular MSCs, they are termed “gate-keepers” of the BM.

In addition to the BM, MSCs can be isolated from adipose tissue, amniotic fluid, peripheral blood, the placenta, and Wharton’s jelly [8, 9]. MSCs are heterogeneous in nature, but they can be expanded as adherent cultures. MSCs are phenotypically well-described, expressing CD29, CD73, CD90, and CD105, while negative for hematopoietic markers such as CD14, CD34, and CD45 [10]. In an unstimulated phase, MSCs could express MHC-II, and this receptor has been shown to be functional, causing the MSCs to exert antigen-presenting properties [11]. Although there are common properties of MSCs, one needs to be cautious that the phenotype and function could be influenced by the age, donor, and culture method.

During the past decade, there is an increase in MSC-based therapies [12]. The therapeutic applications of MSCs arise partly because the microenvironment can change the secretome of MSCs [13, 14]. Additionally, MSCs can be easily isolated and expanded *in vitro* and *in vivo* and can migrate to sites of tissue injury or inflammation [15]. Another advantage is the ability of MSCs to cross allogeneic barrier, indicating that they can be used as “off-the-shelf” cells without requiring HLA compatibility [16]. Further, MSCs can be loaded with therapeutic agents such as microRNA for delivery to sites of inflammation and injury [17]. These diverse properties of MSCs underscore the promise for these cells in therapeutic strategies for a wide range of diseases.

5.2 Immunological Properties of MSCs

MSCs can modify major components of innate and adaptive immune responses, including T-cells, B-cells, natural killer cells, dendritic cells, macrophages, mast cells, and neutrophils. Though MSCs have been thought to be immune-privileged due to their ability to cross allogeneic barriers, MSCs may be immunogenic in certain contexts, thereby making them immune-evasive rather than immune-privileged [18, 19]. Nevertheless, these immunomodulatory properties make MSCs an attractive tool for immunotherapy as well as tissue regeneration. An overview of the interplay between MSCs and components of the immune system is provided in the following text.

5.2.1 T-Cells

A large research effort has been committed toward MSC interaction with effector T-cell subsets. MSCs inhibit T-cell proliferation in mixed lymphocyte cultures and mitogen-stimulated lymphocytes in a dose-dependent and MHC-independent manner [20]. These effects are partially mediated by soluble factors, including prostaglandin E2 (PGE₂), transforming growth factor- β (TGF- β), galectin-1, hepatocyte growth factor (HGF), heme oxygenase-1, and indoleamine 2,3-dioxygenase (IDO), the rate-limiting enzyme in tryptophan catabolism that induces effector T-cell apoptosis and regulatory T-cell proliferation [21–28]. It should be noted that while human MSCs utilize IDO, inducible nitric oxide synthase (iNOS) mediates immune suppression in mice [29]. Contact-dependent mechanisms also play a role, as MSC-expressed FAS ligand (FAS-L) promotes T-cell apoptosis, while ICAM-1 and VCAM-1 enhance adhesion to and suppress proliferation of T-cells [30–32].

MSCs suppress both CD4⁺ and CD8⁺ T-cell proliferation [33, 34]. Studies demonstrated that the cytotoxic capabilities of CD8⁺ T-cells are not reduced after coculture with MSCs [34]. MSCs have also been shown to modulate CD4⁺ T-cell differentiation along T-helper (Th) and regulatory T-cell (T_{regs}) lineages. In mouse models of multiple sclerosis, human BM-derived MSCs (BM-MSCs) diminished Th1 and Th17 responses while increasing Th2 responses [35]. Similar decreases in the Th1/Th2 ratio were observed in mouse models of systemic lupus erythematosus using human adipose tissue-derived MSCs (AT-MSCs) [36]. Further, MSCs inhibited the activation, proliferation, and differentiation of cells induced along Th1 and Th17 lineages, with simultaneous increases in T_{regs} [37]. Other studies have demonstrated a conflicting effect on T-helper cell polarization. In models dominated by Th2 responses, including allergic rhinitis and allergen-driven airway inflammation, Th2 responses were decreased alongside increases in Th1 responses and T_{regs}, respectively [38, 39]. Zhou et al. reported a higher Th1/Th2 ratio in four BM-MSC treated patients with sclerodermatous chronic GvHD [40]. Thus, the polarization of T-helper cell subsets by MSCs seems to be dependent on disease context.

CD4⁺CD25⁺FoxP3⁺ T_{regs} promote immune tolerance, in part through suppression of effector T-cell proliferation and stimulation of IDO release by dendritic cells [41]. MSCs induce T_{regs} via TGF- β , IL-10, PGE₂, and human leukocyte antigen class I molecule HLA-G5 [22, 37, 42–44]. Interestingly, the non-immunomodulatory properties of T_{regs} in tissue repair have been the subject of recent study. T_{regs} can induce cardiomyocyte proliferation in vitro and in vivo, independent of their immunosuppressive function [45]. This regenerative function of T_{regs} has been observed in murine models of the skeletal muscle, lung, and central nervous system injury [46–48]. Further study regarding the influence of MSCs on the regenerative abilities of T_{regs} is required.

5.2.2 *B-Cells*

MSCs impair B-cell proliferation, promote cell cycle arrest in G₀/G₁ phase, and increase B-cell viability, though some studies suggest that these effects require pre-treatment of MSCs with inflammatory cytokines [49–53]. B-cell differentiation into antibody-producing cells is also inhibited by MSCs [49–54]. Both cell-contact-dependent and cell-contact-independent mechanisms have been proposed [49–51, 54]. IDO and programmed cell death protein 1/programmed death ligand-1 (PD-1/PDL-1) were identified as mediators of reduced B-cell proliferation and activation, respectively [52, 54]. Cultures of MSCs and immune cells resulted in a decrease in B-cell antibody production, which was reversed with CD40L antibody, suggesting that MSCs exert their B-cell suppressive effects partially through interactions with T-helper cells [55].

Additionally, recent studies have focused on the ability of MSCs to induce immunosuppressive regulatory B-cells (B_{regs}) [56]. In models using murine BM-MSCs, human BM-MSCs, and human AT-MSCs, increases in IL-10 producing B_{regs} were observed [52, 53, 57].

5.2.3 *Natural Killer (NK) Cells*

MSCs inhibit proliferation of resting NK cells, with a reduced effect when MSCs were added to NK cell cultures after 7 days of IL-2-induced proliferation [58]. MSCs suppress NK cell cytotoxic activity and cytokine release, including interferon gamma (IFN- γ), via IDO, PGE₂, and HLA-G5 [44, 59]. However, MSCs may activate NK receptors Nkp30, NKG2D, and DNAM-1, thus stimulating NK lysis of both autologous and allogeneic MSCs [58]. Petri et al. demonstrated that while early MSC/NK cell interactions activate NK cells, later interactions inhibit NK cell effector function in poly(I:C)-activated MSCs via TGF- β and IL-6 [13]. Interestingly, senescent-like NK cells increased *VEGF* expression in MSCs, and the conditioned media from these MSCs promoted tube formation in human microvascular endothelial cells, suggesting an altered tissue-regenerative capacity of MSCs following interactions with NK cells [13].

5.2.4 *Dendritic Cells*

MSCs impede dendritic cell (DC) maturation via release of IL-6 and PGE₂ [59–61]. In addition to diminished DC function as antigen-presenting cells, murine MSCs further hamper DC migration to lymph nodes via downregulation of CCR7 and CD49d β 1, thus hindering DC priming of naive T-cells [62]. MSC modulation of DC phenotype is not limited to immature DCs, as mature DCs differentiate into a

regulatory DC (regDC) population upon coculture with MSCs [63]. These regDCs exhibit their immunosuppressive functions on lymphocytes in a contact-dependent mechanism via Jagged-2 [64].

5.2.5 Macrophages

Macrophages are a key component of innate immunity and play roles in tissue defense upon injury, as well as tissue repair [65]. Macrophages have been broadly categorized as either classically activated and proinflammatory M1 macrophages or alternatively activated and anti-inflammatory M2 macrophages [66]. MSCs promote M2 macrophage differentiation after coculture, stimulating macrophage release of IL-10 and IL-6, indicative of a M2b phenotype [65]. This interaction was accompanied by increased macrophage phagocytic activity [65, 67]. IDO has been implicated in MSC-induced M2 macrophage polarization [68]. Further, MSCs secrete TNF- α -stimulated gene 6 protein (TSG-6) to diminish inflammation promoted by resident macrophages, as observed in a murine model of zymosan-induced peritonitis [14]. In recent studies, MSC-induced M2 macrophages accelerated cardiac regeneration and spinal cord recovery in animal models of cardiac infarction and spinal cord injury, respectively [69, 70].

5.2.6 Mast Cells

BM-MSCs were shown to inhibit mast cell (MC) degranulation in vitro and in vivo [71]. The in vitro effect was greatest with MSC/MC contact, though MSC-derived PGE₂ was required for this effect in contact-dependent and contact-independent conditions [71]. PGE₂ and TGF- β were found to mediate suppression of MC degranulation in a mouse model of atopic dermatitis using human umbilical cord blood MSCs (hUBC-MSCs) [72]. Further, BM-MSC microvesicles ranging from 50 to 200 nm in size enhanced MC PGE₂ production in vitro and suppressed MC activation in vivo, reducing aneurysmal rupture in mice [73].

5.2.7 Neutrophils

Studies investigating MSC and neutrophil interactions are relatively scarce. MSCs inhibit neutrophil apoptosis via IL-6 and uptake apoptotic neutrophils in an ICAM-1-dependent manner [74, 75]. Additionally, MSCs reduce the respiratory burst of neutrophils via superoxide dismutase (SOD3), thus suppressing neutrophil protease release and extracellular trap formation [74].

5.2.8 *MSC Sources and Delivery*

MSCs are found in perivascular niches of all tissues [76]. Immunomodulatory effects have been demonstrated in MSCs isolated from bone marrow, adipose tissue, umbilical cord blood, and palatine tonsils [72, 77, 78]. Ribeiro et al. compared MSCs from bone marrow, adipose tissue, and umbilical cord matrix (UCM-MSCs) on T-cell, B-cell, and NK cell activities [78]. In this study, AT-MSCs reduced B-cell activation and immunoglobulin production to a greater extent than BM-MSCs, while UCM-MSCs did not have an effect [78]. Similarly, AT-MSCs more significantly prevented T-cell acquisition of lymphoblast morphology compared to BM-MSCs and UCM-MSCs [78]. In another study, conditioned media from UCB-MSCs, but not BM-MSCs, suppressed mast cell degranulation via PGE₂, likely in part due to differences in basal PGE₂ production between MSCs from these two sources [72]. In addition, the route of delivery played a role, as subcutaneous administration of MSCs ameliorated murine atopic dermatitis to a greater degree than intravenous application [72]. Thus, effective therapies will require robust characterization of the properties of MSCs from varying sources.

5.2.9 *Summary*

MSCs display a wide range of immunomodulatory properties utilizing both soluble and contact-dependent factors. Understanding the specific interactions between MSCs and immune responses, as well as the interplay between components of the immune system, is crucial in ameliorating inflammatory diseases. MSCs may also promote a microenvironment conducive to repair, as suggested by their induction of T_{reg} and M2 macrophage cell types [46–48, 69, 70]. Other major considerations include MSC source, dose, route of administration, and cell culture conditions. Indeed, MSCs seemingly must undergo a “licensing” process by IFN- γ and other inflammatory cytokines before exerting immunomodulatory effects [79].

A summary of MSC interactions with components of the immune system is provided in Fig. 5.1. The diagram displays MSC-derived soluble and contact-dependent mediators of immunomodulation, as well as the major effects observed on cell types of the immune response. In the context of an inflammatory microenvironment, MSCs exert immunosuppressive effects.

5.3 **Inflammation-Induced Homing of MSCs**

In order to use MSC-based therapeutics, an understanding of the mechanisms leading to MSC homing and engraftment is fundamental to successfully develop effective treatment strategies. In vivo studies that injected MSCs intravenously showed homing to different organs such as the lungs, liver, brain, and bone marrow [80–82].

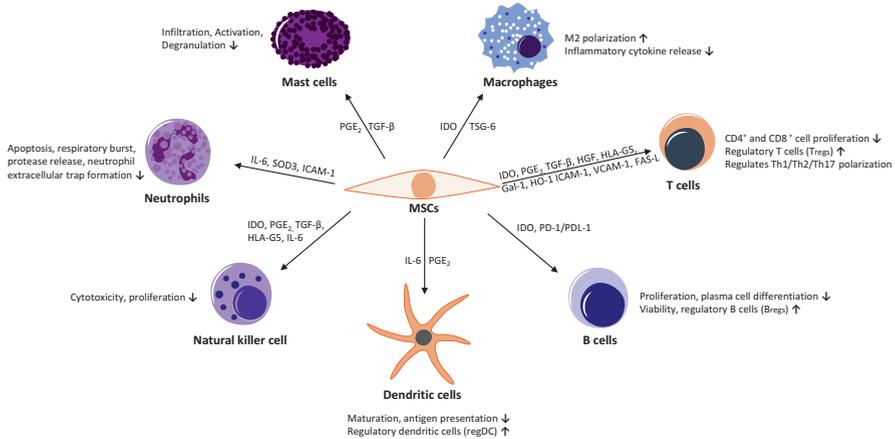


Fig. 5.1 Summary of MSC effects on the immune system and the soluble and contact-dependent factors involved. Abbreviations: FAS-L FAS ligand, Gal-1 galectin-1, HO-1 heme oxygenase-1, HGF hepatocyte growth factor, HLA-G5 human leukocyte antigen-G5, ICAM-1 intercellular adhesion molecule-1, IDO indoleamine 2,3-dioxygenase, IL-6 interleukin-6, MSC mesenchymal stem cell, PD-1/PDL-1 programmed cell death protein 1/programmed death ligand-1, PGE₂ prostaglandin E2, SOD3 superoxide dismutase, TGF-β transforming growth factor-β, TSG-6 TNF-α-stimulated gene 6 protein, VCAM-1 vascular cell adhesion molecule 1

The source of MSCs may determine the homing and engraftment efficiency to organs. MSC homing to various sites is also partly dependent on the expression of integrins, chemokine receptors, and growth factor receptors [83]. Mansilla et al. detected a significant increase in MSC in peripheral blood of acute burn patients compared to healthy donors [84]. This study supported the notion that MSC migrate to the site of injury by responding to inflammatory mediator. These specialized low molecular weight proteins are known as chemotactic cytokines or chemokines. One of the most studied chemokine receptors is for stromal cell-derived factor 1 (SDF-1), C-X-C chemokine receptor type 4 (CXCR4) [85]. In a mouse model of ischemia/reperfusion, SDF-1, through the CXCR4 receptor, enhances MSC homing to the site of injury [86]. The study also showed an increase in hepatic SDF-1, which was responsible for recruiting MSCs. By understanding the mechanism of how MSCs are recruited to a site of injury, scientists will be able to develop efficacious method and are more likely to use MSCs for tissue regeneration.

5.4 MSC-Based Drug Delivery

The ability of MSCs to migrate at sites of inflammation such as injured tissues, arthritic joints, and cancer formed the basis for these cells to be loaded with drugs to treat the injured regions [17]. Examples of such indications include chemotherapy, apoptosis inducers, immunomodulatory agents, virus, and angiogenic factors [17].

The proinflammatory cytokine, tumor necrosis factor-alpha (TNF- α), has been implicated as a promoter of disease pathogenesis in patients with rheumatoid arthritis (RA) [87]. Current therapeutic strategies include administration of TNF- α antibody etanercept, a fusion protein comprised of human soluble TNF receptor II and the Fc portion of human IgG1 [88]. In NOD/SCID mice, MSCs loaded with human soluble TNF receptor reduced serum TNF- α levels faster than etanercept following LPS administration [89]. Further, these transduced MSCs reduced paw swelling in a mouse model of antibody-induced arthritis and reduced knee swelling in a rat model of collagen-induced arthritis [89].

MSCs further showed the potential for drug delivery in the context of cancer therapy [17]. MSCs loaded with functional anti-miR-9 significantly decreased levels of miRNA-9 in glioblastoma cells, thus promoting their chemosensitization [90]. MSCs have additionally been packaged with immunomodulatory agents including IL-2, IL-12, and IFN- β to induce tumor-specific T-cell responses [91–93].

5.5 MSC Secretome

In addition to the role of MSC secretome discussed above, additional effects can occur by secreted biologically active molecules. An established MSC function includes its role as part of the hematopoietic niche to regulate the function of hematopoietic stem cells (HSCs) in the bone marrow [6, 94]. MSC-derived C-X-C motif chemokine ligand 12 (CXCL12), also referred to as SDF-1, is involved in HSC self-renewal and retention in the bone marrow [6, 94, 95].

In the context of tissue repair, MSCs can promote bone regeneration by differentiating into osteoblasts or by secreting angiogenic factors. In vivo studies have suggested effective bone repair by MSC-derived angiogenic factors such as VEGF and EGF [96]. The ability of MSC to home toward chemoattractants and the need to elicit tissue repair, this function could be pathological. Such function is demonstrated by the ability of MSCs to be recruited to tumor sites where they can release similar factors, including TGF- β 1, to promote tumor-associated angiogenesis [97]. In addition to cytokines, MSC-derived exosomes are also potent drivers of angiogenesis [98]. In vitro studies of endothelial cells, cocultured with MSCs, have reported on roles for miRNAs such as miR-222, miR-21, and let-7a in angiogenesis. These miRNAs, when loaded into the MSC-derived exosomes, were responsible for tube-like formation, mobilization, and blood flow [97, 99]. Future work may seek to modify MSCs in a way to safely promote the therapeutic abilities of their secretome. MSCs, transduced with AKT1-expressing lentiviral vector, caused a significant increase in VEGF production, and this improved the vascular density in a swine model of myocardial infarction [100].

5.6 MSCs: Cell Therapy for Crohn's Disease (CD) and Graft-Versus-Host Disease (GvHD)

Since the first successful BM transplant in 1956 by Dr. E. Donnall Thomas, scientists have adapted the method in a growing field of regenerative medicine. The purpose is to uncover new therapeutic advancements with cells to provide replace pharmacological molecules that are likely to have untoward effects. This game-changing era of stem cell therapy has the potential to combat diseases such as CD and GvHD [3, 101]. MSC has been in the spotlight because of their ability to migrate to various sites of the body, including crossing the blood-brain barrier (BBB). MSCs have been shown to elicit immunosuppressive effects which are useful in organ transplantation [3]. During organ transplantation, a patient (host) is the recipients of stem cells from a healthy donor known as “the graft.” In the event of HLA mismatch, the donor's T-cells will elicit an immune response against the host healthy tissues, hence the term graft-versus-host disease. GvHD can either be acute or chronic – either which can potentially be fatal.

The human leukocyte antigen (HLA) class I is surface proteins responsible for identification of “self” [102]. GvHD is an example of an immune-mediated disease that could result in failure of B transplant. MSC treatment is a prime candidate to suppress immune response caused by GvHD [103, 104]. In the acute form of GvHD, MSCs have been shown to inhibit the proliferation and cytotoxicity of immune cells [105].

CD is another immune-mediated disease caused by chronic systemic inflammatory disease of the gastrointestinal tract. This disease occurs when the host immune system defends the body against gut microbiome causing severe injury to the intestine such as ulcers [106, 107]. This inflammatory bowel disease (IBD) can affect the quality of life, and the causes of CD are poorly understood. Studies have reported MSCs to be a useful therapeutic alternative oppose to costly steroids, antibiotics, and anti-TNF agents [107].

In addition to the two trials discussed in this section, there are currently many clinical trials using MSC to treat immune-mediated diseases. We have examined the data in clinicaltrials.gov and have noted more than 20 studies to treat CD with MSCs and more than 35 for GvHD (Fig. 5.2).

5.7 Conclusion

MSCs are a unique cell population with the ability to differentiate into cells of various lineages, home to sites of injury, modulate components of the immune system, and deliver therapeutic agents. These properties make MSCs a tool for tissue regeneration and inflammatory diseases, including Crohn's disease and GvHD. While promising, further research into the mechanisms that promote

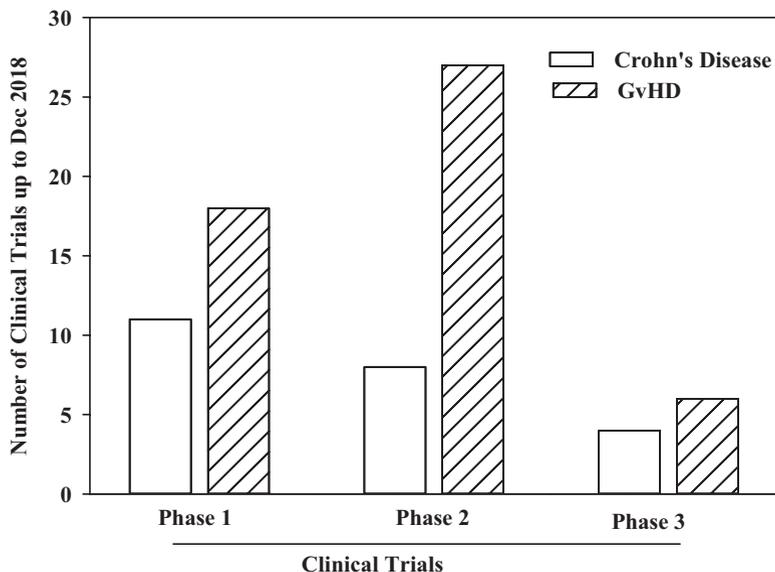


Fig. 5.2 Bar graph depicting the number of clinical trials as of December 2018 that is in Phase 1, 2, and 3

recovery, varying properties of MSCs from different sources, and the effects of culture conditions on MSC migratory potential will be necessary [9]. Finally, MSCs exert a wide range of effects through the release of soluble factors, including cytokines and exosomes. As discussed above, the microenvironment may influence the contents of the MSC secretome, as observed when inflammatory microenvironments promote the release of immunosuppressive factors. Thus, understanding the components of the MSC secretome in both normal and diseased conditions is key to improving MSC therapeutic efficacy.

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Chapter 6

Stem Cells in the Mammalian Gonads



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Abstract Besides spermatogonial stem cells (SSCs) and ovarian stem cells (OSCs), a novel population of pluripotent stem cells termed very small embryonic-like stem cells (VSELs) has been reported in both adult mouse and human testes and ovaries. VSELs and SSCs/OSCs are developmentally linked to each other. VSELs are relatively quiescent, small-sized stem cells that undergo asymmetrical cell divisions (ACDs) whereby they self-renew and give rise to the slightly bigger SSCs/OSCs which in turn undergo symmetrical cell divisions (SCDs) and clonal expansion to form germ cell chains/nests before further differentiation into gametes. Comparison of VSELs and SSCs/OSCs for their potential to differentiate into sperm/oocytes is irrelevant since VSELs only undergo ACD to give rise to SSCs/OSCs that further differentiate into gametes. Being relatively quiescent, VSELs survive oncotherapy and can be manipulated to regenerate nonfunctional gonads of cancer survivors, and thus there is possibly no need to bank testicular/ovarian tissue prior to oncotherapy. Being developmentally linked to the primordial germ cells (PGCs) which are the natural precursors to the gametes, VSELs differentiate into haploid sperm/oocyte-like structures in vitro when cultured on appropriate feeder support, in the absence of a cocktail of growth factors. VSELs express receptors for pituitary and sex hormones (FSHR, ER) and thus get directly stimulated/affected by their circulating levels. Excessive self-renewal of VSELs in the gonads may initiate testicular and ovarian cancers. To conclude, VSELs can be targeted to regenerate the gonads of patients with gonadal insufficiency including cancer survivors and are excellent candidates to differentiate into gametes in vitro.

Keywords Stem cells · Testis · Ovary · Oncofertility · Gametes · Differentiation · Cancer · FSH

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6.1 Current Understanding of Stem Cells in Adult Gonads

Existing understanding is that the mammalian testes harbor spermatogonial stem cells (SSCs) and a recent article describes the isolation protocols for SSCs from the adult human testis and mentions that SSCs are the only stem cells in the testes [1]. Lot of work has been done to show the presence of ovarian stem cells (OSCs) in adult mammalian ovaries which was recently reviewed [2]. However, the concept of two stem cells populations has been described in multiple adult organs, which include quiescent and actively dividing stem cells [3, 4]. The quiescent stem cells, however, remain elusive in several adult organs including the testis, and thus Clevers and Watt [5] in their review on adult stem cells concluded that rather than defining the stem cells by their phenotype should rather be defined by their functions. Our group has extensively reported the presence of a novel population of pluripotent, very small embryonic-like stem cells (VSELs) in addition to SSCs/OSCs in adult mammalian gonads [6].

VSELs are the quiescent stem cells that undergo asymmetric cell divisions whereby they self-renew and also give rise to slightly bigger progenitors (SSCs/OSCs) which in turn undergo symmetric cell divisions and clonal expansion prior to undergoing meiosis and differentiation into gametes [7]. Thus, VSELs are developmentally linked to SSCs/OSCs in the testes and ovaries. Being quiescent in nature, VSELs survive oncotherapy and can be manipulated to restore gonadal function in individuals with gonadal insufficiency including cancer survivors [8]. The observations that VSELs/SSCs/OSCs express receptors for pituitary and gonadal hormones will result in paradigm shift in our basic understanding of endocrinology and gonadal biology. Being developmentally equivalent to primordial germ cells [PGCs, which survive in few numbers throughout life rather than cease to exist during early development] which are natural precursors to gametes, VSELs isolated from the gonads differentiate into sperm and oocytes *in vitro* without any need of growth factors/cytokines to direct their differentiation [9]. Even the bone marrow VSELs can differentiate into male germ cells when cultured on a Sertoli cells bed [10]. The presence of VSELs in various adult tissues also explains why functional germ cells can be obtained from various adult somatic tissues including the pancreas, bone marrow, skin, etc. [11]. Detailed characterization of VSELs and how they compare to ES and PGCs have been compiled in our earlier review [6]. The various aspects of VSELs biology and how they integrate with SSCs/OSCs in the mature gonads will be discussed in this chapter.

6.2 Why the Existing Disbelief in the Presence of VSELs in the Adult Tissues Including Gonads

A recent review [12] discussed the data that has accumulated over time from more than 20 independent groups describing VSELs in various adult tissues since they were first reported in 2006 by Mariusz Ratajczak group from the University of

Louisville, USA. Their small size and presence in very few numbers lead to their accidental loss while processing cells for various experiments. Thus, these stem cells are still struggling to get acknowledged widely by the scientific community [13]. VSELs are located along the basal lamina in the seminiferous tubules of the testes and are interspersed along with the ovary surface epithelial cells but are not visualized easily in paraffin sections of normal testicular/ ovarian sections. They can be easily visualized after being activated, e.g., by FSH in the testis [14] and in smears prepared by gentle scraping of the ovary surface epithelium [15]. Flow cytometry generally used as a tool to study stem cells suffers from experimental reproducibility issues, and VSELs became controversial when few groups could not detect them by flow cytometry [16–20]. Similarly ovarian stem cells (OSCs) have remained highly controversial on technical grounds [21]. Whenever anything new is proposed, it gets strongly opposed by the conventionalists, but persistence in the field will hopefully help arrive at a consensus. Recent review on VSELs shows work done by 20 independent groups confirming the presence of VSELs in adult tissues [12], and Tilly's group has also confirmed the presence of two populations of stem cells including LIN-CD45-SCA-1+ VSELs in mouse ovary [2]. Simplicity, robustness, and repeatability of methods to detect something novel can lead to a paradigm shift in the field. We recently confirmed the presence and enriched VSELs from various adult tissues without using FACS/MACS [22]. Virant-Klun [23] recently showed cortical reaction when the oocyte-like structures obtained by culturing VSELs *in vitro* were exposed to sperm. Thus it may take time to arrive at a consensus, but in addition to the actively dividing SSCs/OSCs, relatively quiescent VSELs also exist in adult mammalian gonads.

6.3 Testicular VSELs

Mammalian testes harbor two populations of stem cells including relatively quiescent, “true stem cells” VSELs and actively dividing “progenitor” SSCs. VSELs in the testis were first reported by Ratajczak's group [24], and few other groups have also suggested the presence of pluripotent stem cells in adult human testis [25–27]. VSELs have been reported by our group in human [28] and mouse [14, 29, 30] testes. Being quiescent, VSELs survive busulfan treatment in the mouse testes, and Kurkure et al. [31] reported the presence of VSELs in azoospermic testis of adult survivors of childhood cancers. We have also observed that both VSELs and SSCs express FSHR [14] and respond to FSH treatment. When exposed to busulfan, testicular VSELs initially increase almost five folds [from about 5000 to 25,000] on day 15 (in an attempt to regenerate the affected testis) but later return to basal levels by day 30 [30] and further when chemoablated testes are exposed to FSH results in almost doubling [$0.045 \pm 0.008\%$ to $0.1 \pm 0.03\%$ of total events studied by flow cytometry] in the numbers of VSELs [14]. Verapamil-sensitive testicular side population also comprises SCA-1-positive stem cells ($5 \pm 0.02\%$ in normal and $8.6 \pm 0.02\%$ in

chemoablated testis). We also found that the VSELs undergo ACD to self-renew and give rise to slightly bigger SSC which undergoes SCD and clonal expansion to form chain-like structures [7, 14].

6.3.1 Integrating VSELs Along with SSCs During Spermatogenesis

Approximately 40 million sperm in mice and 4.4 million sperm in men are produced per gram of testicular tissue on daily basis by the process of spermatogenesis. As per current understanding, SSCs undergo self-renewal and expansion prior to differentiation to maintain the production of large numbers of sperm every day. Confusion prevails regarding the dynamics of the stem cell compartment in the testes as to which are the quiescent “true” stem cells and how to isolate/separate them from actively dividing SSCs. SSCs are heterogeneous in nature with primitive A_s (A_{dark}) and relatively mature $A_{\text{pr}}/A_{\text{al}}$ (A_{pale}) cells with A_s (A_{dark}) cells being the true or “reserve” stem cells that divide very rarely. A_{pale} spermatogonial cells divide regularly and were thus historically defined as the “active” stem cell pool. No specific markers exist to enrich the reserve SSCs despite several attempts to delineate them. *Basically there is no clarity and consensus on the identity of the most primitive and quiescent stem cells in the testes.* The oldest and classical model for stem cells renewal was the A_s model [32] that describes the most primitive A_s spermatogonia which divide and give rise to A_{pr} and A_{al} spermatogonia located along the basal lamina of seminiferous tubules (opposing interstitial arterioles and venules) in mouse testes. However, the A_s are also heterogeneous in nature, and recently two new models were proposed including the “fragmentation” and the “hierarchical” model. Yoshida group proposed the “fragmentation” model based on live cell imaging studies and suggested that A_s , A_{pr} , and A_{al} cells have stem cell properties and interconvert to attain stemness [33]. The “hierarchical” model proposed by Oatley group suggests that A_s spermatogonial cells are heterogeneous in nature and that seminiferous epithelium harbors few “ultimate” ID4⁺/PAX7⁺/BMI1⁺ positive SSCs and large numbers of “transitory” stem cells [34]. Both these models have been criticized on technological grounds and possibly “live cell imaging” method that helped arrive at this understanding focused on spermatogonial cells poised for differentiation rather than on the primitive cells supposed to be located adjacent to the basal lamina [35]. Confusion persists since the presumed primitive SSCs are supposedly located away from the basal lamina. In contrast, VSELs are located along the basal lamina of the seminiferous tubules and are the most primitive, pluripotent stem cells that undergo asymmetric cell divisions to self-renew and give rise to slightly bigger SSCs that in turn undergo symmetric cell divisions and clonal expansion [14] (Fig. 6.1).

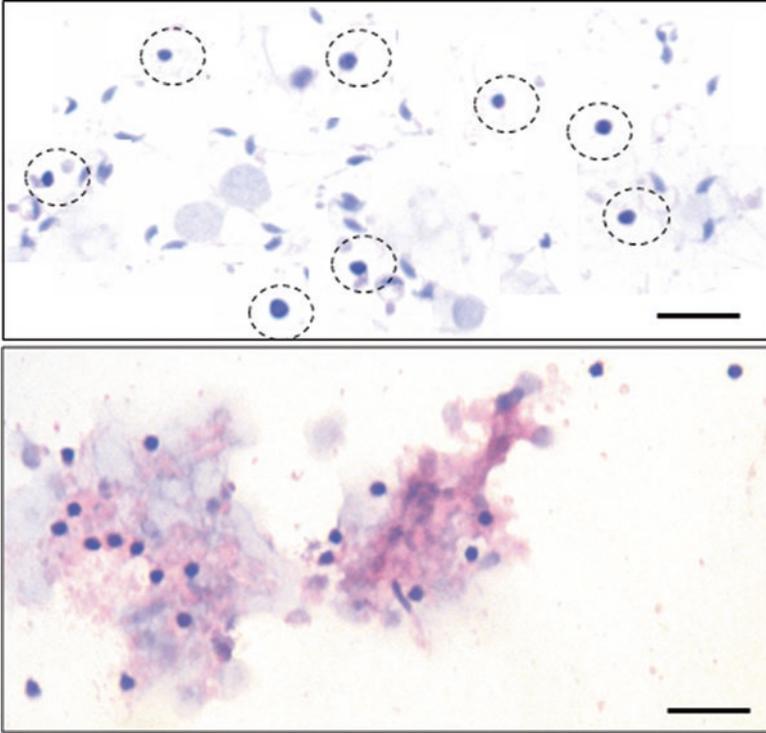


Fig. 6.1 Mouse testicular and sheep ovarian stem cells smears after H & E staining. Small spherical stem cells clearly observed

6.4 Ovarian VSELs

Adult mammalian ovary is believed to have a fixed numbers of follicles at birth that decrease with age, and initial growth of primordial follicles is thought to be gonadotropin independent. Both these beliefs are now being challenged by the stem cells that exist in adult ovaries. Follicles possibly assemble regularly in the ovary surface epithelium/cortical region from the stem cells which also express FSHR, and this process of PF assembly from the stem cells is regulated by FSH. This was recently reviewed by our group [36]. Furthermore, menopause occurs due to altered niche of the stem cells that becomes nonfunctional with age, and thus stem cells differentiation into oocytes gets affected [37].

Tilly group reported ovarian stem cells (OSCs) in mouse ovary surface epithelium [38], and the work done over the years was recently reviewed by their group [2]. In addition to the OSCs, small-sized stem cells in the size range of 3–5 μm were reported in human ovary surface epithelial cell smears [39]. Our group reported two

populations of stem cells including smaller VSELs and slightly bigger OSCs based on OCT-4 expression which was nuclear in VSELs and cytoplasmic in the OSCs [15]. Existing controversies in the field of ovarian stem cells field were recently addressed, and we discussed that VSELs and OSCs are developmentally linked [40]. This is based on our observations that VSELs express nuclear OCT-4 which marks their pluripotent state, whereas SSCs/OSCs express cytoplasmic OCT-4 suggesting that as the pluripotent VSELs enter differentiation, nuclear OCT-4 is no longer required, shifts to the cytoplasm in the immediate progenitors, and gets eventually degraded as the germ cells differentiate into the gametes. Patel et al. [41] observed that sheep ovarian stem cells (VSELs and OSCs) express FSHR, and when exposed to FSH, these stem cells undergo ACD, SCD, and clonal expansion (germ cell nest formation). We had discussed why Lei and Spradling [42] failed to detect germ cell nests in adult mouse ovaries [43]. Parte et al. [44] observed a stimulatory effect of FSH on marmoset, and human OSE and similar stimulatory effect of FSH are also reported on mouse OSE [45]. This novel effect of FSH on mammalian ovary stem cells is mediated via alternately spliced FSHR isoform Fshr3 (a growth factor type 1 isoform that acts via MAPK pathway) in contrast to the canonical Fshr1 that acts via cAMP pathway [46]. Sullivan et al. [47] while studying FSHR expression on different types of follicles by RT-PCR in sheep ovary found that Fshr3 is the most predominant isoform in sheep ovarian follicles. Recently a group from Italy confirmed the presence of small-sized cells along with the OSCs in human ovaries [48], and their findings were discussed [49]. Tilly’s group has also recently confirmed the presence of VSELs in addition to the OSCs in mouse ovaries [2] (Fig. 6.2).

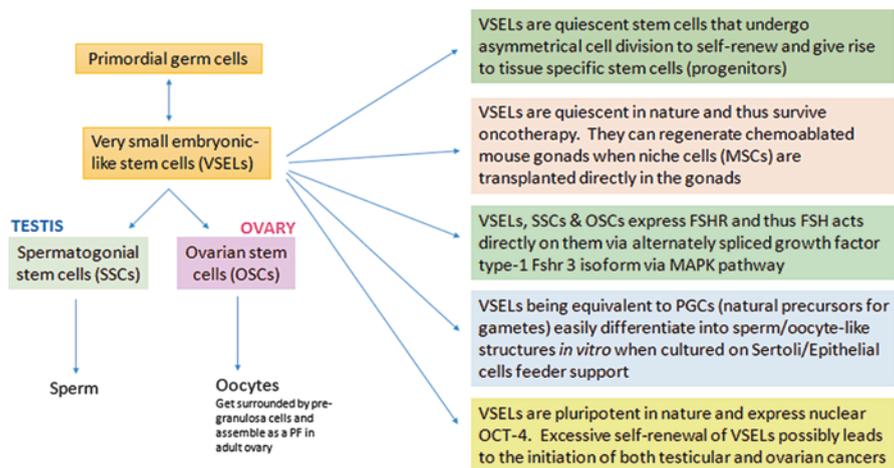


Fig. 6.2 Salient features of gonadal stem cells which include VSELs and SSCs in the testis and VSELs and OSCs in the ovary

6.4.1 *Integrating VSELs/OSCs During Neogenesis and Primordial Follicle Assembly*

Initial evidence supporting stem cells activity and primordial follicle assembly in the OSE was published by our group in adult sheep ovaries [50]. We have also reviewed a possible role of FSH for stem cells activation, to undergo ACD, SCD, and germ cell nest formation prior to differentiating into oocytes [36]. Briefly, the VSELs lodged in the OSE undergo ACD to self-renew and give rise to the OSCs that undergo SCD and germ cell nest formation. The germ cell nests get surrounded by the pre-granulosa cells formed from the epithelial cells by undergoing epithelial-mesenchymal transition. The granulosa cells provide trophic support to the developing oocytes, and this was very nicely demonstrated by our group [51]. Thus stem cells are active in the OSE, and primordial follicles assemble routinely in the OSE. More evidence needs to accumulate in this direction.

6.5 FSHR Expression on Testicular and Ovarian Stem Cells

Expression of FSHR on the testicular [14] and ovarian [41] stem cells reported by our group contradicts the current understanding that FSH acts indirectly on the germ cells via Sertoli and granulosa cells that exclusively express FSHR in the gonads. FSHR action on the stem cells implies that stem cells proliferation/differentiation leading to gametogenesis is a direct action of FSH.

6.5.1 *FSH Action and FSHR Expression Controversy in the Testes*

It is textbook knowledge that LH-stimulated Testosterone production plays a crucial role in regulating spermatogenesis. The role of FSH in regulating spermatogenesis remains an enigma till date since men with inactivating FSHR mutation and male FSHB and FSHR knockout mice maintain normal spermatogenesis and fertility [51]. FSH is thought to stimulate Sertoli cells proliferation peri-pubertally and determines their numbers and thus testicular size. Both T and FSH act via the Sertoli cells to control spermatogenesis. However, recently beneficial effect of treating with high dose of FSH (300 IU every alternate day for 5 months) to oligozoospermic men was reported and led to improved spermatogenesis and pregnancy rates [52, 53]. Oduwole et al. [54] have used double mutant mice (Fshr-CAM/LuRKO with high FSH and minimal T) treated with flutamide (an antiandrogen to completely block T action) and demonstrated a novel role of FSH during spermatogenesis, and these mice remained normal fertile. Thus a new role of FSH during spermatogenesis (in the absence of LH and T) has emerged, and older paradigms have been challenged [55].

As per our understanding, FSH has a crucial role during spermatogenesis that is initiated from a direct action on the stem cells which express FSHR. The existing confusion is rather due to the presence of alternately spliced FSHR isoforms. Men with inactivating FSHR mutations and male FSHB and FSHR knockout mice maintain normal spermatogenesis and fertility not because FSH is not crucial but because FSH acts via alternately spliced FSHR isoform (Fshr3) to bring about spermatogenesis. Various groups in the past only focused on the canonical FSH action mediated through FSHR1 via cAMP pathway. We have shown that FSH receptors are expressed on the testicular stem/progenitor cells (VSELs/SSCs) and FSH acts on the stem cells via alternately spliced Fshr3, and this action of FSH is mediated via MAPK pathway.

6.5.2 FSH Action and FSHR Expression Controversy in the Ovaries

It is widely accepted that primordial follicles growth is gonadotropin independent and FSH acts indirectly on the growing oocyte via granulosa cells that express FSHR. However, emerging data does not support this any longer. Existing belief is based on two studies carried out using sheep and human ovaries in the late 90s. Tisdall et al. [56] carried out RT-PCR and in situ hybridization (ISH) observed that FSHR expression starts in adult ovary from D100 and only in granulosa cells of follicles with 2–3 layers of granulosa cells. Later Oktay et al. [57] collected RNA from human ovarian follicles of different stages and studied FSHR expression by RT-PCR. Both the studies found FSHR expression in growing follicles and not in the immature PF. This led to the existing belief that initial follicle growth is gonadotropin independent. However, both these studies failed to study FSHR isoforms. Primers and probes were designed from 8, 9, and 10 exons. Oktay's group was aware of the FSHR isoforms, but due to limited amounts of cDNA from single follicles, they did not amplify the variants – rather studied only canonical FSHR with well-defined role. In contrast, few other groups have reported FSHR on PF in porcine species. Meduri et al. [58] reported FSHR expression at transcript level in human oocytes by RT-PCR and by autoradiography using [¹²⁵I] human FSH. Zheng et al. [59] reported FSHR in human OSE and PF and primary follicles by ISH. Roy and Albee [60] for the first time demonstrated that FSH is critical for primordial follicle formation. They injected FSH-specific polyclonal antibody in pregnant hamsters, and the ovaries were later studied on day 8. A significant reduction in primordial follicles was observed (2.4% vs. 25%) and further FSHR antibody inhibition could be reversed by treating with PMSG (20 IU). Sullivan et al. [47] took care to use primers specific for Fshr3 and reported it to be the most predominant isoform in sheep ovarian follicles. FSHR expression initially is observed on the ovarian stem cells, whereas the surrounding OSE cells remain negative (refer to Fig. 4F in [7] and Fig. 3 in [41]). The dynamics of FSH during neo-oogenesis from the stem cells and PF assembly was recently discussed (refer to Fig. 12 in [50]).

To conclude, FSH action is not limited to only the gonads, Sertoli cells, and granulosa cells in growing follicles and needs a revisit and more importantly since FSHR expression is also being reported in the hematopoietic system and also on different types of tumors [61].

6.6 Differentiation of Stem Cells into Gametes In Vitro

Making gametes from stem cells for use in infertile clinics is a long-cherished dream of reproductive biologists. So many groups are working globally on this front for several decades now, but the field has not progressed as was anticipated. The challenge is to convert embryonic or induced pluripotent stem cells into PGCs which are the natural precursors to gametes [9]. On the other hand, VSELs are developmentally equivalent to PGCs [12], and thus it has proved very straightforward to differentiate testicular/ovarian VSELs into gametes. In chemoablated testicular cells comprising Sertoli cells and VSELs when cultured on a Sertoli cells bed, VSELs differentiated into sperm after 3 weeks in culture wherein the Sertoli cells provided a feeder support to the differentiating germ cells [29]. Several groups have reported differentiation of oocyte-like structures when ovary surface epithelial cells are cultured wherein the epithelial cells provide feeder support and the VSELs differentiate into oocyte-like structures in humans [15, 39, 48] as well as in mice [62, 63]. In order to prove that indeed it is the VSELs that differentiate into gametes and not few SSCs/OSCs that could have been present in the initial cultures, bone marrow VSELs were cultured on a Sertoli cells bed. Culture over a fortnight resulted in the differentiation of germ cells [10]. The presence of VSELs as a subpopulation in various adult tissues (including mesenchymal cells) explains why various groups have observed germ cells differentiation from so many adult tissues like the bone marrow, skin, pancreas, muscles, amniotic fluid, and endometrium [11]. Virant-Klun [23] exposed the oocyte-like structures to sperm in vitro and found cortical reaction. However, we have debated [9] as to whether we really need to differentiate stem cells into gametes in vitro for clinical use. Regenerating nonfunctional gonads by providing a healthy niche (transplanting autologous mesenchymal cells) may be a better, more practical, and a long-term treatment option rather than onetime use of gametes made in vitro.

6.7 Testicular and Ovarian Stem Cells and Cancer

During testicular development, PGCs on arriving in the gonadal ridge get converted into gonocytes which get enclosed by the Sertoli cells. They continue to proliferate and then get mitotically arrested as spermatocytes. As per current understanding, pre-CIS (carcinoma in situ) gets formed as a result of compromised differentiation of gonocytes. Later during adolescence, the pre-CIS gets converted into a CIS which

over time develops into a full-blown testicular tumor [64, 65]. The reason behind this fetal origin of testicular tumor is to explain expression of embryonic markers in the cancer cells. However, with the presence of a subpopulation of pluripotent embryonic-like stem cells in adult testicular tissue, we need to change our perspective on testicular tumors. It is very likely that VSELs possibly initiate testicular cancer as we have discussed in details [66].

More than 90% of ovarian cancers arise in the surface epithelium [67]. We have earlier discussed how stem cells in the OSE may initiate cancers and a possible role of FSH in the process [46]. Peng et al. [68] have reported high expression of Oct4 and Lin28, in a subpopulation of cells in epithelial ovarian cancer (EOC). Samardzija et al. [69] discussed that enhanced expression of OCT-4A is possibly involved in EOC initiation, progression, and recurrence. Virant-Klun and Stimpfel [70] reported the presence of small-sized VSELs expressing NANOG, SOX2, and SSEA4 in ovarian tissue sections in patients with ovarian cancer. Epithelial-mesenchymal transition (EMT) is considered a major process for the conversion of early-stage ovarian tumors to invasive and metastatic malignancies and promoting the aggressiveness of ovarian cancers [71]. However, we have discussed that it is the stem cells lodged in the OSE that possibly initiate ovarian cancers, whereas EMT of the epithelial cells may lead to the formation of myofibroblasts that exist at the frontal end of cancer during metastasis [7]. Ruan et al. [72] reported that human ovarian cancer cell lines (SKOV3 and A2780) harbor a side population that shows increased OCT-4 expression and OCT-4 was found to increase ovarian cancer progression by activating JAK/STAT signaling pathway.

6.8 Gonadal Stem Cells and Oncofertility

Incidence of cancers has increased in recent times, and majority of the patients get cured due to advances in the treatment. However, a significant fraction of cancer survivors are rendered infertile as a side effect. It is suggested to cryopreserve gonadal tissue prior to oncotherapy, especially in children where sperm/oocytes/embryos cannot be obtained, as a source of germ cells to achieve biological parenthood later on in life. Readers may refer to recent reviews to get an update on this advance [73–77]. Our research on gonadal VSELs has significant relevance to this area since they survive oncotherapy. VSELs have been reported in azoospermic human testes [31, 78] as well as in human ovaries with no follicles [39].

It is well-accepted fact that proliferation/differentiation of stem cells is controlled by their niche (microenvironment) based on where they are located. Thus in order to achieve regeneration, we need to think of both the stem cells and their niche together. Anand et al. [30] carried out microarray studies on Sertoli cells (niche for testicular stem cells) isolated from normal and busulfan treated mouse testes and found that these cells were affected by chemotherapy. Thus, the stem cells (VSELs) survive chemotherapy but are unable to restore spermatogenesis since the niche is compromised. Restoration of spermatogenesis and ovarian function has been

reported by several groups, and a systematic review has also appeared on this strategy [79]. A baby girl has been born by transplanting autologous bone marrow mesenchymal cells in the POF ovaries [80]. We have published few reviews to describe this strategy [6, 9]. More discussions are required in the field to make further advance and help improve the quality life of existing survivors who were deprived of gonadal tissue cryopreservation.

6.9 Emerging Novel Paradigms

1. VSELs exist along with SSCs and OSCs in the testis and ovary, respectively. VSELs are most primitive and pluripotent stem cells that undergo asymmetrical cell divisions to self-renew and give rise to the SSCs/OSCs, which in turn undergo symmetrical cell divisions and clonal expansion (chain or nest formation) prior to further differentiation into gametes.
2. VSELs survive oncotherapy and can regenerate nonfunctional gonads and thus have huge relevance for the field of oncofertility. Restoration of spermatogenesis is the first successful preclinical application of VSELs located in the gonads.
3. Compared to embryonic and induced pluripotent stem cells (ES/iPS), VSELs easily differentiate into gametes when cultured on appropriate feeder support as they are developmentally equivalent to the PGCs, which are natural precursors to the gametes. Thus, the differentiation potential of VSELs is superior to ES/iPS cells.
4. Accumulating literature suggests that altered biology of VSELs possibly initiates testicular and ovarian cancers. It will be of interest to investigate whether altered stem cells and their niche result in various pathologies like reduced sperm count, infertility, POF, PCOS, and menopause.

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Conflict of Interest The authors declare no conflict of interest.

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Chapter 7

Stem and Progenitor Cells in the Pathogenesis and Treatment of Digestive Diseases



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Abstract The global epidemic of chronic degenerative diseases expands rapidly. The pathogenesis of these noncommunicable disorders revolves around innate immunity, microbiome, and stem cell alterations. Understanding the mechanisms behind stem cell biology and their regulatory pathways is a key to understanding the origin of human disease. Stem cells are involved in tissue and organ damage and regeneration. The evidence is mounting that not only eukaryotic cells but also gut microbiota may release extracellular microvesicles that are absorbed from the gut into the portal and systemic circulation. Linking the fields of stem cells, innate immunity and microbiome research opens up new avenues to develop novel diagnostic (e.g., biomarkers), therapeutic (e.g., microbiome modulation, stem cell-based medicines), and prognostic (personalized diets) tools. In this chapter, we present the short overview of various stem and progenitor cells of adult tissues circulating in peripheral blood and their role in the pathogenesis and treatment of digestive diseases. We also briefly discuss the role of host-stem cell-microbial interactions as a new frontier of research in gastroenterology.

Keywords Stem cells · Progenitor cells · VSELs · Regenerative medicine · Microbiome · Biomarkers · Gastroenterology

Pathogenesis of many chronic diseases revolves around innate immunity, microbiome, and stem cell alterations. The cancer risk is influenced by environmental (extrinsic) factors through the processes influencing stem cell divisions [1] and intrinsic (e.g., mutations of somatic stem cells; endogenous auto-, para-, and endocrine mechanisms; alterations of signal transduction or receptor pathways)

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125

factors [2]. Their contribution to chronic disease (e.g., obesity and cancer) has become the key public debate. The global epidemic of chronic degenerative diseases expands rapidly. Dynamic changes in diet and lifestyle and environmental pollution contribute to overwhelming old evolutionary genetically determined door-keeping mechanisms in the digestive tract. The long-term consequences of subtle changes to intestinal and gut epithelium and endothelium (often subclinical) are detrimental to the host but yet difficult to measure. According to the chaos quantum theory, the initial conditions are critical for long-term predictions. For example, the use of nonsteroidal anti-inflammatory drugs (NSAIDs) has been associated with increased rates of myocardial infarction, stroke, and cardiovascular death [3, 4]. NSAIDs interfere with the intestinal mucosa or microbiota and affect gut-vascular permeability [5]. This important observation shapes the light on recent experimental data, where various auto-, para-, and endocrine mechanisms of bacterial and cellular origin might influence distinct organs and the human organism as a whole. Spadoni et al. recently documented that the presence of the gut-vascular barrier (GVB) in the small intestine controls the dissemination of bacteria into the bloodstream [6]. The authors reported a decrease of the wnt/beta catenin-inducible gene *Axin2* (a marker of stem cell renewal) in gut endothelium under the presence of *Salmonella typhimurum* in the small intestine. Similar mechanisms (wnt/beta-catenin genetic system) control the integrity of blood-brain barrier (BBB) [7]. The evidence is mounting that this and other innate immunity-mediated mechanisms govern stem cell mobilization, trafficking, differentiation, and maturation [8]. Understanding the mechanisms behind stem cell biology and their regulatory pathways is key to understanding the origin of human disease. Of research interest are highly proliferative intestinal stem cells possessing continuous ability to regenerate gastrointestinal epithelium as well as studies focusing on various types of bone marrow-derived stem cells (BMSCs), including hematopoietic stem/progenitor cells (HSPC), pluripotent very small embryonic-like stem cells (VSELs), mesenchymal stem cells (MSCs), and endothelial progenitor cells (EPCs). BMSCs are mobilized into peripheral blood in individuals in response to tissue and organ injury as reported, for example, in patients with gastrointestinal (GI) diseases – for example, pancreatic [9] and gastric [10] cancers, myocardial infarction [11], and stroke [12]. Linking the fields of microbiome, innate immunity, and stem cell research opens up new avenues to developing novel diagnostic (e.g., biomarkers), therapeutic (e.g., microbiome modulation, stem cell-based medicines), and prognostic (personalized diets) outcomes. Also of clinical interest is the notion that even minor and subclinical injury to the gut mucosa, associated with stimulation of variety of cellular and microbial molecules, intensifies trafficking of various populations of primitive and more tissue-committed progenitor and stem cells between the bone marrow and digestive system. This can result in variety of either positive or negative consequences with serious impact on the health of an individual. In the era of global rise of obesity and cancer, this topic requires our attention and in-depth exploration.

7.1 Stem and Progenitor Cells in the Gastrointestinal Tract

In the digestive tract, stem cells occupy specific anatomic sites called niches. Within intestinal epithelium structured into crypt-villus units, epithelial TCSCs are located in the lower parts of intestinal crypt similarly to gastric epithelial stem cells localized in the lower parts of gastric glands. TCSCs for esophageal epithelium are in turn situated in the basal layer of epithelium. Liver stem cells are located around the so-called Hering bile ducts, and they have been named “oval cells.” The intestinal villus is paved with epithelium responsible for host protection and absorption of vital nutrients from the GI lumen. The crypt-villus unit is an important part of gut barrier (GB). Other components of GB are i) GI microbiota, ii) gut endothelial cells, iii) gut lymphatic vessels, and iv) tight cellular junctions. Gut barrier in its structure and function resembles blood-brain barrier (BBB) [13]. The gut-brain communication is mediated via blood- and bone marrow-derived cells and their microenvironment. Intestinal stem cells divide continuously and give rise to robustly proliferating transit-amplifying cells maturing to epithelial cells. The fate of mature epithelial cells is their migration to the upper part of the villus, where they undergo apoptosis and intestinal excretion. The intestinal epithelium contains *lgr5*-positive crypt base columnar cells (CBCs), with long-term self-renewal potential and capability of producing more mature absorptive and secretory progenitors [14]. Other type of cells positioned in the intestinal crypt between the stem cell and progenitor zone has been named +4 cells [15]. The real stemness of these cells has been postulated based on genetic lineage tracing including *Bmi1* or *Hopx* markers and investigation into reconstitution of intestinal epithelium following injurious stimuli [8]. Of importance, selected populations of +4 and *lgr5*+ cells are highly resistant to radiation with potential to regenerate epithelium postradiotherapy [16].

Absorptive progenitors give rise into mature enterocytes and M (microfold) cells. Enterocytes form majority of intestinal epithelium with main task of nutrients and water absorption. M cells overlie lymphoid follicles (Peyer’s patches) containing mononuclear cells as well as T and B cells. M cells protrude into and sense gastrointestinal lumen transporting signals to lymphoid cells.

Secretory progenitors produce Goblet cells, Paneth cells, enteroendocrine cells, and Tuft cells. Goblet cells secrete protective layer of mucus over the epithelium, and their aberrations have been implicated in intestinal infections [17], cystic fibrosis [18], inflammatory bowel diseases [19, 20], and some cancers [21]. Paneth cells are guardians of intestinal stem cells at the bottom of the crypt. These cells are also involved in signal transduction involved in crypt base columnar division. Pathology of Paneth cells has been described in neonatal intestinal necrosis (NEC), GI infections, and Crohn’s disease. Enteroendocrine cells (ECs) release variety of hormones (e.g., cholecystokinin, serotonin, ghrelin, somatostatin) into the bloodstream and also form functional and structural unit with vagal nerve. EC are critically involved in gut-brain bidirectional communication. Perturbation of their function might seriously affect physiological and homeostatic gut functions in variety of human diseases [22]. Tuft cells are very rare cells involved in antiparasite intestinal defense.

The whole process of cell division and maturation is influenced by set of various regulatory factors including on and off signaling of Wnt, Hippo, Notch, bone morphogenic protein (BMP), RankL, FGF, EGF, R-spondin signaling, and interleukins (e.g., Il-4, Il-22, Il-25, and Il-13). The proliferation, differentiation, and maturation of gastrointestinal cells are partly regulated by Paneth cells as well as fibroblasts, pericytes, myofibroblasts, smooth muscle, and neural cells. Mesenchymal cells are source of various ligands responsible for division and maturation of gut barrier elements (epithelium, endothelium, and smooth muscle cells). Mesenchymal cells also play important role in intestinal stem cell maintenance and both structural and functional integrity of intestinal epithelium. Epithelial cell ligands also influence the mesenchyme, and this bidirectional communication forms the basis for complex interplays of various components among different compartments of intestinal barrier. The graphical organization of intestinal crypt with stem and progenitors cells is presented in Fig. 7.1.

7.2 Intestinal Stem Cells: Plasticity and Regeneration in Gastrointestinal Tract

At least several questions regarding stem cell pool in intestinal tract remain unanswered. It is unknown, for example, whether within digestive tract besides TCSCs are also present some rare PSCs. Such cells could be a potential population of cells which could play a role as reserve pool of stem cells that supplies TCSCs for intestinal epithelium, in particular, in emergency situations. Of interest are studies where the dedifferentiation process of epithelial cells to intestinal stem cells has been observed. Other studies focusing on gastric chief cells, hepatic cells, and pancreatic acinar cells also reported their dedifferentiation capabilities to more primitive stem cellular state. This mechanism could serve as an alternate mode of securing gastrointestinal tissue stability in response to noxious stimuli.

An open question is also if there are other extraintestinal/extrahepatic/extrapancreatic sources of stem cells which could participate in regeneration/renewal of digestive tract structures, for example, during injuries caused by trauma, inflammation, or other pathologic condition. Such extraintestinal population of small stem cells (e.g., VSELs) could migrate into injured tissues and directly differentiate, for example, into intestinal epithelial cells or could be the source of important paracrine signals (e.g., HSCs, MSCs, EPCs) to support regeneration of damaged intestine.

As already mentioned, PSCs are ideal from regenerative medicine point of view, and according to definition, they have a broad potential to differentiate into cells from all three germ layers. However, true PSCs in adult tissues are extremely rare and are still waiting to be tested in the clinic. In the meantime, various types of stem cells isolated from adult tissues (e.g., bone marrow, mobilized peripheral blood, or umbilical cord blood) are being employed to treat damaged organs.

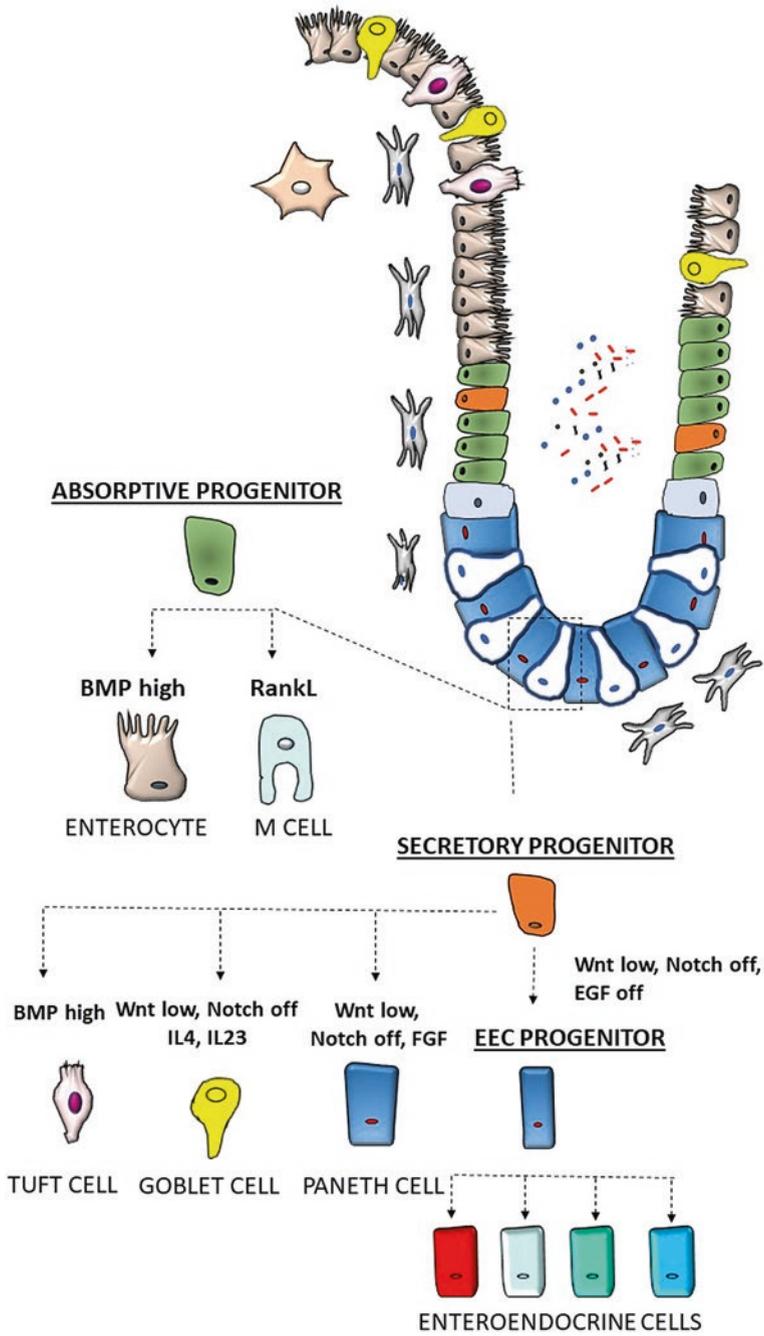


Fig. 7.1 Intestinal stem cell differentiation

Interestingly, while some beneficial effects have been reported following cell-based therapies, there is no solid evidence, particularly in humans, that TCSCs for a given tissue employed to regenerate damaged organs give rise TCSCs for another tissue (e.g., hematopoietic stem cells (HSCs) would transdifferentiate into functional cardiomyocytes in the heart, hepatocytes in the liver, or tubular epithelium cells in the kidney). This contradicts the concept proposed a few years ago that TCSCs (e.g., HSCs) are plastic and may extensively transdifferentiate into cells from different germ layers. Thus, the concept of stem cell plasticity lacks solid experimental support and was never satisfactorily confirmed by independent laboratories.

As a result, the concept of stem cell plasticity or transdifferentiation has been challenged, and some positive effects of stem cell therapies observed in experimental animal models have been explained by alternative mechanisms such as (i) the phenomenon of cell fusion, (ii) the presence of rare population of cells with broader differentiation potential, and (iii) paracrine and endocrine effects of cells employed for therapy. The presence of heterogeneous populations of stem cells in cell preparations employed for cancer diagnosis (e.g., the presence of MSCs, EPC cells, and VSELs in bone marrow or mobilized peripheral blood) as alternative explanation of stem cell plasticity has also been addressed in previous studies [23].

Recent evidence indicates that adult tissues in addition to TCSCs contain population of stem cells that are more primitive and more dormant than a classical stem cell already committed to various tissues. This observation raises several questions such as developmental origin of these cells, their true pluri- or multipotent nature, how they could be efficiently isolated from adult tissues, which surface markers they express, and how their presence affects pool of TCSCs. The phenotype of such dormant cells in adult tissues and expression of some genes characteristic for embryonic stem cells (ESCs), epiblast stem cells (EPSCs), and primordial germ cells (PGCs) suggest their early embryonic origin and their deposition in developing tissues during development as precursors of TCSCs.

Examples of such putative stem cells with broader differentiation potential include (i) mesenchymal stem cells (MSCs), (ii) endothelial progenitor cells (EPCs), (iii) multipotent adult progenitor cells (MAPCs), (iv) marrow-isolated adult multilineage inducible (MIAMI) cells, (v) multipotent adult stem cells (MASCs), (vi) elutriation-derived (Fr25/Lin⁻) stem cells (ELH SCs), (vii) spore-like stem cells, (viii) pluripotent Sca-1 + CD45-c-kit⁻ cells, and (ix) multilineage-differentiating stress-enduring stem cells (Muse SCs). All these stem cell types have been identified by employing different isolation and identification protocols. The similarity in expression of developmental early genes, which is demonstrated in some models their ability to differentiate into cells from more than one germ layer, suggests that they are somehow related to each other and to different degree may represent similar overlapping populations of primitive stem cells that reside in adult tissues and are endowed with broader differentiation potential.

7.3 Stem Cells and Regenerative Medicine in Gastroenterology

Therapeutic strategies based on application of stem cells have been proposed as the alternative therapies of multitude diseases including chronic gastroenterological (GI) disorders. Also, the roots of many GI disorders lie in stem cell pathology. Accordingly, in addition to potential application of stem cells to treat injured organs such as myocardium after heart infarction, brain after stroke, and spinal cord after mechanical injury as well as to treat metabolic disorders (e.g., obesity or diabetes mellitus) or neurodegenerative diseases, stem cell therapies recently become also the focus of interest to gastroenterologists. Stem cells have already been explored to treat several disorders of GI tract, including those affecting the pancreas and liver as well as inflammatory bowel diseases (IBDs). Stem and progenitor cells could also be used in medicine as biomarkers. Examples include inflammatory bowel diseases (IBDs) [24] and cancer [25] including early detection of cancerous, precancerous, or postcancerous lesions in the blood (liquid biopsy) [26]. This idea has recently become reality along with the development of first “liquid biopsy” protocols allowing for identification of cancer stem cells in the human blood [27].

The expanding interest in stem cells is a result of newly developed clinical discipline that is regenerative medicine. It has been proposed that in the future transplantation of entire organs will be replaced by the transplantation of the suspension of stem cells precommitted for the given organ. Such stem cells will have the task to rebuild the injured tissues.

Stem cell according to the definition is able to renew itself and to differentiate into progenitor cells that give rise to one or more cell lineages [28]. Stem cell pool enables to keep the balance of the number of somatic cells within the organism; thus it is responsible for the renewal of somatic cells that are used up with time, as well as is essential for regeneration of the damaged organs and tissues. However, the most important property of stem cell is its ability to self-renew, evidence accumulates that stem cells are very heterogeneous, and describing them by one common definition only could be not feasible.

It is widely accepted that stem cells have a distinct morphology (e.g., small lymphocyte-like appearance), express distinct panel of surface markers (e.g., CD133, CD34, Lin⁻), express low activity of selected metabolic fluorochromes (e.g., Rhodamine123, Pyronin Y, or Hoe3342), or display differences in activity of some enzymes (e.g., aldehyde dehydrogenase) that are helpful in their identification and purification strategies. It is obvious that these properties of stem cells change with their position, which the given stem cells occupy in hierarchy of stem cell compartment and its tissue commitment.

The most valuable for regenerative medicine are pluripotent stem cells (PSCs) that according to the definition may differentiate into cells belonging to all three germ layers (meso-, ecto-, and endoderm). Thus, PSCs have the ability to differentiate into tissue-committed stem cells (TCSCs) such as hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs), and endothelial progenitors (EPCs). All

these population of stem cells have been utilized in the the diagnosis and treatment of gastrointestinal diseases.

7.3.1 Hematopoietic Stem Cells (HSCs)

Hematopoietic stem cells have been evaluated in autologous stem cell transplantation (A-HSCT) protocols in patients with refractory Crohn's disease [29]. A-HSCT uses the patient's own stem cells and is practiced in patients with autoimmune diseases. The rationale behind these trials was based on hypothesis that A-HSCT can reset the immune system and lead to immunological tolerance. *Brierley* et al. in their retrospective survey identified 99 patients in the registry of the European Society for Blood and Marrow Transplantation (EBMT) eligible for cell hematopoietic transplantation. The authors analyzed clinical outcomes of 82 patients enrolled in 19 centers out of 7 countries participating in the study [30]. The authors concluded that A-HSCT was relatively safe and effective in maintaining treatment-resistant Crohn's disease remission. Enrolment of patients into A-HSCT treatment regimens was based on EBMT guidelines on severity of Crohn's disease eligible for stem cell transplantation: (i) active and unresponsive disease despite multiple lines of therapy, (ii) extensive disease where surgical resection would expose the patient to small bowel syndrome risk, and (iii) refractory colonic disease where a stoma is not acceptable to the patient [31]. Incidental reports documenting either long-term or temporary efficacy of A-HSCT in CD patients led European researchers to conduct multicenter randomized controlled trial (ASTIC trial) [32], where all patients underwent stem cell mobilization before random assignment to groups given cyclophosphamide-based HSCT or a control treatment. The primary outcome of the study was to explore how often hematopoietic stem cells led to major improvements associated with complete and symptomatic remission. Exploratory analyses of individual components of the primary (patient off immunosuppressive therapy or biological agents, with a normal CDAI and no evidence of active disease) as well as secondary endpoints suggested significant benefit. In comparison to controls, a significant increase in the proportion of patients able to stop immunosuppressive drugs was noted. It has to be addressed that the benefit of A-HSCT needs to be balanced with adverse events noted in ASTIC trial. Most common adverse events were treatment-related bacterial and viral infections in the 100 days after transplantation. Of note gut colonization by multidrug-resistant Gram-negative bacteria is an independent risk factor for the development of intestinal acute graft-versus-host disease [33], and fecal microbiota transplantation (FMT) in patients with hematological diseases was reported as safe and facilitating eradication of antibiotic-resistant bacteria from the digestive system [34]. Other than infection, smoking and perianal disease at baseline were independent factors associated with the number of serious adverse events [35].

7.3.2 *Mesenchymal Stem Cells (MSCs)*

Mesenchymal stem cells (MSCs) are heterogeneous subset of stromal cells with the capability of modulating immune reactions [36]. As mesenchymal tissue contributes to homeostasis of many tissues, these cells have been implicated in pathogenesis of variety of disease including those affecting the liver and colon. Also the source of MSCs may vary including adipose tissue or bone marrow. MSCs have also been effectively utilized for the treatment of fistulizing Crohn's disease [37], liver failure [38], and fibrosis [39] as well as disorders of gut-brain interaction [40]. The prime evidence of MSC efficacy comes from studies in patients with fistulizing Crohn's disease. The use of MSCs is intriguing in light of epidemiological data where majority of patients with fistulas achieve inadequate medical therapy and require surgical treatment with long-term follow-up [41]. The efficacy of MSC therapy in patients with CD-related fistulas has been confirmed in multicenter, randomized, double-blind phase III trial [42]. In general, locally injected MSCs constitute a safe therapy that rescues refractory CD patients and regains responsiveness to drugs previously proved ineffective [43]. The summary of studies utilizing MSCs of various tissue sources in the treatment of fistulizing Cohn's disease has been presented in Table 7.1. The data are also mounting that MSCs as a heterogeneous population of stromal cells with strong immune-modulating properties may positively influence regeneration of inflamed or fibrotic liver. The beneficial effect of MSCs is mediated mainly via secretion of soluble molecules with immunomodulatory properties. The summary of clinical trials of MSCs in patients with immune-mediated liver disease is summarized in Table 7.2. MSCs as component of cancer microenvironment also contribute to tumor growth and metastasis with strong influence on patient's survival.

7.3.3 *Endothelial Stem Cells (ESCs)*

Endothelial stem cells (ESCs) and endothelial progenitor cells (EPCs) contribute to onset and progression of inflammatory bowel disease [44]. Bone marrow-derived ESCs possess the capability of circulatory migration to the site of endothelial damage. Boltin et al. [44] studied CD patients treated either with biological therapy (infliximab) or immunomodulators and reported a significant increase in the percentage of EPCs of the peripheral mononuclear cells in patients with active disease in comparison to healthy persons. Authors did not find the correlation between EPC percentages and other factors such as age, sex, CDAI, disease duration, duration of biological therapy, or addicts (e.g., smoking). Of interest is the enumeration of ESCs/EPCs in PB that could serve as a surrogate parameter in IBD prognosis and response to therapy. Of importance is the mobilization and activity of stem/progenitor cells that could be affected by numerous factors, including medicines (e.g., antibiotics), proton-pump inhibitors, infection, physical activity, gender, and age [45].

Table 7.1 Representative studies utilizing stem cells in fistulizing Crohn's disease

MSC source	Study (country)	Number of subjects// controls	Location of fistula (n, %)	Dose	Administration protocol	Outcome	Adverse events	Reference
<i>Autologous stem cell therapy</i>								
	García-Olmo et al. (2003) (Spain)	1/0	Rectovaginal (n = 1, 100%)	$9 \times 10^6/2$ ml	Direct injection into rectal mucosa close to the sutured internal opening	Full closure (100%)	None reported	[79]
Adipose tissue	García-Olmo et al. (2005) (Spain)	5/0	Rectovaginal (n = 3); enterocutaneous (n = 5); suprasphincteric perianal (n = 1)*	$3-30 \times 10^6$ per person	Direct injection into the wall of the track (enterocutaneous fistula) and into rectal mucosa closely to previously sutured internal openings (rectovaginal and perianal fistulas)	Week 8: Healing in 6/8 fistulas (75%) nonhealing in 2/8 fistulas (25%) with a decrease in output flow	No acute adverse events (e.g., anaphylaxis, allergic reaction)	[80]

<p>García-Olmo et al. (2009) (Spain)</p>	<p>24/25</p>	<p>Complex perianal (cryptoglandular origin or associated with Crohn's disease)</p>	<p>20×10^6 per person</p>	<p>Direct injection of half of SC into the intersphincteric tracts and those adjacent to the internal opening, and the other half into the tract walls in the direction of the external opening, following curettage and suturing of internal openings</p>	<p>Complete healing in 17/24 (70.8%) and 4/25 (16%) in patients treated with SC and PBO, respectively. 1-year recurrence rate 17.6% (3/24 patients from Tx group)</p>	<p>Totally 11/24 (76.7%) and 17/25 (72.5%) patients in the Tx and PBO groups, respectively, with adverse events. Perianal abscess ($n = 1$) and cholecystitis and cholelithiasis-choledocholithiasis after cholecystomy ($n = 1$) in Tx group. Crohn's crisis and intra-abdominal abscess ($n = 1$) and perianal abscess ($n = 1$) in PBO group</p>	<p>[81]</p>
<p>Lee et al. (2013) (Korea)</p>	<p>33/0</p>	<p>Transsphincteric ($n = 24$, 72.7%); suprasphincteric ($n = 4$, 12.1%); extrasphincteric ($n = 5$, 15.2%)</p>	<p>3×10^7 cells per centimeter of the fistula once (the length not above 1 cm) or twice (the diameter of the fistula was 1 cm, d # 2 cm)</p>	<p>Direct injection into submucosa around the internal opening and fistula tract following thorough curettage, irrigation, and suturing under anesthesia</p>	<p>Complete fistula healing in 27/33 patients (82%) within 8 weeks after SC injection. One -year follow-up: 23/26 (88.46%) patients sustained full closure</p>	<p>Pain ($n = 26$, 60%); anal pain ($n = 7$, 17%), anal bleeding ($n = 3$, 7%)</p>	<p>[82]</p>

(continued)

Table 7.1 (continued)

MSC source	Study (country)	Number of subjects// controls	Location of fistula (n, %)	Dose	Administration protocol	Outcome	Adverse events	Reference
	Cho et al. (2013) (Korea)	10/0	Transsphincteric (n = 5, 50%); suprasphincteric (n = 4, 40%); extrasphincteric fistula (n = 1, 10%)	$1 \times 10^7/2 \times 10^7/3 \times 10^7$ ^b	Direct injection into the fistula tract wall and the mucosa surrounding the internal opening (proportional to the size of the fistula track) following a throughout tract curettage and suturing with 2–0 vicryl	Group 1 (1 × 10 ⁷ cells/ml: Partial closure (n = 3, 100%) Group 2 (2 × 10 ⁷ cells/ml: complete healing at week 8 after injection (n = 2, 50%). Group 3 (4 × 10 ⁷ cells/ml): Complete healing (n = 1, 33%), partial healing (n = 2, 66.6%). Patients with complete healing sustained the effect of SC injection up to 8 months postintervention (6 months)	Pain (n = 3, 30%), diarrhea (n = 2, 20%), enterocolitis, seton application, and infliximab administration for new fistulas unrelated to the target fistula requiring hospitalization (n = 2, 20%)	[83]

<p>Cho et al. (2015) (Korea)</p>	<p>43/0</p>	<p>Transsphincteric (<i>n</i> = 28, 68.3%); suprasphincteric (<i>n</i> = 5, 12.2%); extrasphincteric (<i>n</i> = 8, 19.5%)</p>	<p>3 × 10⁷ cells per centimeter of the fistula once (the length not above 1 cm) or twice (the diameter of the fistula was 1 cm, d # 2 cm)</p>	<p>Direct injection into submucosa around the internal opening and fistula tract following thorough curettage and irrigation and suturing under anesthesia</p>	<p>Complete fistula closure in 27/33 patients (82%). In 23/29 (79.3%) and 21/26 (80.8%) treatment, success was at month 12 and month 24 after SC injection, respectively</p>	<p>53 adverse events in 30 patients (73.2%), in particular: Abdominal pain (<i>n</i> = 5, 17.1%), eczema (<i>n</i> = 3, 9.8%), exacerbation of disease (<i>n</i> = 3, 9.8%), anal inflammation (<i>n</i> = 2, 7.3%), diarrhea (<i>n</i> = 2, 7.3%), and fever (<i>n</i> = 2, 7.3%)</p>	<p>[84]</p>
<p>Dietz et al. (2017) (USA)</p>	<p>12/0</p>	<p>Transsphincteric (<i>n</i> = 8, 66.7%); suprasphincteric (<i>n</i> = 1, 8.3%); intersphincteric (<i>n</i> = 3, 25%)</p>	<p>20 × 10⁶ per plug</p>	<p>Intraoperative placement of the stem cell loaded plug (MSC-MATRIX)</p>	<p>Complete fistula healing in 9/12 (75%) within 3 months postintervention, and in 10/12 patients (83.3%) at 6 months post SC therapy</p>	<p>Debridement of granulation tissue in the fistula tract (<i>n</i> = 1), seroma at the site of fat collection (<i>n</i> = 2), nonserous adverse events due to CD (<i>n</i> = 11)</p>	<p>[85]</p>
<p>Bone marrow</p>	<p>10/0</p>	<p>Perianal (<i>n</i> = 7, 70%); multiple enterocutaneous (<i>n</i> = 3, 30%)</p>	<p>1 × 10⁶ cells per cm of the tract (median: 4 times)</p>	<p>Serial intrafistula injections</p>	<p>Complete fistula healing in 2/3 (66.7%) fully monitored patients. Fistula relapse-free probability: 88% (1 year), 50% (2 years), and 37% (4 years)</p>	<p>23 adverse events: Predominantly abdominal pain, anal inflammation, diarrhea, erythema, nausea, and fever</p>	<p>[43]</p>

(continued)

Table 7.1 (continued)

MSC source	Study (country)	Number of subjects// controls	Location of fistula (n, %)	Dose	Administration protocol	Outcome	Adverse events	Reference
	<i>Allogenic stem cell therapy</i>							
	García-Arranz et al. (2016) (Spain)	10/0	Rectovaginal (n = 10, 100%)	$20 \times 10^6 / 40 \times 10^{6a}$	Direct injection into submucosal area and tract of fistula post curettage and eventually vaginal or rectal flap. After 12 weeks with no improvement, the intervention was repeated	Complete fistula heal in 2/10 patients (20%) at 12 weeks postintervention. S.C. injection fistula healing in 2/8 patients (25%). Fistula curing at any time of the trial in 9/10 patients (90%). Overall treatment success in 3/5 patients fully analyzed (60%) (1 year)	No adverse events, in particular, regarding abnormal tissue and/or inflammation	[86]

Adipose tissue	de la Portilla et al. (2013) (Spain)	24/0	Transsphincteric (<i>n</i> = 17, 70.8%); suprasphincteric (<i>n</i> = 1, 4.2%); intersphincteric (<i>n</i> = 5, 20.8%); extrasphincteric (<i>n</i> = 1, 4.2%)	20 × 10 ⁶ per person	Direct, 2 mm deep injection into the tract walls (half of the total volume in the intersphincteric tracts and those adjacent to the internal opening, the other half in the tract walls in the direction of the external opening) following tract curettage and suturing	Complete closure in 8/21 (38.1%) at week 12 and in 9/16 (56.3%) at week 24	Totally 32 adverse events in 13 patients. Intervention related: Anal abscess (<i>n</i> = 3), pyrexia (<i>n</i> = 1), uterine leiomyoma (<i>n</i> = 1)	[87]
	Panés et al. (2016) (Multicenter)	107//105	Perianal n.s. (100%)	12 × 10 ⁷ cells person	Direct injection into tissue adjacent to fistula tracts and internal openings, following setons removal and suturing	Complete fistula healing at week 24 in 53/107 (50%) patients treated with SC vs 36/105 (34%) who were administered with PBO	Totally 18/103 (17%) and 30/103 (29%) patients in the Tx and PBO groups respectively with adverse events. Anal abscess (<i>n</i> = 6 vs. <i>n</i> = 9) and proctalgia (<i>n</i> = 5 vs. <i>n</i> = 9) in the Tx and PBO groups, respectively	[42]

(continued)

Table 7.1 (continued)

MSC source	Study (country)	Number of subjects// controls	Location of fistula (<i>n</i> , %)	Dose	Administration protocol	Outcome	Adverse events	Reference
	Panés et al. (2018) (Multicenter)	107//105	n.a.	12×10^7 cells per person	n.a.	Complete fistula healing at week 52 in 58/103 (56.3%) patients treated with SC vs 39/101 (38.6%) who were administered with PBO	Totally 79/103 (76.7%) and 74/102 (72.5%) patients in the Tx and PBO groups respectively with adverse events. Study withdrawal in 9 (8.7%) and 9 (8.8%) patients from respective groups	[88]
	Wainstein et al. (2018) (Chile)	9//0	Transsphincteric (<i>n</i> = 8), intersphincteric (<i>n</i> = 1) uch–vaginal (2)*	$10–12 \times 10^7$ per person	Fistula mapping, drainage and seton placement under anesthesia, followed by setons removal after 4–6 weeks and direct injection into internal fistula opening and the fistula tract together with biological plug formation	Complete healing in 10/11 fistulas	None reported	[89]

Bone marrow	Molendijk et al. (2015)	5/5/5/6	Transsphincteric (<i>n</i> = 15); suprasphincteric (<i>n</i> = 1); extrasphincteric (<i>n</i> = 3); intersphincteric (<i>n</i> = 3); superficial (<i>n</i> = 1)*	$1 \times 10^7/3 \times 10^7/9 \times 10^7$ per person	Intralesional fistula tract direct injection following curettage	Week 6: 3/5 patients (60.0%) in group 1, 4/5 patients (80.0%) in group 2, 1/5 patients (20.0%) in group 3, vs 1/6 (16.7%) patients in PBO group. Week 12: 2/5 patients (40.0%) in group 1, 4/5 patients (80.0%) in group 2, 1/5 patients (20.0%) in group 3, vs 2/6 (33.3%) patients in PBO group. Week 24: 4/5 patients (80.0%) in group 1, 4/5 patients (80.0%) in group 2, 1/5 patients (20.0%) in group 3, vs 2/6 (33.3%) patients in PBO group. Week 24: 4/5 patients (80.0%) in group 1, 4/5 patients (80.0%) in group 2, 1/5 patients (20.0%) in group 3, vs 2/6 (33.3%) patients in PBO group	Blood from fistula (<i>n</i> = 1), perianal swelling (<i>n</i> = 6), abscess (<i>n</i> = 4), painful anal sphincters (<i>n</i> = 1), fissura ani (<i>n</i> = 1), anal blood (<i>n</i> = 1), anal pus (<i>n</i> = 1), thrombosed hemorrhoid (<i>n</i> = 1), pimples (<i>n</i> = 3), mild CD activity (<i>n</i> = 2), CD exacerbation (<i>n</i> = 1), abdominal pain (<i>n</i> = 5), diarrhea (<i>n</i> = 2), flatulence (<i>n</i> = 1), nausea (<i>n</i> = 1), vomiting (<i>n</i> = 1), lack of appetite (<i>n</i> = 1), pneumonia (<i>n</i> = 1), common cold (<i>n</i> = 10), otitis (<i>n</i> = 1), headache (<i>n</i> = 2), back pain (<i>n</i> = 1), rosacea (<i>n</i> = 1), cold sore (<i>n</i> = 1)	[90]
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*number of fistulas is greater than number of patients (multiple fistulas in one person)

^aIf no heal occurred second/higher dose was administered

^bDose was escalated upon tolerance, MSC mesenchymal stem cells, *n.a.* not applicable

Table 7.2 Representative studies utilizing stem cell therapy in liver diseases

Study (country)	Liver disease	SC source/dose	Number of subjects/controls	Administration protocol	Outcome (total follow-up)	Reference
Autologous MSC therapy						
Peng et al. (2011) (China)	Liver failure secondary to HBV infection	Bone marrow/ $3.4 \pm 3.8 \times 10^8$ per person	53/105	Direct injection into proper hepatic artery	Improvement: Serum albumin, total bilirubin, prothrombin time, and MELD score. No differences in mortality and HCC incidence. (192 weeks)	[91]
El-Ansary et al. (2012) (Egypt)	Liver cirrhosis secondary to HCV infection	Iliac crest/ 1×10^6 per kg	15/10	Direct injection into peripheral vein	Improvement: MELD score, hemoglobin, serum albumin, direct and indirect bilirubin, incidence of jaundice, encephalopathy, melena, edema, ascites, tremors (6 months)	[92]
Jang et al. (2014) (Korea)	Alcoholic cirrhosis	Bone marrow/ 5×10^7 per person (two times)	12/0	Direct injection into hepatic artery	Improvement: Serum albumin, prothrombin time, child-Pugh and MELD score, histology(12 weeks)	[93]
Mohamadnejad et al. (2007) (Iran)	Liver cirrhosis	Bone marrow/ 3×10^6 per person	4/0	Direct injection into cubical vein	Improvement: MELD score (1 year)	[94]
Kharaziha et al. (2009) (Sweden)	Liver cirrhosis	Bone marrow/ $30-50 \times 10^6$ per person	8/0	Direct injection into peripheral/portal vein	Improvement: MELD score, prothrombin complex, serum creatinine, serum albumin (24 weeks)	[95]
Amer et al. (2011) (Egypt)	ESLV secondary to HVC infection	Bone marrow/ 2×10^6 per person	20/20	Intrasplenic(50%)/intrahepatic(50%)	Improvement: Ascites, lower limb edema, serum albumin MELD score, fatigue scale, performance status (6 months)	[96]

Xu et al. (2014) (China)	Liver cirrhosis secondary to HBV infection	Bone marrow/ $0.75 \pm 0.50 \times 10^6$ per person	20/19	Direct injection into hepatic artery	Improvement: Serum ALT, serum albumin, total bilirubin, MELD score, Treg cells, Th17 cells, Treg/Th17 ratio, Foxp3 and ROR γ t expression, serum IL-17, TNF- α , IL6 (24 weeks)	[97]
Lukashyk et al. (2014) (Belarus)	Liver cirrhosis secondary to HCV infection	Bone marrow/ 1×10^6 per kg	6/0	Direct intraparenchymal injection	Improvement: Fibrinolysis and regeneration of hepatocytes (6 months)	[98]
Suk et al. (2016) (South Korea)	Alcoholic cirrhosis	Bone marrow/ 5×10^7 per person (once or twice)	18/19/18 ^a	Direct injection into hepatic artery	Improvement: Fibrosis, child-Pugh score (both one-time and two-time) (6 months)	[99]
Allogenic MSC therapy						
Zhang et al. (2012) (China)	Chronic HBV infection	Umbilical cord/ 5×10^5 per kg	31/15	Direct injection into peripheral vein	Improvement: Ascites, serum albumin, serum bilirubin, MELD score (1 year)	[100]
Shi et al. (2012) (China)	Chronic HBV infection	Umbilical cord/ 5×10^5 per kg	24/19	Direct injection into peripheral vein	Improvement: MELD score, serum albumin, cholinesterase, prothrombin time, platelet count, serum total bilirubin, alanine aminotransferase (48/72 weeks)	[101]
Fang et al. (2018) (China)	Liver cirrhosis secondary to HBV infection	Umbilical cord/ $4.0-4.5 \times 10^8$ cells per person	50/53	Direct injection into peripheral vein	Improvement: IL6, TNF- α , IL10, T4 cells, T8 cells, Treg cells, B cells, serum albumin, serum AST and ALT, total bilirubin, prothrombin type, MELD score, child-Pugh score (24/48 weeks)	[102]
Wang et al. (2013) (China)	Primary biliary cirrhosis	Umbilical cord/ 0.5×10^6 per kg	7/0	Direct injection into peripheral vein	Improvement: Fatigue, pruritus, serum alkaline phosphatase, g-glutamyltransferase (48 weeks)	[103]

(continued)

Table 7.2 (continued)

Study (country)	Liver disease	SC source/dose	Number of subjects/controls	Administration protocol	Outcome (total follow-up)	Reference
Wang et al. (2014) (China)	Ursodeoxycholic acid (UDCA)-resistant primary biliary cirrhosis	Umbilical cord/ $3-5 \times 10^5$ per kg	10/0	Direct injection into peripheral vein	Improvement: Serum ALT, AST, γ -GT, IgM, IL10, CD8+ T cells, CD4 + CD25 + Foxp3+ T cells. (12 months)	[104]
Liang et al. (2017) (China)	Liver cirrhosis secondary to autoimmune diseases	Bone marrow ($n = 1$)/umbilical cord ($n = 23$)/cord blood ($n = 2$)/ 1×10^6 per kg	26/0	Direct injection into peripheral vein	Improvement: Total bilirubin, serum albumin, prothrombin time, MELD score (2 years)	[105]

Spadoni et al. characterized the endothelial progenitor cells participating in gut barrier structure and function [46]. The authors analyzed the expression of tight junction (TJ) and adherence junction (AJ) proteins and found that, similar to the cerebral endothelium, intestinal endothelial cells (ECs) expressed the main components of TJs, such as occludin, JAM-A, claudin-12, ZO-1, cingulin, as well as VE-cadherin and junctional β -catenin. Blood ECs were FACS sorted based on expression of $CD45^-CD31^+CD105^+LYVE1^-$ out of C57/BL6 mice small intestine and further sequenced for the transcriptional profile. Among all the genes expressed by the intestinal ECs, majority of genes were coding TJ and AJ proteins. Therefore, endothelial progenitor cells seem to be involved in gut barrier functions associated with signaling and transporting cascades. The transcriptome analysis allowed to gain new knowledge on how intestinal endothelium contributes to the metabolism and transport of nutrients and small molecules in the digestive tract and how it responds to infectious stimuli. This study has important diagnostic and therapeutic implications [46] as increased trafficking of EPCs has been observed in patients with active IBD disease and cancerous angiogenesis.

7.4 Circulating Stem and Progenitor Cells in Gastrointestinal Disease

7.4.1 *Inflammatory Bowel Disease and Crohn's Disease*

Crohn's disease is a complex multifactorial inflammatory bowel disease (IBD) with complex and unclear pathogenesis. The evidence indicates a pivotal involvement of innate immunity-mediated mechanisms and defects in stem cell differentiation. Currently, available therapies ranging from anti-inflammatory to immunosuppressive therapies are of limited value due to their scarce efficacy for mucosal healing associated with wide range of toxic adverse events.

Various populations of intestinal progenitor cells have been implicated in IBD pathogenesis, including Paneth and Goblet cell alterations. Of relevance, the autologous hematopoietic cell transplants as well as infusions of mesenchymal stem cells (MSCs) turned out to have beneficial effects in patients suffering from refractory Crohn's disease associated with prolonged remissions. Based on our observations, various populations of stem cells may yield novel diagnostic and therapeutic options for IBD patients.

For the purpose of stem cell identification, the whole peripheral blood (PB)-derived nucleated cell fraction was evaluated against hematopoietic lineage markers (Lin), CD45 antigen, CD133 (APC; clone CD133/1), or CD34 markers.

Our group of collaborators following immunostaining strategy to evaluate the circulation of VSELs (FSC low, SSC low, CD45 negative, lineage markers negative, CD133 positive and FSC low, SSC low, CD45 negative, lineage markers negative, and CD34 positive) and hematopoietic stem cells (HSCs) (CD45 negative, lineage

markers negative, CD133 positive and CD45 negative, lineage markers negative, and CD34 positive).

Also for the first time members of our team demonstrated that in patients with Crohn's disease, various populations of stem cells including MSCs, EPCs, and pluripotent VSELs were mobilized and could be detected in PB [47]. In patients with Crohn's disease, we did not observe significant mobilization of the most primitive CD133+/Lin-/CD45+ and CD34+/Lin-/CD45+ HSCs, which were reported to be mobilized to a high level in other types of organ/tissue injuries such as heart infarct, stroke, or skin burns [48–50]. This could be explained by a fact that due to chronic inflammatory process as seen in IBD patients, the most prominent population of mobilized cells are not the most primitive HSCs but already more differentiated committed hematopoietic clonogenic progenitors. To address this issue better, it will be important to perform clonogenic assays on circulating peripheral blood mononuclear cells to enumerate number of, for example, circulating colony-forming units of granulocyte-monocytes (CFU-GM). These clonogenic hematopoietic progenitor cells could supply in situ in damaged tissue functional granulocytes and monocytes in response to chronic inflammation. Overall, we envision that stem cells mobilized into PB in IBD patients play different biological roles. While VSELs and EPCs are mobilized in an attempt to counteract tissue damage and play some role in regeneration of damaged intestine, MSCs mobilized into and circulating in PB may be involved in some immunomodulation of the inflammatory process. This conjecture requires further study. Furthermore, stem cells may also secrete several growth factors, cytokines, or even membrane-derived microvesicles that accelerate the regeneration process. From therapeutic point of view, it is important to employ pluripotent PSCs that will be able to differentiate into all types of tissues present in the damaged intestine. VSELs, based on current data, could be employed as a population of such potentially PSCs. The proper understanding of the mechanisms that govern stem cell mobilization in regeneration of damaged intestine will help develop more efficient strategies to improve this process (e.g., by promoting mobilization of circulating stem cells).

Several mechanisms have been proposed to orchestrate stem cell mobilization, but still more work is needed to better understand this process. Overall, mobilization has been proposed to be directed by (i) a decrease in adhesive interactions of SCs within their niches in BM (e.g., due to release of proteolytic enzymes or after molecular blockage due to administration of AMD3100, a small molecular antagonist of the CXCR4 receptor), (ii) release of neurotransmitters from the synapses of the nerves that innervate the bone marrow microenvironment, (iii) activation of the coagulation cascade (e.g., release of uPAR), and (iv) activation of complement cascade (e.g., release of C5b-C9 membrane attack complex – MAC). An important role in this process is played also by growth factors (e.g., hepatocyte growth factor/scatter factor (HGF/SF), vascular endothelial growth factor (VEGF)), and some chemokines (e.g., SDF-1).

Mobilization of stem cells in Crohn's patients correlated with the plasma levels of hepatocyte growth factor/scatter factor (HGF/SF) and vascular endothelial growth factor (VEGF), but, somewhat surprisingly, not with the level of

stromal-derived factor – 1 (SDF-1). In fact, these factors have been reported as potent chemoattractants for MSCs (HGF/SF), EPCs (VEGF), and VSELs (HGF/SF). Recent research also indicates the involvement of other factors, in particular, small bioactive lipids that may direct mobilization and trafficking of stem cells to injured organs. Notably, the release of sphingosine-1-phosphate (S1P) correlated with the activation of the complement cascade and formation of the C5b-C9 membrane attack complex (MAC). Based on these observations, the role of complement cascade activation and release of bioactive lipids in stem cell mobilization in patients with IBD requires further study. Similarly, further studies are also needed to see whether an increase in plasma level of bioactive lipids (e.g., sphingosine-1-phosphate [S1P] or ceramide-1-phosphate [C1P]) could play a role in the mobilization of SCs in IBD patients. However, drugs with the capacity to block S1P axis are already on the market and undergo dynamic clinical testing [51].

Some IBD patients who achieve clinical remission without deep mucosal healing mobilize CXCR4+/Lin-/CD45- cells into PB. These patients (i) displayed active mucosal lesions at the time of colonoscopy, (ii) had a potentially worse prognosis, and (iii) had an increased rate of complications. In patients with treatment naïve Crohn's disease, an increase in the number of circulating MSC, EPC, and small primitive cells expressing the VSEL phenotype could be observed. The number of circulating progenitor/stem cells correlates with endoscopic but not clinical scores (unpublished data). Endoscopic scores also better correlate with several stem cell's chemoattractants (e.g., VEGF). Active CD disease is also associated with strong mRNA upregulation of intestinal early differentiation markers (Igr-5 and ASCL-2) in circulating PB mononuclear cells. Higher plasma C5a levels in IBD in comparison to controls were also noted. Of interest is that no correlation between SDF-1 and the number of VSELs has been found.

These results might indicate that enumeration of circulating stem/progenitor cells in peripheral blood could serve as surrogate parameter in accessing treatment efficacy in IBD. It is likely that mobilization of VSELs is associated with active mucosal inflammatory lesions regardless of the clinical manifestation of IBD.

Overall data support the hypothesis that circulating stem cells might be involved in the process of regeneration of intestinal epithelium in IBD and strengthen the importance of achieving deep remission by stratifying treatment protocols. Furthermore, the mobilized and circulating SCs might potentially serve as biomarkers of the rate of intestinal healing and regeneration; however, this hypothesis requires further clinical evaluation.

7.4.2 Gastric and Pancreatic Cancer

Bone marrow-derived cells attracted to gastric mucosa could be a source of gastric malignancy under inflammatory conditions [52]. This observation paved up the foundations of a concept of migrating cancer stem cells (CSCs). Migrating CSCs contributing to gastric malignancy could express the following surface markers:

CD44, CD133 (prominin-1), ABCG2, ALDH1, LGR5, EpCAM, BMI1, CD24, OCT4, SOX2, and NANOG [53]. In general, CSCs possess special functions, and their presence facilitates metastasis and resistance to radiotherapy and chemotherapy [54]. Currently, targeting liver CSCs is viewed as an emerging approach for the treatment of hepatocellular carcinoma (HCC) [55].

Increased trafficking of bone marrow-derived stem cells in patients with pancreatic and stomach cancer has been described by our collaborators [56]. This mobilization was associated with the activation of promobilizing complement cascade and sphingosine-1-phosphate (S1P) in peripheral blood. The biological role of activated stem cells could result from their autocrine and endocrine effects affecting tumor vascularization and stromalization. Paracrine mechanisms could be executed through release of extracellular microvesicles (ExMVs) from circulating stem cells. Recent research also indicates the involvement of other factors, in particular, small bioactive lipids that may direct mobilization and trafficking of stem cells to injured organs. Notably, release of sphingosine-1-phosphate (S1P) correlates with activation of the complement cascade and formation of the C5b-C9 membrane attack complex (MAC). Activation of proteolytic and fibrinolytic complement cascades and release of cleavage fragments (e.g., C5a and desArgC5a fragments) could enhance mobilization of stem cells from their niche in the bone marrow. Moreover, these stem cells can be attracted from the bone marrow (and hypothetically from the intestinal epithelium) in response to tumor-derived plasma chemoattractants, such as stromal-derived factor-1 (SDF-1), vascular endothelial growth factor (VEGF), zonulin, and hepatocyte growth factor (HGF) or sphingosine-1-phosphate (S1P). Of note, stem cells may secrete several growth factors, cytokines, or even membrane-derived microvesicles that accelerate the regeneration process [57]. As the role of non-mutated, normal stem cells in cancer biology is still controversial, we become interested if such cells could be detected in plasma of colorectal cancer patients.

7.4.3 Colon and Rectal Cancer

Colorectal cancer is one of the most common cancers in western countries, with the prevalence increasing with age. Numerous intrinsic and extrinsic factors were proposed to contribute to the development of this malignancy. It is well known that rare putative CSCs may circulate in the peripheral blood in humans with colonic malignancies. Putative colon CSCs may express the following biomarkers: CD44, CD133, ABCG2, ALDH1, LGR5, EpCAM, BMI1, CD24, CD166, CD29, SOX2, SOX9, OCT4, and DCLK1 [53].

A number of circulating stem cells (e.g., VSELs, HSCs, EPCs) has already been evaluated in peripheral blood of patients with colorectal cancer [23]. In contrast to individuals diagnosed with pancreatic and gastric cancer, neither mobilization of early stem and progenitor cells into peripheral blood nor activation of a complement cascade was enhanced in patients with colorectal cancer. Therefore, based on these results, the conclusion has been drawn that trafficking of normal stem cells in cancer

patients was depended on the type of cancer and its ability to activate promobilization pathways.

Of interest, the potential role of paracrine and endocrine effects of pituitary gonadotropins and gonadal sex hormones in the pathogenesis of colorectal cancer has recently been implicated in the pathogenesis of colorectal cancer [58]. We hypothesized that higher levels of pituitary gonadotropins (PtGt) such as follicle-stimulating hormone (FSH) or luteinizing hormone (LH) observed in postmenopausal women in response to loss of gonadal dysfunction in aging contribute to alterations in stem and cancer stem cells and affect the cancer prognosis. In our paper, the authors performed pioneer studies on tissue samples obtained from colorectal cancer patients as well as established human colorectal carcinoma cell lines. It turned out that several pituitary and sex hormone (SexH) receptors were expressed by cancer cells isolated from patient colonic tissues as well as human HTC116 and HTB37 colorectal carcinoma established cell lines. Colorectal cancer cell lines responded to stimulation by pituitary glycoproteins and gonadal sex hormones by increased adhesion and chemotaxis, and this corresponded with activation of signaling pathways from these SexH receptors [58]. It is likely that these mechanisms contribute to potential of cancerous metastases and affect patient's survival time.

7.5 The Emerging Role of Microbiota in the Pathogenesis of Human Digestive Disease

In recent years, significant progress has been made in the field of microbiology related to human body surfaces including gastrointestinal (GI) tract. The pathogenesis of common disorders (e.g., autoimmune disease, diabetes, liver cirrhosis, obesity, and cancer) has been linked to the alterations of gut microbiota. Microbes could affect body homeostasis through numerous pathways and interfere with gut barrier elements such as neuroendocrine and immune systems. The role of microbiome and gut barrier has been described and reviewed in detail elsewhere [59]. Microbes mediate signals acting locally through autocrine and paracrine mechanisms in the gut as well as systematically through endocrine system affecting distant tissues and organs.

7.6 Host Stem Cell and Microbial Interactions as New Frontiers in Gastroenterology

Host microbial and host-viral interactions also affect intestinal stem and progenitor cells on the level of intestinal crypt-villus influencing their self-renewal and differentiation potential. Studies utilizing germ-free and/or antibiotic-treated mice confirmed the hypothesis that microbiota influences intestinal epithelium and endothelium

[60, 61]. For example, the absence of GI bacteria was associated with decreased villus height and crypt depth in the jejunum and ileum but increased villus height and decreased crypt depth in the duodenum. These intestinal regional differences in animal deprived of microbiota versus colonized animal models have been recently described [62]. Gavage of neonatal mice with probiotic *Lactobacillus reuteri* increases migration and proliferation of enterocytes and affects crypt height. Microbial diversity has direct impact on intestinal epithelial homeostasis [63]. Another example concerns *Lactobacillus rhamnosus*, which protects the murine gut epithelium from radiation injury. However, *Lactobacillus rhamnosus* did not affect diversity of microbiota and exerts its protective effects independently of the microbial composition [64]. The presence of this bacterium could have an impact on microbiota function, resulting in increased synthesis of a short-chain fatty acid. For example, butyrate within the crypt bases inhibits expansion of colonic Lgr5+ ISCs and maintains their integrity positively affecting colon fitness [65]. Short-chain fatty acids have been shown to favor growth and budding of mouse intestinal enteroids [66]. The microbiota may exert their regulatory effects on intestinal regeneration via expression of the Nod2 receptor. In doxorubicin-induced intestinal damage, agonist muramyl dipeptide (MDP) improves intestinal epithelial survival and regeneration in a Nod2-dependent manner [67]. The microbiota can also promote transformation of LGR5+ ISCs in colorectal cancer [68, 69] by promoting inflammation [70] triggered by microbes [71, 72]. Recently, microbiota alterations have been linked to colorectal cancer development [73]. The microbiota can directly promote the spread of colon cancer via calcineurin/nuclear factor of activated T-cell-dependent survival and proliferation of CSCs [73]. Several pathogens (e.g., *Salmonella typhimurium* or *Heligmosomoides polygyrus*) could impact the fate of stem cell lineage in the GI tract. *Salmonella* could trigger an increase of Paneth cells and enterocytes and a significant decrease in Lgr5+ ISCs. *H. polygyrus* infections lead to an increase in the abundance of tuft and goblet cells, but do not change the number of Lgr5+ ISCs [143]. Microbiome with its beneficial and harmful habitants is capable of influencing the ISC status [74, 75]. Also, rotaviruses (RV) infect and damage differentiated cells at villus tips without influence on cryptal ISCs. In addition, a recent publication showed that 24-h fasting in mice promoted ISC function by activating a fatty acid oxidation (FAO) program [76]. Intriguingly, the opposite treatment, a high-fat calorie intake, in mice also promotes ISC renewal and represses differentiation [77]. Interestingly, calorie (either low or high) intake acts via an ISC PPAR δ -mediated FAO program that triggers increased circulation of free fatty acids, which contribute to expansion of intestinal stem cells. Dietary lipids modulate ISC function, as has been documented with palmitic and oleic acids promoting intestinal organoid growth [76, 78].

7.7 Final Remarks

Gastrointestinal stem cell research including circulating and cancer stem cells and studies on the role of microbiota in various clinical entities open up a new area of investigation in gastroenterology.

We envision that stem cells and microbiota with by-products are involved in tissue and organ damage and repair. Host-microbe interactions are accompanied by different mechanisms (e.g., autocrine, paracrine or endocrine, immunomodulatory effects, replacement of damaged cells, cytotoxic effects).

Further studies are needed to assess in more details the proper mechanism of cell-host-microbe interactions and their role in the process of tissue regeneration. Also the use of these components as biomarkers is still awaiting elucidation. These questions remain to be addressed on a larger sample of patients in prospective, well-designed clinical trials. The most important is to observe whether the number of normal and cancer stem cells circulating in peripheral blood correlates with disease biology and metastatic potential of cancers and whether these cells have prognostic and therapeutic value. It remains to be tested how microbial factors influence stem cell trafficking and what is the physiological role of recently reported blood microbiome. Recently, the evidence accumulates that not only eukaryotic cells but also gut bacteria may release extracellular microvesicles that are absorbed from the gut into portal and systemic circulation. This phenomenon opens a new area of investigation. Finally, the number of circulating stem cells could be enhanced by systemic infusion or local delivery of these cells purified from the patient's own bone marrow or by increasing or suppressing their number in peripheral blood after administration of various mobilizing or inhibiting agents such as G-CSF and/or the CXCR4 antagonist AMD3100 or SIP agonist fingolimod. The current focus on a role of microbiota in human health and pathology and, in particular, to study its role in gut inflammation, stem cell trafficking, and cancer development will be the source of next-generation diagnostic and therapeutic tools in gastroenterology and gastrointestinal oncology.

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Chapter 8

Stem Cells in Psychiatry



Jolanta Kucharska-Mazur, Mariusz Z. Ratajczak, and Jerzy Samochowiec

Abstract The development of regenerative medicine has provided new perspectives in many scientific fields, including psychiatry. Stem cell research is getting us closer to discovering the biological foundation of mental disorders. In this chapter, we consider the information relating to stem cells and factors involved in their trafficking in peripheral blood in some psychiatric disorders (major depressive disorder, bipolar disorder, schizophrenia, anxiety disorder, and alcohol dependence). The authors also include the implementation of current research regarding neurogenesis in adult brain and induced pluripotent stem cells in investigating concerns in etiopathogenesis of mental disorders as well as the implication of research for treatment of these disorders.

Keywords Stem cell · Depression · Bipolar disorder · Schizophrenia · Anxiety disorder · Complement cascade · SDF-1 · Sphingosine-1-phosphate · Sterile inflammation

8.1 Neurogenesis in Adult Brain

Since the discoveries of Altman [4] and Eriksson [27] concerning neurogenesis in adult brain of mammalian, we have observed a rapid development of research in this field. Undoubtedly, in adult brain, new neurons are generated in the subventricular zone of the anterolateral ventricle and the subgranular zone (SGZ) of the hippocampal

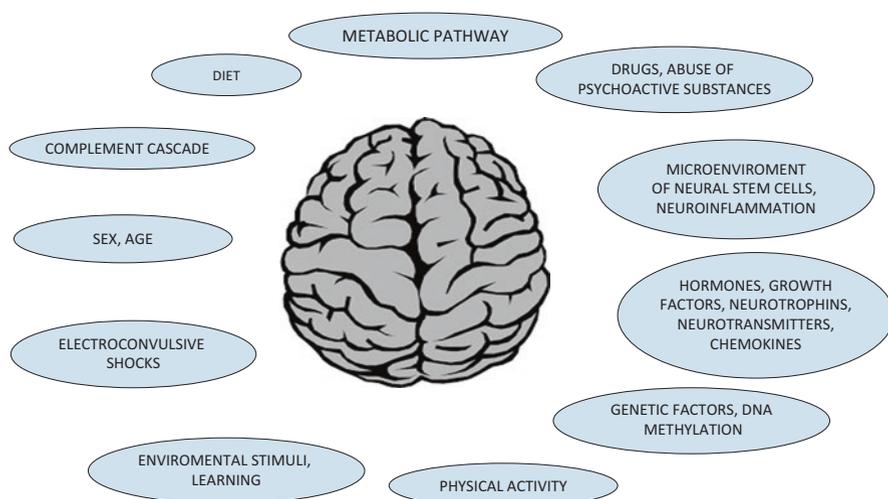
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dentate gyrus. In the subgranular zone, the emerging cells give rise to granule cells [51]. Neuroblasts, forming throughout the entire life in the subventricular zone, migrate along the rostral migratory stream toward the olfactory bulb, where a small part of them differentiates to interneurons, which serve a role in olfactory memory, social interactions, and odorant discrimination [57]. Interneurons of the olfactory bulb may also originate from local progenitor cells [9]. Since interneurons also form in striatum, it can be speculated that their source is also the subventricular zone. The studies of Magnusson et al. [53] allow for the speculation that local astrocytes are involved in their formation. The striatal neurogenesis is attributed a role in the cognitive flexibility [9].

Factors involved in neurogenesis



Neurogenesis possibly also takes place in the area of hypothalamus, and its limitation, e.g., through a diet rich in fats, leads to changes in energy expenditure and changes in the body mass of mice [49, 56]. Insulin-like growth factors (IGFs) take part in the regulation of formation of new neurons. Extreme stress can cause a disruption of this process. Estradiol, leptin, and ghrelin influence the hypothalamic synaptic plasticity, and the effects of these factors are species specific (e.g., rodents vs humans) [76].

Incorrectly proceeding adult neurogenesis may become a cause for many neurodegenerative, neurodevelopmental, and injury-based disorders. Neurogenesis in the hippocampus is influenced by neuroinflammation, physical activity, diet, environmental stimuli, learning, abuse of psychoactive substances, stress, depression, antidepressant medication, sex, and electroconvulsive shocks. The process of formation of new neurons is closely monitored, to avoid the risk of quick depletion of regenerative possibilities or, on the other hand, an exorbitant growth of newly formed cells.

Neurogenesis lasts a lifetime; however, during aging it weakens, which influences the risk of neurodegenerative diseases increasing with age. In human studies, we observe, with aging, a decrease in the number of intermediate progenitors in the dental gyrus [16]. Already in early childhood, the number of proliferating cells in the subgranular zone significantly decreases, and the few remaining are predominantly microglia cells [22]. Gradually, gliogenesis towers over neurogenesis [37]; the density of oligodendrocytes increases as well as the area of myelination in the cortex. The decreased intensity of neurogenesis also causes a decrease of, dependent on it, plasticity of the brain in humans, as well as possibly (lack of conclusive data) cognitive abilities. The decrease of neurogenesis with age is likely connected to disorders Notch/RBPJ signaling [24]. It is worth mentioning that, with age, angiogenesis also weakens, blood flow to the brain decreases, and the concentration of CCL 11 chemokine increases. On the other hand, it is possible to significantly increase neurogenesis in the aging brain of animals, through, e.g., intracerebroventricular fibroblast growth factor 2 infusions, or through decreasing the concentration of β -2-microglobulin, the factor favorable to the process of aging [61, 65, 75].

The external environment affects humans through many factors interacting with peripheral tissue, which communicate with the brain through a range of plasma-derived factors, as well as neural pathways, in turn brain cells affect newly created neurons through neurotransmitters and plasma-derived factors, including gamma-aminobutyric acid (GABA). This influence is not always sustainable. For example, the increase in proliferation and the dendritic spine density of neurons influenced by physical activity quickly fades away after the activity is halted. The factors responsible for tissue regeneration, such as hormone adiponectin, insulin-like growth factor 1 (IGF-1), and vascular endothelial growth factor (VEGF), are acknowledged mediators of physical activity's influence on neurogenesis [25]. According to Bolijn and Lucassen [11] and Eisinger and Zhao [25], angiotensin II, glucocorticoids, leptin, adrenaline, reactive oxygen species, AMP-kinase, peroxisome proliferator-activated receptor (PPAR), PPAR γ coactivator 1 α (PGC-1 α), ciliary neurotrophic factor (CNTF), glycogen synthase kinase 3 beta, diazepam-binding inhibitor, cathepsin B, C-X3-C motif chemokine ligand 1, dynamin-related protein 1, and proinflammatory cytokines may also play a part in this regulation. On the other hand, mediators of influence on neurogenesis rich in environmental stimuli can potentially include kallikrein-related peptidase 8 and Ras protein-specific guanine nucleotide-releasing factor 2 [20, 78], while electroconvulsive shock mediators are general receptor for phosphoinositides 1-associated scaffold and protein growth arrest and DNA damage-inducible beta [52, 84].

It has been shown in studies on rodents that the influence of learning on neurogenesis is significant depending on sex, both in terms of the area which is impacted by learning and the type of tasks performed and chosen strategies ([83]). Under the control of sex, steroids are placed neuronal activity, growth factor expression, nitric oxide signaling, and, indirectly, proliferation, differentiation, and apoptosis of neuronal, endothelial, and glial cells. Their interaction with neurotrophins shapes the neural cell function and plasticity. Estrogens, in human development, affect arborization, synaptogenesis, and circuit formation. They accelerate the progress

of neurodegenerative diseases – women show a more accelerated decline in cognitive functions in Alzheimer’s disease and a more frequent diagnosis of atypical depression and depression with anxiety. Certainly, other sex steroids also influence neurogenesis – for example, progesterone increases mitosis of neural stem cells and neural progenitors, and androgen metabolite DHT increases survival of newly created neurons (for more details, see [48]).

In strict control of neurogenesis, processes on each stage are both paracrine factors and autocrine, membrane-bound factors: Notch, Sonic hedgehog, bone morphogenetic proteins, Wnts, cytokines, and growth factors. They determine proliferation, differentiation to the line of glial or neural lineage, inclusion to neuronal network, and apoptosis [62]. As proven by Chancey et al. [15], glutamatergic and GABAergic signaling remains in close connection in the process of survival of newly formed neurons – an enriched environment causes, with the current participation of GABAergic, activation of NMDA receptors, which in turn allows the premature neurons to react to glutamatergic signaling. Owing it to GABAergic signaling, neurons are integrated into the neuronal network ([79]). Critical period, in which newly formed neurons have an increased synaptic and dendritic plasticity, lasts for 3 weeks after mitosis.

The progress of neurogenesis is significantly influenced by DNA methylation. According to Jobe and Zhao [42], its role is to help discover (1) the diverse cell fates found in the brain and (2) the change in gene expression as an answer to signals received by cells. These processes are linked to the activity of methyl-CpG-binding domain (MBD), DNA methyltransferase (DNMT), and ten-eleven translocation (TET) proteins.

Quiescent neural stems have energy demands considerably different from the demands of proliferating cells that is why the process of neurogenesis is connected to remodeling of metabolic pathways. Presumably, the changes in metabolic pathways not only are linked with the beginning of proliferation but also are specific for particular cells, being created as a result of this process. The functioning of neurons involves large energy expenditure, connected to excitatory signaling: action potentials, the necessity of maintenance and reproduction of ion gradients, as well as energy consumed by the neurotransmitter cycle [5]. In the process of acquiring energy, neurons use lactate, released to the extracellular space as a product of glycolysis in astrocytes [3]. Therefore, differentiating cells of neurogenic lineage requires significant metabolic adaptation [7]. Due to smaller energy requirements, quiescent stem cells, in majority, acquire energy from glycolysis, which also delivers construction elements for many pathways of biochemical synthesis. Homem et al. [34] in animal model studies suggest that entering the proliferation pathway may be caused by the switch from glycolysis to oxidative metabolism. The limitation of glycolysis occurs during the activation of neural stem cells, and the transition of neural stem cells into intermediate progenitor cells, is linked to the upregulation of enzymes of tricarboxylic acid cycle as well as the growth of ATP synthesis related to oxidative phosphorylation. The lack of induction of electron transport chain and oxidative phosphorylation leads to apoptosis of intermediate progenitor cells [8]. The mitochondria influence both proliferation and further phases of neurogenesis,

including shaping of newly formed neurons, through the influence on the redox state of cells and energy control, participation in transfer of electrons and hydrogen, modulation of signaling, and also delivery of products for cell construction [82].

In both primary regions of adult neurogenesis, thyroid hormone influences adult progenitor development; the key role here is played by the balance between liganded and unliganded TR α 1, thyroid hormone receptor isoforms [29].

Since the 1990s, we have known that the complement system plays a crucial role not only in immunological processes but also in intracellular signaling, proliferation, differentiation, neural migration, synapse pruning, and apoptosis of cells [32].

In the early stages of development of the nervous system, impairment of C5a-C5aR1 signaling in the conditions of foliate deficiency leads malfunction in neural tube closure process, because C5aR is involved in regulation: proliferation and differentiation [23]. The importance of this signaling does not end with the moment neural tube is closed, as it is also significant in the process of proliferation of neural progenitor cells of the ventricular zone [19]. On the contrary, complement proteins C1q and C3 control synapse pruning in the developing brain, while C3 in migration of forming neurons [31]. It is thought that the key role in the regulation of the last process is played by C3a, which is a product of MASP1/2 activation of the lectin pathway. It is worth mentioning that in people on the autism spectrum we observe changes in expression of genes C1q, C3, and C4 in the central nervous system [28].

Similarly, significant roles are played by complement factors, especially C3a, also in adult brain. The presence of receptors for complement proteins (C3aR and C5aR1) on neural stem cells and neural progenitor cells suggests that these proteins regulate neurogenesis while also being involved in regenerative processes after damage or hypoxia [64]. For astroglialogenesis, proteins C3a and C1q are crucial [35].

Neurogenesis in hippocampus in given essential role in learning processes, memory, mood regulation, and brain plasticity (understood here as the ability to respond to stimulus and adapt to new conditions), so it is an interesting area for creation of new therapeutic strategies. Perhaps, in the future, it will be possible to take advantage of the migration of neuroblasts into the specific brain areas and use them to fix structural brain damage and posttraumatic damage.

8.2 Affective Disorders

The role of neurogenesis in depression has been known for many years. The premises of this disorder's association with abnormal neurogenesis in hippocampus are, according to Peng and Bonaguidi [61], decreased volume and neurogenesis in hippocampus in people with depression; suppression of neurogenesis through increased level of glucocorticoids, which are secreted in response to chronic stress; stimulation of neurogenesis by selective serotonin reuptake inhibitor (SSRI) antidepressants; delayed effect of this medicine in depression, related to the necessity of forming new neurons; or finally the lack of effectiveness of SSRI, if, due to some

circumstances, neurogenesis is not possible. Consecutively discovered antidepressants, with a mechanism different from monoaminergic mechanism of functionality, also induce neurogenesis, whereby their activity is strongest at the stages of proliferation, maturation, and survival of cell. The level of serotonin seems to be essential here, since it shows a positive correlation with proliferation and survival of forming neurons, while epinephrine works solely at the stage of proliferation of progenitors [46]. Antidepressants influence neurogenesis also through neurotrophic and growth factor. The role in etiopathogenesis of depression is given to a decreased level of BDNF, which is a neurotrophin involved in cell differentiation processes and axon development, which can potentially also induce the death of neuronal cells [67]. Antidepressants may induce BDNF expression [44]. In studies involving rodents, the application of BDNF in the dentate gyrus of hippocampus has a similar effect as application of antidepressants [74]. Involved in the influence of antidepressants on adult neurogenesis are also endothelial growth factor (VEGF), the insulin-like growth factor 1 (IGF1), and fibroblast growth factors (FGFs) [54, 81]. On the contrary, factors such as physical activity or proper diet, influencing neurogenesis, work in a way similar to antidepressants.

It is worth mentioning that factors we link to etiopathogenesis of depression and/or anxiety, such as prenatal stress, early childhood trauma, chronic stress, or disadvantaged living conditions, are also responsible for decreased adult neurogenesis. The connection between chronic stress and neurogenesis creates a vicious cycle. Newly formed neurons, especially in the ventral dental gyrus, are likely responsible for the response modulation to stress ([79]). Antidepressants reverse structural consequences of chronic stress, increasing dendritic arborization and synaptogenesis [72]. Regulation of neurogenesis in the hippocampus is a potential goal of new antidepressive therapies, particularly in drug-resistant depression. One of the suggestions is the encapsulation of mesenchymal stem cells [45].

Mood stabilizers cause increased self-renewal of NSCs, and their delayed effect, much like antidepressants, is explained through their influence on neurogenesis. Therapeutic effect of mood-stabilizing drugs is bound to the activity of Notch signaling, crucial to the self-renewal process of NSCs. Furthermore, in people with affective disorders, we can observe an abnormal function of oligodendrocyte lineage cells in the prefrontal cortex [33]; meanwhile, studies concerning the influence of antidepressants on oligodendrocyte precursor cells (OPC) point to the sensitivity of OPC located in regions linked to affective disorders to stress and therapeutic activity [33].

One of the currently intensively explored fields of science is the role of chronic inflammation in etiology of mental disorders, particularly affective disorders. In patients with MDD, we conclude increased indications of inflammation, including IL-6, components of cascade, as well as tumor necrosis factor (TNF) [58]. Antidepressants affect the concentration of proinflammatory factors, including interleukin 1b, IL-6, TNF α , and interferon, which in turn can impair adult neurogenesis [6]. TNF α decreases proliferation of neuronal progenitors and promotes apoptosis [26]. The application of proinflammatory cytokine, interferon α , relates to

the appearance of depressive symptoms, which in the animal model is related to a decrease in adult neurogenesis [86].

Generally, there is not enough data concerning the activity of chronic peripheral inflammation and proinflammatory cytokines in humans, but in animal models they cause resident microglia activation in the brain, which in turn leads to suppression of proliferation of NSC and apoptosis of progenitors and decreases chance of survival for newly formed neurons and the possibility of incorporating them into neuronal circuits. In diseases connected with such inflammation (e.g., chronic intestinal inflammation, diabetes mellitus), we statistically more often observe depression, cognitive disorders, and learning difficulty which is connected to their influence on adult neurogenesis [17]. Also, bipolar disorder is clearly connected with inflammatory diseases, e.g., cardiovascular disease. Despite relatively few studies on this topic, proinflammatory chemokines are treated as potential peripheral markers for bipolar trait. Numerous studies on bipolar disorder deliver information about the level of IL8, CCL2, and CCL3; however, these results are not conclusive [77].

Studies on people with bipolar disorder (of at least 10-year progression, not treated with lithium salts, in euthymia) have led to interesting discoveries. Well, in these people were found increased concentrations of complement proteins C3a, C5a, and C5b-9 in comparison to healthy people, without the adequate mobilization of stem cells from bone marrow to peripheral blood. Perhaps, such a mobilization happens during a period of exacerbation of the disease. The comparison between groups with bipolar disorder I and bipolar disorder II has shown higher concentration of C5b-9 in BP II [79]. The usage of lithium salts in patients with BP caused suppression in regenerative processes [30]. Chronic inflammation and inadequate regenerative reaction may be a cause for structural changes in the brain of these people. These discoveries require further studies on bigger groups of patients as well as people suffering from major depressive disorder, treated correspondent medication.

New facts concerning etiopathogenesis of depression are delivered by studies that use human-induced pluripotent stem cells (iPSCs), which allow for the recreation of cellular phenotypes, typical in depression, and studying of new antidepressant therapies, as well as a verification of existing views on efficiency of antidepressants [36]. iPSCs is gained through reprogramming of somatic cells, e.g., fibroblasts, to pluripotent stem cells and afterward obtaining from them cells inherent for different tissues, including nervous tissue. In such cells, obtained from people with bipolar depression, changes leading to hedgehog signaling disorder were found [18], potentially related to depression. Specific methods used to obtain serotonergic neurons *in vitro* and their assets were discussed in review of Vadodaria et al. [80].

When it comes to iPSCs obtained from people with bipolar disorder and differentiated to glutamatergic dentate gyrus, neurons show hyperexcitability and upregulated mitochondrial gene expression, elevated mitochondrial function, and smaller mitochondria in comparison to healthy people [2].

However, it is crucial to remember that these kinds of models have many limitations, such as limited reaction to environmental factors (stressors) [12].

8.3 Schizophrenia

Currently, the most often quoted is neurodevelopmental theory of schizophrenia, according to which the onset of this disorder should be searched for in early development phases in humans. In schizophrenia, we observe the presence of genetic risk factors, dysfunction in regulation of HPA axis, neurodevelopmental abnormalities, brain plasticity dysfunctions, and dysregulation of the immune system. Chronic neuroinflammation leads to loss of neurons and the growth of oxidative stress. Neurogenesis is one of neuroprotective factors, which is why its limitation may lead to unfavorable progression of schizophrenia with an elevated deterioration. Studies concerning the effectiveness of adding medication with anti-inflammatory (e.g., COX-2 inhibitors) and antioxidizing effect (e.g., N-acetyl cysteine, vitamin C) in neuroleptic treatment give inconclusive but promising results [43].

In peripheral blood of people with schizophrenia, we affirm an elevated concentration of proinflammatory cytokines and dysfunction in lymphocyte functions. In particular, an elevated concentration of interleukins IL - 1 β , IL-6, and TNF α is seen [21]. Also, an association of schizophrenia with polymorphisms of IL-10 and IL-1 genes is being observed [10, 85]. Inconclusive results are obtained in studies of peripheral concentration of IL-8, CCL2, CCL3, and CCL5 [77].

Crucial meaning in pathogenesis of schizophrenia is being given to complement cascade proteins, mediating in innate and acquired immunity, but also significantly involved in transfer of stem cells [1] as well as in synapse elimination (complement proteins C1q, C4A, and C4B [63]); complement forms a bridge between neurodevelopmental theory and inflammatory schizophrenia. As shown in multicenter genome-wide association studies, led by Schizophrenia Working Group of the Psychiatric Genomics, polymorphisms within genes coding complement proteins C4a and C4B show an association with an elevated risk of schizophrenia, which is being explained with pruning dysfunction in conditions of elevated activity of C4 [71]. On the other hand, Ishii et al. [38] have found significantly elevated concentration of C5 in cerebrospinal fluid in people with schizophrenia, as compared to healthy people. The role of complement system proteins is also confirmed by studies led by Santos S3ria et al. [69], who found an elevated level of C3 serum in patients with schizophrenia, in comparison to euthymic bipolar patients and healthy controls, along the lack of differences in C4 levels. Authors explain it through chronic immune activation in schizophrenia and differences in the pathomechanism of this disorder and bipolar disorder.

In the Pomeranian University of Medicine in Szczecin, a study was conducted on patients with first-episode psychosis (FEP) of the nonaffective character, from the standpoint of mobilizing stem cells from bone marrow to peripheral blood and level of plasma factors influencing their movement. It was found that in the group of people with psychosis, the concentration of VSEL cells (Lin-/CD45-/CD34+) was higher, and the concentration of sphingosine-1-phosphate and complement C3a was lower than in healthy control group. After obtaining a remission of psychotic symptoms, the concentration of VSEL cells and S1P was not changing, and the concentration of C3a was normalized to the level adequate for healthy

people. Elevated expression of genes, being markers for pluripotent stem cells and early nervous lineage cells, and aforementioned differences were proof that FEP is accompanied by systemic regenerative reaction with its simultaneous dysregulation. After neuroleptic treatment, the change in gene expression was being observed, as well as the concentration of C3a, which demonstrates the influence of this kind of treatment on regenerative system. Interesting seems an observation of different concentrations of C3a, S1P, and VSEL between people with an episode of schizophrenic and non-schizophrenic character [47].

Induced pluripotent stem cells have been used for studies on schizophrenia since 2011. Brennand et al. [13] obtained neural progenitor cells from fibroblast of patients with schizophrenia, declaring a decreased connectivity and difference in expression and transcription of genes, leading to decreased neuronal migration, of many disorders in signaling, synaptic transmission, and dendritic spine function [14, 39]. Other studies, using NPC, have allowed to observe changes in mitochondrial structure and function and oxygen consumption as well as increased number of reactive oxygen species in NPC obtained from patients with schizophrenia [60] and also abnormal differentiation to glutamatergic and dopaminergic lineages [68]. iPSC can also be used for studying the effectiveness of neuroleptics [70]. On the other hand, it raises doubts how much reprogrammed cells show the significance of phenotypes in schizophrenic patients (increased risk of mutation), on what level they allow to illustrate the environmental influence, and if they are sufficiently controlled at genetic level. It is worth pointing out that the studies concern individual patients, forming neurons are underdeveloped, and they lack myelination [73].

8.4 Anxiety Disorders

Etiology of anxiety disorders is not exactly known; undoubtedly however their development is influenced by the interaction of genetic factors and environmental factors, with the predominance of the latter.

Part of the information, concerning the influence of chronic stress and antidepressive/antianxiety treatment on neurogenesis, is placed in earlier chapters.

Studies, conducted on people with panic disorder (PD), concerning the behavior of stem cells in peripheral blood and factors involved in their trafficking, have allowed to discover lower concentration of hematopoietic stem cells (HSCs) (Lin-/CD45 +/AC133 +), complement cascade proteins (C3a, C5a, C5b-9), sphingosine-1-phosphate, and stromal cell-derived factor 1 (SDF-1) in test group, in comparison to healthy controls. It is worth mentioning that the regenerative system in people with PD behaves differently from psychotic patients, or with affective disorders [40]. The connection between complement and affective disorders is confirmed by the experiment conducted on mice, in which centrally administrated C5a had an anxiolytic-like effect [55]. On the contrary, studies conducted on animal model, led by Jang et al. [41], suggest a relationship between increased S1P and appearance of medication, through modulating dopaminergic activity in amygdala.

8.5 Alcohol Dependence

The relationship between heavy alcohol consumption and regenerative system is connected with its effect on liver functions – directly and through microbial products. As a result, the liver is at risk of being affected by proinflammatory factors (TNF α , IL-8, and IL-1b). The final result is alcoholic liver disease [59].

The iPS-derived neurons, obtained from people addicted and not addicted to alcohol, provide information on pathophysiology of addiction. They are used, inter alia, for electrophysiological studies and for evaluation of the influence of exposure to alcohol, e.g., on GABA and NMDA receptors [50].

8.6 Sterile Inflammation

The “sterile inflammation” theory explains in what way do chronic inflammatory processes influence the state of the brain. Studies, conducted throughout the last decade, provide increasingly more evidence on the connection between mental disorders and pathological activation of inflammatory factors. In psychotic disorders, in peripheral blood are found proinflammatory factors, including complement system factors, increased concentration of monocytes and neutrophils, and increased reactivity of microglia and astrocytes. Also, depression often coexists with inflammatory processes. The abovementioned information was on prolonged or chronic activation of complement cascade, which releases the inflammatory process in the brain and impairs the functions of this organ. This process is initiated by the mannan-binding lectin pathway of complement cascade activation released by, present in increased numbers in brain tissue, danger-associated molecular pattern (DAMP) mediators. This includes, inter alia, extracellular ATP and high-mobility group box 1 (HMGB1) protein. The inflammatory process is being limited by heme oxygenase 1.

8.7 Summary

Since 1998, research on neurogenesis, stem cells and the factors responsible for their movement has been carried out more and more frequently. They allow, on the one hand, for better understanding of etiopathogenesis of mental disorders, and on the other hand, they create hope in finding peripheral markers for mental disorders [47]. They also bring hope for new therapeutic strategies, based on knowledge about mechanisms leading to pathological changes in the brain of people with mental disorders.

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Chapter 9

The Role of Extracellular Vesicles as Paracrine Effectors in Stem Cell-Based Therapies



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Abstract Stem cells act in a paracrine manner through the secretion of biologically active cargo that acts on cells locally and systemically. These active molecules include not only soluble factors but also extracellular vesicles (EVs) that have recently emerged as a mechanism of cell-to-cell communication. EVs act as vehicles that transfer molecules between originator and recipient cells, thereby modifying the phenotype and function of the latter. As EVs released from stem cells may successfully activate regenerative processes in injured cells, their application as a form of therapy can be envisaged. EVs exert these proregenerative effects through the modulation of relevant cellular processes including proliferation, angiogenesis, oxidative stress, inflammation, and immunotolerance, among others. In this chapter, we review the preclinical studies that report the effect of stem cell-derived EVs in various pathological models of human disease.

Keywords Exosomes · Microvesicles · Extracellular vesicles · Stem cells · Tissue repair · Experimental models · Cell-to-cell communication · EV-based therapy

9.1 Introduction

Stem cells are multipotent undifferentiated cells with the capability to form identical clones, self-renew, and generate differentiated cells. Classification of stem cells is based on their ability to differentiate; for example, embryonic stem cells are able to differentiate into cells of the ectoderm, endoderm, and mesoderm and therefore

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are considered totipotent. Fetal stem cells such as cells of the umbilical cord and adult stem cells exhibit multipotent/unipotent characteristics and are found in tissues of children, adolescents, and adults. The use of embryonic stem cells in humans is currently under review due to ethical concerns together with their possibility to degenerate into malignancies [1, 2]. As mentioned before, a small number of multipotent adult stem cells are present in the tissues which have the ability to engender the specific cell types of the tissue in which they are located. Among various types of adult stem cells identified, the mesenchymal stromal cells (MSCs) have proven to exhibit a promising role in regenerative medicine. Apart from the bone marrow, MSCs can also be found in other tissues such as adipose, dental pulp, umbilical cord blood, amniotic fluid, the placenta, Wharton's jelly, the lung, the thymus, the liver, the pancreas, the spleen, the kidney, as well as the brain [3, 4]. They have the potential to differentiate into cells of mesenchymal origin such as chondrocytes, osteoblasts, endothelial cells, cardiomyocytes, and adipocytes. In addition, MSCs can also differentiate into cells of nonmesenchymal origin such as neuronal cell lineages and hepatocytes [5]. Many studies in the literature have shown that MSCs exert beneficial effects at least partly through a paracrine action as oppose to engraftment within damaged tissues [6]. The presence of injury is considered an important prerequisite for the homing of MSCs to a specific site to exert their regenerative effects. For instance, the administration of MSCs as a preventive treatment in an acute kidney injury model (AKI) did not induce recovery [7]. Furthermore, in a rat gentamicin-induced AKI model, bone marrow-derived MSC did not prevent the damage but repaired it mainly through a paracrine action [7]. Localization of MSCs at the site of injury is driven by various receptor interactions. For instance, SDF-1 has been shown to favor homing of MSCs to the kidney post-ischemia reperfusion injury through the interaction with CXCR4 which is upregulated due to hypoxia in the injured tissue [8]. Moreover, CD44 expressed on the membranes of MSCs has been shown to interact with hyaluronic acid normally hyperexpressed in the injured kidney, thus favoring recruitment of MSCs [9]. Nonetheless, the engraftment of MSCs in most tissues is considered to be transient, and no evidence of *in vivo* transdifferentiation has been reported. In addition, the low number of MSCs detected within kidney tubules and the increased proliferation of tubular cells that survived postinjury suggest that the observed beneficial effect of MSC treatment occurs through a paracrine/endocrine pathway of repair rather than a direct repopulation of tubules. The most compelling evidence of this mechanism of tissue repair comes from the demonstration that conditioned medium from MSCs was also able to reproduce the same beneficial effects of the actual cells [10]. In addition, the beneficial effect of MSCs in experimental models of myocardial infarction was also reported to be due to paracrine factors that promoted endogenous repair by inducing angiogenesis, stabilization of extracellular matrix, and stimulation of myocytes [11–14].

There is now a general consensus that the multipotency ability of MSCs has a minimal contribution towards their therapeutic potential with the paracrine mechanism appearing to be more significant. All stem cells including MSCs secrete a multitude of soluble factors such as growth factors, cytokines, chemokines, and

nucleic acids that can modulate tissue regenerative responses by inhibiting cell apoptosis and fibrosis, favoring cell proliferation and angiogenesis, the expansion of intrinsic tissue progenitors, as well as modulating inflammatory and immune responses [15, 16]. Furthermore, the secretome may act either directly or indirectly on injured cells triggering neighboring cells to also secrete bioactive molecules [16]. Ratajczak et al. [17] through their studies have shown that part of this secretome also includes vesicles that could influence cell-to-cell communication by delivering bioactive molecules including growth factors and nucleic acids that are able to modify the phenotype of recipient cells.

9.2 Stem Cell-Derived Extracellular Vesicles

In recent years, it has become evident that cells from tissues are capable of releasing small vesicles collectively known as extracellular vesicles (EVs). Similar to the cells from which they are derived, these vesicles are composed of a lipid bilayer that encapsulates and protects the internal biologically active cargo composed of proteins and nucleic acids from the external environment [18, 19]. In both physiological and pathological settings, EVs transfer their cargo to other cells located either in the vicinity or systemically through the blood and other organic fluids. EVs mainly comprise of two major types of vesicles (microvesicles and exosomes) with overlapping phenotype and size distribution. It has therefore been recommended by International Society of Extracellular Vesicles (ISEV) to use the term extracellular vesicles to define this heterogeneous group of particles collectively [20]. Microvesicles are vesicles shed directly in the microenvironment from the cell membrane of healthy cells and have a size ranging from 100 to 200 nm. These vesicles differ from preapoptotic microvesicles, which also originate from the cell surface through budding of the plasma membrane but instead are much bigger in size and are mainly derived from cells affected by an injury. In the past, the term microvesicles was used in the literature to indicate vesicles shed from the cell surface of both healthy and diseased cells, and for this reason, the range of size was considered to be from 100 to 1000 nm.

Exosomes are a more homogeneous group of vesicles smaller in size (30–150 nm), which are released extracellularly following the fusion of multivesicular bodies (in which they are formed) with the cell membrane. The endosomal sorting complex required for transport (ESCRT) [21] together with ceramide [22] or tetraspanins has been shown to play a role in the generation and release of exosomes [23]. However, both ESCRT and tetraspanins have also been implicated in the biogenesis of microvesicles. Apoptotic bodies are also considered as a class of vesicles released by apoptotic cells. They have a size range between 1000 and 5000 nm and are mainly enriched with nuclear fragments [24].

In this chapter, we review the data relative to EVs released by stem cells investigating the potential role of their paracrine action. The seminal work of Ratajczak [17] demonstrated that EVs derived from embryonic stem cells could modify the

phenotype of hematopoietic progenitors through the horizontal delivery of proteins and mRNA of transcription factors. Subsequent studies demonstrated that the ability of EVs to transfer information that modified the phenotype and functions of recipient cells was a more general phenomenon observed in multiple cell types and lineages. For instance, endothelial progenitor cell (EPC)-derived vesicles were shown to induce a proangiogenic phenotype in quiescent endothelial cells through the transfer of mRNA coding for proangiogenic factors [25], whereas, in another study, Valadi et al. [26] showed that EVs derived from mastocytes were able to share both functional mRNAs and microRNAs between cells. Through these works, it was therefore confirmed that EVs carry and transfer multitude of cargo including several types of coding and non-coding nucleic acids [27].

Over the last few years, a plethora of studies have been reported that underlined the potential of stem/progenitor EVs to mimic the biological activity of their cellular counterparts. Furthermore, several preclinical studies have also addressed the possible use of these EVs as a cell substitute for therapy [28].

9.3 Regenerative and Anti-Inflammatory Effects of EVs Derived from Stem/Progenitor Cells

EVs from different stem cell types have been investigated in various models of tissue injuries. Reports of these studies have shown EVs to exert proregenerative effects through the modulation of relevant cellular processes such as proliferation, angiogenesis, oxidative stress, inflammation, and immunotolerance (Table 9.1).

9.3.1 Renal Regeneration

Several studies demonstrated that stem cell-derived extracellular vesicles (SC-EVs) have renoprotective properties in various models of kidney injury. The first evidence of the beneficial effect of EVs released from bone marrow MSCs (BM-MSCs) was demonstrated in an AKI model induced by glycerol administration. A single intravenous injection of EVs at the peak of the damage facilitated recovery morphologically and functionally, mainly through the induction of tubular cell proliferation [29]. This effect of EV-MSCs was similar to that induced by the cells from which they were derived, suggesting EVs as a suitable substitute for cell therapy. In an additional study, the effect of MSCs and their EVs was compared in a mouse model of nephrectomy [30]. The data showed that MSC-EVs like their cellular counterpart reduced fibrosis and tubular atrophy. Further analysis of the MSC-EV content revealed that the proliferative effect was exerted by the exosomal fraction through the transfer of mRNAs, miRNAs, and proteins which could influence cell proliferation, as well as regulate different pathways (proproliferative and antiapoptotic), leading to kidney regeneration [31]. In addition, a similar effect in the same model was also observed on

Table 9.1 Therapeutic effects of stem/progenitor cell-derived EVs

Therapeutic applications	Preclinical model	EV sources	Mechanism	References
<i>Renal regeneration</i>	Glycerol-induced AKI	BM-MS HLSC	Tubular cell proliferation Functional recovery	[29, 31, 32]
	Nephrectomy-induced CKD	BM-MS	Reduced tubular atrophy and fibrosis	[30]
	Cisplatin-induced lethal AKI	BM-MS CB-MS	Improved survival, renal function and morphology Reduced oxidative stress and cell apoptosis	[33, 34]
	Gentamicin-induced AKI	BM-MS	Reduced renal dysfunction	[7]
	Ischemia/reperfusion injury	BM-MS CB-MS WJ-MS Renal-MS EPC	Reduction of renal damage and prevention of CKD; suppression of inflammation	[35–39, 41, 42, 48]
	Anti-Thy1.1 glomerulonephritis	EPC	Inhibition of complement-mediated mesangial injury	[43]
	Diabetic nephropathy	Urine-MS	Protection of podocytes and tubular cells from apoptosis	[45, 46]
	Aristolochic acid-induced CKD	HLSC	Inhibition of fibrosis; suppression of inflammation	[47]
<i>Neural regeneration</i>	Traumatic brain injury	BM-MS	Enhanced angiogenesis and neurogenesis; reduced inflammation	[51]
	Retinal degeneration.	BM-MS	Ganglion cell survival and axon regeneration	[52]
<i>Cardiac regeneration</i>	Myocardial ischemia-reperfusion injury.	ESC-MS CPCs CB-MS	Reduction infarct size; angiogenesis and inhibition of inflammation; Protection from apoptosis; Decrease of fibrosis	[55–61]
<i>Hepatic regeneration</i>	CCl4-induced liver fibrosis	CB-MS	Inhibition of epithelial-mesenchymal transition and inhibition of fibrosis	[63]
	Liver ischemia-reperfusion injury	iPS-MS	Hepatoprotection	[66]
	Acute liver failure toxic models	BM-MS HLSC	Inhibition of apoptosis and inflammation; hepatocyte regeneration; enhanced survival	[64, 65, 67–70]

(continued)

Table 9.1 (continued)

Therapeutic applications	Preclinical model	EV sources	Mechanism	References
<i>Regeneration of cutaneous tissue</i>	Burn-induced injury	CB-MSC	Inhibition of inflammation and tissue repair	[71]
	Skin wound	CB-MSC Amniotic-MSC CB-EPC ASC	Neoangiogenesis, wound healing, inhibition of scar formation	[72–74]
<i>Skeletal regeneration</i>	Cartilage damage Osteoarthritis Cardiotoxin-induced muscle injury	ESC-MSC Synovial-MSC iPS-MSC BM-MSC	Restoration of chondrogenesis; migration and proliferation of chondrocytes Muscle regeneration	[77–79]
<i>Lung injury protection</i>	Endotoxin-induced lung injury Monocrotaline-induced lung hypertension	BM-MSC	Inhibition of inflammation and permeability Reversion of pulmonary arterial thickening and right to left ventricle ratio	[80–82]

Abbreviations: *BM-MSC* Bone marrow-mesenchymal stem cells, *HLSC* human liver stem cells, *CB-MSC* cord blood-mesenchymal stem cells, *WJ-MSC* Wharton’s jelly-mesenchymal stem cells, *EPC* endothelial progenitor cells, *ESC-MSC* embryonic-mesenchymal stem cells, *CPCs* cardiac progenitor cells, *iPS-MSC* mesenchymal stem cells differentiated from induced pluripotent stem cells, *ASC* adipose tissue-derived stem cells, *AKI* acute kidney injury, *CKD* chronic kidney disease

administering EVs derived from a liver resident stem-like cells (the human liver stem cells, HLSCs), albeit through a different mechanism of action [32].

EVs obtained from BM-MSC were also tested in a lethal AKI model made by the administration of cisplatin, a drug used to treat oncological patients [33]. Cisplatin-induced nephropathy characterized by the rapid loss of renal function could be lethal or sublethal, depending on the murine strain used and on the dose administered. A single injection of EVs increased mice survival and improved renal morphology and function but did not prevent chronic renal injury. Repeated EV injections, on the other hand, reduced mortality and prevented tubular atrophy [33]. Administration of EVs obtained by cord blood-derived MSCs (CB-MSCs) in the same model protected mice from oxidative stress, stimulated cell proliferation, and reduced cell apoptosis, resulting in improvement of renal function and morphology [34]. Similarly, in an AKI model induced by gentamicin injection, EVs derived from BM-MSCs reduced renal dysfunction [7].

Apart from drug-induced models of AKI, EVs have also been studied in other models of kidney injury. The ischemia/reperfusion injury (IRI) model is a model to mimic kidney injury occurring in many clinical settings as well as in renal transplantation. IRI is usually induced by occluding the renal artery and vein of one or both kidneys for different periods, followed by re-establishing the blood circulation. BM-MSC EVs were administered intravenously in rats immediately after transitory renal ischemia [35–38]. This resulted in the effective reduction of renal damage and improved overall renal function, accelerating tubular cell proliferation [35].

A similar positive effect was also observed after the administration of EVs derived from CB-MSCs [36] as well as from Wharton's jelly-derived MSCs (WJ-MSCs) albeit with different mechanisms of action [37, 38]. For instance, EVs obtained from CB-MSCs accelerated tubular cell proliferation not only through the horizontal transfer of human hepatocyte growth factor (HGF) but also through the induction of HGF in recipient animals. On the other hand, WJ-MSC-derived EVs reduced inflammation and apoptosis via mitochondrial protection. Moreover, other methods of EV administration have also proven to be advantageous. For instance, injecting EVs derived from BM-MSCs in an IRI murine model under the renal capsule exerted the same therapeutic effects observed in other models mainly through the suppression of inflammation [39].

The effect of EVs released by other stem cell sources has also been investigated with similar results. For instance, EVs released by mouse renal MSCs cultured under hypoxic conditions were able to improve peritubular microvascular rarefaction *in vivo* [40, 41]. The effect observed was attributed to several proangiogenic transcription factors shuttled by EVs such as IGF-1, bFGF, and VEGF, which may led to the recovery of kidney morphology and function. MSCs (and their derived EVs) isolated from human adult glomeruli contributed towards the recovery of kidney function by reducing ischemic damage and stimulating tubular cell proliferation. In the same model, the administration of a population of a resident intratubular CD133-positive cell contributed towards renal recovery, but no positive effect was observed on administering EVs derived from these cells [41].

EPCs, a proangiogenic circulating progenitor cell type derived from the peripheral circulation of healthy blood donors, could also be a source of EVs with regenerative properties [42]. Administration of EV-EPCs after IRI induction prevented the development of tissue injury. This functional and morphologic protection conferred was mainly through the enhancement of tubular cell proliferation together with a reduction in apoptosis and leukocyte infiltration. In addition, EV-EPCs banned the progression of acute injury to chronic kidney disease (CKD) by preventing the capillary rarefaction, tubule-interstitial fibrosis, and glomerulosclerosis [42]. EV-EPCs have also been tested in a rat model of experimental anti-Thy1.1 glomerulonephritis induced by complement-mediated mesangial injury. In this model, EV-EPCs inhibited the infiltration of leukocyte, the activation of mesangial cell, and the activation of serum complement and decreased proteinuria improving renal function [43]. Furthermore, in a murine model of AKI induced by IRI, vesicles derived from endothelial cell-forming colonies (ECFC), in particular, exosomes, significantly attenuated renal injury [44]. Hence, various sources of stem/progenitor cell EVs have regenerative healing effects in multiple models of AKI with different mechanisms of action, an avenue that could be exploited for future therapeutic intervention.

Vesicles from various stem cell sources have also been studied in different animal models of CKD. For instance, EVs isolated from urinary MSCs were administrated weekly in a streptozotocin model of diabetic nephropathy (DN) in rats for 12 weeks starting from the onset of diabetes. A reduction in urine volume and microalbumin excretion was observed in mice treated with EVs. Furthermore, EVs were found to be effective in preventing disease progression by conferring protection to podocytes and tubular epithelial cells against apoptosis. Analyses of the EV content revealed

the presence of growth factors (bone morphogenetic protein-7, angiogenin, and transforming growth factor- β 1) that could possibly be the reason for the renal protection [45]. In both the animal models of diabetes (type 1 caused by streptozotocin and type 2 induced by high fatty diet), BM-MSCs and MSC-conditioned medium suppressed immune cell infiltration and reduced interstitial fibrosis and glomerular alteration. Moreover, the direct injection of MSC-exosomes under the renal capsule in the streptozotocin-induced DN model prompted a rapid improvement of renal morphology which was observed 1 to 2 weeks after EV administration [46].

Recently, Kholia et al. [47] demonstrated that HLSC-derived EVs inhibited development of fibrosis in a model of accelerated CKD induced in mice by administration of aristolochic acid. HLSC-EVs were shown to contain a pattern of antifibrotic microRNAs able to downregulate profibrotic genes restoring normal renal function.

Apart from rat and mice animal models, the therapeutic effects of EVs were also observed in larger size animal models. For instance, in a model of metabolic syndrome in pigs with stenosis of renal artery, an intrarenal administration of a single dose of EVs derived from autologous adipose stem cells (ASCs) improved renal inflammation and medullary oxygenation and reduced fibrosis 1 month posttreatment [48]. Hence, there is sufficient evidence in the literature to prove the therapeutic effect of EVs from various stem cells in multiple models of kidney disease. This result could potentially be converted from bench side to bedside in the near future.

9.3.2 Neural Regeneration

Injuries and pathologies leading to the degeneration of the nervous system can be very debilitating. Very little curative measurements and therapeutic interventions are currently available for damage to the nervous system. In order to find a solution to this problem, the therapeutic effect of stem cells and their derived EVs have been investigated on the nervous system (neurons and nerves) with some fruitful results. For instance, EVs derived from MSCs that are naturally rich in miRNA-133b could stimulate neural cells causing a boost in neurite outgrowth [49]. On the other hand, treatment with EVs derived from neuronal cells under differentiation could induce human MSCs to differentiate into neuron-like cells through the transfer of miR-125b, therefore confirming a two-way synergistic interaction [50].

Traumatic brain injury (TBI) mainly due to an external insult involving mechanical force can lead to a temporary or permanent impairment of brain function. Investigating the regenerative potential of stem cell EVs in TBI would therefore be advantageous as a therapeutic avenue. Zhang et al. [51] in a rat model of TBI observed that treatment with EVs not only enhanced endogenous angiogenesis and neurogenesis but also attenuated neuroinflammation. In another model of neurodegeneration, EVs derived from BM-MSCs significantly promoted the survival of retinal ganglion cells together with the axon regeneration [52]. Interestingly, EVs derived from adipose stem cells (ADSCs) have recently been reported to exhibit regenerative activity. In an in vitro setting, EVs derived from the hADSCs enhanced both neuronal survival and proliferation [53], whereas, in an in vivo model of Alzheimer's disease, the positive impact

of ADSC-EVs was attributed to their large payload of neprilysin, a prominent enzyme for the degradation of β -amyloid peptide in the brain. Furthermore, this effect was confirmed by *in vitro* studies which demonstrated that EVs reduced both intracellular and secreted levels of β -amyloid in neuroblastoma cells, therefore making them a potential therapeutic avenue for treatment of Alzheimer [54].

9.3.3 Cardiovascular Regeneration

Cardiovascular diseases are a major cause of death in the western world. Although there has been a remarkable improvement in medical intervention for the prevention and/or treatment of the disease, more is required. EVs from various stem cell sources have been proved to exhibit cardioprotective properties. For instance, EVs derived from MSCs obtained by differentiating human embryonic stem cells decreased infarct size in a murine model of heart-induced ischemia-reperfusion injury [55]. Treatment with EVs enhanced both ATP and NADH levels, limited oxidative stress, and decreased macrophage and neutrophil infiltration allowing the infarcted tissue to recover [56].

In other studies, it has been showed that EVs derived from bone marrow MSCs enhanced cardiac function by stimulation of neoangiogenesis and prevention of inflammatory response both *in vitro* and *in vivo* [57, 58]. A similar effect was also observed in EVs derived from human umbilical cord MSCs (hUC-MSCs), whereby they conferred a cardiotherapeutic effect by promoting angiogenesis and protecting from apoptosis myocardial cells, leading to an overall improvement in cardiac systolic function [59].

EVs derived from cardiac-specific stem cells have also been investigated in different models or heart injuries. EVs isolated from cardiospheres were reported to promote proliferation and inhibit apoptosis of cardiomyocytes when locally injected after ischemic injury [60]. The beneficial effect was correlated to the miR-146a presence within the vesicles. In another study, EVs derived from cardiac progenitor cells (CPCs) were tested in a rat MIR injury model where EVs ameliorated cardiac function by increasing angiogenesis and reducing fibrosis [61].

Moreover, Ratajczak et al. [62] have shown that CD133+ cells secrete several paracrine factors including EVs containing several antiapoptotic and proangiogenic mRNAs including mRNA for kit ligand, insulin growth factor-1, VEGF, bFGF, and interleukin-8. These EVs promoted angiogenesis both *in vitro* and *in vivo* suggesting a potential employment of CD133+ cell-derived paracrine factors in regenerative medicine.

9.3.4 Hepatic Regeneration

The regenerative potential of EVs from different stem cellular sources has also been very well reported in liver disease. For instance, EVs derived from hCB-MSCs were investigated in a model of liver fibrosis induced by carbon tetrachloride (CCl₄).

The EV treatment not only improved liver fibrosis but also conferred protection to hepatocytes by impeding epithelial-to-mesenchymal transition through the downregulation of the TGF- β 1/Smad 2 pathway [63]. Content analyses of EVs derived from hCB-MSCs showed enrichment with the antioxidant glutathione peroxidase 1 (GPX1). A single administration of these EVs rescued mice from acute liver injury, reducing the oxidative stress and the cell death through a GPX1-mediated neutralization of hydrogen peroxide [64]. In a mouse model of acute liver injury induced by CCl₄, EVs obtained from embryonic-derived MSCs, increased hepatocyte repair and regeneration through the upregulation of proteins involved in proliferation (e.g., Cyclin D1) and the Bcl-xL antiapoptotic gene [65]. In another model of liver disease involving hepatic ischemia-reperfusion injury, EVs derived from human-induced pluripotent stem cell-derived MSCs (hiPSC-MSCs) alleviated IRI by inhibiting cellular apoptosis, reducing inflammatory responses and oxidative stress responses [66]. Apart from the liver parenchyma, EVs also have an effect on non-parenchymal cells. For instance, adipose tissue-derived MSC-EVs expressing miR-122 inhibited the proliferation and activation of hepatic stellate cells (HSCs) that play a primary role in the development of liver fibrosis [67].

The effect of MSC-EVs was also evaluated in an immune-induced liver injury setting caused by concanavalin-A. In this model, EV treatment not only reduced serum levels of alanine aminotransferase (ALT) but also exhibited an overall anti-inflammatory effect through the downregulation of proinflammatory cytokines, upregulation of anti-inflammatory cytokines, and also expansion of regulatory T cells [68]. Furthermore, a similar immunomodulatory effect was also observed in a D-galactosamine-/TNF- α -induced lethal mouse model of liver failure whereby EVs derived from BM-MSC reduced hepatic injury and increased survival through the activation of antiapoptotic pathways and downregulation of the inflammatory response [69].

Apart from EVs derived from various sources of MSCs, other tissue-specific stem/progenitor cells have also been effective in liver injury, particularly EVs derived from HLSCs. In an in vitro setting, HLSC-EVs not only stimulated proliferation but also increased the resistance of human and rat hepatocytes to apoptosis. On administering these EVs in a rat model of 70% hepatectomy, acceleration in the morphological and functional recovery of the liver was observed possibly due to an increase in hepatocyte proliferation [70].

9.3.5 Regeneration of Cutaneous Tissue

The effect of stem cell EVs has also been investigated in various models of cutaneous injuries. For instance, EVs produced by hUC-MSCs reverted burn-induced inflammatory response and boosted tissue repair in a model of burn injury. Further studies revealed the attenuation of the inflammatory response to be due to miR-181c shuttled by hUC-MSC exosomes which downregulated the TLR4 signaling pathway [71]. When different concentrations of exosomes from amniotic epithelial cells

were injected subcutaneously in proximity of the wound site in a model of wound healing, migration and proliferation of fibroblasts were observed. This accelerated the healing process of full-thickness skin defect in a dose-dependent manner [72]. Furthermore, application of another source of EVs derived from human umbilical CB-derived EPCs on a similar wound healing assay led to a robust proangiogenic and healing response mainly through the regulation of the Erk1/2 signaling pathway [73]. Flap necrosis is the most frequent postoperative complication in reconstructive surgery. ASCs and their exosomes were able to protect skin flaps during I/R injury through the activation of neovascularization [74].

9.3.6 Skeletal Regeneration

EVs present in MSC-conditioned medium have been reported to accelerate the healing process postfractures [75]. This EV-mediated effect was attributed to increased proliferation as well as osteogenic differentiation of BM-MSCs both in vitro and in vivo principally through the regulation of the PI3K/Akt signaling pathway [76].

Stem cell-derived EVs have also proven to be therapeutic in cartilage damage. For instance, in a rat model of cartilage damage, osteochondral defects were created on bilateral trochlear grooves. One defect was injected with human embryonic MSC-EVs intra-articularly once a week, and the contralateral lesion was treated with the vehicle alone. Complete cartilage healing was observed after EV treatment, whereas only fibrous scar tissues were found in the contralateral defect [77]. In a model of osteoarthritis, EVs derived from human synovial membrane MSCs and from iPS-derived MSCs could attenuate arthritic damage by stimulating migration and proliferation of chondrocytes; furthermore, EVs derived from iPS-derived MSCs were found to be more potent compared to EVs derived from synovial MSCs [78].

Moreover, SC-EVs have also proven to promote skeletal muscle regeneration. Particularly exosomes derived from BM-MSC, which were able to promote muscle regeneration by enhancing angiogenesis and myogenesis. This effect was mediated by miRNAs, in particular, through the transfer of miR-494 [79].

9.3.7 Lung Injury Protection

In a model of endotoxin-induced lung injury in mice, Zhu et al. [80] demonstrated that BM-MSC-EVs decreased inflammation and reduced lung permeability preventing edema. The preventive effect on lung permeability by BM-MSC-EVs was also observed in an ex vivo model of perfused human lung [81]. In a model of monocrotaline-induced pulmonary hypertension in mice, BM-MSC-derived EVs were shown to reverse lung injury based on their cargo of microRNAs [82].

9.4 Role of Extracellular Vesicles in the Immunomodulatory Properties of MSCs

The performance of both innate and adaptive immune response can be influenced by MSCs of different origin to induce a tolerant or a less inflammatory phenotype. MSCs have been reported to suppress the proliferation and activation of lymphocytes, inhibit the activity of natural killer cells (NK) and the maturation of dendritic cells (DCs), and induce expansion of regulatory T cell (Treg) [83]. The immunomodulatory properties of MSCs depend either on direct cell-to-cell interactions or on the secretion of soluble factors.

Various soluble factors implicated in the immunomodulatory effects of MSCs have been identified. For instance, transforming growth factor (TGF)- β 1 and hepatocyte growth factor (HGF) suppress T-cell proliferation [84] as well as interleukin (IL)-10 and indoleamine 2,3-dioxygenase (IDO) [85]. The latter is a rate-limiting enzyme implicated in the catabolism of the tryptophan, an essential amino acid necessary for T-cell proliferation [86]. Another soluble factor released by MSCs on coculturing with T lymphocytes is prostaglandin (PG) E2 synthesized from arachidonic acid either by the constitutive cyclooxygenase-1 (COX-1) or the inducible COX-2 [87]. PGE2 plays a crucial role in the immunomodulatory effect exerted by MSCs, since inhibiting PGE2 production diminishes immunomodulatory properties of MSCs [87]. Another important mediator is nitric oxide (NO) which contributes towards the immunomodulatory effect of MSCs by suppressing T-cell proliferation [88]. Furthermore, PGE2, IDO, and TGF- β 1 can similarly inhibit NK functions, therefore widening the immunomodulatory effects of MSCs [87, 89]. In addition, MSCs also secrete IL-6 that can reverse the maturation of DCs to a more immature phenotype [90]. Apart from inhibiting the proliferation of cytotoxic T cells, TGF- β 1 and PGE2 secreted by MSCs also contribute towards the expansion of Treg [91]. Another soluble factor released by MSCs is HLA-G5, which is involved in the expansion of Treg, as well as in the suppression of allogeneic T-cell proliferation. Moreover, HLA-G5 can also inhibit cytolysis and interferon-gamma (IFN- γ) secretion of NK cells [92]. Apart from T lymphocytes, MSCs can also modulate B-cell function by inhibiting B-cell proliferation and differentiation consequently leading to an impairment of immunoglobulin production [93].

Recently, it has also been shown that, in certain conditions, EVs released by MSCs can act directly on immune cells. For instance, in a mouse model of autoimmune encephalomyelitis, EVs from MSCs pretreated with interleukin-1- β were shown to inhibit the expansion of autoreactive lymphocytes, induced apoptosis of activated T cells, as well as initiated Treg-mediated release of anti-inflammatory cytokines such as TGF- β and IL-10 [94]. Additionally, coculturing monocytes/macrophages with MSCs-EVs increased the levels of the anti-inflammatory cytokine IL-10 and reduced secretion of proinflammatory interleukins (e.g., IL-1 β , IL-6, and tumor necrosis factor TNF- α) [95]. Furthermore, EVs also induced a phenotypic transition of macrophages from M1 to M2 [96, 48].

The effect of MSC-EVs has been studied in various models of immune-mediated diseases. For instance, MSC-EVs upregulated the expression of TGF- β 1 and IL-10 in peripheral blood mononuclear cells isolated from asthmatic patients, which in turn stimulated the proliferation of Tregs, enhancing their immunosuppression capability, therefore leading to an overall mitigation of inflammation in asthma [97]. EVs produced by BM-MSCs mimicked the immunomodulatory effects of MSCs in type 1 diabetes. In particular, they interfered with an antigen-elicited immune response whereby Th1 responses were downregulated *in vitro*, leading to an increased proportion of Tregs and a lower percentage of Th17 cells. In another study, MSC-EV treatment increased T-cell-mediated production of PGE2 and TGF- β , both of which are enriched in EVs at mRNA and protein levels. Therefore, EV-MSCs can increase the proportion of Tregs and restore Th1/Th2 balance in type 1 diabetes [98]. Moreover, MSC-EVs reduced B-cell growth and differentiation as well as the immunoglobulin (IgM, IgG, and IgA) production by cultured peripheral blood mononuclear cells [99].

A direct comparison of immunomodulatory activity of MSCs and their EVs revealed that both MSCs and their EVs had an analogous effect on inhibition of B-cell proliferation, even if EVs had a lower capacity to inhibit B-cell differentiation and immunoglobulin production. In addition, MSCs were more efficient than EVs on inhibiting *in vitro* T-cell proliferation. EVs induced only a slight increase in IL-10 and TGF- β secretion and a slight decrease in granulocyte macrophage colony-stimulating factor (GM-CSF) and IFN- γ secretion by both T and B cells in comparison with the cells of origin [100]. EVs derived from other source of MSCs, such as adipose tissue, have also been investigated on T-cell proliferation and activity. EVs from ASCs inhibited proliferation of both CD4⁺- and CD8⁺-stimulated lymphocytes [101].

The capacity of EVs produced by MSCs to attenuate an aggressive immune response was demonstrated in a preliminary study where EVs were administered to a patient with grade IV acute graft-versus-host disease (GVHD) refractory to therapies. The patient remained stable for 5 months after EV treatment. Despite a progressive increase in EV dose, all administrations were well tolerated without adverse effects. GVHD clinical symptoms rapidly improved after EV treatment [102].

9.5 Conclusion

It is now universally recognized that stem cells may act in paracrine manner on nearby and distant cells by secretion of a multitude of soluble factors such as growth factors, cytokines, chemokines, and nucleic acids. The secretome of stem cells includes also the release of EVs, which may act as carriers for molecules critical for cell stemness. By sharing such molecules, EVs may modify the phenotype of recipient cells by modulating the differentiation state and the cell cycle. The role of this newly described mechanism of cell-to-cell communication in stem cell biology is at present only partially understood. However, a multitude of studies has shown the

possibility to exploit stem cell-derived vesicles for therapy as substitute of stem cells themselves. In fact, several preclinical studies indicate that EV treatment may mimic the beneficial effect of stem cells. EVs were shown to limit injury and improve regeneration in several tissues by inhibiting cell apoptosis and fibrosis, favoring cell proliferation and angiogenesis and expansion of intrinsic tissue progenitors as well as modulating inflammatory and immune responses [15, 16]. Furthermore, EVs may act not only directly but also indirectly triggering neighboring cells to also secrete bioactive molecules [16].

The possibility to perform a stem cell-based therapy without the cells is certainly appealing. However, several problems remain to be solved such as the upscale production of EVs in good manufacture practice (GMP) conditions, the definition of criteria of identity, purity and potency of an EV-based product, and, most importantly, their pharmacodynamics/pharmacokinetics and biosafety.

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Chapter 10

Cellular Therapy for Ischemic Heart Disease: An Update



Hsuan Peng and Ahmed Abdel-Latif

Abstract Ischemic heart disease (IHD), which includes heart failure (HF) induced by heart attack (myocardial infarction, MI), is a significant cause of morbidity and mortality worldwide (Benjamin, et al. *Circulation* 139:e56–e66, 2019). MI occurs at an alarmingly high rate in the United States (approx. One case every 40 seconds), and the failure to repair damaged myocardium is the leading cause of recurrent heart attacks, heart failure (HF), and death within 5 years of MI (Benjamin, et al. *Circulation* 139:e56–e66, 2019). At present, HF represents an unmet need with no approved clinical therapies to replace the damaged myocardium. As the population ages, the number of heart failure patients is projected to increase, doubling the annual cost by 2030 (Benjamin, et al. *Circulation* 139:e56–e66, 2019). In the past decades, stem cell therapy has become a promising strategy for cardiac regeneration. However, stem cell-based therapy yielded modest success in human clinical trials. This chapter examines the types of cells examined in cardiac therapy in the setting of IHD, with a brief introduction to ongoing research aiming at enhancing the therapeutic potential of transplanted cells.

Keywords Myocardial infarction · Heart failure · Recovery · Stem cells · Mesenchymal stem cells · Regeneration · Skeletal myoblasts · Bone marrow mononuclear cells

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195

10.1 Introduction

Advanced revascularization techniques have lowered MI mortality rates in the past decade; however, the incidence of HF is on the rise [1]. After MI, up to 1 billion cardiac cells die in response to ischemic injury, leading to reduced cardiac function, and scar formation, leading to left ventricular (LV) remodeling and HF [2]. Timely reperfusion has improved patient survival after MI, but cannot stop or reverse cardiac damage and the ensuing HF. Currently, heart transplantation is the only viable option for end-stage HF patients to replace the infarcted myocardium. However, organ shortage, serious postprocedural complications, side effects of long-term immunosuppressive therapies, and overall suboptimal patient prognosis make it a suboptimal treatment [3]. Hence, stem cell therapy has been proposed as a novel treatment to repair damaged hearts.

The remarkable discoveries in stem cell biology such as cloning of Dolly the sheep and establishment of first human embryonic stem cell line in the late 1990s bring to light that adult cells have the potential to become totipotent with genetic reprogramming (Fig. 10.1) [4–7]. Fast-forward a decade, Shinya Yamanaka and Kazutoshi Takahashi's breakthrough discovery of induced pluripotent stem cells (iPSCs) established the feasibility of directly reprogramming adult somatic cells into pluripotent cells with transcription factors [8]. These scientific breakthrough ignites the hope to repair or regenerate the damaged adult human tissue, including the least regenerative organs such as the human heart. Since then, different sources and cell types have been investigated for their potential in cardiac repair and/or preservation in the setting of ischemic (ICM) and nonischemic (NICM) cardiomyopathy. The most commonly investigated stem cell candidates are bone marrow- or adult heart-derived stem cells. Several strategies were also developed to generate new cardiomyocytes. These include differentiation of cardiomyocytes from iPSCs [9, 10], stimulation of cardiomyocytes to reenter cell cycle [11, 12], and direct reprogramming of fibroblast to cardiomyocytes [13–15]. This chapter provides an overview of progress in current cardiac stem cell therapy, the reprogramming strategies employed, and concludes with a brief discussion on the researches aiming at refining and enhancing the therapeutic potential of transplanted cells.

10.2 Noncardiac Origin Cell Types

The earliest attempt of cardiac therapy includes transplantation of skeletal myoblasts (SMs) [16–18], bone marrow cells (BMCs) including bone marrow mononuclear cells (BMMNCs), hematopoietic stem cells (HSCs), and mesenchymal stem cells (MSCs). These cells were selected based on their self-renewal capacity, innate mobilization after injury, ability to withstand harsh postinjury environment, proregenerative secretory profiles, and clinical availability.

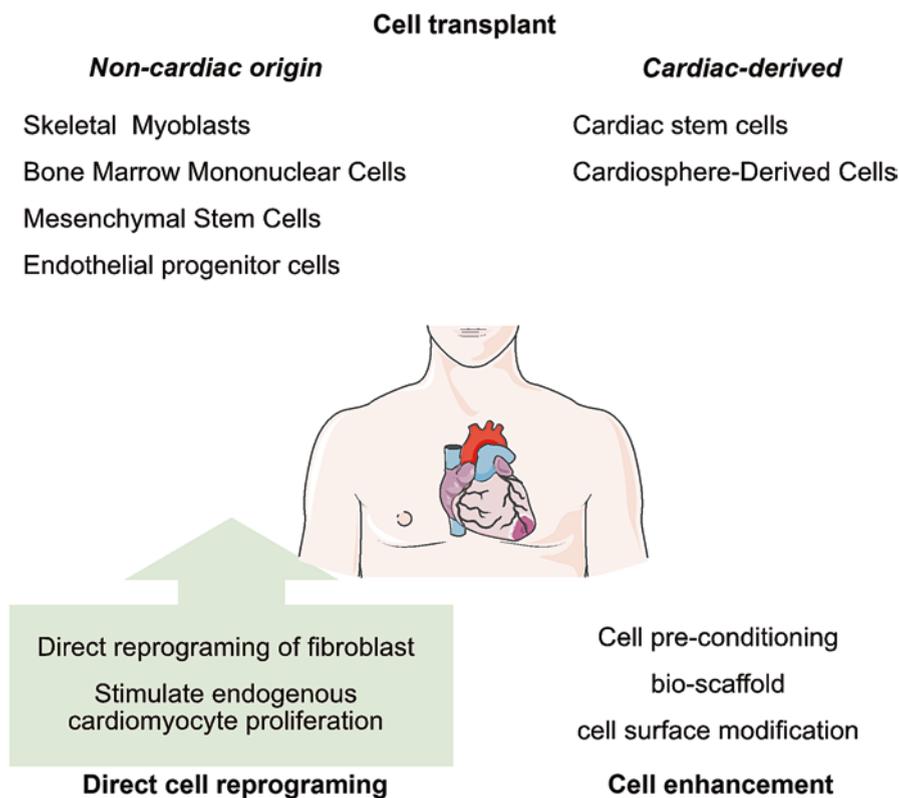


Fig. 10.1 Cell therapy-based approaches for ischemic heart disease have advanced significantly over the last 2 decades and currently range widely from cell transplantation of unmodified cells (cardiac and noncardiac in origin) to the use of induced pluripotent stem cells (with and without cardiac preconditioning) and the direct reprogramming of cardiac fibroblasts. Advances in our biological understanding of cell commitment and their beneficial effects on the ischemic myocardium have resulted in the development of new noncellular strategies such as the use of gene therapy and exosomes in ischemic heart disease and heart failure

10.2.1 Skeletal Myoblasts (SMs)

Skeletal myoblasts (SMs) were proposed for cardiac therapy with the rationale that autologous SMs can be quickly expanded in vitro and are resistant to ischemia [19]. However, the preclinical study showed no electrophysiological coupling between SMs and native cardiomyocytes in rodents [17, 20] and limited evidence of SMs differentiating into cardiomyocytes [21]. While an improvement of left ventricular (LV) function was found in a swine model [22], no exact mechanisms were reported. In the human clinical study, SM transplantation is associated with arrhythmia in severe ICM patients due to the lack of SM integration with native cardiac cells [23, 24].

Given the lack of efficacy in clinical trials and arrhythmogenic side effects, another relatively easily obtained stem cell, BMMNCs, has been investigated a source of cardiac therapy.

10.2.2 Bone Marrow Mononuclear Cells (BMMNCs)

The discovery of recipient-derived cardiomyocytes in sex-mismatched donor hearts after heart transplant spiked the interest of searching for extracardiac progenitor cells capable of regenerating damaged myocardium [25–27]. Later studies suggested that at least part of these cells are of bone marrow origin [28, 29]. Not surprisingly, bone marrow is a rich source of adult stem cells, including BMMNCs and its subpopulation, HSCs. BMMNCs are among the most widely tested cell types in the clinic for heart diseases because it is easy to isolate in a larger quantity with minimal ex vivo manipulation [30–32]. In preclinical studies, BMMNCs transplant yielded promising results [28, 33–37]. Early small clinical studies also supported the preclinical findings in which BMMNC transplant improves LV ejection fraction (EF) (BOOST [38], REPAIR-AMI [39]). However, later trials failed to reproduce these findings [40–42]. In general, BMMNCs had been shown to benefit the injured myocardium after their administration, but the effect was ephemeral with multiple studies showing similar cardiac recovery after 1 year. A recent study with repeated BMMNC administration seems to improve clinical outcome compared with single treatment [43].

10.2.3 Mesenchymal Stem Cells (MSCs)

Discovered in 1970 [44], mesenchymal stem/stromal cells (MSCs) are another widely studied bone marrow-derived cellular subset in cardiac research. MSCs possess several features that make them prime candidates for cardiac therapies. Currently, MSCs are defined by the International Society for Cellular Therapy as self-renewing, multipotent cells that exhibit plastic adherence under standard culture conditions and express CD73, CD105, and CD90 but not CD45, CD34, CD14, CD11b, CD79a, CD19, or HLA-DR surface markers, with in vitro multilineage differentiation capacity [45]. In vitro studies have shown that MSCs can differentiate into different cell types including adipocytes, chondrocytes, and osteocytes [46]. MSCs can be isolated from numerous postnatal organs [47–51]. Among the sources of MSCs, bone marrow [44] and adipose tissue [52] have been the most commonly studied to date [53]. Recently, umbilical cord blood-derived MSCs have gained significant attention in treating patients with acute and chronic ICM, owing to some of their unique properties and their ease of use as an off-the-shelf source of therapy cells [54–58]. MSCs have been considered as hypoimmunogenic due to their cytokine profile and surface antigen expression [19]. This property makes MSCs a prime

candidate for allogeneic transplantation therapy. While some evidence suggested that allogeneic MSCs may lose their immune privilege upon differentiation [19, 59], there are no definitive advantage of autologous MSCs over allogeneic MSCs in clinical studies [60].

In preclinical studies, MSC transplantation after MI improved cardiac function in small and large animal models [61–66]. However, the exact mechanisms involved are not well understood. Early studies found little evidence of MSC myocardial engraftment shortly after transplantation. While some evidence suggested that MSCs can transdifferentiate into cardiomyocytes [67], it is extremely rare *in vivo* [19, 67–69]. Moreover, induced MSC-derived myocytes do not have similar electrical properties to a functional cardiomyocyte [70]. Hence, the primary function of MSCs is largely confined to its secretome, which has been shown to be cardioprotective and immunomodulatory [53, 65, 71, 72]. In a swine model, percutaneous injection of allogeneic MSCs into infarcted heart improved EF and decreased scar size [73]. Besides immunomodulation, MSC transplantation has yield promising results in a chronic model of ischemic heart disease in dogs [74]. In this study, MSC treatment increased vascularity and improved cardiac function. Similar cardiac benefits were reported in other preclinical studies with an overall improvement in EF, cardiac contractility, heart perfusion, and blood vessel density [75–78].

The safety of intravenous injection of allogeneic MSCs was first analyzed in a short-term (6 months) human study. The result demonstrated improvement in EF compared to the placebo group without arrhythmogenicity or tumorigenicity [79]. A later study also supported the safety of autologous bone marrow-derived MSC transplants with up to 5-year follow-up [80]. However, despite the highly promising results in animal models, there is modest benefit observed in human clinical trials.

MSCs have been tested in treating acute MI. In the APOLLO trial, the application of autologous adipose tissue-derived MSCs resulted in improved cardiac perfusion with smaller scar tissue [81]. MSCs have also been tested in the setting of chronic ischemic cardiomyopathy and showed promising therapeutic efficiency [82]. In the POSEIDON trial, transendocardial injection of bone marrow-derived MSCs (BM-MSCs) attenuated pathological cardiac remodeling and reduced scar size, without evidence of immune rejection detected in recipients [82]. When compared to BMMNCs, BM-MSCs transplantation decreased infarct size, improved contractility, and improved overall quality of life as demonstrated in the TAC-HFT trial. However, no significant difference in EF was observed [83]. In 2015, MSC-HF study showed encouraging results with HF patients who received a single dose of autologous MSCs delivered intramyocardially. MSC-treated patients have greater functional improvements at 12 months follow-up. Interestingly, the observed benefit is correlated with increasing MSC dosing [84].

It is worth to note that guided cardiopoiesis has been attempted using MSCs. This is done with the rationale that MSCs can become committed to cardiac differentiation by mimicry of natural cardiogenic signaling. In both murine and swine models, cardiopoietic MSC transplantation improves EF and reduces infarct size compared to controls. Clinically in the C-CURE trial [85], MSCs were preconditioned with a cardiogenic cytokine cocktail for 10 days prior to injection. This cocktail

consisted of transforming growth factor-beta1, bone morphogenetic protein-4, activin A, retinoic acid, insulin-like growth factor-1, fibroblast growth factor-2, alpha-thrombin, and interleukin-6 and was formulated to engage MSC into cardiopoiesis [86]. Increased EF and quality of life were observed in the treated group. No systemic toxicity or adverse effects were observed up to 2 years of follow-up. Based on the results of C-CURE, the phase III CHART trial was conducted in HF patients [87]; however, the initial results did not meet efficacy endpoints. Interestingly, the study revealed an interesting dosage and efficacy relationship with lower dosage being more efficacious [88].

Conclusion Currently, the efficacy of BMC therapy remains controversial as several trials were unable to reproduce the preclinical and clinical benefits [40, 89]. The lack of benefit has been attributed to the difference in trial design and variability in imaging modalities used to assess efficacy endpoints. In 2012, a meta-analysis reviewing over a total of 50 studies (enrolling 2625 patients) found that transplant of adult BMCs improves LV function, reduces infarct size, and improves remodeling in patients with IHD compared with standard therapy [90]. Moreover, BMC transplantation reduces the incidence of death and recurrent MI in IHD patients. Since most inconsistencies regarding BMC comes from randomized controlled trials (RCTs), a 2015 update of meta-analysis examined 48 RCTs of heart repair with BMCs [91]. By identifying and excluding trials with discrepancies, Afzal et al. concluded that BMC therapy improves cardiac function, remodeling, and clinical outcomes even when assessed by the most rigorous methods. Overall, the BMC therapy in IHD patients is associated with modest yet significant improvement in LV ejection fraction [3, 90, 92]. BMC-treated patients also experienced a substantive reduction in all-cause mortality despite numerically small improvements in cardiac parameters. BMC-based clinical trials have effectively established a standard of operation using cell-based therapies in IHC. These results provided a robust basis for the refinement of current BMC-based therapies by enhancing their cardiac engraftment and regenerative potential after transplantation. However, future studies are required to assess the optimal timing for BMC delivery and the patient population who will benefit the most from this novel therapy (Tables 10.1 and 10.2).

10.2.4 Endothelial Progenitor Cells (EPCs)

EPCs represent a heterogeneous population of hematopoietic and nonhematopoietic progenitor cells that participate in vascular repair [93]. Asahara et al. first described EPCs in 1997 when they isolated angioblasts with endothelial lineage potential from human peripheral blood [94]. In general, the expression of surface markers such as CD133, CD34, and VEGFR2 has been adopted to identify EPCs [95]. These cells are also characterized by their ability to differentiate into mature endothelial cells and secrete angiogenic factors [96, 97]. EPCs can be isolated from different sources, including bone marrow, peripheral blood, umbilical cord blood, and other

Table 10.1 MSC-based cardiac regenerative therapies in preclinical studies

Therapeutic source	Cell number	Animal model	Disease model	Delivery method	Time from onset	Results	Ref.
Allogenic BM-MSCs	2.4, 24, 44×10^7	Swine	MI	Endomyocardial	3 days	↓ Infarct size. No does dependent response	[60]
Allogenic BM-MSCs	25, 75, 225, 450×10^6	Sheep	MI	Intramyocardial	1 hour	↓ Scar size. ↑ Cardiac function Data suggesting there is an optimal dosage	[62]
Allogenic BM-MSCs	200×10^6	Swine	ICM	Transendocardial	12 weeks	↓ Infarct size. ↑ EF. ↑ Regional contractility and MBF	[63]
Allogenic BM-MSCs	75×10^6	Swine	MI	Transendocardial	3 days	↓ Infarct size Stimulating endogenous cardiac progenitors	[64]
Allogenic BM-MSCs	200×10^6	Swine	MI	Intramyocardial	3 months	↓ Scar size. ↑ EF	[65]
Allogenic BM-MSCs	200×10^6	Swine	MI	Percutaneous	3 days	↓ Scar size. ↑ Cardiac function. Long-term cell engraftment. No allogenic rejection	[72]
Autologous BM-MSCs	20, 200×10^6	Swine	MI	Left anterior thoracotomy	12 weeks	↑ Regional contractility. ↑ LVEF (high does only). Data suggest there is dose-response effect	[76]

tissues [95, 98]. The procedure to isolate EPCs is straightforward, and thus, EPCs have been largely used both in preclinical and clinical studies for cardiovascular therapy. In both rat and swine models, transplantation of autologous human peripheral blood-derived EPCs improves LV function after MI and myocardial ischemia, respectively [99, 100]. In clinical study, phase I/II randomized trial using peripheral blood VD34+ cells for patients with coronary artery disease and angina showed less angina episodes and increased exercise tolerance at 6 months follow-up [101].

Table 10.2 MSC-based cardiac regenerative therapies in clinical trials

Therapeutic source	Cell number	Trial acronym/ number	Diseases	Delivery method	Time from onset	Results ↓↑	Ref.
Allogenic BM-MSCs	0.5, 1.6, 5.0 × 10 ⁶ / kg	NCT00114452	MI	Intravenous	1–10 days	No arrhythmogenicity. No tumorigenicity	[79]
Autologous BM-MSCs	>10 × 10 ⁶	NTR1553	MI	Intramyocardial	<1 month	No adverse effects	[80]
Autologous AT-MSCs	17.4 ± 4.1 × 10 ⁶	APOLLO/ NCT00442806	MI	Intracoronary	<24 hours	No adverse effects. ↓ Scar tissue. ↑ Perfusion	[81]
Allogenic WJ-MSCs	6 × 10 ⁶	NCT01291329	MI	Intracoronary	5–7 days	↑ EF. ↓ Heart perfusion	[58]
Autologous vs. allogenic BM-MSCs	20, 100, 200 × 10 ⁶	POSEIDON/ NCT01087996	ICM	Transendocardial	–	↓ Scar tissue. ↑ EF	[82]
Autologous BM-MSCs vs. BMMNCs	100, 200 × 10 ⁶	TAC-HFT/ NCT00768066	ICM	Transendocardial	–	No adverse effects. ↓ Infarct size. ↑ Contractility	[83]
Autologous BM-MSCs	77.5 ± 67.9 × 10 ⁶	MSC-HF/ NCT00644410	ICM	Intramyocardial	–	↑ LV mass. ↑ LV function. ↑ EF	[84]
Preconditioned autologous BM-MSCs	6–11 × 10 ⁸	C-CURE/ NCT00810238	ICM	Endomyocardial	–	↑ EF	[85]
Autologous BM-MSCs	>24 × 10 ⁶	CHART-1/ NCT01768702	IHF	Endomyocardial	–	No adverse effects	[87]

Encouraging evidences are also found in meta-analysis analyzing 28 EPC-based trial in patients with ST-segment elevation MI. This study found that EPC treatment significantly reduces infarct size at 12 months after treatment [102]. Recent meta-analysis on three randomized trials including 269 patients receiving CD34+ cell transplant for refractory angina further supports that CD34+ cell therapy lowers mortality and angina frequency without an increase in adverse events [103]. In acute MI patients, intracoronary infusion of EPCs led to a significant improvement in infarct remodeling at 1-year follow-up (TOPCARE-AMI trail) [104]. The PERFECT phase III clinical trial using intramyocardial infusion of 0.5–five million of CD133 + CD133+ EPCs for MI patients showed cardiac functional improvement with higher LVEF at 180 days after treatment. Overall, EPC transplant in clinical trial has demonstrated safety and efficacy. However, it faces similar challenges as BM-MSC, in particular, that varied EPC definition, characterization, and isolation protocol in the literature [95] limit the interpretation of results from clinical studies.

10.3 Cardiac-Derived Stem Cells (CSCs)

10.3.1 Cardiac Stem Cells (CSCs)

CSCs are defined as resident heart cells that show clonogenic, long-term, self-renewal, and multipotent capacity in vitro [105]. CSCs are described as self-adherent clusters when cultured from the postnatal cardiac explant. There is no consensus on defining surface markers for CSCs. Some studies have reported stem cell antigen 1 (SCA1; also known as LY6A), c-kit, and insulin gene enhancer protein ISL1 (also known as islet 1) [106, 107]. Some groups have reported that CSCs can differentiate into fully functional cardiomyocytes [108]; however, recent retractions of papers regarding c-kit+ stem cells that regenerate cardiomyocytes in adult heart question the scientific validity of these findings [109].

10.3.2 Cardiosphere-Derived Cells (CDCs)

CDCs represent a mixed cell population in a 20–150 μm cellular sphere generated from the explant outgrowth cell of heart biopsies [108]. The CDCs are highly proliferative, clonogenic, and multipotent in vitro when cultured on fibronectin [110]. These cells are thought to possess enhanced regenerative capacity through the stimulation of endogenous cardiac cells and/or paracrine mechanisms. CDCs have been tested in vivo using small and large animal models [111–113]. Most studies found that CDCs reduce infarct size, improving LVEF and cardiac hemodynamic in animal models. In a swine MI model, intracoronary delivery of CD105 and c-kit+ CDCs has shown encouraging efficacy and feasibility without immediate safety

concerns at 8 weeks of follow-up [112]. A recent meta-analysis concluded that CDC treatment results in 10.7% improvement in LVEF compared to control animals [114]. This positive outcome has led to the CADUCEUS trial with intracoronary infusion of autologous CDCs for MI patients (with LVEF of 25–45%) [115]. This study found a significant reduction in scar size, increase in viable heart mass, and contractility with CDC treatment at 1-year posttreatment [115, 116]. Interestingly, in swine chronic ischemic cardiomyopathy model, cotransplant with CSCs and MSCs showed greater improvement in cardiac performance with a smaller scar size than treated with either cell type alone; however, the mechanisms responsible for this phenomenon are not well understood [117, 118]. Indeed, more studies are needed to confirm the findings and to elucidate the mechanisms involved in CDC-mediated reduction in infarct size and increased viable myocardium.

10.4 Directed Cell Reprogramming and Induced Proliferation to Generate New Cardiomyocytes

10.4.1 Direct Cardiac Reprogramming of Fibroblast

Srivastava et al. first reported that postnatal cardiac and dermal fibroblasts can be directly reprogrammed into cardiomyocyte-like cells in vitro with a cocktail of transcription factors (GMT: Gata4, Mef2c, and Tbx5) [13]. Subsequent studies from the group demonstrated feasibility of in vivo reprogramming using retroviral delivery of GMT, leading to decreased infarct size and modestly attenuated cardiac dysfunction in a murine model [119]. Recently, nonintergraded approaches were developed to deliver cardiac transcription factors in vivo and offer great potential for clinical application [12, 120, 121]. However, direct cardiomyocyte reprogramming is more challenging with human cells, as most induced human cardiomyocytes remained in partially reprogrammed state [14]. Therefore, addition of transcription factors such as MYOCD, FPM2, ESRRG, and MESP1 to GMT has been developed to enhance reprogramming efficiency in human cells and represent a step toward therapeutic application in the clinic [14].

10.4.2 Stimulate Endogenous Proliferation of Cardiomyocytes

Endogenous adult cardiomyocyte renewal is measurable, but inefficient to repair injured myocardium after MI [122–127]. Recent studies in zebrafish suggest that manipulation of critical cell regulatory pathway can stimulate cardiomyocyte proliferation [128]. Early study of the evolutionarily and functionally conserved Hippo-YAP (Yes-associated protein) pathway revealed its critical roles in maintaining both cardiac health and cardiac regeneration [129–131]. Subsequent studies in altering

the Hippo pathway showed evidence of enhanced cardiac reparative capacity suggesting a potential in reversing the progression of HF in mouse model [131]. Alteration in the level of cyclin proteins, such as cyclin A2, has also been shown to improve cardiac function and increase cardiomyocyte cell cycle reentry after ischemic injury in mice [132]. Similar cardiac protection is also reported in rat and pig models with viral delivery of cyclin A2 to infarcted hearts [133, 134]. More recently, Mohamed et al. demonstrated that overexpression of cell cycle regulators CDK1/CCNB/CDK4/CCND (cyclin-dependent kinase 1/cyclin B1/cyclin-dependent kinase 4/cyclin D1) can activate proliferation of postmitotic cardiomyocytes [12], which further supports the potential of endogenous cardiac repair as a therapeutic strategy.

10.5 Cell-Enhancement Approaches to Refine Current Therapy

With modest clinical success, several strategies are investigated to refine current cardiac therapy. One fundamental limitation in the majority of cell-based therapy is that transplanted cells have poor long-term engraftment in the heart. To enhance the retention and survival of transplanted cells, both scaffold-based and scaffold-free approaches can be utilized. Scaffolds for cardiac cell therapy include decellularized matrices, injectable biomaterials, and cardiac patches made from synthetic or natural hydrogels [135–137]. Scaffold-free constructs such as cell sheets and microtissues have also been developed [138, 139]. Antibody-based strategies have also been proposed to link cells to myocardium [140]. Recently, the development of cardiac-specific peptides has also been developed to target injured heart tissue [141–143].

Another area that has been actively studied is to precondition or pretreat cells prior to transplant. Overexpression of N-cadherin and connexin-43 has been shown to improve electrical coupling after transplantation. Cells preconditioned in hypoxic condition can enhance their therapeutic potential by improving cell survival in the harsh nonrevascularized myocardial environment [144].

10.6 Conclusion

Ischemic heart disease is approaching epidemic levels in the United States and the Western world. Unfortunately, no approved therapies can limit the initial damage after MI or repair the damaged myocardium. The discovery of adult stem cells and their innate ability to proliferate and differentiate sparked excitement in cell-based cardiac therapy. The initial attempt to remuscularize the heart with SMs was abandoned due to ventricular arrhythmias. Subsequent studies in the field focused on both bone marrow-derived and cardiac-derived stem cells, with BMMNCs, MSCs, and CDCs being the most widely studied cell types. Preclinical studies using

BMMNCs and MSCs showed promising results but limited clinical benefits in human studies. CDCs were also investigated with the rationale that organ-matched stem cells might provide better tissue integration. While CDCs showed encouraging results in both preclinical and clinical settings, concerns remain due to retractions of multiple papers that set the foundation of CSC-based therapy. There is also a lack of clear understanding of their mode of action in vivo. The advancement of cell reprogramming techniques has offered new perspective in cardiac therapy using transcription factors and small molecules to directly convert fibroblast into cardiomyocytes. This approach has an advantage in that it bypassed the lengthy ex vivo isolation and expansion of stem cell. However, targeted delivery of these factors to the cardiomyocytes remains a key challenge in this line of approach as off target can generate side effects and decrease treatment efficacy.

Overall, cell therapy holds the potential to repair damaged hearts, but multiple translational and mechanistic studies are needed to refine this revolutionary therapy. Additionally, the selected cell type should be carefully evaluated and fully characterized for optimal dose and timing of administration. Finally, future approaches might include the combinatorial cell delivery concept with repeated sequential administration of cells [145, 146].

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Chapter 11

Vasculogenic Stem and Progenitor Cells in Human: Future Cell Therapy Product or Liquid Biopsy for Vascular Disease



David M. Smadja

Abstract New blood vessel formation in adults was considered to result exclusively from sprouting of preexisting endothelial cells, a process referred to angiogenesis. Vasculogenesis, the formation of new blood vessels from endothelial progenitor cells, was thought to occur only during embryonic life. Discovery of adult endothelial progenitor cells (EPCs) in 1997 opened the door for cell therapy in vascular disease. Endothelial progenitor cells contribute to vascular repair and are now well established as postnatal vasculogenic cells in humans. It is now admitted that endothelial colony-forming cells (ECFCs) are the vasculogenic subtype. ECFCs could be used as a cell therapy product and also as a liquid biopsy in several vascular diseases or as vector for gene therapy. However, despite a huge interest in these cells, their tissue and molecular origin is still unclear. We recently proposed that endothelial progenitor could come from very small embryonic-like stem cells (VSELs) isolated in human from CD133 positive cells. VSELs are small dormant stem cells related to migratory primordial germ cells. They have been described in bone marrow and other organs. This chapter discusses the reported findings from in vitro data and also preclinical studies that aimed to explore stem cells at the origin of vasculogenesis in human and then explore the potential use of ECFCs to promote newly formed vessels or serve as liquid biopsy to understand vascular pathophysiology and in particular pulmonary disease and haemostasis disorders.

Keywords ECFC · Endothelial progenitor cells · VSEL · Very small embryonic-like stem cells · Extracellular vesicles · Pulmonary vascular disease · Haemostasis and thrombosis · Liquid biopsy

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215

11.1 Introduction

New blood vessel formation in adults was considered to result exclusively from sprouting of preexisting endothelial cells, a process referred to angiogenesis. Vasculogenesis, the formation of new blood vessels from endothelial progenitor cells, was thought to occur only during embryonic life. Discovery of adult endothelial progenitor cells (EPCs) in 1997 by Isner's group had major implications for vasculogenic concepts in human but also opened the door for angiogenic therapy [1]. Endothelial progenitor cells contribute to vascular repair and are now well established as postnatal vasculogenic cells in humans [2]. It is now admitted that endothelial colony-forming cells (ECFCs) are the vasculogenic subtype. ECFCs are progenitor cells committed to endothelial lineage and have strong vasculogenic properties in preclinical models of vascularization [2]. They express specific endothelial lineage markers [3] and originate from bone marrow although some organs, and particularly the lung, can serve as a cellular reservoir for ECFCs [2, 4]. ECFCs could be used as a cell therapy product; however, their expansion is quite difficult when they are obtained from adult blood [5, 6]. ECFCs could also be used to prevascularize tissue-engineering construct. Moreover, whatever systemic or local injection, ECFCs have been proposed as vector for gene therapy in several diseases. However, despite a huge interest in these cells, their tissue and molecular origin is still unclear. We recently proposed that endothelial progenitor could come from very small embryonic-like stem cells (VSELs) [7].

This chapter discusses the reported findings from in vitro data and also preclinical studies that aimed to explore stem cells at the origin of vasculogenesis in human and then explore the potential use of ECFCs to promote newly formed vessels or serve as liquid biopsy to understand vascular pathophysiology.

11.2 Endothelial Progenitor Cells Definition in Culture: Future Cell Therapy Product?

Since Asahara first reported the existence of EPCs in peripheral blood, several studies have shown significant heterogeneity among adult ex vivo expanded EPC populations. Likewise, early and late outgrowing EPCs showed comparable in vivo vasculogenic capacity in improving neovascularization in myocardial infarction [8], in vascular graft survival [9], in tumor angiogenesis [10], or in matrigel plug in vivo [11, 12].

Classical isolation methods include adherence culture of total peripheral blood mononuclear cells and the use of magnetic microbeads coated with anti-CD133, anti-CD34, anti-CD14, or anti-CD146 antibodies. At least two types of EPCs have been described [11, 13]. "Early" EPCs or circulating angiogenic cells appear within 4–7 days of culture, are spindle-shaped, and express both endothelial (von Willebrand factor) and leukocyte/monocytic (CD 45 +/- 14) markers, whereas

“late” EPCs or endothelial colony-forming cells (ECFCs) appear after 2–3 weeks of culture and have the characteristic of precursor cells committed to the endothelial lineage; they have a cobblestone pattern in culture, and their long-term proliferative potential or their reactivity for growth factor depends on their origin (umbilical or adult blood) [6, 14–16].

Early EPCs, which express CD45 antigen, were the cells identified by Asahara et al. in 1997 and have been the most studied EPC population between 1997 and 2010. There are many uncertainties as to their origin and their progenitor properties. Elsheikh et al. [17] tried to identify the subpopulation within monocytic cells that exerts “EPC properties.” These authors isolated CD14⁺ monocytic cells and purified those cells that expressed vascular endothelial growth factor receptor 2 (VEGF-R2 or KDR). CD14⁺VEGF-R2⁺ cells but not CD14⁺ VEGF-R2⁻ cells contributed to reendothelialization in mice after denuding injury. These data showed that VEGF-R2 is a fundamental receptor-identifying cell with endothelial capacity. A specific subfraction of circulating CD14⁺ monocytic cells was recently shown to express the stem-cell markers Nanog and octamer-binding transcription factor 4 (oct-4) [18]. These Nanog⁺ monocytic cells were positive for VEGF-R2 and showed low CD34 expression. CD14⁺/CD34^{low}/Nanog⁺ cells appear to represent the active fraction of CD14⁺/VEGF-R2⁺ cells isolated by Elsheikh et al. and confirm the origin of stem cells of a subpopulation of early EPCs [19]. Leukocyte-derived EPC is an area that has been abandoned since no clear endothelial or vessel differentiation has been clearly proved in CD45-positive cells. Multiparametric labeling of cells and new sorting strategies will probably reopen this research field in the next years.

ECFCs are now commonly considered as the true vasculogenic progenitor cell population, and their culture is now consensual with a position paper from vascular biology standardization subcommittee of International Society on Thrombosis and Haemostasis [20]. ECFCs include expression of endothelial cell markers (e.g., CD31, CD144, CD146, EGFL-7, and VEGFR2) and lack of hematopoietic markers (CD45 and CD14) [2, 3]. Several mechanisms have been proposed to explain their vasculogenic effects observed in preclinical studies:

1. The first mechanism is a direct incorporation in host tissue to form vessels. In contrast to early EPC (or circulating/myeloid angiogenic cells), ECFC can graft in tissue or form human vessels in mice models of hind limb ischemia [5, 21].
2. The second mechanism proposed more recently is a paracrine effect. Firstly, ECFCs have been described as non-secreting cells compared to early EPC since they do not secrete VEGF-A and low levels of IL-8 [13]. Since this first description, we know that ECFCs have a secretion potential in inflammatory, stress, or senescence condition for IL-8 [22, 23]. Moreover, they are able to recruit inflammatory cells by secreting other CXCL or CCL molecules [24] or perivascular cells by secreting PDGF-BB so enhancing cell engraftment [25]. Moreover, ECFC has been shown to secrete microvesicle and/or exosomes that could be at the origin of their beneficial effects. This aspect of ECFC secretion of microvesicles and/or exosomes will be treated at the end of this chapter.

3. The third mechanism is a direct supportive effect of mesenchymal stem cells from different origin [26, 27] or perivascular cells [28]. They probably help mesenchymal stem/progenitor cells to differentiate in real perivascular cells by a Notch/jagged-1-induced mechanism [29].
4. The last mechanism proposed is a direct adhesive property to perivascular cells by an endoglin-dependent mechanism. Indeed, ECFC endoglin binding when MSC are co-injected in hind limb ischemia model allows a quicker engraftment of perivascular cells and an accelerated recovery [30, 31].

However, a major barrier to ECFC development as an autologous cell therapy product is their paucity in the peripheral circulation. Attempts have been made to expand ECFCs *ex vivo* by priming cells with growth factors [32–34], peptides [35, 36], platelet lysates [37], hypoxia [38], or acidosis [39]. *In vivo* mobilization has also been tested [40]. ECFCs have been isolated from the blood of patients with critical limb ischemia patients [41], CAD [42], acute MI [43], and pulmonary hypertension [44, 45], but their therapeutic potential has never been tested in clinical trials because of difficulties to obtain and expand them in a sufficient number of cells allowing human injection with clinically approved procedures [40]. However, the main problem for therapeutic use of ECFCs is probably addressing cells to specific area, and expansion of “adult” cells for autologous cell therapy is probably not the way to improve vasculogenesis in adult.

11.3 ECFCs Stemness and Ontogeny of Endothelial Lineage in Human

Despite the fact that ECFCs and mature endothelial cells share a similar phenotype *in vitro*, they show different properties in endothelial homeostasis and repair [2, 3]. Whatever their origin, mature endothelial cells do not retain the ability of revascularization when injected to preclinical models, by contrast to progenitor cells [13, 46, 47]. We and other groups have described for a long time functional difference between ECFCs and HUVECs in terms of proliferation potential and survival after growth factor activation [3, 6, 14], and several authors reported a decreased resistance to apoptosis of mature endothelial cells compared to ECFCs [48]. Moreover, ECFCs were described to be reprogrammed into induced pluripotent stem cells (iPSCs) in a better way than adult endothelial cells [49]. There is therefore a need to understand the link between ECFC stemness and their vasculogenic potential. A relative plasticity of ECFCs has been described, probably related to their stem cell/progenitor nature [50]. Membrane expression of CD34 and/or CD133 in ECFCs is a controversial field [51, 52]. One major marker of stemness of hematopoietic cells is CD34. CD34 is heterogeneously expressed on ECFC in culture, and its expression has been correlated to their vasculogenic properties [53, 54]. However, CD34 is also expressed by many mature endothelial cells in culture and has been used in human tissues to identify mature vessels. Therefore, CD34 is not a good candidate to fully explain the

relationship between vasculogenic potential and stemness. Endothelial progenitors and/or angiogenic population have been also described coming from CD133+ cells [55]. However, ECFC membrane expression of CD133 is negative despite a mRNA expression that can vary along culture and passages [35, 56].

CD133, a Pentaspan membrane glycoprotein, has been used as a stem cell marker for stem cell isolation from several normal and pathological tissues, but its functional involvement is not clearly defined. In a muscle injury rat model, granulocyte colony-stimulating factor-mobilized peripheral blood CD133+ cells can differentiate into endothelial and myogenic lineages [57]. In addition, bone marrow-derived CD133+ stem cell therapy has been used in clinical trials for patients with refractory angina [58, 59], chronic total occlusion and ischemia [60], or myocardial infarction [61]. Thus, we recently described intracellular expression of the stemness marker CD133 in ECFCs [62]. CD133 gene expression inhibition abolishes ECFC vasculogenic effects in hind limb ischemia model. These findings could resume the discrepancies found in the literature concerning CD133 positivity in endothelial progenitors. Our results could explain why circulating cells expressing surface CD133 have not been correlated to ECFCs in several clinical physiological or pathological situations [40, 44, 63] and also why CD133 isolation in cord and adult blood do not give rise to ECFCs [51, 52]. A better approach would be to quantify and/or isolate ECFC by intracellular expression of CD133. Such approach has been partially evocated with cytosolic aldehyde dehydrogenase (ALDH) [64]. Our hypothesis needs to be confirmed by a prospective correlation between circulating cells expressing intracellular CD133 and ECFCs obtained in culture.

In terms of human postnatal vasculogenic stem cells, the two cell types described so far have been isolated from CD133+ populations. The first one is the stem cell isolated from proliferating phase of infantile hemangioma (Hem-SC), using anti-CD133-coated magnetic beads. These cells are scarce, representing between 0.1% and 1% of the cells from hemangioma. Hem-SCs display two essential properties of stem cells: the ability to self-renew and to undergo multilineage differentiation, including endothelial and mesenchymal lineage [65–67]. The second stem cells described to give rise to endothelial cells are very small embryonic-like stem cells (VSELs) [7, 68, 69]. Indeed, VSELs are pluripotent stem cells defined as lineage-negative, CD133-positive, and CD45-negative cells of small size (<6 μm in diameter) [68, 69]. VSELs have a large nucleus-to-cytoplasm ratio, and a high expression level of pluripotency core factors. They could represent a clinically relevant alternative to embryonic stem cells (ESCs) and induced pluripotent stem cells (iPS) for cell therapy since VSELs do not complete blastocyst development and do not form teratomas after transplantation into deficient mice [70, 71]. VSELs are small cells probably at the top of the stem cell hierarchy in adult tissues, able to differentiate into cells from different germ layers. We have previously described that human bone marrow (BM) VSELs isolated from patients with critical limb ischemia were able to differentiate into endothelial cells but also in perivascular cells and to foster post-ischemic revascularization in experimental model of critical limb ischemia [7, 72]. Endothelial cell differentiation has been obtained from VSELs after a step of mesenchymal phenotype as previously described in hemangioma that has been

confirmed by another group [73]. VSEL's ability to differentiate into several lineages could explain the discrepancies observed in the literature in the field of EPCs. Indeed, ECFCs have been firstly described in an elegant study of BM transplantation from Hebbel's group [74]. However, Yoder's group in Indianapolis proposed that these ECFCs directly come from vessels [4, 15] and from CD45-negative cells [5, 51, 52]. VSELs have a huge motility and are theoretically able to migrate from bone marrow to vessels. They have all the characteristics of potential vasculogenic CD45-negative stem cells able to give rise to vessels since they have been described to differentiate in endothelial cells by several independent groups with human, mouse, or rat VSELs [7, 75, 76]. However, further work is required to stimulate the proliferative capacity of VSELs to make them a cell therapy product. The critical expansion step should be to reverse VSEL-quiescent state and expand them. VSELs can now be expanded *ex vivo* in the presence of nicotinamide or valproic acid [69] or in the presence of the small-molecule UM177 [77] without transduction by DNA or RNA or by using supportive feeder cells. These first preclinical steps could lead to clinical trials with expanded cells with or without differentiation step.

Other bone marrow-derived cells have been proposed in human to give rise in endothelial cells. Mesenchymal stem cells (MSCs) have been described in some publication to be able to differentiate into endothelial cells [78] and improve neo-vascularization in vivo [79]. However, no clear proof of this differentiation ability has been convincing, and probably MSCs are just paracrine cells that support angiogenesis [21, 80] by secreting growth factor, modulate immunologic responses, and differentiate into perivascular cells without endothelial differentiation ability [27]. The same kind of results can be proposed for fibrocytes derived from idiopathic pulmonary fibrosis patient's blood [81]. Discrepancy of MSC differentiation ability could come from the presence of MSC derived from VSEL. In 2002, Verfaillie's group [10] also reported that multipotent adult progenitor cells (MAPCs) can be isolated from postnatal human bone marrow. Likewise, these data have been retracted and seem controversial. However, clear differentiation of stem cells in ECs needs to be deeply done in bone marrow cells and postnatal cells, and vasculogenic ability is resumed in Fig. 11.1.

Since ECFC number from adult blood is reduced in contrast to cord blood [20] and has low expansion properties [5, 6], differentiation of human pluripotent stem cells into ECFC-like phenotype and characterization of endothelial differentiation pathways may allow to have enough vasculogenic and highly proliferative blood vessel-forming cells to create newly formed vessels in patients with vascular disease. A protocol to convert human-induced pluripotent stem cells (hiPSCs) or embryonic stem cells (hESCs) into ECFC-like cells has been proposed by Mervin Yoder's group. This neuropilin-1 (NRP-1)-mediated differentiation is a promising tool; however, using hESCs or hiPSCs for regenerative medicine is still a controversial field. VSEL expansion and differentiation protocol could allow us to have an autologous cell therapy product in vascular disease. ECFC ontogeny needs also to be explored according to Peg3/PW1 [82], CD157 [83], and PROCR [84] expression to establish in human a clear hierarchy in endothelial stem and progenitor cells in human.

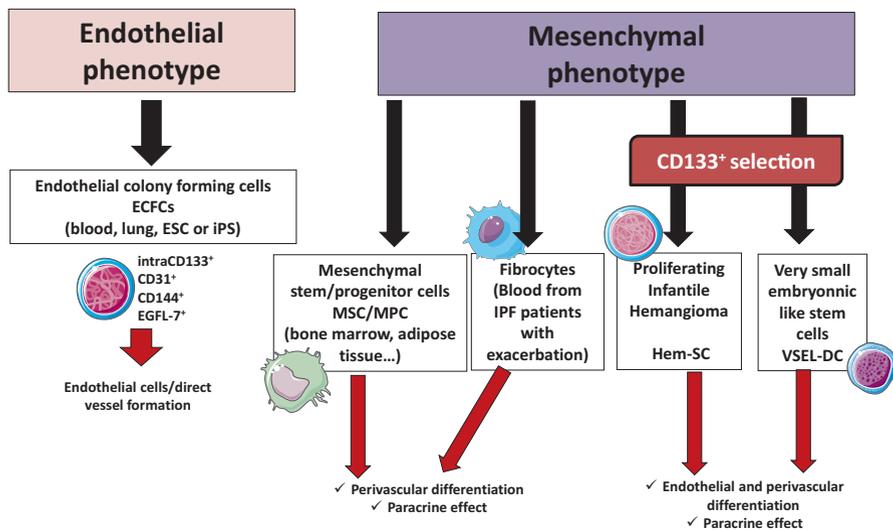


Fig. 11.1 Human postnatal vasculogenic stem and progenitor cells

11.4 ECFC and Secretion of Extracellular Vesicles

More than soluble factors, the release of extracellular vesicles (EV) constitutes a new mechanism for intercellular communication [85]. Indeed, EVs may affect cell function in impaired tissues by horizontal transfer of proteins, mRNA or miRNA. EVs are distinguished on the basis of their size including respectively from small to large vesicles: exosomes, microvesicles, and apoptotic bodies. These effects of MV and exosomes that could recapitulate effects of original cells might justify skipping the use ECFCs and limiting their role as “MVs/exosomes ex vivo producers” which would then be administered to the patient. All the findings demonstrating that benefits of progenitors or stem cells derived from MVs/exosomes can be recapitulated by their sole injection are an enthusiastic cell-free approach that would have clinically relevant advantages in terms of cost and probably also immunological properties. One of the perfect examples in cardiovascular disease has been demonstrated in Pr Menashe’s group in a postinfarct heart failure model. Indeed, EVs from progenitor cells originated from ESCs increase cardiac function recovery in the same way than originated cells. These results support the hypothesis that a paracrine mechanism is enough to enhance chronic heart failure recovery in cell-based therapies [86]. Concerning endothelial MVs, they have been largely described as causative agents or markers of endothelial dysfunction in vascular and cardiovascular disease [87]. More than a biomarker, these endothelial MVs have been described to have several biological effects with both protective and deleterious effects. Indeed, endothelial MVs have been described as anticoagulant, profibrinolytic, and proangiogenic agents, while at the same time, they can act as procoagulant/thrombotic/

inflammatory agents as well as antiangiogenic properties depending of technical condition for isolation and also depending on patient condition [88]. We will focus here on preclinical data about MVs and exosomes from ECFCs.

11.4.1 *Microvesicles from ECFC*

ECFCs can be considered as a liquid biopsy of in situ endothelium (see Chap. 4). Thus, MVs obtained from adult peripheral blood-derived ECFCs could also be a valuable tool to understand vascular disease. Indeed, Pr Dignat-George's group demonstrated that ECFC expanded from cord blood of healthy neonates has angiogenic potential in vitro and promotes blood flow recovery in mouse hind limb ischemia model. However, when obtained from senescent ECFC isolated from low birth weight neonates, the EVs collected in similar culture conditions are able to alter endothelial cell homeostasis in vitro and transfer senescence in target cells [89]. In addition, we recently demonstrated that MVs from ECFC isolated from patients with idiopathic pulmonary fibrosis (IPF) induce profibrotic effects, have higher fibrinolytic properties than controls, and could correlate with IPF severity [90]. Based on these two observations, we can observe that, according to the pathological environment associated to the donor health status, the properties of ECFC in terms of angiogenic or tissue repair potential can be significantly affected. We anticipate that EVs from patient-derived ECFC can have some deleterious effect on vascular system that has to be documented and reversed before their use in an autologous context. More than that, MVs from disease models could help to understand vascular pathophysiology.

As previously described, ECFC may act as cell therapy product in some vascular disease. However, there are several situations with a very low level of ECFC engraftment and injection of ECFC-conditioned media, and in particular, MVs are probably enough to have vascular repair in retinal ischemia [91] or bronchopulmonary dysplasia [92].

11.4.2 *Exosomes from ECFCs*

Exosomes are produced in the endosomal compartment of most eukaryotic cells and have a size between 40 and 100 nm of diameter. These are very small membrane-bound vesicles that are over produced by most proliferating cell types during normal and pathological states. Their levels have been proposed as diagnostic and therapeutic tools in cardiovascular disease or cancer. Exosomes contain a large variety of RNAs and noncoding RNAs (ncRNAs) including miRNAs and long non-coding RNAs but also proteins and lipids, which are representative to their cellular origin and shuttle from a donor cell to a recipient cell. Since 2011 [93], ECFCs have been described to be able to secrete exosomes. Their functions and secretion specificity are specific of cell and clinical situation. Their roles are different from MVs.

Indeed, in senescence associated to premature neonates [89], ECFC has a higher number of MVs, while exosomes are secreted at the same level. In ECFC efficacy, as previously described here, ECFC improves vascular function, and their conditioned media are enough to have beneficial effects. In acute kidney injury (AKI), intravenous injection of ECFC significantly attenuated increases in plasma creatinine, tubular necrosis, macrophage infiltration, oxidative stress, and apoptosis, without cell engraftment in the kidneys [94]. Total conditioned media and exosomes from ECFC reproduce the same effect than original ECFC, while MVs do not have any beneficial effects [94]. ECFC exosomes reduce AKI via transferring miR-486-5p-targeting PTEN and with a CXCR-4/SFD-1 mobilization that helps in exosome uptake [95, 96]. ECFC-derived exosomes have also been described to reduce cardiac fibroblast activation [97] and restore blood-brain barrier continuity in mice after a traumatic brain injury [98].

11.5 ECFC as a Liquid Biopsy to Understand Vascular Disease?

Recently growing interest has been reported for “noninvasive” liquid biopsy as a valuable source for molecular profiling and/or cellular pathophysiology. A biomarker and/or composition of biomarkers capable of detecting vascular abnormalities could help to characterize or deeply understand vascular diseases. ECFCs, after being described in early 2000 from bone marrow [74], have been proposed originating from vascular endothelium [15] and, in particular, lung vessel wall [99]. Since ECFCs have been described to come from vessel wall, they could be considered as a potential liquid biopsy, in particular, in pulmonary vascular disorders. In this chapter, we focus on ECFC involvement in lung diseases and also in haemostasis disorders.

11.5.1 ECFC in Lung Diseases

11.5.1.1 Pulmonary Arterial Hypertension (PAH)

Our team was particularly interested in the endothelial compartment circulating in pulmonary hypertension. It is a rare, rapidly fatal disease in the absence of treatment, characterized by pulmonary vascular obstruction leading to a progressive increase in resistance to blood flow and ultimately to right heart failure. It is defined as mean pulmonary arterial pressure (mPAP) >25 mmHg at rest or >30 mmHg at exercise, measured during right heart catheterization, which is the gold standard for diagnosing pulmonary hypertension [100]. Endothelial dysfunction plays a key role in the development of pulmonary hypertension [101]. This disease is characterized by pulmonary vascular remodeling of small arteries and precapillary arterioles. Plexiform lesions, an uncontrolled proliferation of endothelial cells, are characteristic of idiopathic PAHs, and an antiapoptotic profile of endothelial cells has been described

in the irreversible forms of PAH secondary to congenital heart disease [102]. In order to evaluate the involvement of the circulating endothelial compartment during PAH, we quantified ECFCs but also circulating endothelial cells (CECs) in pediatric PAH. We found strong correlation between CECs and remodeling process or vasodilator therapy efficacy [103–105], while we never found any modification of ECFC level in PAH compared to controls or reversible PAH. However, we demonstrated that prostacyclin analog treprostinil increases ECFC level [104] by a VEGF-A-dependent mechanism [106]. Thus, we do not think that ECFCs are a good biomarker of vascular remodeling process in PAH, and conflicting results in literature probably paved the way of absence of quantitative relationship between ECFC and vessel pathophysiology [107]. However, functional properties of ECFCs are related to PAH vascular function. Indeed, ECFC proliferation has been correlated to disease severity [108] suggesting a link between their function and clinical settings. Moreover, hereditary PAH can be the consequence of bone morphogenetic protein receptor type 2 gene (BMPRII) mutation. ECFC from PAH patients with BMPRII mutations has been shown to have a defective ability to form vessels *in vitro* [45]. Studies on ECFC from patients with BMPRII allowed finding new therapeutic targets like translationally controlled tumor protein (TCTP) [109], miR-124 [110], chloride intracellular channel 4 (CLICL4) [111], or interferon type 1 [112] but also proposed new treatment like chloroquine [113] or BMP9 [114].

11.5.1.2 Chronic Obstructive Pulmonary Disease (COPD)

COPD is associated with chronic airway inflammation including chronic bronchitis and emphysema, characterized by alveolar loss and enlargement. Cigarette smoke is in general at the origin of COPD. The contribution of the endothelial progenitor to COPD pathogenesis is not been fully understood because of the diversity of cells phenotype quantified [115–118]. We recently demonstrated that VSELs (described to be at the origin of endothelial lineage; see earlier in this chapter) were mobilized in COPD patients with PaO₂ under 92% [119]. Concerning ECFC, their number seem decreased in most chronic pulmonary diseases [120], but more interesting is their abnormal function in terms of adhesion [121] or senescence [122]. Indeed, Pr Anna Randi's group evidenced that ECFC from smokers and COPD patients had an accelerated aging due to epigenetic dysfunction [122]. They also found that miR-126-3p is downregulated in ECFCs isolated from COPD patients and promotes increased DNA damage at the origin of endothelial dysfunction [123].

11.5.1.3 Idiopathic Pulmonary Fibrosis (IPF)

Idiopathic pulmonary fibrosis (IPF) is a devastating disease characterized by obliteration of alveolar architecture, resulting in declining lung function and ultimately death. We and others have previously demonstrated that EPCs [63, 124, 125] and,

in particular, ECFCs are downregulated in stable IPF but increased significantly in patients with impaired gas transfer [diffusing capacity of the lung for the carbon monoxide (DLco) <40%] [63]. ECFCs from IPF patients could participate to vascular remodeling in fibrotic lung diseases by a direct vasculogenic effect but also by cooperating with fibrocytes, a cell type well known to contribute to organ fibrosis [81]. Cell therapy approaches have been proposed in several chronic lung diseases; thus, we tested ECFC injection in fibrogenesis induced by bleomycin in nude mice. Mice were injected with ECFCs isolated from cord blood or IPF patients. We assessed morbidity, weight variation, collagen deposition, lung imaging by microCT, Fulton score, and microvascular density. No modulation of fibrosis or vascular density during fibrogenesis or when fibrosis was constituted was observed with ECFCs whatever their origin [126]. We then postulated that ECFCs might behave as a liquid biopsy in IPF patients, and we have demonstrated that senescent and apoptotic states were increased in ECFCs from IPF patients as shown by galactosidase staining, p16 expression, and annexin V staining but also increased interleukin-8 secretion [22]. We showed that IL-8 secretion from ECFCs of IPF patients induced migration of neutrophils in vitro and in vivo in a matrigel implant model in nude mice. To check clinical relevance of these results, we showed an infiltration by neutrophils in IPF lung biopsies, and we found, in a prospective clinical study, a higher level of neutrophils in peripheral blood in IPF patients with a poor prognosis [22]. Finally, we also demonstrated that microparticles released from ECFC isolated from IPF patients compared to controls had an increased plasminogen activation and could stimulate fibroblast migration [90], suggesting involvement of ECFC-derived endothelial microparticles to pulmonary fibrogenesis. Altogether, our results are in favor of a real correlation of ECFCs in IPF with the phenotype observed in lungs, while no active process by injecting them in mice is observed [127]. So, ECFCs in IPF are clearly a “liquid biopsy” of vessels.

11.5.2 ECFC in Haemostasis and Thrombosis

11.5.2.1 von Willebrand Disease (VWD)

von Willebrand factor (vWF) is a glycoprotein, highly involved mainly in haemostasis and bleeding disorders, produced uniquely by endothelial cells and megakaryocytes. Its quantification or labeling is routinely used in pathology lab to identify vessels. vWF is increased during angiogenesis since fibroblast growth factor-2 and vascular endothelial growth factor have been shown to increase its expression in a variety of endothelial cells [128]. vWF has been used to affirm endothelial origin of ECFCs and more recently explored mainly by Pr Anna Randi's group to explore endothelial dysfunction in patients with VWD. Congenital forms of VWD are due to mutations in the vWF gene. There are three subtypes: types 1 and 3 with quantitative defects (respectively partial and total for types 1 and 3) and type 2 with qualitative defects. ECFC culture in patients with VWD evidenced involvement of

vWF in angiogenesis processes [129, 130]. Indeed, significant enhancement of in vitro tube formation, proliferation, and migration was observed in ECFCs isolated from VWD patients [131], with a variability in different subtypes of VWD [132, 133]. These studies in VWD are also pointing a role of ECFC as potential liquid biopsy since they allowed studying vascular function of different defect in vWF that could explain angiogenic disorders like angiodysplasia in type 2 patients. ECFC in VWD could also help to understand physiology of vWF storage in endothelial cells and help to decipher between desmopressin (which induces release of vWF from endothelial cells) or replacement therapy in VWD treatment. Finally, gene therapy of VWD type 3 has been proposed by using ECFCs as a cellular vehicle for therapy [134]. These approaches also proposed to cure hemophilia (factor VIII) or anemia (erythropoietin) are still at a very preliminary step for a potential clinical application [135, 136].

11.5.2.2 Myeloproliferative Neoplasms (MPN)

Initial studies in patients with Philadelphia chromosome-positive or with Janus kinase 2 V617F ($JAK2^{V617F}$) mutation in MPN were found only in hematopoietic cells and not in ECFC [5, 137, 138]. We recently confirmed this result with ECFCs from a patient double mutated with *BCR-ABL1* and $JAK2^{V617F}$ mutations that lack these two mutations [139]. However, Teofili et al. found in a subgroup $JAK2^{V617F}$ mutated patients with thrombotic events that ECFC could express the mutation [140]. Another group also found a regulation of ECFCs in subpopulation of patient with thrombotic disorder in nonactive MPN [141]. Thus, as found in lung disorders and VWD previously, probably in MPN, ECFCs could be marker of a “clinical vascular phenotype” and be the reflection of prothrombotic state or disease evolution from a hematopoietic disorder to a systemic disorder. Another consequence of these studies is about stem cell at the origin of hematopoietic and vascular lineage. Probably mutation present at the origin only affects hematopoietic stem cells, while after a disease evolution perhaps, mutation – i.e., or at least $JAK2^{V617F}$ – can appear on putative hemangioblast or potentially on VSELs at the origin of both lineages. Hypothesis of the prothrombotic phenotype of endothelial cells that could acquire $JAK2^{V617F}$ mutation has been described by two French groups [142, 143]. Indeed, both groups, with different in vitro models, found an increased expression of P-selectin associated with prothrombotic, proinflammatory, and proadhesive phenotype of endothelial cells.

11.5.2.3 Hereditary Hemorrhagic Telangiectasia (HHT)

Mutations in endoglin or activin receptor-like kinase-1 (*ACVRL1/ALK1*) genes can give rise to hereditary hemorrhagic telangiectasia (HHT). These patients present epistaxis, telangiectases, and arteriovenous malformations in the lung,

brain, or liver. ECFC from HHT patients had impaired angiogenesis potentially related to decreased endoglin expression [144, 145]. We explored in our group endoglin involvement in ECFC angiogenic potential and found that endoglin is an adhesive molecule necessary for vessel stabilization and ECFC regenerative potential [30, 31, 146].

11.5.2.4 Venous Thromboembolism (VTE) Disease

Thrombus resolution has been described to be related to endothelial progenitor mobilization [147, 148]. ECFCs have been also involved in fibrin infiltration in vitro, and thrombin could be a chemoattractant for ECFC [149]. Indeed, ECFC has a whole panel of hemostatic receptor and molecule including inducible tissue factor [150], fibrinolytic properties [149], or thrombospondin-1, largely described as a platelet aggregation stabilizer and an active partner for vWF [151, 152]. We also described that ECFC expresses thrombin receptor PAR-1 [6, 23, 24, 35, 36, 149]. Indeed, thrombin involvement is essential in thrombosis. Activation of its main receptor PAR-1 by thrombin- or PAR-1-activating peptide (which mimics the effect thrombin on its PAR-1 receptor without causing cleavage) on cord blood ECFC favored all stages of angiogenesis that are the cell proliferation, migration, and differentiation [6, 23, 24, 35, 36, 149]. Thrombin has also been described to induce endothelial differentiation from bone marrow mononuclear cells [153]. PAR-1 activation on ECFCs improves angiogenesis in vitro by activating the pathways of angiopoietin 2, which promotes cell proliferation, and that of SDF-1/CXCR4, which promotes differentiation. In addition, the modulation of the PAR-1 on ECFCs by an autologous fibrin network that can constitute a matrix enables ECFCs to acquire anticoagulant and antifibrinolytic properties in addition to their angiogenic properties [149]. It is recognized that leukocytes play an important role in this recanalization of the thrombus. Thus, thrombin, which is also a proinflammatory factor, and interleukin-8 (IL-8) have angiogenic properties by interaction with its CXCR1 and CXCR2 receptors. Indeed, IL-8 is strongly expressed in early EPCs, and its level in the conditioned medium is unchanged after activation of PAR-1. On the other hand, the secretion of IL-8 by ECFC, very weak under basal conditions, is strongly increased after activation of the PAR-1 and induces the Boyden chamber migration of the early EPCs. These results suggest that thrombin receptor activation on these ECFCs allows cooperation between different angiogenic cells during paracrine-mediated neovascularization. We further explain how PAR-1 activation in ECFC could interact with inflammatory cells and demonstrated a large panel of chemotactic gene expression increase and an effect of ECFC-conditioned media on leukocyte recruitment at ischemic sites through a COX-2-dependent mechanism [24]. We finally showed that thrombin PAR-1-activating peptide was able to increase inflammatory cell recruitment on other postnatal vasculogenic stem cell. Indeed, activation of infantile hemangioma tumor-derived stem cells (HemSCs) incubated with PAR-1-ap increased leukocyte recruitment in a Matrigel® implant model in

nude mice [24]. In addition, ECFCs obtained from healthy volunteers are able to express TF following stimuli such as TNF- α [150, 154]. ECFC stimulation by TNF- α also induces the generation of procoagulant microparticles [150], which may represent an angiogenic but also thrombogenic vector. Hubert et al. demonstrate in a well-recognized cremaster arterial laser-induced injury model of thrombosis that neutrophils present at the site of thrombus can recruit ECFCs [155]. Involvement of ECFC in VTE has been demonstrated by the group of Dr. Alvalrado-Moreno [156, 157]. In recurrent and unprovoked VTE, they described dysfunctional ECFC and proposed an association with these defects and the risk of thrombotic events. One of their findings is an increased proinflammatory cytokine secretion of ECFC from VTE patients [156]. Further studies are required to determine whether dysfunctional ECFCs are involved in thrombus formation or recanalization. However, in this VTE situation, we have one more time an ECFC phenotype different from control that could be associated with endothelial dysfunction found in thrombotic patients.

11.6 Conclusion

After several years of inconsistency and ambiguity about endothelial progenitor cell definition, ECFCs have been largely described as the progenitor cell linked to clear vasculogenic potential and vascular diseases, and now, standardization protocols are on the way [20]. Ontogeny of these cells needs a lot of effort yet to determine which stem cell is at the origin of endothelial lineage in human adult [68]. New technologies allowing multiparametric evaluation of heterogeneous cell populations could probably help us to decipher different cell population and explore new marker of vascular stem cells (Peg3/PW1, PROCR, or CD157) in human vasculogenesis. ECFC involvement as a liquid biopsy is now very clear, and we have with ECFC probably the perfect tool to explore vascular disease and find new therapeutical targets or at least demonstrate pathophysiology of vessel abnormalities. Several clinical applications have been proposed as a cell therapy product, a vector for gene therapy, or a tissue-engineering strategy. Recent research about hemocompatibility of biomaterials, in particular, with development of a bioprosthetic total artificial heart [158–160], could open a new area of biomaterial bioengineering with ECFCs and/or endothelial stem cells.

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Chapter 12

Epidermal Stem Cells



Anna M. Lenkiewicz

Abstract A multilayered epithelium to fulfil its function must be replaced throughout the lifespan. This is possible due to the presence of multipotent, self-renewing epidermal stem cells that give rise to differentiated cell lineages: keratinocytes, hairs, as well as sebocytes. Till now the molecular mechanisms responsible for stem cell quiescent, proliferation, and differentiation have not been fully established. It is suggested that epidermal stem cells might change their fate, both due to intrinsic events and as a result of niche-dependent extrinsic signals; however other yet unknown factors may also be involved in this process. Given the increasing excitement evoked by self-renewing epidermal stem cells, as one of the sources of adult stem cells, it seems important to reveal the mechanisms that govern their fate. In this chapter, we describe recent advances in the characterisation of the epidermal stem cells and their compartments. Furthermore, we focus on the interplay between epidermal stem cells and extrinsic signals and their role in quiescence, proliferation, and differentiation of appropriate epidermal stem cell lineages.

Keywords Epidermal stem cells · Transit-amplifying cells · Cell division and proliferation · Self-renewal

12.1 Introduction

A multilayer epithelium, known as the epidermis, together with the dermis and subcutaneous tissue forms the skin, whose main functions are protection against insults, infection, and dehydration, as well as enables thermoregulation and sensory perception.

The epidermis built mainly by keratinocytes is the outermost of the three layers of the skin. It consists of a number of coating, which include stratum basale,

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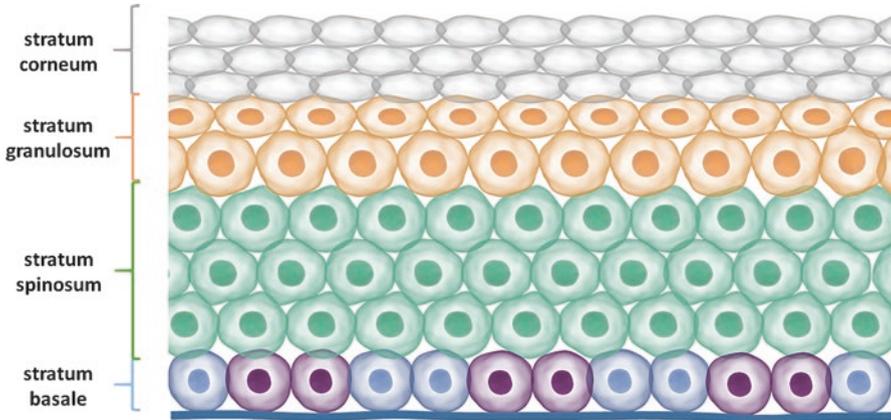


Fig. 12.1 Organisation of interfollicular epidermis

stratum spinosum, stratum granulosum, stratum lucidum (only on palms and soles), and stratum corneum (Fig. 12.1). Each of the mentioned layers has a different function. Stratum basale composed mainly of interfollicular epidermal stem cells, and undifferentiated proliferative progenitors ensure replenish of basal layer and what's more give rise to nonproliferative, transcriptionally active spinous and granular keratinocyte layers [1, 2]. The keratinisation process that occurs in stratum spinosum and stratum granulosum leads to the formation of up to 30 layers of polyhedral, flattened, enucleated corneocytes that are continuously shedding from the skin [1, 3].

The epidermis is punctuated by hair follicles and glandular structures that are created by hair follicle bulge stem cells and sebaceous stem cells, respectively [3, 4]. Both adnexal structures and interfollicular epidermis to fulfil their functions must be replaced throughout the lifespan of an organism. Thus, resident self-renewing epidermal stem cells are essential for ensuring continuous renewal of the epidermis.

Interfollicular epidermis is a stratified structure. Self-renewing stem cells (blue) and transit-amplifying cells (purple) reside within the stratum basale. Detachment from basal layer initiates cell differentiation that goes through three stages: spinous layers, granular layers, and stratum corneum layers that are shed from the skin surface.

12.2 Epidermal Stem Cell Proliferation and Differentiation

Given the increasing excitement evoked by self-renewing epidermal stem cells, it seems important to understand the molecular mechanisms responsible for their proliferation and differentiation. Constant turnover of epidermal cells and healing in case of injury require reservoirs of stem cells.

Local tissue microenvironment capable of housing and maintaining one or more stem cells is called stem cell niche [1, 5]. In response to the microenvironment of the niche, epidermal stem cells may remain quiescent or start to divide, proliferate, and finally differentiate into lineage-specific cells. In addition to a major primary signal from niche that acts directly on stem cells, it is suggested that additional signals acting indirectly may also play an important role in preserving stem cell properties [6]. Hence, interaction between the stem cells, their niche, and additional signals has a significant impact on the maintenance of an appropriate number of stem cells [1, 5, 6].

12.2.1 Models of Epidermal Stem Cell Proliferation

Stem cells have the ability to self-renew providing permanent turnover of epidermal cells during a lifetime. To fulfil their function, stem cells must divide and differentiate creating lineage-specific cells. Epidermal stem cells exhibit a high degree of spatial organisation, cell clustering, and motility. It was indicated that stem cells undergo symmetric and asymmetric divisions during the course of organogenesis [7, 8], thus safeguarding against excessive stem cell proliferation and providing tissue homeostasis (Fig. 12.2). When the population of stem cells require self-renewal, a symmetric division occurs and two stem cell daughters are produced [9]. Accordingly, in postnatal mammalian epidermis, the planes of mitosis are oriented such as to produce two daughter cells in the basal layer [10, 11].

In contrast, asymmetric division creates one stem cell daughter and one transit-amplifying cell (TA cell). Till now it is unclear whether TA cells have multilineage differentiation potential or whether they are lineage-restricted [9, 12]. By analogy to

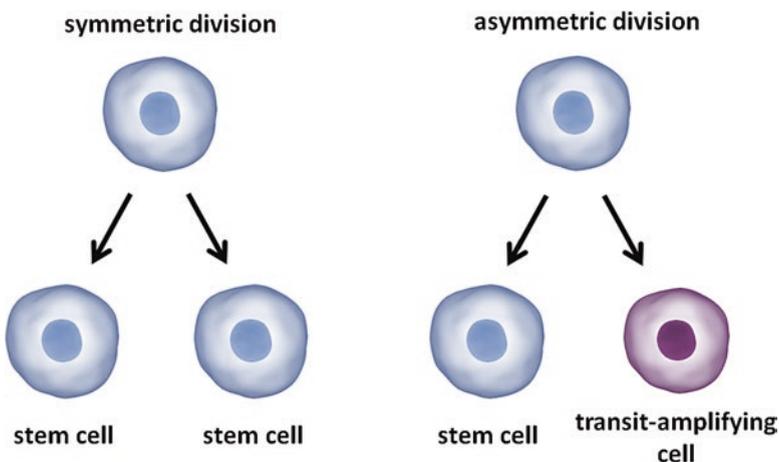


Fig. 12.2 Models of epidermal stem cell division

haematopoietic system, it could be possible that each TA cell is committed to differentiate along one particular lineage: interfollicular epidermis, hair follicle, or sebaceous gland. However, it could be also possible that TA cell lineage choices are determined by extracellular conditions or local microenvironment of niche [13]. Despite the fact that TA cell potential is undetermined, as a result of subsequent rounds of their division, a number of differentiated progeny cells are formed, and the epidermis may be replaced [14].

Asymmetric division of stem cells may be a consequence of several events. It is well known that cells are capable of asymmetrically segregating proteins, organelles, as well as nucleic acids during division [7, 15]. Thus, it seems that asymmetric localisation of cell polarity and/or fate regulators might be one of the causes of asymmetrical division of stem cell. Another way is to vertically orient the kariokinetic spindle [15, 16]. In this case, one daughter cell remains in the basal (undifferentiated) layer, and the other is exposed to extrinsic signals in the suprabasal layer that inhibits its self-renewal properties. Accordingly, analysis of the oesophageal epithelium cell divisions comprising just like the epidermis, multiple keratinocyte layers, revealed a subpopulation of cells that undergo asymmetric divisions, producing one undifferentiated basal cell and one suprabasal differentiating daughter [17].

Epidermal stem cell (blue) division can occur either symmetrically or asymmetrically. Symmetric division gives rise to two identical daughter cells (blue). Asymmetric division of epidermal stem cell creates one stem cell daughter (blue) and one more differentiated, transit-amplifying cell (purple).

12.2.2 Models of Epidermal Stem Cell Differentiation

Asymmetric division of stem cell creates one stem cell daughter and one TA cell which give rise to nonproliferative, mature effector cells. Mechanism leading to stem cell differentiation and factors responsible for this process have not been fully established. However, two models of epidermal stem cell differentiation have been proposed (Fig. 12.3) [12, 18]. The hierarchical model assumes that along with stem cell divisions the self-renewal potential is limiting, which results in increased differentiation of descendant cells. This model suggests that the number of stem cell clones will remain unchanged during a lifetime, since only the stem cells are long-lived.

Stochastic model implies that the division of one stem cell leads to the creation of one of three separate outcomes. In the first case, stem cell division may create one differentiated cell that departs from the basal layer and one remaining in the basal layer, constantly dividing stem cell daughter. However, two progenitor cells and two differentiated cells may also arise from the division of one stem cell. To the above, stochastic model assumes that the fate choices are random and that the number of stem cell clones will vary in size.

Both presented models suggest maintenance of multipotent epidermal stem cells by self-renewal and generation of more differentiated cells, based on differences in

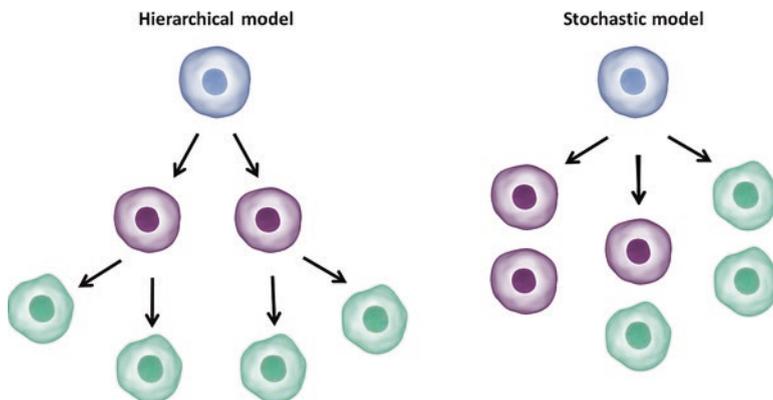


Fig. 12.3 Models of epidermal stem cell differentiation

the stem cell division. However, latest data highlight the importance of intra- and extracellular cues in the process of stem cell differentiation [12, 19–21]. The following subchapters will discuss the influence of signalling pathways on the differentiation of epidermal stem cell lineages.

Hierarchical model of epidermal stem cell (blue) differentiation assumes that rare stem cell divisions generate rapidly dividing more differentiated, transit-amplifying cell (purple), which then give rise to differentiated cells (green). Stochastic model of differentiation proposes that division of one epidermal stem cell leads to the creation of one of three separate outcomes: two transit-amplifying cells, one transit-amplifying cell, and one differentiated cell or two differentiated cells.

12.3 Interfollicular Epidermis Stem Cells

The interfollicular epidermis is a multilayered epithelium constantly renewed throughout life. It provides the first line of defence against pathogens or environmental toxins as well as retains fluids in the body.

A highly organised structure of interfollicular epidermis provides constant access to newly formed differentiated cells, called keratinocytes (Fig. 12.1). This process takes place in several stages. Proliferation of stem cells and TA cells takes place within the basal layer (stratum basale). Detaching of cells from basal layer initiates terminal differentiation program of their progeny, directing cells gradually towards the three, upper, distinct layers of the epidermis: stratum spinosum, stratum granulosum, and finally stratum corneum. In the last stage, keratinocytes lose their nuclei and become flattened, what makes them easier to shed from the surface of the skin. The column, located between stratum basale and corneum, formed by stem cell/TA cells and keratinocytes at various stages of differentiation is known as epidermal proliferative unit (EPU) [22]. The EPU confirms hierarchical model of stem cell

differentiation wherein basal layer provides microenvironment with all required signals for stem cell renewal and/or activation and proliferation to create a distinct spatial unit in tissue, ensuring permanent renewal of the epidermis. Lineage-tracing experiments on mice and spatial differences in expression levels of stem cell markers also confirmed the existence of the hierarchy of different progenitor populations with distinct properties, allowing to distinguish interfollicular stem cells from their progeny [23]. While undifferentiated basal stem cells express keratins K5 and K14, spinous and granular cells express keratins K1 and K10 and involucrin. In turn, differences in b1-integrin expression level allow to distinguish epidermal cells based on their clonogenicity [23]. In addition to those mentioned above, several molecules with high expression such as a6 integrin, notch signalling ligand Delta1, CD200, melanoma-associated chondroitin sulphate proteoglycan, and Lrig1 (leucine-rich repeats and immunoglobulin-like domains 1) as well as low expression of CD71 can be specified as markers of interfollicular epidermis stem cells [23–28].

The literature reports contradictory data in the context of the regenerative capacity of interfollicular stem cells. While some reports indicate that stem cells of the basal layer do not regenerate lost hair follicles [29, 30], other groups give evidences about the regenerative capacity of interfollicular stem cells to form hair follicle with stem cells capable of clonal growth and K15 and CD34 expression [31, 32]. Despite these discrepancies, current knowledge suggests that epidermal stem cell's regenerative capacity may be functionally interconvertible as a result of changes in the niche microenvironment and/or cell-cell interactions. These assumptions were confirmed by experiments showing that dermal papilla affecting interfollicular epidermal stem cells induces their differentiation towards formation of hair follicles and sebaceous glands [33].

12.3.1 Molecular Mechanisms Responsible for Interfollicular Stem Cell Maintenance, Proliferation, and Differentiation

Till now the molecular mechanisms responsible for stem cell quiescent, proliferation, and differentiation have not been fully established. However, the complexity of their regulation can be compared to spider's web, in which different pathways intersect in many different ways, and touching the web at any one point will impact on multiple threads. It is suggested that self-renewing stem cell might change their fate, both due to intrinsic events, such as DNA damage or telomere shortening, and as a result of niche-dependent extrinsic signals. Thus, constellation of all intrinsic and extrinsic signals regulates the balance of self-renewal and differentiation in epidermal stem cells (Fig. 12.4) [2, 5].

To this day, very little is known about the mechanism and essential signalling factors regulating interfollicular stem cell maintenance. However, it is suggested that quiescence might be a result of inhibition or blockade of extrinsic signals

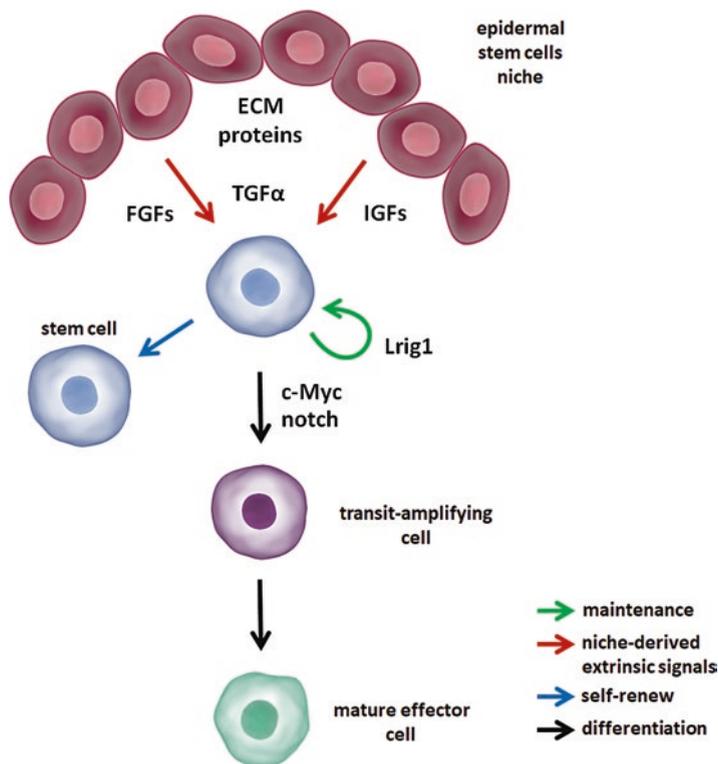


Fig. 12.4 Model of molecular mechanisms responsible for interfollicular stem cell maintenance, proliferation, and differentiation

coming from niche microenvironment. Accordingly, recent data point on Lrig1 (leucine-rich repeats and immunoglobulin-like domains 1) as a potential factor associated in regulation of interfollicular stem cell quiescent [27, 28]. Overexpression of Lrig1 via decreased epidermal growth factor (EGF) receptor levels results in reduced proliferation maintaining interfollicular stem cells in nondividing state.

Interfollicular stem cell proliferation might be regulated via several mechanisms based on niche-dependent extrinsic signals, differences in cell adhesion, or circadian rhythm. Epithelial stem cells are connected with underlying basement membrane via $\alpha 3\beta 1$ and $\alpha 6\beta 4$ integrin/laminin-5 interactions. The closeness of these layers causes interactions between epithelium and dermis. And so, dermal fibroblasts secreting a number of mitogens such as fibroblast growth factor-7 (FGF-7), FGF-10, insulin-like growth factors (IGFs), and EGF ligands and transforming growth factor- α (TGF- α) promote the proliferation of epidermal stem cells localised in stratum basale [19–21, 34, 35]. Experiments on mouse models confirmed these assumptions indicating that epidermis lacking IGF1R receptor has impaired basal epidermal proliferation [21]. Moreover, ectopic expression of FGF-7 in epidermal cells induces their hyperproliferation [34]. The same phenomenon is observed after

excessive TGF- α activation or Mig6 deletion, factors responsible for regulation of EGF receptor (EGFR) signalling [35, 36]. Also in human, reduction of expression of EGFR antagonist, Lrig1, results in keratinocyte proliferation in culture [28]. All these data confirm the importance of fibroblast-derived mitogens in stem cell proliferation.

It is suggested that extracellular matrix (ECM) proteins play a key role as niche components, allowing on adhesion of stem cells to their niche and increasing their proliferative capacity. Indeed, impairment of β 1, β 4, and α 6 integrins or their ECM ligand laminin-5 in mice compromises membrane proliferation or results in skin blistering, respectively [12, 37]. Furthermore, in human stratum basale, increased expression of β 1 integrin in a slow-cycling population of cells with a high colony-forming capacity was observed, confirming the role of ECM proteins also as a stem cell marker [38]. Expression of ECM proteins, α 6 or β 1 integrins, in human keratinocytes can be regulated via epigenetic factors such as histone methyltransferase EZH2 [39]. Hence, it is possible that epigenetic modifications altering the attachment of basal cells to basement membrane regulate epidermal stem cell proliferation and differentiation [40, 41]. Confirming this hypothesis, genetic studies demonstrate essential role of EZH1 and EZH2 methyltransferases and JMJD3 demethylase in epidermal homeostasis and differentiation, respectively [42, 43].

The behaviour of epidermal stem cell may be also regulated by circadian rhythms. In mice, increased epidermal stem cell proliferation is observed during night and correlates with decreased level of reactive oxygen species. These observations can be explained by the fact that inhibition of daytime proliferation protects against UV- and free-radical-dependent DNA damage. Accordingly, deregulation of expression of core clock genes (e.g. Arntl1) induces proliferation and differentiation during daytime impairing stem cell colony-forming efficiency [44].

The interfollicular stem cell differentiation involves two fairly well-understood mechanisms: first, in which cells lose their attachment to the basement membrane in consequence moving upwards called delamination or, second, already-mentioned asymmetric division resulting in suprabasal progeny and proliferative basal cell formation.

The difference in cellular adhesiveness resulting from the clustering and motility variables between stem cells and their progenies may be a consequence of changes in expression of integrins in these cells. It was indicated that stem cells have higher level of integrin expression than TA cells, which are less cohesive and more motile. This phenomenon is a result of c-Myc activation that stimulates proliferation and differentiation of interfollicular stem cells via their exit from the epidermal stem cell niche, by repression of cell adhesion genes, including the α 6 and β 1 integrin subunits [45]. Moreover, it was indicated that c-Myc stimulates TA cell division by increased expression of RNA methyltransferase, Misu (NSun2), since knockdown of Misu blocks Myc-dependent keratinocyte proliferation.

Recent data highlights the role of composition of the underlying basement membrane that may influence on cell motility. Greater delamination of interfollicular stem cells as well as their asymmetric division requires Notch signalling. Cell transition from basal to spinous layer is possible via ligand-receptor interactions between

located basally Jagged and expressed suprabasally receptors Notch1, Notch2, and Notch3 [46]. In consequence, γ -secretase-dependent Notch cleavage occurs resulting in release of intracellular domains (NICD1, NICD2, and NICD3) [47]. Finally, binding of nuclear NICD domains to transcription factors regulates the expression of target genes responsible for delamination process. Indeed, recent data indicates that constitutive expression of Notch signalling in the basal epidermis results in reduced integrin expression, detachment of epidermis from underlying dermis, and expansion of spinous layers [48]. What is more, activation of the Notch pathway plays an important role in the asymmetric division of basal stem cells. Impairment of core components of asymmetric division prevents Notch-dependent spinous cells formation, thus confirming the key role of this signalling pathway in maintaining balance between stem cell self-renew and differentiation.

To sum up, multidirectional interactions between an interfollicular stem cell, dermal cells, and the underlying basement membrane play a pivotal role in maintenance, proliferation, and differentiation of this epidermal stem cell layer.

Epidermal stem cell niche provides appropriate microenvironment for stem cell maintenance or proliferation. Dermal fibroblasts secrete a number of mitogens such as fibroblast growth factor-7 (FGF-7), FGF-10, insulin-like growth factors (IGFs), EGF ligands, and transforming growth factor- α (TGF- α) to promote the division of epidermal stem cells localised in the stratum basale. Moreover, interactions between extracellular matrix (ECM) proteins, β 1, β 4, and α 6 integrin, and their ligand laminin-5 allow adhesion of stem cells to their niche and increase their proliferative capacity. Lrig1-dependent interfollicular stem cell quiescence results from inhibition or blockade of extrinsic signals coming from niche microenvironment. Interfollicular stem cell differentiation might be regulated via several mechanisms based on intrinsic signals, differences in cell adhesion, or circadian rhythm.

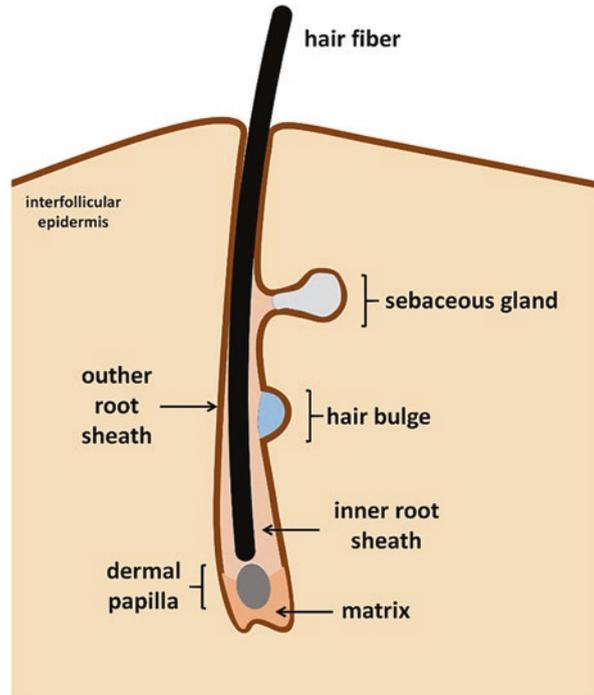
12.4 Hair Follicle Bulge Stem Cells

Hair follicles are an integral part of the epidermis. In addition to generating hairs, they play a pivotal role in thermal regulation and serve as anchors to sensory neurons, arrector pili muscles, and blood vessels.

Hair follicle is a constantly remodelled miniorgan; therefore, in its structure it requires the presence of fully functional epithelial stem cells (Fig. 12.5). Three phases of hair growth could be distinguished – anagen, catagen, and telogen [49]. During the hair growth cycle, epithelial stem cells located within the bulge region migrate to the base of the follicle regenerating hair bulb via new hair matrix and outer root sheath companion layer creation [50, 51]. The next phases of the hair cycle are apoptosis-dependent epithelial regression (catagen) and a subsequent period of relative quiescence (telogen), after which a new anagen hair bulb is regenerated and the old hair is removed.

The first identified adult epidermal stem cells were bulge cells, located between the sebaceous gland and the arrector pili muscle insertion point [52–54].

Fig. 12.5 Organisation of hair follicle



The identity of individual stem cells within the hair bulge and the nature of their niche have not yet been fully determined. It is suggested that stem cells of the bulge region display the greatest *in vitro* growth capacity and clonogenicity compared to cells from other regions of the hair follicle and epidermis. Their lineage-independent multipotent character confirmed experiments in which bulge cells were capable of forming almost all epithelial cell types: hair follicle; hair shaft including the outer root sheath, matrix, medulla, and sebaceous glands [50, 52, 55–59]; as well as nonepithelial cells like neurons or Schwann cells [60, 61]. Interestingly, elegant studies indicate that although bulge stem cells have high regenerative capacity, they are not responsible for maintaining interfollicular epidermis [62]. Consistent with this, Ito group have shown that depletion of bulge cells in mouse model results in loss of hair follicle structures but not of the interfollicular epidermis [59], confirming the hypothesis about separate origin of these two types of epithelial stem cells.

The epidermis is punctuated by hair follicles and glandular structures that are created by hair follicle bulge stem cells and sebaceous stem cells. Stem cells located within the bulge region, at the site of arrector pili muscle insertion, can migrate giving rise to new matrix cells and sebaceous gland. Moreover, these cells participate in epidermal wound healing.

Presumably, remarkable flexibility in generating new adnexal structures in adult epidermis owes distinct populations of self-renewing bulge cells. Accordingly, clonal labelling experiments demonstrated the presence of distinct populations of

self-renewing bulge cells creating individual lineages at the base of the hair follicle as well as distinct clonal organisation along both the proximodistal and radial axes of the follicle [63]. Notwithstanding, it is worth pointing out that regenerative capacity of bulge cells, as well as interfollicular stem cells, may be regulated via changes in the niche microenvironment. Thus, lineages selected by stem cell progeny can be restricted under physiological conditions by local environmental cues, while under pathological conditions they might show their full regenerative potential. Furthermore, cell-cell interactions also play an important role in regenerative capacity of hair follicle. Both the production of the hair shaft in anagen and the initiation of a new hair cycle at telogen are the result of reciprocal interactions between the dermal papilla and the overlying epithelial cells [19].

Due to the high regenerative abilities of bulge stem cells, it is important to identify marker molecules applicable to isolate this epidermal stem cell. It was observed that during differentiation of hair bulge stem cells, the expression of several genes is silencing. While hair follicle stem cells located in basal layer surrounding the telogen club hair as well as anagen bulge region expressed K15 and high levels of b1-integrin [64], differentiated hair matrix keratinocytes concomitantly lose their ability to express K15 and decrease expression of b1-integrin. Hence, expression of K15, b1-integrin, and other proteins as CD200 can act as a reliable marker of hair follicle stem cells [26].

12.4.1 Molecular Mechanisms Responsible for Hair Follicle Bulge Stem Cell Quiescence and Differentiation

Epidermal stem cells must be maintained in their undifferentiated state to provide hair follicle regenerative capacity for the entire duration of the individual's lifetime. This is possible due to very strict regulation of growth and/or differentiation of bulge cells via intercellular signal transduction. In physiological conditions, it is necessary to maintain an environment that inhibits growth of hair follicle bulge; however, when stem cells do not receive appropriate signals for growth and/or differentiation, it results in failure to replenish mature skin tissue or loss of hair. On the other hand, excessive stimulation of epidermal stem cell growth or differentiation of progenitor cells can cause neoplastic formation.

Regulation of growth or differentiation of bulge cells requires interplay between acting oppositely on intercellular signal transduction. Therefore, quiescent stem cells will inhibit intercellular pathways that promote proliferation and differentiation of hair follicle, and vice versa, differentiating hair follicle cells will not activate the quiescence-related pathways. Only ensuring the balance between pro- and anti-growth factors provides optimal conditions for maintaining cells in appropriate state (Fig. 12.6).

Transcriptional profiling of human and mouse hair follicle bulge cells indicated intercellular signal transduction that are pivotal to maintain a growth inhibitory environment for hair follicle bulge cells [30]. Latest data indicated that Wnt signal-

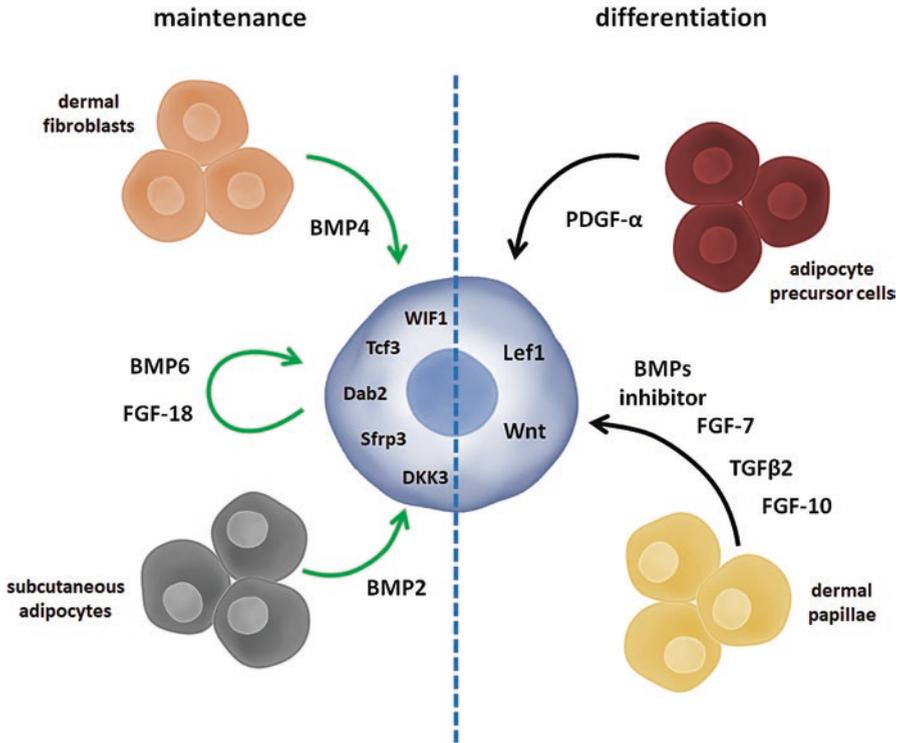


Fig. 12.6 Model of molecular mechanisms responsible for hair follicle bulge stem cell quiescence and differentiation

ling pathway plays an important role in hair follicle morphogenesis, proliferation, as well as hair cycling [65]. Therefore, upregulation of transcripts involved in the inhibition of the Wnt pathway could be one of the ways to ensure quiescent state of hair follicle bulge stem cells [66]. Indeed, two of the most important growth inhibitors that are overrepresented in the human hair follicle bulge encode the Wnt inhibitors – Wnt inhibitory factor-1 (WIF1) and Dickkopf-3 (DKK3), while in mouse they are DKK3, Disabled-2 (Dab2), and secreted frizzled-related protein-1 (Sfrp1) [65, 67–69]. Also overexpression of lymphocyte enhancer factor-1 (Lef-1) induces abnormal hair follicle formation both in interfollicular and oral epithelium; thus, silencing of this gene restrains the formation of mature hair precursors during embryogenesis [70, 71]. Furthermore, it was demonstrated that regulation of epithelial stem cell differentiation is negatively controlled by transcription factor-3 (Tcf-3) [72]. It is suggested that Tcf-3-dependent activation of genes related to quiescent state promotes a transcriptional program shared by embryonic and postnatal stem cells [72]. Quiescent epidermal stem cell phenotype is also supported by factors secreted from stem cells progeny and dermal cells. The influence of secreted factors

is particularly visible during telogen, when bulge cells produce BMP6 and FGF-18 while dermal fibroblasts and subcutaneous adipocytes BMP4 and BMP2, respectively [26, 30, 73–75]. In turn, at the anagen stage, expression of quiescent factors decreases giving way to dermal cell-dependent activating factors and in consequence hair follicle stem cell proliferation. Here, dermal papillae express BMP inhibitor noggin (NOG), FGF-7, FGF-10, and tumour growth factor β 2 (TGF β 2), and adipocyte precursor cells secrete platelet-derived growth factor α (PDGF- α) [26, 30].

Hair follicle stem cells are maintained in quiescent state for most of the hair cycle and only proliferate in early anagen. The Wnt/ β -catenin signalling pathway is responsible for the proliferation of hair follicular stem cells. It plays an important role in hair follicle morphogenesis as well as hair cycling [65]. The level of β -catenin activation, the key effector of Wnt signalling, is important in regulating hair follicle growth and controls differentiation of epidermal stem cell lineages [33, 65, 66, 76]. This protein is accumulated in activated hair germ; hence, without β -catenin, hair follicle is arrested in telogen. High level of β -catenin activation induces hair follicle differentiation, whereas inhibition of its activation favours interfollicular epidermal distinction. This phenomenon may be a result of β -catenin-dependent upregulation of Sonic hedgehog (Shh) signalling, a potent mitogen secreted by hair matrix that is essential for hair growth and morphogenesis [78]. Latest data indicate that moderate activation of β -catenin combined with Shh inhibition blocks de novo hair follicle formation, while inhibition of Shh signalling in response to a high level of β -catenin activation improves hair follicle morphogenesis [78].

Depletion of circadian clock proteins in mice results in delaying anagen entry, while their knockout has only a mild effect on hair cycle progression. These observations, together with available data indicating that expression of several circadian clock genes fluctuates throughout the day in bulge stem cells, matrix, and dermal papillae, suggest that circadian rhythms may influence in yet unrecognised manner on hair follicles [79–81].

Regulation of growth or differentiation of bulge cells requires interplay between acting oppositely on intercellular signal transduction. Quiescent epidermal stem cell phenotype is supported by factors secreted from stem cell progeny (BMP6, FGF-18), dermal fibroblasts (BMP4), and subcutaneous adipocytes (BMP2). Upregulation of transcripts involved in the inhibition of the Wnt pathway, such as Wnt inhibitory factor-1 (WIF1) and Dickkopf-3 (DKK3) or Disabled-2 (Dab2) and frizzled-related protein-1 (Sfrp1) in human and mouse, respectively, could be a one of the ways to ensure quiescent state of hair follicle bulge stem cells. Regulation of epithelial stem cell differentiation is also negatively controlled by transcription factor-3 (Tcf-3). Hair follicle bulge stem cell differentiation depends on intercellular Wnt signalling pathway and extrinsic signals from adipocyte precursors and dermal papillae cells. Wnt signalling pathway induces hair follicle morphogenesis, proliferation, as well as hair cycling, while dermal papillae express BMP inhibitor noggin (NOG), FGF-7, FGF-10, and tumour growth factor β 2 (TGF β 2), and adipocyte precursor cells secrete platelet-derived growth factor α (PDGF- α).

12.5 Sebaceous Gland Stem Cells

Sebaceous glands together with sebaceous duct are located above the hair follicle bulge and play an important role in thermoregulation of the body. Moreover, by secreting sebum they provide hydration for the skin and its waterproofing, as well as protect the body against microorganisms.

The development of the sebaceous glands is related to the differentiation of hair follicles and epidermis [82]. When stratum intermedium becomes apparent (10–12 week of foetal life), hair germs are developed extending downwards into the dermis, while sebaceous gland rudiments appear on the posterior hair peg surface. At a later stage of development, sebaceous glands arise from hair follicles in a cephalocaudal direction, finally creating cells that are identical to those located in stratum basale or hair follicular bulge. Although the sebaceous gland stem cells are the least characterised among the different populations of epidermal lineages, latest data indicate their pivotal role in the continuous regeneration of sebocytes that form the sebaceous gland and secrete sebum [66, 83–86]. The peripheral layer of the sebaceous gland is covered by stem cells that express K14 [87]. The maturation of this line takes place towards the centre of the sebaceous gland. Differentiation of sebaceous gland stem cells results in enhanced synthesis and accumulation of lipids that leads to increased cell volume. In the next step, sebocytes undergo apoptosis, lyse, and release their entire content into the sebaceous duct and then on the skin's surface (Fig. 12.7).

Sebaceous gland stem cells and cells located in the hair follicle, in response to injury, can generate differentiated epithelial lineages [55, 57, 58, 88]. So far, the regenerative capacity of sebaceous gland stem cells is still under investigation. It is suggested that regenerative abilities of sebaceous gland stem cells may result from interdependency with hair follicles. Consistent with this, when one organ collapses, the other is also often lost [89]. However, other data suggest that sebaceous gland stem cells can be marked specifically over multiple hair cycles, indicating that

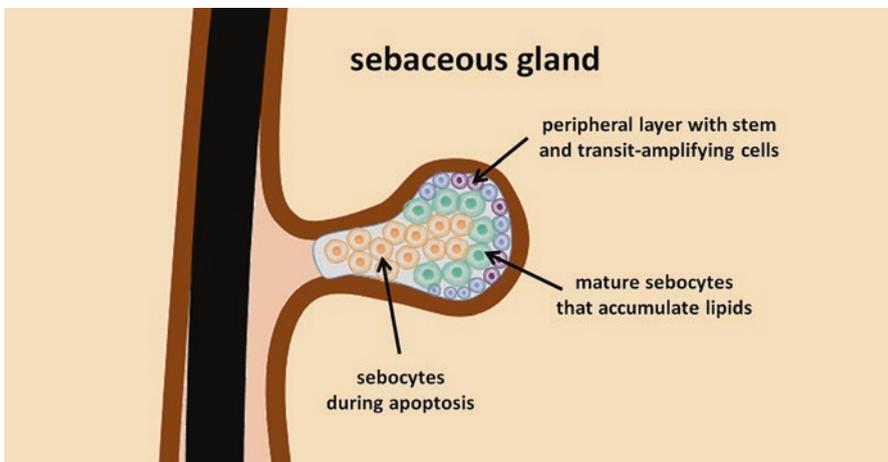


Fig. 12.7 Model of sebaceous gland stem cell differentiation

population of self-renewing cells maintain the sebaceous gland of the hair follicle bulge independently [86, 90].

Sebaceous glands arise from hair follicles in a cephalocaudal direction, finally creating cells that are identical to those located in stratum basale or hair follicular bulge. The peripheral layer of the sebaceous gland is covered by stem and transit-amplifying sebaceous gland cells. The maturation of sebaceous gland stem cells takes place towards the centre of the sebaceous gland. Differentiation of sebaceous gland stem cells results in increased cell volume via enhanced synthesis and accumulation of lipids. In the next step, sebocytes undergo apoptosis, lyse, and release their entire content into the sebaceous duct.

12.5.1 Molecular Mechanisms Responsible for Sebaceous Gland Stem Cell Proliferation and Differentiation

Till now the molecular mechanisms responsible for proliferation and differentiation of sebaceous gland stem cells are not fully established; however, latest data suggest that factors such as c-Myc, hedgehog signalling, B-lymphocyte-induced maturation protein-1 (Blimp1), β -catenin, and dominant-negative mutant form of Lef1 (DNLeF1) may play a pivotal role in this process (Fig. 12.8) [77, 88].

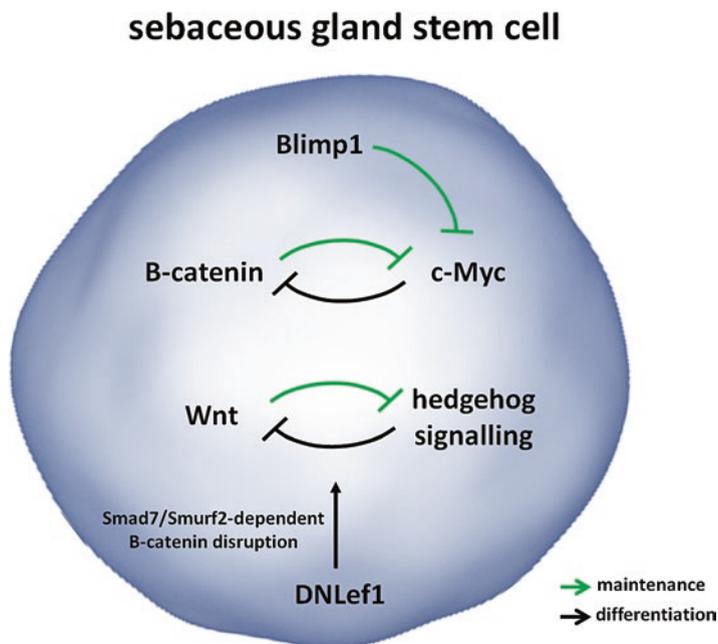


Fig. 12.8 Model of molecular mechanisms responsible for sebaceous gland stem cell proliferation and differentiation

Experiments on mouse models and cell culture systems revealed that c-Myc and β -catenin exert opposing effects on sebocyte proliferation and differentiation. While expression of c-Myc inhibits hair follicle formation [57] favouring differentiation of stem cell lineages to the interfollicular epidermis and sebaceous gland [90], β -catenin reduces sebocyte differentiation inducing de novo hair follicle morphogenesis [57, 66, 85].

Moreover, it is suggested that transcriptional repressor Blimp1 by negative regulation of c-Myc affects sebaceous gland stem cell distinction. Thus, it may act as a marker of small cluster of sebaceous gland progenitor cells that localise at the base of the glands, as well as differentiating cells in the interfollicular epidermis and hair follicle [57, 91].

Obviously, the involvement of hedgehog signalling in sebocyte proliferation cannot be underestimated. Consistent with this, constant activation of hedgehog signalling results in excessive ectopic sebocyte development, while inhibition of this important signalling pathway suppresses sebocyte proliferation [88].

Similar to hair follicle bulge stem cells, it is suggested that inhibition of Wnt signalling may be required for lineage specification of sebaceous gland stem cells. DNLEF1 may be responsible for this process on several ways. First, DNLEF1-dependent decrease of Wnt signalling stimulates sebocyte and interfollicular epidermal differentiation, simultaneously suppressing hair follicle differentiation [83]. Second, it was indicated that DNLEF1 via Smad7/Smurf2-dependent disruption of β -catenin signalling results in sebaceous gland hyperplasia [92]. Furthermore, DNLEF1 regulates proper sebaceous development and sebocyte proliferation by providing a delicate balance between Wnt and hedgehog signalling [83]. The importance of DNLEF1 in sebaceous gland stem cell lineage specification confirmed the report that mutations of human DNLEF1 in mice and human result in sebocyte tumorigenesis [93].

Blimp1- and β -catenin-dependent inhibition of c-Myc, as well as Wnt-dependent repression of hedgehog signalling, is responsible for sebaceous gland stem cell quiescence. Sebaceous gland stem cell differentiation is maintained by DNLEF1 via Smad7/Smurf2-dependent disruption of β -catenin signalling providing a delicate balance between Wnt and hedgehog signalling.

12.6 Epidermal Stem Cells in Skin Regeneration

Skin regeneration requires rapid response of epidermal stem cells, to restore compromised barrier and repair skin damage. There are three phases of wound healing, including inflammation, tissue formation, and tissue remodelling [94–96]. Inflammation created by injury leads to platelet aggregation and recruitment of innate immunity cells that clear the wound site from dead cells and pathogens [97]. Moreover, recruited leukocytes including neutrophils, macrophages, mast cells, and T cells via secreted cytokines and growth factors stimulate healing processes [98]. Angiogenesis and migration and proliferation of keratinocytes and dermal fibroblasts occur due to TGF- β , IGF, and FGF secretion. These factors stimulate

synthesis of ECMs and if necessary generate new hair follicles. Tissue formation is possible due to covering the wound by macrophages and granulation tissue over which epidermal stem cells reepithelialised the wound. Moreover, tissue formation requires activation of adipocyte precursor cells that generate mature adipocytes important for fibroblast recruitment [95, 99]. In the last phase, tissue remodelling is a result of epidermis and dermal fibroblast ECM protein deposition that strengthens the repaired tissue.

Nowadays, the usage of cultured human keratinocytes in the treatment of burns is a standard [100–103]. However, it is worth noting that despite the great success of autologous transplants that provide epidermal barrier protection, they do not generate appendages, hair follicles, and sebaceous glands that are necessary for the proper functioning of the epidermis. In light of the above, great importance should be attached to strategies aimed at inducing mentioned appendages. Interestingly, recent data suggests that hair follicle can be generated from neonatal dermis-derived fibroblasts [104]. Moreover, induction of epidermis Wnt signalling together with mesenchymal BMP inhibition may also trigger hair follicle formation. In turn, recent data indicate that sebaceous gland, as well as hair follicle and epidermis, can be regenerated from adult hair shaft stem cells transplanted into immunocompromised mice.

All these data confirmed that although the potential and behaviour of epidermal stem cells are under control of a number of niche-dependent signalling pathways, there are not definitively established during life. As a result of epidermis regeneration, changes in the microenvironment of the niche occur releasing the full potential of stem cells. Understanding of regulatory signals within the niche and their involvement in epidermal stem cell behaviour.

allow the discovery of new therapeutic strategies for regenerative medicine.

12.7 Conclusion

Current knowledge indicates that the key effect on the regulation of epidermal stem cell fate is their niches and local microenvironment. The past years have brought rapid progress in revealing the molecular basis of these interactions; however, with each new report, the complexity of regulation of quiescence, proliferation, as well as differentiation of epidermal stem cells is even more puzzling. Although much remains to be investigated, both self-renewal and multilineage differentiation of epidermal stem cells cause these cells to be attractive for studying as well as broadly understood as regenerative medicine. Comprehending on biological processes that promote stem cell's self-renewal and senescence may help to develop new anticancer therapeutic strategies, since tumorigenic cancer stem cells often have phenotypic and functional characteristics similar to normal stem cells in the same tissue. Moreover, examination of age-related changes responsible for the decline of epidermal regenerative potential can be a milestone in understanding the regulation processes and will allow manipulating stem cell behaviour during the treatment of wound healing, loss of skin plasticity, or wrinkling.

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Chapter 13

Stem Cells in Lungs



Andrzej Ciechanowicz

Abstract The respiratory system plays an essential role for human life. This system (like all others) undergoes physiological regeneration due to many types of stem cells found both in the respiratory tract itself and in the alveoli. The stem cell hierarchy is very extensive due to their variety in the lungs and is still not completely understood.

The best described lung stem cells are alveolar type II cells, which as progenitor lung stem cells are precursors of alveolar type I cells, i.e., cells that perform gas exchange in the lungs. These progenitor stem cells, which reside in alveoli corners, express high levels of surfactant protein C (SFTPC). Despite the fact that type II pneumocytes occupy only 7–10% of the lung surface, there are almost twice as many as alveolar type I cells occupying almost 95% of the surface.

Other stem cells making up the lung regenerative potential have also been identified in the lungs. Both endothelial, mesodermal, and epithelial stem cells are necessary for the lungs to function properly and perform their physiological functions.

The lungs, like all other organs, undergo an aging process. As a result of this process, not only the total number of cells changes, the percentage of particular types of cells, but also their efficiency is reduced. With age, the proliferative potential of lung stem cells also decreases, not just their number. This brings about the need to increase the intensity of research in the field of regenerative medicine.

Keywords Alveolar type II cells · Type II pneumocytes · Bronchoalveolar lung stem cells · Club cells · Lung 3D organoid culture · Lung regeneration · Lung aging · Lung development · Lung stem cells · Respiratory system

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13.1 Introduction

A crucial condition for the survival of every living cell (with the exception of anaerobic bacteria) is a constant supply of oxygen. The human body can survive several weeks without the supply of energy components, a few days without water, but only a few minutes without delivery of oxygen contained in the air. Thus, unlike chemical reserves (proteins, lipids, or carbohydrates) and water, which is the main component of cells and circulating fluids, oxygen reserves are so small that the supply, transport, and exchange of this gas must be carried out continuously. The lungs are the main organ of the respiratory system and function to exchange gas, taking in oxygen and expelling carbon dioxide [1].

13.1.1 *Respiratory System Macroscopic Structure*

Air enters the nasal passages and passes down the trachea and successively finer branches of the conducting airways (bronchioles) until it reaches the alveolar network where gas exchange occurs [2]. The lung is an organ consisting of the right lung and the left lung. The right lung consists of three lobes (i.e., upper, middle, and lower). The left lung consists of two lobes (i.e., upper and lower). In each lobe, there is a further separation of the lung structure through the connective tissue on the so-called lobules. Air enters every lobe through the terminal bronchioles, which are further separated into bronchioles, alveolar ducts up to the alveoli (their number is estimated at about 750 million in humans).

13.1.2 *Airways and Lung Microscopic Structure*

The mammalian lung is comprised of proximal and distal compartments that form two spatially, structurally, and functionally distinct anatomical regions [3, 4]. The proximal compartment is a “tree” of progressively branching airway tubules that accomplish ventilation, transmitting air into and out of the distal compartment, a densely packed aggregate of thin-walled air sacs termed “alveoli” where gas exchange take place. Each of the alveoli consists of a thin layer of epithelial cells exposed on the one hand to atmospheric air and on the other hand to a dense network of capillary blood vessels. The alveolar compartment has a seamless honeycomb-like pattern on cross-section, which is remarkable given that it represents a packing together of alveoli emanating in clusters from different terminal bronchioles like grapes of a stalk [3]. Because of this seamless appearance and the incredible density of alveoli, it is difficult to distinguish where one cluster (acinus) ends and another begins. As a result, neither the absolute nor relative position of a given alveolar sac along the proximo-distal axis can be accurately inferred [3].

Despite the high complexity of the distal structure of the lungs, the surface of the alveoli consists mainly of only two types of cells that simultaneously fulfill the key functions for the survival of the body.

The tracheobronchial airways are composed predominantly of ciliated, Club, and basal cells as well as lesser number of goblet and neuroendocrine cells. Submucosal glands are restricted to the highest reaches of the cartilaginous tracheal airway. In the distal lung, the bronchiolar epithelium is composed largely of Club and ciliated cells with intermittent clusters of neuroendocrine cells, whereas type I and type II alveolar epithelial cells make up the gas exchange surface of the alveoli [5] (Fig. 13.2).

13.2 Stem Cells of Respiratory System

One definition of regeneration is the ability of an organ to regrow a fully functional replacement following catastrophic loss. Lungs are slow-turnover organs that are highly quiescent at steady state. However, the lungs have a tremendous ability to repair epithelial damage following acute injury and contain multiple, highly plastic, stem cell populations [2].

Over the past few years, significant advances have been made in understanding the processes that generate lung progenitors and regulate lung development and differentiation. Constantly improved research techniques have allowed for a sophisticated approach to genetic manipulation, line tracking, genome screening, and imaging. Thanks to the knowledge gained from these studies, the role of particular signaling pathways was better understood, which translated into a better understanding of the pathogenesis of lung diseases and how these pathways affect the regeneration of injury.

Deepened knowledge about the signaling pathways of various pathological processes gave rise to the development of new cellular therapies. This has opened up a whole new field of research and new perspectives for the potential use of cellular therapies in tissue engineering and regenerative medicine in lung diseases.

13.2.1 Diversity of Respiratory System Stem Cells

In order to accommodate diverse functions, the human lung possesses several specialized cell types [1]. Adult stem cells (ASCs), in contrast to embryonic stem cells (ESCs), have increasing degrees of fate restriction. ASCs are also called somatic stem cells and differentiate into limited range of cell lines (i.e., multipotent) or can generate only one cell line (i.e., unipotent) [6]. The processes of repair and regeneration of adult tissues after various types of lesions or during growth are performed by a pool of ASCs found in almost all adult organs. In tissues with a high proliferative potential, such as, bone marrow, ASCs are organized according to a classical,

established hierarchy, producing terminally differentiated cells [7]. However, in tissues with a significantly lower proliferative potential, such as lung tissue, there is no classical hierarchy of stem cells. In the nonclassical hierarchy, ASCs do not participate in the normal renewal of lung tissue but are activated in the case of general tissue damage or depletion of lung progenitor cells [3, 5] (Fig. 13.1).

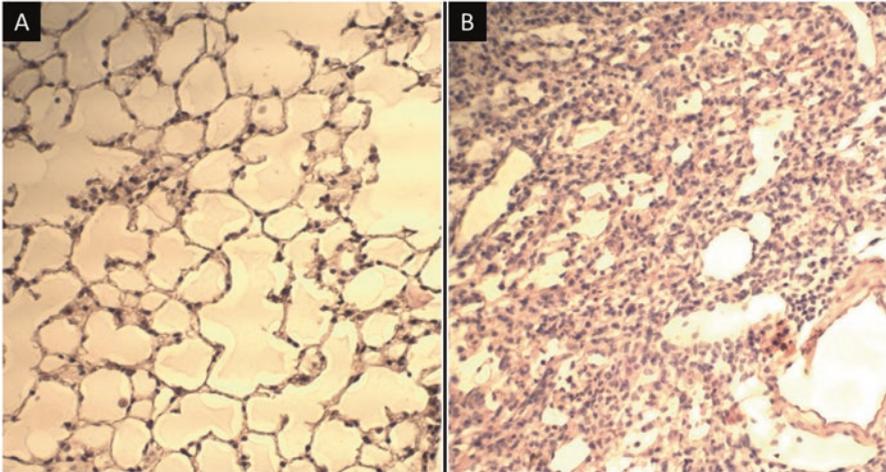


Fig. 13.1 Photograph of lungs of wild-type mice stained by H & E technique. Panel **A** shows a photograph of healthy lungs. Panel **B** shows a photograph of the lungs that were fibrotic in response to the lung damage factor (bleomycin)

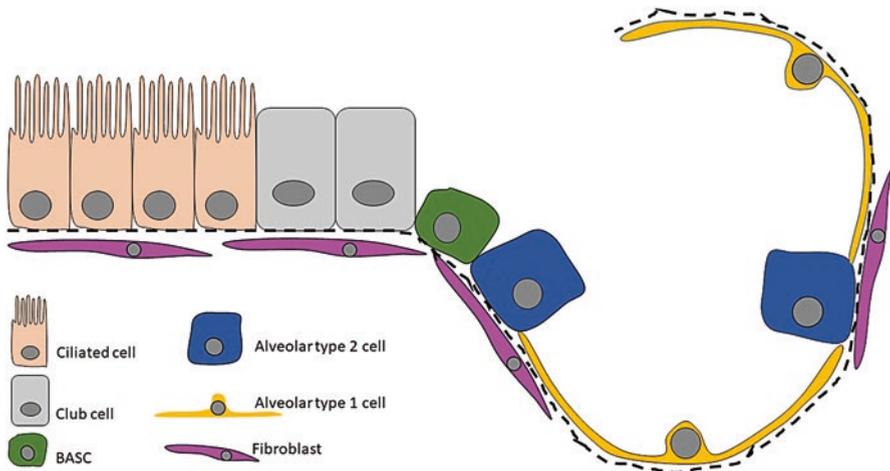


Fig. 13.2 The simplified diagram shows the distribution of major types of lung cells in the distal airway and the pulmonary alveolar

In the complex microscopic structure of the lungs, there are many niches that are colonized by epithelial progenitor cells. The type of cells within each niche, as well as their location, is a matter of dispute among many scientists. The reason for this is their very large diversity, described in many studies [8–19]. It has been shown that in fully developed lungs, there are populations of multipotent endogenous stem cells. These cells are located in niches which microenvironment regulates their proliferation and differentiation potential in the injured organ. Despite the different laboratory methods used to characterize pulmonary stem cells, the combination of genetic techniques with lineage tracing is most often used as the smallest probability of error.

Depending on the specificity of a given niche in the distal lung, there are different cell populations and their precursors in them.

13.2.1.1 Lung Endothelial Progenitor Cells

Pulmonary endothelial progenitor cells (EPCs) are a population of resident in lungs and circulating progenitor vascular cells [20]. These cells can be described by a phenotype CD31+, CD34+, CD45-, CD144+, and CD309+. EPCs express also endothelial nitric oxide synthase and von Willebrand factor [20]. These two populations of EPCs do not have well-described markers that can definitely distinguish them from each other. As a result, it is difficult to determine the physiological functions of each of these populations and their role in the reconstruction of damaged tissue. Additional difficulties in identifying the scope of their regenerative potential are caused by the fact that these cells are very few [21]. Despite this, studies have been described that prove both their direct proliferative potential in lung endothelial regeneration processes as well as their paracrine interaction using exosomes [20, 22–24]. Some authors also claim that these cells might have a protective role in chronic obstructive pulmonary disease (COPD) by alleviating inflammatory infiltration, inhibiting proteolytic enzyme activity, decelerating apoptosis, and improving antioxidant activity [25].

13.2.1.2 Alveolar Type II Cells

Each alveolus contains cuboidal type 2 epithelial cells (AEC2) called also alveolar type 2 cells (AT2) or type II pneumocytes, which reside in alveoli corners, expressing high levels of surfactant protein C (SFTPC) [26]. Type II pneumocytes occupy around 7–10% of the alveolar surface area SFTPC-positive AT2s function as progenitor cells and have key role in mediating epithelial repair in the alveoli [27]. Type II pneumocytes can differentiate into AT1 cells or divide and proliferate into new AT2 cells (self-renewal). In the resting state, type II cells are quiescent, with a normal turnover time of 2–3 weeks [28]. Most scientists claim that type I pneumocytes are completely differentiated because there is little evidence that type I pneumocytes

can divide. In case of the alveolus injury, AT2 cells start intense division processes, covering the damaged epithelium with AT1 cells [4, 28–32].

Despite the fact that type II pneumocytes occupy only 7–10% of the lung surface, there are almost twice as many as alveolar type I cells occupying almost 95% of the surface [27, 33]. Moreover, AT2 cells are a source of pulmonary surfactant that is necessary for proper lung function [34]. Insufficient amount of pulmonary surfactant, which sometimes occurs in premature newborns, can lead to neonatal respiratory distress syndrome (RDS), which until recently was one of the main causes of premature neonatal deaths [34, 35].

13.2.1.3 Alveolar Type I Cells

Alveolar type 1 (AT1) cells are somatic cells that do not display any stem cell characteristics. According to the different versions of the lung stem cell hierarchy presented in the literature, they can be derived from many different types of cells. However, by far the most evidence is provided by the hypothesis that they arise primarily from AT2 cells, but also (rarely) from BASCs.

While AT2 cells produce lung surfactant proteins to reduce the surface tension of alveoli, AT1 squamous cells basically constitute a barrier between blood and lung air space. They form a tight epithelium sealed by protein intercellular connections, that is, tight junction complexes. AT1 represents more than the normal part of the air-blood barrier that contains the epithelium, endothelium, and their complex basement membranes. These cells regulate and control the fluid homeostasis in the alveolar wall, and especially AT1 contain various ionic and aqueous channels and tight junction proteins in its cell membrane. This affects the active protection of the body against external factors, such as inhaled toxins, molecules, or microorganisms. While paracellular transport is thus limited, the transport of solutes occurs mainly via transcellular pathways, that is, by passive diffusion or by active transport carried out by the transporter proteins [36–43].

13.2.1.4 Bronchoalveolar Stem Cells

Bronchoalveolar stem cells (BASCs) are the cells of the borderline of the airways and the alveoli. Bronchoalveolar stem cells are located in the niche BADC (bronchioalveolar duct junctions) and are characterized by bronchiolar markers as well as alveolar markers. BASCs may give rise to an alveolar type II cells, alveolar type I cells, and Club cells (Fig. 13.3). They proliferate in response to damage to both the airways and the alveoli. BASCs have been identified in mice and have not yet been confirmed in humans [44]. Stimulation of BASCs by airway injury resulted in an increase in tumor number and size, further implicating BASCs in tumorigenesis [44].

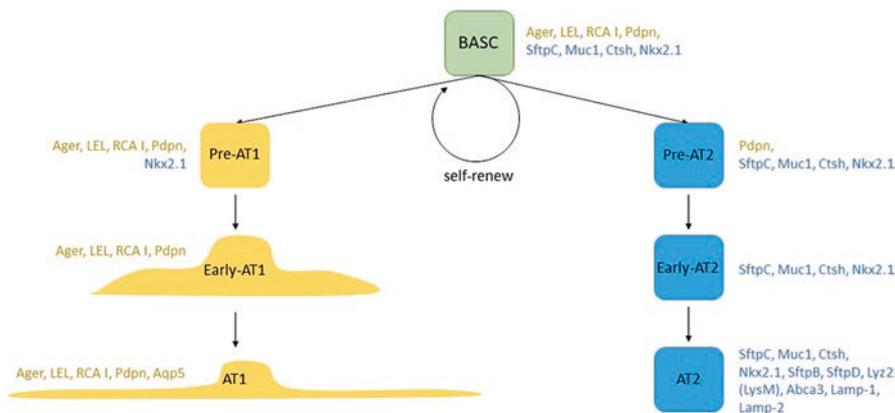


Fig. 13.3 The diagram presents the possibility of bronchoalveolar stem cell differentiation. The figure also presents the characteristic marker proteins characteristic of each of the descendant lines

13.2.1.5 Club Cells

In small mouse airway, Club cells are stem cells and capable of long-term self-renewal to generate new ciliated cells [2]. Club cells (previously called Clara cells) are located in the epithelium of the distal airways. Club cells settle BADJ, NEB (neuroendocrine bodies), and PSMCs (parabronchial smooth muscle cells) niches [45–47]. The molecular mechanisms that control steady-state Club cell turnover are not well defined [2]. Airway Club cells can be specifically depleted by systemic administration of naphthalene which they convert to cytotoxic metabolites via Cytochrome p450 (Cyp2f2) [2]. Cyp2f2⁻ Club cells located at airway branch points and adjacent to neuroendocrine bodies survive naphthalene injury and proliferate to repopulate the bronchial epithelium [2, 48–50]. There is Club cell heterogeneity at steady state, and new evidence suggests that Uroplakin3a⁺ (Upk3a⁺) Club cells, which are somewhat enriched near neuroendocrine bodies, are more likely than the bulk club cell population to self-renew and produce ciliated cells [2, 51, 52].

13.2.1.6 Mesenchymal Stromal Cells

Mesenchymal stromal cells (MSCs) also called marrow stromal cells are mesenchymal tissue-derived multipotent stem cells, with characteristic surface marker phenotype CD13⁺, CD44⁺, CD73⁺, CD90⁺, CD105⁺, CD106⁺, CD124⁺, and CD45⁻ [53]. MSC cells, thanks to the ability to differentiate into different types of mesenchymal cells (including bone, cartilage, and adipose cells) and easily available sources of their acquisition (bone marrow, umbilical cord blood, placenta, Wharton’s jelly, peripheral blood, adipose tissue, and muscle), are a good subject research in the field of regenerative medicine, including lung regenerative medicine. The MSCs do not occupy any specific niches in the lungs, and their distribution in this organ is even in all lung lobes.

Among the MSCs located in the lung, there are three main groups:

1. *Side population (SP)* – This population is characterized by phenotype CD45-, CD11b-, CD34-, CD14-, CD44+, CD90+, CD105+, CD106+, CD73+, and Sca-1+. In addition, high-expression telomerase is observed. SP cells have mesenchymal, vascular, hematopoietic, endothelial, but first of all epithelial potential [54, 55].
2. *Multipotent MSCs* – The population of these cells is characterized by the use of both surface proteins (CD44+, CD73+, CD90+, CD105+, and CD106+) as well as the expression of intracellular proteins (vimentin, ICAM-1, collagen IV, and lamin). Multipotent MSCs have the potential to proliferate towards osteocytes, adipocytes, fibroblasts, chondrocytes, and smooth muscle cells [5, 56].
3. *Fibroblastic progenitor cells* – These cells, which have the potential to differentiate towards fibroblasts, osteocytes, lipofibroblasts, and chondrocytes, are described using CD31+, CD34+, CD144+, CD309+, and CD45- cell membrane proteins as well as intracellular protein groups, including endothelial nitric oxide synthase (ENOS) and von Willebrand factor [5, 56].

13.2.2 Endodermal Origin of the Lung and its Stem Cells

The vast majority of research on lung development is currently carried out on organoid cultures and embryos (in the case of animals). Smith and Cochrane in their oncology research were the first to use the term organoid to indicate a pathological and tumorlike tissue mass formed in a human infant [57, 58]. Since then, this term has been increasingly used in scientific publications as an in vitro research model for organ-like cell cultures. Over time, the definition of the word “organoid” evolved to describe the in vitro 3D structure (Fig. 13.6) derived from single stem cells isolated from the body [58]. The research carried out on organoids was not limited only to the pathophysiology of the systems concerned, but began to develop towards the study of physiology, cell biology, medicine, and pharmacy.

Lung development is predominantly characterized using the mouse model [2]. During lung development in the mouse, progenitors of anterior foregut endoderm undergo directed differentiation to establish distinct respiratory epithelial cell compartments [5]. The beginning of the respiratory system appears already in the third week (in human) of embryonic development in the form of a vague in the ventral wall of the upper digestive tract, from which a few days later two pulmonary buds develop: the right is divided into three bronchi and the left two. Gradually, bronchial buds are divided into more and more generations and at the same time pulmonary circulation develops. By the sixteenth week, approximately 17 bronchial generations are created, taking the trachea as a generation number 0. Then the lungs lose glandular structure, and the surrounding interstitial tissue becomes highly vascularized and gradually form elements of the primary wafer (respiratory), covering the next three generations (from 17 to 19), which are the acute bronchioles in the mature lung. After the 28th week, next three generations of bubbles (20–22) with type I and II

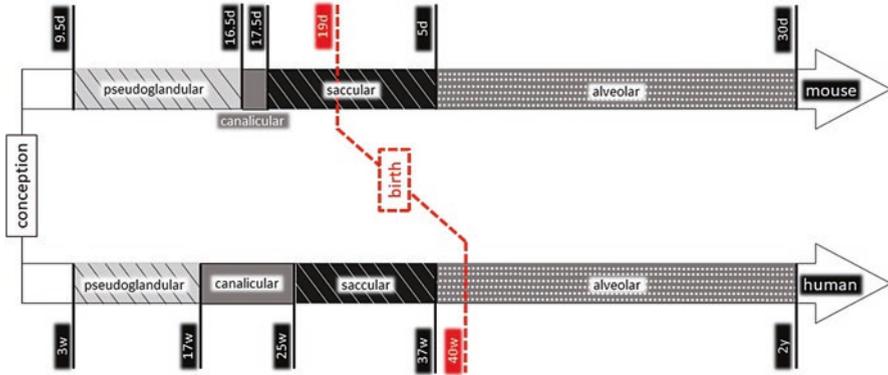


Fig. 13.4 Schematic representation of the order and duration of lung developmental stages in mice and human. Abbreviations: d day, w week, y year

pneumocytes are formed. At birth, the lungs are not yet fully developed and the further formation of bronchioles and vesicles lasts for about two months. More and more bubbles are formed not only in the alveolar ducts but also in bronchioles.

The respiratory progenitor cell specification and overall lung development begin earlier after conception in humans compared to mice. The proportional length of individual stages of lung development (Fig. 13.4) in mice and humans varies; however, the processes taking place in these stages are the same. During the pseudoglandular stage, there is a pulmonary development involving the formation of branching and lung asymmetry. At the end of this phase, the lung with its structure resembles a simple gland, in which individual tubules lined with epithelial cells are separated from each other by a thick layer of mesenchyme.

In the canalicular phase (Fig. 13.5), the branching of the airways ends, the mesenchyme becomes thinner, which leads to approximation between blood vessels and epithelial tubules. During the saccular stage the distal lung expands to form primitive air sacs, and bipotent stem cell differentiate into type I and type II pneumocytes. During the alveolar phase, fibroblasts and endothelium form primary septa in the air sacs, causing the formation of premature alveoli. Immediately after, secondary septa develop from crests of tissue containing capillary and stromal cells that migrate in from the walls and subdivide alveoli [59]. This process takes (depending on the species) from several weeks to several years.

13.2.3 Changes in Lung According to the Aging Processes

The lungs, like all other organs, undergo an aging process. As a result of this process, not only the total number of cells changes, the percentage of particular types of cells, but also their efficiency is reduced. With age, the proliferative potential of lung stem cells also decreases, not just their number. This brings about the need to increase the intensity of research in the field of regenerative medicine.

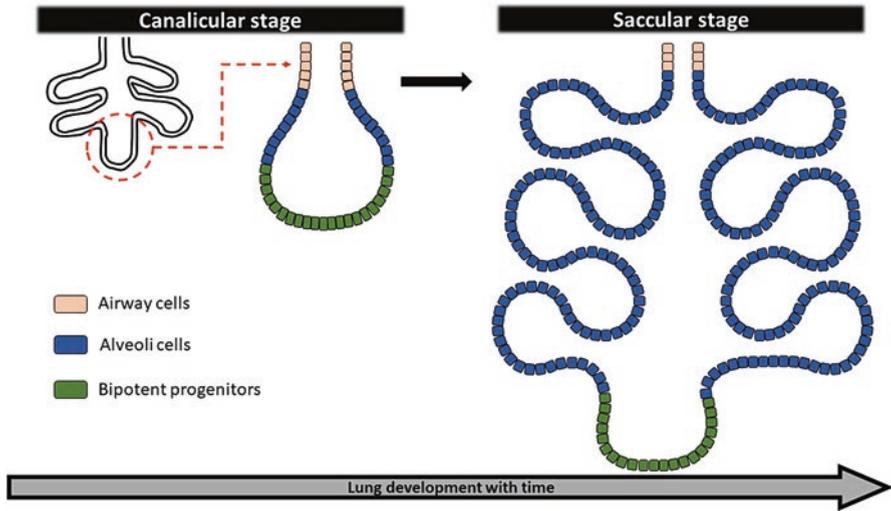


Fig. 13.5 Schematic representation of lung development during canalicular stage and saccular stage

A special strain of rapidly aging mice was created to study the aging process and changes in the functioning of individual organs caused by aging. Wild-type mice survive an average of 11.9 to 17.5 months, while senescence-accelerated mice (SAM) only 12–14 weeks. During such a short period of life, this strain of mice exhibits many elements that are symptoms of aging (e.g., hair loss, behavioral changes, osteoporosis, senile amyloidosis) [60]. Due to the fact that lung screening of SAM mice showed lesions characteristic of lungs of other strains in senile age, they can be successfully used in lung age studies in a much shorter time [61–65].

As the mouse ages, a number of changes take place in the lungs. One such change is the increasing lung volume. The observed increase is linear and occurs throughout the lifetime of the mice and can be doubled in relation to the volume in the first month of life [64, 66].

In addition to the increase in total lung volume, an increase in the area of the intra-alveolar pores from $0.083 [10^{-2} \text{ m}^2]$ at the age of 1 month to as much as $0.357 [10^{-2} \text{ m}^2]$ in the 28th month of life of mice is also observed [66]. Observed enlargement of the pores may occur as a result of the decrease of elasticity and, consequently, the rupture of tissue strands between adjacent pores, especially in the aging organ. Huang et al. conclude that the loss of lung elasticity may be due to the delayed increase in collagen synthesis in relation to the loss of elastin in the parenchyma of the aging lungs of C57BL/6 J mice [67]. In addition, Sueblinvong et al. showed that in the 24-month-old C57BL/6 mice, there was an increased expression of MMP-2, MMP-9, and TGF- β receptor 1 at the mRNA level, demonstrating the phenotype of early fibrosis [68].

In aging mice, an increasing number of immune cells in the bronchoalveolar lavage are also observed with age. According to Higashimoto et al., the total number

of cells increases from $0.93 \pm 0.02 \times 10^5$ at the age of 1.5 months to $7.49 \pm 0.01 \times 10^5$ at the age of 26 months [69]. Nevertheless, the overall percentage composition of individual cell fractions varies between these time points. Both the percentage of neutrophil and lymphocyte populations increases from $0.1 \pm 0.6\%$ to $1.9 \pm 0.96\%$ and $1.82 \pm 0.6\%$ to $2.83 \pm 0.61\%$, respectively. Only the percentage of macrophages slightly decreases from $97.0 \pm 0.6\%$ to $94.8 \pm 1.3\%$. The cause of these changes may be the aging of the immune system and the greater involvement of phagocytic cells in maintaining lung sterility [69]. In addition, in older mice, there is an increase in the expression of genes associated with inflammation, that is, CD20, Burkitt lymphoma receptor 1, CXCR-3, provirus integration site for Moloney murine leukemia virus-2, CD72, IL-8RB, C-Fgr, and CD8 β [70].

Chen et al. reported that also the concentration of glutathione (GSH) in the lungs of aged C57BL/6 mice is reduced with age by as much as 30%. Due to the fact that GSH is mainly responsible for carrying out detoxification processes, it is concluded that its ability in the lungs decreases with age [71].

With age, not only the number of cells in the immune system in the bronchoalveolar lavage changes but also the cells that build the lung tissue. In studies conducted on young (6 weeks) and old (26 months) Fischer 344 rats, a very distinct difference in the composition of the lungs was observed. The number of alveolar type I, interstitial, and vascular endothelial cells increased with age from 30 ± 4 to $59 \pm 7 \times 10^6$, from 114 ± 7 to $130 \pm 15 \times 10^6$, and from 253 ± 22 to $263 \pm 26 \times 10^6$ cells, respectively [72]. The amount of alveolar type II cells increased very slightly, that is, from 54 ± 3 to $57 \pm 8 \times 10^6$ cells. In order to the increasing number of other lung cells, the percentage of AT2 among all lung cells decreases, thus leading to a decrease in the regenerative potential of the lungs with age [72] (Fig. 13.6).



Fig. 13.6 The photograph shows a 3D organoid culture, carried out in a matrigel. These cultures are run through 16–21 days. The presented culture was established from BASCs and type II pneumocytes in the presence of pulmonary fibroblasts. Organoids formed from BASCs were marked with green arrows, while organoids formed from type II pneumocytes were marked with blue arrows

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Chapter 14

Targeting Purinergic Signaling and Cell Therapy in Cardiovascular and Neurodegenerative Diseases



Roberta Andrejew, Talita Glaser, Ágatha Oliveira-Giacomelli, Deidiane Ribeiro, Mariana Godoy, Alessandro Granato, and Henning Ulrich

Abstract Extracellular purines exert several functions in physiological and pathophysiological mechanisms. ATP acts through P2 receptors as a neurotransmitter and neuromodulator and modulates heart contractility, while adenosine participates in neurotransmission, blood pressure, and many other mechanisms. Because of their capability to differentiate into mature cell types, they provide a unique therapeutic strategy for regenerating damaged tissue, such as in cardiovascular and neurodegenerative diseases. Purinergic signaling is pivotal for controlling stem cell differentiation and phenotype determination. Proliferation, differentiation, and apoptosis of stem cells of various origins are regulated by purinergic receptors. In this chapter, we selected neurodegenerative and cardiovascular diseases with clinical trials using cell therapy and purinergic receptor targeting. We discuss these approaches as therapeutic alternatives to neurodegenerative and cardiovascular diseases. For instance, promising results were demonstrated in the utilization of mesenchymal stem cells and bone marrow mononuclear cells in vascular regeneration. Regarding neurodegenerative diseases, in general, P2X7 and A_{2A} receptors mostly worsen the degenerative state. Stem cell-based therapy, mainly through mesenchymal and hematopoietic stem cells, showed promising results in improving symptoms caused by neurodegeneration. We propose that purinergic receptor activity regulation combined with stem cells could enhance proliferative and differentiation rates as well as cell engraftment.

Roberta Andrejew and Talita Glaser equally contributed to the work.

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Keywords Purinergic signaling · Cell therapy · Cardiovascular diseases · Neurodegenerative diseases · Clinical trials

Abbreviations

6-OHDA	2,4,5-Trihydroxyphenethylamine
AD	Alzheimer's disease
ALS	Amyotrophic lateral sclerosis
AMP, ADP, ATP	Adenosine 5' mono-, di-, triphosphate
A β	Amyloid beta
BBG	Brilliant Blue G
BM	Bone marrow
BM-MNC	Bone marrow-derived mononuclear cell
CNS	Central nervous system
COX-2	Cyclooxygenase-2
EAE	Experimental autoimmune encephalomyelitis
EC	Endothelial cell
EDSS	Expanded Disability Status Scale
ESC	Embryonic stem cell
FGF	Fibroblast growth factor
FRS	Functional Rating Scale
GABA	γ -Aminobutyric acid
GDNF	Glial cell line-derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
HSC	Hematopoietic stem cell
hUC-MSC	Human umbilical cord-derived mesenchymal stem cell
IFN- γ	Interferon gamma
IL-1 β /2/6/9/10/17	Interleukin 1 β /2/6/9/10/17
iPSC	Induced pluripotent stem cell
MNC	Mononuclear cell
MOG	Myelin oligodendrocyte glycoprotein
MPTP	1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
MSC	Mesenchymal stem cell
MSC-NTF	MSC-secreting neurotrophic factors
NPC	Neural progenitor cell
NSC	Neural stem cell
PD	Parkinson's disease
PKA/C	Protein kinase A/C
PLP	Proteolipid protein
PMS	Progressive multiple sclerosis
RRMS	Relapsing-remitting multiple sclerosis

SGZ	Subgranular zone
SMC	Smooth muscle cell
SOD1-G93A	Superoxide dismutase 1 mutant (glycine 93 changed to alanine)
SOD1	Superoxide dismutase 1
Sox1-GFP ESC	Sox1-green fluorescent protein embryonic stem cell
SVZ	Subventricular zone
Tg APP/PS1	Double transgenic mice expressing a chimeric mouse/human amyloid precursor protein (Mo/HuAPP695swe) and a mutant human presenilin 1 (PS1-dE9)
Tg2576 mice	Transgenic mice expressing the human amyloid precursor protein gene carrying the Swedish mutation (HuAPP695swe)
TNF- α	Tumor necrosis factor-alpha
UDP, UTP	Uridine di-, triphosphate
VEGF	Vascular endothelial growth factor

14.1 Introduction

The term “purinergic neurotransmission” has emerged after the identification of adenosine 5'-triphosphate (ATP) as a transmitter of non-adrenergic and non-cholinergic inhibitory nerves in guinea pig *taenia coli*, by Geoffrey Burnstock [1]. Following re-examination of Dale's Principle and based on the observation that in sympathetic nerves, noradrenaline is released together with ATP, the co-transmission hypothesis was formulated in 1976 [2]. This concept is now well established also in enteric, parasympathetic, and sensory-motor nerves. Besides, there is evidence of ATP co-release with acetylcholine, γ -aminobutyric acid (GABA), dopamine, serotonin (5-hydroxytryptamine), glutamate, and noradrenaline in the central nervous system (CNS) [3], acting on specific plasma membrane proteins known as purinergic receptors.

In 1978, a pharmacological basis (in line with the second messenger system and molecular structure) was proposed to distinguish two types of purinergic receptors: (a) selective for adenosine (called P1, with four subtypes currently cloned, namely, A₁, A_{2A}, A_{2B}, and A₃) and (b) selective for ATP/ADP (called P2) [4, 5]. In 1985, P2 purinoceptors were divided between two families, defined as P2X ionotropic ligand-gated ion channel receptors, activated by ATP (with seven subtypes currently characterized, namely, P2X1-7) and P2Y metabotropic G protein-coupled receptors (with eight subtypes currently characterized in mammals, namely, P2Y1,2,4,6,11,12,13,14), activated by ATP, uridine triphosphate (UTP), adenosine diphosphate (ADP), uridine diphosphate (UDP), or UDP-glucose [6–10].

Several studies have focused on the purinergic system, due to the pivotal role of purine and pyrimidine nucleotides as well as the nucleoside adenosine as extracellular messengers regulating several physiological and pathological processes [11]. Purinergic receptors appear early in evolution, as evidenced by studies showing cloned

receptors in primitive invertebrates (which resemble mammalian P2X receptors) and ATP signaling in plants [12–15]. Numerous cell types physiologically release ATP (e.g., in response to mechanical distortion or hypoxia) [16], which is transported to the extracellular milieu via vesicular release, ATP-binding cassette (ABC) transporters, connexin or pannexin hemichannels, maxi-ion channels, or P2X7 receptors [17]. Then, they undergo degradation by ecto-nucleoside triphosphate diphosphohydrolases (ecto-NTPDases, which can hydrolyze ATP to ADP or AMP), ecto-nucleotide pyrophosphatase/phosphodiesterases (ecto-NPPs, which hydrolyze ATP to AMP), ectoalkaline phosphatases (which degrade AMP into adenosine), and ecto-5'-nucleotidases (which convert AMP into adenosine) [18].

Short-term events involving purinergic signaling include acute inflammation, chemoattraction, secretion, neurotransmission, and neuromodulation [19]. Long-term events include cell proliferation, migration, differentiation, and death during numerous processes, such as development, regeneration, cancer, and aging [20, 21].

Metabotropic G protein-coupled P1 receptors are activated by extracellular adenosine and divided into four subtypes (A_1 and A_{2A} , with high affinity to adenosine; A_{2B} and A_3 , with low affinity to adenosine). A_{2A} and A_{2B} receptors are coupled to G_s proteins, while A_1 and A_3 receptors interact with G_i proteins. Crystallization studies and structure-based drug design of these receptors indicate the presence of the following structural properties: seven transmembrane α -helices (and one short membrane-associated helix), an extracellular amino-terminus (N-terminus) with one or more glycosylation sites, and an intracellular carboxy-terminus (C-terminus) with phosphorylation and palmitoylation sites as well as three extracellular and three intracellular loops [22–25].

P2X1-7 receptors are a family of trimeric ATP-gated ion channels, permeable for Na^+ , K^+ , and Ca^{2+} [26, 27], and are ubiquitously expressed in eukaryotic tissues. They present the following structural properties: two transmembrane domains (TM1, involved with channel gating, and TM2, lining the ion pore); short intracellular N- and C-termini; an extended extracellular loop, with conservation of ten cysteine residues (forming a series of disulfide bridges and an ATP-binding site); and ionic pore subunits (with a dolphin-shaped structure) assembling homomultimers or heteromultimers (e.g., P2X1/2, P2X1/4, P2X1/5, P2X2/3, P2X2/6, P2X4/6) [28]. Investigation of the molecular mechanism of P2X receptor gating showed that these receptors range between different functional states in response to ATP (i.e., closed, open, and desensitized states), as revealed by X-ray structures from different species (including humans), and chemical optogenetic approaches [29–33]. Activation of P2X receptors promotes an increase in the intracellular Ca^{2+} levels that leads to the activation of several protein kinases [34–37] and the release of vesicular neurotransmitters [38–40].

P2Y1,2,4,6,11,12,13,14 receptors are G protein-coupled receptors, activated, with different sensitivities, by the nucleotides ATP, UTP, UDP, and UDP-glucose [41], presenting the following structural properties: seven hydrophobic transmembrane domains and an extracellular N-terminus and intracellular C-terminus, besides three extracellular loops (with four cysteine residues which form two disulfide bridges) and three intracellular loops [42–49]. The intracellular signaling pathways

induced by P2Y_{1,2,4,6} receptors activation depend on G_q protein and phospholipase C- β activation, generating inositol 1,4,5-triphosphate, that increases intracellular Ca²⁺ levels, and diacylglycerol, resulting in activation of protein kinase C (PKC) [50]. P2Y_{12,13,14} receptors activate G_i proteins, which results in adenylyl cyclase inhibition and reduced levels of intracellular cyclic adenosine monophosphate (cAMP). Activation of both G_q and G_s proteins by the P2Y₁₁ receptor generates an increase of intracellular Ca²⁺ and cAMP levels [51].

Considering the numerous diseases lacking a definitive treatment, stem cells appear as a promising field within regenerative medicine [52, 53]. In this sense, several strategies have been used in an attempt to repair damaged tissues and organs. One is the mobilization of stem cells present in specific niches in the patient's organism (such as in the brain, blood, and bone marrow (BM)). A second approach is based on engraftment of stem cells, which had been locally (i.e., by intracerebral transplantation) or systemically (intravenously or intra-arterially) injected. Transplantation may use cells of the patient (autologous transplantation) or another individual (allogeneic transplantation). Within this scenario, in addition to the perspective that these transplantations may promote tissue protection or repair, stem cells are potent tools for studies of cell differentiation, identification of therapeutic targets and drug toxicity, as well as etiology of genetic diseases [54].

To be considered a stem cell, a cell must meet specific requirements, including the ability to self-renew over long periods and be capable of self-renewing and specializing themselves by differentiating into more than one cell type, being classified as totipotent, pluripotent, or multipotent according to their differentiation potential [55]. Totipotent stem cells, capable of originating embryonic and extra-embryonic cells, correspond to cells from the zygote stage and cells resulting from its initial cell divisions (up to the morula stage). Pluripotent stem cells, capable of originating cells from the three germ layers, include embryonic stem cells (ESCs), embryonic germ cells, embryonal carcinoma cells, as well as induced pluripotent stem cells (iPSCs). On the other hand, fetal stem cells, neonatal (such as umbilical cord stem cells), and adult stem cells (such as BM stem cells and neural stem cells (NSCs)) are considered multipotent due to the limited number of cell types that they can originate [56, 57].

Before reaching their fully differentiated state, stem cells generate an intermediate cell type, called precursor or progenitor cell, which is usually already "compromised" to differentiate along a particular path of cellular development, originating cell types with specialized functions. The niche or microenvironment, in which the stem cells are found, also contributes to their proliferation, migration, and differentiation due to the presence of soluble factors, such as growth factors and chemokines, extracellular matrix molecules, and cell-to-cell interactions [58–66].

The organism's ability to repair and replace some cells and tissues, but not others, is a subject that intrigued researchers for several years. Thus, the discovery of the existence of stem cells in the adult organism has opened up new perspectives for the understanding of tissue repair mechanisms and the development of more effective therapeutic strategies [67–69].

Adult stem cells are rare and proliferate slowly, being found in specific niches [70–76], where their primary function is the maintenance of tissue homeostasis, being also able to generate precursors and differentiated cells [77–80]. Due to the limited number of stem cells in the adult organism and their difficult obtainment, it is often necessary to purify and expand these populations *in vitro* to enable their use in therapies [81–83]. On the other hand, the therapeutic use of adult stem cells offers several advantages, such as a reduced risk of tumor formation in comparison to pluripotent stem cells [84]. The possibility of performing autologous transplants may avoid possible ethical or legal issues [85] and reduces the risk of rejection after transplantation. With the considerable increase in life expectancy, one of the significant challenges for performing more effective autologous transplants and therapies based on the mobilization of endogenous progenitors is controlling stem cell senescence. Thus, it is essential to understand the mechanisms involved in the loss or reduction of the repair capacity of these cells during aging and the best strategies to prevent or reverse this scenario [86–88].

Mesenchymal stem cells (MSCs) are self-replicating cells found in almost all tissues (located mainly in perivascular niches), such as BM [89, 90], adipose tissue [91], umbilical cord [92–94], menstrual blood [95, 96], placenta [97], liver [98], lung [99], kidney [100], skeletal muscle [101], amnion [102], and dental pulp [103, 104]. These cells may differentiate into osteoblasts, adipocytes, and chondroblasts [105]. Among MSC sources, the BM is the most studied. These cells were described in 1968 by Friedenstein and colleagues [90], who defined them as “colony-forming unit fibroblasts.” In this biological niche, MSCs constitute a subgroup of multipotent stromal cells, representing only 0.01–0.001% of the total nucleated cells [106, 107], generating the connective tissue of BM. Moreover, MSC provide support for the maintenance and self-renewal of hematopoietic stem cells (HSCs), which give rise to all types of blood cells, protecting against apoptotic stimuli and avoiding their differentiation [84, 108, 109].

Despite their heterogeneous distribution in the organism and the lack of a global specific marker, populations of MSC obtained from different sources have several common characteristics that are used as minimum criteria for the identification of this cell type [105]: fibroblast morphology, multipotentiality (differentiation in osteocytes, chondrocytes, and adipocytes under specific stimulus), adherence to plastic *in vitro*; expression of specific surface markers (ecto-5'-nucleotidase/CD73, CD90, and CD105), and absence of expression of hematopoietic lineage markers (CD34, CD45, CD19, and HLA-DR). Different markers have been described later as present in MSC, such as CD29, or absent, such as CD11b [107].

Some authors suggest that MSC with perivascular location would be pericytes (as in the BM and liver sinusoids), supported by the correlation between blood vessel density and number of MSC (both decaying throughout the individual's life) [110, 111]. The pericytes also present several characteristics that fulfill the “operational” definition of MSC [112–118] and are mostly in a quiescent state in adult tissues. However, in certain circumstances, they may become activated, being able to proliferate and migrate locally, secrete trophic factors, and modulate the immune system [119, 120]. This activation can be induced during events such as angiogenesis,

wound healing, inflammation, and tissue remodeling [121–123]. The primary function of pericytes is the stabilization of vessels and control of blood flow within them [124]. Further, these cells are essential regulators of the blood-brain barrier properties, as permeability [125, 126].

MSCs play an essential role as trophic mediators, releasing substances that favor tissue protection and regeneration [111, 127], as antiapoptotic, anti-inflammatory molecules, and trophic factors (vascular endothelial growth factor (VEGF) brain-derived neurotrophic factor, among others) [128–131]. Transplantation of these cells may also stimulate the recruitment of local progenitors and their subsequent differentiation into neural cells [84, 132]. Another essential feature of these cells for therapeutic use is their ability to migrate to damaged areas, including in the CNS [133–135].

MSCs also modulate the immune response [136], since they exert immunosuppressive effects, inhibiting the proliferation of T [137, 138] and B [139] lymphocytes, interfering in the process of dendritic cell maturation [129], and acting on natural killer cytotoxicity [140]. Other mechanisms involved in paracrine signaling promoted by these cells include the transfer of extracellular vesicles (microvesicles and exosomes) or even healthy mitochondria to damaged or compromised cells [141–150]. Exosomes are small vesicles (40–100 nM in diameter) originating from the exocytosis of multivesicular bodies and may contain messenger RNAs, micro RNAs, and proteins. Moreover, microvesicles (50–2000 nM in diameter) originate from plasma membrane budding and may contain messenger, silencing and micro RNAs, as well as proteins and lipids [151–155].

The generation of neurons from undifferentiated cells (neurogenesis) persists throughout adult life in mammals, mainly in two regions: the subgranular zone (SGZ) of the hippocampal dentate gyrus [156] and the subventricular zone (SVZ) of the lateral ventricles [157].

The SVZ consists of four cell types: ependymal cells (type E cells) that are in direct contact with the ventricular lumen [158] and NSC (type B cells), which are multipotent, capable of self-renewal [159–161], and generate transient-amplifying cells (type C cells), which in turn originate migratory neuroblasts (type A cells). Type A cells migrate along the rostral migratory pathway to the olfactory bulb, where they differentiate into granule and periglomerular neurons [160, 162].

In the hippocampal dentate gyrus, an NSC population divides continuously in the SGZ (the region bounded by the hilus and the granule cell layer), giving rise to progenitor cells. These cells differentiate into granule neurons after migrating to the granule cell layer [163, 164]. Neurogenesis in the hippocampal dentate gyrus occurs analogously to that in the SVZ, involving cells with astrocyte characteristics that have a slow cycle of division (type B cells), immature precursors (D cells), and migratory neuroblasts (G cells) [165].

One of the aspects that allowed the discovery of stem cell existence in adult SVZ was the observation that an SVZ cell subpopulation when dissociated and cultured in the presence of growth factors as fibroblast growth factor (FGF)-2 was able to form cell clusters called neurospheres [159]. These primary neurospheres, after dissociation, generate secondary neurospheres and, after growth factor removal, differentiate into neurons, astrocytes, and oligodendrocytes [157]. The division of

NSC can occur symmetrically, generating two stem cells, or asymmetrically generating a stem cell and a progenitor cell compromised with differentiation [55].

There is still considerable controversy regarding the true identity of the NSC. Some studies suggest that a subpopulation of ependymal cells (CD133⁺) may represent a population that remains in the quiescence state longer than the NSC in the SVZ [166]. These cells would be activated under specific conditions, being able to differentiate into type B, C, and A cells to restore cell population in SVZ, as occurs during the aging process in mammals [167]. During development, ependymal cells also generate the type B cells [166].

The physiological role of neurogenesis in SVZ has been associated with olfactory processing [168–170], and in SGZ, directly related to recognition memory and emotion (e.g., anxiety and depression) [171, 172], being both modulated by several extrinsic and intrinsic factors [173–175].

The derivation of stem cells from human blastocysts [176] and obtaining iPSC by ectopic expression of specific transcription factors [177, 178] ushered in a new era of stem cell research. The enormous differentiation potential of pluripotent stem cells has created revolutionary possibilities in the context of regenerative medicine [179]. These include the differentiation of these cells into specific cell types and the creation of artificial organs to treat a variety of diseases, as well as applications such as the study of early human development events and drug toxicity tests [180–183]. However, some ethical, technical, and safety aspects need to be taken into account for the use of pluripotent stem cells in clinical practice [184–187]. Moreover, in some countries, there are legal restrictions on obtaining embryonic or fetal stem cells from embryos discarded during *in vitro* fertilization procedures or from aborted human fetuses, even when used for research purposes [188, 189]. In this scenario, iPSCs bypass these issues using patients' easily accessible differentiated cells, such as fibroblasts and peripheral blood cells, dedifferentiated into a pluripotent state.

Although the high proliferation rate of pluripotent cells allows the *in vitro* expansion of obtained cells, they are prone to form teratocarcinomas (which contain cells derived from the three germ layers) [190, 191]. Thus, one of the challenges is to ensure that, after *in vitro* differentiation, there is no remnant undifferentiated cell in the cell pool to be transplanted, since it could generate a tumor [192–194]. In this sense, the use of biomaterials in association with traditional culture matrix represents a new approach to improve the differentiation and maturation of iPSC [195, 196], as well as the survival of engrafted cells [197].

The lack of histocompatibility [198], which potentially results in the rejection of transplanted cells, is another critical aspect to be considered when choosing a stem cell source. In this case, the use of iPSC offers a significant advantage, since it allows the accomplishment of autologous transplants. The cellular reprogramming technology also allows the establishment of models for disease studies, since reprogrammed cell types induced to differentiation allow *in vitro* recapitulation of events occurring in the course of the disease [199, 200].

Although very promising, the study of iPSC is a very new field of research. Therefore, several challenges still need to be overcome for the clinical use of these cells, such as the efficient and safe generation of pluripotent cells [201]. Thus, it is

fundamental to understand the molecular mechanisms involved in reprogramming [202–206], allowing the characterization of the cell types and defining the most favorable conditions for this process that induce fewer DNA changes [207–211].

The choice of the appropriate cell type to be reprogrammed is also essential. Nonhereditary mutations that occurred throughout the patients' life could be retained in iPSC and their derivatives, which may facilitate tumor formation. The cell differentiation stage also seems to influence reprogramming efficiency directly [211]. Thus, the use of neonatal, adult or progenitor stem cells [212, 213] can represent an excellent strategy to increase the efficiency of reprogramming when compared to specialized adult cells.

As previously demonstrated, cell therapy appears as a new therapeutic alternative for various diseases, with promising results [214–218]. In this sense, several strategies using different types of stem cells and their derivatives have been investigated [219–223], with the aim of reversing or delaying disease progression through several mechanisms, such as (1) cell replacement, directly replacing the lost cell type [224–226]; (2) trophic support, promoting the survival of affected cells and endogenous recruitment of progenitors [227–229]; and (3) modulation of inflammation [230, 231]. A system largely involved in these processes is the purinergic signaling, acting on cell survival, migration, differentiation and in the inflammatory modulation. Thus, therapies based on modulation of the purinergic system have also shown relevant results [232–234]. In this chapter, we selected diseases with clinical trials using cell therapy or purinergic receptor activity modulation, concluded or in progress. We discuss these approaches as therapeutic alternatives for the most deadly or common diseases, like neurodegenerative and cardiovascular ones.

14.2 The Cardiovascular System

Cardiovascular diseases are highly prevalent and have a high rate of disability and mortality. The cardiovascular system is the main transportation system of the animals. The pump responsible for the distribution of nutrients, metabolites, oxygen, CO₂, and antibodies through the whole body is the heart, and failures in the function of this organ are usually lethal.

In 2016, the main global cause of death was due to ischemic heart disease and stroke, equivalent to 17.9 million/year, which accounts for 31% of total global deaths. They have been the leading cause of death for the last 15 years [235] and are more prevalent in low- and middle-income countries. In addition, the main diseases that alter heart function are coronary heart disease (that affects blood vessels supplying the heart muscle); rheumatic heart disease (damage to the heart muscle and heart valves from rheumatic fever, caused by streptococcal bacteria); and congenital heart disease (malformations of heart structure existing at birth) [235].

Vascular diseases are those that affect the blood vessels. The blockage of blood flow through heart by fatty deposits is the main cause of heart attacks. Some risk factors can favor the development of this pathology, like tobacco use, unhealthy diet

and obesity, sedentary life, alcohol abuse, hypertension, diabetes, and hyperlipidemia. Among the most studied vascular diseases are stroke, peripheral artery disease, and critical limb ischemia. In this topic, we focus on coronary artery disease and ischemia. Thus, a better understanding of vessel wall structure is essential to understand the benefits of purinergic receptors activation modulation and cell therapy in cardiovascular diseases.

Vascular system is formed by vessels with different compositions. All vessel types of the vascular system present a layer of endothelial cells (EC) that limit the vascular lumen. Arteries and veins have an outer tunica adventitia layer consisting of extracellular matrix, fibroblasts, and pluri- or multipotent cells. Additionally, the arteries have an intermediate layer of smooth muscle cells (SMC) accompanied by elastic lamellar tissue or pericyte cells [236].

As in other tissues, several signaling pathways are involved in the process of migration, proliferation, differentiation, and apoptosis in the formation and repair of blood vessels. The process of forming new vessels from a pre-existing vessel as a consequence of lesions is called angiogenesis. In this process, angiogenic stimuli induce the migration of a leading EC to initiate the new vascular branch. Thereafter, subsequent ECs proliferate and form internal vacuoles which subsequently fuse to the vacuoles of adjacent cells forming a new vascular lumen.

Among the main stimuli in this process are the vascular endothelial growth factor and stromal-derived factor-1 α , released by lesions such as ischemia and tumors, which induce the release of pluri- or multipotent cells from vascular niche [236, 237]. These cells include endothelial progenitor cells, SMC progenitors, HSC, and MSC and can differentiate into EC and SMC for vascular regeneration. Additionally, endothelial progenitor cells are found in small amounts in circulating blood, involved in rapid regeneration processes [237].

In the *in vitro* differentiation process, pluripotent cells as iPSC or ESC are initially induced to differentiate into mesodermal progenitor cells. This process is described to be achieved through embryoid body formation or in monolayer culture with the addition of mesodermal-inductive factors. Subsequently, the addition of specific growth factors induces differentiation into EC, SMC, or other vascular cell types such as MSC [238–240]. *In vitro* differentiation models contribute to the understanding of vascular regeneration process and the search for tools to induce regeneration.

The investigation of extracellular purines in the cardiovascular system, mainly the heart, has been extensive. Dating from 1929, studies described coronary dilator effects of AMP and later adenosine, ATP and ADP, even before the purinergic signaling discovery [20, 241–243]. In 1936, Drury has already published a review summarizing the early data about the system [241]. Similarly, some studies described ATP as three times more potent to modulate cardiac rhythm when compared to adenosine [241, 244–246]. The increase in heart rate caused by activation of A₁ receptors appears to be mediated by its action on the CNS, while the decrease in blood pressure by the activation of A_{2A} receptors appears to be mediated in the periphery [247, 248]. Therefore, treatments of coronary diseases, like angina, were based on ATP and AMP injections already in 1940s [249–254].

Within the heart, adenine nucleotide concentration in the extracellular heart space is variable. In accordance, EC, red blood cells, and activated platelets can release ATP [16, 255–258], upon stimuli like hypoxia and ischemia, and shear stress [242, 257, 259–268] to dilate the vessels and increase the blood flow. Although, the levels of detected ATP range from 1 to 40 nM in the coronary lumen [269, 270] due to efficient degradation of ATP by ecto-enzymes (mainly ecto-5'-nucleotidase), resulting in adenosine accumulation. In addition, long-lasting treatment with P1 receptor antagonist caffeine augments heart rate and resting blood pressure [271]. A₁ receptor-induced heart rate increase in mouse is more pronounced in males than in females [272].

Besides the heart rate and coronary dilatation, purinergic receptors are extensively present in cardiomyocytes. First, adenosine-promoted actions on cardiomyocytes may mediate negative chronotropic effect through A₁ receptor activation, which is upregulated upon chronic ingestion of theophylline diet [273]. A₁ receptor activity may inhibit the activation of α 1-adrenergic receptors in cardiac sarcolemma Na⁺/H⁺ exchanger [274] and is related to increases of nitric oxide and cyclic guanosine monophosphate levels [275] preventing mitochondrial damage [276].

Adenosine, through A_{2A} receptor stimulation, enhanced contractility of cardiomyocytes and isolated perfused hearts and through A_{2B} receptor activation induced nitric oxide release [277–279]. Even though A₃ receptor expression was not detected in the atrium, it can induce apoptosis of cardiomyocytes from newborn rats [280, 281].

Furthermore, ATP also plays some roles in cardiomyocytes. For example, during hypoxia, acidosis, or adrenaline action, cardiomyocytes release ATP [261, 263, 282], increasing intracellular Ca²⁺ levels and contractility of ventricular and atrial areas. This occurs mainly upon P2Y₂ receptor activation, which leads to inositol 1,4,5-triphosphate production and cytosolic Ca²⁺ mobilization upregulating PKC and PKA activity [283–285].

Other P2 receptor-coding mRNAs were detected in the human heart, like P2X₁, P2X₃, and P2X₄ receptors, while in the human fetal heart mainly P2X₁, P2X₃, P2X₄, and P2Y₂, P2Y₄, and P2Y₆ receptors are expressed [286–289]. Reports about P2 receptors in the heart suggested that P2X₄ and P2X₇ receptors are expressed in the t-tubular network of ventricular cells [290].

Stretching input can induce cell death of cardiomyocytes through ATP release. P2X₇ receptor activity mediates apoptosis in this process [291], while the P2X₄ receptor enhances myocyte contractility as shown by transgenic mice models that overexpress the P2X₄ receptor [292–295]. P2Y₂ and P2Y₄ receptors were described to regulate the cardiac contractility of young rats. ATP and UTP trigger P2Y₂ receptor-mediated Ca²⁺ release via G protein activation, inducing the opening of heteromeric transient receptor potential cation channels 3/7 and inducing a sustained nonspecific cationic current in neonatal rat myofibroblasts [296–299].

Some P2 receptors have also been described participating in pro-fibrotic responses in cardiac fibroblasts from mouse and rat, and, in this case, P2Y₂ and P2Y₆ receptor agonists lead to thickening of scar tissue following inflammation or injury [300, 301]. Lu and Insel described that fibrotic response depends on the balance of ATP and adenosine signaling, which have opposite effects, by inducing and inhibiting, respectively [302].

Coronary diseases usually lead to heart attack. In this case, ATP is released from coronary EC and red blood cells during hypoxia resulting in blood flow changes [258, 303]. ATP can also be released as a response of acetylcholine, bradykinin, serotonin, and ADP [304], causing maximal coronary vasodilatation in humans [305] by hyperpolarizing EC [306–308], after triggering the purinergic receptors identified as important in this vasodilation: P1, P2Y1, P2Y2, and P2Y4 receptors [309–313]. However, the most expressed and sensitive receptors in coronary are P2X1 and P2Y2 receptors [314, 315], participating even in the control of blood flow during exercise [316–318]. A₁, A_{2A}, and A₃ receptors can also modulate the release of nitric oxide, which is a classical vasodilator [319–322].

A_{2A} receptor agonism has been used in clinical procedures of coronary artery stenosis diagnosis in patients unable to perform the exercise stress test, due to its capability of coronary vasodilatation [323].

In pathophysiological processes with endothelium damage, platelets under aggregation release ATP, ADP, and UDP. As mentioned, these compounds activate P1 and P2 receptors that control several processes, including cellular proliferative states. For example, endothelial progenitor cells derived from ESC express P2X4, P2X6, and P2X7 as well as P2Y2, P2Y4, P2Y11, P2Y13, and P2Y14 receptors. Endothelial progenitor cell proliferation is inhibited by ATP or UTP [324]. Moreover, it is known that adenosine A₃ receptor activation induces neovascularization in tumor cells, supporting tumor growth and infiltration in adjacent healthy tissues, and induces differentiation to EC [325]. While A₃ receptor blockade inhibits maleficent stem cell transformation after engraftment, pretransplantation treatment of stem cells with an A₃ receptor agonist *in vitro* facilitates EC differentiation. Moreover, activation of adenosinergic receptors modulates the response to injury, such as A₁ receptor activation, that induces EC barrier enhancement in hypoxia environment, preventing vascular leak – a key pathophysiological mechanism of vascular diseases [326].

Adenosine receptors modulate SMC proliferation as well. A_{2B} receptor activation reduces coronary SMC proliferation, besides inhibiting collagen synthesis by vascular SMC [327, 328]. Thus, A_{2B} receptor agonists may protect against vessels occlusion by reducing extracellular matrix synthesis. The stimulation of P2Y receptor apparently induces opposite effects in SMC. P2Y1, P2Y2, P2Y4, and P2Y6 receptor activation induces SMC proliferation [329–331].

Besides acting on the proliferative state of SMC, purinergic receptors modulate vessel contraction. A study with P2Y6 receptor knockout (KO) mice showed decreased contractile aorta effects induced by UDP and UTP evidencing the role of P2Y6 receptor in blood vessels [332]. The P2Y12 receptor antagonist clopidogrel is extensively used as an antiplatelet aggregation agent for treating vascular diseases. However P2Y12 receptor activation seems to also regulate contraction of arteries, along with P2Y1, P2Y6, and P2Y14 receptors [333–335]. Finally, A_{2A} and A_{2B} receptor activation increases vasodilatation and blood flow [322]. A core component of ischemia and heart attack is cardiomyocyte death, which is usually replaced by a fibroblastic scar [336]. The reestablishment of myocardium mass with minor scars is the desirable treatment option.

Stem cell therapy for ischemia and heart failure has evolved quickly in the past decade due in vitro and in vivo preclinical research as well as some clinical trials. Both embryonic and adult stem cells have been studied in cardiac and vascular repair. However, adult stem cells overcome many ethical concerns. In order for effective transplantation of MSC, these cells must provide a new source of functional cardiomyocytes or contribute to the establishment of a new vascular network, which can nourish the site of injury for recovery of ischemic myocardium [337, 338].

Some animal experiments using MSC in hearts after ischemia showed many beneficial properties regarding heart function, such as attenuation of myocardial scar, improved ventricular function, and increased myocardial perfusion due to enhanced vascular density [339–342].

The MSC's capability to induce vasculogenesis and angiogenesis has been reported in different studies, which showed that MSC can be derived from endogenous SMC or pericytes [112, 343–345]. Additionally, injection of STRO-1-positive cells, a MSC marker, contributed to increased neovascularization and myocardial ejection fraction in the myocardial infarcted rat [112].

Studies investigating the potential of injected MSC in differentiating into undesired cell types or disorganized tissue structures in the myocardial infarction recovery have yet revealed little results. Anywise, the use of cells committed to the cardiac cell line would provide a better approach for restoring the cardiomyocyte population. Previous works demonstrated that some chemicals could convert MSC to cardiac precursors, such as 2–4 weeks of exposure to 5-azacytidine, or to dexamethasone and ascorbic acid [346], bone morphogenetic protein-2, and FGF-44 [347] or even coculture with cardiomyocytes [348]. Differentiation efficiency is measured by the development of intercellular connections by intercalated discs and spontaneous cell contractility.

Interestingly, immunohistological assays have also evidenced in vivo differentiation into cardiomyocytes after xenogeneic, autologous, or allogeneic transplantation in various animal species [339, 340, 342, 349–351]. However, since engrafted cells do not acquire the full phenotype's characteristics, the process is incomplete [340, 342, 351–353]. Some groups have claimed that engrafted MSC actually fuse with the preexistent cardiac cells instead of differentiating [354, 355].

The induction of vascular regeneration in vascular diseases promotes a decrease in ischemia- and hypoxia-damaging effects and avoids thrombosis and embolism [237]. The use of mobilizing agents to recruit stem cells from the BM to circulatory blood is a widely studied therapy for vascular repair. In 1991, granulocyte colony-stimulation factor induced the repair of mechanically wounded endothelial monolayers in vivo and promoted angiogenesis in vivo [356]. Clinical trials using granulocyte colony-stimulating factor in vascular diseases were conducted, including ischemic stroke with increased stem cell migration to the injured site and recovery [357]. A more direct approach involves cell therapy. The injection of different progenitor cells proved to increase repair in vascular diseases due to post graft differentiation and cytokine and growth factor release.

MSC are one of the largely studied stem cell population for vascular regeneration. They can be obtained from the saphenous vein and BM and expanded in vitro for

engraftment [358]. For instance, studies showed that plaque rupture, a complication resulted from atherosclerosis, was repaired by BM-MSc therapy, reducing stroke risk [359]. In animal models of limb ischemia, treatment with BM or adipose tissue MSC improved post-ischemia angiogenesis and consequent reestablishment of perfusion [360, 361]. Likewise, treatment with modified MSC encapsulated with microbeads presented similar results [362]. In diabetic patients with limb perfusion, intramuscularly injected BM-MSc induced an increased lower limb perfusion [363]. A range of studies combined MSC with growth factors or antiapoptotic agents in ischemia models, presenting more prominent therapeutic results [364–366].

As mentioned, MSC can be found in the BM, in the fraction of mononuclear cells (MNCs), along with other nonhematopoietic and hematopoietic cells. A range of clinical studies indicate BM-MNC as a tool for vascular regeneration, with different efficacy outcomes in comparison to MSC grafts depending on the analyzed parameters [367]. Grafts of EC derived from iPSC also showed promising results in animal models of limb ischemia or peripheral arterial diseases by enhancing perfusion recovery [368–370]. When induced to MSC differentiation, transplanted iPSC enhanced perfusion and reduced muscle fibrosis and tissue loss [371]. Associated with a biocompatible matrix, iPSC-derived EC cell therapy showed increased survival of injected cells and promoted neovascularization. Thus, cell therapy seems to be a promising tool in vascular regeneration.

As we described above, both stem cells and adenosine can improve the regeneration of heart and vascular diseases. Following this line, adenosine attenuates inflammation in the in situ damaged area during the healing process, while mediating cardioprotective, vasodilatory, and angiogenic responses [372–376]. Moreover, A_{2A} receptor stimulation by adenosine produced by CD73 enhanced proliferation and decreased apoptosis of MSC [377–381]. Notwithstanding, ATP usually triggers inflammatory/stress signals mediated by P2 receptors [382].

Previous studies showed that spontaneous release of ATP by MSC via hemichannel gap junctions induced activation of P2Y1 receptors triggering cytosolic Ca^{2+} oscillations, while activation of P2X receptors led to pronounced proliferation rates [383]. Cell density-dependent Ca^{2+} oscillations in rat MSC were also detected upon stimulation with UTP, demonstrating a correlation between P2Y2 receptor and cell cycle progression [384].

At the site of injury, ATP can upregulate proliferation and migration of human cardiac fibroblasts through P2Y2, P2X4, and P2X7 receptors, leading to undesired scar tissue [385]. In addition, the P2X1 receptor inhibited proliferation of human coronary SMC by increasing NR4A1 expression, hampering angiogenesis and coronary recovery after ischemia [386]. Therefore, inhibition of these receptors should be beneficial during recovery.

Adenosine, differently from ATP, enhanced vasculogenesis in cardiac microvascular tissue. A_{2B} receptor stimulation in cardiac fibroblasts promoted remodeling after myocardial infarction and ischemic injury by tuning basal levels of collagen and protein synthesis [387, 388]. Moreover, adenosine can protect rat cardiomyocytes from apoptosis induced by angiotensin II exposure [389].

Finally, upregulation of P2Y₄ and P2Y₁₄ receptors is important for early lineage commitment to vascular cell types by both endothelial and SMC differentiation [390]. The main findings concerning therapeutic approaches with stem cells and purinergic modulation are described in Table 14.1. Moreover, the role of purinergic receptors and stem cells in the cardiovascular system is represented in Fig. 14.1.

14.3 Multiple Sclerosis

Multiple sclerosis (MS) affects approximately 2.5 million people worldwide, mostly women, presenting a variable prevalence from high levels in North America and Europe (>100/100,000 inhabitants) to low rates in Eastern Asia and sub-Saharan Africa (2/100,000 population) [391, 392]. Although MS-specific causes are unknown, its etiology has been consistently associated with genetic susceptibility [393] and environmental factors such as immunoglobulin G seropositivity to Epstein-Barr virus nuclear antigen, infectious mononucleosis, and smoking [394].

This autoimmune disease presents a biphasic course, which initiates with relapsing-remitting multiple sclerosis (RRMS) and evolves to progressive multiple sclerosis (PMS). RRMS is characterized by repeated acute episodes of focal inflammation within the CNS called relapses, leading to the development of neurological disabilities and demyelinating lesions detectable on magnetic resonance imaging (MRI) scans, which may be partially or completely restored with the improvement of neuroinflammatory responses after several weeks [395]. After 20–25 years, 70–90% of patients with RRMS progress to PMS, when the acute inflammatory events tend to occur less frequently, but the repeated damage to the CNS results in long-lasting disabilities [395]. The occurrence of many relapses after disease onset associated to early appearance of disabilities or large numbers of demyelinating lesions in MRI predicts a poor prognosis [396, 397].

Symptoms onset of this debilitating disorder usually occurs between the age 20 and 40 years [398], i.e., affects the most productive age group, which implies a high social and economic burden for MS [399]. No cure is currently available for MS; only symptomatic treatment is performed [400]. Thus, the understanding of participation of intracellular signaling and purinergic receptor activity modulation in this disease could highlight new approaches for MS treatment.

The participation of P2X₇ receptors in MS have been proposed, since their activation increases neuroinflammatory response, which is a main feature of MS outcome. Moreover, these receptors are important mediators in astrocytes and axon-oligodendrocyte communication, necessary for myelin formation and repair [401]. Corroborating these data, *postmortem* analysis of MS patients revealed increased P2X₇ receptor levels in microglia [402], in astrocytes [403], and in oligodendrocytes [404]. In accordance, MS treatment with glatiramer acetate (an approved drug for MS treatment) reduced P2X₇ receptor and interleukin 1-beta (IL-1 β) expression in patients' monocytes [405]. Moreover, a P2X₇ receptor polymorphism which results in gain of function was identified in MS patients [406]

Table 14.1 Clinical trials with therapeutic approach with stem cells and purinergic modulation

Disease	Stem cell type/target	Delivery method	Phase	Outcome	Clinical trial ID
Acute coronary syndrome	Endothelial progenitor cells/ticagrelor (P2Y12 receptor antagonist)	Oral	Phase IV	Ticagrelor significantly increased the number of circulating endothelial progenitor cells and decreased inflammatory cytokines, such as IL-6 and TNF- α diabetic acute coronary syndrome patients	NCT02487732
Ischemic cardiomyopathy	BM-derived mononuclear cell	Transendocardial	Phase II	No differences were found	NCT00824005
Acute heart failure	Rolofylline (A ₁ receptor antagonist)	Intravenous	Phase III	No benefits were found	NCT00328692, NCT00354458
Acute myocardial infarction	Adenosine	Intravenous	Phase II	Infarct size was reduced upon adenosine infusion, following a dose-response relationship	AMISTAD-II
	BM-derived mononuclear cell	Intracoronary	Phase III	No beneficial effect was detected after 6 months.	NCT01569178
Atherothrombosis and atherosclerosis	Clopidogrel (P2Y12 receptor antagonist)	Oral	Phase III	Clopidogrel plus aspirin-induced effects was not different from aspirin alone in reducing myocardial infarction, stroke, or death from cardiovascular causes	NCT00050817
Peripheral artery disease	Ticagrelor or clopidogrel	Oral	Phase III	Use of ticagrelor in patients with peripheral artery disease lowered the rate of ischemic and all-cause stroke	NCT01732822
Multiple sclerosis	Autologous HSC	Intravenous	Phase I	No results posted	NCT03113162
			Phase II	No results posted	NCT00273364, NCT00342134
			Phase III	No results posted	NCT03477500

Autologous HSC	Intravenous	Phase II	Transplantation has distinctive effects on CD4 ⁺ and CD8 ⁺ T cell repertoires. CD4 ⁺ T cells were dominantly present before treatment, but they were undetectable following reconstitution, and patients largely developed a new repertoire. In contrast, dominant CD8 ⁺ clones were not effectively removed, and the reconstituted CD8 ⁺ T cell repertoire was created by clonal expansion of cells present before treatment	NCT00288626
			After 3 years, HSC transplantation without maintenance therapy induced sustained remission of active RRMS and was associated with improvements in neurologic function. Moreover, treatment was associated with few serious early complications or unexpected adverse events	
			After 5 years, HSC transplantation without maintenance therapy induced long-term sustained remissions of active RRMS	
			No results posted	NCT03342638
			HSC transplantation fully halted all detectable inflammatory activity in the CNS of patients with MS for a prolonged period in the absence of any ongoing disease-modifying drugs. Furthermore, many of the patients had substantial recovery of neurological function despite their disease's aggressive nature	NCT01099930
			No results posted	
			No results posted	NCT00040482
			No results posted	NCT00497952
			Results under review (submitted in February 2019)	NCT00469378
			No results posted	NCT01377870, NCT01606215

(continued)

Table 14.1 (continued)

Disease	Stem cell type/target	Delivery method	Phase	Outcome	Clinical trial ID
	Autologous BM-derived MSC	Intravenous	Phase I	No results posted	NCT03778333, NCT03069170
			Phase I/II	No results posted	NCT02035514, NCT01745783
	hUC-MS	Intrathecal	Phase I/II	No results posted	NCT01895439
			Phase I/II	No serious adverse events were reported. Symptom improvements were most notable 1 month after treatment in EDSS scores, as well as in bladder, bowel, and sexual dysfunction, in walk times and general perspective of a positive health change and improved quality of life. MRI scans of the brain and the cervical spinal cord showed inactive lesions in 83.3% subjects after 1 year	NCT02034188
	Autologous adult human MSC	Intravenous	Phase I/II	Evidence of structural, functional, and physiological improvement after treatment in some visual endpoints is suggestive of neuroprotection	NCT00395200
	Autologous MSC	Intravenous	Phase II	No results posted	NCT02239393
			Phase I/II	No results posted	NCT03326505
	Allogenic umbilical cord-derived stem cells	Intrathecal	Phase I	The treatment was safe and well tolerated. No serious adverse events were reported. Minor adverse events were observed, including transient fever and mild headaches usually resolved in less than 24 hours. Patients demonstrated improved median values in EDSS scores, improved muscle strength and bladder function	NCT03355365
	MSC-derived neural progenitors	Intrathecal	Phase I	The treatment was safe and well tolerated. No serious adverse events were reported. Minor adverse events were observed, including transient fever and mild headaches usually resolved in less than 24 hours. Patients demonstrated improved median values in EDSS scores, improved muscle strength and bladder function	NCT03355365
	Autologous MSC	Intravenous	Phase I	Autologous MSC transplantation in MS appears to be feasible, safe, and well tolerated. No treatment-related severe or serious adverse events were reported	NCT00813969

	Autologous adipose-derived MSC	Intravenous	Phase I/II	No results posted	NCT01056471
	Cryopreserved autologous BM adult MSC	Intravenous	Phase I/II	No results posted	NCT02495766
Parkinson's disease	Umbilical cord-derived MSC	Intravenous	Phase I	No results posted	NCT03550183
		Intrathecal and intravenous	Phase I/II	No results posted	NCT03684122
	Allogenic BM-derived MSC	Intravenous	Phase I/II	No results posted	NCT02611167
Amyotrophic lateral sclerosis	BM-MS	Intravenous or intrathecal	Phase I	During follow-up, the patients did not present any adverse effects. MSC transplantation evidenced to be safe and feasible	NCT01759797
	Adipose-derived MSC	Intravenous	Phase I	No results posted	NCT02492516
	Autologous MSC	Intrathecal	Phase I	No results posted	NCT01142856, NCT01609283
			Phase I	No results posted	NCT02987413
			Phase I/II	No results posted	NCT02917681
	iPSC of ALS somatic cells	Intravenous	Phase I/II	No results posted	NCT02290886
		In vitro study	Case control	iPSC were differentiated into motor neurons	NCT00801333
	Mesenchymal BM stromal cells secreting neurotrophic factors	Intramuscular or intrathecal	Phase I/II	During follow-up period, treatment was safe and well tolerated. The patients presented a reduction in the rate of progression of the forced vital capacity and ALS FRS-revised score following intrathecal or intrathecal + intramuscular injection	NCT01777646 and NCT01051882

(continued)

Table 14.1 (continued)

Disease	Stem cell type/target	Delivery method	Phase	Outcome	Clinical trial ID
Alzheimer's disease	Autologous adipose-derived MSC	Intrathecal	Phase II	No results posted	NCT02017912
	Autologous adipose-derived MSC	Intrathecal	Phase II	No results posted	NCT03268603
	Autologous BM MSC	Intrathecal	Phase I/II	18-month follow-up showed that the transplantation was safe and well tolerated. In the second phase, MSC slowed down disease progression measured by the ALS Functional-Rating Scale, forced vital capacity and weakness scale parameters	NCT03828123
	Human umbilical cord blood-derived MSC	Hippocampal and right precuneus injections	Phase I	MSC transplantation by stereotactic injection was safe, feasible, and well tolerated	NCT01297218
	Allogenic MSC	Intravenous	Phase I	No results posted	NCT02600130
	hUC-MSC	Intravenous	Phase II	No results posted	NCT02833792
	Autologous adipose-derived MSC	Intraventricular	Phase I/II	No results posted	NCT02672306
	Human umbilical cord blood-derived MSC	Intraventricular	Phase I/II	No results posted	NCT02054208
	Autologous adipose-derived MSC	Intravenous	Phase I/II	No results posted	NCT03117738
	Autologous BM-MNC	Intra-arterial	Phase I/II	No results posted	NCT03172117
	Autologous BM-MNC	Intra-arterial	Phase I	Satisfactory clinical improvement was found. Serial clinical, laboratory, electroencephalogram, and imaging evaluations showed no procedure-related adverse events. Satisfactory clinical improvement occurred in 6/20 (30%) patients following 90 days. Eight patients (40%) showed a good clinical outcome	NCT00916266

Autologous MSC and neuro-induced MSC	Intravenous administration of MSC and intrathecal injection of neuro-induced MSC	Phase I/II	The treatment was safe and well tolerated, without presenting severe adverse effects. Some patients achieved remission or became responders to antiepileptic drugs	NCT02497443
Autologous adipose-derived MSC	Intrathecal	Phase I	No results posted	NCT03676569

ALS amyotrophic lateral sclerosis, *BM* bone marrow, *BM-MNC* bone marrow-derived mononuclear cells, *CNS* central nervous system, *EDSS* Expanded Disability Status Scale, *FRS* Functional Rating Scale, *HSC* hematopoietic stem cells, *hUC-MSC* human umbilical cord-derived mesenchymal stem cells, *IL-6* interleukin 6, *iPSC* induced pluripotent stem cells, *MNC* mononuclear cells, *MRI* magnetic resonance imaging, *MS* multiple sclerosis, *MSC* mesenchymal stem cells, *RRMS* relapsing-remitting multiple sclerosis, *TNF- α* tumor necrosis factor-alpha

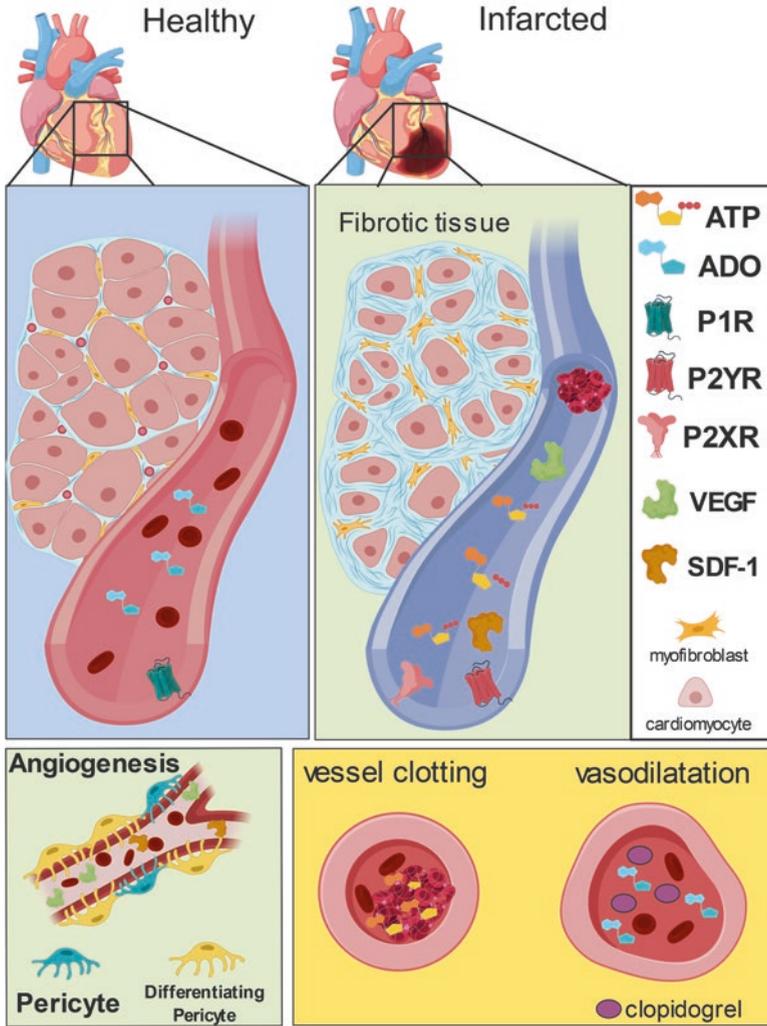


Fig. 14.1 Purinergic receptors and stem cell in cardiovascular system. In the healthy heart, ATP levels in coronary are rapidly converted in adenosine, promoting vasodilatation and correct blood flow through P1 receptor activation. Upon ischemia, due to clogging of coronary vessel by blood clot, blood and vessel cells secrete high amounts of ATP, VEGF, and SDF-1 in the extracellular medium, activating P2 receptors that at short timing cause apoptosis of cardiomyocytes (by P2X7 receptor) and higher rate of heart contractility (P2Y2 receptor), while at longer timing worsen fibroblast scar forming the fibrotic tissue. After ischemia, pericyte stem cells are activated by VEGF and SDF-1 to differentiate for originating alternative vessels for tissue blood support. P2Y12 receptor promotes the formation of blood clots, and the inhibitor of this receptor, named clonidogrel, is used as treatment. Since adenosine can also promote the vessel dilatation, both adenosine and clonidogrel can be used to avoid ischemia and strokes

while P2X7 receptor loss of function induced by another polymorphism provided a twofold protective effect against MS outcome [407].

Animal studies demonstrated that the administration of P2X7 receptor antagonist, Brilliant Blue G (BBG), during experimental autoimmune encephalomyelitis (EAE) chronic phase attenuated symptoms and tissue damage related to MS by increasing remyelination and restoring normal axon conduction velocity [404]. Accordingly, EAE induction in P2X7 receptor KO mice resulted in a decreased number of apoptotic lymphocytes in the CNS, increased expression of interferon- γ in the spinal cord [408], lower production of endocannabinoids, and reduced axonal damage [409] in comparison to wild-type animals. Moreover, P2X7 KO mice present lower frequency of EAE development and reduced astrocyte activation. Altogether, these studies indicate that P2X7 receptor inhibition may have beneficial advantages for MS.

Based on these evidences and in the effects induced by P2X7 receptor activation, the following mechanism was proposed for the involvement of P2X7 receptor in MS: increased cell death observed in MS results in a massive ATP release and consequent P2X7 receptor stimulation in microglia and macrophages, leading to IL-1 β production and cyclooxygenase-2 (COX-2) activation [402, 410]. Both processes intensify cell death and, therefore, MS symptoms. In accordance with the prospect, the use of the P2X7 receptor antagonists periodate-oxidized ATP and BBG for MS treatment was patented (EP1655032 B1).

During neuroinflammation, P2Y12 receptor stimulation in microglia seems to have a neuroprotective effect by preventing oxidative stress induced by apoptosis as well as to control pro-inflammatory cytokine release [411]. Accordingly, postmortem studies showed that P2Y12 receptor expression is decreased in highly demyelinated brain areas of MS patients [412]. Moreover, these receptors were found to be co-localized with myelin-binding proteins and astrocytes, which suggests a role for these receptors in remyelination [412]. Corroborating this data, P2Y12 receptor KO mice presented an enhanced EAE phenotype associated to increased levels of IL-17A in serum and enhanced number of T-helper cells (Th) in spleen and CNS [413]. Therefore, P2Y12 receptor stimulation supposedly has beneficial effects in MS treatment, since it is involved in Th-cell population balance and cytokine release control.

Previous studies suggest that A₁ receptor stimulation exerts beneficial effects in MS by decreasing tumor necrosis factor-alpha (TNF- α) levels, since this pro-inflammatory factor induces demyelination and is considered an initiating factor for MS development [414]. Accordingly, *postmortem* analysis demonstrated lower expression of A₁ receptors in glial populations of MS patients [415]. Corroborating these results, EAE induction in A₁ receptor KO mice leads to a severe progressive-relapsing form of MS associated to demyelination, axonal loss, and microglial activation [416]. In this study, chronic caffeine (P1 receptor antagonist) administration upregulated A₁ receptor expression in microglia along with reduction of EAE severity, which was further enhanced by concomitant treatment with adenosine amine congener, an A_{1A} receptor agonist [416]. Accordingly, caffeine decreased the risk to induce neuroinflammatory response and had neuroprotective and anti-inflammatory

effects in MS patients and an EAE animal model [417, 418]. Therefore, A₁ receptor stimulation is suggested to induce neuroprotective effects in MS.

Altogether, inhibiting neuroinflammatory response through P2X7 receptor blockade or A₁ receptor stimulation and increasing P2Y₁₂ receptor-mediated remyelination are alternative pharmacological targets for MS treatment. These receptors are also important for stem cell differentiation.

Disease-modifying therapies, including interferons, immunosuppressants, corticosteroids, and monoclonal antibodies, mainly targeting cells and mediators of the adaptive immune system, have been largely employed in MS treatment [419–421]. These therapies are efficient in reducing the frequency and intensities of relapses, but fail in inducing regenerative effects [420]. Stem cell therapy is an effective approach for regenerative medicine, and the use of HSC and MSC for MS treatment is under clinical evaluation [422, 423].

HSC, present in the peripheral blood and the BM, give rise to all hematopoietic cell lineages including erythrocytes, leukocytes, platelets, as well as to innate, and adaptive immune system cells [424]. HSC transplantation aims to replace or rebuild the patient's hematopoietic system, which is especially useful in autoimmune diseases as MS, since it may eliminate autoantigen-reactive T and B lymphocytes, reconstituting the self-tolerance [425]. Preclinical studies indicated that HSC transplantation could induce positive effects in animal models of MS, including EAE [426]. Accordingly, the use of HSC transplantation in patients with MS has been investigated by clinical trials (Table 14.2) since 1997 [472]. Studies have described the beneficial effects of HSC engraftment in MS patients, especially in RRMS [473–476]. However, HSC transplantation seems to be not effective in PMS [477], which is plausible since the neurological disability observed in this phase is mainly caused by axonal atrophy, and not by inflammatory process.

MSC derived from several adult tissues induced benefic effects in acute and chronic MS animal models (Table 14.2). Clinical data also supports the positive effect of MSC on MS treatment (Table 14.1). In 2007, a pilot study conducted in ten PMS patients showed a mild improvement in clinical symptoms of MS after autologous MSC treatment [478]. In 2009, the successful treatment of a MS patient with human umbilical cord-derived MSC (hUC-MSC) transplantation was firstly reported [479]. In these studies, autologous MSC treatment improved or stabilized clinical symptoms as well as avoided the progression of cerebral lesions in most of the patients, which was evaluated by MRI [478–480]. Moreover, MSC transplantation showed a safe profile since the observation of MS patients along one year detected no significant adverse effects [481]. Mild adverse events, such as headache, fever, nausea, vomiting, and weakness in the lower limbs, have been reported. These features highlight MSC transplantation as a promising alternative for MS treatment.

Both MS development and stem cell differentiation are influenced by purinergic signaling. In view of that, modulation of this system could help stem cell engraftment in MS patients.

Purinergic signaling modulates HSC self-renewal, expansion, and proliferation. In vivo and in vitro studies showed that ATP induces proliferation of HSC [482, 483]

Table 14.2 Preclinical findings with stem cell-based therapy

Disease	Stem cell source	Specie	Animal model	Delivery method	Outcome	References
Multiple sclerosis	Autologous BM-derived MSC	Mice	EAE	Intravenous	Ameliorated EAE, decreased inflammatory infiltrates, and demyelination in the CNS	[427]
			PLP-induced relapse remittent EAE	Intravenous	Ameliorated EAE, fewer relapses, decreased number of inflammatory infiltrates, reduced demyelination, and axonal loss through pathogenic inhibition of T and B cell responses	[428]
	Human BM-derived MSC	Mice	MOG and PLP-induced EAE	Intravenous	Functional recovery in chronic and relapsing-remitting EAE, reduced the extent of damage and increased oligodendrocyte lineage cells in lesion areas possibly via reduced T-helper cell 1 and 17 as well as increased IL-4-producing T-helper cell 2 cells	[429]
	Human adipose tissue MSC	Mice	MOG-induced EAE	Intraperitoneal	MSC derived from older donors are less effective than younger donors	[430]
			MOG-induced EAE	Intraperitoneal	MSC from obese donors could not suppress clinical signs of EAE and inflammation in the CNS	[431]
	Human adipose tissue MSC and human BM-derived MSC	Mice	MOG-induced EAE	Intraperitoneal	Ameliorated EAE, reduced tissue damage, inflammatory infiltrates, and serum levels of IFN γ and IL-12	[432]
	Human amniotic MSC	Mice	MOG-induced EAE	Intraperitoneal	Ameliorate EAE severity, attenuated inflammatory response (decreased production of IFN- γ , TNF- α , IL-1 β , and IL-17 in the spleen and CNS), and may promote the remyelination by increasing the production of neurotrophic factors	[433]

(continued)

Table 14.2 (continued)

Disease	Stem cell source	Specie	Animal model	Delivery method	Outcome	References
Parkinson's disease	Human iPSC	Rat	6-OHDA	Intra-striatal	Cells differentiated in midbrain dopaminergic neurons and improved the motor behavior	[434]
	BM-derived MSC supplemented with basic FGF	Rat	Rotenone	Intra-striatal	Reduced the motor impairments and prevented the death of dopaminergic neurons, while basic FGF was able to intensify the neuronal differentiation and its therapeutic effects	[435]
	Human umbilical cord mesenchymal stromal cells	Rat	Rotenone	Intra-striatal	The cells migrated to substantia nigra, prevented dopamine neurons from degeneration, dopamine terminals from loss and improved the motor performance	[436]
	hUC-MSC and VEGF-expressing hUC-MSC	Rat	Rotenone	Intra-striatal	Induced a reduction in motor dysfunction, restoration of tyrosine hydroxylase-positive cells in the striatum and substantia nigra. VEGF-expressing hUC-MSC improved these parameters and increased the dopaminergic differentiation of hUC-MSC in vivo	[437]
	Autologous iPSC	Monkey	MPTP	Intra-striatal	Improved motor function, induce a remarkable and complete reinnervation, protection of dopaminergic neurons, and extensive outgrowth	[438]
	iPSC cell clone O9	Rat	6-OHDA	Intra-striatal	The cells were positive to dopamine markers, increased tyrosine hydroxylase-positive cells, and improved motor function	[439]
	iPSC from monkeys	Rat	6-OHDA	Intra-striatal	Promoted behavioral recovery	[440]
	Autologous iPSC from <i>rhesus</i> monkey	Monkey	MPTP	In the striatum and substantia nigra	Cells survived for up to 6 months and could differentiate in neurons, astrocytes, and oligodendrocytes	[441]

Amyotrophic lateral sclerosis	Human MSC-expressing GDNF	Rat	SOD1-G93A	Bilateral muscle injections	Improved denervation of neuromuscular junctions, prevented the loss of motoneurons, increase survival in 28 days and disease progression	[442]	
	Human MSC	Mice	SOD1-G93A	Intra-parenchymally into the lumbar spinal cord	Prevented astrogliosis and microglial activation, delayed the loss of motor neurons and improved motor performance	[443]	
		Human	ALS patients	Multiple intraspinal thoracic injections	There was no evident disclosure, but four patients presented a delay in the decline of the forced vital capacity at ALS FRS score	[444]	
	Mice MSC	Mice	SOD1-G93A	Intravenous	Improved survival and motor functions. Decreased astroglial and microglial activation, accumulation of ubiquitin agglomerates in the spinal cord, and reverted disease progression	[445]	
	ALS patient MSC	Mice	SOD1-G93A	Intrathecal	Higher dose increased the survival and delayed the decline of motor functions and increased motor neurons number	[446]	
	Spinal neural progenitors derived from iPSC of ALS patients	Mice	Homozygous for severe combined immunodeficiency disease mutation	Cervical spinal cord	Induced loss of motor neurons and non-motor neurons and impaired the motor performance	[447]	
	Glial-rich NPC derived from human iPSC	Mice	SOD1-G93A	Lumbar spinal cord	Improved clinical scores of lower limbs and prolonged lifespan	[448]	
	NPC	Rat	A β -infused mice	Hippocampal	NPC migrated to the disease site, reduced microgliosis and the expression and secretion of pro-inflammatory cytokines	[449]	
	Alzheimer's disease						

(continued)

Table 14.2 (continued)

Disease	Stem cell source	Specie	Animal model	Delivery method	Outcome	References
	NSC	Mice	Aged triple transgenic mice	Hippocampal	Rescued spatial learning and memory deficits without altering A β or tau pathology	[450]
	BM-derived MSC	Rat	Isoproterenol-induced amnesic rats	Intravenous	Rapid regain of memory in rats treated with single intravenous administration of BM-derived MSC and oral administration of galantamine loaded solid lipid nanoparticles for 21 days	[451]
			Aged rat and ibotenic acid treatment	Hippocampal	Increased learning ability and memory in both age- and ibotenic acid-induced memory impairment	[452]
		Mice	Tg APP/PS1 mice	Hippocampal	Reduced the deposition of A β and memory impairment	[453]
			Tg APP/PS1 mice	In the lateral ventricle	Favored the neovascularization and diminished senile plaques, reduced cognitive deficits, and counteracted memory deficits	[454]
	hUC-MSC	Human	Tg2576 mice	Intravenous	Reduced hippocampal oxidative stress by decreasing the level of malondialdehyde, increasing the level of nitric oxide, enhancing the activity of superoxide dismutase and neuronal nitric oxide synthase	[455]
			Tg APP/PS1 mice	Intrahippocampal	Decreased A β plaques by cell migration toward A β deposits	[456]
			Tg APP/PS1 mice	Intrahippocampal	Decreased A β deposition and improved memory by activating M2-like microglia and modulating neuroinflammation	[457]
	Placenta-derived MSC	Human	A β -infused mice	Intravenous	Regulated neuronal death, neurogenesis, hippocampal glia cell activation	[458]
	Human MSC	Human	Tg APP/PS1 mice	Intravenous	Recovered the decreased neuronal development and inhibited neurite outgrowth.	[459]

Epilepsy	Human MSC with a knockdown of adenosine kinase	Human	Kainic acid mice model	Intrahippocampal	Alleviated acute seizure-induced cytotoxicity	[460]
	BM-MNC	Rat and mice	Pilocarpine mice model	Intravenous	Significantly reduced epileptogenesis	[461]
		Mice	Pilocarpine mice model	Intravenous	Decreased seizure frequency and duration and decreased levels of pro-inflammatory cytokines. Protected against neuronal loss and gliosis and stimulated the proliferation of new neurons	[462]
	Striatal precursor cells	Rat	Lithium-pilocarpine mice model	Intravenous	Reduced the frequency of seizures and improved the learning and long-term spatial memory impairments	[463]
			Kainic acid mice model	Intrahippocampal	Decreased the frequency of seizures	[464]
	NSC	Rat	Temporal lobe epilepsy model	Intrahippocampal	Reduced seizure frequency and duration, despite no changes occurring in cognitive function	[465]
			Kainic acid rat model	Intrahippocampal I	Decreased frequency of abnormal spikes. Most of the transplanted NSC differentiated into GFAP-positive astrocytes	[466]
	Medial ganglionic eminence interneuron progenitors	Mice	GABA _A receptor (α_4 subunit deletion)	In the motor cortex	Increased GABA release onto cortical pyramidal neurons and reduced seizure activity	[467]
	Autologous MSC	Rat	Pilocarpine rat model	Intrahippocampal	MSC displayed a long survival time, increased A ₁ receptors expression, and decreased A _{2A} receptors expression	[468]
	(continued)					

Table 14.2 (continued)

Disease	Stem cell source	Specie	Animal model	Delivery method	Outcome	References
	Sox 1-GFP ESC	Mice	Pilocarpine mice model	Intrahippocampal	The transplanted cells functionally integrated into epileptic hippocampal circuitry	[469]
	Human NSC	Mice	Lithium-pilocarpine mice model	Intravenous	Suppressed spontaneous recurrent seizure formation. Transplanted NSC differentiated into hippocampal GABA interneurons	[470]
	BM stromal cells	Rat	Pilocarpine rat model	Intravenous	Structural and functional improvement	[471]

6-OHDA 2,4,5-trihydroxyphenethylamine, *AD* Alzheimer's disease, *ALS* amyotrophic lateral sclerosis, *Aβ* amyloid beta, *BM* bone marrow, *BM-MNC* bone marrow-derived mononuclear cells, *CNS* central nervous system, *EAE* experimental autoimmune encephalomyelitis, *FGF* fibroblast growth factor, *FRS* Functional Rating Scale, *GABA* γ -aminobutyric acid, *GDNF* glial cell line-derived neurotrophic factor, *GFAP* glial fibrillary acidic protein, *hUC-MSC* human umbilical cord-derived mesenchymal stem cells, *IFN- γ* interferon gamma, *IL-1 β* /4/12/17 interleukin 1 β /4/12/17, *iPSC* induced pluripotent stem cells, *MOG* myelin oligodendrocyte glycoprotein, *MPTP* 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, *MSC* mesenchymal stem cells, *NPC* neural progenitor cells, *NSC* neural stem cells, *PLP* proteolipid protein, *SOD1-G93A* superoxide dismutase 1 mutant (glycine 93 changed to alanine), *Sox1-GFP ESC* Sox 1-green fluorescent protein embryonic stem cells, *Tg APP/PS1* double transgenic mice expressing a chimeric mouse/human amyloid precursor protein (Mo/HuAPP695swe) and a mutant human presenilin 1 (PS1-dE9), *Tg2576 mice* transgenic mice expressing the human amyloid precursor protein gene carrying the Swedish mutation (HuAPP695swe), *TNF- α* tumor necrosis factor alpha, *VEGF* vascular endothelial growth factor

and UTP promotes proliferation and migration of HSC [483, 484] while adenosine potentiates the stimulatory effect of growth factors and cytokines on HSC proliferation and differentiation [485]. In vitro data revealed that HSC store ATP in vesicles, releasing it through a calcium-sensitive manner, and present functional P2X receptors [482]. According to this study, pharmacological blockade of P2X receptors restrained hematopoietic progenitor proliferation, but not myeloid differentiation in cell culture [482]. A role of P2Y receptors in the differentiation and maturation of HSC has also been proposed, highlighting the involvement of P2Y11 receptor activation in the granulocytic differentiation of promyelocytes and in the maturation of monocyte-derived dendritic cells [486].

MSC proliferation may also be modulated by purinergic signaling. According to studies, treatment with the P2 receptor antagonist pyridoxalphosphate-6-azophenyl-2',4'-disulfonate (PPADS) or the selective P2Y1 receptor antagonist 2'-deoxy-N6-methyladenosine3',5'-bisphosphate (MRS 2179) increased human MSC proliferation [487].

Furthermore, P2X7 receptor activation has been associated with the increased neuroinflammatory response observed in MS [402, 410], which may lead to rejection after stem cell transplantation. Indeed, increased ATP levels and neuroinflammatory effects were associated to unsuccessful neural progenitor cell (NPC) engraftment [488].

In view of that, a possible treatment for MS could combine HSC/MS transplanted with drugs targeting purinergic receptors to achieve better results regarding cellular survival, differentiation, and integration, culminating in a more effective tissue repair. Additionally, a P2X7 receptor antagonist could be used to decrease neuroinflammatory response and thus the probability of rejection after stem cell transplantation. The role of purinergic receptors and stem cells in MS is shown in Fig. 14.2.

14.4 Parkinson's Disease

Parkinson's disease (PD) is classically associated with widely death of dopaminergic neurons of the substantia nigra pars compacta [489]. However, cell loss is also present in locus coeruleus, raphe nuclei, nucleus basalis of Meynert, dorsal nuclei of the vagus, and some other catecholaminergic neurons in the brain stem, such as in the ventrosegmental area [490]. Diagnosis of PD occurs with the onset of motor symptoms, such as bradykinesia, rigidity, and tremor, but the pathogenesis can be preceded by a prodromal stage of 20 years or more, which is characterized by non-motor symptoms [491]. Advanced stages of the disease present dysphagia, postural instability, falls, freezing of gait, and even psychosis [491]. The motor deficits are mainly attributed to degeneration of dopamine neurons of the nigrostriatal pathway [492]. Nevertheless, nowadays it is known that other cell type dysfunction generates hyposmia, autonomic dysfunction, hallucinations, depression, sleep disturbances, and cognitive decline [493].

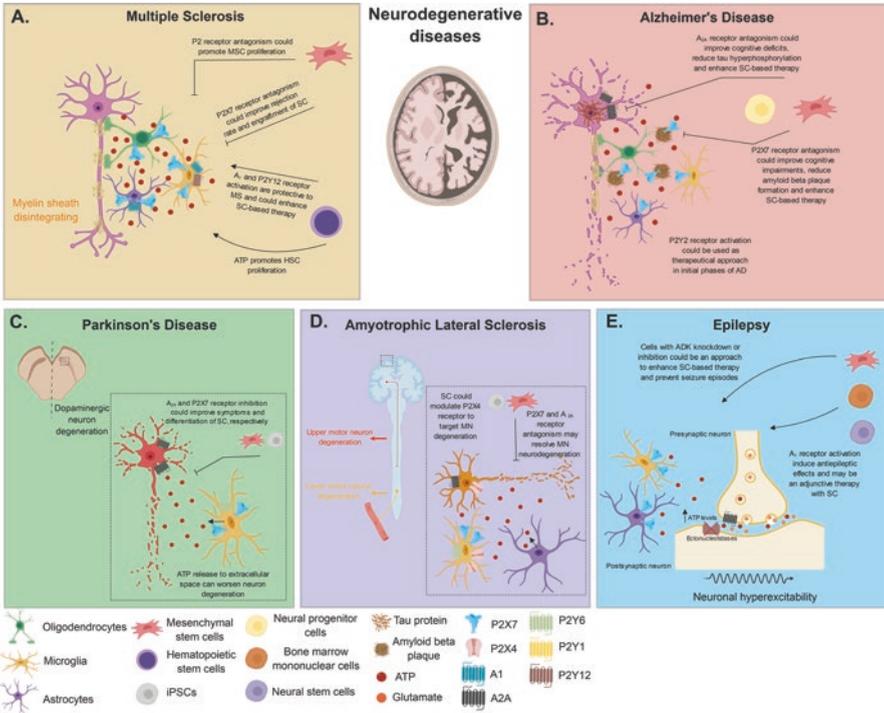


Fig. 14.2 Purinergic-based therapy can enhance the beneficial therapy based on stem cells regeneration in neurodegenerative diseases. (a) MS pathology presents an increase of P2X7 receptor expression in microglia, astrocytes, and oligodendrocytes. Increase of cell death results in a massive ATP release and consequent P2X7 receptor stimulation in microglia. Postmortem studies showed that P2Y12 receptor expression is decreased in highly demyelinated brain areas, and P2Y12 receptor stimulation in microglia is suggested to have a neuroprotective effect. Moreover, postmortem analysis also demonstrated lower expression rates of A1 receptors in the glial population of MS patients. (b) A_{2A} receptor expression is increased in cortical regions in AD pathology while A_{2A} receptor blockade may prevent cognitive deficits and tau hyperphosphorylation. Postmortem studies revealed an increase in expression of P2X7 receptors in Aβ plaques and around Aβ plaques. P2Y2 receptor stimulation may have a neuroprotective effect in AD. A₁ receptors are differentially expressed across brain areas. (c) Dopaminergic neuron death causes an extensively release of ATP. This purine can induce sustained P2X7 receptor activation which can generate a large pore in the plasmatic membrane, resulting in disruption of ion balance and cell death, worsening PD pathophysiology. A_{2A} receptor expression is increased in the early stages of the disease and antagonist seems a valuable treatment approach. (d) Studies have shown that P2X4, P2X7, and P2Y6 receptor expression levels were upregulated in the ALS pathophysiology. P2X4 receptor antibody specifically recognized degenerating motor neurons in rat model of SOD1G93A. Adenosine levels are increased in cerebrospinal fluid of ALS patients. A_{2A} receptor expression is increased in spinal cord of SOD1G93A mice and in postmortem human samples from ALS patients. A_{2A} receptor antagonism may induce a neuroprotective effect MNs. (e) In epilepsy, ATPase activity may be reduced in hippocampal slices. P2X7 receptor expression appeared to be upregulated in hippocampi of pilocarpine-induced chronic epileptic rats. A₁ receptor activation has been shown to induce antiepileptic effects. A₁ receptor activation may have anticonvulsant effects, whereas A_{2A} receptor activation appears to have proconvulsant effects. MSC: mesenchymal stem cells; HSC: hematopoietic stem cells; SC: stem cells; MS: multiple sclerosis; AD: Alzheimer's disease; MN: motor neurons; ADK: adenosine kinase; iPSC: induced pluripotent stem cells. Arrows: release of ATP

The pathophysiology is marked by the accumulation of the presynaptic protein α -synuclein with aggregate inclusions, named Lewy bodies [494]. Besides, PD features include mitochondrial dysfunction, neuroinflammation, oxidative stress, and excitotoxicity [495]. The mechanisms underpinning these events are not yet established yet. However, it is known that PD can be triggered by sporadic or familial nature. Despite genetic predisposition are rare forms of PD, there are 14 genes associated with disease onset [496], mainly leucine-rich repeat kinase 2 (LRRK2), α -synuclein (α Syn), β -glucocerebrosidase (GBA), and several PARKIN genes [494].

Neuroinflammation is well-established in the pathogenic process, once postmortem samples showed elevated levels of IL-1 β , IL-2, IL-9, and TNF- α in the striatum and increased levels of TNF- α in the substantia nigra [497–499]. Moreover, increased levels of IL-6 in plasma were linked to a higher risk to develop PD [500]. Several studies indicate immunological commitment in PD pathogenesis in patients and rodent models [501–504]; however, extracellular ATP signaling is also seen as a pro-inflammatory element that is being widely studied in PD.

Cell death causes extensive release of ATP into the extracellular matrix, prompting to more apoptosis, microglial activation, and cell survival mechanisms through P2 receptor activation [505]. This purine can induce sustained P2X7 receptor activation, generating a large pore in the plasmatic membrane, resulting in disruption of ion balance and cell death [506] and worsening the neurodegenerative state. A study showed that A-438079, a P2X7 receptor antagonist, prevented DA depletion in the striatum induced by the injection of 2,4,5-trihydroxyphenethylamine (6-OHDA), a rat model of PD [507]. Other studies demonstrated that treatment with BBG, a non-selective P2X7 receptor antagonist, could reverse the loss of dopaminergic neurons in the substantia nigra [508] as well as microglial activation [509] in animal models of PD. Moreover, pretreatments with BBG and A-438079 were able to decrease rotational behavior induced by apomorphine in the 6-OHDA rat model, indicating recovery from cell death. In vitro analysis indicated that BBG could control synaptotoxicity, neurotoxicity, and gliosis [510]. However, these neuroprotection mechanisms demonstrated through inhibition of P2X7 receptors were not reproducible in a work that used in vitro and in vivo models of PD based on P2X7 receptor KO mice. This was possibly due to compensatory mechanisms, such as increase in P2X4 receptor expression levels [511].

Recently, it was evidenced that sporadic PD patients presented higher gene expression of P2Y6 receptors in peripheral blood MNC when compared with control individuals and multiple system atrophy patients [512]. Thus, P2Y6 receptor expression may be explored for a differential diagnosis distinguishing PD and multiple system atrophy [512].

Another receptor with great evidence of involvement in PD is the A_{2A} receptor, exerting excitatory actions [513] in the basal ganglia, where it is prominently expressed, and facilitating the release of glutamate and regulating dopamine neurotransmission [514, 515]. The A_{2A} receptor forms a heteroreceptor with the D2 dopaminergic receptor in the striatum. This functional heterodimer has an allosteric inhibitory interaction, once adenosine binding to the A_{2A} receptor decreases the affinity of dopamine to D2 receptors [515]. This receptor revealed increased expression in the early stages of the disease [516]. A_{2A} receptor KO in the α -synuclein PD model

prevented the decrease of striatal dopamine content and the loss of dopaminergic neurons [517]. In the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model, antagonism of A_{2A} receptor reestablished the ability of microglia to respond to injury, exerting neuroprotective effects possibly through modulation of microglial process extension in response to cell death [518]. These findings are summarized in Table 14.2.

Based on the well-established A_{2A} receptor mechanism in turning down dopamine release in the one of most affected brain structures in PD and promising data obtained in preclinical studies, several clinical trials emerged to test the therapeutic potential of A_{2A} receptor antagonism in PD patients. Preladenant, a potent and selective A_{2A} receptor antagonist, was tested in phase IIb and phase III trials. The appearance of symptoms and motor function fluctuations were reduced in the phase IIb study [519]. However, in the phase III trial preladenant had no efficacy in improving symptomatology compared to placebo as monotherapy [520] or adjunctive therapy with levodopa [521]. Other studies with A_{2A} receptor antagonists, such as V81444, are in progress to test safety and potential modulatory effects in cognition function and blood flow (NCT02764892). Istradefylline, a selective A_{2A} receptor antagonist, showed several promising results that present efficacy and safety to be used with levodopa or other anti-Parkinsonian therapies [522, 523]. Several phase II and phase III studies are being conducted to show efficacy mainly as adjunctive treatment with levodopa/carbidopa. In a 12-week phase II study, it was demonstrated that istradefylline was effective to reduce the “off” state, without an increase in the “on” state, and was tolerated as treatment together with levodopa [524]. Other phase II studies are in progress to verify if istradefylline at 20 mg/day or 40 mg/day concentrations could be effective in the treatment of early or advanced PD, as adjunctive therapy or monotherapy (clinicaltrials.gov number NCT00455507, NCT00456586, NCT00199355, NCT00199433, NCT00250393). Moreover, phase III trials also are being conducted to test the potential adjunctive therapy of 10 mg/day, 20 mg/day, or 40 mg/day istradefylline doses in moderate to severe PD (clinicaltrials.gov number NCT00955526, NCT00199407, NCT00199394, NCT00199420, NCT01968031, NCT00203957) and with the intention to evaluate long-term tolerability and safety (NCT00199368, NCT00955045, NCT02610231, NCT00957203). In fact, istradefylline was approved in Japan as an adjunctive treatment with levodopa, demonstrating the capacity to enhance the anti-parkinsonian propriety of this currently used drug [525]. These results are summarized in Table 14.1. Moreover, there are also clinical trials in initial phases, which are testing other A_{2A} receptor antagonists for PD treatment, and those that were discontinued, such as trials based on preladenant and vipadenant application [526, 527].

Little is known about the functional role of other purinergic receptors in PD. There are evidences that P2Y1 and P2X1-6 receptor subtypes are expressed in rat brain slices of substantia nigra and ventral tegmental area [528]. Finally, a study based on pharmacologic blockade or genetic knockdown of P2X1 receptor expression showed inhibition of ATP-induced α -synuclein accumulation and/or aggregation in an in vitro model [529].

MSC are being applied in PD models due to their regenerative properties. Growth factors, such as basic FGF, can enhance neuroprotection and expansion and

differentiation of multipotent stem cells [435]. Using these tools, rotenone-induced hemiparkinsonian rats were transplanted with human BM-MSC supplemented with basic FGF [435]. MSC treatment reduced motor impairments and prevented death of dopaminergic neurons, while basic FGF intensified neuronal differentiation and its therapeutic effects [435]. In the same animal model, hUC-MSC were transplanted into the corpus striatum and migrated to the substantia nigra in vivo [436]. These cells prevented degeneration of dopamine neurons and terminals and improved the motor performance on the apomorphine test [436]. In another work of Xiong and collaborators using rotenone model, the striatal infusion of hUC-MSC or hUC-MSC expressing vascular endothelial growth factor induced a reduction in motor dysfunction and restoration of tyrosine hydroxylase (TH)-positive cells, a marker of dopaminergic neurons, in the striatum and substantia nigra [437]. Vascular endothelial growth factor-expressing hUC-MSC enhanced the behavioral performance and neuroprotection, as well as increased the dopaminergic differentiation of hUC-MSC in vivo [437].

Dopamine progenitors derived from human iPSC were transplanted into 6-OHDA injured rats. The cells survived and differentiated in midbrain DA neurons, as well as improved motor behavior [434]. Similar results were found in vitro cultured iPSC lineage. These cells grafted into the striatum, inducing an increase in tyrosine hydroxylase-positive cells and also expressing other dopamine markers, and improved motor function in the same animal model [439]. Midbrain dopaminergic neurons derived from monkey iPSC promoted behavioral recovery when transplanted into striatum of 6-OHDA rats [440]. Recent studies advanced to autologous transplantation of iPSC-derived NPC into rhesus monkey injured with MPTP [441]. It was demonstrated these cells survived for up to 6 months and could differentiate in neurons, astrocytes, and oligodendrocytes [441]. Autologous transplantation of iPSC into the putamen of MPTP-injured cynomolgus monkey was tracked for 2 years [438]. In this study, iPSC-derived midbrain-like dopaminergic neurons engrafted and survived, improved motor function, and induced remarkable and complete reinnervation, protection of dopaminergic neurons, and extensive outgrowth in a nonhuman primate model of PD [438].

Clinical translation to verify the therapeutical potential of stem cells engraftment in the treatment of PD patients is still growing. There are few evidences of clinical trials hitherto. Currently, more clinical trials in initial processes are being conducted, as demonstrated in Table 14.1. Figure 14.2 summarizes the role of purinergic receptors and stem cells in PD.

14.5 Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease which presents the death of upper motor neurons (neurons that project from the cortex to the brainstem and spinal cord) and lower motor neurons (neurons that project from the brainstem or spinal cord to muscle) [530, 531]. This loss of neurons causes progressive paralysis of voluntary muscles presenting spasticity and atrophy [530], and

commonly at final stages, the patients exhibit respiratory insufficiency and die from respiratory failure [532]. Besides the motor symptoms, ALS patients can also present cognitive alterations and psychiatric disorder comorbidities, such as schizophrenia or bipolar disorders [530]. Commonly, the patient dies within 2–5 years from diagnosis [533]. U.S. Food and Drug Administration (FDA) has approved riluzole as treatment that was shown to prolong patient survival by 2–3 months with increased effectivity at early stages of disease [534, 535].

ALS is exhibited mostly as a sporadic disease, while approximately 10% of patients are affected with the familial form [536]. The most common genetic mutations are located in the genes encoding superoxide dismutase 1 (SOD1), C9ORF72, sarcoma translated in liposarcoma (encoding RNA-binding protein FUS, also known as TLS), and TARDBP (encoding TAR DNA-binding protein 43, TDP-43). These genes are responsible for up to 70% of all cases of genetic inheritance [532, 537]. Besides these molecular identifications, the etiology of motor neurons' death is unknown. Evidences have pointed to pathophysiological mechanisms that comprehend mitochondrial dysfunction, oxidative stress, aberrant RNA metabolism, dysfunctions in protein folding, glutamatergic excitotoxicity, axonal structure and function, oligodendrocyte degeneration, impairment in growth factors, and inflammation [532, 538]. Clinically, the neuropathological hallmark of ALS is the aggregation and accumulation of ubiquitylated protein inclusions in motor neurons [532].

There is an important association between ALS and neuroinflammation. Several studies demonstrate the presence of activated microglia and macrophages of brain parenchyma, as well as infiltrates of lymphocytes in motor cortex, brainstem, corticospinal tract, and anterior horn of the spinal cord [539–541]. Another study showed that ALS patients presented an increase of microglia activation specifically in supplementary motor areas in the motor cortex [542]. Preclinical studies evidenced a deficit in microglia number and no differences in astrocytes number at presymptomatic stages and an increase in subpopulations of activated microglia and glial fibrillary acidic protein (GFAP) labeling at early-symptomatic stage [543]. These data demonstrate that glial cells are responsible for initiating the process leading to motor neuron loss [543]. Moreover, several studies indicate that microglial responses are essential for the neuronal damage in ALS rodent models [544–547].

Microglial cells express mainly P2X4, P2X7, P2Y6, P2Y12, and P2Y13 receptors, which drive activation, chemotaxis, and migration processes [548]. A study using cells of SOD1^{G93A} mice, a familial animal model of ALS, found that P2X4, P2X7, and P2Y6 receptors were upregulated in the immortalized culture and primary microglial cells from these animals [549]. BzATP, a preferential agonist of P2X7 receptor, enhanced the microglial polarization to reactive state and increase the TNF- α and COX-2 levels. In this sense, microglial cells that expressed SOD1^{G93A} and had been preactivated with BzATP were neurotoxic. Corroborating these results, all mechanisms were prevented by P2X7 receptor antagonists, evidencing the important role of P2X7 receptor in the microglial involvement in ALS pathophysiology [549]. Besides, P2X7 receptor stimulation in SOD1^{G93A} microglia cells enhanced oxidative stress, which was decreased by reactive oxygen species inhibitors [550]. Beyond the microglial role, SOD1^{G93A} astrocytes present increased

extracellular ATP signaling, maintaining the microenvironment aberrantly activated [551]. P2X7 receptor activation in spinal cord astrocytes promoted motor neuron death, while pharmacological inhibition of P2X7 receptor or modulation of ATP levels by treatment with apyrase restored the neurodegeneration [551]. In postmortem spinal cords of patients with sporadic ALS, microglial cells/macrophages presented augmented P2X7 receptor and COX-2 immunoreactivities [402].

Transgenic SOD1^{G93A} mice treated with an allosteric activator of P2X4 receptors extended mice lifespan in almost 10% [552]. P2X4 receptor antibody specifically recognized degenerating motor neurons in the rat model of SOD1^{G93A}, demonstrating a valuable tool to identify neurodegeneration in this model [553]. Similarly, P2X4 receptor antibodies were able to recognize the misfolded mutant SOD1^{G93A} expressed only in degenerating neurons. When the antibody was administered intraventricularly, it promoted microglial and astrocyte activation [554]. These data suggest a relevant participation of P2X4 receptors in promoting neuroinflammation.

Beyond P2X receptors, adenosine receptors seem to play an important role in the ALS pathophysiology. Adenosine levels are increased in the cerebrospinal fluid of ALS patients [555]. Another clinical data points to systemically adenosine mechanism through the A_{2A} receptor, once its expression is upregulated in lymphocytes from ALS patients [556]. In a preclinical study using spinal cord cell culture, A_{2A} receptor antagonism induced a neuroprotective effect in motor neurons possibly through reduction of TrkB receptor activation [557]. This neuroprotective effect could be explained by the presence of brain-derived neurotrophic factor in ALS pathogenic processes [557, 558]. Ng and collaborators demonstrated that A_{2A} receptor expression increased in spinal cord of SOD1^{G93A} mice at symptomatic stages and in postmortem human samples from patients with ALS at the end stage [559]. Moreover, *in vitro* analysis evidenced death of ESC-derived motor neurons under treatment with adenosine. Corroborating these results, A_{2A} receptor antagonism and partial genetic deletion (A_{2A}AR+/-) prevented ESC-derived motor neuron death and slowed down disease progression in the SOD1^{G93A} animal model [559]. Further, treatment with an agonist of A_{2A} receptors led to enhancement of neuromuscular transmission at the presymptomatic stage and had no effect at the symptomatic phase in SOD1^{G93A} mice [560]. In contrast to the previous report, chronic caffeine intake reduced SOD1^{G93A} mice survival, impaired motor performance, and promoted down-regulation of A_{2A} receptor in the wild-type mice spinal cord [561]. The observed effects could be due to the nonselective antagonist of the A_{2A} receptor by caffeine. However, these conflicting results require further investigations in this field.

There is growing number of literature regarding therapeutic effects of stem cells to revert neurodegeneration processes. Due to the substantial loss of motor neurons in ALS pathophysiology, many preclinical and clinical studies have been carrying out with the aim of counteracting this neurodegeneration. A preclinical study used human MSC constructed to secrete glial cell line-derived neurotrophic factor (GDNF), which were transplanted bilaterally into three muscles type [442]. The transplanted cells survived and released GDNF into skeletal muscle, improving the denervation of neuromuscular junctions, preventing the loss of choline acetyl-transferase-positive

motor neurons and increasing survival in 28 days and disease progression in SOD1^{G93A} rats [442]. Another study based on injection of human MSC into cisterna magna and intraparenchymally into the lumbar spinal cord in SOD1^{G93A} mice revealed that only lumbar spinal transplantation prevented astrogliosis and microglial activation, delayed the loss of motor neurons, and improved motor performance [443]. SOD1^{G93A} mice intravenously administrated with allogeneic MSC presented improved survival and motor functions and decreased astroglia and microglial activation, TNF- α and IL-1 β expression, as well as accumulation of ubiquitin agglomerates in the spinal cord [445]. Moreover, MSC treatment reverted disease progression parameters [445]. A different type of preclinical study transplanted human MSC from ALS patients into the cisterna magna of SOD1^{G93A} mice, and the higher cell concentration increased the survival and delayed the decline of motor functions, as well as increased motor neurons number [446].

A different cell type for promising cell transplantation treatment is the iPSC. In order to comprehend the ALS pathology, iPSC-derived NPC from sporadic ALS patients were transplanted into the spinal cord of homozygous mice for severe combined immunodeficiency disease mutation [447]. NPC from ALS patients differentiated into astrocytes and induced loss of motor neurons and non-motor neurons and impairment in the motor performance. These data demonstrated the involvement of glial cells in the pathogenic process and that neurodegeneration is not restricted to motor neurons, once it is preceded by non-motor neuron death [447]. To study the therapeutic properties of iPSC, glial-rich NPC derived from human iPSC were transplanted in lumbar spinal cord of SOD1^{G93A} mice, improving clinical scores of lower limbs and prolonged lifespan [448]. Summarized preclinical findings are presented in Table 14.2.

Autologous transplantation of MSC through intraspinal injections at thoracic level, performed in nine ALS patients, was monitored for four years [444]. Although there is no definitive conclusion, the patients did not present toxicity, abnormal cell growth, or negative reactions, and four patients presented a delay in clinical scores measured by forced vital capacity at the ALS functional rating scale score [444]. A recent phase I clinical trial was performed with autologous MSC transplantation in ALS patients to verify the safety and feasibility of intravenous or intrathecal injections [562]. There were no reports of adverse events during the follow-up period, indicating that BM-MSc engraftments are safe and feasible [562]. Results from 23 ALS patients of a phase I/IIa clinical trial showed the safety of a single intrathecal injection of autologous MSC by lumbar puncture [563]. Moreover, 18 months of follow-up indicated beneficial effects in some patients: a reduction in ALS functional rating scale 3 months after application, persisting for up to 6 months, and remaining stable in forced vital capacity and weakness scale parameters [563]. Another approach to study stem cell-based therapy was conducted by Petrou and collaborators, who performed phase I/II and IIa clinical trials using autologous MSC-secreting neurotrophic factors (MSC-NTF) [564]. Initially, MSC-NTF treatment was safe and well tolerated. In the phase I/II, ALS patients, who received intrathecal injections, showed an improvement in the monthly progression of ALS functional rating scale score and forced vital capacity. However, these beneficial

effects were not observed in patients who received injections intramuscularly. In the phase IIa trial, the patients received MSC-NTF via intrathecal and intramuscular administrations and the treatment had a more substantial improvement in the ALS functional rating scale and forced vital capacity [564].

Several clinical trials are being conducted to establish the potential therapeutic properties of MSC and iPSC. Phase I and II studies are described in Table 14.1, while several other trials are underway and do not have final results yet.

It is already known that purinergic receptors play an important role in stem cell development, migration, and differentiation. Few studies show the role of P2X7 receptors on embryo development in vivo, although it is demonstrated in vitro that the P2X7 receptor agonist BzATP induced proliferation of ESC [565]. However, during the differentiation phase, P2X7 receptor inhibition enhanced neuroblast differentiation [565]. In mouse NPC cultures, BzATP promoted apoptosis and necrosis, which was reverted by ablation of P2X7 receptor expression [488]. Thus, it was proposed that these cytotoxic properties might be balancing NPC numbers and subsequently number of neurons [566]. Functions of A_{2A} receptor in stem cell mechanisms are controversial, once its activation inhibited oligodendrocyte progenitor differentiation [567] and genetic deletion impaired proliferation and differentiation of mouse BM-MSC [568]. Recent studies indicate that the A_{2A} receptor is playing a role in neurogenesis, as its stimulation was able to promote neurogenesis and reduced neuronal damage [569]. In this sense, A_{2A} receptor genetic ablation induced cognitive impairment with a decrease of neuronal proliferation in the hippocampus and synaptic protein expression [570]. However, this mechanism is not totally understood, once pharmacological inhibition of A_{2A} receptor also enhanced hippocampal neuroblast proliferation [571].

In this sense, P2X7 and A_{2A} receptor functions are altered in PD and ALS pathogenesis as aforementioned. Thus, therapy based on purinergic receptor activity modulation may act together with stem cell-based therapy, in order to improve stem cell integration and survival after transplantation. The role of purinergic receptors and stem cells in ALS is represented in Fig. 14.2.

14.6 Alzheimer Disease

Alzheimer's disease (AD) affects about 15 million of people worldwide [572]. In the United States of America, more than four million people are suffering from AD, and there is an estimation that this prevalence will triplicate in 2050 [572]. Clinical symptoms of AD patients comprise progressive deterioration of cognitive function, memory loss, psychiatric disorders, dementia, and loss of the ability to learn, to make judgments, and even to communicate [573, 572].

The major AD features are the accumulation of extracellular senile amyloid-beta ($A\beta$) plaques and intracellular neurofibrillary tangles [574, 575]. These plaques appear years prior to AD symptom appearance. The $A\beta$ plaque is generated by cleavage of the amyloid precursor protein (APP) at the N- and C-terminal end by

β - and γ -secretase, respectively [576]. Two types of A β peptides are produced by γ -secretase cleavage: 40 or 42 amino acids long, named A β 40 and A β 42, respectively [575]. This amino acid differences lead to A β 42 precipitation and generate fibrils more easily than A β 40 does [577]. Concerning the total secreted A β , only 10% is A β 42 (Vassar and Citron 2000), although in vitro experiments have shown that this fraction is increased during pathology [573, 578]. An interesting study using transgenic mice expressing both types of A β proved that A β 42 promoted amyloid deposition whereas A β 40 was able to inhibit deposition [579].

Several evidences indicate the involvement of P1 receptors in AD, mainly A₁ and A_{2A} receptors. *Postmortem* analysis showed that A₁ receptor expression levels are significantly reduced in the hippocampus of AD [580–582], whereas the expression of these receptors are upregulated in frontal cortex both in early and advanced stages of AD [583]. Studies showed that the loss of A₁ receptors is responsible for cell death in the CA1 region [584, 585]. Accordingly, A₁ receptor activation induced tau phosphorylation and its translocation toward the cytoskeleton of human neuroblastoma cells (SH-SY5Y cells) that naturally express A₁ receptors [586], which would be related to AD progression. However, the activation of A₁ receptors in cultured cells impaired the formation of A β fragments found in neurofibrillary tangles [586], which suggests that A₁ receptor stimulation could slow down the progression of AD-related neurodegeneration. Therefore, the role of A₁ receptors in AD is controversial and requires additional studies to be clarified.

A_{2A} receptor expression is increased in cortical regions in animal models [587] and in cortical tissues of patients with AD [583, 586]. According to studies, A_{2A} receptor blockade may prevent memory and cognitive impairment observed in AD. Oral administration of a selective A_{2A} receptor antagonist improved spatial memory and reduced tau hyperphosphorylation in mice [588]. The administration of either caffeine or an A_{2A} receptor antagonist prevented the memory deficits induced by intracerebroventricular injection of A β in mice [589]. In addition, administration of A β in A_{2A} receptor knockout mice did not evoke any learning deficits or synaptotoxicity [587]. In view of that, diminished A_{2A} receptor activity is associated to improved cognitive function in vivo.

P2 receptors, especially P2X7 and P2Y2 receptors, are also involved in AD. The P2X7 receptor has been largely described in the literature as a key stimulator of neuroinflammatory response, which is involved in the development and progression of AD. Postmortem studies revealed increased expression of P2X7 receptors in A β plaques of AD patients [590] and around A β plaques in a transgenic mouse model of AD [590]. Accordingly, the intracerebral injection of amyloidogenic peptide increased P2X7 receptor levels in fetal human microglial cell culture [590]. Interestingly, the treatment with A β 42 increased intracellular calcium concentration, release of ATP, IL-1 β , and IL-18, and plasma membrane permeability in microglial cell culture from wild type, but not from P2X7 receptor KO mice, suggesting that the neuroimmune response induced by A β depends on P2X7 receptors [591]. Corroborating these results, systemic administration of the P2X7 receptor antagonist BBG decreased spatial memory impairment and cognitive deficits induced by intrahippocampal injection of A β [592] and diminished the formation of

A β plaques in the hippocampus of transgenic mice used as AD model [593–595]. Altogether, these results suggest that P2X7 receptor-induced neuroinflammatory responses are involved in AD.

P2Y2 receptors are endogenously activated by ATP or UTP leading to the stimulation of Gq protein-mediated signaling [596] and increasing neuroplasticity [597–601]. UTP-mediated P2Y2 receptor stimulation increased the non-amyloidogenic processing of APP and neurite outgrowth [602–604], as well as enhancing the uptake and degradation of A β 1-42 in mouse microglia [456]. Accordingly, haploinsufficiency of P2Y2 receptor in a transgenic mouse bearing human APP with Swedish and Indiana mutations increased plaque load, enhanced A β levels in the cerebral cortex and hippocampus of transgenic mice, and led to neurological deficits within 10 weeks of age [605]. Moreover, the immunoreactivity of P2Y2 receptor, but not of P2Y4 or P2Y6 receptors, is selectively decreased in the parietal cortex of AD patients and is correlated with neuropathological scores and synapse degeneration [606]. Taken together, these results suggest that P2Y2 receptor stimulation may have a neuroprotective effect in AD.

Neurogenesis in adulthood is responsible for replenishing the neuronal tissue that is lost due to aging; thus, it is also involved in the establishment of the memory and new synaptic connections [607]. Impairment of NPC proliferation is directly connected to AD pathophysiology [573, 607–609] and has been already vastly reviewed [610–613], describing the hippocampus, cortex, and the SVZ as the main brain structures affected in AD.

As a matter of fact, most of the studies showed decreased neurogenesis in AD mouse models that carry A β plaques [614, 615], like mice overexpressing Swedish and Indiana human A β precursor protein mutations [616, 617]. Different forms of the A β 42 need high doses to impair neurogenesis; for instance, both monomeric and fibrillary A β 42 forms were described in this context [452, 613]. In addition, researchers also investigated the role of A β 42 plaques in vitro by using human NPC derived from patients reprogrammed iPSC [453, 457, 618, 619] and observed elevated rates of apoptosis in mature neurons with A β 42 accumulation [453, 613]. Since neurogenesis is intensively related to AD, many preclinical studies were based on treatment of AD animal models with stem cells and showed promising results, which are summarized on Table 14.2.

In particular, Ryu et al. reported migration of NPC to the site of the disease after cell transplantation into the brain of rats [449]. Moreover, NPC decreased microgliosis and secretion and expression of pro-inflammatory cytokines [449], leading to increased expression of MAP-2 (mature neuronal marker) and neuroprotection [449].

Blurton-Jones and colleagues showed that NSC transplanted into the hippocampus recover the spatial learning and memory deficits of aged 3xTg-AD mice [450]. Brain-derived neurotrophic factor secreted by NPC was responsible for the increased hippocampal synaptic density [450].

Besides NPC, MSC have also been tested in AD models [451–453, 455, 457, 459, 618, 620]. In this case, Misra and collaborators tested the combination of BM-MSc intravenous administration with oral administration of galantamine

hydrobromide (acetylcholinesterase inhibitor)-loaded solid lipid nanoparticles in rats with AD [451] resulting in memory improvement of the animals following 21 days of treatment. hUC-MSC were also used for cell transplantation in a mice AD model (Tg2576) [455] resulting in increased neurogenesis in the hippocampus and consequently progress in cognition [455]. Moreover, other studies proved the use of hMSC transplantation showing neuroprotective effects in AD mouse models [452, 619, 620].

Furthermore, Lee and colleagues showed chemo-attractive induction of microglia by CCL5 after MSC transplantation into the hippocampus [453]. Yang et al. previously differentiated MSC into neurons upon tricyclodecan-9-yl-xanthogenate treatment [457]. Deepening understanding, investigation about the influence of hMSC coculture with NSC from 5XFAD mice, reporting neurite outgrowth and neuronal differentiation by paracrine effects of activin A [459].

Besides preclinical trials, several clinical trials are being conducted to establish the potential therapeutic properties of stem cells for treatment of AD. The studies are described in Table 14.2, whereas several are underway and do not have final results yet (clinicaltrials.gov number NCT02600130, NCT02833792, NCT02672306, NCT02054208, NCT03117738, NCT03172117).

A phase I study (clinicaltrials.gov number NCT01297218) used hUC-MSCs for transplantation into mild-to-moderate AD patients by bilateral stereotaxic injection into the hippocampus. Some light adverse symptoms like dizziness, headache, and postoperative delirium were registered within 12 weeks [458]. Some clinical trials focus on allogeneic transplantation, turning it easier to get the access to MSC as well as avoiding immune rejection (NCT02600130, NCT02833792, NCT03117738). However, other trials focus on using cells that have better survival properties, like hUC-MSC compared to placebo (NCT02672306, NCT02054208, NCT03117738).

Figure 14.2 represents the role of purinergic receptors and stem cells in AD.

14.7 Epilepsy

Epilepsy is a disease caused by the occurrence of at least two seizures that comprises tongue biting, falling, incontinence, or concomitant epileptic abnormalities in the electroencephalogram [621]. Thus, it affects around 0.5–1.0% of the population [622]. A seizure occurs due to uncontrolled neuronal discharges and imbalance of synaptic excitation and inhibition [623] following hyperexcitability of the cerebral cortex neurons [622, 624].

Neurons present a transmembrane potential of -60 mV due to a high intracellular potassium concentration and a high extracellular sodium concentration [625, 626]. Membrane depolarization is promoted upon a misbalance in ion concentrations caused by the opening of transmembrane ion channels [625]. Sodium-potassium ATPase blockade impairs the chemical and electrical gradients promoting the seizures in epilepsy [625, 627]. Moreover, glial cells can also control extracellular ion concentration, affecting seizure activity [628, 629]. Therefore, both ion pumps

and glial cells can be potential targets for novel anticonvulsants [625]. In addition, inflammation of the central nervous system can also induce epileptogenesis [630, 631]. For instance, infiltration of inflammatory cells across the blood-brain barrier into the hippocampus can cause seizures [631, 632]. Interestingly, MS patients that have intracortical lesions, which cause considerable cortical inflammation, show increased risk for developing epilepsy [631, 633].

Previous investigations suggest the function of purinergic signaling in epilepsy. An animal model prone to seizures showed increased extracellular ATP levels, probably due to reduced ATPase activity in hippocampal slices [634]. Furthermore, it was described that microinjection of ATP analogs into the prepiriform cortex of rodents induces generalized motor seizures [635]. Intracellular calcium measurements revealed a biphasic response, indicating the existence of P2X7 receptors [636]. It was supposed that ATP could be facilitating epilepsy development through P2X7 receptors, once they appeared upregulated in hippocampi of pilocarpine-induced chronic epileptic rats [636, 637]. Further, immunoreactivity patterns of P2X7 were similar to mossy fiber sprouting at the dentate gyrus of epileptic animals [636]. These results imply that the P2X7 receptor may be involved in the pathophysiology of temporal lobe epilepsy [636].

It was postulated that adenosine receptors could modulate presynaptic excitability and epileptogenesis [638]. A₁ receptors are differentially expressed across brain areas: they are upregulated in the neocortex of *postmortem* brains of patients [639] and downregulated in the temporal cortex and hippocampus in postmortem brains of patients [640] and in the nucleus reticularis thalami of rats [641]. It was shown that hippocampal adenosine delivery can reduce the frequency of spontaneous seizures in chronic epileptic rats [642] and that selective adenosine A₁ receptor activation in a mice model of pharmacoresistant epilepsy blocks spontaneous seizures [643, 644]. Recently, it was reported that acute administration of 5 mg/kg of caffeine, a nonselective P1 receptor antagonist, significantly reduced seizure threshold [645]. The authors described that different doses of caffeine promoted different effects [645]. Apparently, the effect of higher caffeine doses is mediated through the nitric oxide pathway [645]. The capability of adenosine in seizure suppression could be due the inhibitory effect of A₁ receptors, which inhibit glutamatergic transmission [646].

The postictal state is an anomalous condition that occurs between the beginning of seizure and the return to the resting state of the patient [647, 648]. The release of adenosine can mediate seizure arrest and postictal refractoriness [646]. Extracellular adenosine levels in hippocampus during spontaneous-onset seizures in humans seem to be equivalent to those that have been reported to suppress seizure activity in animals *in vivo* [649]. In this study, electrodes were implanted for 10–16 days in the hippocampi of four patients to test, whether during the seizures extracellular adenosine reaches levels capable of depressing epileptiform activity [649, 650]. All the seizures propagated to the two hemispheres and adenosine concentrations reached 65 μM , which depressed epileptiform activity [649]. These data indicate that adenosine is capable of mediating seizure arrest and postictal refractoriness [649].

MSC can recruit other surrounding cells to help in tissue repair in CNS pathologies, such as stroke, traumatic brain injury, and epilepsy [651, 652]. Regarding epilepsy, a large number of articles demonstrate the supporting role of adenosine as an endogenous anticonvulsant agent involved in antiepileptic and antiapoptotic functions, also participation in neurogenesis promotion [653]. Although numerous adenosine agonists effective anticonvulsants in animal models of epilepsy, they often present serious systemic adverse events [654].

Promising data concerning the antiepileptic effects of adenosine, mainly through the A₁ receptor, led to an alternative strategy consisting of designing cells with adenosine kinase (ADK) expression knockdown. This strategy can provide large amounts of adenosine in loco, limiting its action to the foci of seizure [652]. In this sense, brain implants that release adenosine showed seizure [655] and epileptogenesis suppression [656]. Similarly, adenosine-releasing implants were effective in seizure suppression in a rat model of temporal lobe epilepsy, demonstrating that activation of central adenosine A₁ receptors suppressed seizure activity in a mouse model of drug-resistant epilepsy [642]. It was concluded that the local delivery of adenosine into the brain is effective to treat intractable seizures [642]. An interesting study that used different approaches to modulate ADK expression showed that genetic downregulation of this enzyme suppressed spontaneous recurrence of seizures, whereas overexpression evoked recurrent seizures in mice [657]. Fedele and collaborators generated a tool to the therapy based on stem cells for seizure modulation by disrupting both alleles of the ADK in mouse ESC [658]. ESC were differentiated into NPC and implanted into intrahippocampal fissure of rats [658]. The cells were able to produce a adenosine concentration, which was sufficient for seizure suppression [658]. In another work, these cells promoted anti-epileptogenic effects [656, 659]. Human adenosine-releasing stem cells and hMSC were engineered to reduce ADK expression and transplanted into the mice hippocampus prior to induction of status epilepticus [460]. These transplanted cells reduced the seizure duration by 35% and CA3 neuronal cell loss by 65% [460]. Similar approaches were used in other studies, showing that transplantation of cells from different sources, including mouse ESC, hMSC, and immortalized fibroblasts, also engineered to release adenosine, suppressed seizures [660].

As in other neurodegenerative diseases, stem cell-based therapies provide a tool for cell replacement in epilepsy treatment research. Ex vivo gene therapy can be performed, generating genetically modified cells to release therapeutic compounds, such as neurotransmitters and neurotrophic factors [643].

Several sources of multipotent stem cells for transplantation are being studied in animal models of epilepsy, including NSC and NPC. In a rat model of status epilepticus induced by kainic acid, Jing and colleagues found that hippocampal NSC transplantation reduced irregular spikes and hippocampal excitability as well as spontaneous recurrent seizures [466]. Moreover, most of the transplanted NSC differentiated into astrocytes [466]. In this same animal model, hippocampal engraftment of striatal precursor cells treated with FGF-2 and caspase inhibitor, that increase cell survival, reduced chronic recurrent seizures during 9–12 months after treatment [464]. This same group also injected NSC extracted from embryonic

ganglionic eminence into the hippocampus, diminishing seizure frequency and duration without improving cognitive function [465]. These cells, when transplanted in a GABA A receptor $\alpha 4$ subunit KO mice model of epilepsy, differentiated into GABAergic interneurons, increased GABA release in cortical pyramidal neurons and reduced seizure activity as well [467]. Moreover, human NSC transplanted intravenously into rats with status epilepticus induced by lithium-pilocarpine injection also presented anti-epileptogenic effects [470]. NSC differentiated into hippocampal GABAergic interneurons and terminated spontaneous recurrent seizures [470]. In pilocarpine-induced epileptic mice, transplanted mouse ESC-derived NPC differentiated into GABAergic interneuron subtypes expressing calcium-binding proteins parvalbumin, calbindin, or calretinin [469]. These neurons are the most affected ones in severe temporal lobe epilepsy, either by cell death induction or even by protective mechanisms, indicating a promising therapy for this condition [469].

Another source of multipotent stem cells used to overcome epilepsy symptoms in mice are BM cells. Mice injected with lithium-pilocarpine and intravenously treated with GFP⁺-BM-MNC presented a reduction on epileptogenesis, suppression of early spontaneous recurrent seizures, and improvement of cognitive impairments [461, 463]. Later, the same group reported that this treatment, besides reducing seizure frequency and duration, also decreased pro-inflammatory cytokine (TNF- α , IL-1 β , and IL-6) levels and increased anti-inflammatory cytokine IL-10 levels [462]. Finally, BM stromal cell transplantation also decreased the number of seizures of pilocarpine-induced rats [471]. All animal data are resumed in the Table 14.2.

Some clinical trials are being conducted to establish potential therapeutic properties of stem cell treatment of epilepsy. The studies are described in Table 14.1, although several trials are underway and do not yet have final results. A phase I study (NCT00916266) investigated the use of autologous BM stem cells for the treatment of medically refractory temporal lobe epilepsy patients. They evaluated seizure frequency, hippocampal volume, and cognitive performance. The results showed clinical improvement in 30% of the patients 90 days after treatment, and 40% of the patients had good clinical outcome [661]. Another study on phase I and II (NCT02497443) analyzed the use of autologous MSC to treat resistant symptomatic epilepsy. Patients received one intravenous injection of MSC and a following intrathecal injection of MSC previously induced to neuronal differentiation. Data showed that 30% of patients did not present seizures in the following 1 year or more and other 50% became responders to antiepileptic drugs. Researchers concluded that MSCs are safe and promising for cell therapy of antiepileptic drug-resistant patients with refractory epilepsy [662]. Finally, a recent phase I study (NCT03676569) evaluated the use of MSC derived from adipose tissue in patients with autoimmune-determined refractory epilepsy. Cells were administered by intrathecal infusion. The neurological status, brain MRI, cognitive function, and antiepileptic effects were monitored for 24 months, but no data is available yet.

It is known that MSCs have an important participation in the preservation of mesodermal tissue homeostasis throughout the adult body [390]. It is known that the inhibitory effect of adenosine system is impaired in epileptogenesis and that A_{2A} receptor activation by increased adenosine production is involved in MSC-promoted

effects. Thus, Huicong and coworkers [468] discovered that MSC transplantation into lithium-pilocarpine-induced epileptic rats improved the imbalanced expression between A_1 and A_{2A} receptors, highlighting the importance of purinergic receptors in cell therapy [468].

The involvement of purinergic receptors and stem cells in epilepsy is shown in Fig. 14.2.

14.8 Conclusion

Stem cell therapy has been consistently appealing in the past years, mainly due to the promise of recovering tissues with limited regeneration. Stem cells that overcome some ethical and religious concerns, like MSC and iPSC, propose a new alternative to cure diseases, mainly the most common ones that are responsible for high death rates or patients' disabilities. Cell therapy has been intensively studied and largely applied in clinical trials in the past decades; however, bone marrow transplantation for leukemia treatment is still the only successful existing cell therapy. The reasons for such low efficiency is the lack of direction of stem cell fate commitment, maintenance of the cells at the site of injury, or even the adverse effects caused by the secretion of some factors by the injected stem cells, including extracellular unphosphorylated nucleosides like adenosine and phosphorylated ones like ATP and ADP.

In this chapter, we focused on describing the correlation of purinergic signaling and stem cells in cardiovascular diseases (Fig. 14.1: ischemia and thrombosis) and in some neurological diseases (Fig. 14.2: multiple sclerosis, Parkinson's disease, amyotrophic lateral sclerosis, Alzheimer's disease, and epilepsy), highlighting their clinical applications. First, in the cardiovascular system, adenosine through activation of A_{2A} receptors is largely used as a vasodilator that contributes to avoid heart attack and stroke, while ATP favors agglomeration of platelets through P2Y12 receptor activation that can clog vessels, causing ischemia. ATP secreted upon ischemia enhances heart contractibility and cell death by activation of P2Y2 and P2X7 receptors, respectively. Activation of P2Y2 and P2Y6 receptors worsen the scar tissue formed by myofibroblasts, aggravating the heart status. Overall, adenosine signaling promotes a healthier heart, whereas ATP favors injury and inflammation. Moreover, some clinical trials show positive repair effect of stem cell and/or purinergic signaling modulators injection in cardiovascular diseases, such as rolofyl-line, ticagrelor, and clopidogrel.

In neurological diseases, P2X7 receptor blockade or A_1 receptor stimulation as well as increasing P2Y12 receptor expression mediated remyelination in MS. Caffeine intake and blockade of A_{2A} receptor in AD prevented the memory deficits, associating improved cognitive function in vivo to inhibited A_{2A} receptor activity. Meanwhile, A_{2A} receptor is responsible for the loss of striatal dopaminergic neurons in PD, and its istradefylline is already being tested in clinical trials. In the case of ALS, P2X7 receptor plays an important role in activating microglia and

causing inflammation that kills the motor neurons. Therefore, P2X7 and A_{2A} receptor functions are altered in PD and ALS pathogenesis imposing a promising therapeutic tool. In epilepsy, adenosine reduces seizures, mainly through A₁ receptor activity, which inhibits glutamatergic transmission.

The concomitant administration of MSC in cardiovascular system with purinergic P2Y₂, P2X7, and P2Y₁₂ receptor antagonists in the cardiovascular system under P2X7 and A_{2A} receptor blockage, and A₁ and P2Y₁₂ receptor stimulation, would enhance the success rate of cell therapy.

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Chapter 15

Plausible Links Between Metabolic Networks, Stem Cells, and Longevity



Magdalena Kucia and Mariusz Z. Ratajczak

Abstract Aging is an inevitable consequence of life, and all multicellular organisms undergo a decline in tissue and organ functions as they age. Several well-known risk factors, such as obesity, diabetes, and lack of physical activity that lead to the cardiovascular system, decline and impede the function of vital organs, ultimately limit overall life span. Over recent years, aging research has experienced an unparalleled growth, particularly with the discovery and recognition of genetic pathways and biochemical processes that control to some extent the rate of aging.

In this chapter, we focus on several aspects of stem cell biology and aging, beginning with major cellular hallmarks of aging, endocrine regulation of aging and its impact on stem cell compartment, and mechanisms of increased longevity. We then discuss the role of epigenetic modifications associated with aging and provide an overview on a most recent search of antiaging modalities.

Keywords Stem cells · Aging · IGF system · Sirtuins · VSELs · Imprinted genes · Caloric restriction

15.1 Introduction

Cellular senescence was initially described in a seminal study by Hayflick and colleagues (1961) [1] in which they observed that normal human fibroblasts had a limited capability to proliferate in culture [1–3]. Normal cells were able to enter a state of irreversible growth arrest after serial cultivation in vitro; meanwhile, cancer cells did not enter such growth arrest and proliferated indefinitely. It is well

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recognized that after cells reach the Hayflick number of divisions, their proliferative capability is exhausted that is reflected by shortening of telomeres [4–6]. Shortening of telomeres leads to telomere dysfunction and genetic aberrations and subsequently impacts cell proliferation that may end as replicative senescence [7, 8]. Telomeres are bound by a specific multiprotein complex known as shelterin [9]. A major function of this complex is to prevent the access of DNA repair proteins to the telomeres. Otherwise, telomeres would be “repaired” as DNA breaks leading to chromosome fusions. Due to their restricted DNA repair, DNA damage at telomeres is prominently persistent and highly efficient in inducing senescence and/or apoptosis [10]. Telomerase deficiency in humans is associated with premature development of pulmonary fibrosis, aplastic anemia, and dyskeratosis congenita, which involve the decrease of regenerative capacity of various tissues [11]. Aging of an organism is manifested by a progressive decline in cellular maintenance, and repair processes, resulting in the gradual loss of hemostasis and functionality of tissues/organs over time. At the cellular level, aging is often manifested as structural and functional alterations in cellular constituents such as DNAs, proteins, and lipids.

An important mechanism in the aging process is the generation of reactive oxygen species (ROS) that contribute to replication stress and oxidative DNA damage [12]. The ROS, generated in mitochondria as a product of oxidative phosphorylation, induce DNA damage including induction of the DNA double-strand breaks that can cause accumulation of mutations [13]. Accumulation of unrepaired or incorrectly repaired DNA lesions increases genomic instability that includes point mutations, translocations, chromosomal gains, and losses. It should be noted that hyaluronic acid, which is major constituent of stem cell niche, by intercepting ROS protects stem cells from oxidative damage by exogenous oxidants [13, 14].

Aging and some aging-related diseases are associated with impaired protein homeostasis or proteostasis [15]. All cells count on precise mechanisms that regulate protein homeostasis to maintain a stable and functional proteome. A progressive deterioration in the ability of cells to preserve the stability of their proteome occurs with age and contributes to the functional loss characteristic of old organisms [16]. Protein homeostasis involves mechanisms for the stabilization of correctly folded proteins – most prominently, the family of heat-shock proteins – and mechanisms for the degradation of proteins by the proteasome or the lysosome [17, 18]. All molecular chaperones and proteases, together with MOAG-4, that act through an alternative pathway, function in coordinated fashion to restore the structure of misfolded polypeptides or to remove and degrade them completely, thus preventing the accumulation of damaged structures and assuring the continuous renewal of intracellular proteins. A number of animal models support a causative impact of chaperone decline on longevity. In particular, transgenic worms and flies, overexpressing chaperones, are long-living [19, 20]. For example, overexpression of the small mitochondrial Hsp22 extends *Drosophila* life span and increases resistance to oxidative stress [19]. Also, mutant mice deficient in the carboxyl terminus of Hsp70-interacting protein (CHIP) [21] exhibit accelerated aging phenotype, whereas long-lived mouse strains show a marked upregulation of some heat-shock

proteins [22]. Chronic expression of unfolded, misfolded, or aggregated proteins contributes to the development of some age-related pathologies, such as Alzheimer's disease, cataracts, and Parkinson's disease [15].

15.2 Stem Cell and Aging

It would be impossible to summarize shortly the great extent of aspects of the biology of aging that pertain to stem cells. Here we briefly address how aging is manifested at the cellular level and how stem cell properties and function may change during aging. One hypothesis in regard how human age is so-called the "stem cell hypothesis" [23–25]. Accordingly, it is proposed that an essential mechanism of aging is that as stem cell become older, they either become quiescent or die or both. The human body relies on a variety of rare stem cells which function to replace somatic cells and tissues as they get damaged or are affected by pathological changes. Replacement of lost somatic cells progressively declines with age due to stem cell aging and attrition.

15.2.1 Hematopoietic Stem Cells

Advancing age is accompanied by a number of pathophysiological changes in hematopoietic system, resulting in a diminished production of adaptive immune cells [26] and in an increased incidence of anemia [27] and myeloid malignancies [28]. The ability of human HSC to give rise to primitive progenitors declines with age, suggesting a progressive loss of stem cell activity with age [29]. Studies on aged mice have revealed an overall decrease in cell-cycle activity. During mammalian ontogeny, hematopoietic precursor cells are first found in the yolk sac and then in the fetal liver [30]. Also human umbilical cord blood is a rich source of hematopoietic precursor cells [31], which most likely represent a developmental stage intermediate between fetal liver and adult bone marrow. The ability of human HSCs to give rise to progenitor cells declines during ontological transitioning from fetal liver, to umbilical cord blood, to adult bone marrow [32], suggesting a progressive deprivation of stem cell activity with age [29]. A retrospective analysis of 6978 bone marrow transplantations facilitated by the National Marrow Donor Program from 1987 to 1999 has been conducted to study the effects of various donor characteristics including possible effects of donor age on recipient outcome. Age was the only donor trait significantly associated with overall and disease-free survival [33]. It was demonstrated that the use of younger donors may lower the incidence of graft-versus-host disease (GVHD) and improve survival after bone marrow transplantation [33]. Overall, these studies suggest that the proliferative and regenerative potential of human HSCs decline with age and that declined stem cell activity is largely cell intrinsic [29]. This hypothesis has been corroborated in studies performed in animal models.

Inside of the hematopoietic system, the ratio of differentiated progenitors differs with age. Hematopoietic stem cells (HSCs) from both old humans and aged mice display an increased propensity to differentiate along myeloid rather than the lymphoid lineage [34, 35]. Such decline in lymphoid differentiation potential of HSC with age is not caused by a change in the differentiation potential of individual HSCs per se but rather by a preferential selection of distinct subset of HSCs over time [25, 36–38]. Importantly, the differential capacity of HSCs from old mice to give rise to lymphoid and myeloid progeny was found to be transplantable [29, 35].

Moreover, although pediatric leukemias tend to originate in lymphoid compartment, the leukemias that manifest in old individuals tend to be myeloid in origin. This latest result suggests that the changes in malignant capacity of hematopoietic progenitors mirror the change in lineage potential of HSCs during aging [34, 35, 39, 40]. Using a murine model of chronic myeloid leukemia, an adult-onset malignancy that arises from transformation of hematopoietic stem cells by the BCR-ABL(P210) oncogene, it has been demonstrated that young bone marrow cells that were transformed with BCR-ABL(P210) initiated both a myeloproliferative disorder (MPD) and B-lymphoid leukemia, whereas BCR-ABL(P210)-transformed old BM cells recapitulated the human disease by inducing an MPD with rare lymphoid involvement [41]. These data demonstrated that aging affects patterns of leukemogenesis and indicates that the effects of senescence on hematopoiesis are more extensive than previously appreciated [41].

15.2.2 Neural Stem Cells

Although most neurons are postmitotic, slowly cycling NSCs sustain neurogenesis in specific regions of the mammalian brain, the subventricular zone (SVZ) of the lateral ventricles and the subgranular zone (SGZ) of the dentate gyrus of the hippocampus [42–44] during adulthood. Adult NSCs in the SVZ generate neurons that migrate to the olfactory bulb to subsequently become granule and periglomerular neurons that are essential for the maintenance of the olfactory bulb, whereas neurons of granulate cell layer of the adjacent dentate gyrus are important for the memory and learning [25]. Activity of NSCs is believed to be important for sensory functions such as olfaction as well as cognitive functions as memory and learning [45]. Therefore, decline in both sensory and cognitive functions in the elderly implicates age-associated NSC decline. In fact, a number of NSCs decrease with age, which contributes to decreased neurogenesis [46, 47]. Using BrdU-labeling approach together with neural markers, it has been demonstrated that both the number of NSCs and their proliferative capacity decline with age in rodents [46, 48–50], in both subventricular zone of the lateral ventricle and the subgranular zone of the dentate gyrus in the hippocampus. The observed decline correlates with gradual loss of cognitive and olfactory functions [46, 47, 50]; however, it is unclear whether attenuated neurogenesis during aging is directly correlated to the neural stem cells or to the neurogenic niche. Compared with NSC from young rodent, NSCs from aged animals exhibit a marked decline in self-renewal capacity both in

in vitro neurosphere assays and in vivo tracing studies [25, 51]. The declining function of NSC with age has been linked with increased genomic instability [52] and induction of p16^{Ink4a} expression [53]. Additionally, the spectrum of tumor types in the brain changes with age [29], with neuroblastomas and medulloblastomas predominating in pediatric patients, and tumors of glial origin linked with aged individuals. It will be important to determine whether changes in developmental repertoire of NSC during ontogenesis indicate the predisposition to the different spectrum of brain tumors presented during different stages of life.

The molecular studies identified critical genes or signaling pathways that regulate the self-renewal of NSCs. It was demonstrated that HMGA2-deficient mice and FoxO3 knockout mice have reduced number of NSCs in central and peripheral nervous system [51, 54]. Both chromatin-interacting protein HMGA2 and FoxO transcription factors that are necessary for enhanced longevity of insulin pathway mutants in invertebrates [55, 56] play a role into the maintenance of NSC homeostasis with age. FoxO proteins modulate NSC homeostasis by regulating the expression of cell-cycle regulatory proteins, such as cyclin D1, polo-like kinase 2, and inhibitor of DNA binding 1 [57]. HMGA2 expression declines in stem cells, including HSC and NSC, with age [54, 58]. The association of FoxO3 polymorphism with longevity in humans has been reported, leading to hypothesis that FoxO3 also regulates life span in mammals [59–61]. The potential link between FoxO3 and longevity in multiple species will be discussed in the next section of this chapter. It remains to be determined whether these proteins play causal role in NSC depletion during normal aging.

15.2.3 Intestinal Epithelial Stem Cells

The mammalian intestine is covered by a single layer of epithelial cells that is renewed every 4–5 days [62]. The gut is anatomically divided into the small intestine and the colon. The intestinal epithelium is the most vigorously self-renewing tissue of adult mammals [63]. The intestinal epithelium is organized by crypts and villi and four differentiated cell types that reside within the epithelium — goblet cells, enteroendocrine cells, Paneth cells, and enterocytes [63]. Proliferative cells reside in the crypts of Lieberkuhn, epithelial invasions into the underlying connective tissue. The crypts harbor stem cells and their progeny, transit-amplifying cells. Transit-amplifying cells spend approximately two days in the crypt, in which they divide 4–5 times before they terminally differentiate into the specialized intestinal epithelial cell types [63]. Slow-cycling cells, as candidate intestinal epithelial stem cells (ISCs), have been described to reside at the +4 position relative to the base of the crypts [64–66]. Putative ISCs were described as Lgr5⁺ (*leucine-rich-repeat-containing G protein-coupled receptor 5*) cells that express also Ascl2, both targets of Wnt signaling, that reside not only in the intestine, but at the crypt base in the colon epithelium and at the gland base at the bottommost region of stomach epithelium [67, 68]. In the murine small intestine, ISCs are responsible for production of about 250 cells per crypt per day [64].

Age-related changes in the intestinal epithelium have been described [69, 70]. Studies of the murine gastrointestinal tract have shown that villi are generally larger but cellularity is reduced in old animals. The number of crypts also declines over time. These age-related histological changes appear to be accompanied by functional changes in response to DNA damage [70]. The cells from aged mice at or near the position of stem cells within the crypts of Lieberkuhn are more susceptible to apoptosis under stress condition and display decreased regenerative potential despite an age-dependent increase in the number of clonogenic crypt cells [70]. Aging also appears to induce epigenetic changes in the gut epithelium. Increased average crypt DNA methylation was reported in both intestinal and colon crypts from older humans [71]. The extent to which the age-related increase in DNA methylation impacts the transcriptome of gut epithelial cells remains to be determined. Interestingly, analysis of methylation patterns by a quantitative model to distinguish between immortal or niche stem cell lineages. Crypt methylation patterns were more consistent with stem cell niches than immortal stem cell lineages. Human large and small intestine crypt niches appeared to have similar stem cell dynamics, but relatively less methylation accumulated with age in the small intestines [72]. Moreover, the identification of small intestine and colon Lgr5⁺ stem cells provides the stimuli for studies on molecular mechanisms driving normal intestinal stem cell aging and subsequent events required for development of colon cancer, since age is undoubtedly the number one risk factor for colon cancer.

15.3 Extended Longevity of Mice with GH Deficiency, Resistance, and Deletion of GH Receptor

The insulin-like growth factor (IGF) system impacts cellular development by regulating proliferation, differentiation, and apoptosis and is an attractive therapeutic target in cancer. The IGF system is complex, with two ligands (IGF1, IGF2), two receptors (IGF1R, IGF2R), and at least six high-affinity IGF-binding proteins (IGFBPs) that regulate IGF ligand bioavailability [73]. This family includes proinsulin, insulin, IGF-1, and IGF-2, which display high amino acid sequence homology and share similar ternary structure [74]. IGF-1 acts in an autocrine/paracrine and endocrine manner and is secreted by hepatocytes, which produce 75% of serum IGF-1 [75]. In the liver *Igf-1* gene expression is mainly regulated by pituitary gland-derived growth hormone (GH), although insulin and nutrition also affect its expression [74, 76]. Consecutively, endocrine/serum IGF-1 regulates pituitary GH production through a negative feedback loop. *Igf-2* gene expression is GH independent and, as will be discussed later in this chapter, is tightly regulated by parental imprinting [77, 78]. The biological effect of IGF-1 and IGF-2 is mediated by the type 1 IGF receptor (IGF-1R) that is a membrane-bound tyrosine kinase heterotetramer. IGF-1R shares 60% homology with the insulin receptor A and B (IR-A, IR-B) but differs in ligand specificity and affinity and in transmission of downstream signals [74, 79]. IGF2R specifically binds IGF2, but

lacks an intracellular tyrosine kinase domain [80]. While IGF2R cannot initiate downstream signaling cascades, IGF2-IGF2R complexes undergo cellular trafficking, potentially regulating extracellular IGF2 levels and providing an indirect mechanism to influence cellular behavior [81]. The circulating levels of IGF1 and IGF2 are regulated by high-affinity interactions with IGFBPs [82]. To date, 6 high-affinity IGFBs have been characterized [83, 84], and their distribution is tissue dependent and affected by several factors, for example, age, nutrition, exercise, and pregnancy [85–87]. IGF-1 plays a pivotal role in fetal development, adolescent growth, and adult tissue homeostasis [74]. Together with insulin and growth hormone, IGFs impact glucose and lipid metabolism and thereby regulate body composition. However, GH and IGF-1 act very differently on glucose and lipid metabolism; GH blocks insulin action, promotes lipolysis, and impedes lipogenesis, whereas IGF-1 has opposing effect [88, 89].

After reaching adulthood, secretion of GH and IGF-1 declines continuously to very low levels in those aged ≥ 60 [88, 90] – phenomenon known as “somatopause” [91].

With the development of gene-targeting approaches in animal models, the roles of IGF system in normal physiology and aging have become more explicable (Table 15.1).

15.3.1 *Snell Mice*

In 1929, George Snell discovered a recessive mutation that in a homozygous state caused drastic retardation of postnatal growth and a diminutive adult phenotype [92], which was later shown to be related to defective development of the anterior pituitary and absence of growth hormone (GH)-producing cells [93]. The Snell dwarf mouse that is characterized by pituitary hypoplasia and GH, PRL, and TSH deficiencies has a point mutation (W261C) within the *Pit1* gene, affecting the third helix of the POU homeodomain [94–96]. This abrogates binding of Pit1 to its target promoter sequences [92, 94]. Several mutations and deletions of the POU1F1 gene have been identified in humans with combined pituitary hormone deficiency (CPHD) [97, 98]. Mean, median, and maximum life span of Snell dwarf mice ($Pit1^{dw}/Pit1^{dw}$) is increased by 40–50% in DW/J females [95]. Interestingly, Snell dwarf mice, in addition to increased longevity, have improved glucose metabolism [99], delayed of multiple signs of senescence, including decreased osteoarthritis [100].

15.3.2 *Ames Mice*

Brown-Borg et al. reported that Ames dwarf mice live approximately 50% longer than their normal siblings and hypothesized that observed extension of their longevity is due to deficiency of GH [101, 102]. When Ames dwarf mice are

Table 15.1 Phenotypic characteristic of selected mouse strains with altered GH/IGF axis and its effect on life span

Strain	Gene	Life span (change in %)	Background strain	BW	Insulin sensitivity	Blood IGF-1	Tumor incidence
Ames [101, 288–291]	<i>Prop-1</i>	F + 68 M + 49	Heterogenous	↓	↑	↓	↓
Snell [92, 93, 96, 99, 290, 292]	<i>Pit-1</i>	F and M + 42	DW/J × C3H/HeJ	↓	↑	↓	↓
<i>lit/lit</i> [96, 112, 293, 294]	<i>Ghrhr</i>	F + 25 M + 23	C56BL/6 J	↓	↑	↓	↓
GHR KO [117, 290, 295–297]	<i>Ghr/bp</i>	F + 21 M + 40	129Ola × BalbC	↓	↑	↓	↓
PAPPA KO [148, 298]	<i>Pappa</i>	F and M + 38	C57BL6 × 129SV/E	↓	?	NC	↓
Bovine GH transgenic mouse [299, 300]	<i>Gh</i>	M – 45%	ND	↑	↓	↑	↑
<i>Igf1^{+/-}</i> [137]	<i>Igf1r</i>	F + 33 M NS	129/J	↓	↓	↑	ND
<i>Igf1r^{+/-}</i> [138]	<i>Igf1r</i>	NS	C57BL/6	↓	↓	NC	↔
Klotho [301]	<i>Klotho</i>	F1 and F2 + 19 ^a M1 + 20 M2 + 31	C57BL/6 × C3H	NC	↓	NC	ND
<i>Irs1^{-/-}</i> [155, 157, 158]	<i>Irs1</i>	F + 17 M NS	C57BL/6	↓	↓	NC	ND
<i>Irs2^{-/-}</i> [155]	<i>Irs2</i>	F – 26 M – 84	C57BL/6	NC	↑	ND	ND

Significant changes in the various characteristics of mouse mutant as compared with control mice are presented

Abbreviations: ↑ increase, ↓ decrease, ↔ no change, *F* female, *M* male, *NC* no change, *ND* no data available

^aTwo transgenic mouse strains [1 and 2] were investigated

subjected to caloric restriction, their life span is increased even further [88, 103]. Ames dwarf mice are homozygous for a recessive spontaneous mutation prophet of pituitary factor-1 (*Prop1*) and resemble mice homozygous for the Snell's dwarf mutation (*Pit1dw*) [45, 104, 105]. *Prop1* encodes a paired-like homeodomain protein that is expressed specifically in embryonic pituitary and is necessary for Pit1 expression in cells producing GH, PRL, and thyroid-stimulating hormones (TSH) [106, 107]. Homozygous Ames dwarf mutant mice show growth retardation after the first postnatal week, and weight at 2 months is only about one-half normal [105]. Mutations of the pituitary transcription factor gene POU1F1 (the human homolog of mouse Pit1) are responsible for deficiencies of GH, prolactin, and thyroid-stimulating hormone (TSH) in man, while the production of adrenocorticotropic hormone (ACTH), luteinizing hormone (LH), and follicle-stimulating hormone (FSH) is preserved. These particular mutations (*Prop1df* and *Pit1dw*)

affect development of other pituitary cell types (lactotropes, thyrotropes); therefore Snell and Ames animals also exhibit circulating prolactin and thyrotropin deficiencies [108]. What is of equal importance in Ames, Snell, and Laron dwarf mice is their exceptionally extended health span, that is, these animals display a longer period of life free of age-related diseases [109].

15.3.3 *lit/lit* Mice

In 1976 Beamer described the first “little” mouse (*lit/lit*) that is GH deficient owing to a mutation in *Ghrhr* gene, which encodes the GH-releasing hormone receptor [110]. Mice homozygous for the little spontaneous mutation (*Ghrhrlit*) are characterized by a deficiency in pituitary growth hormone (GH) along with prolactin and growth retardation [96, 111]. The *lit/lit* mice reach ~50% of the size of unaffected siblings and are characterized by increased adiposity [112]. The life span of *lit/lit* mice is significantly increased; females live approximately 25% and males 23% longer compared to wild-type littermates [96].

15.3.4 *Ghr*^{-/-} Mice

The *Ghr*^{-/-} mouse, also known as the Laron dwarf mouse, is produced by targeted disruption of the GH receptor and GH-binding protein-encoding gene [113]. The Laron mouse is referenced to the similarities with human Laron syndrome [114, 115]. Mice homozygous for this deletion are born normal-sized but reach less than 50% of the adult weight of wild-type littermates [88]. *Ghr*^{-/-} mice despite elevated GH levels in blood have very low IGF-1 levels (~20% of those of control mice) [116] since these animals do not secrete IGF-1 from the liver because of a lack of functional GH receptors on hepatocytes. Also, their fasting glucose concentration and their fasting insulin level are reduced throughout life span. Mean life span of female and male Laron dwarf mice are 21% and 40% longer, respectively, compared to wild-type control animals [88, 117]. In fact, one *Ghr*^{-/-} mouse lived nearly 5 years and currently holds the record for the longest-lived laboratory mouse [118]. Similarly, long living are also GH-releasing hormone-deficient mice (*Ghrh*^{-/-}) that also are characterized by a very low level of circulating IGF-1.

15.3.5 *RasGRF1*^{-/-} and *S6 K1*-Deficient Mice

RasGRF1 is a Ras-guanine nucleotide exchange factor implicated in a variety of physiological processes including learning and memory and glucose homeostasis. RAS has been shown conclusively to exert a role in aging in yeast [119–121].

Mutations that decrease the activity of the RAS/Cyr1/PKA pathway extend longevity and increase stress resistance in yeast by activating transcription factors Msn2/Msn4 and the mitochondrial superoxide dismutase [120]. It has also been reported that Ras genes are a major homeostatic device in the regulation of the life span of *S. cerevisiae* [122]. The longevity-modulating function of IGF-I in *C. elegans* displays a signal bifurcation involving the Ras ortholog Let-60, consistent with a role for RAS signaling downstream of IGF-IR. As reported, *RasGrf1*^{-/-} mice display reduced IGF-1 circulation level and smaller adult body size compared to normal wild-type littermates [123] and extended longevity [124].

Both RasGRF1 and ribosomal protein S6 kinase 1 are downstream signaling targets of GH/INS/IGF pathway. S6 K1 is a substrate of mTORC1 that has been shown to modulate life span in invertebrates [125–131] and mice [132–134]. Reduced nutrient signaling, achieved either by caloric restriction (CR) or genetic inhibition of the mTOR pathway, can robustly extend life span in many different species and protect against multiple age-related disorders [174]. Recently it was demonstrated using S6K1 floxed mice (*S6K1*^{fl/fl}) that hepatic S6K1 partially regulates the life span of mice with mitochondrial complex I deficiency [135].

15.4 Longevity of Mice with Disruption of Genes Affecting IGF-1 Signaling

15.4.1 *Igfr*^{+/-} Mice

Whereas mice lacking GH and the GH receptor are viable and long-lived, most IGF-1 receptor knockout mice (*Igfr*^{-/-}) die at birth [136]; therefore studies on the role of IGF-1 and IGF-1R in aging have concentrated on GH-deficient and GH-resistant mice as well as mice with nonlethal tissue-specific IGF-1/IGF-1R genetic disruption (Table 15.1). Since IGF-1 mediates many of GH effects, it seems likely that extension of longevity in Snell dwarf, Ames dwarfs, *lit/lit* mice, and *Ghr*^{-/-} mice may be due to reduced IGF-1 signaling. Accordingly, heterogenous deletion of the IGF-1R (*Igf1r*^{+/-}) results in a marked reduction in IGF-1-induced intracellular signaling and 33% longer mean life span of female [137, 138]. No significant change in life span was observed in male *Igfr*^{+/-} mice [137]. Partial inactivation of the IGF-1 receptor gene solely in the brain affects development of GH-secreting cells in the pituitary gland resulting to reduced adult body size and significant extension of longevity [139].

15.4.2 *Papp-A*^{-/-} Mice

The significant role of IGF-1 in the regulation of mouse longevity is strongly supported by findings in animals with deletion of pregnancy-associated plasma protein-A (PAPP-A) [140–142]. PAPP-A is a proteolytic enzyme that was discov-

ered to increase local insulin-like growth factor (IGF) availability for receptor activation through cleavage of inhibitory IGF-binding proteins (IGFBPs), in particular IGFBP-4 [143–147]. Both male and female PAPP-A knockout (KO) mice on chow diet live 30–40% longer than wild-type (WT) littermates, with no secondary endocrine abnormalities [148]. It is important to note that PAPP-A KO mice have normal levels of circulating IGF-I (and GH) and their phenotype reflects reduction in local IGF action [148]. Deletion of PAPP-A leads to increased levels of IGF-binding proteins with the subsequent reduction of bioavailable IGF-1 in the tissues [146, 149].

15.4.3 *Irs1*^{−/−} and *Irs2*^{−/−} Mice

Insulin receptor substrates (IRS) are a family of cytoplasmic adaptor proteins that transmit signals from the insulin and IGF-1 receptors to elicit a cellular response. IRS-1, the first member of the family to be identified, was initially characterized as a 185-kD phosphoprotein in response to insulin stimulation [150, 151]. IRS-2 was discovered as an alternative insulin receptor substrate, initially named 4PS, in insulin-stimulated cells derived from *Irs1*^{−/−} mice [152, 153]. IRS-1 and IRS-2 are ubiquitously expressed and are the primary mediators of insulin-dependent mitogenesis and regulation of glucose metabolism in most cell types. *Irs1*^{−/−} mice are born ~70% the size of wild-type mice and remain small throughout their lives and display insulin resistance with defects in insulin signaling mainly in skeletal muscle [154]. Female *Irs1*^{−/−} have a 17% increase in mean life span, but no significant difference is detected in male *Irs1*^{−/−} mice compared to wild type [155, 156].

Irs2^{−/−} mice display profound insulin resistance that is observed in skeletal muscle and liver tissue. Unlike *Irs1*^{−/−}, *Irs2*^{−/−} mice exhibit a progressive development of a type 2 diabetic phenotype [157]. At birth *Irs2*^{−/−} mice are slightly (10%) smaller than wild-type littermates [158]. Heterozygous or homozygous deletion of *Irs2* or selective deletion of *Irs2* in the brain resulted in extension of mouse longevity [156]. However, it is difficult to determine whether the impact of reducing IRS expression on longevity is due to suppression of IGF-1 signaling, insulin signaling, or maybe both. Although IGF-1 and insulin affect cell function differently, signaling by both hormones involves activation of insulin receptor substrates.

15.4.4 *Several Metabolic Pathways Related to Cellular Senescence and Aging*

The somatotrophic axis in mammals comprises the growth hormone (GH), which is produced by anterior pituitary, and its secondary mediator, insulin-like growth factor 1 (IGF-1), produced in response to GH by many cell types, most notably by hepatocytes [10]. The intracellular signaling pathway of IGF-1 is the same as that evoked by insulin, which informs cells of the glucose presence. Therefore, IGF-1

and insulin are known as the “insulin and IGF-1 signaling” (IIS) pathway. Remarkably, the insulin/IGF1 signaling pathway is the best characterized pathway affecting longevity [159–161]. Among IIS multiple targets are the FOXO family of transcription factors and mTOR complexes, which are also involved in aging and conserved through evolution [10] [121, 160, 162]. In *C. elegans* and *D. melanogaster*, organisms in which the insulin and IGF1 pathways converge on a single receptor decreased insulin/IGF1 signaling that increases life span by as much as twofold [160, 163]. The IIS signaling pathway is highly conserved between worms, flies, and mammals [121, 161, 164]. As demonstrated in Fig. 15.1, one of the intermediates

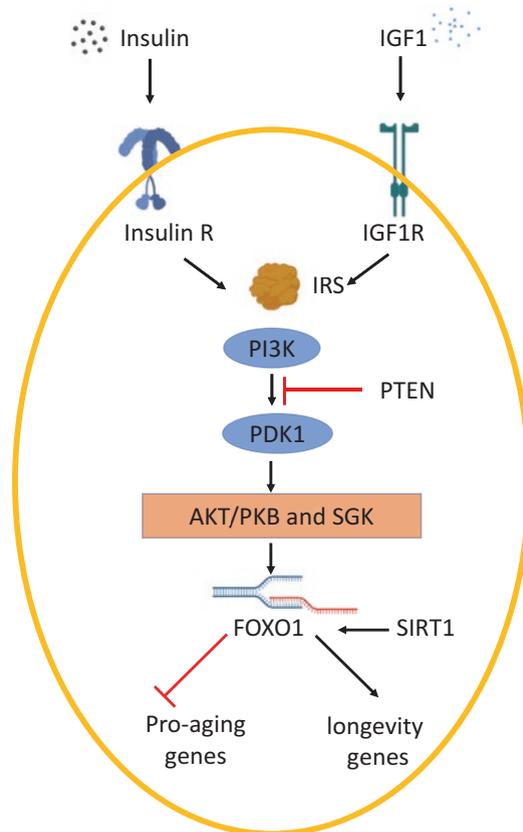


Fig. 15.1 Insulin/IGF1 signaling in the regulation of aging. On binding of insulin and IGF1 to respective receptors, the tyrosine kinase activities of IR and IGF1R are activated and phosphorylate phosphoinositide 3-kinase (PI3K). The phosphorylated PI3K synthesized phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P₃) and PtdIns(3,4)P₂ [302], which in turns activate 3-phosphoinositide-dependent protein kinase-1 (PDK1) [161]. Subsequently, PDK1 phosphorylates and activates AKT/protein kinase B (PKB) and the serum/glucocorticoid-regulated kinase (SGK) [161, 165]. These kinases target FoxOs and block their transcriptional activity. Inhibition of the insulin/IGF1 pathway allows FoXOs for positive regulation of the expression of antiaging genes and negative regulation of the pro-aging gene expression [55, 303, 304]

in the insulin/IGF-1 signaling cascade is the activation of the serine/threonine kinase AKT/protein kinase B (PKB) and the serum/glucocorticoid-regulated kinase (SGK) [165]. Worms and flies both have a single ortholog of the mammalian insulin and IGF-1 receptor tyrosine kinases called DAF-2 and insulin receptor (InR), respectively [166–168]. Upon binding of one of a family of insulin-like peptides [169], the tyrosine kinase activates DAF-1 and InR. Activated DAF-2 and InR subsequently phosphorylate phosphatidylinositol 3-kinase (PI3K). Detailed information of endocrine regulation of aging are discussed in a review paper by Russell and Kahn [161].

From the aging perspective, the most important AKT/PKB and SGK substrates appear to be the class O of forkhead box transcription factor (FoxOs) [163]. Phosphorylation of FOXO by AKT leads in translocation of FoxOs to the cytosol and inactivation of its pro-longevity transcriptional targets that include genes involved in defense against oxidative stress and encoding molecular chaperons [170]. Mutations that decrease signaling through insulin/IGF-1 reduce the phosphorylation of FoxOs, resulting in their nuclear translocation, where FoxOs modulate expression of genes that increase life span [166, 168, 171–173].

Figure 15.2 displays pathways activated through the GH-, INS-, and IGF-receptors as part of nutrient-sensing response. As demonstrated these pathways converge on the serine/threonine kinase mTOR (mechanistic target of rapamycin). mTOR plays an essential role in regulating growth and metabolism in response to signals from insulin and IGF signaling, the cellular energy sensor AMPK (AMP-activated protein kinase), proinflammatory cytokines, amino acids, and oxygen level [174, 175]. It is well accepted that mTOR modulates the ratio between anabolic and catabolic processes in response to nutrient availability and overall cellular energy status [176]. mTOR is the catalytic subunit of two distinct complexes: mTORC1 and mTORC2 [175]. mTORC1 phosphorylates a diverse set of targets involved in protein biosynthesis, metabolism, and transcriptional regulation, while mTORC2's most prominent targets are regulatory kinases of the AGC family. mTORC1 signaling is stimulated by growth factors and amino acids and is inhibited by low cellular energy levels and hypoxia. Conserved protein regions involved in target recognition in mTORC1 are located in the Raptor subunit. mTORC1 is inhibited by rapamycin and its analogs that leads to inhibition of mRNA translation and protein synthesis due to negative effect of the two mTORC1 substrates – S6 K1 and eukaryotic translation initiation factor 4E-binding protein (4E-BP1) [174] – and it also represses autophagy through multiple substrates. This puts mTOR (TORC1) complex at center stage as an evolutionary conserved regulator that integrates information from multiple upstream nutrient and energy sources to regulate metabolic processes, somatic maintenance, and life span. In fact, it was demonstrated that mTOR signaling influences both reproduction and longevity. In yeast, worms, and flies, manipulation of TOR by genetic ablation or by rapamycin or its analogs extends life span [125, 177, 178]. With respect to downstream mTORC1 substrates, reducing levels of the effector S6 K1 can also extend life span. As mentioned in previous paragraph, mice with a mutation in S6 K1 display an extended life span, perhaps because loss of S6K1 mimics some aspects of caloric restriction.

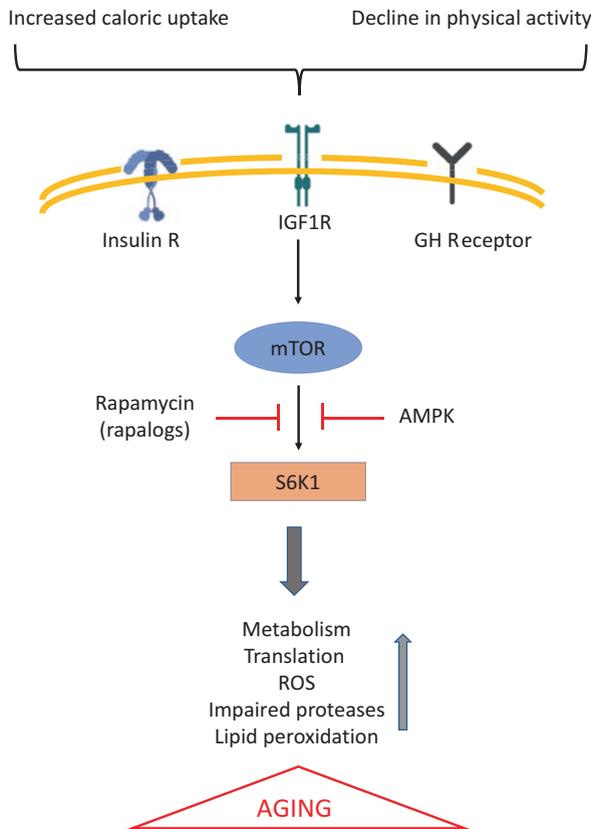


Fig. 15.2 INS/IGF/GH signaling-dependent metabolic pathways impact aging in all somatic cells and stem cells. Parallel pathways in *C. elegans* and *D. melanogaster* play a critical role in the regulation of aging. A high caloric diet and decline in physical activity enhance INS/IGF/GH signaling in cells in mTOR/mTORC1-dependent manner. The mTORC1 functions as a nutrient/energy/redox sensor that regulates energy resources, nutrient availability, and oxygen abundance. The main role of mTORC is to activate and control translation of protein. However, over time, this subsequently leads to damaging, mTOR-activated intracellular processes due to oxidative damage, epigenetic changes in methylation, decrease proteases, lipid peroxidation, and inhibition of autophagy. The beneficial effect of metformin and berberine – AMPK activators that inhibit mTORC1 and direct mTOR inhibitors (rapamycin and rapalogs) – are indicated and discussed

These mice are sensitized to insulin because of a decrease in the phosphorylation of threonine residues in insulin receptor substrate 1 resulting in altered insulin signaling [179]. Importantly, this advantageous effect of caloric restriction or S6K1 mutation on life span can be explained by reduces mTORC1 activity downstream of GH/INS/IGF signaling (Fig. 15.2). It also has been reported that the mTORC1 complex is negatively regulated by AMPK, a key sensor of cellular energy status, that is activated in response to cellular energy depletion leading to downstream effects generally associated with the induction of catabolic pathways together with repression of anabolic

pathways [180–182]. It was recently demonstrated that AMP-activated protein kinase (AMPK) and dietary restriction promote longevity in *C. elegans* via maintaining mitochondrial network homeostasis and functional coordination with peroxisomes to increase fatty acid oxidation (FAO) [183]. AMPK overexpression or its activation by plant-derived compounds, such as metformin, berberine, or some chemically synthesized small-molecule activators, has been reported to extend life span in experimental animal models [184, 185]. This effect is due to AMPK-mediated inhibition of GH/INS/IGF signaling in mTORC1-dependent manner (Fig. 15.2) [13]. Given that AMPK is activated by different approaches like physical activity or CR and modulates many apparent beneficial adaptations, various studies have attempted to evaluate whether AMPK activators can serve as exercise or CR mimetics to improve health. In sedentary mice, it was reported that 4 weeks of treatment with AICAR, AMPK agonist, significantly enhanced running endurance [186]. The antidiabetic drug, that is believed to be an AMPK activator, increased life span in yeast and mice [187–189], but not in rats. However, it is unknown whether AICAR and/or metformin is capable to recapitulate these effects in humans. Finally, the exact mechanism by which AMPK activation modulates aging is unclear; its ability to inhibit mTOR signaling may be critically important. Several clinical trials are currently undertaken using mTOR inhibitors, such as rapamycin or its rapalogs, as well as AMPK activators, including metformin and berberine, to extend human life span [190–192]. Systematic long-term studies have to be completed in order to collect definitive data. Notably, direct target for metformin and berberine is respiratory complex I of electron transport chain in mitochondria. Inhibition of this complex by either of these drugs prevents formation of ATP and thereby leads to an increase of AMP/ATP ratio that provides trigger-activating AMPK and subsequently results in inhibition of mTOR signaling [193].

15.5 Epigenetic Mechanisms of Longevity and Aging: Unexpected Role of Sirtuins

Sirtuins are a class of NAD⁺-dependent deacetylases, which deacetylate both histones and wide range of proteins [194]. In mammals the sirtuin family contain seven proteins (SIRT1–SIRT7), which vary in cellular localization, enzymatic activity, and tissue specificity [195]. SIRT1 is mainly localized in the nucleus but is also present in the cytoplasm. Its nuclear export signal allows shuttling to the cytosol under special circumstances, for example, when the insulin pathway is pharmacologically inhibited. SIRT3, SIRT4, and SIRT5 have a mitochondrial targeting sequences, and their localization in mitochondria was confirmed in various experimental settings [196, 197].

SIRT1, the most extensively studied member of the sirtuin family, targets a number of substrates that regulate DNA damage, stress response, mitochondrial biogenesis, and glucose and lipid metabolism [198, 199]. SIRT2 controls cell cycle and glucose and lipid metabolism [200]. Cytosolic SIRT2 deacetylates tubulin [201] and more

importantly also deacetylates partitioning defective 3 homolog (PAR3) [202]. SIRT3, SIRT 4, and SIRT 5 regulate ATP production cell signaling, metabolism, and apoptosis. More recently, it became apparent that SIRT3 also affects oxidative stress defense by protecting cells from reactive oxygen species (ROS) [203–205]. Mitochondrial protein SIRT4 ADP ribosylates GDH, thereby inhibiting its activity and blocking amino acid induce insulin secretion [206, 207]. SIRT4 also regulates fatty acid oxidation in hepatocytes and myocytes [207]. The only target described for SIRT5 is CPS1, deacetylation of which during fasting activates ammonia detoxification through the urea cycle [208]. SIRT6 is involved in genomic DNA stability and repair and is crucial in metabolism and aging [209]. SIRT7 was reported to activate RNA polymerase I transcription, although its protein substrate is still unidentified [210]. Phylogenetic analysis revealed that mammalian sirtuins can be divided into 4 classes: SIRT1–SIRT3 belong to class I, SIRT4 to class II, SIRT5 to class III, and SIRT6 and SIRT7 to class IV [194].

Originally, sirtuins were described as nicotinamide adenine dinucleotide (NAD)-dependent protein type III histone deacetylases (HDACs) [194, 211]. HDACs remove acetyl groups on histones, which allows these protein to wrap DNA around core histones of nucleosome more tightly [212]. Mammalian SIRT1 shares the highest similarity to Sir2, a protein that was demonstrated to play an essential role in the regulation of yeast replicative life span [213]. The enzymatic reaction catalyzed by sirtuins requires NAD⁺ as a substrate. NAD⁺ level is determined by the nutritional state of the cell; therefore, NAD⁺ controls adaptive responses to energy stress by modulating activity of sirtuins and downstream effectors [198].

Of the seven mammalian sirtuins, SIRT1 is the closest homolog of yeast Sir2 and is the most-studied mammalian sirtuin. SIRT1 predominantly localizes to the nucleus and exhibits several pleiotropic effects apart from its role in histone deacetylation, regulates energy metabolism in the hypothalamus, and is induced by a negative energy balance [159]. Respectively, it may deacetylate p53 and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1 α) and thus inhibit apoptosis and enhance mitochondrial biogenesis and function [159]. Furthermore, SIRT1 deacetylates the inflammatory regulator nuclear factor- κ B (NF- κ B) [214], which may be an essential player in insulin resistance and metabolic syndrome. SIRT1-regulated acetylation of FOXO transcription factors (FOXO1, FOXO3a, FOXO4) is believed to selectively direct these factors to certain cellular targets and to regulate cell metabolism (lipid and glucose metabolism) and stress responses [215]. SIRT1 has been associated with glucose and lipid metabolism. Studies in pancreatic beta cells revealed that SIRT1 positively regulates insulin secretion through inhibition of uncoupling protein-2 (UCP2) [216]. Additional studies demonstrated that SIRT1 inhibits acetyl-CoA carboxylase and fatty acid synthase by activation of AMP-activated protein kinase (AMPK) and suppresses lipid accumulation in liver [217]. Other, newly identified function of SIRT1 includes neuroprotection, liver regeneration, and delayed replicative senescence of fibroblasts [218–220].

The yeast protein Sir2 was described as a longevity protein [221]. Also *C. elegans* SIR-2.1 [222] and fly SIR2 [223] were implicated in life span regulation and were believed to mediate the beneficial effects of caloric restriction on life span. This role

of sirtuins was seemingly verified by the beneficial effects of the sirtuin-activating compound resveratrol on life span of yeast [224], but studies in *Drosophila melanogaster* and *C. elegans* provided a skeptical perspective on the possibility that sirtuin mediates the effect of resveratrol and caloric restriction on longevity [225].

Although there is no consistent evidence showing that SIRT1 level and/or activity declines with age, activating this pathway might still be beneficial in preventing some manifestation of aging and affect health span in a pleiotropic manner. Resveratrol, a polyphenolic compound found in grapes, may activate SIRT1 [226] and may function as a CR mimetic [227]. Resveratrol treatment increases the life span of high-fat-fed mice, which had improved insulin sensitivity, mitochondrial function, and survival [228]. Recently, treatment of obese mice with synthetic activator of SIRT1 – SRT1720 – resulted in similar improvement in survival as were noted in resveratrol-treated group of animals [229]. Upregulation of SIRT1 by SRT1720 or SRT3025 had beneficial effect on extending life span and expanding HSC in wild-type mice [13, 230]. Mice with inducible hematopoietic SIRT1 knock-out displayed accelerated hematopoietic aging since SIRT1 ablation promoted stress-induced loss of epigenetic and genomic hematopoietic stem and progenitor cell maintenance [231]. This latest result indicates that SIRT1 may be a guardian of HSC during adulthood. Overall, studies suggest that both natural and synthetic SIRT1 activators may improve health and survival, though the belief that these effects are SIRT1 dependent is limited at best.

15.6 Aging and Longevity from the Perspective of VSELs Residing in Adult Tissues

Aging is accompanied by a decline in tissue/organ function, regeneration, and repair. A substantial part of this decline is created by the deterioration of stem cells residing in adult tissues as discussed above.

Our group demonstrated that adult tissues including murine and human bone marrow, mobilized peripheral blood, and human umbilical cord blood (UCB) contain a population of pluripotent *Oct4*⁺ Sca-1 + lin-CD45⁻ (murine) and *Oct4*⁺ AC133 + lin-CD45⁻ (human) very small embryonic-like stem cells (VSELs) [232, 233]. We have also postulated that these primitive pluripotent stem cells (PSCs) originate from early epiblast-derived migrating primordial (PGC) – like cells are deposited during early stages of embryonic development in various tissues as a source of tissue committed stem cells in adulthood [234]. Molecular analysis of murine BM-derived VSELs has revealed that VSELs express several genes characteristic for epiblast stem cells (EpiSC) like *Fgf5*, *Gbx2*, and *Nodal* and genes involved in germ line specification (*Stella*, *Blimp1*, *Fragilis*, *Nanos3*, *Prdm14*, and *Dnd1*) [235, 236]. Chromatin immunoprecipitation analysis revealed that the *Stella* promoter in BM-isolated VSELs displays transcriptionally active histone modifications (H3 hyperacetylation, H3Ac, and trimethylated-lysine-4 of histone 3, H3K27me3) [235, 237] and was less enriched for transcriptionally repressive histone modifications (dimethylated-lysine-9 of histone 3, H3K9me2, and trimethylated-lysine-27 of histone 3, H3K27me3) [235, 237].

Based on developmental origin of VSELs, we postulated that these cells similarly like PGCs may modify methylation of imprinted genes that prevents them from uncontrolled proliferation and explains their quiescence in adult tissues [170, 235, 238, 239]. It is well documented that imprinted genes play a crucial role in embryogenesis, fetal growth, totipotency of the zygote, and pluripotency of early stem cells [78, 240–244]. The expression of imprinted genes is regulated by DNA methylation on differentially methylated regions (DMRs), which are CpG-rich *cis*-elements in their loci [77, 78, 245, 246]. We have demonstrated that freshly isolated BM VSELs erase the paternally methylated imprints for *Igf2-H19*, *RasGRF1* loci [238], but at the same time VSELs display hypermethylation on maternally methylated ones: *Igf2R*, *Kcnq1-p57^{Kip2}*, and *Peg1* [238]. As a result of these novel epigenetic modifications in the methylation state of DMRs in paternally imprinted genes, VSELs highly expressed growth-repressive genes (*H19*, *p57^{Kip2}*, and *Igf2R*) and at the same time downregulate growth/proliferation-promoting genes (*Igf2* and *RasGRF1*) [238, 239]. Furthermore, as demonstrated by ingenuity pathway (IPA) and canonical pathway analysis, VSELs isolated from murine BM display low expression levels of the components of the IGF-1 signaling cascade compared to hematopoietic stem cells, specifically *FOS*, *JUN*, *JAK1*, *KRAS*, *SOS2*, serum response factor (*Srf*), suppressor of cytokine signaling 3 (*SOCS3*), and *SHC1* [247]. The leading-edge gene subset analysis revealed that several genes commonly involved in the mitogenic growth factor signaling pathways (e.g., ERK1/MAPK, TRKA, and PI3K) are repressed in VSELs [247]. Also, the cell cycle and insulin-signaling-inhibiting miRNA expression pattern of VSELs contribute to their quiescent state [248]. In particular, VSELs highly express as mentioned above, H19 noncoding RNA that gives rise to miRNA-675-3 p and miR-675-5 p, both of which negatively affect expression of IGF-1R [249] and INS R [250], which in turn plays an important role in somatotrophic signaling. We have confirmed high expression of both miRNAs in murine BM-isolated VSELs [248]. Additionally, we have detected high expression of miRNA-184, which exhibits a complementary sequence to the mRNA sequence of *RasGRF1* gene that may contribute to attenuation of IIS in VSELs [248]. Since expression of IGF2 may be regulated at the miRNA level [251, 252], we demonstrated that VSELs exhibit upregulation of miR-292-5 p, miR-125 b, and miR-665, all of which negatively affect IGF2 expression [248].

The changes summarized above in expression of imprinted genes and miRNA species in murine VSELs subsequently lead to perturbation of IIS by downregulation of *i*) IGF2, which is autocrine factor involved in proliferation of VSELs, and *ii*) RasGRF1, which is a GTP-exchange factor (*guanine exchange factor*) important for intracellular signaling from activated IGF-1R and insulin receptor [170, 239, 253]. Additionally, hypermethylation of DMRs on the maternal chromosome encoding IGF-2R [238] negatively affects IIS in VSELs, since IGF2R serves as a decoy receptor that prevents IGF-1 from binding to IGF-1R. As reported, miRNA-15b plays an inhibitory role in IGF2R expression, and our studies revealed that miRNA-15b is strongly downregulated in murine VSELs [248].

Therefore, epigenetic reprogramming changes observed in VSELs lead to decrease in GH/INS/IGF signaling in these small cells, keeping them quiescent and preventing from premature depletion in adult tissues [13, 237, 238, 248]. In mice,

the number of VSELs in the bone marrow declines with age [253, 254], and studies assessing the abundance of VSELs in bone marrow of GH-related mutant animals, in which level of IGF-1 in circulation is dramatically suppressed, revealed that the number of VSELs is higher in Ames dwarf and GHRO mice than in normal littermates [254, 255]. In contrast, the number of VSELs was reduced in GH transgenic mice, which are characterized by chronically elevated IGF-1 level in circulation [256]. Hormonal replacement therapy with IGF-1 in GHRKO animals and with GH in Ames dwarf mice resulted in significant decrease of VSELs in their bone marrow, similarly as treatment of normal mice with large doses of GH [255]. This results clearly demonstrated that in animals with GH deficiency or GH resistance and the consequent suppression of plasma IGF-1 levels, the abundance of VSELs in the bone marrow is increased, likely reflecting limited differentiation and/or other mechanisms involved in age-dependent depletion of primitive stem cells [257].

Additionally, changes in epigenetic reprogramming observed in VSELs may be additionally enhanced by caloric restriction [258], physical activity [259, 260], and administration of certain drugs, including metformin, berberine, or rapamycin [13, 261] as well as activators of AMPK and of SIRT-1 [230, 231, 262]. An inhibition of SIRT-1 by valproic acid or nicotinamide has been recently demonstrated to play an important role in promoting efficient expansion of human LT-HSCs [263–266].

15.7 In Search of Antiaging Modalities

The only intervention that invariably has been demonstrated to increase life span from nematodes to primates is caloric or dietary restriction (CR) [162, 267, 268]. In 1935, McCay et al. published report showing that caloric restriction in rats, when implemented after puberty, extended median and maximum life span and attenuated the severity of chronic disease [269]. Subsequently, reports have shown that CR, defined as a reduction in caloric intake below usual *ad libidum* intake without malnutrition, decreases aging and increases maximum life span in yeasts, flies, worms, fish, and rodents [267, 270–273]. The age when CR is initiated, the severity of restriction, and the strain or genetic background of the animals determine the severity of life extension [270]. Studies on mouse models have shown that restricting caloric intake by 40–60% increases life span by 30–50% and lowers the onsets of age-related loss of function and diseases, including neurodegeneration [121, 274]. How CR extends life span is still be fully elucidated [267, 275], but several studies demonstrate that sensing of nutrient levels is important. It is inclined that the lack of specific dietary amino acids mediates the effect of CR rather than the restriction of caloric intake alone [276]. For example, several studies indicate that CR retards or delays the age-associated decline in cognition while several other reports find CR to be ineffective in this regard [267]. It was demonstrated that a diet low in the essential amino acid methionine advances life span in the mouse and reduces age-related pathologies [277]. CR delays the onset and/or slows the progression of most age-associated diseases, including neoplastic, immune, and degenerative diseases [267, 278, 279].

In humans, dietary restriction provides major and sustained beneficial effects against obesity, insulin resistance, oxidative stress, inflammation, and left ventricular diastolic dysfunction [121, 280], similarly with the metabolic and functional changes observed in CR rodents [270]. Several randomized, controlled intervention trials have evaluated the effect of CR on aging-related variables in nonobese adults. The results from one study found that 25% reduction in calorie intake for 6 months decreased insulin resistance, visceral fat mass, body temperature, metabolic rate, and levels of oxidative stress marker [281–283]. Another trial revealed that 20% reduction in calorie intake for 12 months reduced visceral fat mass, decreased level of circulating inflammatory markers, and improved insulin sensitivity [284, 285]. Despite many similarities in the metabolic adaptation to CR observed in rodents and humans, it is not known if such restriction affects maximum life span in humans. In fact, it was proposed that CR can only minimally extend maximum life span in human and non-human primates because of differences in “metabolic stability,” “evolutionary entropy,” and “dietary reaction norms” between species [270, 286, 287].

15.8 Conclusion

In animal models, mutations resulting in suppressed GH/IGF-1 axis with reduced GH/IGF-1 signaling actually increase life span. Snell and Ames dwarf mice with defects in anterior pituitary function due to Pit-1 and PROP-1 mutations, respectively, exhibit severely reduced insulin, IGF-1, glucose, and thyroid hormone levels, female infertility, and increased longevity. Lit/Lit dwarf mice with mutations in the extracellular domain of the GHRH receptor had reduced serum IGF-1 levels, increased adiposity, and ~25% increased longevity. Heterozygous IGF-1 receptor gene disrupted mice have a 50% reduction in receptor levels, and a 33% increased life span in females, who are not dwarf. Caloric restriction, another mechanism of decreasing circulating IGF-1 levels, also prolongs the life span in several species. We also presented data indicating that increase in GH/INS/IGF signaling has accelerating effect on stem cell exhaustion including VSEs. Therefore, by targeting GH/INS/IGF signaling by specific inhibitors, we may be able to develop new, potent, and side-effect-free therapeutic strategies that could extend maximum life span of humans.

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Index

A

A_{2A} receptor, 288
Acetylcholine, 277
Activated by ATP, 277
Acute brain injury, 80
Acute kidney injury (AKI), 223
Adenine nucleotide concentration, 285
Adenosine, 288
Adenosine 5'-triphosphate (ATP), 277
Adenosine A₃ receptor activation, 286
Adenosine diphosphate (ADP), 277
Adipocytes, 176
Adipose stem cells (ASCs), 182
Adipose tissue-derived MSCs (AT-MSCs), 95
Adipose tissues, 4, 8
Adjunctive therapy/monotherapy, 308
Adrenocorticotrophic hormone (ACTH), 362
Adult BM, 50
 stem cell compartment, 54, 58–60
 YS to, 56–57
Adult brain
 neurogenesis, 159–163
Adult gonads, 110
 OSCs, 110
 pituitary and gonadal hormones, 110
 quiescent stem cells, 110
 SSCs, 110
 VSELs, 110
Adult stem cells (ASCs), 2, 4, 81, 263, 264, 279, 280
Adult tissues, 2, 8, 9, 13–16
 VSELs, 371–373
Age-related macular degeneration (AMD), 24
 retina, 3

Aging

 and aging-related diseases, 356
 antiaging modalities, 373, 374
 biology, 357
 and cellular senescence, 365–369
 and epigenetic mechanisms of longevity, 369–371
 heat-shock proteins, 356
 HSCs, 357, 358
 IGF (*see* Insulin-like growth factor (IGF) system)
 IGF-1 signaling, 364–365
 insulin/IGF1 signaling, 366
 ISCs, 359, 360
 mechanism, 357
 NSCs, 358, 359
 organism, 356
 protein homeostasis, 356
 ROS, 356
 sirtuins, 369–371
 in somatic cells, 368
 in stem cells, 368
 VSELs, 371–373
Aging process, 269–271
Airways
 and lung microscopic structure, 262, 263
Alanine aminotransferase (ALT), 184
Aldehyde dehydrogenase (ALDH), 62, 219
Allogeneic transplantation, 279
Alveolar type 1 (AT1) cells, 266
Alveolar type 2 cells (AT2), 265, 266
Alveoli, 262
Alzheimer's disease (AD)
 A β 42, 315
 A β plaque, 313, 314
 A₁ receptors, 314

- Alzheimer's disease (AD) (*cont.*)
- A_{2A} receptor, 314
 - chemo-attractive induction of microglia, 316
 - clinical symptoms, 313
 - features, 313
 - hUC-MSCs, 316
 - neurogenesis, 315
 - NPC, 315
 - NSC transplanted, 315
 - P1 receptors, 314
 - P2 receptors, 314
 - P2X7 receptor, 314
 - P2Y2 receptors, 315
 - preclinical trials, 316
 - purinergic receptors, 316
 - stem cells, 316
- Ameliorate behavioral deficits, 84
- Ameliorate neuropsychiatric disorders, 80
- AMP-activated protein kinase (AMPK), 8, 369
- Amyotrophic lateral sclerosis (ALS)
- adenosine levels, 311
 - A_{2A} receptor, 311
 - autologous MSC transplantation, 312
 - BzATP, 310
 - clinical trials, 312, 313
 - genetic mutations, 310
 - in vitro analysis, 311
 - iPSC, 312
 - microglia activation, 310
 - microglial cells, 310
 - molecular identifications, 310
 - motor symptoms, 310
 - MSC treatment, 312
 - MSC-NTF, 312
 - and neuroinflammation, 310
 - neuropathological hallmark, 310
 - NPC, 312
 - P2X receptors, 311
 - P2X7 receptor, 310, 311
 - pathophysiology, 311
 - purinergic receptors, 313
 - SOD1^{G93A} mice, 312
 - sporadic disease, 310
 - transgenic SOD1^{G93A} mice, 311
 - transplanted cells, 311
 - upper/lower motor neurons, 309
- Angiogenesis, 280, 284
- Angiogenic therapy, 216
- Animal models, 356
- Antiaging modalities, 373, 374
- Anti-apoptosis, 26
- Antibody-induced arthritis, 100
- Anti-CD133-coated magnetic beads, 219
- Antidepressants, 164
- Antigen-presenting cells, 96
- Anti-inflammatory cytokines, 186
- Anti-Parkinsonian therapies, 308
- Anxiety disorder, 162, 164, 167
- Aorta-gonad-mesonephros (AGM), 50, 51, 54–57, 67
- APOLLO trial, 199
- Apomorphine test, 309
- Apoptosis inducers, 99
- A₁ receptor stimulation, 297, 298
- Astrocytes, 297
- Asymmetrical division, 242
- ATP-binding cassette (ABC) transporters, 278
- Autoimmune disease, 289
- Autoimmune encephalomyelitis, 186
- Autologous stem cell transplantation (A-HSCT), 132
- Autologous transplantation, 32, 81, 279, 280
- Azoospermic testis, 111
- B**
- B1-integrin expression, 244
- Basic fibroblast growth factor (bFGF), 82
- B-cells, 96
- Berberine, 8
- Bioactive lipids, 9
- Biomarkers, 126, 131, 147, 148, 151
- Biomaterials, 282
- Bipolar disorder, 165, 166
- Blastocysts, 2, 11
- Blastula, 10
- Blood islands, 53
- Blood-brain barrier (BBB), 83–85, 126, 127
- BM microenvironment, 60, 61
- BM-derived MSCs (BM-MSCs), 95
- Bone marrow (BM), 4, 8, 50, 94, 263
- Bone marrow cells (BMCs), 196, 200
- Bone marrow mononuclear cells (BMMNCs), 198
- Bone marrow MSCs (BM-MSCs), 178, 180–182, 184, 185, 187
- Bone marrow-derived CD133+ stem cell therapy, 219
- Bone marrow-derived cells, 220
- Bone marrow-derived stem cells (BMSCs), 126
- Bone morphogenetic protein (BMP-4), 15
- Bone morphogenetic protein receptor type 2 gene (BMPRII) mutation, 224
- Bradykinesia, 305
- Brain damage, 84
- Brain metastasis, 84
- BrdU-labeling approach, 358

- Brilliant Blue G (BBG), 297
 Bronchoalveolar stem cells (BASCs), 266
 Burst-forming unit of erythrocytes (BFU-E), 59, 60
 Burst-forming unit of megakaryocytes (BFU-Meg), 59
- C**
 C5a-C5aR1 signaling, 163
 Caloric restriction (CR), 362, 364, 367, 370, 373, 374
 cAMP pathway, 114
 Cancer
 mammalian gonads, 117, 118
 NSCs, 84–86
 Cancer risk, 125
 Cancer-associated genes, 29
 Capillary rarefaction, 181
 Carboxyl terminus of Hsp70-interacting protein (CHIP), 356
 Carboxylesterase (CE), 85
 Cardiac progenitor cells (CPCs), 183
 Cardiac-specific stem cells, 183
 Cardiac stem cells (CSCs), 203
 Cardiomyocytes, 13, 176
 direct cardiac reprogramming, 204
 endogenous proliferation, 204, 205
 Cardiopoiesis, 199
 Cardiosphere-derived cells (CDCs), 203, 204
 Cardiovascular diseases
 adenine nucleotide concentration, 285
 adenosine, 285, 288
 adenosine A₃ receptor activation, 286
 animal trials, 287
 A₁ receptor activity, 285
 A_{2A} receptor, 286
 A_{2B} receptor, 286
 ATP, 284, 285, 288
 clinical trials, 289–295
 coronary dilatation, 285
 coronary diseases, 286
 disability, 283
 extracellular purines, 284
 heart, 285
 heart function, 283
 heart rate, 285
 immunohistological assays, 287
 in vitro differentiation models, 284
 induce cell death, 285
 ischemic heart disease and stroke, 283
 MNCs, 288
 mortality, 283
 MSCs, 287, 288
 P2 receptor-coding mRNAs, 285
 P2 receptors, 285
 P2Y₄ and P2Y₁₄ receptors, 289
 P2Y₆ receptor knockout (KO) mice, 286
 pathophysiological processes, 286
 pre-existing vessel, 284
 purinergic receptors, 296
 SCs, 296
 signaling pathways, 284
 stem cell therapy, 287
 stromal-derived factor-1a, 284
 therapeutic approaches, 289–295
 vascular diseases, 283, 284
 vascular endothelial growth factor, 284
 vascular regeneration, 287
 Cardiovascular regeneration, 183
 Cartilaginous tracheal airway, 263
 Catalogue of Somatic Mutations in Cancer (COSMIC) database, 29
 CD34, 218
 CD133, 218, 219
 CD133+ populations, 219
 Cell adhesion, 245
 Cell clustering, 241
 Cell-cycle activity, 357
 Cell density-dependent Ca²⁺ oscillations, 288
 Cell division and proliferation
 ESCs, 241, 242
 Cell source, 30–32
 Cell surface antigens, 63
 Cell surface markers, 63
 Cell therapy, 283
 CD, 101
 clinical trials, 283
 GvHD, 101
 Cell therapy approaches, 225
 Cell therapy product, 222
 Cell-to-cell communication, 177, 187
 Cell-to-cell interactions, 279
 Cellular reprogramming technology, 282
 Cellular senescence, 355
 and aging, 365–369
 Central nervous system (CNS), 80
 Ceramide-1 phosphate (C1P), 57
 Chaos quantum theory, 126
 Chemoablated testis, 112
 Chemoattractants, 57
 HSCs, 61, 62
 Chemokine receptors, 99
 Chemokines, 9, 99, 279
 Chemotactic cytokines, 99
 Chemotherapy, 85, 99
 Chloride intracellular channel 4 (CLICL4), 224

- Chondrocytes, 176
 Chromosomal aberrations, 25, 26
 Chronic disease, 126
 Chronic inflammation, 164
 Chronic kidney disease (CKD), 181
 Chronic obstructive pulmonary disease (COPD), 224, 265
 Chronic peripheral inflammation, 165
 Chronic stress, 163
 Chronic total occlusion, 219
 Cigarette smoke, 224
 Circadian rhythm, 245
 Circulating angiogenic cells, 216
 Circulating endothelial cells (CECs), 224
 Cisplatin-induced nephropathy, 180
cis-regulatory elements, 29
 Clara cells, 267
 Clinical application
 genomic instability, 34–36
 Clinical research, 80, 86, 87
 Clinical trials
 A_{2A} receptor, 308
 allogeneic transplantation, 316
 autologous MSC transplantation, 312
 cell therapy, 283
 granulocyte colony-stimulating factor, 287
 HSC, 298
 in vivo preclinical research, 287
 MSC-NTF, 312
 stem cell/purinergic signaling modulators
 injection, 320
 therapeutic properties, 313, 319
 therapeutical potential, 309
 Clinical vascular phenotype, 226
 Clonogenicity, 248
 Clonote, 5, 10
 Club cells, 266, 267
 c-Myc activation, 246
 c-Myc regulator gene, 81
 Cognitive deficits, 83
 Cognitive disorders, 165
 Collagen-induced arthritis, 100
 Colon cancer, 148, 149
 Colony-forming unit fibroblasts, 280
 Colony-forming unit of basophils (CFU-Baso), 59
 Colony-forming unit of eosinophils (CFU-Eo), 59
 Colony-forming unit of erythrocytes (CFU-E), 59
 Colony-forming unit of granulocyte and monocytes (CFU-GM), 59, 60
 Colony-forming unit of granulocytes (CFU-G), 59
 Colony-forming unit of mix lineages (CFU-Mix), 58, 60
 Colony-forming unit of monocytes (CFU-M), 59
 Colony-forming unit–granulocyte-erythrocyte-monocyte-megakaryocyte (CFU-GEMM), 58
 Combined annotation-dependent depletion score, 30
 Combined pituitary hormone deficiency (CPHD), 361
 Common lymphoid progenitor (CLP), 52, 53, 58, 59
 Common myeloid progenitor (CMP), 52, 58, 59, 65
 Comparative genomic hybridization, 26
 Complement cascade, 166–168
 Complement proteins, 163
 Congenital heart disease, 283
 Copy number variations (CNVs), 26, 27
 Cord blood-derived MSCs (CB-MSCs), 180, 181
 Coronary diseases, 286
 Coronary heart disease, 283
 CPT-11, 85, 87
 Crohn's disease (CD), 134–141, 145–147
 MSCs, 101, 102
 Crypt base columnar cells (CBCs), 127
 Crypt-villus unit, 127
 Cutaneous tissue, 184, 185
 C-X-C chemokine receptor type 4 (CXCR4), 99
 C-X-C motif chemokine ligand 12 (CXCL12), 100
 Cyclooxygenase-2 (COX-2) activation, 297
 Cytokines, 9, 96, 98, 100, 102
- D**
 DAF-2, 367
 Dale's Principle, 277
 Danger-associated molecular pattern (DAMP), 168
 Delamination, 246
 Dendritic cells (DCs), 96, 97, 186
 Dendritic spine density, 161
 Depression, 160, 162–165, 168
 Dermal fibroblasts, 247
 Diabetic nephropathy (DN), 181
 Dickkopf-3 (DKK3), 250, 251
 Differentiation, 110, 112–115, 117–119
 Digestive diseases
 BMSCs, 126
 chaos quantum theory, 126
 GVB, 126

- mechanisms, 126
- microbiota, 149
- regulatory pathways, 126
- stem cell biology, 126
- Direct cardiac reprogramming fibroblast, 204
- Disabled-2 (Dab2), 250, 251
- Disease-causing SNPs, 30
- Disease-modifying therapies, 298
- Distal lung, 263
- DNA breaks, 356
- DNA damage, 244
- DNA methylation, 162
- DNA methyltransferase (DNMT), 162
- DNA methyltransferase 3B (DNMT3B), 26
- DNA methyltransferase 3-like (DNMT3L), 15
- Donor-derived hematopoietic stem cells, 29
- Dopamine, 277
- Dopamine progenitors, 309
- Dormant cells, 130
- Driver mutations, 29
- Drug delivery
 - MSCs, 99, 100
- Drug-induced models, 180
- Drug resistance, 85
- Drug toxicity tests, 282

- E**
- Early EPCs, 216
- Ecto-5'-nucleotidases, 278
- Ectoalkaline phosphatases, 278
- Ectodermal cells, 94
- Ecto-nucleotide pyrophosphatase/ phosphodiesterases (ecto-NPPs), 278
- EGF receptor (EGFR), 246
- Elutriation-derived (Fr25/Lin⁻) stem cells (ELH SCs), 130
- Embryogenesis, 5, 6
- Embryonic stem cells (ESCs), 2, 10, 67, 130
 - blastocysts, 11
 - blastula, 10
 - fertilized oocytes, 11
 - histocompatibility genes, 11
 - human embryos, 4
 - in humans, 176
 - and iPSCs, 4, 8, 16
 - vs. NSCs, 80
 - patient-derived bare nucleus, 11
 - and PSCs, 5
 - reproductive cloning, 11
- Embryonic tissues, 67
- Endogenous adult cardiomyocyte, 204, 205
- Endogenous stem cells, 80
- Endosomal sorting complex required for transport (ESCRT), 177
- Endothelial cells, 100, 176
- Endothelial colony-forming cells (ECFCs)
 - adult blood, 220
 - BM transplantation, 220
 - cell markers, 217
 - cell therapy product, 216
 - cellular reservoir, 216
 - characterization, 220
 - culture, 217
 - development, 218
 - EV, 221–223
 - hematopoietic markers, 217
 - in vivo mobilization, 218
 - lineage, 216
 - liquid biopsy (*see* Liquid biopsy)
 - mechanisms, 217, 218
 - ontogeny, 218–220
 - phenotype, 220
 - prevascularize tissue-engineering construct, 216
 - stemness, 218–220
 - therapeutic use, 218
 - vasculogenic progenitor cell population, 217
 - vasculogenic properties, 216
- Endothelial lineage
 - ECFC
 - ontogeny, 218–220
 - stemness, 218–220
- Endothelial MVs, 221
- Endothelial progenitor cells (EPCs), 14, 50, 53, 55, 126, 130, 200, 203
 - adult, 216
 - CD14⁺ monocytic cells, 217
 - CD45 antigen, 217
 - definition, 216–218
 - gastroenterology, 133, 145
 - isolation methods, 216
 - leukocyte-derived, 217
 - lung, 265
 - properties, 217
 - types, 216
 - vascular repair, 216
- Endothelial progenitor mobilization, 227
- Endothelial stem cells (ESCs)
 - gastroenterology, 133, 145
- Enterocytes, 127
- Enteroendocrine cells (ECs), 127
- Epiblast stem cells (EPSCs), 130
- Epidermal growth factor (EGF), 82, 245
- Epidermal proliferative unit (EPU), 243

- Epidermal stem cells (ESCs)
 differentiation, 242, 243
 division and proliferation, 241, 242
 hair follicle bulge stem cells, 247–251
 hierarchical model, 243
 interfollicular (*see* Interfollicular ESCs)
 properties, 241
 sebaceous gland stem cells, 252–254
 self-renewing, 240
 stem cell niche, 241
 stochastic model, 243
- Epidermis, 239, 240
- Epilepsy, 316–320
- Epithelial ovarian cancer (EOC), 118
- Epithelial-mesenchymal transition (EMT), 118
- Estrogens, 161
- Etiopathogenesis, 164, 165
- European Society for Blood and Marrow
 Transplantation (EBMT), 132
- EV-based therapy, 188
- EV-EPCs, 181
- Exogenous NSC transplants, 82
- Exosomes, 177, 181, 182, 184, 185, 281
 ECFCs, 222, 223
- Expansion of HSCs, 65, 66
- Experimental autoimmune encephalomyelitis
 (EAE), 297
- Experimental models, 176
- Extracellular matrix (ECM), 246
- Extracellular matrix molecules, 279
- Extracellular microvesicles (ExMVs),
 9, 148
- Extracellular vesicles (EV)
 apoptotic bodies, 221
 cardiovascular disease, 221
 cell function, 221
 coding and non-coding nucleic
 acids, 178
 definition, 177
 endothelial MVs, 221
 EPC-derived vesicles, 178
 ESCRT, 177
 exosomes, 177, 221–223
 external environment, 177
 immunomodulatory properties, MSCs,
 186, 187
 lipid bilayer, 177
 microvesicles, 177, 221, 222
 MVs/exosomes *ex vivo* producers, 221
 paracrine action, 177
 physiological and pathological
 settings, 177
 SCs (*see* Stem cell-derived extracellular
 vesicles (SC-EVs))
- Extraembryonic ectoderm, 53
- F**
- Fatty acid oxidation (FAO), 150, 369
- Fertilized oocytes, 11
- Fetal alcohol spectrum disorders (FASD)
 NSCs, 83–84
- Fetal alcohol syndrome, 83
- Fetal liver (FL), 50
- Fetal stem cells, 81, 176, 279
- Fetus's swift growth, 84
- Fibrinolytic properties, 227
- Fibroblast
 direct cardiac reprogramming, 204
- Fibroblastic progenitor cells, 268
- Fibrogenesis, 225
- First-episode psychosis (FEP), 166
- Flow cytometry, 111
- Fluorescence-activated cell sorting (FACS), 7
- Follicle-stimulating hormone (FSH), 15, 111,
 113–116, 118, 149
- Forkhead box transcription factor (FoxOs), 367
- FoxO proteins, 359
- FR25 fraction containing small cells, 64
- Fragmentation model, 112
- Frizzled-related protein-1 (Sfrp1), 251
- FSHR
 OSCs, 116, 117
 testicular stem cells, 115, 116
- G**
- GABAergic signaling, 162
- Galectin-1, 95
- Gametes, 110, 114, 119
in vitro, 117
- Gamma-aminobutyric acid (GABA), 161
- Gastric cancer, 147, 148
- Gastroenterology
 clinical discipline, 131
 EPCs, 133, 145
 ESCs, 133, 145
 host stem cell, 149, 150
 HSCs, 132
 IBDs, 131
 identification and purification strategies, 131
 microbial interactions, 149, 150
 morphology, 131
 MSCs, 133
 progenitor cells, 131
 PSCs, 131
 stem cells, 131
- Gastrointestinal disease
 CD, 145–147
 colon and rectal cancer, 148, 149
 gastric and pancreatic cancer, 147, 148
 IBDs, 145–147

- Gastrointestinal tract
 plasticity, 128–130
 regeneration, 128
 stem and progenitor cells, 127–128
- G-banding, 25
- Gene therapy, 85
- Genetic predisposition, 307
- Genetic reprogramming, 196
- Genomic aberrations in iPSCs
 chromosomal aberrations, 25, 26
 CNVs, 26, 27
 point mutations, 27–29
- Genomic instability, 4, 13, 16
 clinical application, 34–36
 iPSCs (*see* Induced pluripotent stem cells (iPSCs))
 reprogramming methods (*see* Reprogramming methods)
 somatic cells, 24
- Germ cell nests, 114
- Glial fibrillary acidic protein (GFAP), 310
- Glioblastoma, 85
- Glioblastoma cells, 100
- Glomerulosclerosis, 181
- Glucocorticoid-induced TNF receptor, 82
- Glucocorticoids, 163
- Glutamate, 277
- Glutathione peroxidase 1 (GPX1), 184
- Glycogenesis, 161
- Goblet cells, 127
- Good manufacture practice (GMP), 188
- Grafts, 80, 81, 86
- Graft-versus-host disease (GVHD), 187, 357
 MSCs, 101, 102
- Granular layers, 240
- Granulocyte-macrophage progenitor (GMP), 65
- Growth factors, 279
- Growth hormone (GH)
 anterior pituitary, 365
 deficiency, 360–364
 deletion, 360–364
 and insulin, 361
 receptor, 360–364
 resistance, 360–364
- Gut barrier (GB), 127
- Gut-brain communication, 127
- Gut-vascular barrier (GVB), 126
- H**
- Hair follicle bulge stem cells
 adnexal structures, 248
 anagen, 247
 basal layer, 249
 catagen, 247
 cell-cell interactions, 249
 clonal labelling, 248
 clonogenicity, 248
 differentiation, 249–251
 in vitro growth capacity, 248
 marker molecules, 249
 miniorgan, 247
 molecular mechanisms, 249–251
 organisation, 247, 248
 quiescence, 249–251
 regenerative capacity, 249
 sebaceous gland, 247
 telogen, 247
 thermal regulation, 247
- Heart failure (HF)
 heart transplantation, 196
 and IHD, 197
 and LV remodeling, 196
- Heart-induced ischemia-reperfusion
 injury, 183
- Heart transplantation, 196
- Heat-shock proteins, 356
- Hebbel's group, 220
- Hemangioblasts, 53
- Hemangioma (Hem-SC), 219
- Hematogenic endothelium, 53
- Hematopoiesis, 49–55, 57–59, 62, 66, 67, 69
- Hematopoietic BM, 51
- Hematopoietic cells, 2
- Hematopoietic islands, 53
- Hematopoietic markers, 94
- Hematopoietic niche and retention, 60, 61
- Hematopoietic progenitor cells (HPCs)
 circulating blood and lymph, 52
 CLP, 52
 CMP, 52
 differentiation, 59
 markers, 65
 murine and human, 53
 pharmacological agents, 52
 specification, 59
- Hematopoietic progenitors, 65
- Hematopoietic stem cells (HSCs), 167
 adult BM, 52
 aging, 357, 358
 application, 3
 BM microenvironment, 50
 circulating blood and lymph, 52
 clinical expansion, 66
 clinical settings, 68
 compartment, 50
 constant number, 7
 definition, 50
 development, 52
 developmental journey, 51

- Hematopoietic stem cells (HSCs) (*cont.*)
 developmental origin, 53–56
 embryonic and adult tissues, 50
 embryonic tissues, 67
 expansion, 65
 gastroenterology, 132
 in hematopoietic transplants, 3
 immunodeficient mice, 68
 in vitro and in vivo assays, 66, 67
 in vivo assays, 66
 markers, 62, 63
 MHC-I antigens, 66
 mobilization process, 61
 molecular analysis, 66
 murine and human, 53
 murine embryonic tissues, 67
 murine FL-derived HSCs, 67
 murine pre-HSCs, 67
 NOD/SCID mice, 68
 pharmacological agents, 52
 phenotype, 56–57
 purify and isolate, 63, 64
 self-renewal, 50, 280
 SRCs, 68
 symmetric and asymmetric division, 50, 51
 untired travelers, 51
 xenotransplants, 68
 YS-derived murine, 66
 YS to adult BM, 56–57
- Hematopoietic stem/progenitor cells (HSPCs), 126
 in BM microenvironment, 60, 61
 niche and retention, 60, 61
- Hematopoietic system, 358
- Hematopoietic transplants, 65
- Heme oxygenase-1, 95
- Hemostasis and thrombosis
 HHT, 226
 MPN, 226
 VTE disease, 227, 228
 VWD, 225, 226
- Hepatic regeneration, 183, 184
- Hepatocyte growth factor (HGF), 95, 148, 181, 186
- Hereditary hemorrhagic telangiectasia (HHT), 226
- Hering bile ducts, 127
- Hierarchical model, 112
- Histone deacetylase-3 (HDAC) inhibitor, 65
- HLSC-derived EVs, 182
- Hormone adiponectin, 161
- Host rejection, 84
- Host stem cell, 149, 150
- Human blastocysts, 282
- Human embryonic stem cells (hESCs), 220
- Human fetal tissues, 86
- Human leukocyte antigen (HLA), 101
- Human leukocyte antigen (HLA)-homozygous iPSC lines, 24
- Human NSCs, 85
- Human postnatal vasculogenic stem cells, 219
- Human umbilical cord blood MSCs (hUBC-MSCs), 97
- Human umbilical cord MSCs (hUC-MSCs), 183, 298
- Human-induced pluripotent stem cell-derived MSCs (hiPSC-MSCs), 184
- Human-induced pluripotent stem cells (hiPSCs), 220
- Huntington's disease, 80
- I**
- Idiopathic pulmonary fibrosis (IPF), 222, 224, 225
- IGF-1 signaling
Igfr^{+/–} mice, 364
 and insulin, 366
Irs1^{–/–} and *Irs2*^{–/–} mice, 365
Papp-A^{–/–} mice, 364, 365
- IGF-binding proteins (IGFBPs), 360, 361
- Immune response, 94, 98, 101
- Immunohistological assays, 287
- Immunomodulatory agents, 99
- Immunomodulatory effects, 98
- Immunotherapy, 94
- Imprinted genes, 372
- In vitro assays, 50, 56, 58, 59, 66, 67
- In vivo assays, 50, 53, 58, 59, 62, 64–66, 69
- In vivo mobilization, 218
- Indoleamine 2,3-dioxygenase (IDO), 95, 186
- Induced pluripotent stem cells (iPSCs), 165, 196
 adult tissues, 12
 AMD, 24
 cardiomyocytes, 13
 characteristics, 24
 clinical application, 34–36
 clinical safety, 13
 clinical trial, 24, 25
 and ESCs, 4, 24
 genetic aberrations, 24, 25
 genomic aberrations (*see* Genomic aberrations in iPSCs)
 HLA-homozygous iPSC lines, 24
 mouse fibroblasts, 12
 multigerm layer, 11
 mutagenesis, 13

- mutations, 29, 30
 - patient-matched pluripotent stem cells, 24
 - phenotype, 29, 30
 - potential risks, 13
 - RPE, 24
 - Yamanaka factors, 12
 - Inducible nitric oxide synthase (iNOS), 95
 - Inducible tissue factor, 227
 - Inflammation-induced homing, MSCs, 98, 99
 - Inflammatory bowel diseases (IBDs), 101, 131, 145–147
 - Inflammatory pathways, 82
 - Injecting stem cells, 84
 - Insulin receptor (InR), 367
 - Insulin receptor substrates (IRS), 365
 - Insulin/IGF1 signaling pathway, 366
 - Insulin-like growth factor (IGF) system
 - Ames mice, 361, 363
 - endocrine/serum, 360
 - gene-targeting approaches, 361
 - Ghr*^{-/-} mice, 363
 - IGFBPs, 360, 361
 - ligands, 360
 - lit/lit* mice, 363
 - phenotypic characteristics, 361, 362
 - RasGRF1^{-/-} and S6 K1-deficient mice, 363, 364
 - receptors, 360
 - Snell mice, 361
 - somatopause, 361
 - Insulin-like growth factor 1 (IGF-1), 161
 - Integrative multi-omics approach, 30
 - Interferon type 1, 224
 - Interfollicular ESCs
 - and adnexal structures, 240
 - b1-integrin expression, 244
 - differentiation, 244–247
 - EPU, 243
 - keratinocytes, 243
 - lineage-tracing experiments, 244
 - maintenance, 244–247
 - molecular mechanisms, 244–247
 - organisation, 240
 - pathogens/environmental toxins, 243
 - proliferation, 243–247
 - regenerative capacity, 244
 - Interleukin (IL)-10, 186
 - International Society of Extracellular Vesicles (ISEV), 177
 - Intestinal epithelial stem cells (ISCs), 359, 360
 - Intestinal stem cells, 127–130
 - Intracellular signaling pathway, 365
 - Intracranial neoplasm, 84
 - Intraparenchymal injections, 84
 - Ischemia/reperfusion injury (IRI) model, 180
 - Ischemic cardiomyopathy (ICM), 196
 - Ischemic heart disease (IHD)
 - cardiomyocytes, 204–205
 - CDCs, 203, 204
 - cell-enhancement approaches, 205
 - cell therapy-based approaches, 196, 197
 - CSCs, 203
 - HF (*see* Heart failure (HF))
 - ICM/NICM, 196
 - MI (*see* Myocardial infarction (MI))
 - noncardiac origin (*see* Noncardiac origin cell types)
 - stem cell biology, 196
 - Ischemic stroke therapy, 83
 - Istradefylline, 308
- J**
- Janus kinase 2 V617F (JAK2^{V617F}) mutation, 226
 - JNK/p38 MAPK pathway activation, 82
- K**
- Keratinisation process, 240
 - Keratinocytes, 239, 243
 - Kit ligand (KL), 15
- L**
- Lactobacillus reuteri*, 150
 - Lactobacillus rhamnosus*, 150
 - Lamina-associated domains (LADs), 28
 - Leucine-rich repeat kinase 2 (LRKK2), 307
 - Leukocyte-derived EPC, 217
 - Lineage-restricted progenitor cells (LRP), 65
 - Lineage-tracing experiments, 244
 - Liquid biopsy
 - ECFCs
 - biomarker, 223
 - hemostasis and thrombosis, 225–228
 - lung diseases, 223–225
 - Live cell imaging method, 112
 - Liver diseases, 142–144
 - Liver stem cells, 127
 - Lobules, 262
 - Long-term repopulating HSCs (LT-HSCs), 50, 55
 - Lrig1 (leucine-rich repeats and immunoglobulin-like domains 1), 245
 - Lung diseases
 - COPD, 224
 - IPF, 224, 225
 - PAH, 223, 224

- Lung injury protection, 185
- Lungs
- aging process, 269–271
 - and airways, 262, 263
 - development, 263, 268–270
 - endodermal origin, 268–270
 - energy components, 262
 - EPCs, 265
 - microscopic structure, 262, 263, 265
 - progenitors, 263
 - regeneration, 263, 265
 - respiratory system (*see* Respiratory system)
 - slow-turnover organs, 263
 - stem cells, 266, 269
 - 3D organoid culture, 268, 271
 - types, 264
- Luteinizing hormone (LH), 15, 149
- Lymphocyte enhancer factor-1 (Lef-1), 250
- M**
- Macrophages, 97
- Mammalian gonads
- adult gonads, 110
 - cancer, 117, 118
 - FSHR (*see* FSHR)
 - gametes, 117
 - and oncofertility, 118, 119
 - OSCs, 117, 118
 - testicular, 117, 118
 - VSELs (*see* Very small embryonic-like stem cells (VSELs))
- Mammalian ontogeny, 357
- Mammalian ovary stem cells, 114
- Mammals, 51
- Marrow adipose tissue (MAT), 51
- Marrow-isolated adult multilineage inducible (MIAMI) cells, 130
- Marrow stromal cells, 267
- Mass destruction, 5
- Mast cells (MCs), 97
- MCAo rat model, 82
- Mean pulmonary arterial pressure (mPAP), 223
- Mechanistic target of rapamycin (mTOR), 367
- Megakaryocytes, 59
- Membrane attack complex (MAC), 148
- Mental disorders, 164
- Mesenchymal cells, 128
- Mesenchymal stem cells (MSCs), 126, 130, 133, 220
- adipocytes, 198
 - advantage, 94
 - allogeneic, 199
 - APOLLO trial, 199
 - autologous, 199
 - B-cells, 96
 - BM, 94, 280
 - cardiac regenerative therapies
 - in clinical trials, 202
 - in preclinical studies, 201
 - cardiac therapies, 198
 - cardiopoiesis, 199
 - CD, 101, 102
 - CD29, 94
 - CD73, 94
 - CD90, 94
 - CD105, 94
 - cell types, 94
 - characteristics, 280
 - chondrocytes, 198
 - clinical applications, 9
 - colony-forming unit fibroblasts, 280
 - components, 94
 - cytokine profile, 198
 - DCs, 96, 97
 - definition, 198
 - delivery, 98
 - drug delivery, 99, 100
 - favoring recruitment, 176
 - GvHD responses, 101, 102
 - immune response, 281
 - immunomodulation, 199
 - immunomodulatory properties, 94, 98, 186, 187
 - immunotherapy, 94
 - in vitro studies, 198
 - inflammation-induced homing, 98, 99
 - licensing process, 98
 - localization, 176
 - macrophages, 97
 - maintenance, 280
 - markers, 280
 - MCs, 97
 - multipotency ability, 176
 - myocardial engraftment, 199
 - neutrophils, 97
 - NK cells, 96
 - operational definition, 280
 - pericytes, 280, 281
 - populations, 280
 - POSEIDON trial, 199
 - primary function, 199
 - properties, 94
 - respiratory system, 267, 268
 - secretome, 100
 - self-replicating cells, 280

- soluble and contact-dependent factors, 98, 99
 - sources, 98
 - swine model, 199
 - TAC-HFT trial, 199
 - T-cells, 95
 - therapeutic agents, 94
 - therapeutic applications, 94
 - tissue-regenerative capacity, 96
 - transplantation, 199
 - trophic mediators, 281
 - Mesodermal cells, 94
 - Mesodermal-inductive factors, 284
 - Metabolic fluorochromes, 62, 63
 - Metabotropic G protein-coupled P1 receptors, 278
 - Metformin, 8
 - Methyl-CpG-binding domain (MBD), 162
 - 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model, 308
 - Mice animal models, 182
 - Microbial interactions, 149, 150
 - Microbiome, 125
 - Microbiota
 - GI tract, 149
 - Microglial activity, 83
 - Microglial cells, 310
 - Microvesicles, 177, 281
 - ECFCs, 222
 - miR-124, 224
 - miR-381 overexpression, 82
 - Mitochondria, 162
 - Mitogens, 245
 - Mobilization process, HSCs, 61
 - Mobilized peripheral blood (mPB), 3, 4, 8
 - Monoclonal somatic cells, 28
 - Mononuclear cells (MNCs), 63, 64, 288
 - Monopotent stem cells, 6
 - Mood stabilizers, 164
 - Motor deficits, 305
 - Motor performance, 309
 - MPO+ infiltration, 82
 - MSC-EVs, 178
 - MSC-secreting neurotrophic factors (MSC-NTF), 312
 - Multilayer epithelium, 239
 - Multilineage-differentiating stress-enduring (Muse) cells, 7
 - Multilineage-differentiating stress-enduring stem cells, 130
 - Multiple sclerosis (MS), 95
 - A₁ receptor stimulation, 297, 298
 - autoimmune disease, 289
 - development, 298
 - disease-modifying therapies, 298
 - etiology, 289
 - HSCs, 298, 305
 - MSCs, 298–305
 - neuroinflammation, 297
 - P2X7 receptor polymorphism, 289
 - P2X7 receptors, 289, 297, 305
 - P2Y12 receptor, 297
 - PMS, 289
 - postmortem analysis, 289
 - RRMS, 289
 - symptoms, 289
 - Multiple-hit hypothesis, 29
 - Multipotent, 279
 - Multipotent adult progenitor cells (MAPCs), 7, 130, 220
 - Multipotent adult stem cells (MASCs), 7, 130, 176
 - Multipotent MSCs, 268
 - Multipotent progenitor cells (MPCs), 7, 65
 - Multipotent stem cells (MPSCs), 6
 - definition, 2
 - ectoderm, 6
 - endodermal, 6
 - germ layers, 6
 - postnatal tissues, 12
 - types, 7
 - Murine embryonic tissues, 67
 - Murine multipotent progenitor (MMP), 65
 - Murine VSELS, 58
 - Muscle activation, 83
 - MVs/exosomes ex vivo producers, 221
 - Myelin-binding proteins, 297
 - Myeloid lineage, 59
 - Myeloproliferative disorder (MPD), 358
 - Myeloproliferative neoplasms (MPN), 226
 - Myocardial infarction (MI), 219
 - cardiac function, 199
 - death and recurrent, 200
 - MSCs, 199
 - patient survival, 196
 - ST-segment elevation, 203
 - swine MI model, 203
 - Myocardium, 4
- N**
- Nanog⁺ monocytic cells, 217
 - National Marrow Donor Program, 357
 - Natural killer (NK) cells, 96, 186
 - Ncx1-KO embryos, 55
 - Neonatal intestinal necrosis (NEC), 127
 - Neural progenitor cell (NPC), 167, 305
 - Neural regeneration, 182, 183

- Neural stem cells (NSCs), 281, 282
 - aging, 358, 359
 - cancer, 84–86
 - characterization, 81
 - clinical applications, 87
 - CNS, 80
 - vs. ESCs, 80
 - exogenous, 80, 82
 - FASD, 83–84
 - grafts, 80
 - human fetal tissues, 86
 - in vivo models, 82
 - ischemic insult, 80
 - ischemic stroke, 82
 - low-density PNPC transplants, 80
 - neuroprotective mechanism, 86
 - in OGD, 82
 - preclinical experiments, 80
 - SDF-1, 82
 - self-renewal, 80
 - single dosage, 80
 - sources, 87
 - stroke, 81–83
 - translational studies, 86
 - transplantation, 80, 81
 - VEGF, 82
- Neural transplantation, 80, 84
- Neuroblasts, 160
- Neurodegenerative diseases
 - ALS (*see* Amyotrophic lateral sclerosis (ALS))
 - etiology, 279
 - SGZ, 281
 - SVZ, 281, 282
- Neurodegenerative disorders, 80
- Neurogenesis
 - in adult brain
 - aging, 161
 - autocrine, 162
 - C5a-C5aR1 signaling, 163
 - complement proteins, 163
 - complement system, 163
 - dendritic spine density, 161
 - DNA methylation, 162
 - estrogens, 161
 - external environment, 161
 - GABAergic signaling, 162
 - glycogenesis, 161
 - glycolysis to oxidative metabolism, 162
 - hippocampus, 163
 - hypothalamus, 160
 - IGFs, 160
 - in hippocampus, 160
 - lactate, 162
 - mammalian, 159
 - membrane-bound factors, 162
 - metabolic adaptation, 162
 - neuroblasts, 160
 - olfactory bulb, 160
 - paracrine factors, 162
 - primary regions, 163
 - proliferation, 161
 - quiescent neural stems, 162
 - rodents, 161
 - sex steroids, 162
 - SGZ, 159
 - subventricular zone, 159
 - tissue regeneration, 161
- Neuroinflammation, 297
- Neurons cells, 248
- Neuropilin-1 (NRP-1)-mediated differentiation, 220
- Neuropsychiatric disorders, 84, 86
- Neurotransmitters, 161
- Neutrophils, 97
- Next-generation sequencing (NGS), 26
- Niche-dependent extrinsic signals, 245
- Nicotinamide, 8
- Nicotinamide adenine dinucleotide (NAD)-dependent protein type III histone deacetylases (HDACs), 370
- Nigrostriatal pathway, 305
- Noncancerous skin cells, 31
- Noncardiac origin cell types
 - BMMNCs, 198
 - EPCs, 200, 203
 - MSCs (*see* Mesenchymal stem cells (MSCs))
 - SMs, 197, 198
- Noncoding regions, 29
- Noncoding RNAs (ncRNAs), 222
- Non-integrating vectors, 13
- Nonischemic cardiomyopathy (NICM)
 - cardiomyopathy, 196
- Non-motor symptoms, 305
- Non-proteolytic enzymes, 61
- Nonsteroidal anti-inflammatory drugs (NSAIDs), 126
- Nonsynonymous/nonsense, 29
- Noradrenaline, 277
- Normal cells, 355
- Notch signaling, 164
- Notch/RBPJ signaling, 161
- Nuclear energy, 5
- Nuclear factor kappa B (NFkB), 82
- Nuclear Oct-4A, 14
- Nuclear transfer, 10, 11
- Nucleic acids, 242
- Nutrient-sensing response, 367

O

Odorant discrimination, 160
 Olfactory memory, 160
 Oligodendrocyte precursor cells (OPC), 164
 Oligodendrocytes, 161
 Omnicytes, 7
 Oncofertility, 119
 and mammalian gonads, 118, 119
 Osteoblasts, 176
 Oval cells, 127
 Ovarian stem cells (OSCs)
 in adult mammalian ovaries, 110
 cytoplasmic OCT-4, 114
 FSHR, 116, 117
 human ovaries, 114
 mammalian gonads, 117, 118
 mouse ovaries, 114
 mouse ovary surface epithelium, 113
 neogenesis and primordial follicle
 assembly, 115
 small-sized stem cells, 113
 VSELs, 110, 114
 Ovarian VSELs, 113–115
 Ovary, 111, 113, 114, 116, 117, 119
 OX-42+ microglia, 82
 Oxygen and glucose deprivation (OGD), 82

P

P2X ionotropic ligand-gated ion channel
 receptors, 277
 P2X1-7 receptors, 278
 P2Y metabotropic G protein-coupled
 receptors, 277
 P2Y1,2,4,6,11,12,13,14 receptors, 278, 279
 P2Y12 receptor, 297
 Pancreatic cancer, 147, 148
 Paneth cells, 127, 128
 Panic disorder (PD), 167
 Paracrine action, 177
 Paracrine effects, 4, 9, 13
 Parental skin fibroblasts, 26
 Parental somatic cells, 27
 Parkinson's disease (PD), 80
 A_{2A} receptor, 307, 308
 adjunctive therapy/monotherapy, 308
 cell death, 307
 cell loss, 305
 clinical translation, 309
 diagnosis, 305
 dopamine progenitors, 309
 dopaminergic neurons, 305
 functional role, 308
 genetic predisposition, 307
 IL-6, 307
 iPSC, 309
 istradefylline, 308
 mechanisms, 307
 motor deficits, 305
 MSCs, 308, 309
 neuroinflammation, 307
 neuroprotection mechanisms, 307
 P2X7 receptor, 307
 P2Y6 receptor, 307
 pathophysiology, 307
 pharmacologic blockade/genetic
 knockdown, 308
 sporadic patients, 307
 Passage-induced mutations, 29
 Passenger mutations, 29
 Patient-matched pluripotent stem
 cells, 24
 Pentaspan membrane glycoprotein, 219
 Pericytes, 280, 281
 Peripheral blood (PB), 50
 Pharmacological mobilization, 52
 Phenotypes, iPSCs, 29, 30
 Photothrombotic stroke model, 82
 Pituitary factor-1 (*Prop1*), 362, 363
 Pituitary gonadotropins (PtGt), 149
 Plasma-derived factors, 161
 Plasticity, 128
 Pluripotency, 26
 Pluripotent cells, 282
 Pluripotent Sca-1+CD45-c-kit- cells, 130
 Pluripotent stem cells (PSCs), 111, 128, 131,
 279, 282, 371
 adult tissues, 13–16
 definition, 2, 9
 ESCs, 10, 11
 germ layers, 5, 6
 in vitro and in vivo criteria, 9, 10
 iPSCs, 11–13
 population, 7
 postnatal tissues, 2, 12
 proximal epiblast, 50
 Pluripotent/multipotent stem cells, 58
 Point mutations, iPSCs
 classification, 27
 in vitro culture, 27
 NGS technology, 27
 passage-induced mutations, 29
 preexisting mutations, 27, 28
 protein-coding regions, 27
 reprogramming-induced mutations, 28
 POSEIDON trial, 199
 Preclinical research, 80, 83, 84, 86
 Precursor cell, 279

- Pregnancy-associated plasma protein-A (PAPP-A), 364
- Primordial germ cells (PGCs), 7, 14, 15, 53–56, 58, 130
- Progenitor, 283
- Progenitor cell, 279
- Progenitor SSCs, 111
- Progressive multiple sclerosis (PMS), 289
- Proinflammatory cytokines, 100, 165
- Pro-inflammatory T-cell activity, 82
- Prostaglandin E2 (PGE₂), 95
- Protein homeostasis, 356
- Protein kinase B (PKB), 367
- Protein-coding mutations, 30
- Protein-coding point mutations, 29
- Protein-coding regions, 29
- Psychiatric disorders, 83
- Psychiatry
 - affective disorders, 163–165
 - alcohol dependence, 168
 - anxiety disorders, 167
 - neurogenesis in adult brain, 159–163
 - schizophrenia, 166–167
 - sterile inflammation, 168
- Pulmonary arterial hypertension (PAH), 223, 224
- Pulmonary vascular disease, 223
- Purinergic neurotransmission, 277
- Purinergic receptor activity modulation, 283
- Purinergic receptors, 277, 313
 - evolution, 277
 - selective for adenosine (P1), 277
 - selective for ATP/ADP (P2), 277
- Purinergic signaling
 - AD (*see* Alzheimer's disease (AD))
 - adult stem cells, 280
 - ALS (*see* Amyotrophic lateral sclerosis (ALS))
 - cardiovascular diseases (*see* Cardiovascular diseases)
 - epilepsy, 316–320
 - in vivo and in vitro studies, 298
 - iPSCs, 282
 - long-term events, 278
 - mechanisms, 283
 - metabotropic G protein-coupled P1 receptors, 278
 - MS (*see* Multiple sclerosis (MS))
 - MSCs, 280, 281
 - neurogenesis, 281
 - nonhereditary mutations, 283
 - NSCs, 282
 - P2X1-7 receptors, 278
 - P2Y1,2,4,6,11,12,13,14 receptors, 278, 279
 - PD (*see* Parkinson's disease (PD))
 - purine and pyrimidine nucleotides, 277
 - purinergic receptors, 277
 - short-term events, 278
- Putative PSCs, 7
- Q**
- Qualitative defects, 225
- Quantitative defects, 225
- Quiescent ESCs, 250, 251
- Quiescent neural stems, 162
- Quiescent stem cells, 110
- R**
- Randomized controlled trials (RCTs), 200
- Rat gentamicin-induced AKI model, 176
- Reactive oxygen species (ROS), 356
- Rectal cancer, 148, 149
- Red marrow, 51
- Regeneration, 196, 198, 200–203
- Regenerative medicine, 8, 128
 - adult stem cells, 4
 - clinical, 8
 - ExMVVs, 4
 - gastroenterology (*see* Gastroenterology)
 - goals, 3
 - and mechanisms, 5
 - PSCs (*see* Pluripotent stem cells (PSCs))
 - secretome, 4
 - therapeutic procedure, 4
- Regulatory DC (regDC), 97
- Regulatory pathways, 126
- Relapsing-remitting multiple sclerosis (RRMS), 289
- Renal regeneration, 178–182
- Reproductive cloning, 11
- Reprogramming methods
 - cell source, 30–32
 - culture conditions, 34
 - delivery factors, 32, 33
 - factors, 33
 - genomic instability in iPSCs, 30, 31
 - SCNT, 33
- Reprogramming-induced mutations, 27, 28
- Respiratory distress syndrome (RDS), 266
- Respiratory system
 - ASCs, 263, 264
 - AT1, 266
 - AT2, 265, 266
 - BASCs, 266
 - club cells, 267

- definition of regeneration, 263
 - endodermal origin, 268–270
 - lung EPCs, 265
 - macroscopic structure, 262
 - MSCs, 267, 268
 - Retinal pigment epithelial (RPE), 24
 - Rheumatic heart disease, 283
 - Rheumatoid arthritis (RA), 100
 - Rodent stroke models, 82
 - Rotaviruses (RV), 150
 - Rotenone model, 309
 - Rotenone-induced hemiparkinsonian, 309
- S**
- Sca-1⁺c-kit⁺Lin⁻ (SKL) cells, 64
 - Schizophrenia, 166–167
 - Schwann cells, 248
 - SCID mouse-repopulating cells (SRCs), 68
 - SCNT-ESCs, 33
 - SDF-1, 176
 - Sebaceous gland, 247
 - Sebaceous gland stem cells
 - characterization, 252
 - development, 252
 - differentiation, 252–254
 - hair cycles, 252
 - hair follicle, 252
 - molecular mechanisms, 253, 254
 - peripheral layer, 252, 253
 - posterior hair peg surface, 252
 - proliferation, 253, 254
 - regenerative capacity, 252
 - thermoregulation, 252
 - Secreted extracellular microvesicles (ExMVs), 4, 8, 9
 - Secreted frizzled-related protein-1 (Sfrp1), 250
 - Secretion
 - EV, 221–223
 - Secretome, 4, 177
 - MSCs, 100
 - Selective serotonin reuptake inhibitor (SSRI) antidepressants, 163
 - Self-renewal, 241, 242, 244, 255
 - Self-renewing ESCs, 240
 - Self-renewing stem cells, 240, 244
 - Senescent-like NK cells, 96
 - Serine, 367
 - Serotonin, 164, 277
 - Serum/glucocorticoid-regulated kinase (SGK), 367
 - Sex hormone (SexH) receptors, 149
 - Sex steroids, 162
 - Shelterin, 356
 - Side population (SP), 64, 268
 - Signaling lymphocyte activation molecule (SLAM) markers, 63
 - Signaling pathways, 263
 - Single nucleotide polymorphism (SNP) arrays, 26
 - Single-cell zygote, 2
 - Single-dose treatment, 80
 - Sirt-1, 15
 - Sirtuin family contain seven proteins (SIRT1–SIRT7), 369, 370
 - Sirtuins, 369–371
 - Skeletal muscle biopsies, 4
 - Skeletal myoblasts (SMs), 197, 198
 - Skeletal regeneration, 185
 - Skin epithelium, 4, 8
 - Skin fibroblast-derived iPSCs, 31
 - Skin fibroblasts, 12, 30
 - Skin regeneration, 254, 255
 - Small-molecule UM177, 15
 - Social behaviors, 83
 - Social interactions, 160
 - Somatic cell nuclear transfer (SCNT), 33
 - Somatic cells, 24, 357, 368
 - Somatic stem cells, 263
 - Somatopause, 361
 - Sonic hedgehog (Shh) signalling, 251
 - Spatial organisation, 241
 - Spermatogenesis
 - SSCs, 112
 - Spermatogonial stem cells (SSCs)
 - cytoplasmic OCT-4, 114
 - description, 110
 - FSHR, 111
 - in mature gonads, 110
 - progenitor, 111
 - spermatogenesis, 112
 - VSELs, 110, 114
 - Sphingosine-1 phosphate (PLc), 57
 - Sphingosine-1-phosphate (S1P), 61, 148, 166, 167
 - Spinal cord motor neurons, 83
 - Spinous layers, 240
 - Sporadic disease, 310
 - Spore-like stem cells, 7, 130
 - Stage-specific embryonic antigen (SSEA), 14, 58
 - Stem cell aging hypothesis, 357
 - Stem cell alterations, 125
 - Stem cell-based therapy, 299–304
 - Stem cell daughters, 241, 242

- Stem cell-derived extracellular vesicles (SC-EVs)
 cardiovascular regeneration, 183
 cutaneous tissue, 184, 185
 hepatic regeneration, 183, 184
 lung injury protection, 185
 neural regeneration, 182, 183
 renal regeneration, 178–182
 skeletal regeneration, 185
 therapeutic effects, 179–180
- Stem cell homing
 chemoattractants, 61, 62
- Stem cell niche, 241
- Stem cell plasticity, 130
- Stem cell purification, 68
- Stem cells (SCs)
 adult tissues, 2
 and adenosine, 288
 and aging (*see* Aging)
 biological organization, 2
 biology and technology, 4
 classification, 175
 clinical settings, 4
 developmental hierarchy, 5, 6
 engraftment, 279
 ESCs (*see* Embryonic stem cells (ESCs))
 HSCs (*see* Hematopoietic stem cells (HSCs))
 IHD (*see* Ischemic heart disease (IHD))
 in lungs (*see* Lungs)
 mammalian gonads (*see* Mammalian gonads)
 mobilization, 279
 molecular biology, 3
 molecular composition, 4
 MPSCs (*see* Multipotent stem cells (MPSCs))
 niche/microenvironment, 279
 properties, 2, 3
 PSCs (*see* Pluripotent stem cells (PSCs))
 psychiatry (*see* Psychiatry)
 self-renew, 279
 TCSCs (*see* Tissue-committed stem cells (TCSCs))
 therapeutic application, 8, 9
 therapeutic effects, 311
 three-dimensional structures, 4
 tissue development, 5–8
 tissue engineering, 3
 tourism, 5
 TSCs (*see* Totipotent stem cell (TSCs))
 types, 283
 zygote, 2
- Stem cell therapies, 80, 81, 84
- Stem cell transplantation, 80, 83
- Stemness marker, 219
- Sterile inflammation, 168
- Stochastic model, 242
- Stratum corneum layers, 240
- Streptozotocin model, 181
- Stroke, 80
 NSCs, 81–83
- Stromal cell-derived factor 1 (SDF-1), 99, 148, 167, 284
- Subgranular zone (SGZ), 81, 159, 281, 358
- Subventricular zone (SVZ), 81, 159, 281, 282, 358
- Swine model, 199
- System-wide chemotherapeutics, 85
- T**
- TAC-HFT trial, 199
- T-cells, 95
- Telomerase, 356
- Telomere shortening, 244
- Ten-eleven translocation (TET) proteins, 162
- Teratoma formation, 9, 11, 16
- Testicular stem cells
 FSHR, 115, 116
 mammalian gonads, 117, 118
- Testicular VSELs, 111–113
- Testis
 adult human, 110, 111
 azoospermic, 111
 chemoablated, 112
 FSH, 111
- T-helper cell polarization, 95
- Therapeutic cloning, 5, 10, 11
- Threonine kinase AKT, 367
- Thrombospondin-1, 227
- Thyroid-stimulating hormones (TSH), 362
- Tissue-committed stem cells (TCSCs), 127, 128, 130
 adult cells, 6
 adult tissues, 6
 clinical data, 8
 cytostatic drugs, 8
 definition, 7
 hemato-/lymphopoietic cells, 6
 mesenchyme, 9
 monopotent, 2, 6, 7
 properties, 8
 radio-chemotherapy, 8
 sympathetic ganglions, 6

- types, 8
 - UCB-derived, 8
 - Tissue development
 - biology and genetics, 5
 - embryogenesis, 5, 6
 - ESCs, 5
 - FACS, 7
 - mass destruction, 5
 - MPSCs, 6
 - PSCs, 5
 - and regenerative medicine, 8
 - totipotent stem cells (TSCs) (*see* Totipotent stem cells (TSCs))
 - VSELs, 7
 - Tissue regeneration, 161
 - Tissue repair, 176, 184
 - Tissue repair mechanisms, 279
 - TNF- α -stimulated gene 6 protein (TSG-6), 97
 - Totipotency, 2
 - Totipotent stem cells (TSCs), 2, 5, 279
 - Tracheobronchial airways, 263
 - Transcription factor-3 (Tcf-3), 250, 251
 - Transcriptional profiling, 249
 - Transforming growth factor- β (TGF- β), 95, 186
 - Transit-amplifying cell (TA cell), 240–243, 253
 - Transition-dominant pattern, 28
 - Translationally controlled tumor protein (TCTP), 224
 - Transmission electron microscopy, 58
 - Transversion-dominant pattern, 28
 - Traumatic brain injury (TBI), 80, 83, 182
 - Trisomy 12, 25, 26
 - True stem cells, 111
 - Tubule-interstitial fibrosis, 181
 - Tuft cells, 127
 - Tumor-associated angiogenesis, 100
 - Tumor-derived plasma chemoattractants, 148
 - Tumour growth factor β 2 (TGF β 2), 251
 - Tumor necrosis factor-alpha (TNF- α), 100, 297
 - Type I alveolar epithelial cells, 263
 - Type II alveolar epithelial cells, 263
 - Type II pneumocytes, 265, 266
 - Tyrosine hydroxylase (TH)-positive cells, 309
- U**
- UDP-glucose, 277
 - Umbilical cord blood (UCB), 4, 8, 50
 - Umbilical cord blood cells, 31
 - Umbilical cord stem cells, 279
- Unrestricted somatic stem cells (USSCs), 7
 - Uridine diphosphate (UDP), 277
 - Uridine triphosphate (UTP), 277
- V**
- Vascular cell adhesion molecule-1 (VCAM-1), 60
 - Vascular diseases, 283, 284
 - Vascular endothelial growth factor (VEGF), 82, 148, 161, 284
 - Vascular regeneration, 287
 - Vascular system, 284
 - Vasculogenesis, 216
 - Venous thromboembolism (VTE) disease, 227, 228
 - Verapamil-sensitive testicular side population, 111
 - Very small embryonic-like stem cells (VSELs), 7, 12–16, 52, 54–58, 65, 66, 69, 126, 219
 - adult tissues, 110, 111
 - basal lamina, 111
 - bone marrow, 110
 - characteristics, 220
 - characterization, 110
 - definition, 219
 - flow cytometry, 111
 - hemangioblast, 226
 - human BM, 219
 - LIN-CD45-SCA-1+ VSELs, 111
 - mesenchymal phenotype, 219
 - nucleus-to-cytoplasm ratio, 219
 - oocyte-like structures, 111
 - OSCs, 111
 - ovarian, 113–115
 - proliferative capacity, 220
 - quiescent stem cells, 110
 - SSCs, 112
 - SSCs/OSCs, 110
 - stem cell hierarchy, 219
 - testicular, 111–113
 - von Willebrand disease (VWD), 225, 226
 - von Willebrand factor (vWF), 225, 226
- W**
- Wharton's jelly-derived MSCs (WJ-MSCs), 181
 - Whole exome sequencing (WES), 27
 - Whole genome sequencing (WGS), 26
 - Wnt/beta-catenin genetic system, 126

Wnt/ β -catenin signalling pathway, 251
Wnt inhibitory factor-1 (WIF1), 250, 251
Wnt signalling pathway, 249–250
Wound healing, 281

X

Xenotransplants, 68

Y

Yamanaka factors, 12
Yolk sac (YS), 50, 51, 53, 55–57, 66, 67

Z

Zonulin, 148
Zygote, 2