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Regenerative Medicine and Stem Cell Biology

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Regenerative Medicine and Stem Cell Biology

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Preface

The field of stem cells and regenerative medicine has expanded to include many disciplines in biology, medicine, physics, material science, biomedical engineering, and nanotechnology. This multidisciplinary approach necessitates acquiring knowledge that is contextual, practical, and focused on these new disciplines. This book provides an overview of the basic concepts of stem cell research and the important topics in the field that are of interest to students and also to researchers and physicians. The topics have been selected carefully to fulfill both the theoretical and practical aspects of stem cell research, in an approach that is beneficial to researchers who are interested to specialize in the field or to complement their research in other fields.

The introduction provides an overview of stem cells and the facts and hype about their usage in the clinic. Some diseases are prescribed stem cell transplantation as routine therapy, especially those of hematopoietic origin. Other therapeutic approaches are still experimental. At the forefront of diseases treated with stem cells are hematological disorders, leukemia, lymphoma, hemoglobinopathies, and immune deficiencies. Research on hematopoietic stem cells has been pioneering in delivering reliable therapy for blood disorders, and it comes as no surprise that almost all of the FDA-approved stem cell products are also of hematopoietic origin. The chapters on adult stem cells cover hematopoietic stem cells, mesenchymal stromal cells, endothelial progenitor cells, and pericytes and provide the reader with a good basis for understanding the biology and applications of these important cells.

The chapters on the epigenetic regulation of stem cells, cancer development and its regulation by cancer stem cells and associated stromal cells cover the molecular mechanisms that govern stem cell development and differentiation in health and disease. The same theme extends into the chapter on the use of stem cell therapy in the treatment of metabolic disorders, which provides a much-needed insight on regenerative therapy in the clinical setting, with a focus on diabetes as the most prevalent metabolic disease.

Many landmark experiments, from cloning frogs in the 1960s and mammalian cloning in the 1990s to the current direct cellular reprogramming and gene editing provided a more flexible and broader understanding of stem cell biology and of cell biology in general.

Characterization of the embryonic stem cells and Yamanaka's pioneering experiments in reprogramming somatic cells into induced pluripotent cells made stem cell therapy more achievable. It is now becoming more possible to manipulate mature cells on the genetic and epigenetic levels, to reverse their development and to regenerate their differentiation potential. This revolution in cell biology has not been matched unfortunately with a comparable clinical revolution, where patients have directly and similarly benefited from these unprecedented advances.

Advances in biotechnology, nanotechnology, and bioprinting have opened the doors to unlimited possibilities in regenerative medicine. Using natural and synthetic scaffolds fulfills the structural foundation of any organ on which cells are seeded and coaxed to differentiate and develop into the desired tissues. Bioprinting, 3D culture techniques, organ-on-a-chip, and other technical advances expanded the applications of stem cells well into personalized medicine. In vitro disease modeling and testing drugs on patient-specific tissues undoubtedly present a leap in precision medicine. Chapters 10 and 11 discuss tissue engineering with detailed examples of bioscaffold preparation in the form of the decellularized human amniotic membrane. After its use with success in skin and corneal grafts, its attractive anti-inflammatory and antimicrobial properties and low immunogenicity support its use as a scaffold for stem cell growth and differentiation. Detailed protocol for bioscaffold preparation and other protocols for isolation and culture of mesenchymal stromal cells and induced pluripotent stem cells are also detailed.

The book concludes with a reminder for young scientists of following the basics of the scientific method, of adherence to ethical practices in their research, and of frequently questioning the methods and goals of their research. These practices tie directly with the introduction on the benefits of stem cell research and its applications, to maximize the hope and minimize the hype in this promising field.

Giza, Egypt Nagwa El-Badri

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Introduction and Basic Concepts in Stem Cell Research and Therapy: The Facts and the Hype

1

Mohamed Essawy, Shaimaa Shouman, Shireen Magdy, Ahmed Abdelfattah-Hassan, and Nagwa El-Badri

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List of Abbreviations

(ACI)	Autologous Chondrocyte Implantation
(ADSCs)	Adipose-derived stem cells
(ALL)	Acute Lymphoblastic Leukemia
(AMD)	Age-related Macular Degeneration
(AML)	Acute Myeloid Leukemia
(BM)	Bone Marrow
(BM-HSCs)	Bone Marrow Hematopoietic Stem Cells
(BM-MSCs)	Bone Marrow Mesenchymal Stem Cells
(CAR)	Chimeric Antigen Receptor
(CBT)	Cord Blood Transplantation
(CFU-F)	Colony Forming-Unit Fibroblast
(CLL)	Chronic Lymphoblastic Leukemia
(CLP)	Common Lymphoid Progenitor
(CML)	Chronic Myeloid Leukemia
(DLI)	Donor Leukocyte Infusion
(DM)	Diabetes Mellitus
(DMT1)	Type 1 Diabetes Mellitus
(DMT2)	Type 2 Diabetes Mellitus
(ECM)	Extracellular Matrix
(ESCs)	Embryonic stem cells
(FTSG)	Full-thickness Skin Graft
(G-CSF)	Granulocyte Colony-stimulating Factor
(GvHD)	Graft versus Host Disease
(GVL)	Graft Versus Leukemia
(HSCs)	Hematopoietic Stem Cells
(HSCT)	Hematopoietic Stem Cell Transplantation
(HSPCs)	Hematopoietic Stem/Progenitor Cells
(iPSCs)	Induced Pluripotent Stem Cells
(ISSCR)	International Society for Stem Cell Research
(MS)	Multiple Sclerosis
(MSCs)	Mesenchymal Stem Cells
(NSCs)	Neural Stem Cells
(OA)	Osteoarthritis
(PB)	Peripheral Blood
(PD)	Parkinson's Disease
(PRP)	Platelet-rich Plasma
(RIC)	Reduced-intensity Conditioning
(RPE)	Retinal Pigment Epithelial
(SCNT)	Somatic Cell Nuclear Transfer

(STSG) Split-thickness Skin Graft
(UCB) Umbilical Cord Blood
(UC-HSCs) Umbilical Cord Hematopoietic Stem Cells
(UC-MSCs) Umbilical Cord Mesenchymal Stem Cells

What You Will Learn in This Chapter

This chapter provides the introduction and overview of stem cells, their definition, origin, and applications. It illustrates the unique properties of stem cells, such as potency, multilineage differentiation potential, self-renewal, and resistance to senescence and apoptosis. It provides a brief description of stem cell research, and its current applications in cell therapy, bone marrow transplantation, tissue engineering and its modern and diverse applications. These will cover approved human stem cell products, and therapies based on cells or their derivatives. Finally, the chapter will cover the gap between research and clinical applications, and concludes with the facts, hope, and hype in stem cell research and development.

1.1 What Is a Stem Cell?

A stem cell is an unspecialized and undifferentiated cell that has a remarkable capacity for self-renewal and the ability to undergo prolonged periods of cell division, both in vitro and in vivo. Stem cells are also capable of asymmetrical division into two non-identical daughter cells with distinctive and different fates. Among the earliest evidence of the existence of stem cells were the breakthrough studies conducted in the early 1960s, when the radiation physicist, James Till, joined with the hematologist, Ernest McCulloch, to study the effects of radiotherapy on hematological cancers in the bone marrow. Among their findings, Till and McCulloch identified a self-renewing population of hematopoietic cells originating in the bone marrow that were capable of generating all blood cell lineages; they named these progenitors "stem cells" [1–3].

Unlike other types of cells, stem cells have the capacity to differentiate into various specialized cells and cell lineages under defined physiological, pathological, and/or experimental conditions. The regenerative capacities are high among younger individuals; aging is associated with lower regenerative potential [4–6]. Moreover, in a mature organism, some organs, such as the blood and intestinal epithelium, maintain a higher rate of regeneration throughout life, whereas other organs, including the heart and pancreas, have limited potential for repair [7]. Stem cells can be classified based on their differentiation capacity into totipotent, pluripotent, multipotent, oligopotent, and unipotent cells, as shown in Fig. 1.1. Totipotent stem cells exhibit the highest capacity for differentiation of any cell in an entire organism, the notable example of this phenomenon is the zygote (i.e., a fertilized egg) which has the capacity to give rise to all embryonic and extraembryonic

structures [8, 9]. Pluripotent stem cells, such as embryonic stem cells (ESCs), are somewhat less potent and are capable of generating embryonic tissues only (i.e., the three germ layers, mesoderm, endoderm, and ectoderm [10]). Lineage specific multipotent stem cells such as mesenchymal stem cells (MSCs) and hematopoietic stem cells (HSCs) have a more restricted capacity for differentiation and give rise to their specific tissues and cell types [11]. Oligopotent stem cells are even more restricted but maintain the capacity to differentiate into specific cells within specific tissues. A good example of an oligopotent stem cell is the common lymphoid progenitor (CLP), which can give rise to T lymphocytes, B lymphocytes and natural killer cells [12, 13]. Unipotent stem cells are the most restricted, as they are capable of generating cells of a single lineage; examples of unipotent stem cells include epidermal stem cells of the skin [14, 15], myogenic precursors [16], and spermatogonial stem cells [17].

It is generally understood that the capacities for self-renewal and differentiation diminish as cells become more specialized. However, this dogma was recently challenged by the successful reprogramming of fully differentiated somatic cells into a pluripotent-like state in the form of somatic cell nuclear transfer (SCNT) [18] and likewise via the induction of pluripotent stem cells (iPSCs), first described in 2006 [19].

1.2 Origin and Types of Stem Cells

Stem cells are classified as embryonic or adult stem cells based on their source of origin (as shown in Fig. 1.1). Tissues associated with pregnancy, including the placenta, amniotic fluid, umbilical cord, and Wharton's jelly, among others, are all rich in stem cells. Likewise, iPSCs are cells produced by the direct reprogramming of somatic cells into pluripotent stem cells. A comparison of the properties of embryonic, adult, and iPSCs is presented in Table 1.1.

1.2.1 Embryonic Stem Cells (ESCs)

ESCs can be collected from the inner cell mass of pre-implantation embryos 3–5 days following fertilization. ESCs are pluripotent cells that have the capability to divide for extended periods of time and to differentiate into cells of each of the three germ layers [10, 20]. This robust differentiation potential qualifies ESCs as the best-known source of cells that can be used to generate fully differentiated cells for cell therapy applications [21, 22]. Ethical concerns related to the destruction of human embryos have hampered the full application of ESCs, which are isolated from spare/discarded embryos that were generated to support in vitro fertilization (IVF) procedures and not from healthy in utero-implanted ones [23–25].

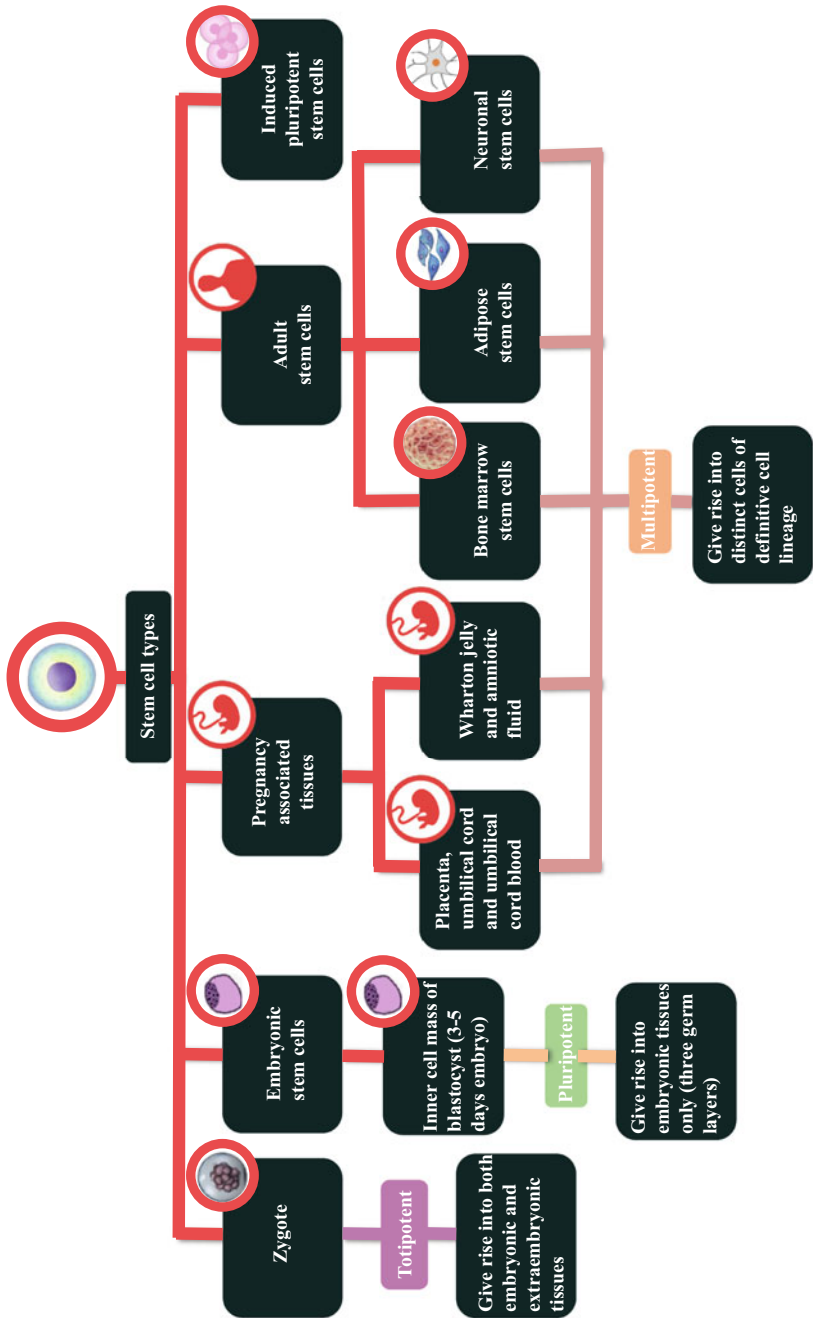

Fig. 1.1 The origins and types of stem cells

Table 1.1 General comparison between embryonic stem cells, adult stem cells, and iPSCs

Cell type	Embryonic stem cells	Adult stem cells	iPSCs
Origin	Pluripotent cells derived from the inner cell mass of the blastocysts [10, 43]	Multipotent cells derived from adult tissues [37, 44–46]	Somatic cells reprogrammed into embryonic-like pluripotent stem cells [19, 47]
Self-renewal capacity	High [10, 43]	Limited [37, 44–46]	High [19, 47]
Potency	Pluripotent [10, 43]	Multipotent [37, 44–46]	Pluripotent [19, 47]
Differentiation	Can differentiate into cells of each of the three germ layers [10, 43]	Restricted lineage differentiation [37, 44–46]	Can differentiate into cells of each of the three germ lineages [19, 47]
Surface markers	Pluripotency markers (OCT4, SOX2, NANOG, SSEA-3, SSEA-4, TRA-1-60, and TRA-1-81 [10, 43, 48]	Specific markers of adult tissue-derived stem cells. For example, MSCs express CD90, CD73, and CD105 along with a negative expression for the hematopoietic markers CD45, CD3, CD19, CD11, CD79α, and human leucocyte antigen-DR (HLA-DR) [46, 49]	Pluripotency markers (OCT4, SOX2, NANOG, SSEA-4, and KLF4 [19, 47, 50]
Spontaneous oncogenic transformation	Present [10, 43]	Absent [37, 44–46]	Present [19, 47]
Immune response	Strong [51, 52]	Strong for allogeneic, but not for autologous cells [53–55]	Strong, but can be minimized for autologous cells [56]
Ethical concerns	Yes [24, 51]	No [57]	Minimal [58]

1.2.2 Adult Stem Cells

Somatic or adult stem cells are rare populations of undifferentiated cells that are found among their differentiated counterparts throughout the adult body. These cells contribute to tissue homeostasis, as they serve as a source of raw material for repair and/or replacement of injured or dead cells [5]. Adult stem cells have only a limited range of differentiation potential when compared with ESCs. Examples of adult stem cells include the following:

- **Mesenchymal Stem Cells (MSCs)**

MSCs are adherent fibroblast-like cells when cultured in vitro. They were first isolated from the bone marrow [26, 27], where they are most abundant. They produce colony forming-unit fibroblast (CFU-F), when cultured in vitro and are distinguished by the capacity to differentiate into osteocytes, chondrocytes, and adipocytes. There are numerous sources of MSCs including bone marrow [28], adipose tissue [29], dental pulp [30], and synovial membranes [31].

- **Hematopoietic Stem Cells (HSCs)**

HSCs have been isolated from the bone marrow; they have the capacity for self-renewal as well as the ability to differentiate into all blood cell lineages [3]. They are widely used clinically in HSC transplantaion for treating various blood disorders and malignancies.

- **Neural Stem Cells (NSCs)**

NSCs are found in the central nervous system; they have the potential to differentiate into both neuronal and non-neuronal glial cells [32]. As such, they have been used clinically in efforts to repair injuries sustained by the nervous system [33, 34]. Currently, the use of NSCs for treating neurodegenerative diseases is under investigation [35].

1.2.3 Other Stem Cells

The discovery of stem cells in the human umbilical cord blood (UCB) paved a new and useful source of progenitors; notably umbilical cord blood hematopiotic stem cells (UCB-HSCs) have become a viable source of autologous bone marrow stem cells. UCB-HSCs are capable of differentiating into multiple hematopoietic lineages, in addition to their capacity for long-term self-renewal [36, 37]. Clinically, UCB stem cells have been employed successfully as HSC transplants in 1988 [38]. As such, parents in some countries now routinely bank the UCB of newborns so as to have a source of HSCs in the advent of any childhood hematological disorders or malignancies. Likewise, as noted earlier, MSCs have been identified in extraembryonic tissues, including Wharton's jelly [39], amniotic membrane and placenta [39, 40], and amniotic fluid [41].

1.2.4 Induced Pluripotent Stem Cells (iPSCs)

iPSCs are generated in vitro in an effort to imitate the potential of ESCs by effectively reversing the differentiation of somatic cells (e.g., skin fibroblasts) in order to become pluripotent [19, 42]. The discovery of iPSCs was driven at least in part by the need to identify ESC-like pluripotent stem cells for clinical use which could be generated without

raising strong ethical concerns. Many ongoing efforts are aimed at improving current reprogramming approaches so as to enhance the current clinical applicability of iPSCs.

1.3 Stem Cell Therapies: The Present and the Future

The remarkable potential of stem cells, including their capacities for self-renewal and differentiation, has led to their use in numerous clinical applications, including cell-based therapies [59], drug discovery [60], and tissue engineering [61]. The ultimate goal of stem cell-based therapies is to treat, repair, or replace diseased tissues or organs with ones that are new, healthy, and functional [62, 63]; numerous applications of this type are presented in Fig. 1.2. Therefore, stem cells are currently featured in several thousand ongoing clinical trials focused on disease treatment.

Most of these protocols focus on the use of stem cells for treating hematological disorders, including myeloid leukemia; lymphoma; sickle cell anemia; immune deficiencies; β-thalassemia [64–67]; wound healing and skin injuries [68]; neurological disorders, such as Parkinson's diseases and spinal cord injury [69, 70]; autoimmune disorders, such as multiple sclerosis, rheumatoid arthritis, Crohn's disease, and type-1 diabetes [71–74]; and cardiac diseases, including ischemic heart disease [75]. Promising trials, which focus on the use of stem cells to treat ocular disorders, including macular degeneration and retinitis pigmentosa [76, 77], and bone diseases, including osteosarcoma,

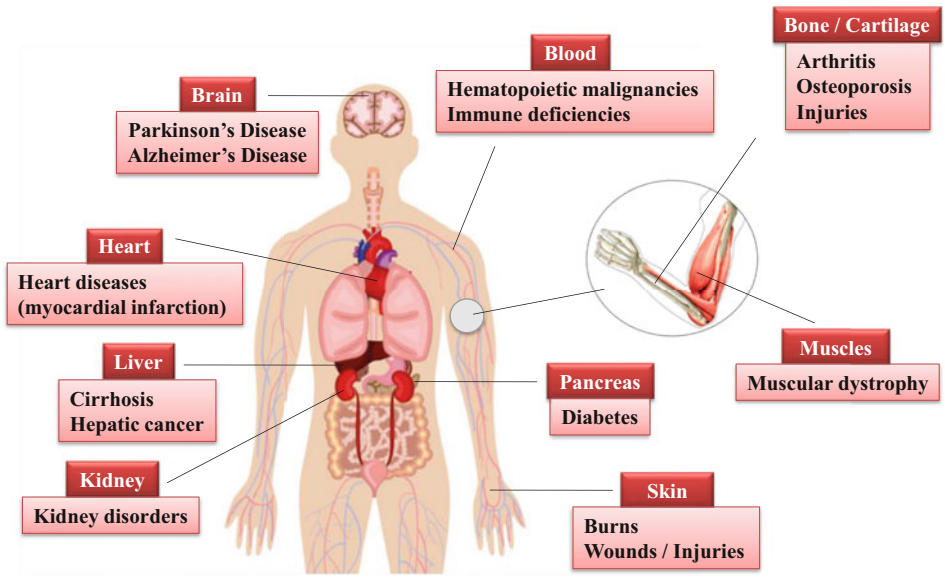

Fig. 1.2 Stem cell therapy for chronic diseases

osteoporosis, and osteoarthritis, are also in progress [78, 79]. So far, only a handful of the U.S. Food and Drug Administration (FDA) approved stem cell products are available for clinical use, including allogeneic cord blood hematopoietic stem/progenitor cells for treating hematological and immunological disorders (https://www.fda.gov/vaccines-blood-biologics/cellular-gene-therapy-products/approved-cellular-and-gene-therapy-products). Currently approved stem cell-based therapies are listed in Table 1.2.

Table 1.2 Approved human stem cell-based products

Approved products	Used stem cell type	Indications	Approval status	Approved by
ALLOCORD	Allogeneic cord blood hematopoietic progenitor cell	Used in conjunction with an appropriate preparative regimen for hematopoietic and immunologic reconstitution of patients with inherited or acquired disorders of the hematopoietic system or as a result of myeloablative treatment.	Approved	Office of Tissues and Advanced Therapies of the FDA (USA)
CLEVECORD				
DUCORD				
HEMACORD				
HPC, Cord Blood				
HPC, Cord Blood—MD Anderson Cord Blood Bank				
HPC, Cord Blood—Life South				
HPC, Cord Blood—Blood works				
HOLOCLAR	Ex vivo expanded autologous human corneal epithelial cells containing stem cells	Treatment of adult patients with moderate to severe unilateral or bilateral limbal stem cell deficiency due to physical or chemical ocular burns.	Conditional Approval	European Medicines Agency (EU)
ZYNTEGLO	Autologous $CD34^+$ hematopoietic stem cells transduced with lentiviral vector encoding the human $beta^{A\text{-}T87Q}$-globin gene	Treatment of beta thalassemia.	Conditional Approval	

1.3.1 Routine Stem Cell Therapy for Hematopoietic Disorders

1.3.1.1 Hematological Malignancies

Transplantation of unmodified or genetically modified HSCs derived from different sources offers a promising approach to the reconstitution or replacement of diseased cells. Cell therapies for hematological disorders, such as hemoglobinopathies (e.g., sickle cell anemia) and blood malignancies (e.g., leukemia and lymphoma), have undergone substantial development over the past few decades, as in the examples discussed below [80].

Leukemia

Leukemias are a group of white blood cell malignancies classified by the World Health Organization (WHO) based on genetics, morphology, immunophenotype, and clinical features [81, 82]. Interestingly, one of the earliest known cases of leukemia was identified based on the findings from an Egyptian skeleton in dating back to 2160–2000 BCE [83]. Leukemias are classified into several major subtypes, including acute myeloid leukemia (AML), acute lymphoblastic leukemia (ALL), chronic myeloid leukemia (CML), and chronic lymphoblastic leukemia (CLL) [84]. Chemotherapy was an initially effective treatment for childhood ALL when first attempted in 1948; unfortunately, disease typically relapsed ultimately leading to death [85, 86]. Currently, the standard treatment includes combination chemotherapy to destroy the defective hematopoietic system followed by hematopoietic stem cell transplantation (HSCT) [87, 88]. This approach is particularly indicated for recurrent disease, and can be introduced shortly after first-line treatment with chemotherapy [89, 90]. HSCs can be derived from the bone marrow (BM), umbilical cord blood (UCB), or peripheral blood (PB) [91]. The first successful allogeneic human bone marrow transplantation (BMT) performed in patients with leukemia following optimized radiation and chemotherapy doses resulted in a Nobel Prize in Medicine for Dr. E. Donnall in 1990 [92]. However, histocompatibility mismatching and graft rejection resulted in high relapse rates; as such, the disease relapsed and the success rate was low [93]. Among the efforts made to improve these outcomes, donor leukocyte infusions (DLI) were introduced, by providing immune cells pre-collected from the anticipated HSC donor following myeloablation in patients undergoing leukemia treatment; the goal was to establish donor chimerism and thereby preventing graft rejection [94]. Although, DLI was effective in managing disease relapse, it was related to the development of graft *versus* host disease (GvHD) in treated patients, resulting from the activity of effector donor T-cells [95]. Reduced-intensity conditioning (RIC) was also applied in an effort to control graft *versus* host disease (GvHD), while enhancing the graft *versus* leukemia effect (GVL), thereby maintaining engraftment and eradicating malignancy [96]. The use of less aggressive RIC and non-myeloablative conditioning reduces the overall toxicity and mortality associated with conditioning prior to transplantation, especially in older patients [96].

The relatively recent inclusion of UCB as a source for HSCs overcame the challenges associated with an attempt to locate an HLA-matched allogeneic donor [97]. UCB cells

were also less immunogenic and also easy to collect; UCB cells cryopreserved for decades still support the efficient recovery of HSCs [98]. However, UCB maintains comparatively fewer HSCs with respect to adult weight; as such, two bags of cord blood are typically required in order to obtain a sufficient yield of HSCs for transplantation into a single patient [99–101]. Nonetheless, a long-term follow-up of the Eurocord–European Group for Blood and Marrow Transplantation study revealed encouraging results. The study evaluated the outcome of UCB transplantation for 147 children, among whom 74% had been diagnosed with acute leukemia. In these patients, the cumulative incidence of neutrophil recovery was 90% at 2 years post-transplantation, the incidences of acute and chronic GvHD were reported to be 12% and 10%, respectively. At 5 years post-transplantation, the cumulative incidences of relapse and non-relapse mortality were 47% and 9%, respectively; the probability of disease-free survival was 44%. These results stand in strong support of UCB banking and the use of cord blood units to facilitate HLA-identical cord blood transplantation (CBT) [102].

PB-HSCs can be collected by noninvasive means; this provides a safe procedure for both the donor and recipient who can then undergo more rapid engraftment [103]. Administration of recombinant granulocyte colony-stimulating factor (G-CSF) stimulates the release of endogenous HSCs from the BM and into the blood. Currently, about 80% of all allogeneic transplantations are performed using stem cells derived from the PB of adult patients [104]. Similarly, recent developments in targeted therapy approaches have resulted in improved outcomes and can eliminate the negative sequelae associated with indiscriminate cytotoxic myeloablation. Genetically modified T-cells that express antigen-specific chimeric antigen receptor (CAR) will target leukemic cells while sparing those that are otherwise normal [105]. The FDA has approved the use of autologous genetically modified CD19-lymphocyte cells (CAR T-cells) for the treatment of relapsed ALL and diffuse large B-cell lymphoma [106].

Sickle Cell Anemia

In addition to traditional HSC transplantation, it is now possible to manipulate the diseased cells by removal, addition, or alteration of specific DNA sequences in order to correct defective or mutated genes. High efficiency and precise genetic manipulation or gene editing of the human genome has recently become possible with the use of the method known as clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 [107]; this procedure is outlined in Fig. 1.3. CRISPR/Cas9 was used to restore the normal blood cell phenotype by repairing $CD34^+$ hematopoietic stem/progenitor cells (HSPCs) from patients diagnosed with sickle cell anemia, a disorder that typically results from a single nucleotide substitution within a β-globin gene [108]. The gene-edited HSPCs were transplanted back into the patient's BM to function as a source of healthy autologous red blood progenitors; using this method the disease undergoes genetic correction, and graft rejection is evaded [108].

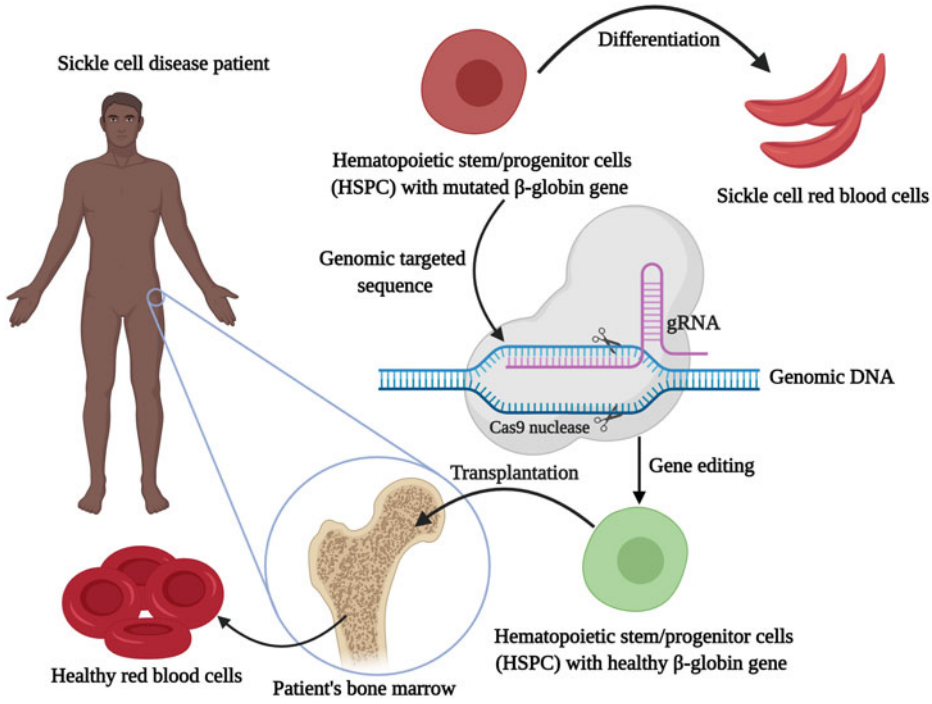

Fig. 1.3 Illustration of CRISPR/Cas9 gene editing

1.3.2 Stem Cell Therapy in Clinical Trials

1.3.2.1 Skin Injuries and Wound Healing

Skin is the largest organ in the body and a major part of the integumentary system that covers and protects the human body [109]. Physical, chemical, and biological factors can all disrupt skin integrity. Depending on the depth of injury, skin wounds can be epidermal, or they can involve either partial or full skin thickness [110]. The natural healing mechanisms are compromised by third- and fourth-degree burn injuries; this presents a significant challenge for both the surgeons and patients. Over the past century, the gold standard for treating burns has been grafting of healthy skin. Skin grafting can include split-thickness skin graft (STSG) and full-thickness skin grafts (FTSG) [111, 112]. Skin grafting involves the transfer of healthy skin (autograft or allograft) comprised of the epidermis and a portion of the dermis to the site of injury; problems arise when there is not enough healthy skin, a failure to treat deep wounds, a poor cosmetic outcome, and limited strength of grafted skin when compared with the original skin at the affected site [113]. Skin engineering thus represents an attractive alternative. Autologous keratinocytes or fibroblasts are cultured on a scaffold, in some cases, a scaffold alone is implanted into the wound to improve healing [114]. This technique results in the regeneration of both the epidermal and

dermal layers; however, this method did not facilitate the regeneration of skin appendages, including hair, nails and skin glands. Of note, traditional skin grafting also failed to regenerate skin appendages; however, pigmented melanocytes and neural and vascular tissues were recovered using this method, an outcome that was not achieved using the engineered skin [114]. Skin replacements can be generated using cellular or acellular scaffolds; based on the composition of the skin-substitute [115]. Acellular skin-substitutes are biodegradable scaffolds (e.g., collagen, elastin, and silicon, among others) that facilitate wound healing by recruiting fibrocytes and vascular cells in vivo and by inhibiting granulation and scar formation. The most common acellular skin-substitutes currently approved by the FDA and undergoing review in clinical trials include Integra® [116], Alloderm™ [117], and NovoSorb™ BTM (Biodegradable Temporizing Matrix) [118]. Cellular skin-substitutes that contain epidermal cell sheets include Dermagraft® and Apligraf®; these products were approved by the FDA for the treatment of diabetic foot ulcer [118, 119]. ReCell® is an FDA-approved commercial cell spray device that provides autologous keratinocytes designed to heal second-degree burns. ReCell® works by facilitating enzymatic digestion of the patient's healthy skin in order to harvest keratinocytes, which are then sprayed over the wound [120, 121]. Commercially available skin-substitutes are still far from perfect. The cells frequently fail to integrate; show poor vascularization, weak mechanical integrity, and scar formation; and are subjected to immune-mediated rejection [109]. Indeed, there are no completely functional skin-substitutes available at this time; of particular note, there is a great need for a functional skin-substitute that can undergo rapid vascularization. Recent advances in stem cell therapy, nanotechnology, tissue engineering, and microfluidics paved the way for improved skin tissue engineering focused on deep wound healing [122]. Bioscaffolds for skin engineering must all be biocompatible, nontoxic, non-immunogenic, biodegradable, and sufficiently porous so that free exchange of gases and nutrients can occur through a neo-vascularized functional skin-substitute [123]. The cell source for the engineered skin also has a significant impact on the outcome. For example, ESCs can be differentiated into both keratinocytes [124] and fibroblasts [125], but direct clinical applications of these cells are hampered by instability and concerns with respect to the functionality of the resultant tissues. Adipose-derived stem cells (ADSCs) can also differentiate into keratinocytes, fibroblasts, and other skin components; ADSCs also produce extracellular matrix (ECM) which is rich in growth factors and cytokines that enhance healing [126–128]. The ADSC secretome contains vascular endothelial growth factor (VEGF), growth differentiation factor (GDF-11), and transforming growth factor (TGF-β); all of these act on macrophages, fibroblasts, and endothelial cells and lead to limiting the immune responses, enhancing cell proliferation, and promoting angiogenesis at the transplantation site [129]. Clinical applications of autologous ADSCs are still under investigation for healing diabetic foot ulcers (NCT02092870, see https://www.clinicaltrials.gov) [130]. Furthermore, methods used to generate three-dimensional skin grafts using iPSC-derived keratinocytes and fibroblasts remain promising [131].

1.3.2.2 Osteoarthritis

Osteoarthritis (OA) is a chronic degenerative disease characterized by deterioration of joint articular cartilages; this results in exposed subcondylar bones and leads to friction, pain, and synovitis [132]. Globally, OA is currently estimated as the 11th highest contributor to adult disability; this results largely from pain, stiffness, and impaired mobility due to disease affecting the knees, feet, hands, and spine joints [133]. Non-surgical approaches for treating OA include intra-articular injections of corticosteroids, hyaluronic acid "viscosupplementation," or autologous platelet-rich plasma into the deteriorating joints [134–136]. These approaches are designed to alleviate pain, but they do not treat the underlying cause of mechanisms associated with OA [137]. Joint surgery for OA varies from whole knee replacement (arthroplasty) to minimally invasive arthroscopic techniques such as microfracture or microdrilling [138–140]. The aforementioned arthroscopic techniques involve the generation of multiple small fractures within the affected joint, promoting the recruitment of progenitor cells from the underlying BM which then undergo differentiation into chondrocytes [139]. The drawbacks of these approaches include the formation of an inferior form of cartilage that lacks mechanical durability [138].

Alternative cell-based approaches have been applied, including osteochondral transplantation and soft tissue grafting [141]. Among the problems associated with these approaches, outcomes have included poor grafting and integration, calcification of the grafts, and limited number of available donor tissues [142, 143]. Accordingly, more effort has been directed toward autologous/allogeneic chondrocyte implantation (ACI) [144]. Currently, there are numerous phase III clinical trials involving ACI that include the expansion of autologous or allogeneic chondrocytes, followed by grafting into the deformed lesion [145]. As an example, a phase III clinical product that is now commercialized with the brand name Chondrosphere® utilizes scaffold-free spheroids of chondrocytes obtained from autologous articular cartilage that are introduced for use to treat cartilage defects associated with hip injuries (NCT01222559) [146]. The challenges currently encountered include increased susceptibility of the donor to OA after tissue sampling in normal joints and an overall insufficient number of harvested chondrocytes. Likewise, expanded chondrocytes may undergo dedifferentiation and lose their ability to generate cartilage matrix [147].

MSCs have also emerged as a promising source of cells for this application owing to their robust capacity for expansion and chondrogenic differentiation [148, 149]. In addition, MSCs secrete a variety of cytokines and growth factors with anti-inflammatory effects [150]; these cytokines may function to counteract the inflammatory processes associated with OA. Autologous bone marrow-derived MSCs have been used to repair full-thickness cartilage defects in two cases [151]. In this study, BM was aspirated from the iliac crests and cultured until adherent MSCs had undergone several expansion passages. Cultured MSCs were then collected, embedded in a collagen-gel scaffold, and transplanted onto the surface of the defective articular in the knee joint. Symptoms were relieved at 6 months, and both male and female patients were satisfied with the outcomes during the 4 years following transplantation [151]. MSCs derived from the umbilical cord, placenta,

Wharton's jelly or amniotic membrane all have shown promise with respect to novel treatments for patients diagnosed with OA [152–154]. In particular, UC-MSCs exhibited higher proliferative, clonogenic, anti-inflammatory, and chondrogenic potential compared with MSCs from maternal-derived decidua or BM [155]. CARTISTEM® is a commercialized product that utilizes UC-MSCs for the treatment of cartilage deterioration in patients with OA; it is currently approved for a phase III clinical trial with the goals of evaluating safety and expanding its indications for use (NCT01041001, NCT01626677). Recently, phase II clinical trials have been initiated to assess the role of ADSCs for the treatment of patients with OA (NCT02838069) [78].

1.3.3 From Bench to Bedside

1.3.3.1 Diabetes Mellitus (DM)

Diabetes mellitus (DM) is a chronic inflammatory metabolic disorder that results in sustained hyperglycemia due to defects in insulin production (Type I), insulin utilization (Type II), or a combination of both [156]. Type I DM (T1DM) is an autoimmune disease, wherein activated immune cells attack insulin-secreting β-cells in the pancreas, resulting in insulin deficiency [157]; contrarily, type II DM (T2DM) is characterized as a chronic inflammation state that ultimately leads to insulin resistance, reduced insulin secretion, β-cells exhaustion, and apoptosis [158–160]. Untreated DM leads to severe complications that can be life-threatening and have significant impact on numerous major organs including the kidneys [161], heart [162, 163], eyes [164, 165], and nervous system [166].

Patients with diabetes attempt to regulate their blood glucose levels and to maintain values at or near normal limits with dietary control [167], hypoglycemic drugs [168], and lifestyle changes [169]. However, these traditional methods often fail to maintain normoglycemia in the long run [170]. Islet transplantation (also known as Edmonton protocol) was developed in 1999 to provide more β-cells and thus increase insulin production for patients diagnosed with T1DM [171–173]. However, the use of this approach was limited due to the risks associated with the surgical procedure [174], the need for long-term immunosuppressive therapy [175], a shortage of organ donors [176], and only limited impact with respect to achieving insulin independence [177].

Stem cell-based therapy provides a new approach for the management and treatment of DM. First, this approach can create a virtually unlimited supply of insulin-producing cells [178–181]; other applications focus on restoring β-cell function [182], modifying immune dysregulations, and reversing the associated metabolic complications [183]. Pluripotent ESCs were successfully differentiated into β-cells in vitro [184, 185]; results in vivo revealed that insulin production and normal blood glucose level were sustained at 3 months post-transplantation [186]. Despite these promising results, there are few clinical trials addressing this approach, and there is currently no reliable information on its safety or efficacy (https://www.clinicaltrials.gov/).

Considering the different embryological origins of MSCs and pancreas, MSCs showed variable responses to the efforts made toward differentiating them into pancreatic β-cells. For instance, BM-MSCs failed to adopt functional characteristics of β-cells when cultured in vitro [187]; contrarily, ADSCs revealed some genetic and morphological similarities to pancreatic cells [188, 189]. However, MSC-mediated immunomodulation and inhibition of autoimmune progression may be achieved by educating autoreactive T lymphocytes, an approach in which the autoreactive T-cells are being regulated to be less reactive to the patient's own islet cells, thereby reducing the extent of β-cell destruction in patients diagnosed with T1DM [181, 190, 191]. Moreover, for T2DM patients, the transplantation of autologous MSCs would reduce the associated inflammatory reactions and promote pancreatic healing [181, 192, 193]. Several clinical trials (NCT03343782, NCT01068951 and NCT01759823) demonstrated that autologous BM-MSC transplantation was a promising approach, as it coupled long-term efficacy and safety vis à vis the diabetic microenvironment [194–196]. Results from a limited number of trials for T1DM patients revealed improved clinical outcomes in patients treated with UC-MSCs than in those treated with BM-MSCs, although BM-HSCs were more effective than UCB-HSCs [197]. Despite the fact that stem cell therapy may ultimately overcome many of the well-known limitations of traditional DM therapy, more clinical trials are still required. At this time, short follow-up periods, small number of patients, missing control groups, and lack of standardization of the transplantation protocols were major setbacks for some of the clinical trials [196, 198].

1.3.3.2 Multiple Sclerosis (MS)

Multiple sclerosis (MS) is a chronic, autoimmune, inflammatory, and neurodegenerative disorder of the central nervous system [199]. MS is characterized by demyelination with axonal loss and long-term progressive disability due to disease exacerbation with the inflammatory microenvironment that enhances local oxidative stress and hypoxia [200, 201]. Several pharmacological and non-pharmacological therapies are currently approved for the treatment of MS; however, these treatments may only delay disease progression and reduce the severity of its symptoms [202]. Consequently, therapies that promote remyelination of injured axons remain among the challenges.

HSCT has been used to treat MS following high dose chemotherapy for immunosuppression [203]; this modality aims to reboot the immune system and eliminates autoreactive T- and B-cells, thereby facilitating the generation of a new and tolerant immune system [203]. HSCT has since become an alternative option for the treatment of other autoimmune-related diseases as well [204–207]. Despite the improvements observed in some MS patients, the high risk of chemotoxicity and immune deficiency in this patient cohort remains an important drawback to widespread implementation [208, 209].

MSCs have unique immunomodulatory and anti-fibrotic properties [210, 211] and are thus attractive choices for the development of targeted treatments for MS. Autologous BM-MSC transplantation resulted in diminished production of pro-inflammatory cytokines in association with improved vision and movement in patients diagnosed with MS

[212, 213]. In another trial, UC-MSC transplantation resulted in improvements in physical movement with fewer side effects [214]. However, the potential therapeutic effects and mechanism of action of these cells require further investigation.

1.3.3.3 Parkinson's Disease (PD)

PD is the second most prevalent neurodegenerative disease worldwide with an incidence that increases with age [215]. Characterized by gradual death of the dopaminergic neurons in the substantia nigra of the brain, PD leads to motor nerve impairment and reduction in the capacity for voluntary movements [216]. The exact cause of PD remains under investigation, however, the gene encoding α-synuclein (SNCA) was found to be involved with the abnormal accumulation of Lewy bodies inside neurons [217, 218]. There is currently no cure for PD; however, specific drugs are reasonably effective in restoring dopamine concentrations, as well as improving motor neuron function and relieving symptoms characteristic of PD. Nevertheless, these medications are often associated with off-target adverse events in long-term use [219, 220]; this limits their overall efficacy.

Pluripotent stem cells have the capacity to differentiate into dopaminergic neurons in vitro [221–223]. ESCs underwent efficient differentiation into midbrain dopaminergic neurons. When grafted into the striatum, these cells promote motor improvement, improved graft survival, and reduced levels of teratoma formation in mice [224]. A phase I/II clinical trial is currently underway, which aimed to investigate the safety and efficacy of neural precursor cells generated from human ESCs (NCT03119636) [225]. In addition, iPSCs are also promising candidates, in terms of the possible generation of dopaminergic neurons for transplantation to treat PD [226]. A personalized medicine approach revealed that differentiated dopaminergic neurons generated from autologous iPSCs could limit the progression of PD for 18–24 months [227]. A clinical trial designed to evaluate the efficacy of this approach in PD patients is currently ongoing (NCT00874783) [47].

Administration of MSCs that differentiated into dopaminergic neurons resulted in improved movement after transplantation using PD mouse models [228, 229]. Interestingly, MSCs were also found to exert a neuroprotective effect via their capacity to regulate both autophagy and α-SNCA expression, thereby rectifying PD brain-microenvironment [230]. In addition, the introduction of MSC-associated secretory factors and exosomes was associated with outstanding results in PD animal models [231–233]. BM-MSCs are the most commonly used cells in clinical trials; administration of autologous and allogeneic BM-MSC transplantation resulted in improved movement in three of seven patients; another two patients tolerated a reduction in PD drugs following BM-MSC transplantation [234]. No serious health concerns were reported during the 12–36-month trial; these findings encourage further testing of the BM-MSC transplantation in a larger number of patient cases [234]. Recently, administration of UC-MSCs resulted in promising outcomes in experiments conducted using PD animal models [235–237]; two clinical trials exploring both the efficacy and safety of this approach are ongoing (NCT03684122 and NCT03550183).

Administration of NSCs also resulted in positive outcomes with respect to treatment of PD; these cells released neurotrophic factors that enhance neural functions and promote their migration to the site of the lesion, thereby facilitating repair of damaged tissue [238]. One clinical trial (NCT03815071) is currently testing the efficacy of administration of autologous NSCs to patients diagnosed with PD; more trials are required in order to evaluate the long-term efficacy and safety of the use of NSCs under these conditions.

1.3.3.4 Age-related Macular Degeneration (AMD)

Age-related macular degeneration (AMD) is an incurable disease resulting in the gradual loss of vision in one or both eyes [239, 240]. The macula, which is the central part of the retina, contains the photoreceptors (rods and cones) and is essential for central vision, perception of details, and differentiation among colors within a field of vision [241, 242]. Retinal pigment epithelial (RPE) cells are supportive cells that provide nutrition to retinal photoreceptors. In macular degeneration, RPE cells degenerate and fail to support the retina, resulting in the loss of central vision, blurred visual fields, and diminished capacity for color discrimination [240]. Macular degeneration exists in both wet exudative and dry non-exudative forms [239]. The dry type is associated with thinning and death of the RPE cells and is associated with yellow deposits (drusen), whereas the wet type involves the formation of new blood vessels and bleeding beneath the retina [240].

The current treatment for AMD focuses on delaying its progression, via the administration of antioxidants or anti-VEGF for patients diagnosed with dry or wet AMD, respectively [243–246]. While these therapies result in slight improvements in retinal function, they do not restore degenerating RPE cells. As such, preclinical studies have focused on transplantation of retinal progenitor sheets in an effort to replenish RPE cells in the injured area of the eye; this approach has shown promising results by improving vision in mice [247–250].

Recently, the use of pluripotent stem cells for the repair of macular damage gained much attention. ESCs can differentiate in vitro into photoreceptor cells [251] that can then be transplanted into the eyes of an individual diagnosed with AMD; through this method, human ESC-derived RPE cells were injected directly into the injured eye. The results of preliminary studies revealed that this method is safe and that there is little immune rejection of the transplanted cells; the ESC-derived RPE cells were genetically stable, did not generate tumors, and maintained strong differentiation to >99% pure RPE cells (NCT01345006 and NCT01344993) [77, 252]. However, concerns regarding genetic instability and the potential for tumorigenesis when administering pluripotent stem cells for the treatment of AMD were recently addressed [253, 254]; a recent study aimed to validate the safety of ESC-derived RPE cells through genomic analysis [255]. Furthermore, iPSC cell lines were recently differentiated into three-dimensional retinal organoids which may be useful for replacing damaged photoreceptors [256]. Reprogramming of autologous skin fibroblasts into iPSCs, then their differentiation into RPE cells, has also been investigated (Clinical trial UMIN000011929) [257].

Although MSCs were tested repeatedly for their capacity to differentiate into neuronal cells or photoreceptors [258, 259], recent studies revealed that these cells should not be used to treat AMD. Despite the absence of appropriate preclinical studies, some physicians rushed forward and use MSCs in AMD treatment protocols; this unfortunately led to several incidents of complete blindness. As but one example, a 2017 report described the case of a 77-year-old woman who received autologous adipose MSC injections into both eyes, at a clinic in Georgia; she experienced bilateral retinal detachment and complete blindness at 3 months following the procedure [260].

1.4 Stem Cell Therapies: Facts, Hope and Hype

Stem cell therapies are among the most exciting and revolutionary medical advances of the twenty-first century. They are frequently described in the media as a "wonder-cure" or "cure-all." Indeed, clinical applications of stem cells are increasing in number worldwide as its research progresses and matures. It remains important, however, to balance patients' needs and desires with the fact that there are currently no well-established clinical outcomes from any stem cell-based protocol. Unfortunately, several clinicians have undertaken a "rogue" approach by misusing stem cell therapy and providing services to patients that go beyond currently approved applications [261]. Moreover, false marketing and unsubstantiated advertising in almost all media outlets feature unapproved stem cell therapies for conditions ranging from mild cosmetic enhancements to cure for intractable organ failure.

By 2018, more than 430 established enterprises in the USA were promoting numerous variants of stem cell therapy (all types of stem cells for so many diseases) in more than 710 clinics distributed in various states [262]; these numbers indicate a profound increase over those reported only 2 years earlier (i.e., during 2016 [263]). Taking together, these findings indicate an increasing trend toward embracing uncontrolled and unproven stem cell therapies. Moreover, in a study conducted in 2017, researchers found that only 43.6% of a total of 408 funding campaigns focused on stem cell therapy reported true and verifiable information in terms of efficacy, and only 8.8% mentioned the risks associated with their use [264]. Most of these businesses asserted scientific legitimacy by referring to published articles in journals with little or no scientific peer-review, and provided false claims regarding their involvement and relationship with preclinical research conducted at reputable research centers [265].

Warnings are issued constantly by the FDA, the U.S. Centers for Disease Control (CDC), Euro Stem Cell, the International Society for Stem Cell Research (ISSCR) as well as other international stem cell consortiums regarding the premature use of stem cells in clinical sittings. These cautions are fully justifiable, since claims of efficacy and safety of several uncontrolled and improperly identified stem cell therapies are portrayed with optimistic messages; that often ignore the associated risks and/or potential for adverse reactions [266, 267]. As such, there is a compelling need to increase patients' awareness of

what therapies are actually clinically approved as opposed to what is currently advertised inappropriately.

Some forms of stem cell therapy, particularly the use of HSCs for hematopoietic disorders, have been the subject of extensive research, are clinically proven, and have been established as routine standard of care. The skin stem cells used for treating severe burns have shown considerable promise as well as treating immune deficiencies and solid cancers. However, other modalities featuring stem cells are still under experimental investigation and have not yet been approved for clinical use.

Validated clinical trials are required in order to provide the utmost guarantee of safety and efficacy prior to the approval of any new drug, or therapy; stem cells are certainly no exception. Despite the enormous number of research articles published each day regarding the potential of stem cells and stem cell therapy, the absence of clear, verifiable information can lead to tragedy. For example, various incidences were reported in macular degeneration patients who developed blindness, retinal detachment and intraocular bleeding, following adult stem cell-based therapy [260, 268]. Moreover, we do not yet have clear information documenting the genetic stability of ESCs, nor do we have a handle on their capacity for sustained reproducible differentiation. The use of iPSCs may overcome some of these limitations; yet, we have a long journey of research is still required to prove its safety and efficacy range. Indeed, in 2008, Yamanaka advised against the "hype" associated with iPSCs and declared that it would be quite dangerous to predict the safety of this technology with respect to clinical trials and applications [269].

Numerous factors should be considered when designing stem cell therapies. For example, an important obstacle when considering the use of umbilical cord derived stem cells is the cost of cord blood banking; these must meet the international standard regulations for the collecting, storage, and use of UC blood for transplantation [270] as well as any and all associated legal regulations [271]. At this time, the UC blood banking industry has begun to decline due to the high costs associated with its implementation. This will certainly have an impact on the future availability and therefore the use of UC derived stem cells [272].

In conclusion, the hope place in stem cells remain strong; this is certainly warranted given the opportunity to use their powerful potential to develop new cures for acute and chronic diseases. With more clinical data and improved standardization, stem cells may be safely used for treating an ever-expanding list of diseases. However, the public needs to be aware that this will take some time and that they need to be wary regarding the advertised "hype" associated with this exciting cutting-edge field. Patients are encouraged to be cautious and to look for validated and credible information before deciding to undergo an unapproved and unproven stem cell-based therapy.

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Take Home Message

- The biology of stem cells in tissue homeostasis and development has made it the prospect for the field of regenerative medicine.
- Stem cell potency is more pronounced in embryonic tissues compared to adult cells. In the adult tissues, stem cells are widely distributed throughout the body including, but not limited to, the bone marrow, adipose tissue, intestine, skin, synovial membrane, and dental pulp.
- Reprogramming somatic cells by induced pluripotent stem cell (iPSC) technology, gene editing, and applying modern techniques of nanotechnology and bioprinting have all made it possible for extensive applications of adult stem cells in regenerative medicine.
- Hematopoietic stem cells transplantation (HSCT) is already a routine practice, and has secured FDA approval for its cellular products to treat hematological diseases.
- Research is still in progress for wound healing and osteoarthritis treatment using stem cells.
- Preclinical and clinical studies showed new hope in treating incurable chronic diseases like multiple sclerosis, macular degeneration, Parkinson's Disease, and diabetes mellitus with stem cells.
- FDA, CDC, ISSCR and other stem cell societies and institutes are regularly warning about the misused stem cell therapy away from their approved applications to minimize patients' risks.
- Various types of stem cells need more clinical investigations to test their safety and efficacy before being clinically translated.
- Patients have to be cautious about the credibility of any cell-based medical application; and especially before undergoing stem cell therapy.

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Embryonic and Pluripotent Stem Cells

2

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What You Will Learn in This Chapter

This chapter will focus on pluripotency as a key feature in determining the differentiation potential of cells and the importance of embryonic and pluripotent stem cells in research and their promising applications in regenerative medicine. It also includes a brief description of the major findings on embryonic stem cells' derivation, characterization, and differentiation. The differences between naÿve and primed pluripotency will be highlighted, and the in vitro growth conditions contribute to these differences. The chapter will also cover major findings in nuclear reprogramming and the developments in induced pluripotent stem cell technology. Finally, we will conclude with the limitations of embryonic stem cells in clinical applications and areas for future research.

2.1 Introduction

2.1.1 Pluripotency

Pluripotency is defined by two main characteristics, self-renewal and potency. Self-renewal describes the ability of cells to divide almost infinitely, or to divide long-term in culture, resulting in two daughter cells with distinct cellular fates, where one of the daughter cells maintains the pool of undifferentiated cells [1]. The ability of a cell to differentiate into different cell types that exhibit different characteristics than the mother cell is called cell plasticity [2]. Pluripotent stem cells show a substantial degree of plasticity as they can give rise to cells of the three embryonic germ layers: ectoderm, mesoderm, and endoderm. However, pluripotent stem cells have limited potential to give rise to extraembryonic tissues, particularly the placenta (Fig. 2.1) [2]. Nonetheless, a newly derived type of pluripotent cell, known as extended potential pluripotent stem cells (EPSCs), has been shown to recapitulate both embryonic and extraembryonic tissues [3]. Multiple types of pluripotent stem cells can be derived from different embryonic stages and tissues, as well as artificially from direct reprogramming of somatic cells [2, 4]. Pluripotency is also a highly dynamic process, where interchange between naïve and primed states can occur [4]. The

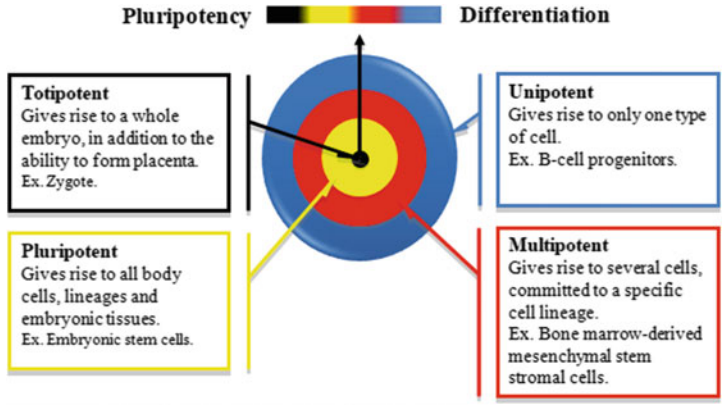

Fig. 2.1 Different cell potency abilities

unique characteristics of pluripotent cells have led to exploration of novel therapeutic approaches. Moreover, the advances in reprogramming of somatic cells into a more naïve pluripotent state have paved the way for clinical applications with limited constraints [5].

The molecular hallmark for determining pluripotency is the expression of specific transcription factors that halt the activation of lineage-specification genes, leaving pluripotent cells in a quiescent state. The core transcription factors that control pluripotency include the octamer-binding transcription factor 4 (Oct-4), which is also known as POU5F1 [6], the homeodomain transcription factor (Nanog) [7], and the protein SRY-box transcription factor 2 (Sox2), which is related to high-mobility group (HMG) proteins [8]. Other important characteristics of pluripotency include high expression of telomerase reverse transcriptase, which plays a major role in the regulation of cellular life span [9]. In addition cell-surface antigens, such as stage-specific embryonic antigen-3 (SSEA-3), SSEA-4, and CD9 tetraspanin, together with positive intracellular enzyme alkaline phosphatase activity, also play a role in regulating cellular life span [10–14]. However, the most robust methods to determine pluripotency are functional assays. The principle of these assays depends on the ability of pluripotent cells to recapitulate the three germ layers in vitro or in vivo. Examples include (1) in vitro differentiating cell aggregates, termed embryoid bodies [15], (2) in vivo teratoma formation [16], and (3) in vivo chimera formation [17]. These assays are explained in more detail in this chapter.

2.1.2 Historical Overview of Pluripotency

Cell reprogramming is the process of induced cell transformation from one specific cell type to another [18]. For many decades, numerous techniques have been developed in cell

reprogramming and induced pluripotency. Cellular differentiation was thought to be unidirectional and irreversible (i.e., from immature pluripotent to more mature differentiated cells), like a ball rolling down from the top of a mountain to the bottom. However, this concept was challenged, and it is now known that reprogramming to obtain pluripotency can be achieved using a variety of approaches, including nuclear transfer, cell fusion, and direct reprogramming. Recent work has identified that cell fate is not irreversible, and that it is a plastic or reversible (i.e., the ball can roll back upwards from the bottom to the top of the mountain).

The early work of John B. Gurdon in 1962 demonstrated, in lower animal models, that reprogramming can be achieved by nuclear transplantation [19]. In these experiments, a nucleus of a somatic cell from the intestine of a frog was implanted into an enucleated unfertilized frog egg. This egg then generated an adult tadpole. This process of somatic cell reprogramming to the pluripotent embryonic state led to "rejuvenation" and was termed somatic cell nuclear transfer (SCNT) [19]. These early experiments showed that the nucleus of mature cells could be reprogrammed to generate an entirely new animal without sexual reproduction. These experiments fundamentally changed the perception of biology and reproduction and were the basis for the mammalian cloning experimentation that followed (see, Table 2.1, and Fig. 2.2). For many years it seemed that it was not possible to clone mammals. However, Ian Wilmut's research group cloned Dolly the sheep, born on July 5, 1996 from a mammary cell of an adult sheep [20]. Later, Gurdon and Yamanaka shared the 2012 Nobel Prize in Physiology and Medicine for their innovative contributions to the field of cellular reprogramming.

In 1981, Martin Evans, Matthew Kaufman [26], and Gail Martin [16] established the first self-renewable and pluripotent embryonic stem cell (ESC) lines derived from preimplantation mouse embryos. When immune-deficient mice were injected with these cells,

Table 2.1 Therapeutic cloning compared to reproductive cloning

Therapeutic cloning	Reproductive cloning
1. Cloning by SCNT for reprogramming [20]	1. Cloning either by: – SCNT for reprogramming [20] or – Embryo splitting: The IVF cattle embryo at 4-cell stage is divided into 3 or 4 identical cells, and each cell is then developed into healthy monozygotic calves [21]
2. Intended to isolate patient-derived ESCs from embryos created in vitro without later transfer into the uterus [22]	2. Intended to implant the embryo into a female uterus to obtain a whole organism [21]
3. Offers great promise for regenerative medicine through the production of autologous nuclear transfer embryonic stem cells (ntESC) [22]	3. Offers a great option for cloning of livestock especially the genetically engineered animal (e.g., human coagulation factor IX in the milk of transgenic sheep) [23]
4. Applicable for both animal and humans but with some ethical constraints [24]	4. Banned for humans but applicable to animals [25]

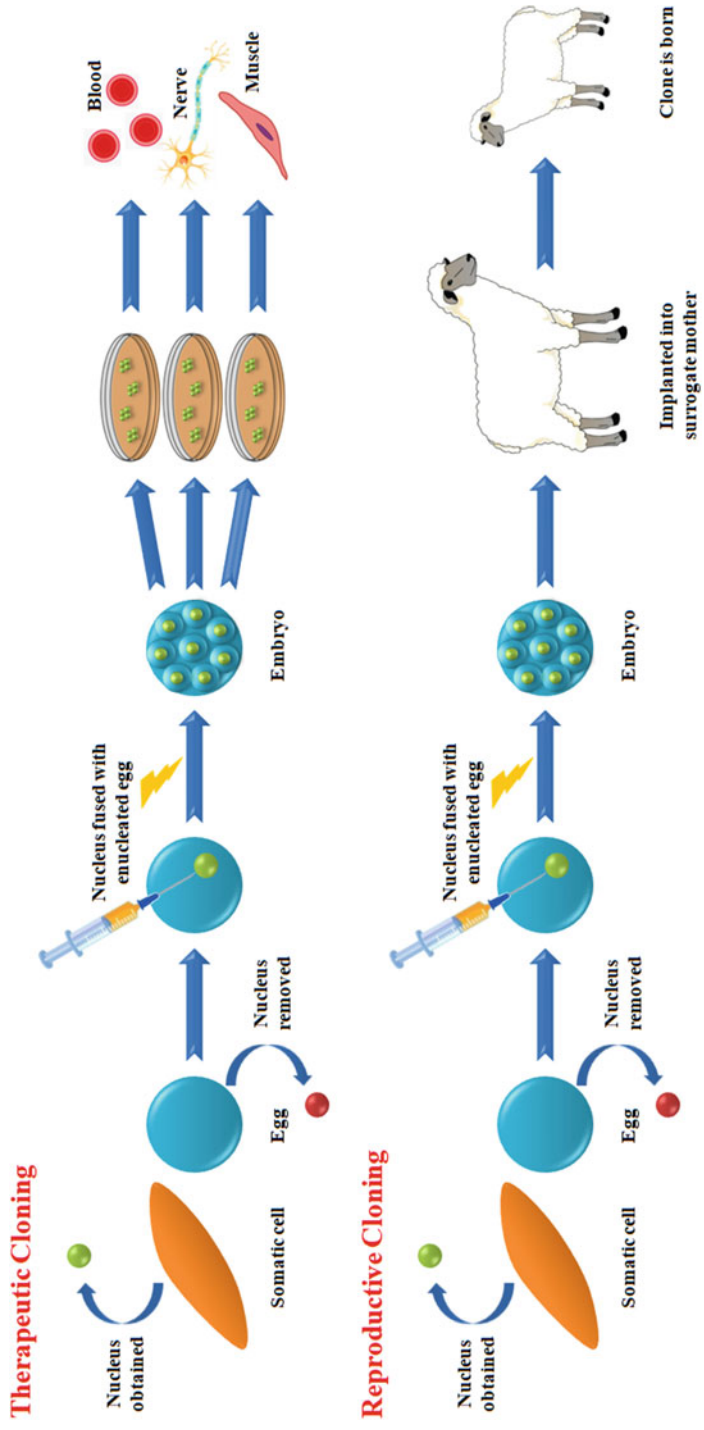

Fig. 2.2 Therapeutic cloning and reproductive cloning

they generated teratomas or teratocarcinomas, which at that time, became one of the hallmarks of pluripotency. In addition, these ESCs were shown to contribute to the formation of chimeric mice after being injected into mouse blastocysts [17]. In 1998, the research group of James Thomson and Jeffrey Jones isolated the first human ESCs from the inner cell mass of blastocysts produced by in vitro fertilization [15]. In the same year, Austin Smith and colleagues described the culture conditions and factors that are important for the in vitro maintenance of ESC pluripotency [27].

In 2001, cell fusion of ESCs and somatic adult thymocytes produced hybrid cells [28]. And as a result, ESCs could effectively reprogram thymocytes into a more embryonic state. This gave the ability to form chimeras, in addition to the tri-lineage differentiation potential. The pluripotency-associated Oct-4 gene, which is normally suppressed in thymocytes, was up-regulated following fusion with ESCs, and epigenetically, the chromatin was less condensed into a more transcriptionally accessible state. These two factors induced the reprogramming of the heterokaryons into a more pluripotent state [28]. However, these hybrid cells still retained some of their somatic characteristics, which represented a challenge for their use in clinical applications. This study gave clues about the possibility of the existence of reprogramming factors, which could lead to ESC-like cells via direct use on somatic cells, without the need for mammalian embryos.

Davis and colleges showed possible direct reprogramming of somatic cells into embryonic-like stem cells. Embryonic mouse fibroblasts treated with 5-azacytidine, an inhibitor of DNA methylation, generated myoblasts as shown by ectopic expression of the muscle-specific gene (MyoD) [29]. In 2006, Yamanaka and Takahashi reported a seminal discovery in which they created induced pluripotent stem cells (iPSCs) from mouse fibroblasts by combisning of four reprogramming factors. These factors included Oct 3/4, Sox2, Klf4, and c-Myc (OSKM, also known as the Yamanaka factors).These factors were used to generate pluripotent cells from somatic fibroblasts using a viral delivery system [30]. One year later, Thomson and colleagues generated human induced pluripotent stem cells (hiPSCs) from human fibroblasts using another set of reprogramming factors, Oct 3/4, Nanog, Sox2, and Lin 28 (ONSL) [31]. Different approaches for reprogramming somatic nuclei are illustrated in (Fig. 2.3).

2.2 Pluripotent Stem Cells

2.2.1 Pluripotent Stem Cell State: Naïve Compared to Primed

Pluripotent stem cells (PSCs) can be isolated from vertebrates, including mice and humans, based on their tissue of origin and developmental stage (Fig. 2.4). PSCs are further classified as “naïve” or “primed” (Table 2.2), based on their ability to produce all somatic and germline cells, as well as their in vitro growth conditions [33]. The in vitro growth conditions include colony morphology, growth characteristics, culture requirement for maintenance of the pluripotent state, gene expression, and the global state of DNA

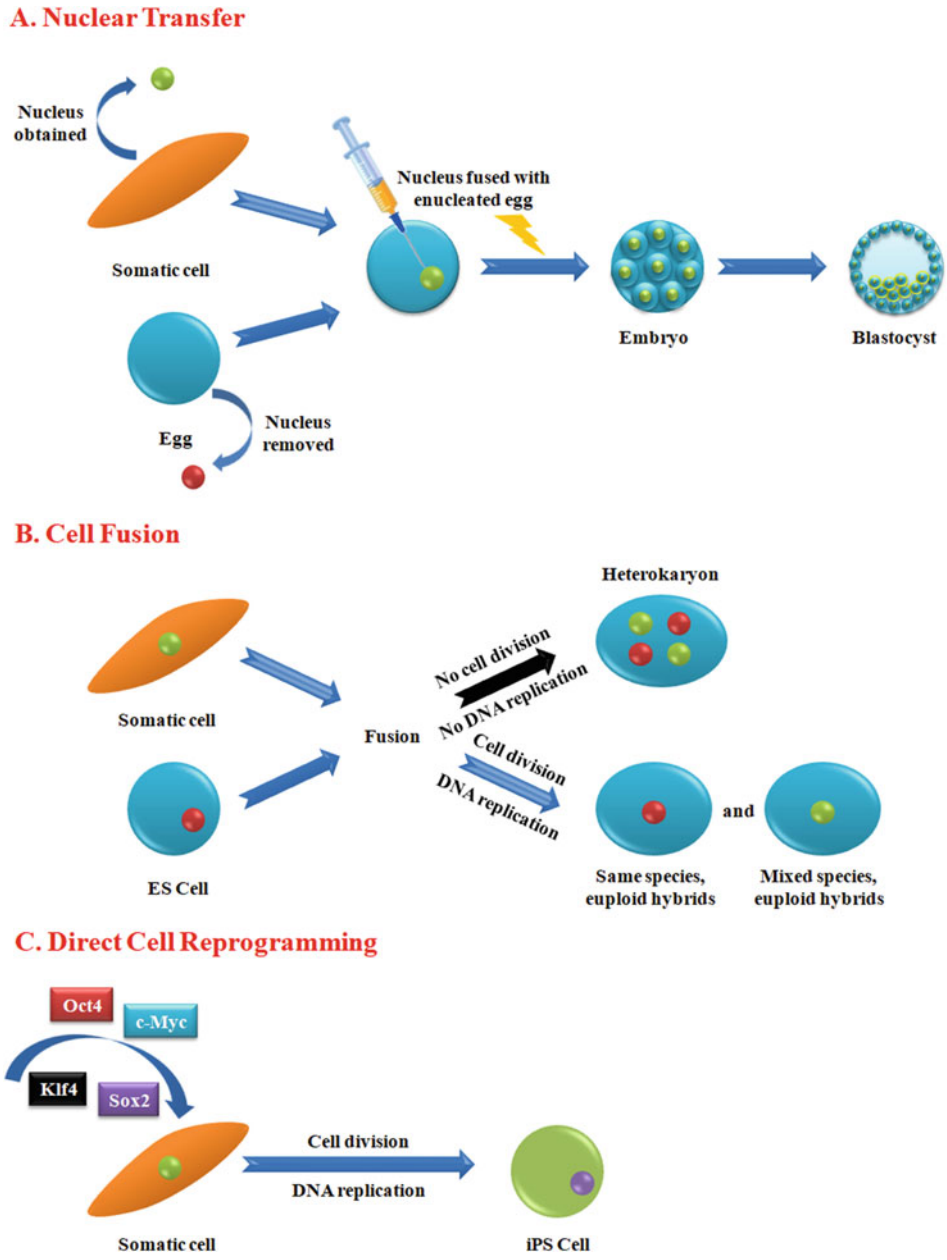

Fig. 2.3 Different approaches for cellular reprogramming

methylation. Furthermore, the naïve or primed classification can be based on chimera formation or X chromosome inactivation in female cells [33]. We could resemble chimeras as "a mosaic painting during the Byzantine era" which the body of developing organisms is

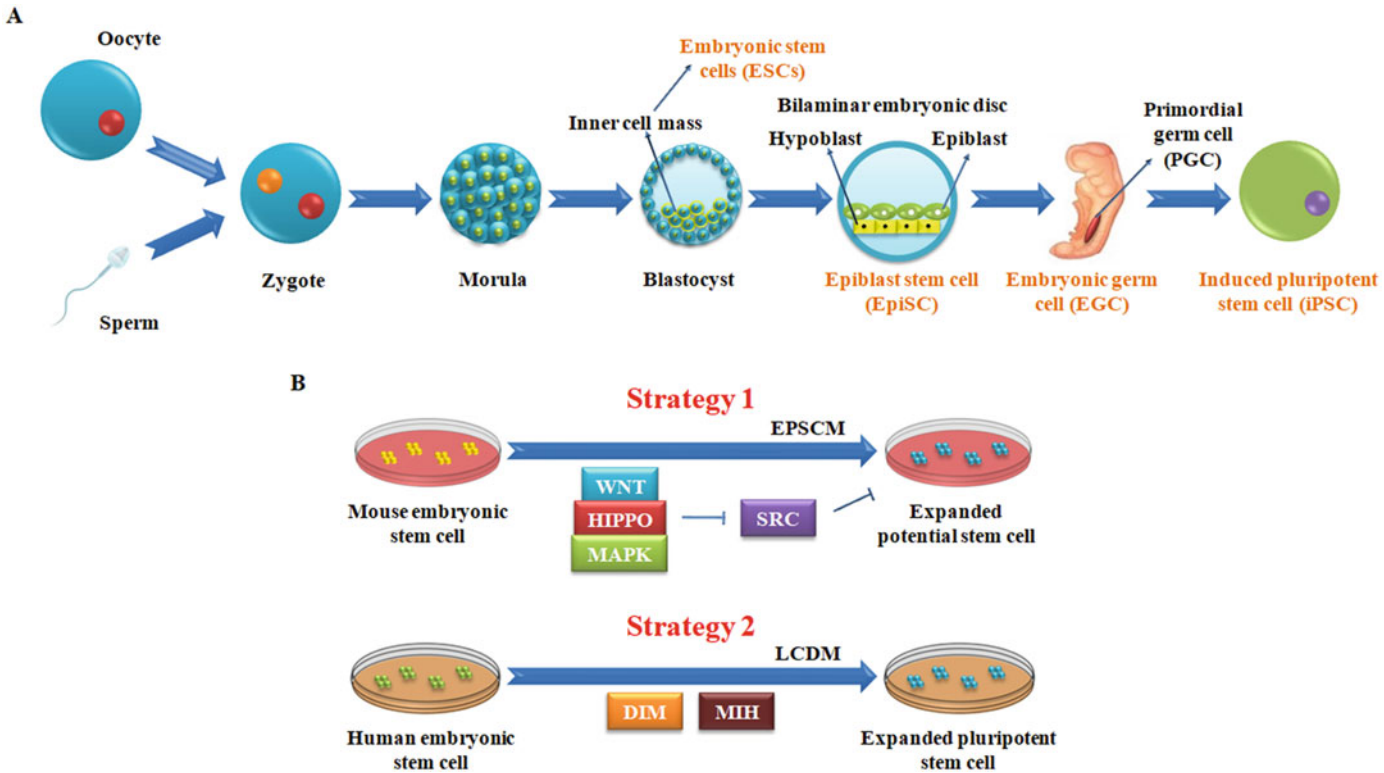

Fig. 2.4 (**a**) Stages of deriving different types of pluripotent stem cells in mice and humans. (**b**) Current strategies to obtain expanded potential stem cells (EPSCs)

Table 2.2 Naïve and primed pluripotent stem cells

Cell type	mESCs	mEpiSCs and hESCs
Origin	ICM of an early blastocyst [26]	Post-implantation epiblast of the mouse embryo [32], ICM of the human embryo [15]
Pluripotency state	Naïve [33]	Primed [33]
Teratoma formation	Present [34]	Present [34]
Blastocyst chimeras	Present [35]	Absent [35]
Epigenetic state	Global hypomethylation [36]	Global hypermethylation [36]
Expressed genes	High expression of Oct4 (or POU5F1), Nanog and ESRRβ (or ERR2) [36]	Oct4, Sox2, Nanog, Fgf5, Brachyury, and Otx2 [34, 37].
X-chromosome inactivation status	Both X chromosomes are active [36]	One X chromosome is inactive [34]
Clonogenicity	High [33]	Low [33]
Oct4 enhancer usage	Distal [38]	Proximal [38]
Response to LIF/STAT3	Self-renewal [34, 37]	None [34, 37]
Response to Fgf2	Differentiation [34, 37]	Self-renewal [34, 37]

composed of distinct cell populations with different genetic origins, resulting from the fusion of more than one zygote. Chimera studies have been used to assess the developmental potency and fate of different embryonic cell lines based on their ability to participate in embryonic development after injection into a blastocyst [39]. Inactivation of one X chromosome randomly occurs at an early stage of female embryonic development to ensure dosage compensation between both genders regarding sex-linked genes expression [40]. However, female naïve pluripotent cells reactivate both X chromosomes (XaXa), in contrast to primed pluripotent cells which have only one active (XaXi) chromosome [41].

The *naïve state* represents a cellular state that is similar to the preimplantation inner cell mass. This can be described as PSCs in a "ground state" that are free of any lineage commitment, and therefore not constrained epigenetically. In contrast, the *primed state* is representative of the post-implantation epiblast cells (EpiSCs) that are more committed toward lineage-specific developmental pathways and are epigenetically restricted [33]. To date, the naïve state has been achieved in mouse ESCs (mESCs), but not in human ESCs (hESCs), even though both were derived from preimplantation embryos [15]. It is still unknown whether the reconversion of PSCs from primed to a more naïve state is direct, or

involves a transitional state. Overall, more evidence suggests that human and mouse PSCs are not identical, and that differences in gene expression and culture requirements could affect the pluripotency states in vitro and therefore could lead to different outcomes in terms of PSC-based therapies.

2.2.2 Switching Between Pluripotency States: Naïve to Primed and Back

Different states of pluripotency of mESCs (in vitro) correspond to in vivo embryonic development. This means that naïve and primed states are not categorical states, but rather, represent successive molecular snapshots during embryonic development [4]. Therefore, questions have been raised about how these cells could be converted back from the primed state to the naïve one. One crucial factor that is involved in this process is the culture conditions that the ESCs are exposed to after isolation, which has proved to be critical in determining their fate. Since hESCs or EpiSCs are in a near primed state, several attempts have been made to reset the pluripotency of hESCs back to a more naïve state, similar to that of mESCs [33]. These attempts included forced resetting through transgenic induction of Oct4, Klf2 and Klf4 [42], or Nanog and Klf2 [43] in the presence of 2i/ leukemia inhibitory factor (LIF) culturing conditions, or by simply manipulating the culture conditions to reset "genetically unmodified" hESCs into naïve ESCs [44, 45]. These attempts have enabled successful direct derivation of naïve hESCs from the inner cell mass (ICM) by adding different growth factors and small molecules such as LIF, FGF2, Activin A, GSK3 inhibitors, STAT3 inhibitors, ROCK inhibitors, and MEK inhibitors [45]. In all of the forced or non-forced previous attempts, the naïve hESCs that were generated met the naïve criteria of mESCs.

Epigenetic modifications also determine the pluripotency state as the mESC (naïve) genome is globally hypomethylated, whereas the EpiSCs (primed) genome is hypermethylated [4]. The pattern of histone modification especially on the gene promoter region is different in naïve cells, which prefer to use the distal enhancer for Oct4 gene transcription, while the proximal enhancer is primarily used in the primed cells. This difference suggests that there are histone modifications that change chromatin structure and accessibility in order to regulate transcription [38]. In humans, the epigenetic status of ESCs is considered to be primed, since it is similar to mouse EpiSCs [4]. However, recent work has identified that the primed hESC state can be reversed back to the naïve state through epigenetic resetting via transient histone deacetylase inhibition [46]. Therefore, non-transgenic naïve hESCs can be obtained via either manipulation of the culture conditions or epigenetic resetting. However, it is still debatable whether it is acceptable to unify the definition of naïve/primed pluripotency in human ESCs (and possibly other species) based on ESC characteristics identified in rodents. Instead, identifying species-specific characteristics for naïve/primed ESCs may be necessary.

2.3 Types of Pluripotent Stem Cells

2.3.1 Embryonic Stem Cells (ESCs)

ESCs are isolated from the ICM of early preimplantation embryos, at E3.5 in mice [26], or from human blastocysts [15]. When mESCs were isolated and cultured under proper conditions, including essential growth factors, feeder cells, or feeder-free medium in addition to proper incubation, they maintained their naïve pluripotency state for a long time. Cultured human ESCs (hESCs) were less naïve (more primed) than mESCs, and differed in their culture requirements (Fig. 2.4, Table 2.2) [36]. To date, it is not clear whether the differences between mESCs and hESCs are only due to the culture conditions or they are also due to other factors. The in vitro maintenance of naïve mESCs requires LIF signaling, while hESCs depend mainly on FGF2 and TGFβ1/Activin2 signaling, and not LIF [36]. Reports suggested that LIF with two inhibitory small molecules, CHIR99021 and PD0325901 (called 2i), inhibited the mitogen-activated protein kinases (MAPK)/extracellular signal-regulated kinases (ERK) pathway and the glycogen synthase kinase 3β (GSK3β) pathway. Inhibition of both pathways stabilized the ground state of mESCs. Naïve mESCs express various pluripotency markers, including OCT-4 (Pou5f1), NANOG, and Esrrβ (Err2), in addition, they lack the X-inactivation state in female cells. On the other hand, hESCs express high levels of some naïve pluripotency markers, such as NANOG, PRDM14, REX1 (or ZFP42), and E-cadherin, however, hESCs also show some of the primed cell characteristics, including low expression of KLF17 and DPPA3, lack of exclusive nuclear localization of TFE3, lack of hypomethylation and tendency of a pre-X-inactivation in female ESC lines [36]. Therefore, using conventional direct derivation and culture approaches, hESCs are less naïve than mESCs but more naïve than mouse epiblast stem cells (a primed state of mESCs). To achieve naïve hESCs, in a similar state to naïve mESCs, scientists adapted the culture conditions to reset isolated hESCs in vitro into a more naïve state. This suggests that the culture conditions do affect the state of pluripotency, which will also affect the outcome of using ESCs in clinical practice.

2.3.2 Epiblast Stem Cells (EpiSCs)

EpiSCs are isolated from the epiblasts of post-implantation embryos in mice (between E5.5 and E7.5) [32]. However, due to ethical considerations, no EpiSCs have been obtained from human embryos [37]. Mouse EpiSCs (mEpiSCs) share some similarities with mESCs but are classified as a different type of PSCs, based on several cellular and molecular differences (see Table 2.2). Similar to mESCs, mEpiSCs are pluripotent, as they give rise to all three germ layers and germ cells, and can form teratomas when they are injected into immune-deficient mice. However, similar to hESCs, the pluripotency state of mEpiSCs is primed [34]. The characteristics of mEpiSCs are also similar to those of hESCs in some aspects, including the inability to survive as a single-cell clone after trypsinization, and

inactivation of one X chromosome in the female genome [34]. The nucleus of mEpiSCs is similar to that of mESCs, which is large relative to the cytoplasmic content, but the cellular morphology is epithelial and grows as a monolayer resembling hESCs [34]. Moreover, mEpiSCs have limited ability to produce chimeras, as compared to mESCs [35]. The canonical pluripotency factors (i.e., Yamanaka factors, including Oct4, Nanog, and Sox2) are expressed by both ESCs and EpiSCs. However, mEpiSCs express specific markers for the differentiated epiblast (or post-implantation epiblast), such as Fgf5, Brachyury, and Otx2. In vitro maintenance of mEpiSCs in the undifferentiated pluripotency state requires supplementation of Activin A and fibroblast growth factor 2 (FGF2); but not LIF, as is the case in hESCs [34, 37].

2.3.3 Embryonic Germ Cells (EGCs)

Embryonic germ cells (EGCs) are PSCs that are derived from unipotent primordial germ cells (PGCs), the precursors of the germ cell lineage [47]. In early development at the time of gastrulation, a small group of cells known as PGCs are assorted to later form oocytes or spermatozoa, depending of the sex of the embryo (Fig. 2.4). The development of the PGCs has been studied extensively in mouse models, but not in humans due to ethical concerns. Around day E6.25, and shortly before gastrulation, PGCs are initially developed in the epiblast, and later, at E10.5, they migrate to the extraembryonic mesoderm and then through the mesentery of the hindgut to colonize the genital ridges [47–49], however, stray PGCs can lead to teratoma formation. PGCs can be identified by tissue non-specific alkaline phosphatase activity [12] and the expression of surface-specific embryonic antigens (SSEA-1,-3,-4), mouse vasa homolog (Mvh or VASA), and intracellular proteins (Stella or Dppa3, OCT-4, NANOG, Fragilis, and Blimp1 among others) [50, 51]. Based on these findings, it was important to address how unipotent PGCs develop into pluripotent EGCs. Although the answer is not fully understood, in vitro epigenetic reprogramming (dedifferentiation) could provide an explanation. Culturing of PGCs derived from mice at E8.5 to E12.5 in the presence of LIF, basic fibroblast growth factor (bFGF), and stem cell factor on feeder cells [52, 53] gave rise to EGCs. The pluripotency of these cells was then confirmed in vitro, by the formation of embryoid bodies that contained cells from all three germ layers. These EGCs were positive for both alkaline phosphatase and SSEA-l markers, resembling pluripotent embryonic stem cell. EGCs formed teratomas in vivo when injected into immunocompromised mice, and contributed to chimeric mouse tissue, including the germline [53]. Interestingly, under mESCs culture conditions, the derived EGCs were indistinguishable from mESCs, and seemed to share a similar transcriptome, but had somewhat different epigenetic imprinting [54, 55]. On the contrary, PGCs could neither form embryoid bodies in vitro nor contribute to mouse chimeras, and stopped proliferation after a certain number of divisions [53].

2.3.4 Induced Pluripotent Stem Cells (iPSCs)

Ethical concerns over using human ESCs have encouraged researchers to look for alternative, less controversial, sources of stem cells for clinical applications. Induced pluripotent stem cells (iPSCs) were developed from mice in 2006 and humans in 2007 [30, 31]. IPSCs derived from adult somatic cells (e.g. skin), which were genetically reprogrammed to ESCs-like state by transgenic expression of specific transcription factors, including OCT-3/4, SOX-2, KLF-4, c-MYC (Yamanaka factors, see Figure 2.3c). These factors, which are highly expressed in ESCs, reactivated the developmental signaling network that is necessary for initiating and maintaining an ESC-like pluripotency. Once this pluripotency was activated, these iPSCs were capable of tri-lineage differentiation, contributing to chimeras and teratoma formation, similar to ESCs [30, 31].

2.3.5 Extended Potential Pluripotent Stem Cells (EPSCs)

Extended or expanded potential stem cells (EPSCs) and extended pluripotent stem cells (EPS) are totipotent stem cells that were established from mice and humans in 2017 (Figure 2.4b). The two involved research groups reported that these cells have blastomere-like features, and may be even superior to ESCs in terms of their contribution to the three germ layers and extraembryonic tissues [3, 56]. One group screened more than 100 small molecules that can convert primed hESCs into a more naïve state (as mESCs), and the resulting cells were EPS [3]. They depended on the fact that mESCs were maintained on the ground state upon treatment with LIF, CHIR99021, and PD0325901 (2i) [36]. These cells were also supplemented with another two small molecules, minocycline hydrochloride (MiH), and dimethindene maleate (DiM), that inhibit PARP1 and muscarinic, histamine receptors, respectively. The established culture medium was called LCDM, which stands for (**L**IF, **C**HIR 99021, **D**iM, and **M**iH). Either hESCs or human iPSCs (hiPSCs) were cultured in LCDM medium and attained a more naïve state for up to 50 passages. This more naïve state was indicated by the expression of the distal OCT-4 enhancer and the lack of expression of the repressive epigenetic marker (H3K27me3) for X-chromosome inactivation. The chimera assay showed that these cells exhibited high plasticity beyond ESCs, where these cells were not only contributing to three embryonic lineages, but also to extraembryonic tissues. EPSCs, derived from eight-cell stage mouse embryos (i.e., earlier than the stage used for ESC derivation), were developed by the other research group. These EPSCs were also derived from mESCs or somatic reprogrammed pluripotent cells (i.e., iPSCs). Similar to iPSCs, EPSCs depended mainly on the manipulation of the in vitro culture conditions in order to block blastomere differentiation through targeting specific signaling pathways causing trophectoderm/ICM segregation. These conditions include culturing the cells (8 cell-stage blastomeres, ESCs, or iPSCs) in serum-free medium that contains LIF and a cocktail of small molecules. These culture conditions aim to block MAPK/ERK, Src, and Wnt/Hippo/Tnks1/2 signaling,

which are implicated in the early segregation of embryonic cells into ICM or trophectoderm. The expanded/extended potential relates to the ability of a single cell to form both the extraembryonic and embryonic tissues in the chimera assay. The clinical applications of these cells are still under investigation [3, 56].

2.4 Human Embryonic Stem Cells (hESCs)

2.4.1 Isolation of hESCs

Human ESCs are derived from the ICM of the blastocyst, which is obtained after 4–6 days of fertilization (Fig. 2.5). The zygote (day 0–1 post-fertilization) is considered totipotent, as it gives rise to all embryonic and extraembryonic structures (fetal membranes, umbilical cord, and placenta). Cultured hESCs exhibit self-renewal and multi-lineage differentiation potential into the three germ layers: ectoderm, mesoderm, and endoderm. Human ESCs are primarily isolated from IVF embryos [57]; however, this has elicited ethical concerns as it raises the probability of premature embryonic destruction [58]. During IVF, high quality embryos are usually transferred to mother's womb, but the remaining "spare" embryos (of variable quality) are either frozen or donated for research; for ESC derivation or cell line creation [57]. The creation of human blastocysts via IVF for the sole purpose of ESC

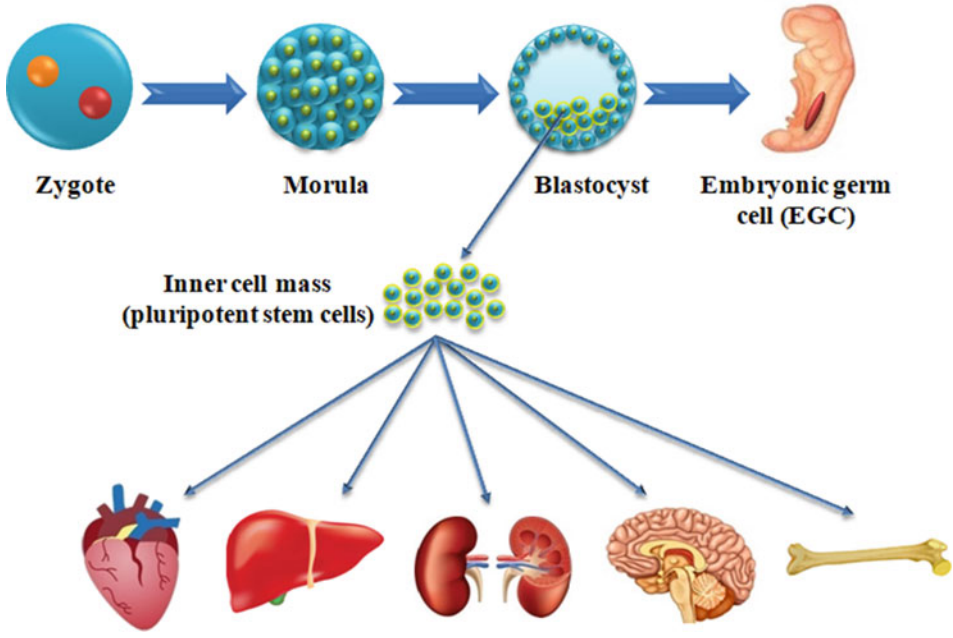

Fig. 2.5 Embryonic pluripotent stem cells derived from the inner cell mass of an embryo

derivation is highly controversial [58]. Various techniques have been developed in order to isolate hESCs, either from fresh or frozen IVF products [15, 59, 60]. Immunosurgery and mechanical dissection through microsurgery were some of the first methods used to isolate embryonic stem cells from the ICM [15, 60–62]. More recently, laser technologies were embloyed; where a laser beam of high thermal and cutting potential is used to cut into the blastocyst to obtain the ICM for ESCs isolation [63, 64].

Immunosurgery is based on the vulnerability of the mouse blastocyst to complement-dependent antibodies. Solter and Knowles were the first to perform this technique, and they found that it was possible to digest the outer trophoblast layer of the blastocyst using complement-dependent antibodies that bind to the trophoblastic outer membrane antigens. The activated complement reaction leads to lysis of the outer trophoblasts and exposure of the ICM, which is then collected from the deformed trophoblasts for further culture [62]. This technique has been used to successfully isolate hESCs using animal serum and complement [15, 65]. However, to avoid the use of animal-origin substances, microsurgery was proposed in order to isolate ESCs via performing surgery under the microscope on preimplantation blastocysts. Microsurgery has been performed either mechanically, using specialized needles under a stereomicroscope, or by using a laser to cut the blastocyst and separate the ICM from the trophectoderm layer [60, 64].

2.4.2 Culture of hESCs

Primitive and undifferentiated hESCs require the presence of specific factors to maintain their growth and undifferentiated state. Therefore, hESCs are cultured either on feeder cells or in feeder-free media, in which feeder cells are replaced by LIF [15, 59, 60, 66, 67]. Earlier cultures of hESCs used mitotically inactivated murine embryonic fibroblasts (MEFs) feeder cells to provide the cultured cells with growth factors and cytokines that are essential for their in vitro maintenance [15, 68, 69]. However, transfer of infectious pathogens or animal-derived viral particles to cultured hESCs has been reported when using MEFs, which renders cultured stem cells clinically useless [70–72]. To avoid contamination of hESCs with animal-based products, feeder cells of human origin have been proposed as a substitute for MEFs. Human cells from various sources, such as umbilical cord, endometrial and placental cells [73–76], as well as human-derived growth factors have been shown to be more effective at maintaining the in vitro growth of hESCs [66, 77, 78]. Use of animal serum or serum replacements that contain animal proteins, such as bovine serum albumin [79], elicited a type of immune rejection when used clinically [79, 80]. Xeno-free culture conditions have been critical for clinical applications. One approach to culture hESCs under feeder-free conditions is to provide growth factors and small molecules. Mouse ESCs were cultured feeder-free when supplemented with LIF [81]; however, hESCs still required a feeder cell layer. In 2001, the first successful feeder-free culture of hESCs was achieved by sub-culturing hESCs colonies (which were initially cultured on feeders) on a matrigel or laminin matrix supplemented with MEF-conditioning

medium, with human basic fibroblast growth factor (bFGF) [67]. Later, cultures supplemented with bFGF, activin, and noggin maintained the growth and proliferation of hESCs [82–85]. Another feeder-free and xeno-free hESCs culture was achieved using a chemically defined medium, containing bFGF, LiCl, γ-aminobutyric acid (GABA), pipecolic acid, and TGFβ [86]. In addition, alternative feeder-free approaches have included encapsulation of hESCs in hydrogels [87, 88] or 3D scaffolds made of biocompatible natural polymers [89]. All of these feeder-free approaches have resolved some of the limitations of hESCs use in therapy and allowed higher-scale production. However, these approaches still do not fully recapitulate the in vivo environment for growth and propagation of hESCs. More research is needed for proper large-scale development of hESCs for therapeutic purposes.

2.4.3 Characterization of hESCs

Human ESCs form homogenous round-shaped colonies with defined borders that can be propagated for a long time in culture [90, 91]. ESCs have distinguished nuclear structure and less cytoplasm than somatic cells, and form embryoid bodies upon culturing in vitro [92]. When grafted in severe combined immune-deficient (SCID) mice, ESCs form teratomas [15]. Teratomas are heterogeneous tissues of highly differentiated cells from all the three germ layers, including the ectoderm, mesoderm, and endoderm, and provide a robust evidence of ESC's tri-lineage differentiation potential [16]. Another functional property of ESCs is the formation of chimeras [17]. The chimera assay is based on xeno-transplantation of ESCs into a blastocyst. The injected cells are then tracked to study their contribution in the subsequent developmental stages of the embryo [17], See Table 2.3**.**

Pluripotency is maintained in ESCs by defined pluripotency markers that prevent their differentiation [95–97]. Phenotypic identification of ESCs using the most common markers is shown in Table 2.4. Undifferentiated ESCs express stage-specific antigen–3 (SSEA-3), and SSEA-4, in addition to the glycoprotein tumor recognition antigens TRA-1-60 and TRA-1-81 [10, 15, 102, 103]. ESCs also express the pluripotency markers OCT-4 and Nanog [31, 95, 99, 104]. Oct-4, Sox-2, and Nanog are described as the three master regulators of pluripotency in ESCs [95]. The forced reactivation of these key pluripotency regulators has reversed somatic cells to their pluripotent embryonic-like state. Yamanaka's group was able to reprogram somatic fibroblasts to iPSCs using these regulators [98]. However, it is important to note that neoplastic cells and embryonal carcinoma cells express similar pluripotency markers [102, 105]. Therefore, for a safer clinical use, these findings suggest the importance of continuous monitoring/characterization of in vitro cultured hESCs, in order to distinguish normal ESCs from cells that underwent neoplastic progression or began spontaneous transformation to undesirable cells [106, 107].

Table 2.3 Functional assays for pluripotency assessment

Embryoid body formation and differentiation	Teratoma formation	Chimera formation
Embryoid bodies (EB) are three-dimensional aggregates of pluripotent cells generated by culture of ESCs on ultra-low attachment plates in the absence of the self-renewal cytokine LIF [26]. Another method used is the induction of EB formation by the hanging drop technique. EB formed after several days can be transferred and cultured under the appropriate conditions to develop into various cell types [93]	The pluripotent cells such as ES can give rise to teratomas, composed of three germ layer tissues upon injection into immune-deficient SCID mice. After transplantation, the resulting labeled teratomas can be monitored and analyzed using molecular imaging approaches to determine the derivatives of the embryonic germ layers [16]	Microinjection is one of different techniques that have been developed to generate chimera in animal model like mice. ESCs-derived from ICM of black donor mice's blastocyst at day four is microinjected into blastocyst cavity of pre-implanted albino recipient mice. These manipulated blastocysts are then implanted into surrogate mother. The contribution of donor cells to the recipient mouse germline can be determined by examining the F1 offspring for albino and black color distribution which would reflect chimera [17, 94]

Table 2.4 Embryonic stem cell markers

Marker	Type	Role in pluripotency	Reference
Oct-4	POU family transcription factor (homeodomain protein)	Maintain self-renewal and pluripotency. Transcriptional patterns play a role in specifying ESC identity. Oct-4/SOX-2 complex found to have a fundamental role in gene expression and regulatory control in ESCs	[31, 95, 98–100]
Sox-2	HMG-box transcription factor		[31, 95, 98, 106]
Nanog	Homeodomain proteins		[31, 95]
SSEA-3	Glycolipid carbohydrate antigens	Antigens are specifically expressed on ESCs, EGCs, and human teratocarcinoma cells. Any decrease in the expression of these antigens is associated with differentiation patterns and development	[11, 13, 101, 102]
SSEA-4	Glycolipid carbohydrate antigens		[11, 101, 102]
TRA-1-60	Keratan sulfate-related antigens		[10, 102, 103]
TRA-1-81	Keratan sulfate-related antigens		[10, 102]

2.4.4 Differentiation of ESCs

As discussed earlier in this chapter, pluripotency is the primarily trait of ESCs [15]. Pluripotency is always linked to a high potential to further differentiate into different cell types [2]. As the cell differentiates, its functional specification prevails and pluripotency diminishes [2, 97]. Expression of pluripotency markers, including Oct-4, SOX-2, and Nanog maintains the pluripotent state of ESCs by controlling the expression of differentiation gene cascades. Accordingly, lack or loss of these pluripotency markers initiates the differentiation of ESCs, which is accompanied by the expression of differentiation markers [96, 97]. For example, differentiation can be driven by the activation of polycomb repressive complexes and microRNAs that regulate or switch-off pluripotency regulators of ESCs [97, 108, 109]. In addition, growth factors, epigenetic state, and cell-to-cell signals cooperatively determine which lineage specification the differentiated ESCs will go through [110–112].

In vivo, cells of the ICM, which is the origin of ESCs, divide to give rise to all cell types in the body, leading to complete structural and functional body mass. Similarly, ESCs can differentiate in vitro into specialized cells of any of the three germ layers, including the ectoderm, mesoderm, and endoderm, in the presence of the proper stimuli and growth factors [15, 113]. In the absence of self-renewal, ESCs cultured in vitro can spontaneously differentiate as aggregates of cells or embryoid bodies [114]. Cells of the embryoid bodies have been shown to adopt in vivo-like temporal and spatial differentiation patterns. Ectodermal-like cells appear first, followed by endodermal cells, and further differentiation and specification of mesodermal cells [115, 116]. Germ layer cells further differentiate into more specialized cells, such as cardiomyocytes [117], hepatocytes [118], neurons, astrocytes and oligodendrocytes [119, 120], and ovarian follicle-like cells [121].

Factors that affect the differentiation of hESCs in vitro include the seeding density, pH, temperature, and most importantly the components of the culture medium and growth factors. Hepatocyte growth factor (HGF) and nerve growth factor (NGF) can induce differentiation into the three germ layers. While activin A and transforming growth factor (TGF)-β are essential to induce mesodermal differentiation, other factors such as bone morphogenic proteins (BMP)-4, retinoic acid (RA), basic fibroblast growth factor (bFGF), and epidermal growth factor (EGF) can all promote ESC differentiation into both mesodermal and ectodermal lineages [92].

2.5 Applications of Human Embryonic Stem Cells

The use of hESCs allows the advancement of our understanding of disease etiology and also shows great promise for the development of novel therapeutic approaches. There are currently over 40 clinical trials using hESCs that are registered on the NIH Clinical Trials website (https://clinicaltrials.gov/, as of the 16th of May 2020, using “embryonic stem cells” as the search criteria). These studies include using hESCs to generate retinal pigment

epithelium in order to treat ophthalmic diseases, such as age-related macular degeneration and retinitis pigmentosa [122]. These studies also include those targeting cardiac diseases. In particular, severe ischemic heart failure was treated using hESC-derived cardiac progenitor cells that were combined with a fibrin scaffold and grafted onto the epicardium of the infarcted area [123]. Clinical trials have also used hESCs to target neurodegenerative diseases, such as Parkinson's disease (PD) and spinal cord injury (SCI) [124], as well as type 1 diabetes [125]. However, to date, there are no approved FDA products that are based on hESCs [126]. Research is now focused on using hESCs for disease modeling and regenerative medicine, where animal models have failed or are still inappropriate for these purposes.

2.5.1 Disease Modeling

ESCs have been used to model disease through development of disease-specific cells that carry relevant aberrations or mutations. Human ESCs are either modified using gene editing or induced to acquire chromosomal aberrations via manipulatng in vitro cell culture conditions [127, 128]. Disease-specific ESCs may also be directly isolated from defective IVF embryos carrying genetic diseases or chromosomal aberrations. Preimplantation genetic diagnosis and genetic screening are two methods used to identify embryos with monogenic disorders or chromosomal abnormalities [129, 130].

In 2004, human-derived ESCs were successfully genetically engineered to model Lesch–Nyhan disease through the induction of a mutation in the hypoxanthine phosphoribosyltransferase 1 (*HPRT1)* gene using homologous recombination [128, 131]. Development of successful hESC disease models was also performed for Fragile X Syndrome [132] and Turner's syndrome [127]. Examples of methods for developing disease models include gene editing techniques, where Zinc finger nucleases were used to mediate site-specific modifications in the ESC genome with high efficiency [133–136]. In addition, transcription activator-like effector nucleases (TALENs) were made to induce genomic modifications and exploit the potentials of hESCs in disease modeling [137]. Gene editing has also been performed in ESCs to model X-linked severe combined immunodeficiency (X-SCID) disorder [133]. More recently, clustered regularly interspaced short palindromic repeat-associated protein 9 (CRISPR-Cas9) technology [134, 138], SCNT [139], and iPSCs [30] have been used as practical alternatives to hESCs use for disease modeling.

2.5.2 Regenerative Medicine

Human ESCs can generate various types of differentiated cells for cell replacement therapies and can be also used in clinical trials for disease treatment. Below are some examples of their use in the clinic:

2.5.2.1 hESCs and Spinal Cord Injury

In 2009, FDA approved the first hESC-based phase I clinical trial using hESC-derived oligodendrocyte progenitor cells (GRNOPC1, ClinicalTrials.gov Identifier: NCT01217008, known as Geron's trial) for the treatment of acute spinal cord injury (SCI) patients. The treatment protocol included the injection of two million GRNOPC1 cells into affected SCI patients within 7–14 days post-injury, followed by the administration of immune-suppressants for 46 days. Both animal studies and preclinical data have shown that GRNOPC1 cells have the potential to regenerate injured cord and promote motor recovery in SCI patients. After enrolling five patients with SCI, no adverse effects were observed. Safety was measured through assessment of the frequency and severity of adverse events occurring within 1 year of injection. However, no improvement was reported in motor or sensory responses in enrolled SCI patients [124]. This trial was terminated in 2011, and another study using hESC-derived oligodendrocyte progenitor cells (now called AST-OPC1) to treat SCI has been initiated (ClinicalTrials.gov Identifier: NCT02302157).

2.5.2.2 hESCs and Diabetes

In 2014, a phase 1/2 clinical trial for type 1 diabetic patients was sponsored by ViaCyte and CIRM (ClinicalTrials.gov Identifier: NCT02239354). The study tested a new product (called VC-01) that contains stem cell-derived pancreatic islet replacements in order to treat type 1 diabetes mellitus. Pancreatic endoderm cells derived from hESCs (PEC-01 cells) were encapsulated in an inert biomaterial in order to protect them from attack by the immune system. The encapsulated "islets" were expected to act as an artificial pancreas in order to effectively control blood glucose levels. The capsule was surgically implanted under the patient's skin and was expected to mature over several months and start producing insulin. The tolerability, therapeutic dose, and safety were evaluated in the first cohort group. After 24 months, the product that was implanted showed promising results and had minimal adverse effects (related to the surgery) and no immunological sensitivity. The cells had prolonged survival, and their ability to differentiate into pancreatic islet cells was determined using immunohistochemical staining for NKX6–1, insulin, and glucagon markers. Importantly, no off-target tumors were observed. This study suggests that the use of ESCs may be a new effective approach to treat chronic autoimmune diseases, such as type 1 diabetes [125].

2.6 Induction of Pluripotency: Needs and Challenges

Human ESCs have a great potential to treat many degenerative diseases [140]. However, translating hESCs for use in the clinic has been challenging for a variety of reasons. Ethical controversies about the derivation and use of hESCs in research, as well as in the clinic, are still a significant obstacle to advances in this field [141]. Additionally, the use of these cells also leads to immune challenges [142] and other issues of safety and functional efficacy

[143]. Safe and ethically accepted alternatives to ESCs for therapies in regenerative medicine have been developed by researchers. As such, iPSCs have been established to model diseases and have been used for drug discovery. Newer gene editing technologies and direct differentiation protocols are also less controversial and more effective source of pluripotent cells for regenerative medicine purposes [144].

Take Home Message

- Pluripotent stem cells can be differentiated in vitro to all three germline lineages, excluding the extraembryonic tissue.
- Embryonic stem cells are obtained from the inner cell mass of the blastocyst, while induced pluripotent stem cells are obtained by reprogramming of adult somatic cells.
- New approaches enabled scientists to create extended or expanded potential stem cells (EPSCs), which can form both extraembryonic and intraembryonic tissues.
- Approaches to cellular reprogramming; include nuclear transfer or cloning, cell fusion, and direct reprogramming, paved the way for discovering pluripotency.
- Pluripotency hallmarks include unregulated expression of pluripotent genes, in vitro embryoid bodies formation, in vivo teratoma formation, and in vivo chimera formation.
- Pluripotency exists in two different states, primed and naïve. Human ESCs are more primed than murine ESCs, however the former can be induced to naïve state under certain culture conditions and genetic manipulations.
- ESCs have been proposed for disease modeling and drug discovery, however, the obstacles are ethical concerns and teratoma formation.

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Hematopoietic Stem Cells and Control of Hematopoiesis

3

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Abbreviations

5FU	5-fluorouracil
β-TCP	β-TriCalcium phosphate
(AGM) region	Aorta–gonad–mesonephros region
ALL	Acute lymphocytic leukemia
AML	Acute myeloid leukemia
AML1	Acute myeloid leukemia-1 protein
BCL-2	B-cell lymphoma 2
BM	Bone marrow
Bmi-1	Polycomb complex protein 1
BMPs	Bone morphogenic proteins
Cbfa2	Core-binding factor subunit alpha-2
Cbx7	Chromobox protein homolog 7
CDKs	Cyclin-dependent kinases
CFU	Colony-forming unit
CIBMTR	The Center for International Blood and Marrow Transplant Research
CML	Chronic myeloid leukemia
Ezh1	Enhancer of zeste homolog
(FDCP)-mix	Factor-dependent cell Paterson-mix
FGF	Fibroblast growth factor
FL	Flt3 ligand
G-CSF	Granulocyte colony-stimulating factor
GVHD	Graft-versus-host disease
GVL	Graft-versus-Leukemia
HE	Hemogenic endothelium
HECs	Hemogenic endothelial cells
HIF-1	Hypoxia-inducible factor-1
HPCs	Hematopoietic progenitor cells

HSCs	Hematopoietic stem cells
IFNs	Interferons
IGF	Insulin-like growth factor
IGFBP2	IGF-binding protein 2
ILs	Interleukins
IRF2	Interferon regulatory factor-2
Irgm-1	Immunity-related GTPase family M protein-1
KitL	c-kit/kit ligand
KSL cells	$Kit^{+}Sca^{+}lin^{-}$cells
LIF	Leukemia inhibitory factor
LPS	Lipopolysaccharides
LTC	Long-term culture
LTC-ICs	Long-term culture-initiating cells
LT-HSCs	Long-term HSCs
MDS	Myelodysplastic syndrome
MIP-lα	Macrophage inflammatory protein lα
MLL	Mixed lineage Leukemia
MPPs	Multipotent progenitors
MPR	Melphalan, prednisone, lenalidomide
Msi2 protein	Musashi-2 protein
NGF-β	Nerve growth factor β
PB	Peripheral blood
PcG protein	Polycomb-group protein
RBCs	Red blood cells
Runx1	Runt-related transcription factor-1
Sca-1	Stem cell antigen-1
SCF	Stem cell factor
SCL	Stem cell leukemia
SDF-1	Stromal cell-derived factor-1
SET1A	SET domain-containing protein 1A
SLAM	Signaling lymphocyte activation molecule
STAT1	Signal transducer and activator of transcription 1
ST-HSCs	Short-term HSCs
TGF-β	Transforming growth factor-β
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TPO	Thrombopoietin
TrxG protein	Trithorax-group protein
UCB	Umbilical cord blood
VCAM-1	Vascular cell adhesion molecule-1
VLA-4	Very late antigen-4

What You Will Learn in This Chapter

This chapter presents the evolving concept of hematopoietic stem cells (HSCs) while focusing on the primitive and definitive hematopoiesis processes. It also demonstrates the unique properties of HSCs as well as their classification in terms of potency and differentiation potential. Moreover, it includes a brief description of HSCs characterization and regulation. Additionally, the hematopoietic hierarchy tree, showing the classical hematopoiesis hierarchy and specific clonal analysis for each cell type is highlighted. Finally, it will discuss the current therapeutic applications and potential of HSCs and concludes with the novel outcomes from ongoing HSC research which continue to redefine and refine our knowledge and provide a venue for endless improvements in HSC based clinical therapeutics.

3.1 Bone Marrow (BM) and Its Microenvironment

Hematopoietic stem cells (HSCs) are multipotent cells that are the universal progenitors of all blood cell lineages generated by hematopoiesis. Further research into the biology of HSCs will be of great importance towards improving our understanding of physiological hematopoietic processes as well as pathological conditions, including leukemia and lymphomas. Hematopoiesis is initiated at an early stage of embryogenesis and remains in progress until death; as such, it will be essential to understand both prenatal (embryo–fetus) and adult hematopoiesis. Ethical concerns associated with invasive investigations of hematopoiesis in human embryos have created the need for model organisms. To this end, developmental biologists among others have introduced model systems, which include chick embryos, mice, and zebrafish (*Danio rerio*). These comparative approaches have revealed many fundamental concepts underlying hematopoiesis and have led to the development of therapeutics that are now used to treat blood disorders and cancers. Given the overall conservation of genetic programs controlling hematopoiesis among vertebrates, studies carried out in the zebrafish model have provided us with dramatic new in vivo insights into this process [1].

3.1.1 Sites of Hematopoiesis

In mammals, hematopoiesis occurs at different anatomical positions during development from early stage embryo to adulthood. In mammalian embryos, the earliest stage, also known as primitive hematopoiesis, occurs in the yolk sac. The hematopoietic precursors, called hemangioblasts, have limited capacity for self-renewal and differentiate into a limited number of cell lineages, including endothelial cells, nucleated red blood cells (RBCs), and macrophages. This stage is followed by definitive hematopoiesis wherein

HSCs undergo translocation to the aorta–gonad–mesonephros (AGM) region. During mid-gestation, hematopoiesis transitions mainly to the liver and, to a lesser extent, to the spleen and thymus. Finally, BM becomes the primary site of hematopoiesis during late gestation and in adulthood [2]. Definitive hematopoiesis includes HSCs and hematopoietic progenitors derived from them; this process leads to the production of enucleated RBCs and the full set of myeloid and lymphoid lineages. HSCs are one of the several cellular components of the BM; their developmental niche includes all hematopoietic cells derived from HSCs and vascular cells and extracellular matrix. To achieve a larger understanding of hematopoiesis, study of this dynamic microenvironment remains critical. Approaches used for the study of hematopoiesis include in vivo imaging and ex-vivo analysis known as "bone marrow (BM)-on-a-chip"; in the latter case, processes that take place within the BM are studied using a three-dimensional (3D) scaffold on a microchip. Unique biomaterial-based 3D scaffolds have been recently used to generate systems that mimic the interaction of HSCs with the 3D structure of the BM microenvironment [3]. One such study featured a scaffold comprised of macro- and micro-porous printed β-tricalcium phosphate (β-TCP), a bioceramic used for bone tissue engineering, combined with Matrigel® (β-TCP/Matrigel®); this matrix provided an ideal support for the study of hematopoietic cell recruitment, proliferation, and differentiation and for remodeling of the extracellular matrix. In addition, upon transplantation in murine models, this scaffold promoted neovascularization and provided a functional extramedullary BM niche, which recapitulated both osteogenesis and hematopoiesis [4].

3.1.2 Anatomy of the Bone Marrow

In long bones, BM can be found within the diaphysis and the metaphysis. BM fills the medullary cavity of the diaphysis; the shaft of compact bone that provides physical support for the BM and a site for mineral storage and locomotion. BM can also be found inside the cavities of cancellous bone (also known as trabecular or spongy bone) that include primary and connected secondary trabeculae in the metaphysis. The porous structure of the cancellous bone provides strength and flexibility and is comparatively lighter in weight. The BM coexists with a complex vascular and neural network and is tightly associated with the dynamic bone environment at which new bone tissue is added, removed, or remodeled from spongy to compact and vice versa [5].

3.1.3 Types and Morphology of BM

The BM is a soft and gelatinous-like tissue as it contains primarily hematopoietic cells and adipose tissue; the nature of these components defines the type of the marrow. Hematopoietic red marrow is the primary site of active hematopoiesis; it is comprised of abundant progenitors and mature RBCs, white blood cells, platelets, and adipose tissue.

Red marrow can be found inside virtually all bones of neonates but becomes less widespread with increasing age. In adults, red marrow is confined to axial skeletal structures, including the skull, vertebrae, ribs, sternum, pelvic bones, and in the proximal metaphysis of the humerus and femur.

The second type of BM is the yellow marrow, which includes primarily adipose cells accompanied by islands of hematopoietic tissue. There is a dynamic balance between the two types of marrow throughout the life span of an individual. The results of several studies suggest that the yellow marrow could, at least in part, revert to red marrow in response to specific erythropoietic stimuli [6]. Yellow marrow has been described as a "buffering tissue," which facilitates the expansion or regression of hematopoietic cells within the bone [7].

3.2 Hematopoiesis and the Hematopoietic State

As typical stem cells, HSCs have the capacity for self-renewal and the ability to differentiate and give rise to all blood cell lineages. In the mouse embryo, precursors of hemogenic endothelial cells (HECs) go through intermediate stages of development to form the first HSCs in the AGM region [8].

3.2.1 Tracing Hematopoiesis Throughout Development

3.2.1.1 Primitive and Definitive Hematopoiesis

Large nucleated RBCs and macrophages are generated in the yolk sac as a result of a primordial wave of blood formation, known as primitive hematopoiesis [9]. Adult-type hematopoiesis swiftly replaces the primordial wave, which occurs in the AGM region [10]. At this point during embryogenesis, a tube developing into a single aorta is created after the lateral plate mesoderm undergoes migration and comes into contact with the endoderm. This process is followed by the emergence of HSCs in the ventral wall of the dorsal aorta near the AGM region. Subsequently, the fetal liver, thymus, spleen, and, eventually, BM are overtaken by HSCs that are capable of long-term self-renewal to establish definitive hematopoiesis (Fig. 3.1) [11].

3.2.1.2 Extraembryonic Hematopoiesis

Extraembryonic hematopoiesis is among the earliest stages of primitive hematopoiesis. At embryonic day (E) 7.0 in mice, the first hematopoietic progenitors can be identified in the yolk sac [12]. Hematopoietic activity can also be detected in the umbilical arteries and in the allantois, but not in the umbilical veins [13]. These findings support the hypothesis that HSCs originate mainly during arterial development. It remains unclear whether placental HSCs originate de novo or via colonization from earlier sites of hematopoiesis at the time that circulation is initiated or both [14, 15].

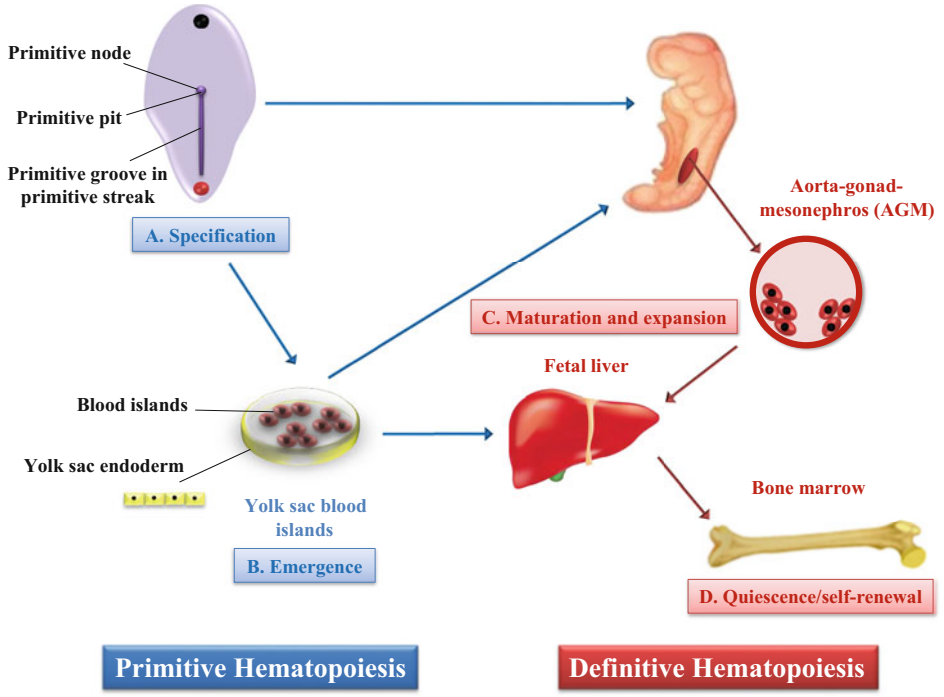

Fig. 3.1 Initiation of primitive and definitive hematopoiesis during development

3.2.1.3 Mesoderm to Hemangioblast

Hematopoietic precursor cells were first discovered nearly 100 years ago from studies of total chick blastoderms cultured on cover slips and from explant cultures of the posterior sections of blastoderms during the gastrulation phase; these cells were designated as angioblasts or hemangioblasts. In both types of experiments, the hemangioblasts were shown to be the precursors of both endothelial and hematopoietic cells [16]. Findings from these early studies carried out in chick embryos are fundamental to our current understanding of the concept of a hemangioblast; these findings remain correct through the present time.

In mice, migrating mesoderm is generated by means of gastrulation that takes place at E-6.5 [17]. The mesoderm differentiates into distinct populations with different developmental fates. In chick embryos, the mesodermal cells from the posterior primitive streak were the source of the initial blood islands [18]. All developing mesodermal cells are marked by a transcription factor and member of the family of T-box genes known as Brachyury. Detection of Brachyury^{+} cells declines once they are patterned and directed toward the generation of blood, connective tissues, endothelium, and skeletal or cardiac muscles [16, 19]. The hematopoietic potential of individual cells in the mouse epiblast, primitive streak, and early yolk sac was established by Padrón-Barthe et al. [19]. In vivo clonal analysis identified specified independent epiblast populations (before gastrulation) such as early

yolk sac blood and endothelial lineages, and the hemogenic activity was similar in both the embryonic hemogenic endothelium (HE) and a subpopulation of the yolk sac endothelium. Padrón-Barthe et al. [19] also characterized the appearance of the HE in the yolk sac, which ultimately gave rise to hematopoietic precursors showing markers related to definitive hematopoiesis.

3.2.1.4 Hemangioblast to Hemogenic Endothelium

It has been proposed that HSCs may be generated from hemangioblasts via formation of an HE intermediate [20]. This hypothesis was based on observations that localized the HE at a site adjacent to the hemangioblasts. Vogeli et al. [21] exploited advancements in single-cell resolution fate mapping of the late blastula and gastrula of zebrafish and confirmed the existence of hemangioblasts in vivo via the emergence of the bi-potential progenitors, which were capable of generating both hematopoietic and endothelial cells adjacent to the lining of the ventral mesoderm. The in vitro transformation of hemangioblasts/blast colony-forming cells into hematopoietic cells was characterized as a two-step process. Initially, the hemangioblasts generated a tightly adherent cell layer, which primarily expressed endothelial cell markers (thus comprising a transitory HE stage) after 24 h, later, at 36–48 h of culture, these cells became non-adherent, rounded, and initiated the formation of hematopoietic blast colonies [20].

3.2.1.5 Transition from Hemogenic Endothelium to Definitive Hematopoietic Progenitors or Pre-hematopoietic Stem Cells (Pre-HSCs)

Before final differentiation into HSCs, a second intermediate stage of hematopoietic precursor cells (pre-HSCs) arises from the HE. These pre-HSCs are found at various sites within the embryo, including the dorsal aorta, the vitelline and umbilical arteries, the yolk sac, and the placenta [22]. Runt-related transcription factor-1 [*Runx1*, also known as core-binding factor subunit alpha 2 (*Cbfa2*) and acute myeloid leukemia 1 protein (*AML1*)] is a critical factor that promotes differentiation of these hematopoietic progenitors from the HE; mutations in this gene are associated with numerous blood disorders.

3.2.1.6 Development and Differentiation of HSCs

Once sites of definitive hematopoiesis have been established, HSCs will maintain themselves and also have the capacity to differentiate into hematopoietic progenitor cells (HPCs); these latter cells ultimately give rise to multipotent progenitors (MPPs) and provide the embryo/fetus with the blood cell lineages, which are essential to support rapid growth and development. While the MPPs gradually lose their self-renewal potential, they maintain their capacity to promote multipotential differentiation into adult hematopoietic [23].

3.2.1.7 Cell Fate Choice

Upon undergoing cell division, HSCs can proceed along two distinct pathways; they can undergo self-renewal to produce new HSCs or they can differentiate and produce daughter

cells that have the capacity to mature into committed blood cells and cell lineages [24]. Once HSCs divide, they have the option of proceeding along one of several downstream cell fate pathways; the choice is made during the process of cell division. In this regard, symmetric division, asymmetric division, and symmetric commitment are among the possible patterns resulting from HSC division. Asymmetric division permits HSCs to balance their capacity for self-renewal with commitment and differentiation. A single HSC can give rise to two daughter cells with different functions, cell cycle kinetics, and/or multilineage capacity using a strategy called clone splitting [25]; this mechanism generates one cell that is committed to differentiation and another that maintains the capacity for self-renewal and HSCs pool. By contrast, symmetric division of HSCs gives rise to two daughter cells of the same type and potential. In other words, symmetric division can generate either two stem cells that remain capable of self-renewal or two progenitor cells that have completed their first step toward commitment and differentiation. These strategies are both tightly controlled to achieve a critical balance between self-renewal and differentiation [26]. Whereas, symmetric commitment is an essential pathway of cell division when rapid regeneration of damaged tissue is required, as both daughter cells can generate committed hematopoietic progenitors [27].

3.3 The Evolving Concept of the Hematopoietic Stem Cell

Concepts focused on our understanding of HSCs have undergone significant evolution; HSCs were the first stem cells to be discovered, and, due to their importance with respect to treatment of blood and neoplastic diseases, these cells were the first to be used clinically through BM transplantation. As such, HSCs have been the subject of substantial interest and remain of critical importance in research programs focused on biomedical sciences and regenerative medicine.

3.3.1 Properties of HSCs

3.3.1.1 Self-Renewal

HSCs undergo self-renewal to maintain the pool of undifferentiated cells throughout the life of the organism while preserving their capacity to differentiate [28]. Most of HSCs remain dormant; this serves to preserve balanced hematopoiesis and to protect the pool of HSC from succumbing to exhaustion. Only a finite number of HSCs enter the cell cycle and differentiate and mature into blood cells [29]. Several pathways are involved in promoting HSC self-renewal; we consider here the pathways that are most critical and best characterized. Among these, Notch-mediated signaling plays an important role in supporting HSC-mediated self-renewal. Activation of the Notch pathway by the ligands Delta and Jagged led to increasing HSC pool in vivo via enhancing the capacity for self-renewal (as evaluated by sequential BM transplantation experiments) and prevented

differentiation in vitro [30]. Importantly, Notch signaling is also a critical mechanism underlying osteoblast-mediated support for HSCs; osteoblasts activated by parathyroid hormone expressed Jagged-1 and promoted increased capacity for self-renewal among HSCs in experiments carried out in vivo [31]. Also important is c-Myc, a transcription factor and an oncogene that has been described as a master regulator of genes involved in protein synthesis, cell cycle, and cancer metabolism [32]. Activation of c-Myc occurs downstream of both Notch and homeobox family member HoxB4 signaling; this pathway supported in vitro self-renewal of murine $Lin^{-}Sca\text{-}1^{+}$HSCs cultured with stem cell factor (SCF), Fms-related receptor tyrosine kinase 3 (Flt3) ligand, and interleukin (IL)-6 for 28 days via upregulation of cell cycle genes (*c-myc*, cyclin-D2, cyclin-D3, cyclin-E, and E2F1) and increased telomerase activity [33].

The Wnt signaling pathway is also indispensable for the regulation of HSCs; forced expression of β-catenin, a core component of the Wnt signal transduction pathway, led to a 100-fold increase in the number of cultured HSCs and increased expression of both Notch-1 and HoxB4 [34]. Wnt3a is an essential factor promoting self-renewal of HSCs; deficiency of Wnt3a led to irreversibly impaired hematopoiesis due to reduced numbers of HSCs and reductions in their capacity for long-term repopulation [35]. However, there are contradictory data vis à vis Wnt and its role in promoting HSC regulation; it is clear that the role of Wnt pathway in hematopoiesis is complex and will require ongoing and careful exploration. Indeed, Luis et al. [36] recently reported that different levels of Wnt activation led to different outcomes with respect to HSC regulation. Specifically, self-renewal required only limited activation of Wnt signaling, while hematopoietic differentiation resulted from intermediate levels; once levels exceeded those associated with physiologic activation, both self-renewal and differentiation were impaired.

Smad-mediated signaling is another important pathway, which regulates hematopoiesis. Ligands associated with this pathway include those of the transforming growth factor-β (TGF-β) family, which includes TGF-β and bone morphogenetic proteins (BMPs) among other factors. TGF-β is a potent inhibitor of HSC growth and is considered to be an important regulator of HSC quiescence in vivo [37]. TGF-β-related inhibition is probably related to altered levels of cytokine receptor expression on HSCs together with the upregulation of cell cycle inhibitors, including p21, p27, and p57 [38–40]. By contrast, BMP-4 promoted self-renewal of cultured HSCs in vitro, while diminished levels of BMP-4 levels facilitated their differentiation [41].

Fibroblast growth factor (FGF) signaling has also been implicated in the regulation of HSC development and function. Both FGF-1 and FGF-2 support long-term culture (LTC) and the repopulation potential of HSCs identified in unfractionated BM cells; however, these factors were ineffective in experiments performed with $Lin^{-}Sca\text{-}1^{+}c\text{-}Kit^{+}$HSCs [42]. Deletion of FGF receptor 1 (*Fgfr1*) had no apparent impact on steady-state hematopoiesis; however, recovery was impaired in these mice in response to BM injury with 5-fluorouracil (5FU) [43]. This research group also reported that deletion of *Fgf-2* also had no impact on steady-state hematopoiesis, although this factor proved to be essential for HSC/HPC proliferation and recovery via its capacity to induce the expansion of stromal cells, increase

the production of SDF-1, and suppress the expression of CXCL12 in BM [44]. Likewise, FGF-mediated signaling was essential to suppress BMP activity in the AGM region during embryogenesis to establish an HSC niche; these actions were mediated via activation of BMP antagonists noggin2 and germlin1a [45]. Taken together, current findings suggest that FGF regulates hematopoiesis and HSCs indirectly via its role in supporting BM stromal cells.

Regulation of hematopoiesis by the insulin-like growth factor (IGF) pathway has also been explored; however, current findings are contradictory in nature. For example, while some studies revealed that IGF-1 functioned as a "silent killer" of pluripotent stem cells upon prolonged exposure [46], others reported that IGF-1 supports the osteoblastic niche and leads to improved levels of long-term HSC engraftment [47]. Moreover, IGF-binding protein 2 (IGFBP2) was described as an important factor serving to promote HSC survival [48].

The involvement of all these pathways provides redundancy in the process of HSCs self-renewal, probably ensuring that if one pathway has problems, other pathways could compensate/cover up the deficiency in order to maintain lifelong normal hematopoiesis.

3.3.1.2 Asymmetric Division

Asymmetric division results in two daughter cells that are not physically, molecularly, and/or functionally identical. The fact that all mature blood cells originate from HSCs with a single phenotype led to the assumption that both HSCs and HPCs were capable of asymmetric division. This hypothesis was confirmed by the discovery of four distinct segregating proteins, including CD53, CD62L/L-selectin, CD63/lamp-3, and CD71/transferrin receptor, and their roles during mitosis of in vitro cultured $CD34^{+}CD133^{+}$ HSCs/HPCs [49]. Furthermore, HSCs (c-kit^{+}Sca-$1^{+}$$Lin^{-/lo}$ $CD34^{-}$) isolated from transgenic Notch reporter mice (wherein green fluorescent protein is highly expressed in putative HSCs and undergoes downregulation as the cells which begin to differentiate) were capable of both symmetric and asymmetric division [50].

In this context, a first-level asymmetric division occurs when HSCs choose to undergo division into two daughter cells; one of the daughter cells serves to maintain the pool of undifferentiated HSCs and the other generates a progenitor cell that is no longer capable of self-renewal and that has initiated the differentiation process (i.e., an HPC). Given that HSCs have the capacity to generate all hematopoietic lineages, other differentiated progenitors will result from differential activation by cytokines or growth factors (as will be discussed later in this chapter); these observations contribute to the second level of asymmetric division. The differentiating daughter cells will continue to grow and to undergo additional asymmetric divisions so as to generate single-potential progenitor cells; these progenitors then divide symmetrically to generate the appropriate blood cell lineage.

3.3.1.3 HSC Heterogeneity

HSCs were the first stem cells to be isolated and characterized; they were initially considered to be a homogeneous population of cells, a perception that persisted for many years. However, due to recent technological advances, including functional assays, immunophenotyping, and genetics, this perception has changed. The HSC pool is now known to be heterogeneous. Interestingly, differences reported with respect to their capacity for in vivo repopulation and transplantation were largely due to the properties of distinct HSC subfractions; among these differences, the distinct HSC subfractions can promote differences in reconstitution kinetics, duration of repopulation, differentiation potential, cell cycle status, and the capacity for self-renewal [51–53]. As but one example, use of a flow-assisted cell sorting technique revealed differential expression of phenotypic markers associated with the signaling lymphocyte activation molecule (SLAM) family, including CD150, CD48, CD229, and CD244, in what was previously assumed to be a highly purified, homogeneous pool of $Kit^{+}Sca^{+}lin^{-}$ HSCs, also known as KSL cells. These findings led to further subdivision of what was then understood to be a heterogeneous population of KSL cells into more homogeneous HSC and HPC populations with different capacities for self-renewal and repopulation [54]. An improved understanding of HSC heterogeneity will promote the discovery of specific markers for appropriate subfractionation of HSCs; this will facilitate an improved understanding of their localization within distinct BM niches and will likewise improve the accuracy of current fate mapping and lineage-tracing approaches.

Potential Factors Contributing to HSC Heterogeneity [55]:

- Differences with respect to embryonic origin: During early embryonic development, both pre-HSCs and HSCs originate from distinct mesodermal and/or endothelial cells detected within sites associated with primitive hematopoiesis.
- Different developmental signals: Different inductive signals could be generated at unique embryonic sites, including the yolk sac, AGM, liver, or developing placenta. Cells may respond to different signals encountered during HSC migration between the multiple embryonic sites and/or from within the circulation.
- Intrinsic factors: In the absence of external stimuli, HSCs may have the capacity to control their lineage commitment and heterogeneity by upregulating or downregulating individual or groups of genes and/or receptors, thereby facilitating differential responses to external stimuli.
- Microenvironmental and extrinsic factors: HSCs and their progenitors are detected in distinct locations within the adult BM; each location may be capable of activating HSCs in a different fashion, depending on the signals, factors, and stromal cell types present within the tissues.

3.3.1.4 Plasticity

Plasticity is a critical feature that defines the nature of HSCs; this term implies that a stem cell can transcend its lineage boundary and give rise to different cells and tissues. The past

four decades witnessed many reports of the capacity of HSCs to differentiate into cell types typically associated with other tissues, including those that are not only mesodermal but also ectodermal and endodermal in origin; these cells include the muscle, heart, brain, and liver. As such, HSCs were perceived as a feasible, ethical, and promising source of raw material, which might be used to develop cell-based therapies for various diseases [56–59]. However, the limits of HSC-associated plasticity have recently been challenged. For example, many of these studies featured cells that were not pure populations of HSCs but a mix of different cells, also, many of these studies focused only on phenotypic markers and did not include functional analyses or in vivo tracking of these cells or their progeny [60, 61]. Thus, controversies remain as to whether or not HSCs possess this profound degree of flexibility.

However, clearly, HSCs maintain intra-hematopoietic and/or hematopoietic lineage plasticity; in other words, it is clear that committed hematopoietic cells are able to be reprogrammed to facilitate production of blood cells from another lineage. As an example of this phenomenon, overexpression of the GATA-1 transcription factor in murine myeloid leukemia cells led to their transformation into erythroid and megakaryocyte-like cells; this is largely understood as proof of myeloid–erythroid plasticity [61], together with various other similar examples [62]. Lineage plasticity may also contribute to HSC heterogeneity as discussed in Sect. 3.3.1.3.

3.3.1.5 Migration

As discussed in an earlier section, HSCs migrate from one anatomical site to another during embryogenesis until ultimately reaching sites of adult hematopoiesis; well-regulated and active hematopoiesis was maintained at each site. In mammals, HSCs first appear in the yolk sac and then migrate to the AGM region before reaching the fetal liver; as a final step, these cells take up residence in the BM. Other species feature alternative sites of lifelong active hematopoiesis; while adult hematopoiesis takes place in the long bones and the spleen of mice [63], this process takes place in the liver in frogs [64], and in the kidneys of zebrafish [65].

Even after the HSCs reach sites that maintain adult hematopoiesis, some HSCs and HPCs undergo constant migration from this niche into peripheral circulation and back. Interestingly, peripheral blood and lymph both contain twice as many HSCs/HPCs early in the morning when compared to later hours at night; these results suggest that their release is governed by a circadian rhythm [66–68]. In addition, more circulating HSCs/HPCs were identified during intense exercise [69], and secondary to acute myocardial infarction-induced inflammation [70] and among patients with cardiovascular disease [71].

Several approaches have been used successfully to induce this migratory behavior in vivo. For example, CXCR4 receptor blockade with the selective agent, AMD 3100, led to deactivation of signaling mediated by CXCL12 (also known as stromal cell-derived factor-1 or SDF-1). This blockade promoted mobilization of HSCs and HPCs from their BM niches and ultimately their release into the circulation [72]. Granulocyte colony-stimulating factor (G-CSF) also mobilizes HSCs and HPCs from their BM niche via various means

[73], including activation of c-kit/kit ligand (also known as SCF) and counteracting the impact of very late antigen-4 (VLA-4, also known as α4β1 integrin) and its ligand vascular cell adhesion molecule-1 (VCAM-1). G-CSF also counteracts signaling via the CXCL12/CXCR4 axis; it serves to suppress osteoblast maturation and expression of CXCL12, leading to a state wherein HSC quiescence is maintained in the BM niche [73]. Furthermore, hypoxia was also implicated in this process; a gradient of hypoxia-inducible factor-1 (HIF-1) promoted the upregulation of CXCL12 (SDF-1) expression and the migration and homing of HSCs/HPCs into ischemic tissues [74]. Accordingly, mobilization of HSCs/HPCs has been targeted clinically using CXCR4 antagonists, G-CSF, or erythropoietin to generate as much as a 100-fold increased yield of HSCs and HPCs from peripheral circulation to improve stem cell transplantation outcomes in clinical practice [75]. It is thus clear that "quiescent" HSCs actively migrate and return to their original niches; this raises the question as to whether HSCs "choose" their niche and/or whether their niche attracts and calls to them. This question calls for further investigation.

3.3.2 Other Sources of HSCs

As HSCs have extensive migratory potential, it was plausible to consider the possibility that they might reside outside their BM niches. Indeed, HSCs and HPCs are found in both peripheral blood (PB) and umbilical cord blood (UCB) as rare populations of cells (typically 1:100,000 when defined as $CD34^+$ $CD38^-$ $CD45RA^-$ $CD90^+$ $CD49f^+$ $Rhodamine^{lo}$) that are capable of colony formation in vitro and long-term repopulation in vivo [76, 77].

One of the earliest clues regarding the presence of HSCs and HPCs in the peripheral circulation was revealed from an experiment carried out in 1965. In this study, mice tails were shielded during whole body irradiation and the spleen was recolonized by hematopoietic cells from the tail [78]. Several subsequent studies reported successful hematopoietic recovery in response to administration of hematopoietic cells from PB in baboons, dogs, and humans [79–83]. As discussed earlier, mobilization of HSCs into the peripheral circulation is now an approved clinical practice and is used to increase the yield of HSCs for subsequent transplantation.

Another important source of HSCs is UCB. The first description of the existence of HSCs at this site was in 1978 in a study that reported that myeloid forming colonies could be generated in vitro from cultured UCB cells [84]. However, important differences were reported that distinguished HSCs/HPCs isolated from UCB from those characterized in BM. Among these differences, UCB HSCs ($CD34^+CD38^-$) responded more effectively to hematopoietic cytokines and generated seven times as many progeny cells as did BM HSCs [85].

3.3.3 Characterization of HSCs

Characterization of HSCs depends on the expression of various cell surface markers, in addition to functional assays (in vitro and in vivo).

3.3.3.1 Cell Surface Antigen Markers

Cell surface antigens are widely used to characterize various hematopoietic cells; however these markers are not exclusive for HSCs or HPCs, as some are normally expressed on other cells of the body. However, a combination of certain markers is essential for the isolation of relatively pure HSCs population, commonly used are $CD117^{high}Sca\text{-}1^{+}Lin^{-/low}CD90^{low/-}$ [86], $Lin^{-}CD34^{+}CD38^{-}Rhodamine^{low}$ (77), or $Sca\text{-}1^{+}Lin^{-}CD117^{+}CD34^{-/+}$ [51]. Lineage (Lin) negative cells are hematopoietic cells that do not express any of mature blood cells' markers; such as, CD3, CD11b, CD45R, Gr-1 (Ly6G), or Ter119 (Ly76). Table 3.1 shows cell surface markers known to be expressed on HSCs and various progenitors.

3.3.3.2 In Vivo Assays for the Evaluation of Hematopoietic Stem and Progenitor Cells

Establishing assays to identify the different populations of progenitor cells of HSCs progressed from stem cells to their downstream functional cells is a major challenge. It has been found that the quantitative measurement of the potential of multipotent HSCs to proliferate could be measured by in vivo colony-forming units assay for spleen (CFU-s), which was first established by Till and McCulloch. They used this in vivo functional assay for further studying of the macrophages, granulocytes, erythroid cells, and megakaryocytes found in the spleen of irradiated animals in order to know which primitive progenitor cells in mouse BM has the ability to form them [97]. Interestingly, it has been shown that multiple CFU-s cells can be formed from one CFU-s cell indicating that CFU-s shows a high level of self-renewal [98]. The multilineage property of CFU-s made it to be identified as the most primitive HSCs along many years. Furthermore, CFU-s cells have shown a possession of different capacities of self-renewal that can form collectively a heterogeneous cell population. This can be reflected through studying CFU-s-8, that can form the eighth day's colonies in the studied irradiated spleen, and CFU-s-12, that can form the twelfth day's colonies in the studied irradiated spleen, which in turn shows a higher primitivity of CFU-s-12 than CFU-s-8 [98].

The competitive repopulation assay is considered the gold standard for the quantitative measurement of HSCs activity [99] as shown in Fig. 3.2. In this assay, the number of stem cells is expressed as competitive repopulating units (CRUs) and has been measured through comparing the repopulation activity of HSCs from unknown source against other HSCs with known number. The limiting dilution competitive repopulation assay (LDCRA) allows for higher accuracy in determining the CRU frequency or HSCs frequency [100] in addition to the ability of limiting dilution of HSC transplantation after the transplantation of small numbers of HSCs into marrow-ablated recipient mice for higher

Table 3.1 Cell surface markers known to be expressed on HSCs and various progenitors

Cell type markers	Human LT-HSCs	Human ST-HSCs	MPP	CMP	CLP	MEP	GMP	CFU-Mk	BFU-E	Megakaryocyte
Lin	−	−	−	−	−	−	−	−	−	
CD34	−	+	+	+	+	+	+	+	+	+
CD38	−	−	−	+	+	+	+	−	+	−/+
CD90 (Thy-1)	+	+	−		−					
CD45RA	−	−	−	−	+	−	−	−	−	
c-kit (CD117)	+	+	+	+	Low	+	+			
Sca-1 (Ly6A)	+	+	+	−	Low	−	−			
Flk2 (CD135)	−	−	+		+					
CD49f	+		−							
IL-3Ra (CD123)				Low/+		−	+			
CD10					+					
CD127(IL7Ra)					+					
Flt3	−	−/+								
CD201	+									
CD150 (Slamf1)	+		−							
CD48	−		−							
FcyRII/III				Low		−	+			
CD41										+
CD42b										+
MPL (CD110)										+
References	[87]	[88]	[87, 89]	[90, 91]	[92]	[90, 91]	[90, 91]	[93, 94]	[95, 96]	[93]

Long-term HSCs (LT-HSCs), Short-term HSCs (ST-HSCs), Multipotent Progenitor (MPP), Common Myeloid Progenitor (CMP), Common Lymphoid Progenitor (CLP), Megakaryocyte–Erythroid Progenitor (MEP), Granulocyte–Macrophage Progenitor (GMP), Colony-Forming Unit–Megakaryocyte (CFU-Mk), and Burst-Forming Unit–Erythroid (BFU-E). Markers accompanied with "+" or "–" symbol indicate that the specific cell type expresses (+) or lacks (−) this marker. In some cases, markers are accompanied with "high" or "low," indicating different degrees of expression. Empty cells indicate that there is no information in the literature relevant to this marker

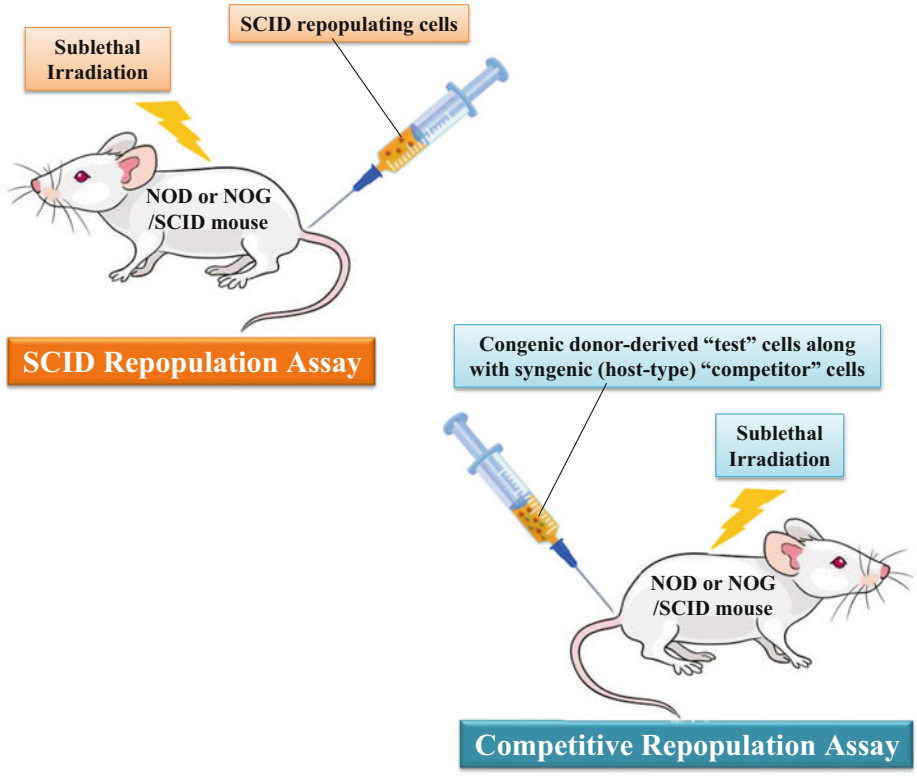

Fig. 3.2 Functional characterization of HSCs. Xenotransplantation is used to detect repopulating SCID cells using non-obese diabetes/severe combined immunodeficiency (NOD/SCID) or NOD with common gamma receptor deficiency (NOG/SCID) mice subjected to sublethal radiation. The competitive repopulation assay included congenic donor-derived test cells that were expected to contain HSCs along with synergic (host-type) competitor cells that were both transplanted in mice subjected to sublethal irradiation

sensitivity in the measurement. In this competitor assay, single-hit Poisson distribution is used for the estimation of HSCs frequency which obtained through making dilution series of HSCs of unknown source and comparing it against a defined number of BM cells, then in each cell dose, the number of negative mice which cannot make HSCs repopulation is measured [101].

3.3.3.3 In Vitro Assays for the Evaluation of Hematopoietic Stem and Progenitor Cells

Colony-Forming Unit Assays

In this assay, the number and types of mature cells identified on the basis of morphological and phenotypic criteria are used to classify and count the colonies derived from progenitor

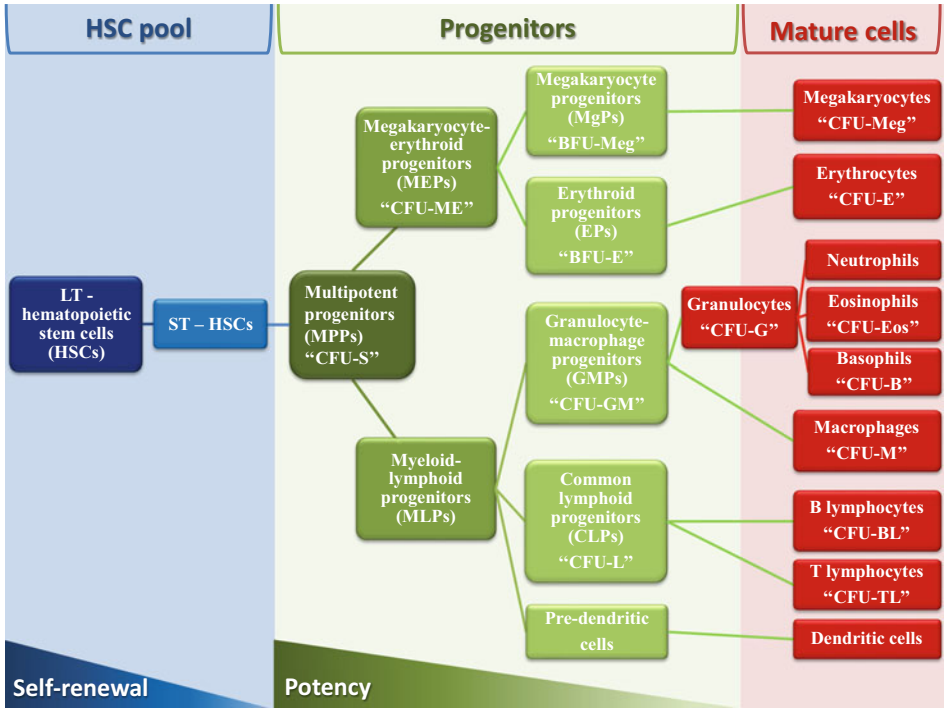

Fig. 3.3 The hematopoietic hierarchy tree, showing the classical hematopoiesis hierarchy and specific clonal analysis for each cell type

cells. Multipotential and lineage-restricted progenitors of the erythroid, granulocytic, and macrophage lineages are detected most frequently by colony-forming unit (CFU) assays (Fig. 3.3); megakaryocyte and B lymphoid progenitors can be identified under selective culture conditions. Most of CFUs detected in BM, blood, and other tissues are progenitors with restricted capacity for self-renewal and hematopoietic repopulating potential in vivo [102].

Long-Term Cultures

LTC assays are used to detect and enumerate HPCs and permit more accurate assessment of HSCs self-renewal than CFU assays. These assays were initially established for primitive progenitors of myeloid (i.e., granulocyte, macrophage, erythroid, and megakaryocyte) lineages [103, 104]. They were later modified to support the growth of B lymphoid and NK cell progenitors [105, 106]. Hematopoietic cells are cultured on an adherent monolayer of primary stromal cells or on immortalized stromal cell lines. Specialized culture media are used to sustain functions, including survival, self-renewal, proliferation, and differentiation of long-term repopulating HSCs for a period of several weeks [107, 108]. The cells identified in LTC assays are recognized as LTC-initiating cells (LTC-ICs); these cells have

the capacity to produce differentiated CFUs in these stroma-supported cultures for at least 5 weeks (>4 weeks for mouse cells). This design guarantees that any CFUs that existed in the initial cell sample develop into terminally differentiated progeny [107].

3.4 Regulation of Hematopoietic Stem Cells

It is essential to maintain the HSCs pool in order to have the capacity to replenish the circulation with mature blood cells throughout life. As such, it is critical to maintain a fine balance between self-renewal and differentiation.

3.4.1 Regulatory Molecules and the HSC Niche

HSCs are regulated by both intrinsic and extrinsic factors; both types of factors create specific microenvironmental niches wherein HSCs grow and develop [109]. For example, stem cell leukemia (SCL) is a transcription factor, which plays a critical role in regulating HSC quiescence, survival, and self-renewal [110]. SCL is involved in controlling long-term competence of HSCs and their G_0–G_1 transition via direct regulation of the expression of Id1 and the cell cycle regulator or Cdkn1a; both of these factors contribute to HSC quiescence [111]. Cyclins and cyclin-dependent kinases are also involved in the regulation of HSCs; for example, cyclin-dependent kinase (CDK)6 regulates the timing of HSC exit from the quiescent state. Self-renewing long-term HSCs (LT-HSCs) do not express CDK6, while non-renewing short-term HSCs (ST-HSCs) express high levels of CDK6, which facilitates their rapid entry into the cell cycle in response to mitogenic stimulation. Enforced expression of CDK6 in LT-HSCs forces their exit from the quiescent state [112]. Musashi-2 (Msi2) is another regulatory protein that plays a key role in regulating HSC quiescence and in maintaining the balance between symmetric and asymmetric divisions and the capacity for self-renewal required to maintain normal hematopoiesis [113]. Moreover, telomerase is expressed at low levels in HSCs isolated from adult BM; levels of this enzyme increase once HSCs begin to differentiate and proliferate [114].

Epigenetic regulation constitutes another important regulatory feature. Treatment of HSCs with different chromatin-modifying agents resulted in either maintenance (i.e., in response to valproic acid) or expansion (i.e., in response to trichostatin A and 5-aza-2′-deoxycytidine) [115]. Moreover, Bmi-1, a member of the polycomb protein group that promotes transcriptional suppression via histone modifications and chromatin remodeling, was identified as a crucial epigenetic determinant for maintaining the capacity for HSC self-renewal [116]. Knockdown of Bmi-1 had no impact on the development of the embryonic hematopoietic system but served to reduce the capacity for self-renewal among HSCs and their long-term repopulation capacity, leading to postnatal pancytopenia [117].

The HSC niche is the environment surrounding the cells; the HSC niche provides major contributions to their external regulation. This niche has many different cell types, including stromal cells (e.g., mesenchymal stromal cells, osteoblasts, fibroblasts, adipocytes, and endothelium) and supporting cells (e.g., lymphocytes, macrophages, and neurons); other components include the extracellular matrix, cytokines, and growth factors. The niche thus provides suitable conditions that support maintenance and differentiation of HSCs. It was recently reported that HSCs may be located in endosteal, perivascular, and vascular niches in the BM microenvironment [118]. Stromal cell-derived factor-1 (SDF-1 or CXCL12) is an important example of a stromal factor produced in the adult marrow by osteoblasts, endothelium, and other perivascular stromal cells that have proved to be essential vis à vis HSC viability and migration [119]. It has recently become clear that CXCL12-CXCR4 signaling is a critical feature underlying migration of HSCs and HSPCs into the BM [68, 120]; disruptions in this signaling pathway lead to HSC mobilization and depletion from the BM [72]. Other important extrinsic factors that support HSC maintenance and expansion include SCF, thrombopoietin, angiopoietin-1, angiopoietin-like proteins, IGF-2, and fibroblast growth factor-1 [121, 122]. Furthermore, in addition to inflammatory mediators, the ambient oxygen level and signals from the central nervous system also contribute to the regulation of HSC fate.

3.4.2 Role of Inflammation

Inflammation leads to increased numbers of blood cells in the peripheral circulation, especially leukocytes; this is directly related to BM output. Inflammatory mediators and cytokines act directly within the BM microenvironment to dictate the fate of HSCs and their progeny. Therefore, it should not be surprising that inflammation plays an important role in regulating hematopoiesis. Of the vast array of inflammatory cytokines, interferons (IFNs), interleukins (ILs), tumor necrosis factor (TNF), and Toll-like receptor ligands (TLR ligands) are among the most prominent of the factors that regulate HSC self-renewal, differentiation, and repopulation potential [123]. Table 3.2 includes a list of different cytokines and their impact on HSC self-renewal and differentiation.

HSCs (phenotypically identified as $Kit^{+}Sca^{+}lin^{-}CD150^{+}CD48^{-}$) escaped quiescence in response to IFNα administration; these cells actively entered the cell cycle and began to proliferate. This response was achieved via the upregulation of both signal transducer and activator of transcription 1 (STAT1, a transcription factor) and stem cell antigen-1 (Sca-1, a cell surface protein). Moreover, HSCs devoid of Sca-1, STAT1, or IFNα receptor were unresponsive to IFNα stimulation [135]. Furthermore, loss of interferon regulatory factor 2 (IRF2, a transcriptional repressor of IFNα) led to a larger fraction of cycling/proliferating HSCs with reduced potential for repopulation; the latter was restored after the IFNα receptor was disabled [136]. IFNγ is typically produced in response to chronic infection; this pro-inflammatory mediator has also been reported to promote an increase in in vivo

Table 3.2 Impact of various cytokines on maintaining quiescence and/or capacity for self-renewal of HSCs

Hematopoietic cytokine	Function on HSCs	References
Stem cell factor (SCF, steel factor, mast growth factor or kit ligand)	Maintains and stimulates self-renewal	[124]
Thrombopoietin (TPO)	Maintains and stimulates self-renewal	[125]
Chemokine receptor type 4 (CXCR4)	Self-renewal inhibition and quiescence induction	[126]
Granulocyte-colony stimulating factor (G-CSF)	Quiescence induction and stimulation	[127, 128]
Angipoietin-1 (Ang-1)	Self-renewal induction	[129, 130]
Interleukin-3 (IL-3)	Self-renewal and survival maintenance	[131, 132]
Interleukin-6 (IL-6)	Enhances the proliferation and differentiation	[133]
Fms-related receptor tyrosine kinase 3 ligand (Flt3 ligand)	Stimulates the proliferation and differentiation of HSCs	[134]

proliferation and also the repopulation potential of LT-HSCs ($Kit^+Sca^+lin^-CD150^+$) via activation of IFNγ receptor 1 and STAT1 [137].

Various cytokines and interleukins serve to regulate hematopoiesis [138] by acting on HSCs and other hematopoietic progenitors. In an attempt to determine the most important mediators that promote self-renewal of putative BM HSCs ($CD34^+CD38^-$), 16 cytokines were tested alone or in combinations, including IL-1, IL-3, IL-6, IL-7, IL-11, IL-12, TNFα, Flt3 ligand (FL), thrombopoietin (TPO), erythropoietin, G-CSF, GM-CSF, SCF, macrophage inflammatory protein lα (MIP-lα), nerve growth factor β (NGF-β), and leukemia inhibitory factor (LIF) [124]. IL-3, SCF, and FL all served to increase the capacity for self-renewal among putative HSCs when each was used alone (the most effective was FL); the combination of three factors was even more effective. After stimulation of HSC differentiation by TPO, IL-3 was the most effective in this role when used alone or in combination with SCF, FL, and either IL-6, G-CSF, or NGF-β. TNFα had a negative impact on the capacity of HSCs to undergo self-renewal [124].

BM HSCs ($Kit^+Sca^+lin^-Flk2^-$ or $Kit^+Sca^+lin^-IL7Ra^-$) express the pattern recognition receptors, Toll-like receptors 2 and 4. In vitro activation by their respective ligands (Pam3CSK4 and lipopolysaccharide [LPS], respectively) led to activation of the MyD88 downstream intracellular adapter protein; this ultimately led to myeloid expansion [139]. Moreover, repeated in vivo administration of small doses of LPS resulted in TLR4 activation and defective self-renewal and repopulation potential of HSCs [140]. $CD34^+$ HSCs/HPCs isolated from human BM expressed TLR4, TLR7, TLR8, and TLR9 [141], and human UCB cells expressed TLR1, TLR2, TLR3, TLR4, and TLR6 [142]. The activation of these TLRs on isolated progenitor cells promoted myeloid differentiation.

G-CSF is essential for normal granulopoiesis and functions via stimulation of the common myeloid progenitors. The absence of G-CSF limited the repopulation potential of BM cells and reduced their contributions to the myeloid lineage [143]. By contrast, enhanced G-CSF signaling promoted by a mutant G-CSF receptor was associated with higher levels of HSC proliferation via upregulation of the transcription factor, STAT5 [144]. Administration of G-CSF also led to an increased number of HSC ($Kit^+Sca^+lin^-CD34^-Flk2^-CD41^-$or $Kit^+Sca^+lin^-CD150^+CD48^-CD41^-$ cells) both in the circulation and in the BM, although it resulted in a reduced potential for repopulation. These effects were achieved via activating both TLR and G-CSF receptors; as noted earlier, TLR2, TLR4, and MyD88 signal adapter contribute to HSC expansion, loss of repopulation activity, and quiescence [145].

The role of TNF with respect to the regulation of HSCs is complex and not yet well-understood. In vitro, the administration of TNFα resulted in decreased proliferation and repopulation potential of putative HSCs ($CD34^+CD38^{-/low}$); these findings resulted from the activation of the p55 TNF receptor [146]. In contrast, in vivo findings remain somewhat contradictory. Interestingly, deletion of two TNF receptors (Tnfrsf1a and Tnfrsf1b, also known as p55 and p75, respectively) resulted in no changes in the numbers of HSCs ($Kit^+Sca^+lin^-Flk2^-$) but yielded improved long-term repopulation potential [147]. In contrast, older mice devoid of the Tnfrsf1aor p55 receptor (but not of Tnfrsf1b or p75) showed increased numbers of erythroid and myeloid progenitors and a four-fold reduction in the repopulation potential of HSCs [148]. As such, the complex pleiotropic functions of TNF and its role in host immunity might be extended to the regulation of HSCs as well.

In addition to the direct effects of these inflammatory mediators, many of them have an indirect impact on HSC regulation via actions targeting the BM environment. G-CSF acts indirectly on HSCs by suppressing CXCL12 expression in BM niche stromal cells; this leads to mobilization of HSCs into the circulation [149]. Likewise, TLR-mediated activation of freshly isolated BM $CD34^+$ progenitors in vitro via ligands including immune-stimulating siRNAs or the TLR7/8 ligand R848 led to the production of many cytokines (IL1-β, IL-6, IL8, TNFα, GM-CSF) and induced myeloid differentiation [141]. This differentiation pathway may be promoted by indirect means, via the actions of newly released cytokines in coordination with direct TLR immune-mediated signaling.

Finally, the duration of exposure to inflammatory mediators and the chronicity of the associated inflammatory pathology should also be considered. Short-term inflammatory signals may be beneficial with respect to activating hematopoiesis; however, chronic inflammation can exhaust the BM and the HSC pool [135]. Prolonged inflammation may thus result in BM failure [150] and potentially malignant transformation [151]. Compelling new evidence suggests that HSCs can escape inflammatory exhaustion by re-establishing quiescence [152]. In the case of IFNs, this response involves the transcription factor IRF2 [136] and immunity-related GTPase family M protein-1 or Irgm-1 [153]; however, the full mechanisms underlying this response have yet to be identified.

3.4.3 Role of Oxygen/Hypoxia

Oxygen tension has been recently proposed as a regulator of HSCs and HPCs; the BM niche wherein HSCs and HPCs reside has been described as hypoxic [154, 155]. Recent studies revealed that HIF-1α, a factor that undergoes upregulation in response to hypoxic conditions, promotes the differential expression of cell proliferation and survival genes; these include IGF, cathepsin D, matrix metalloproteinase-2, urokinase plasminogen activator receptor, fibronectin-1, cytokeratin (CK)-14, CK-18, CK-19, vimentin, transforming growth factor α [156, 157], vascular endothelial growth factor (VEGF) [158], and erythropoietin [159]. Administration of G-CSF resulted in stabilization of HIF-1α and increased production of VEGF in the BM [160]. HIF-1α resulted in increased levels of CXCL12 [74] and elevated levels of CXCR4 receptor expression [161]; it also protects HSCs/HPCs from damage caused by overproduction of mitochondrial reactive oxygen species [162].

3.4.4 Role of the Nervous System

The BM environment is heavily enriched with neuronal connections; as such, it has long been proposed that the nervous system may also contribute to the regulation of the HSC niche and likewise of hematopoiesis. Several β_2-adrenergic signals were found to be essential for G-CSF-induced mobilization of HSCs and HPCs; blockade of these signals by 6-hydroxydopamine (i.e., via chemical sympathectomy) or by β-blockers such as propranolol served to reduce G-CSF-induced HSC mobilization [68]. Neurotransmitters such as norepinephrine also regulate hematopoietic cell migration via activation of Wnt signaling in $CD34^+$ cells, by increasing Sca-1^+c-Kit^+Lin^- HSC mobilization [163], and by increasing the expression of both CXCR4 and VCAM-1 [164].

3.4.5 Role of Apoptosis

Apoptosis plays an important role in promoting homeostasis. B-cell lymphoma 2 (BCL-2), an anti-apoptotic protein, was overexpressed in an IL-3-dependent hematopoietic progenitor cell line, the murine hematopoietic nonleukemic factor-dependent cell Paterson (FDCP)-Mix. The transfected FDCP-Mix cells could be maintained in in vitro culture without the need for additional IL-3; cells that had not undergone transfection died via apoptosis in the absence of exogenous IL-3 [165]. Similar in vivo approach using BCL-2-overexpressing transgenic mice revealed 2.4 times more HSCs in the BM when compared to HSCs/HPCs from wild-type mice. Furthermore, the HSCs from BCL-2-overexpressing transgenic mice experienced superior in vitro survival and similar in vivo engrafting potential [131]; they were also capable of survival in response to lethal irradiation [166]. Both the in vitro and in vivo approaches suggested a role for apoptosis in regulating the survival of HSCs/HPCs, although conclusive evidence is still needed.

3.5 The Hematopoietic Hierarchy

The differentiation of HSCs to mature myeloid and lymphoid cells occurs in a stepwise fashion beginning with multipotent, oligopotent, and bipotent cells and ending with fully differentiated cells; this pathway forms the classical hierarchical tree of hematopoiesis [167]. LT-HSCs are at the top of this hierarchy and represent a very small percent (up to 0.2%) of the entire BM cell pool [168], HSCs gradually lose their capacity for self-renewal (ST-HSCs) and become more and more restricted with respect to their differentiation potential. This tree eventually ends with functionally mature blood cells, as shown in Fig. 3.3.

However, current thinking suggests that the hematopoietic system developed in association with mammalian evolution; as such, it will be difficult to constrict our current understanding within the classical organization or hierarchical framework. Moreover, the fact that self-renewing HSCs along with other committed progenitors comprise a large part of the hematopoietic cell pool defies the idea of a simple hematopoietic hierarchy. Importantly, recent evidence suggests that several committed single-lineage progenitors were derived directly from multipotent HSCs; these observations highlight the fact that HSCs have the capacity to produce blood cells in a flexible yet efficient manner [169, 170]. In newer hierarchical models, HSCs do not remain at the top of the hierarchy, but play an overall more dynamic roles toward the goal of supporting normal lifelong hematopoiesis [171].

3.6 Epigenetic Control Over HSCs

Epigenetics does not only play an important role during early development, but is also essential for tissue homeostasis. The self-renewal or differentiation of HSCs depends on different gene expression patterns, which are, in part, the result of epigenetic changes that expose or conceal different genomic regions. Consequently, different chromatin-modifying proteins, such as Polycomb-group (PcG) and Trithorax-group (TrxG) proteins, were recently considered critical epigenetic regulators of HSC self-renewal and differentiation. Of the PcGgroup, Polycomb complex protein 1 (Bmi-1) [116], Enhancer of zeste homolog 1 (Ezh1) [172] and Ezh2 [173] were shown to promote self-renewal of HSCs by suppressing cell cycle inhibitors; and thus preventing cell cycle arrest, senescence, and apoptosis. While Chromobox protein homolog 7 (Cbx7) [174] maintained self-renewal via suppressing the expression of lineage-specific genes. Of the TrxG proteins, Mixed Lineage Leukemia (MLL or Histone-lysine N-methyltransferase 2A) was essential for HSC self-renewal and repopulation potential [175], and SET domain-containing protein 1A (SET1A or Histone-lysine N-methyltransferase SETD1A) was shown to protect HSC self-renewal during stress conditions via activating DNA damage recognition and repair pathways [176]. In addition, marked epigenetic differences were found in aged HSCs contributing

to their lower differentiation and repopulation potential [177]. Epigenetic modifiers that play an important role in HSCs self-renewal or differentiation are described in Table 3.3.

The applicability of epigenetics was achieved by altering the chromatin structure of in vitro cultured HSCs. A mixture of 5-aza-2′-deoxycytidine (5aza, DNA methyltransferases inhibitor) and trichostatin A (TSA, histone deacetylase inhibitor) led to increasing putative BM-HSCs ($CD34^+$) self-renewal and repopulation potential [191]. In addition, valproic acid (histone deacetylase inhibitor) enhanced the expansion of in vitro cultured putative HSCs ($CD34^+$ cells) from BM, BP, or UCB [192].

3.7 Bone Marrow Transplantation (BMT)

The first experimental evidence of the stem cell theory was demonstrated by Ernest A. McCulloch and James E. Till when they performed BM transplantation into irradiated mice [97, 193]. Myeloid multilineage colonies were produced in the spleen of the transplanted mice from these cells where the number of injected cells being proportional to the number of colonies. The multilineage potential of single bone marrow cells (the so-called CFU-S, Colony-Forming Unit in the Spleen) was confirmed by such experiments [98]. Nevertheless, these cells are not identified as true stem cells with a multipotent potential and self-renewal capability, which in that case was limited. Henceforth, the first successful stem cell transplantation was performed by E. Donnall Thomas on identical human twins in 1957 [194]. After this transplantation, the long-term repopulation with the production of new blood cells was confirmed to be as a result of intravenous injection of bone marrow cells. Moreover, transplantations were performed on Yugoslavian nuclear workers (whose bone marrows were injured by irradiation) by the oncologist Georges Mathé [195] who also performed successful allogeneic bone marrow transplantation on a leukemic patient [196]. For more than 50 years, patients with blood-related disorders have been treated with such transplantations. Adult HSCs can now be exceedingly enhanced with a mixture of numerous surface markers. Transplantation protocols in the case of many blood-related diseases, such as leukemia, include different sources of HSCs such as bone marrow, cord blood, or mobilized peripheral HSCs. However, major obstacles include the low number of HSCs in these tissues. Furthermore, reproducing the reported in vitro conditions and permitting proficient HSC expansion without prompting cell differentiation are still very complicated [197].

Use of cord blood as a source of HSCs [198, 199] and new regimes which allowed haploidentical transplantation [200] further facilitated current therapeutic approaches while limiting the undesired consequence of graft-versus-host disease. These approaches are increasingly making the option of allogeneic transplantation available to patients who otherwise do not have a matched-related or volunteer-unrelated donor source of stem cells as shown in Fig. 3.4.

Table 3.3 Epigenetic modifiers that regulate HSCs self-renewal or differentiation

Protein [other names]	Gene	Effect on HSC	References
DNA (cytosine-5)-methyltransferase 1 [Dnmt1, DNA methyltransferase HsaI or DNA MTase HsaI]	*DNMT1*	Required for HSCs self-renewal, niche retention and progression from multipotent to myeloid progenitors. Deletion leads to pedigree skewing into myelopoiesis and defective self-renewal	[178, 179]
DNA (cytosine-5)-methyltransferase 3 (A and B) [Dnmt3a/b, DNA methyltransferase HsaIIIA/B or DNA MTase HsaIII A/B]	*DNMT3* (A and B)	Essential for HSCs self-renewal, Dnmt3a deletion increases HSCs life span	[180, 181]
Methylcytosine dioxygenase TET1 [Ten-eleven translocation 1 gene protein]	*TET* (1 and 2)	TET1 deficiency increases HSCs self-renewal potential TET2 deletion results in improving HSCs self-renewal and improving myelopoiesis	[182, 183]
Isocitrate dehydrogenase [NADP] cytoplasmic (IDH 1) or mitochondrial (IDH2) [Cytosolic or mitochondrial NADP-isocitrate dehydrogenase]	*IDH* (1 and 2)	Required for TET2 cofactors	[184]
Polycomb complex protein 1 (Bmi-1)	*BMI1*	Important for HSCs self-renewal	[116]
Histone-lysine N-methyltransferase EZH (1 and 2) [Enhancer of zeste homolog 1 and 2, Ezh1 and 2]	*EZH* (1 and 2)	Ezh1 important for HSCs self-renewal and prevents senescene Ezh2 preserves self-renewal and prevents exhasution of HSCs	[172, 173]
Chromobox protein homolog 7 [Cbx7]	*CBX7*	Imporatant for self-renewal of HSCs	[174]
Chromobox protein homolog 2, 4 and 8 [Cbx2 Cbx4 and Cbx8]	*CBX2*, *CBX4* and *CBX8*	Overexpression leads to differentiation and exhaustion of HSCs	[174]
Histone-lysine N-methyltransferase SETD1A (SET1A or SETD1A)	*SETD1A*	Protects HSCs self-renewal during stress	[176]
Histone-lysine N-methyltransferase 2A [Mixed Lineage Leukemia, MLL, MLL1 or Trithorax-like protein]	*KMT2A*	Essential for HSCs self-renewal and repopulation potential	[175, 185]

(continued)

Table 3.3 (continued)

Protein [other names]	Gene	Effect on HSC	References
Histone-lysine N-methyltransferase, H3 lysine-79 specific [DOT1-like protein, Histone H3-K79 methyltransferase]	*DOT1L*	Important for embryonic erythropoiesis and maintenance of adult populations of HSCs and HPCs	[186, 187]
Histone H2A deubiquitinase MYSM1 [Mysm1, 2A-DUB, MPN domain-containing protein 1]	*MYSM1*	Involved in HSCs quiescence and self-renewal	[188]
Histone-lysine N-methyltransferase SETDB1 [H3-K9-HMTase 4, ESET, SET domain bifurcated 1]	*SETDB1*	Important for HSCs function	[189]
Polycomb-group protein ASXL1	*ASXL1*	Associated with polycomb chromatin-binding protein Loss results in reduced self-renewal and impaired hematopoiesis	[190]

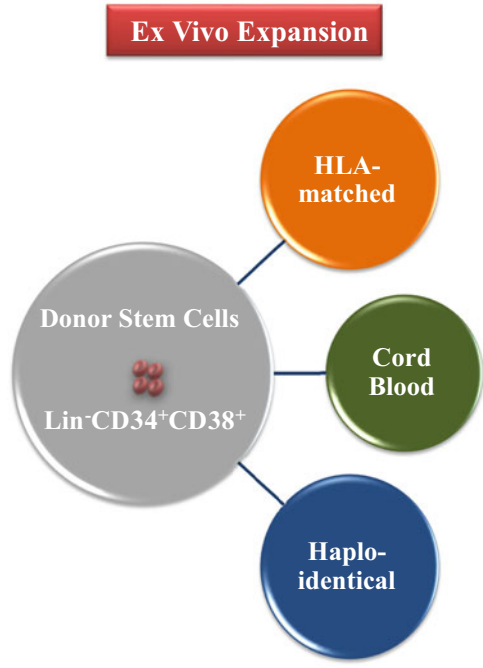

Fig. 3.4 Human HSC transplantation therapy. HLA-matched adult, cord blood or haploidentical adult donor stem and progenitor cells (usually $CD34^+$ enriched cells) are transplanted intravenously following conditioning therapy to permit engraftment of donor marrow into the recipient

3.7.1 Diseases Currently Treated by HSCs

3.7.1.1 Multiple Myeloma

According to the Center for International Blood and Marrow Transplant Research (CIBMTR), the majority of hematopoietic stem cell transplants are autologous. Overall survival and progression free survival were amplified in patients younger than 65 years old on a protocol of initial consolidation therapy with melphalan followed by autologous stem cell transplantation and lenalidomide maintenance therapy [201]. Administration of high-dose of melphalan plus stem cell transplantation demonstrated a favorable outcome compared with consolidation therapy with melphalan, prednisone, lenalidomide (MPR), and it also showed a better outcome in patients who received a maintenance therapy with lenalidomide.

3.7.1.2 Hodgkin and Non-Hodgkin Lymphoma

In cases of recurrent lymphomas (HL and NHL) that showed no response to initial conventional chemotherapy, using a protocol in which chemotherapy was followed by autologous SCT showed favorable outcome. Schmitz and colleagues demonstrated, in a randomized controlled trial, that a high-dose chemotherapy with autologous SCT resulted in better 3-year outcome compared to aggressive conventional chemotherapy in relapsed chemo-sensitive Hodgkin lymphoma [202]. However, there was not a significant difference between the two groups in overall survival. According to CIBMTR, the number of HSC transplant recipients comes second after multiple myeloma.

3.7.1.3 Acute Myeloid Leukemia (AML) and Myelodysplastic Syndrome (MDS)

In patients with AML who fail primary induction therapy and do not achieve complete response, allogeneic SCT could improve outcome and prolong overall survival [203]. The study recommended that early HLA typing for patients with AML could help if they fail induction therapy and are considered for BMT. Allogenic stem cell transplant is considered being curative in cases of disease progression and is only indicated in intermediate- or high-risk patients with MDS.

3.7.1.4 Acute Lymphocytic Leukemia (ALL)

Allogeneic SCT is indicated in refractory and resistant ALL cases when induction therapy fails for a second time in inducing remission. Some studies suggest an increased benefit of allogeneic HSC transplant in patients with high-risk ALL including patients with Philadelphia chromosome and those with t(4, 11) chromosomal translocation [204].

3.7.1.5 Chronic Myeloid Leukemia/Chronic Lymphocytic Leukemia

Combining hematopoietic SCT with available treatments like tyrosine kinase inhibitors has shown high cure rates with low adverse risk profile. SCT is reserved for patients with the refractory disease to first-line agents in CML.

3.7.1.6 Myelofibrosis, Essential Thrombocytosis, and Polycythemia Vera

Allogenic SCT demonstrated an improvement in outcomes in patients with myelofibrosis and those diagnosed with myelofibrosis preceded by essential thrombocytosis and polycythemia vera [205].

3.7.1.7 Solid Tumors

Autologous SCT is considered the standard of care in patients with germ cell tumor (testicular tumors) that are refractory to chemotherapy (after the third recurrence with chemotherapy). HSCT has shown promising outcomes in cases of medulloblastoma, metastatic breast cancer, and other solid tumors [206].

3.7.2 Complications of HSCT

Most of the grafts used for HSCT are either whole bone marrow or sorted $CD34^+$ stem and progenitor cells. In both cases, the contamination of HSCs with other $CD34^+$ non-hematopoietic cells, or even tumor cells, leads to higher incidence of graft-versus-host disease (GVHD) and less graft-versus-leukemia (GVL) effects following allogeneic transplantation [207]. On the other hand, the use of very pure HSCs populations was effective with less GVHD [208, 209], however, it is rarely used in clinical practice, as this implies more labor and importantly costs.

3.8 The Future

Research defining the nature and regulation of HSCs has permitted the manipulation of hematopoiesis regulators in ways that have revolutionized the current treatment options for blood disorders and the use of stem cell transplants. A deeper understanding of HSCs self-renewal and differentiation mechanisms, the cell-fate choices, and intrinsic/extrinsic regulators of HSCs is still missing. Novel outcomes from ongoing HSC research continue to redefine and refine our knowledge and provide a venue for endless improvements in HSC based clinical therapeutics. This includes improvements in HSCs isolation, labeling and sorting, in vivo imaging, together with recent microfluidics, organ-on-chip and omics approaches. For example, improved approaches that use gene-editing of HSCs to facilitate the transplantation of "corrected" allogeneic/syngeneic cells, thus achieving personalized therapy/medicine as shown in Fig. 3.5. However, important challenges remain, which include, developing robust methods to maintain HSCs in vitro (mimicking their in vivo niche) both to accelerate ongoing research and to increase cell numbers for large-scale therapeutics [210].

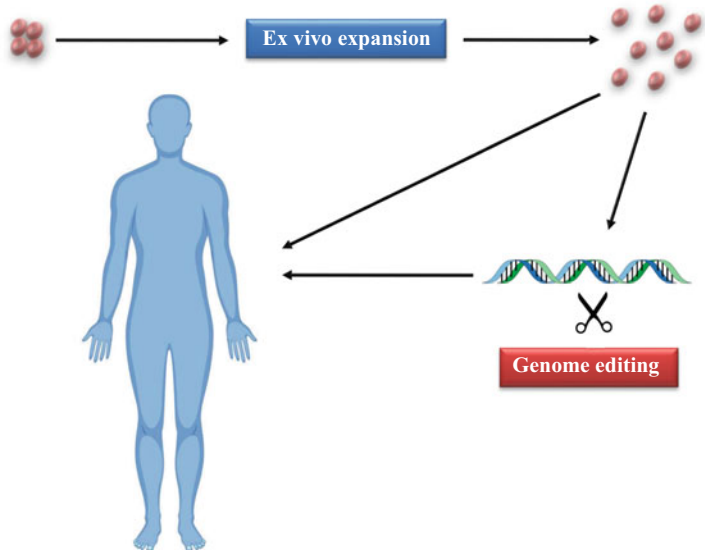

Fig. 3.5 Future directions and applications of HSC research including Ex-vivo stem cell expansion and genome editing of HSCs

Take Home Messages

- Hematopoietic stem cells (HSCs) are pluripotent cells responsible for producing all blood cell types via the process of hematopoiesis.
- Hemangioblast is an embryonic stem cell that gives rise to blood vessels and universal blood stem cells (which give myeloid and lymphoid precursors).
  - Myeloid precursors form several types of differentiated cells including red blood cells (erythrocytes), platelets (megakaryocytes), mast cells, and myeloblasts (basophils, neutrophils, eosinophils, monocytes).
  - Lymphoid precursors form natural killer cells and lymphocytes (B and T lymphocytes).
- Once HSCs divide, they have the option of entering any downstream cell fate pathway giving different blood cell types as needed. Symmetric division, asymmetric division, and symmetric commitment are considered the possible patterns of division of HSCs.
- HSCs control their self-renewal and differentiation, and the fine balance is assured by various intrinsic and extrinsic factors.

(continued)

- Growth factors regulate the growth, differentiation, and function of cells of the hematopoietic cells such as:
  - Erythropoietin: stimulates red cells production.
  - Thrombopoietin: stimulates platelet production.
  - G-CSF: stimulates granulocyte production and activates neutrophil function.
- In order to characterize HSCs, an array of phenotypic markers should be used together with functional assays.
- HSCT is a procedure where HSCs are given to a recipient with the intention of repopulating/replacing their partially or totally damaged hematopoietic system; after radiation, chemotherapy, or other BM damaging conditions.
  - In autologous transplantation, the patient's own HSCs are obtained and freeze-stored for later use. After chemotherapy or radiation is complete, the harvested HSCs are thawed and returned to the patient.
  - In allogeneic transplantation, HSCs are obtained from a donor, ideally a brother or a sister with similar genetic makeup. If the patient does not have a suitably matched sibling, an unrelated person with a similar genetic makeup may be used. Under some circumstances, a parent or a child who is only half-matched can also be used; this is termed a haploidentical transplant.

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Adult Stem Cells: Mesenchymal Stromal Cells, Endothelial Progenitor Cells, and Pericytes

4

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Abbreviations

AD-MSCs	Adipose-derived MSCs
AFP	Alpha-fetoprotein
ang-1	Angiopoietin 1
ANG-2	Angiopoietin-2
ASCs	Adult stem cells
BDNF	Brain-derived neurotrophic factor
bFGF	Basic fibroblast growth factor
BM	Bone marrow
BM-MSCs	Bone marrow-derived MSCs
BMP4	Bone morphogenetic protein 4
BPD	Developing bronchopulmonary disease
CCL-2	(C-C motif) ligand 2
CFU-F	Colony-forming unites-fibroblast
CNS	Nervous system
CVD	Cardiovascular disease
CXCL12	C-X-C motif chemokine 12
DMEM	Dulbecco's Modified Eagle's medium
ECM	Extracellular matrix
ECs	Endothelial cells
ECs	Endothelial cells
eEPCs	Early EPCs
EMT	Epithelial to mesenchymal transition
EOC	Endothelial outgrowth cells
EPCs	Endothelial progenitor cells
EPO	Erythropoietin
ESCs	Embryonic stem cells
FGF	Fibroblast growth factor
G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte-macrophage–colony-stimulating factor
HIF	Hypoxia-inducible factor
HSCs	Hematopoietic stem cells
HSCs	Hepatic stellate cells
IDO)	Indoleamine 2,3-dioxygenase

IGF-1	Insulin-like growth factor-1
IL-(num.)	Interleukin (num.)
INF-	Interferon gamma
ISCT	Society for cellular therapy
KDR	Kinase insert domain receptor
M-CSF	Erythropoietin, macrophage colony-stimulating factor
MMPs	Metalloproteases
MNCs	Mononuclear cells
MSCs	Mesenchymal stromal cells/Mesenchymal stem cells
NG2	Neural/glial antigen 2
NO	Nitric Oxide
OECs	Outgrowth endothelial cells
OPN	Osteopontin
P-(num.)	Cell passage number
PAH	Pulmonary arterial hypertension
PCs	Pericytes
PDGF	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor
PDT	Population doubling time
PGE2	Paracrine factors such as Prostaglandin E2
PSGL-1	Glycoprotein ligand-1
ROCK	Rho Kinases
ROS	Reactive oxygen species
SA-β-Gal	Senescence-associated beta-galactosidase
SDF-1	Stromal-derived factor-1
TACT	Therapeutic angiogenesis by cell transplantation
TGF-β1	Transforming growth factor β1
TGF-β	Transforming growth factor beta
TNF-α	Tumor necrosis factor alpha
TSG-6	TNF-stimulated gene 6
UC-MSC	Umbilical cord -derived MSCs
VEGF	Vascular endothelial growth factor
α-SMA	α-smooth muscle actin

What You Will Learn in This Chapter

In this chapter, you will learn the origin, characteristics, and function of adult stem cells, and the difference between adult stem cells and their embryonic counterparts. Adult stem cells play an important role in maintaining homeostasis, tissue repair, healing, and regeneration. They have become a favorite source for extensive experimentations and clinical trials because of their unique biological and functional criteria, and practical isolation and culture methods. The chapter focuses on mesenchymal stromal cells, endothelial progenitor cells, and pericytes in terms of their biology and functional properties.

4.1 Adult Stem Cells (ASCs)

Adult stem cells (ASCs) are multipotent somatic cells in an undifferentiated state, representing a small percentage of cells within adult specialized mammalian tissues [1–3]. **ASCs** have been detected in almost all tissues including the bone marrow, liver, teeth, testes, ovaries, gut, heart, brain, and skeletal muscle. ASCs are responsible for tissue repair, regeneration, and homeostasis. ASCs reside quiescently in niches that support them structurally and maintain them in an undifferentiated state [3, 4]. When activated by intrinsic or extrinsic signals, such as those elicited by cell injury or cell loss, ASCs become activated and undergo asymmetric division [5]. The first cell of the progeny is lineage-committed and can proliferate and differentiate into a specialized cell as their native origin. The second daughter cell remains undifferentiated to support the long-term maintenance of the stem cell pool [3, 6]. In many tissues, ASCs do not directly differentiate into fully specialized cells, but differentiate into intermediate, partially differentiated progenitor cells. Progenitor cells in turn differentiate into more lineage-committed progenitors, or terminally differentiated, fully specialized cells [7–9]. Compared with embryonic stem cells, ASCs can only give rise to a more limited array of differentiated cell types. Figure 4.1 summarizes the main differences between ASCs and their embryonic counterparts.

ASCs can be extracted from most tissues in the body, including bone marrow, fat, and peripheral blood. In this chapter, we will focus on three important types of ASCs: mesenchymal stromal cell, endothelial progenitor cells, and pericytes.

4.2 Mesenchymal Stromal Cells

Mesenchymal stromal cells (MSCs) are multipotent mesodermal cells that were first described by Alexander Friedenstein [10], as a sub-population within the bone marrow [11]. MSCs are characterized by their fibroblast-like spindles and adherence to plastic. They form fibroblast-like colonies (colony-forming unites-fibroblasts, CFU-F), when cultured at low seeding density, under standard culture conditions [12, 13]. CFU-Fs acquire the characteristics of endothelial cells (ECs) when grown under endothelial culture conditions [14]. MSCs characteristically reside in the perivascular niche, which enables them to be more dynamic and easily migrate within the circulatory system toward injured tissues for maintenance and repair. They also migrate via the lymphatic system and thus play a role in repair during inflammation [15–18].

4.2.1 MSCs: Sources and Origin

MSCs reside in almost all organs and are considered a strategic store for the repair or replacement of degenerated tissues [19] (Fig. 4.2). MSCs are commonly isolated for experimental purposes from the bone marrow [20, 21] and adipose tissue [22]. The sternum and the iliac crest are the main sources of bone marrow aspirates for stem cell collection

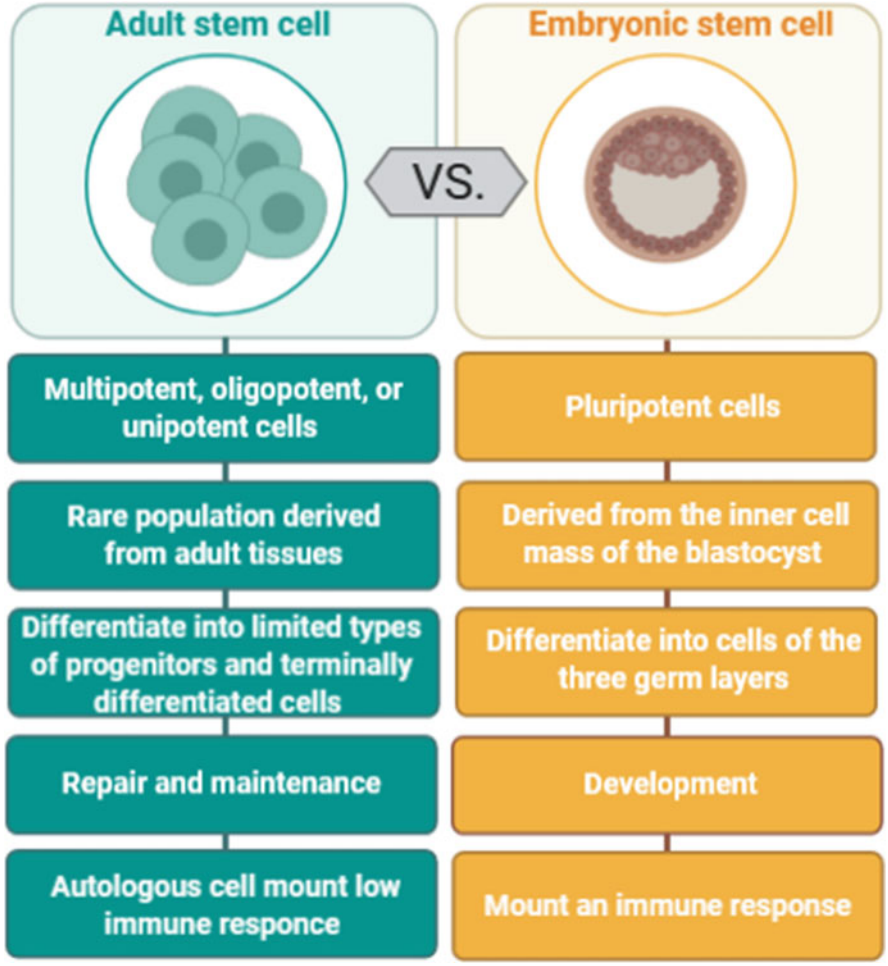

Fig. 4.1 Differences between embryonic and adult stem cells

[23], as both are equally enriched for mononuclear cells (MNCs) [24]. Adipose tissue represents another rich source for MSCs. They are commonly isolated from the subcutaneous adipose tissue, visceral fat, and infrapatellar fat pad during surgical operations related to laparotomy or meniscectomy, or as a byproduct of liposuction [25, 26]. ASCs show some variations based on their origin. For example, those isolated from subcutaneous tissues showed higher proliferation, as well as more chondrogenic and osteogenic differentiation potential than those isolated from visceral fat [27, 28]. On the other hand, ASCs derived from the infrapatellar fat displayed higher chondrogenic differentiation compared with those derived from subcutaneous tissue [29–31].

MSCs were also successfully isolated from the synovial fluid [32] and muscles [33, 34]; these cells exhibited a capacity to regenerate musculoskeletal defects. Other sources of

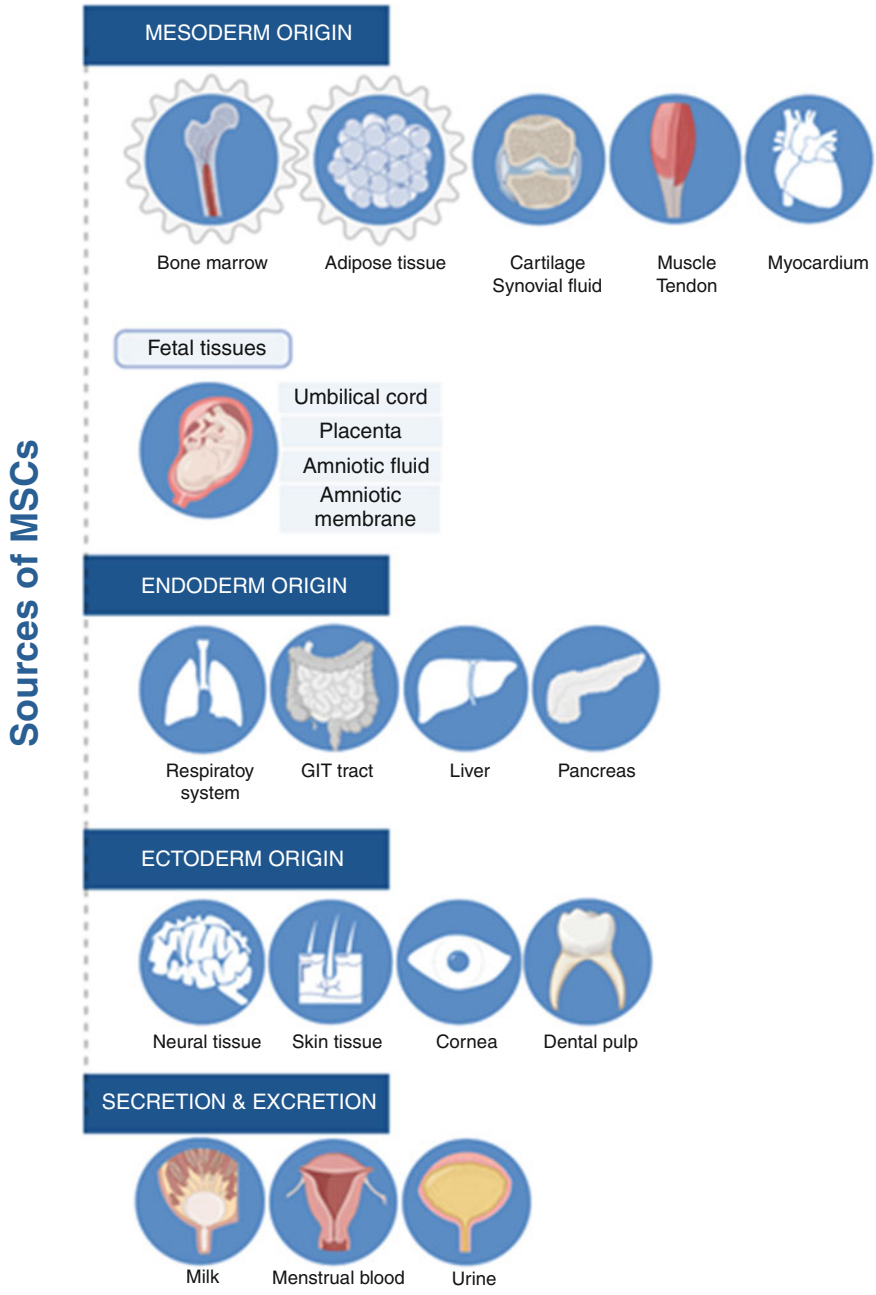

Fig. 4.2 The sources of MSCs: MSCs have mesoderm origins and can be isolated from tissues of endoderm, ectoderm, and mesoderm origin

MSCs include the placenta [35], umbilical cord [36–38], amnion [39], and amniotic fluid [40]. MSCs isolated from fetal tissues were reported to be of low immunogenicity and have a higher regenerative capacity than adult MSCs [36, 41].

MSCs were also isolated from organs of endodermal origin such as the liver [42–44], pancreas [45, 46], and intestines [47, 48]. Furthermore, they were isolated from dental pulp [49], the nervous system [50, 51], corneas [52], and hair follicles [53]. Recently, MSCs were isolated from breast milk [54, 55], urine [56], and menstrual blood [57, 58]. Because of their wide distribution throughout the body, MSCs can be obtained through non-invasive methods with relative ease, and can be harvested in sufficient numbers to identify their properties [59] and investigate their potential clinical application [60, 61].

4.2.2 Characterization of MSCs

The minimal criteria for defining human MSCs, as determined by the International Society for Cellular Therapy (ISCT), include adherence to plastic, and in vitro differentiation potential into chondrogenic, osteogenic, and adipogenic lineages. MSCs express the CD90, CD73, and CD105 differentiation surface markers but do not express the hematopoietic markers CD45, CD3, CD19, CD11, CD79α, and human leucocyte antigen-DR (HLA-DR) [62].

The tissue from which MSCs are isolated should be taken into consideration during their characterization. For example, bone marrow-derived MSCs (BM-MSCs) are positive for CD105, CD73, CD106, CD90, CD44, CD10, CD13, CD146, CD140, and CD271, and negative for the hematopoietic lineage markers (CD45, CD3, CD19, CD11, CD79α). BM-MSCs also show higher osteogenic and chondrogenic differentiation capacity compared to adipose-derived MSCs (AD-MSCs). The latter display higher proliferation and adipogenic differentiation, and have higher expression of CD49d along with lower expression of Stro-1, compared to BMSCs [63, 64]. Table 4.1 summarizes the main differences between BM-MSCs and AD-MSCs.

MSC source, extraction methods, culture conditions, and cell passage numbers all affect their efficiency in clinical applications. These factors may alter their genetic profile, morphology, plasticity, differentiation, and proliferation capacities. These alterations contribute to the heterogeneity of MSC populations, resulting in inconsistent findings in both the laboratory and the clinic [76]. The transcriptional patterns of MSCs also vary depending on their source and surrounding conditions [62]. These large number of variables have made it difficult to characterize MSCs based solely on their phenotype and have necessitated the inclusion of functional criteria for their identification.

Table 4.1 Differences between AD-MSCs and BM-MSCs

		AD-MSCs	BM-MSCs
Colony formation	Clonal efficiency [65]	Consistent until passage 20	Decrease starting from passage 10
	CFU-F [66]	Lower than BM-MSCs	Higher than AD-MSCs
Differentiation	Adipogenic [65, 67]	Retained through passages until passage 20 and shows modest alteration afterwards	Decreases significantly after passage 10 and is completely lost at passage 15
	Osteogenic [65]	Retained for an extended period in culture	Lost after passage 10
	Chondrogenic [65]	Retained up to passage 10	Retained up to passage 5
	Hematopoietic differentiation support [68]	Maintained human early and committed hematopoietic progenitors in vitro and support their complete differentiation toward myeloid and lymphoid lineages	Lower efficiency than AD-MSCs
Proliferation and senescence	Senescence [69]	Very low senescence ratio within early passages compared to BM-MSCs	Higher senescence ratio in early passages compared to AD-MSCs
	Yield from the same amount of tissue [70]	500-fold higher	Lower
	Telomerase activity [65]	A modest decrease in telomerase activity between passages 1 and 10	Significant decrease in telomerase activity between passages 1 and 10 compared to AD-MSCs
Cytokines and chemokines	VEGF [67]	(+)	(+++)
	IL-6 [67]	(+++)	(+)
	TGF [71]	(++)	(+)
Immunophenotype	CD106 [72]	(+)	(++)
	CD34	$CD34^+$ in freshly isolated cells and gradually declines with expansion [73]	$CD34^-$ in cultured MSCs [74, 75]
	CD146 [67]	Decrease with expansion	Maintained
	CD49d [63, 64]	(++)	(+)
	PW1 [67]	(+)	(−)

(−) Negative expression; (+) Positive expression; (++) Higher expression; (+++) Significantly high

4.2.3 Biological Functions of MSCs

4.2.3.1 Proliferation

MSCs undergo a limited number of mitotic divisions to self-renew and maintain their tissue of origin before undergoing senescence. It was reported that MSCs subject to the standard Hayflick phenomenon. Hayflick limit means that the normal cell is able to replicate for limited number of times before undergoing sentence and programmed death [77]. The source of MSCs is a defining factor in their proliferation rate. Lu et al. demonstrated that BM-MSCs have significantly slower population doubling times compared with umbilical cord-derived stem cell UC-MSCs. The mean doubling time of UC-MSCs in passage 1 (P1) is approximately 24 h and remained almost constant until P10. In contrast, the mean doubling time of BM-MSCs is 40 h, and increased considerably after P6 [78]. The population doubling time was reported to be the shortest in MSCs from neonatal sources, such as the umbilical cord, compared to those derived from adult tissue [79]. Kern et al. have reported that BM-MSCs have the lowest population doubling number between passage 4 and passage 6 compared with AD-MSCs and UC-MSCs [69]. They also reported that UC-MSCs possess the highest ratio of MSCs undergoing senescence within early passages compared with BM-MSCs and AD-MSCs [69]. On the other hand, Jin et al. demonstrated that UC-MSCs could be cultured for significantly longer periods and exhibit a greater expansion capacity than AD-MSCs. The latter had the shortest culture time and lowest growth rate. The growth of both BM-MSCs and AD-MSCs was arrested at passage 11–12, whereas UC-MSCs kept proliferating until passage 14–16 [66].

The proliferation of the early passages of MSCs is controlled via the Wnt/β- catenin signaling pathway and depends on the O_2 level. Hypoxic conditions modulate hypoxia-inducible transcription factors, which control a large set of downstream genes that are involved in cell cycle progression. Hypoxia was found to enhance MSC proliferation in comparison with normoxic condition [80, 81]. Moreover, in vivo hypoxic conditions were reported to maintain the viability of MSCs, and protect them from the effects of reactive oxygen species (ROS) and mitochondrial stress [82]. As MSCs aged and approach cell death, accumulated senescence-associated DNA damage, ROS, and shortened telomeres are detected; the cells also display morphological changes that include larger size and irregular shapes. Furthermore, the cessation cell division is associated with augmented expression of senescence-associated beta-galactosidase SA-β-Gal [83, 84].

4.2.3.2 Migration and Homing

Homing refers to the capability of MSCs to migrate toward their original tissue niche and reside there [85]. MSCs migrate to injury sites and differentiate there into local tissue cells [86–88], and release a cytokine and growth factor-rich secretome that promotes tissue repair and regeneration [89]. Under physiological conditions, MSC migration is an organized process that is controlled by signals from the surrounding niche [90]. Trans-membrane integrins, cadherins, cytokines, and growth factor receptors initiate a signaling cascade to potentiate the Rho family of GTPases, especially RhoA. RhoA plays an

important role in the modulation of actin cytoskeletal rearrangement. It also activates Rho kinases (ROCKs), which in turn promote Myosin II activation, stabilizing the polymerization of actin filaments and increasing cell contractility [91]. Contractile MSCs are capable of migrating to blood vessels and can pass through the endothelial wall, directing themselves toward the target tissue [92]. In the presence of an injury, the MSC migration patterns toward the injured tissues can be mediated by cytokines and growth factors such as stromal cell-derived factor-1(SDF-1) [93], osteopontin [94], basic fibroblast growth factor (bFGF) [95], vascular endothelial growth factor (VEGF) [96], insulin-like growth factor-1 [97], platelet-derived growth factor (PDGF) [98], and transforming growth factor β1 (TGF-β1) [99]. Moreover, mechanical factors such as extracellular matrix stiffness [100], mechanical stretch [101], and shear stress [102, 103] all modulate MSC migration. The migratory and homing potential of MSCs is of importance in MSCs therapeutic applications because uncontrolled migration could contribute to the dissemination of the pathological condition, which has been documented in some cancers [104–106].

4.2.3.3 Trophic Properties of MSCs

MSCs proliferate and differentiate to provide elements of the stroma, which are essential for the support and repair of tissues and organs [107–111]. In the bone marrow, MSCs are essential for the growth, proliferation, and differentiation of hematopoietic stem cells (HSCs) [112]. This trophic function of MSCs is mediated by cell–cell interactions, as well as the secretion of growth factors and other mediators. Trophic properties of a cell describe their potential to exert an indirect activity upon the cells in vicinity via secreted bioactive molecules [113]. The secretome of MSCs includes cytokines, such as IL-6 and IL-37, and growth factors including platelet-derived growth factor (PDGFR), erythropoietin, macrophage-colony-stimulating factor (M-CSF), and granulocyte-colony-stimulating factor (G-CSF) [114, 115]. Ball et al. have reported that the released trophic factors support better engraftment and performance of HSCs co-transplanted with MSCs [116]. Similarly, the MSC-conditioned culture medium was found to enhance tissue repair and regeneration [117]. For example, brain-derived neurotrophic factor released from MSCs was demonstrated to activate neural progenitors in brain lesions and promote neurogenesis [118, 119], while CXCL12 and ang-1 promoted angiogenesis [120–123]. The trophic effect of MSCs may be also achieved via the release of extracellular vesicles that act as inter-cellular shuttles, carrying various secretome cargo like exosomes [124].

4.2.3.4 MSCs and Immunosuppression

MSCs have important immune modulation functions that are primarily mediated by released soluble paracrine factors such as prostaglandin E2 (PGE2), interleukin 6 (IL-6), the chemokine (C-C motif) ligand 2 (CCL-2), G-CSF, bone morphogenetic protein 4 (BMP4), TGF-β, and extracellular vesicles [125, 126]. MSCs express HLA-Class I but not HLA- Class II antigens, and lack the co-stimulatory molecules CD40, CD80, and CD86 [127]. However, pro-inflammatory cytokines such as TNF-α, INF-γ, and IL-1B can activate MSCs, increase their HLA-Class I expression, and induce the expression of

HLA-Class II antigens [128, 129]. Moreover, the secretome of the MSCs contains myriad anti-inflammatory factors, such as IL-10, and TGF-β [130–133]. MSCs were also reported to affect the innate and adaptive immune system. For example, the co-culturing of MSCs with T-lymphocytes induced T-lymphocytes apoptosis. This action is regarded as one mechanism by which MSCs exert their immunosuppressive potential [134, 135]. The immunosuppressive action of MSCs could be achieved also via many other mechanisms, including the recruitment of immune suppressive cells such as IL-10-producing dendritic cells, B cells, as well as $CD4^+CD25^+$ $FOXP3^+$ T regulatory cells. Furthermore, MSCs can suppress macrophage-released IL-6 and TNF-α via PGE2 and indoleamine 2,3-dioxygenase (IDO) secretion [136, 137]. The multilevel immunosuppressive action of MSCs makes them suitable for ameliorating and overcoming the immune rejection that is experienced after solid organ transplantation [138, 139].

4.2.3.5 Multipotency and Differentiation

MSCs are multipotent cells that differentiate into lineages such as osteoblasts, chondrocytes, adipocytes, myocytes, as well as other cell lineages. The in vitro differentiation of MSCs into adipogenic, osteogenic, and chondrogenic cells is routinely used for the identification of human multipotent MSCs. Furthermore, the ability of MSCs to differentiate in vitro into ECs [140], vascular smooth muscle [141], and myocytes [142] has also been reported. MSCs could be induced to differentiate in vitro into adipocytes when cultured in a medium supplemented with indomethacin, dexamethasone, insulin, and 1-methyl-3- isobutylmethylxanthine. The Wnt/B-catenin signaling pathway was found to be highly active to induce the commitment of MSCs toward pre-adipocyte formation during the early stages of differentiation. However, this signaling pathway is turned off later in the differentiation process to allow for the maturation of the adipocytes [1, 143]. This differentiation could be assessed by measuring the levels of the resultant adipocyte-specific markers, including enzymes such as PPAR-γ and the lipoprotein lipase enzyme [144]. Furthermore, the appearance of fat droplets is a significant indicator of the successful adipogenic differentiation process [145, 146].

To induce chondrogenic differentiation, MSCs are cultured in a medium that includes TGF-β III, linoleic acid, transferrin, insulin, selenium acid, ascorbic phosphate, dexamethasone, and pyruvate [147–149]. BMP-2 and TGF-β1 were also used to enhance chondrogenic differentiation. Chondrogenic differentiation can be assessed by measuring the levels of released collagen type II and other proteoglycans through immunohistochemical staining [150]. Osteogenic differentiation is enhanced by treating MSCs with ascorbic acid, B-glycerophosphate, and dexamethasone, resulting in osteoblast formation. Osteoblasts can be detected by measuring the levels of alkaline phosphatase and mineralized calcium deposits in the cells [151].

FGF, PDGF, and TGF-β are a set of key regulators in MSC differentiation, whose modulation, up-regulation, or inhibition could diminish cell proliferation. For example, the downregulation of TGF-β was found to be linked to increased adipogenic and osteogenic differentiation, while blocking chondrogenic differentiation. Additionally, PDGF

inhibition and the diminished expression of FGF receptors were found to be related to lower osteogenic differentiation and the inhibition of osteogenic differentiation potential [147].

The differentiation of MSCs into multiple cell types of mesodermal and endodermal origin has been described. Inducing the differentiation of MSCs into hepatocytes could be achieved in two stages. First, MSCs were cultured in IMDM supplemented with nicotinamide, basic fibroblast growth factor (bFGF), and hepatic growth factor (HGF). Then, transferrin, oncostatin M, insulin, dexamethasone, and selenium were added [152, 153]. By the end of the differentiation process, the resultant hepatocytes can be characterized by measuring the release of unique liver proteins such as albumin and alpha-fetoprotein (AFP). The differentiation of MSCs into a cholinergic nerve [154], myocytes [142], pancreatic β-cell-like cells [155], and insulin-producing cells [156] has also been reported.

Mesenchymal Stem Cell or Mesenchymal Stromal Cell?

In 2005, a statement by the International Society for Cell and Gene Therapy (ISCT) stipulated that the terms "mesenchymal stem cells" and "mesenchymal stromal cells" are not equivalent, and cannot be used interchangeably, as they represent two different cell populations [157]. According to the statement, one of the main differences is that mesenchymal stem cells constitute a population that shows progenitor properties in terms of differentiation and self-renewal [115, 158]. However, the stromal counterpart refers to a bulk heterogeneous population that includes fibroblasts, myofibroblasts, and a small population of stem/progenitor cells [159, 160], but does not include hematopoietic or endothelial cells. The heterogeneity of mesenchymal stromal cells makes them demonstrate specific homing [161], secretory, and immunomodulatory criteria [162] that are more relevant to MSC-based clinical therapies [163].

The overlap between the two terms could be attributed to the use of the "MSCs" acronym, which can be expanded to imply mesenchymal stromal cells, mesenchymal stem cells, multipotent stem cells, and medicinal signaling cells. However, the ISCT recommends the use of the MSCs acronym for mesenchymal stem cells because it has been used for decades. The ISCT defines MSCs through the following minimal criteria: their adherence to plastic, the expression of CD73, CD90, and CD105, the lack of expression of the hematopoietic and endothelial markers CD11b, CD14, CD19, CD34, CD45, CD79a, and HLA-DR, and in vitro adipogenic, chondrogenic, and osteogenic differentiation potential [164]. Later on, in 2019, the ISCT issued a new statement that the previous minimal MSCs criteria are not definitive (164). For example, the lack of CD34 expression was typically used as one of MSCs' defining criteria; however, various reports demonstrated that CD34 expression widely

(continued)

depends upon cell source and passage, and they stated that MSCs tend to be more $CD34^+$ under in vivo compared to in vitro conditions [165, 166].

The ISCT MSC committee recommended that the MSC acronym remains in use, but it should be coupled with the tissue of origin like BM-MSCs for bone marrow origin, AD-MSCs for adipose tissue origin, and UC-MSCs for cells originating from the umbilical cord, because MSCs from different tissues exhibit varied phenotypes, functions, and secretomes [71, 167, 168]. The MSC committee also recommended that the use of the MSC acronym should be annotated with functional definitions. Furthermore, the term mesenchymal stem cell should not be used without solid functional in vivo and in vitro evidence to prove the self-renewal and differentiation potential. Indeed, they see that CFU-F progenitor assays and in vitro tri-lineage differentiation assays are indications for the progenitor status but are not sufficient to demonstrate the self-renewal capacity of mesenchymal stem cells in the absence of in vivo data [159]. As for the mesenchymal stromal cell, the committee recommended the evaluation of their trophic factors secretion [113, 169], their modulatory effect on immune cells [170–172], and other relevant criteria such as angiogenesis modulation [173–176] to reflect the multimodal properties of the mesenchymal stromal cell heterogeneous population. They have published an article [162] that discusses the immune assays for the assessment of mesenchymal stromal cells, and recommended that assays should include quantitative RNA analyses of selected genes, flow cytometry of cell surface markers, protein analysis of the MSC secretome, and the characterization of exosomes and/or microRNA [177–180].

4.3 Endothelial Progenitor Stem Cells

4.3.1 History, Definition, and Origin

EPCs constitute multiple cell types that can differentiate into mature ECs. Unlike other progenitor cells, EPCs share some common features with stem cells such as clonogenicity, self-renewability, and differentiation potential [181, 182]. EPCs were first isolated in 1997 by Asahara et al., from human peripheral blood by a molecular isolation technique in which surface-antigen magnetic beads were used to isolate specific peripheral blood mononuclear cells ($PBMC^{CD34+}$ or $PBMC^{Flk1+}$ cells) on fibronectin culture plates [181]. More recently, several studies on harvesting EPCs from different sources used either direct isolation from human bone marrow (HBM), human umbilical cord blood (UCB), or human peripheral blood (PB), or indirectly by transdifferentiation form other somatic cells such as neural, dental, cardiac, or adipose tissue [115, 183–188].

4.3.2 EPCs Characterization

EPCs share many common cell surface markers with HSCs, in addition to numerous common genes affecting both hematopoietic and endothelial cell development. It was thus suggested that HSCs and EPCs originate from a common precursor, the hemangioblast [189–191]. Surface markers used to isolate and characterize EPCs include CD34, CD146, CD45, CD115, CD14, CD133, VEGFR1, VEGFR2 (or KDR) [115]. EPC's phenotype differs based on its source. For example, $CD133^+$ and $CD34^+$ EPCs cells isolated from UCB were higher in number than those isolated from adult PB [192, 193]. Other studies showed that cells expressing CD34 or VEGFR-2 markers generated the most mature ECs [194]. Importantly, EPCs have been described as a population of circulating $CD34^+$ cells that can differentiate ex vivo into cells with endothelial cell-like characteristics [195]. Various studies have reported that EPCs are heterogeneous populations comprising multiple subpopulations. EPCs can differentiate into two different subpopulations; early EPCs (eEPCs) similar to EPCs identified by Asahara and et al. and late EPCs known as outgrowth ECs [196–198]. Both types have different features and biological properties that are summarized in (Table 4.2).

Table 4.2 Differences between early and Late EPCs

	"Early" EPCs	"Late" EPCs
Nomenclature	– Early EPCs (eEPCs) – Pro-angiogenic circulating hematopoietic stem/progenitor cells	– Endothelial colony-forming cells (ECFCs) – "Late" EPCs – Endothelial outgrowth cells (EOC)
Lifespan [199]	Short lifespan up to 3 to 4 weeks [181, 200, 201]	Long lifespan and rapid proliferation [14, 196, 202]
Proliferation [197]	Minimal proliferative capacity	– Significantly higher proliferative potential reaching 28 population doublings (PDs) in 40 days with a doubling time of approximately 34 hours.
Colony formation [198]	– Colonies are produced in 4–6 days after the initial seeding of mononuclear cells – Colonies are characterized by discrete cell aggregates	– Colonies are produced 3–4 weeks after seeding [197]
Immunophenotype [197, 199]	CD45 (+) CD31 (+) CD105 (+/−) CD146 (+/−) CD14 (+) CD34 (+) CD117 (+)	CD45 (−) CD31 (+++) CD105 (++) CD146 (++) CD14 (−) [203] CD34 (++) CD117 (++) CRLR/RAMP-2 (AM1) [204]

(continued)

Table 4.2 (continued)

	"Early" EPCs	"Late" EPCs
Morphology [198, 205]	– Appear within 4 to 7 days of culture with spindle-like morphology; have limited proliferation potential	– Develop after 2 to 3 weeks of culture with a cobblestone appearance [158]
Differentiation [199]	– Heterogeneous cells that are differentiated from hemangioblasts – Early EPC can differentiate into late EPCs [206]	– Homogeneous and well-differentiated cells – Considered to be mature endothelial cells – Differ from mature endothelial cells in terms of proliferation rate and cell senescence – OECs are committed to an endothelial lineage [197]
Gene Expression Profile (200)	– von Willebrand factor (vWF) is not expressed – VEGFR-2 (+)	– Express von Willebrand factor (vWF) – VEGFR-2 (++)
In vitro Function [199]	– KDR (+) – NO (+) – VE-cadherin (+) – Lack tube-forming capacity [197]	– KDR (++) – NO (++) – VE-cadherin (++) – Higher tube formation efficiency – Higher angiogenic properties in vitro [207]
In vivo Function [199]	– Contribute to neovasculogenesis primarily by secreting the angiogenic cytokines that help recruit resident mature endothelial cells and induce their proliferation and survival – No significant difference in contribution to neovasculogenesis in the ischemic limb – A limited degree of engraftment and incorporation into new vessels from early EPCs [203]	– Enhance neovasculogenesis by providing a sufficient number of endothelial cells based on their high proliferation potency – No significant difference in contribution to neovasculogenesis in the ischemic limb – Higher capacity to form de novo vessels in vivo [203]

(+) positive, (+/−) positive or negative, (++) higher, (+++) significantly high

4.3.3 Action Mechanism

The formation of new blood vessels by EPCs necessitates their mobilization, migration, adhesion, and differentiation. In case of vascular occlusion, EPCs have been shown to sense altered (low or oscillatory) shear stress, and as a result increase the expression of pro-oxidant enzymes, which are mediated principally by the transcription factor, NF-κB [208]. Hypoxia can be sensed by ECs in several ways, most notably by the hypoxia-inducible factor and nitric oxide (NO). They both mediate the activation of several signaling

pathways, which powerfully orchestrates the cellular response to low oxygen levels when activated. As a result, different growth factors, cytokines, and chemokines are released, mediating EPC mobilization from the BM [209, 210]. These factors include (VEGF), fibroblast growth factor (FGF-2), granulocyte-macrophage-colony-stimulating factor (GM-CSF), and granulocyte-colony-stimulating factor (G-CSF), as well as angiopoietins [211]. VEGF appears to induce a fast EPCs mobilization from the BM, a phenomenon which has been described in burn patients [212]. However, EPCs were found to have the ability to release VEGF after homing, and generate a local angiogenic response [213]. There are various isoforms of VEGF including VEGF-B, VEGF-C, VEGF-D, but it remains unclear whether there are differences in their effect on EPC regulation. Other factors such as erythropoietin (EPO) can also mobilize EPCs [214]. Granulocyte-macrophage-colony-stimulating factor (GM-CSF) and its related cytokine, granulocyte-colony-stimulating factor (G-CSF), both display mobilizing activity, although they are less potent than VEGF or SDF-1 [215].

The adhesion of EPCs to an injured vessel wall is crucial. This occurs through the interaction of the glycoprotein ligand-1 (PSGL-1) expressed on EPCs with the P-selectin expressed on platelets [213]. EPCs play an important physiological function by acting as the main reservoir of ECs, due to their ability to move into the injury site to preserve the integrity of the endothelium [216]. The contribution of EPCs to vascularization has been demonstrated in animal models and humans [213]. Additionally, the reduction in the number of circulating EPCs and/or alterations in their functions associated with various factors might have a marked impact on endothelium function as well as cardiovascular disease (CVD) onset, complications, and consequently in the survival of individuals with CVD [217].

4.3.4 Clinical Applications

EPCs-based therapy is considered to be a promising endothelial regeneration for several diseases including cardiovascular failure, chronic renal failure, pulmonary diseases, in addition to ischemia related conditions and connective tissue disorders [215, 218]. They also play an important role in tissue engineering by their ability to vascularize engineered tissues, which could be useful for personalized medicine [219]. EPCs are utilized for multiple applications because they could differentiate into both continuous and discontinuous capillaries in the liver and skeletal muscles [219–221]. They could also outperform a vascular-derived endothelium in vascular network formation and possess a comparable permeability to the endothelium vessels [222–229]. The contribution of EPCs to vascularization has been demonstrated in animal models and humans [213]. Additionally, the number of circulating EPCs and/or alterations in their functions associated with various factors might have a marked impact on endothelium function and CVD onset, complications, and consequently in the survival of individuals with CVD [217].

4.3.4.1 EPCs as a Biomarker

Studies have shown that the number and function of circulating EPCs can act as biological markers for vascular function and cumulative cardiovascular risk [216, 230, 231]. The number of EPCs varies depending on the disease. For example, a decrease in the number of EPCs was found to be associated with chronic kidney disease [232], coronary artery disease [233], pulmonary hypertension [234], rheumatoid arthritis [235], and hypertension [236], and a dramatic decrease in EPC proliferation and functional deterioration was found in diabetes mellitus type 1 and type 2 patients [237, 238]. On the other hand, patients with acute myocardial infarction [231] and ischemic-related conditions [239] have an increasing number of circulating EPCs due to their mobilization from the bone marrow. This suggests the close relationship between the status of the ECs and EPCs functionality and mobilization. This relation gives EPCs a clinical advantage over the use of other CVD biomarkers that only correlate with end-tissue damage or stress, such as creatine kinase-MB (CK-MB) [240], troponin [241], or the causative agents like oxidized low-density lipoprotein (oxLDL) [242] and CRP [243].

4.3.4.2 EPC Transplantation

BM-derived EPCs have homing signals to the site of ischemia in animal models. Kalka et al. tested the effect of injecting ex vivo-expanded human EPCs in mice with ischemic limbs [244]. After the infusion of the EPCs, a significant number of the infused cells were detected in newly formed vessels in mice, and a corresponding increase in the rates of blood flow recovery and capillary density were also observed [244]. In another experiment, donated human $CD34^+$ cells were injected in rats with myocardial infarction. The cells were also tracked and detected in newly formed capillaries, and significant induction of neoangiogenesis was also reported [245]. Similarly, Schuh et al. injected human BrdU-labeled isolated EPCs directly into the border infarct zone 4 weeks after acute myocardial infarction was induced in a rat model. Their results showed a significant increase in the left ventricle developed pressure, the coronary blood flow rate, and the neovascularization rate of blood vessels [246]. EPC transplantation trials extended rapidly to human patients due to their promising therapeutic potential in improving vascularization and endothelial integrity [247]. Kudo et al. conducted a clinical trial on two patients with critical limb ischemia, in which they were injected with peripheral blood-derived $CD34^+$ EPCs. An increase in the feet oxygen pressure, improvement of symptoms, and formation of new collateral blood vessels were observed in the injected patients [248]. Another trial was conducted on 11 patients with myocardial infarction. Here, the patients were injected with a combination of bone marrow-derived autologous MSCs and EPCs. Most of the cases showed improved myocardial contractility and repair in myocardial scars [249]. Based on the previous studies and many others, it was deduced that EPC transplantation has therapeutic potential to improve vascularization. However, further investigations should be carried out to overcome the limitations related to their isolation, characterization, purity, culturing conditions, and to optimize their route of injection [246, 250] .

4.3.4.3 Pulmonary Diseases

Many studies have reported the therapeutic role of EPCs and their role as biomarkers for endothelial tissue injury, especially in pulmonary arterial hypertension and chronic obstructive pulmonary disease [251, 252]. Endothelial injury and dysfunction are the major risk factors for the development and progression of both conditions [253, 254]. When endothelial tissue is damaged but ECs fail to repair the damage, inflammatory cells migrate to the injury site and the subendothelium is exposed to the effects of growth factors and other mediators, resulting in intimal proliferation and blood coagulation [255]. Therefore, the availability and mobilization of EPCs in the lungs might be an effective mechanism for lung tissue regeneration and protection. For example, Yamada et al. conducted a clinical trial on 23 patients with pneumonia during both acute and convalescent phases. Patients received autologous peripheral blood-derived EPCs. Results demonstrated that a sufficient number of EPCs enabled patients to recover from pneumonia and improved the associated fibrotic damage to the lungs [256]. EPCs play a role not only in lung tissue repair but also in its early development [257]. Impaired EPC mobilization, recruitment, and engraftment were reported in premature murine pups exposed to moderate hyperoxia, resulting in impaired alveolar and vascular growth [258]. In humans, preterm infants who expressed lower numbers of EPCs at birth were reported to have an increased risk of developing bronchopulmonary disease [257].

4.4 Pericytes: Biological Characteristics and Physiological Roles

4.4.1 Pericyte Discovery and Location

Pericytes (PCs) are the third example of ASCs. PCs or perivascular cells were described almost 150 years ago based on their anatomical location surrounding the endothelium of microvascular capillaries [259, 260]. PCs are also known as mural cells because of their location within the blood vessel, and as “Rouget cells” Charles Rouget, who first described them [261]. They are distributed throughout the body in different tissues at different densities depending on the location. For example, the ratio of PCs to ECs varies from 1:100 in striated muscles to 1:3 in the central nervous system (CNS), and1:1 in the retina, respectively [260, 261]. PCs have acquired different names according to their tissue of residence. For example, they are known as “Ito cells” or hepatic stellate cells, in the liver, they are known as mesangial cells in the kidney, and in the bone marrow, they are called adventitial reticular cells [262, 263].

The basement membrane (BM) separates the majority of the pericyte-endothelial interface, although both cell types come in contact at certain points via micro-holes in the BM. The size and number of pericyte-endothelial contacts vary between tissues, but approximately 1000 contacts have been identified for a single endothelial cell. The cells may make contact via peg-socket junctions, in which PC cytoplasmic projections (pegs) are inserted into endothelial invaginations (pockets). Adhesion plaques constitute another contact

mechanism, and occur between microfilament bundles attached at the pericyte plasma membrane and electron-dense material in the corresponding endothelial cytoplasm [264, 265]. Adhesion plaques, as the name suggests, function to facilitate pericyte adherence to ECs, while peg-and-socket contacts allow the diffusion of molecules and ions between the cytoplasm of the two cell types [264]. Adhesion plaque contacts include fibronectin deposits, while peg-and-socket contacts are secured via the tight, gap, and adherence junctions that contain N-cadherin and β-catenin [266].

4.4.2 Pericyte Ultrastructure, Characterization, and Origin

Pericytes are fibroblast-like cells with distinguishable nuclei, low cytoplasmic content, and several long processes surrounding the endothelial wall. Mature PCs are embedded within the BM of microvessels, which are formed by both pericytes and ECs. Pericytes located on the outer surface of blood capillaries interact with underlying ECs and are covered in the same BM [267]. Pericyte processes are typically connected with more than one endothelial cell via adhesion plaques as well as with peg-and-socket contacts, which permit direct contact between the two cell types [268, 269]. This feature, which was first identified by transmission electron microscopy, differentiates primary and secondary pericyte processes [267].

Based on their location in the blood vessels, PCs are characterized as pre-capillary, mid/true-capillary, and post-capillary PCs [270]. Mid-capillary PCs are distinguished by a lack of α-smooth muscle actin (α-SMA) within the cell and by their elongated and spindle-like shape. Pre- and post-capillary PCs are shorter, more stellate in shape, and have varying amounts of α-SMA [271].

Phenotypically, PCs can be characterized by the expression of a combination of antigens including platelet-derived growth factor receptor-b (PDGFR-b), neural/glial antigen 2 (NG2), α-SMA, CD146, CD90, and CD105, and absence of CD56, CD45, and CD31 [272, 273]. Since PCs lack a specific marker, tracking their lineage is a challenging process [266]. Studies have reported that PCs originate either from the mesoderm or ectoderm based upon their anatomical location [274, 275]. Neural crest fate mapping models have indicated that PCs in the CNS, retina, and thymus originated from differentiated neural crest-derived cells [276, 277].

On the other hand, the vascular mural cells in coelomic organs such as the lungs [278], gut [279], and liver [280] derive from the mesothelium [261]. Mesothelial cells were thus proposed to undergo the epithelial to mesenchymal transition (EMT) before migrating to these organs to differentiate into PCs [262]. However, PCs were also proposed to arise directly from ECs and bone marrow [281, 282]. Furthermore, it has been suggested that PCs residing in the same tissue are heterogeneous and have different origin [283]. For example, Chen et al. reported that coronary PCs originated from endocardial cells after undergoing EMT, but some retinal PCs may be derived from the bone marrow and the neural crest [284].

4.4.3 Pericytes Physiological Roles

4.4.3.1 Angiogenesis

Angiogenesis refers to the formation of new blood vessels from pre-existing ones and is an important process in tissue repair and healing [285]. Stem cells that stimulate angiogenesis process and enhance the sprouting of new vessels have great potential in the as therapeutics for ischemic diseases. Pericytes are excellent candidates for vascular regeneration based on their contribution to vessel growth and stabilization [286]. Extensive research has shown the vital role that PCs play in angiogenesis [287, 288], and their interactions with the ECs to maintain the blood vessel integrity and stability has been elucidated [289–291]. The absence of PCs was shown to be associated with the rupture of blood capillaries [292] and vessel damage [293]. Physiologically, most blood vessels are quiescent in adults; however, angiogenesis can be activated during wound healing [294–297] as well as during tumor growth [298, 299]. Consequently, PCs have been targeted for pharmacological therapy.

4.4.3.2 Initiation of Neovascularization

During embryogenesis, angiogenesis involves the secretion of PDGF-B from ECs,which attracts PDGF-B receptor (PDGFR-B)-expressing PCs that reside in the newly formed vessels [300]. This process is important in maintaining the vessels' functionality and integrity, as a lack of PDGF-B or PDGFR-β in mice embryos was shown to be associated with hemorrhaging, vasodilation, and embryonic lethality [292, 301]. Neovascularization is initiated via the activation of quiescent vessels responding to different chemokines, or angiogenic signals including angiopoietin 2 (ANG-2) and VEGF [302].

Neovascularization comprises vessel formation, stabilization, and maturation [264, 303]. Vessel formation is initiated by the surrounding endothelial cells' secretion (ECs) of angiopoietin-2 (ANG-2), which inhibits Tie-2 receptors which inhibit the ANG/Tie signaling pathway. Inhibition of the ANG/Tie signaling pathway permits the detachment and migration of PCs to reside in the endothelial layer, enhancing new angiogenic activity [304]. Furthermore, both PCs and ECs secrete metalloproteases (MMPs) that degrade the BM to facilitate cell detachment [304]. The detachment of PCs is followed by phenotypic changes to their quiescent state including process shortening, an increase in their volume, and the initiation of proliferation [305]. In parallel, VEGF acts in combination with the ECs that lose their junctions, to increase the endothelial layer permeability and permit the passage of plasma proteins to the extracellular matrix (ECM) [306]. This is followed by EC migration toward the nascent ECM responding to different angiogenic factors. EC migration is directed by the tip cell, which is a single endothelial cell with low proliferation and a high migration rate along the VEGF gradient [307]. VEGF signaling could be enhanced by the expression of VEGF receptor 1 (VEGFR1) on PCs [308]. The tip cell migration is followed by the migration of stalk cells and neighboring ECs, to form the lumen that facilitate the growth of the sprouting vessel [306].

The process of vessel maturation is initiated by angiopoietin-1 (ANG-1) secretion from the PCs in the absence of ANG-2. ANG-1 expression allows TIE-2 receptors on ECs and PCs to be activated, which consequently activates ANG/Tie signaling pathway for vascular stabilization and maturation. Moreover, PCs are recruited to stabilize the primitive vessel via different signals, including from the PDGF-β signaling pathway [309, 310]. The newly enhanced PCs and ECs support the vessel maturation by paracrine factors such as ANG-1and TGF beta. These vessel maturation signals could promote the formation of the endothelial barrier as well as the re-attachment of the PCs and the suppression of EC migration [261].

4.4.3.3 Differentiation

Pericytes are multipotent ASCs that can differentiate into cells from different lineages through induction by specific growth factors [311]. Pericytes have been shown to differentiate into adipocytes [312], osteoblasts [313], chondroblasts [312], fibroblasts [314], smooth muscle cells [314], and neural cells [315]. Their ability to differentiate into multiple cell types supports their application in regenerative medicine [316]. For example, under hypoxia or ischemic conditions, PCs differentiate into vascular cells or neural cells and microglia following an ischemic stroke [317]. Furthermore, microvascular PCs showed angiogenic and cardio myogenic behavior in the myocardium of patients under hypoxic conditions [272]. Studies in mice with an infarcted heart have shown the potential of epicardial PCs to differentiate into coronary mural cells in an autologous transplantation setting [318]. Moreover, some PCs were shown to differentiate into macrophages and dendritic cells, supporting their function in immunological diseases [319, 320].

4.4.3.4 Regulation of Blood Flow

Pericytes play a vital role in regulating the blood flow and the vascular capillary diameter through their ability to stimulate vasoconstriction and vasodilation, depending on to the physiological state [260, 263, 264, 321, 322]. This contractility is mediated by a combination of contractile proteins such as α-SMA, myosin, vimentin, and tropomyosin [323]. PCs act via paracrine signals to regulate their contraction and relaxation and coordinate with ECs to regulate the contractility of blood vessels [324]. The oxygen level also contributes to this regulation, as hyperoxia was reported to enhance pericyte contraction in vitro, while high levels of carbon dioxide induced relaxation [263] . These data support the postulation that vessels contract when the oxygen level is sufficient and dilate responding to insufficient oxygen, accommodating the metabolic state [325]. The vasomotion of PCs serves to regulate the hemodynamic regulation and to maintain the permeability of the blood capillaries [326–328]. The capacity of PCs to relax or contract is determined by several factors [329]. PCs have a rough surface with multiple processes and lamellar folds, and can surround and squeeze the ECs [330], while the distinctive cytoskeleton acts as a contractile apparatus [326]. Immunohistochemical analysis has also demonstrated that PCs express a

combination of contractile proteins [331, 332]. Finally, the pericytes' expression of the contractile proteins depends on the tissue requirement of the vascular supplement [333, 334].

Take Home Message

- Adult stem cells are somatic stem cells in an undifferentiated state that exist in small proportions among most adult specialized tissues.
- Adult stem cells can be extracted from most tissues in the body, including the bone marrow, fat, peripheral blood, umbilical cords, and placental tissue.
- Adult stem cells are multipotent and can differentiate only to specific types of cells, unlike their embryonic counterparts, which are pluripotent and can differentiate into all derivatives of the three primary germ layers.
- $CD90^+$, $CD73^+$, $CD105^+$, $CD45^-$, $CD3^-$, $CD19^-$, $CD11^-$, $CD79\alpha^-$, and human leucocyte antigen-DR (HLA-DR) are the most common phenotype to characterize MSCs, however, according to ISCT they are not definitive and minor changes could be observed according to MSCs source.
- According to ISCT, without solid functional in vivo and in vitro evidence to prove the self-renewal and differentiation potential of the cells, the term mesenchymal stem cell should not be used.
- The therapeutic potential of MSCs is enhanced by their multipotency, immunomodulatory, and trophic properties.
- Unlike other progenitor cells, EPCs have some common features with stem cells such as clonogenicity, self-renewability, and multi-differentiation potential.
- EPCs could be isolated from hematopoietic and non-hematopoietic sources such as peripheral blood, cord blood and tissue, bone marrow, and some other adult tissues. They are also classified into early and late EPCs.
- Alterations in the number and functions of EPCs are significantly associated with cardiovascular, pulmonary diseases, and cancer, which makes them potential predictive biomarkers.
- Pericytes are fibroblast-like cells with distinguishable nuclei, low cytoplasmic content, and several long processes surrounding the endothelial wall.
- Pericytes play a vital role in neovascularization and blood flow regulation.

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Cancer Stem Cells and the Development of Cancer

5

Nehal I. Ghoneim, Rania Hassan Mohamed, Alaa Gamal, Shireen Magdy, and Nagwa El-Badri

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What You Will Learn in This Chapter

The dynamic processes during the various cancer stages of initiation, progression, and invasiveness are all influenced by cancer stem cells (CSCs). Increasing evidence suggests that eradicating CSCs might effectively cure multiple types of cancers. This chapter will discuss the different perspectives of CSC concept starting from their history and origin to their implications in the multistep cancer development. We will highlight the genetic and epigenetic modifications of CSCs, and their correlation with tumor progression, metastasis, immune evasion, and resistance to anti-cancer treatments.

5.1 Cancer Stem Cells' Origin and Heterogeneity

Cancer is an uncontrolled division of abnormal cells in the body. It happens when genes controlling basic cellular functions and cell division mutate, resulting in a random cellular proliferation and tumor formation [1, 2]. Cancerous tumors are treated by surgical excision, chemotherapy, immunotherapy, and/or radiotherapy; however, relapse is usually common [3, 4]. Tumor invasiveness, recurrence, and metastasis all contribute to high morbidity and mortality. Within individual tumors, there is a heterogeneous, highly self-renewing, and pluripotent population of cells known as cancer stem cells (CSCs). This small population of CSCs is believed to contribute to cancer virulence, spread, metastasis, recurrence, and resistance to conventional treatment [5–9]. Several theories on the origin and the development of CSCs have been put forth (Fig. 5.1); nevertheless, CSCs are still not fully understood.

5.1.1 The Embryonic Origin of CSCs

The role of undifferentiated cells in cancer was first recognized in the late nineteenth century. The theory of the embryonic origin of cancer cells, called "embryonic rests theory," was first described by Julius Cohnheim in 1877. Cohnheim hypothesized a

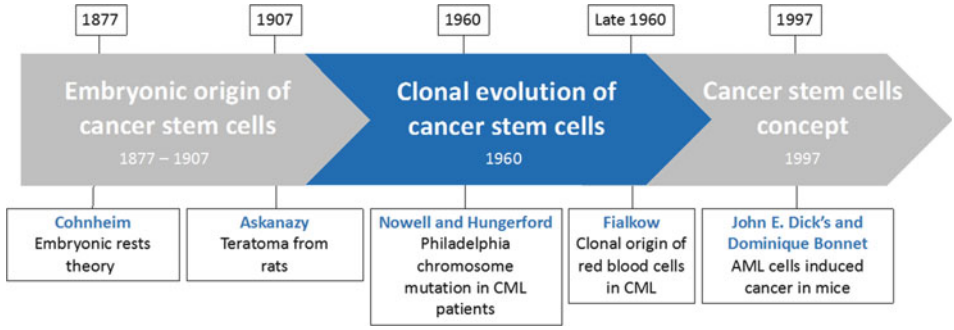

Fig. 5.1 Historical overview of cancer origin theories

common origin of all tumor cells based on the presence of embryonic rests that have remained unused from the time of embryonic development [10, 11]. Cohnheim postulated that if these cells were to receive a steady blood supply, they would begin to grow uncontrollably due to their embryonic nature. This uncontrolled growth results in the formation of tumor masses that constitute a developmental error [11].

Experiments to validate Cohnheim's theory met with very limited success, as reimplanted embryonic cells mostly displayed normal behavior [10]. However, Max Askanazy was able to obtain teratomas that resembled the tumor type hypothesized by Cohnheim. Hence, teratomas became the favored model for differentiating abnormal from normal cell proliferation [10, 11]. In 1907, Askanazy first used the term "stem cells" to describe the unused embryonic residues that he presumed to be hurled in the early developmental stages [11]. In 1930, medulloblasts, which resembled the germinal zones in the embryonic cerebellum, were suggested to support the embryonal rest concept. These medulloblasts failed to differentiate properly and instead formed different types of medulloblastomas [12, 13]. In 1960, Pierce et al. found higher mitotic activity in the undifferentiated cells in teratocarcinomas and considered these cells to be "teratoma stem cells" [14]. However, the differentiated cells that appeared in the teratocarcinoma after the proliferation of embryonal carcinoma were similar to those seen during normal embryogenesis [15]. Cohnheim's theory that teratocarcinoma contains both differentiated and undifferentiated cells led to the proposal that the undifferentiated cells are multipotent cancer cells [16].

5.1.2 Clonal Evolution of Cancer

In the 1960s, many questions remained concerning the origin of CSCs for which the embryonic rests theory was insufficient to answer. How many normal cells contribute to the formation of tumor masses? Do tumor cells originate from a single ancestral cell that transformed from normal to abnormal? Is there a large population of normal cells that

undergo a transformation, and each develops a distinct subpopulation of cells within the tumor mass? The clonal evolution theory of tumor cells, therefore, evolved, based on the similarity of genetic and biochemical markers between tumor cells [17].

The clonal evolution theory states the following: if a particular gene mutation is present in all the tumor cells, this would suggest that all these tumor cells originated from a single mutated cell; this type of tumor is designated as monoclonal. In contrast, if a tumor mass contains different subpopulations of cells with different gene mutations, this would suggest that all tumor cells originated from various cells, and this tumor would be classified as polyclonal [17].

In the 1960s, Nowell and Hungerford discovered the Philadelphia (Ph) chromosome in patients with chronic myeloid leukemia (CML), resulting in the classification of this malignancy as monoclonal [18–20]. In the late 1960s, Fialkow demonstrated the monoclonal origin of blood cell lineages present in patients with CML [21]. His studies focused on female patients with CML, who were heterozygous for the X-linked glucose-6-phosphate dehydrogenase (*G6PD*) gene. Normal females heterozygous for different isoforms of *G6PD* alleles expressed approximately equal distributions of each isoform all over their somatic tissues. Conversely, in female CML patients heterozygous for *G6PD*, all neoplastic cells were found to contain only one isoform. These results showed that all mature blood cells of female CML patients with heterozygous *G6PD* originated from a single mutated cell, expected to be a mutated hematopoietic stem cell (HSC) [21].

5.1.3 The Concept of CSCs

By the early 1990s, teratocarcinoma was no longer regarded as the favorite model for studying issues in cell differentiation, as the results were not applicable in studying other types of cancer [22]. In 1994, T. Lapidote reported that a few rare cells of acute myeloid leukemia (AML) were capable of initiating leukemia after transplantation into mice. These cells also displayed high self-renewal activity, which is characteristic of stem cells [23]. In 1997, John E. Dick and Dominique Bonnet found that the tumor cells that appeared after transplantation were composed of a heterogeneous population of both nontumorigenic and tumorigenic cells, similar to the cells of the initial tumor, and they suggest that these cells were originated as a hierarchy from hematopoietic stem cells [24]. These data showed that the process of tumor development is similar to stem cell proliferation. CSCs from the transplanted tumor produced two different cell populations: one identical to the transplanted cells (self-renewal), and the other a less tumorigenic but a more differentiated cell. These cells have been identified in many solid tumors, including breast, brain, and colon cancers [6, 9, 25]. Concurrent with the reemergence of the CSC concept, it is now believed that tumors are hierarchically organized, similar to normal cells. This organization can be maintained by a population of cells responsible for tumor formation, known as "cancer stem-like cells" or "cancer-initiating cells." These cells have the same characteristics of normal stem cells, especially self-renewal, the ability to produce more

differentiated cells, and unlimited growth. However, CSCs produce fewer differentiated cells, which stops the differentiation process at a particular stage [26].

5.2 Heterogeneity of the Tumor Mass

Tumor heterogeneity refers to the various genetic and molecular characteristics of tumor cells. Heterogeneity can be classified as inter-tumoral heterogeneity and intra-tumoral heterogeneity.

5.2.1 Inter-Tumoral Heterogeneity

Inter-tumoral heterogeneity describes the observed variations between tumors of different tissues and cell types, including variations both between tumors of the same tissue from different patients, and variation between different tumors within the same individual. These differences are recognized by studying histology, gene and protein expression profiles, and other blood- and tissue-specific markers [27, 28].

5.2.2 Intra-Tumoral Heterogeneity

Intra-tumoral heterogeneity describes the observed variations within a single tumor by studying the phenotypic and genotypic profiles of the cells within the tumor mass [27, 29]. Intra-tumoral heterogeneity develops upon genetic and epigenetic alteration in tumor cells, leading to asymmetrical division, abnormal cell growth, metastasis, recurrence, and resistance [30–32]. Intra-tumoral heterogeneity has been validated by many experiments that demonstrated a difference in genetic, epigenetic, and cellular markers between cells within the same tumor [33–36].

Due to the heterogeneous clonal subpopulation within tumor masses, the method of cancer treatment can be more challenging. A treatment may be effective for one type of cancer cell, but not another. Long-term combination therapy could thus be effective in targeting both CSCs and other differentiated tumor cells [29, 37–39].

5.2.2.1 Models of Intra-Tumoral Heterogeneity

Two models are currently used to explain the origin of tumoral heterogeneity. They are known as the clonal evolution (CE) model, or stochastic model, and the CSC, or hierarchical model [28, 29, 40] (Fig. 5.2).

In the CE model, cancer cells are almost homogeneous and have equal genetic instability [41, 42]. Phenotypic heterogeneity in this model results from the exposure to intrinsic and extrinsic factors. Under these influences, some tumor cells acquire stemness and self-renewal behavior over time, due to the accumulation of genetic and epigenetic alterations

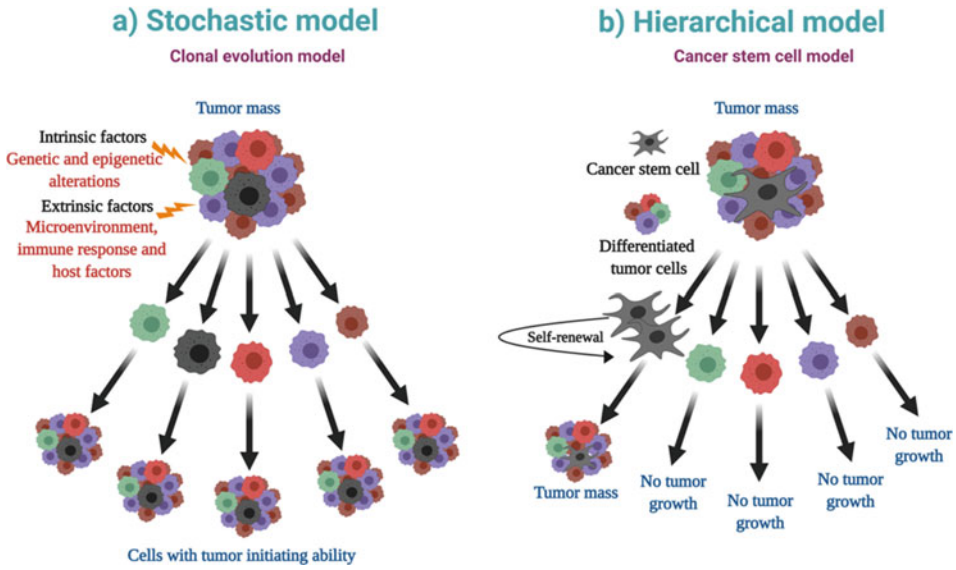

Fig. 5.2 Models of intra-tumoral heterogeneity. (**a**) *Clonal evolution model [Stochastic model]*: All tumor cells within the tumor mass are homogeneous biologically. However, they are different functionally due to intrinsic and extrinsic factors. Some tumor cells acquire the self-renewal and initiation of tumor formation abilities due to the accumulation of genetic and epigenetic alterations. (**b**) *Cancer stem cell model [Hierarchical model]*: A unique type of cells within the tumor, which are called cancer stem cells (CSCs) and acquires the mutations to be tumor initiator in addition to self-renewal ability. CSCs will give rise into new CSCs, which have the potential to divide unlimitedly producing new tumor cells and CSCs, and differentiated tumor cells which loss the potential to produce new tumor cells

[28, 29]. According to this model, isolation of tumorigenic subpopulations is not precise, since each cell has the potential to become tumor-initiating cell. Many current cancer treatment approaches are based on this model and target all cells that have the potential to initiate tumors [41]. Although this model has been widely accepted, it does not explain treatment resistance or recurrence of the same cancer in patients.

In the cancer stem cell model, CSCs in the tumor are hypothesized to be already acquiring the tumorigenic mutations to be tumor initiators and will give rise to terminally intermediate progenitors and differentiated progeny [28, 29, 40, 43]. This model may have more experimental support than the CE model [44]. Due to the biological and functional diversity between tumor cell populations, it is possible to isolate cancer initiator cells if the correct biomarkers are identified. Under this model, any residual CSCs that survived the cancer treatment course will generate a new cancer population, which explains tumor recurrence. Therefore, some current therapeutic approaches focus on targeting CSCs to find potential permanent solutions [41, 42, 45–47].

5.3 Factors Enhancing Cancer Initiation

The contribution of CSCs to cancer initiation occurs after the continuous exposure of normal stem, progenitor, or differentiated cells to mutagens and stress factors, such as high concentrations of reactive oxygen species (ROS), reactive nitrogen species (RNS), lipid peroxidation products (LPPs), or inflammatory chemokines and cytokines [48–52]. This prolonged exposure may result in the accumulation of genetic mutations as well as the failure to repair these mutations, leading to the conversion of mutated cells into CSCs [51, 52]. A recent study demonstrated the association between nuclear localization of cyclooxygenase-2 (COX2) and the upregulation of stemness markers, such as Oct4, Oct3, and CD44v6 [53]. COX2 promotes inflammation by activating prostaglandin E2 (PGE2) signaling, leading to activation of stem cells or CSCs [54]. Chronic inflammation, induced by microbes, stress, or diet, was a major culprit in these mutations. Infection with bacteria such as *Helicobacter pylori* or parasites such as *Schistosoma haematobium* was shown to initiate tumors following chronic inflammation. Inflammation leads to mutation of stem cells, in which NF-κB pathway activation results in upregulation of inducible nitric oxide synthase (iNOS), which contributes to 8-nitroguanine formation, a potential mutagenic DNA lesion. It was reported that 8-nitroguanine exists in Oct3/4-positive stem cells in cancer tissues. Thus, chronic inflammation plays a key role in cancer initiation via nitrative DNA damage [51, 54–56].

5.4 Characterization of CSCs

CSCs are characterized based on cell surface markers and overexpression of some transcription factors (TFs). Each tissue expresses its own unique CSC markers. Since TFs expressed in pluripotent cells (OCT4, SOX2, KLF4, Nanog, and SALL4) are not expressed in somatic cells, overexpression of these markers in tumor cells suggests a presence of CSCs [57]. Importantly, the level of expression of these transcription factors has been shown to be associated with tumor survival, meaning that the identification of these TFs could be utilized in cancer prognosis [58]. Oct4, SOX2, Nanog, SALL4, and c-Myc expression levels were shown to correlate with poor diagnosis of some cancers, such as bladder cancer, lung adenocarcinoma, colorectal, glioma, and hepatocellular carcinoma [59–63]. KLF4 overexpression also correlated with aggressive early stage of breast cancer [64].

The most well-documented markers for CSCs include CD44 and CD133 [63]. Both CD44 and CD133 are membrane glycoproteins that play several roles in migration, metastasis, and chemoresistance [30, 65, 66]. Other CSC cell surface markers include TRA-1-60, SSEA-1, EpCam, ALDH1A1, Lgr5, CD13, CD19, CD20, CD24, CD26, CD27, CD34, CD38, CD44, CD45, CD47, CD49f, CD66c, CD90, CD166, TNFRSF16,

CD105, CD133, CD117/c-kit, CD138, CD151, and CD166. Other CSC markers that are not expressed on the cell surface and are not TFs include ALDH, Bmi-1, Nestin, Musashi-1, TIM-3, and CXCR. The enzyme aldehyde dehydrogenase (ALDH) increases the rate of irreversible oxidation of cellular aldehydes in the cytoplasm. The expression of ALDH1A1 by CSCs results in expansion, self-renewal, and proliferation of the CSCs population [67]. BMI1, Nestin, and Musashi-1 proteins also play roles in CSC self-renewal, maintenance, and malignancy. Nestin enhances the proliferation of blood vessels sustaining the angiogenesis process [68, 69].

CSC maintenance, self-renewal, and differentiation are controlled by several signal transduction pathways, including Hedgehog (HH), Notch, JAK/STAT, and Wnt/β-catenin. The HH pathway is involved in activation and nuclear localization of TFs, which then activate the genes responsible for survival, proliferation, epithelial–mesenchymal transition (EMT), and angiogenesis, as well as self-renewal of CSCs [70]. The Notch signaling pathway is involved in the induction of EMT, acquiring chemoresistance, tumor immunity, and maintenance of the CSC population [71, 72]. The JAK-STAT signaling pathway activates cytokines such as IL-6, which in turn activates IGF, bFGF, EGF, and EGFR, which are also responsible for EMT induction. The JAK-STAT signaling pathway is also involved in activation of telomerase in CSCs [73]. Wnt/β-catenin signaling is important in CSCs generation and self-renewal via stimulation of de-differentiation of cancer cells [74]. Dysregulation of Wnt/β-catenin signaling was shown to be associated with expansion of the CSC population [75]. Tables 5.1 and 5.2 illustrate the CSC surface markers and transcriptional factors.

5.5 CSCs and Dormancy

Dormant cells are those cells appearing as not proliferating, but they sustain their viability. There is growing evidence that tumor dormancy represents the proverbial other side of the coin to the concept of CSCs. Many features are common between CSCs and dormant tumor cells; both are minimal residual cells capable of metastasis, can survive tumor therapy, and promote tumor relapse in the appropriate pro-tumor microenvironment [115]. Also, cell cycle control and modulation of angiogenic and immune microenvironment are biological mechanisms that impact both cells in similar ways [116]. Lately, and in contrast to previous hypotheses that they are indeed cancer dormant cells, CSCs can be classified into dormancy-competent CSCs (DCCs), cancer-repopulating cells (CRCs), dormancy-incompetent CSCs (DICs), and disseminated tumor cells (DTCs) [117]. Repeated cycles of primary tumor therapy induce tumor cell plasticity to resist the therapy and enter dormancy. This state can be maintained for years or even decades. These dormant residual cells can exit dormancy to lead neoplastic relapse, differentiation, and metastasis and facilitate distant tumor progression and growth [117, 118]. Accumulation of several genetic mutations in DCCs drives deprivation of their dormancy potential, then these cells become CSC of more ability to initiate tumor growth (DICs) [117]. A dormant cell population in tumors can be

Table 5.1 Surface markers of cancer stem cells

Cell surface marker	Alternative names	Malignancy	Reference
CD44	Extracellular matrix receptor III (ECMR-III)	Osteosarcoma Ovarian Pancreatic Prostate Leukemia Bladder Breast Colon Gastric Glioma/Medulloblastoma Head and Neck	[76–78]
CD133	Prominin-1	Breast Colon Glioma/Medulloblastoma Liver Lung Melanoma Ovarian Pancreatic	[79, 80]
CD24		Breast Colon Liver Ovarian Pancreatic	[81–83]
CD15	Lewis X	Glioma/Medulloblastoma	[84]
CD117	C-kit	Leukemia Lung Ovarian	[85–89]
CD90	Thy1	Glioma/Medulloblastoma Liver Lung Breast	[90–94]
CD38	Cyclic ADP ribose hydrolase	Myeloma Leukemia	[95–98]

heterogeneous and comprise a non-CSC population, but nevertheless they are resistant to therapy. Cancer stemness and tumor dormancy intersect in many key characteristics such as plasticity, heterogeneity, and regulation, but more studies are required to understand their regulatory mechanisms.

Table 5.2 Cancer stem cells' transcriptional factors

Transcription factor	Alternative name	Malignancy	Reference
OCT4	Oct3/4 or POU5F1	Leukemia Brain Lung Bladder Ovarian Pancreas Prostate Renal Seminoma Testis	[99, 100]
SOX2		Brain Breast Lung Liver Prostate Seminoma Testis	[101–106]
KLF4	Kruppel-like factor 4	Leukemia Myeloma Brain Breast Head and neck Oral Prostate Testis	[107–109]
Nanog		Brain Breast Prostate Colon Liver Ovarian	[110, 111]
C-MYC		Leukemia Lymphoma Myeloma Brain Breast Colon Head and neck Pancreas Prostate Renal Salivary gland Testis	[112]

(continued)

Table 5.2 (continued)

Transcription factor	Alternative name	Malignancy	Reference
SALL4		Leukemia Breast Liver Colon Ovarian Testis	[113, 114]

5.6 CSCs' Metabolomics

CSCs have special metabolic profile of glucose [119]. Unlike differentiated cancer cells that preferably use glycolysis as their main metabolic pathway, CSCs exhibit glycolysis and oxidative phosphorylation (OXPHOS) [120]. Hypoxia and glucose level are responsible for this metabolic switch, allowing the maintenance of CSCs [121]. It was proved that upregulation of glycolytic enzymes such as GLUT1, HK-1, and PDK-1 is important for CSC immortalization [122]. Furthermore, the glucose utilization, lactate synthesis, and ATP content in CSCs are higher than cancer cells [119, 123]. Switching of CSCs from OXPHOS into glycolysis promotes stemness via acquiring of the pluripotency markers [124].

Interestingly, OXPHOS is a preferred metabolic pathway in CSCs [125]. CSCs have high mitochondrial mass and membrane potential. This results in enhanced mitochondrial reactive oxygen species (ROS) and higher rates of oxygen consumption [126, 127]. The bulky mitochondria promote self-renewal [128], metastatic potential, and resistance to DNA damage [129]. This high mitochondrial mass is associated with upregulation of fatty acid oxidation (FAO) and OXPHOS genes [126]. FAO induction contributes to self-renewal, survival, and drug resistance of CSCs [130]. Nanog was shown to induce activation of FAO genes contributing to vigorousness of the aforementioned CSC characteristics [131, 132]. Furthermore, CSCs make use of the glycolytic metabolic wastes of the differentiated cancer cells such as lactate, which incorporate in OXPHOS to produce more energy [131].

In a nutshell, there are still controversial data regarding CSC metabolism, with some suggesting that the cells are predominately glycolytic over their utilization of oxidative phosphorylation pathways, while other data suggest the opposite. We here conclude that CSCs prefer OXPHOS pathway rather than glycolysis due to its tremendous energy production capabilities. While in hypoxic microenvironment, the CSCs metabolically switch into glycolysis.

5.7 Role of CSCs in Tumor Growth, Angiogenesis, and EMT

As tumor size increases, the need for oxygen and blood supply also increases. Aggressive recruitment of new blood vessels is one mechanism that CSCs use to nourish and supply the tumor with sufficient oxygen and nutrients. CSCs modulate the tumor niche by activating signaling pathways such as Notch, Wnt, and Shh pathways, which are responsible for the maintenance of stemness, angiogenesis, and long-term cell survival [133]. CSCs also send signals to recruit and alter the function of the surrounding tumor-associated cells (TACs) in the favor of tumor progression [134]. These TACs are composed of stromal cells, such as cancer-associated fibroblasts (CAFs), mesenchymal stromal cells (MSCs), and endothelial cells, along with immune cells, including tumor-associated macrophages (TAM), tumor-associated neutrophils (TANs), and myeloid-derived suppressor cells (MDSCs) [43, 52, 135, 136]. CSCs and TACs collectively form a lattice that serves to maintain CSC function and cancer aggressiveness, proliferation, and recurrence [137].

Epithelial to Mesenchymal Transition (EMT)

Is a reversible process whereby epithelial cells lose its cell polarity, adhesion properties, and transform into mesenchymal cells acquiring invasive properties.

CAFs have higher capacities than normal fibroblasts to proliferate and stimulate the production of more extracellular matrix (ECM) proteins. CAFs also secrete a number of growth factors and cytokines, such as transforming growth factor-β1 (TGF-β1), which is a key player in regulation of epithelial–mesenchymal transition (EMT) in cancer cells and cancer stem cells (CSCs) [138–142]. They also secrete chemokine (C-X-C motif) ligand 12 (CXCL12) and stromal-derived factor-1 (SDF-1) [143, 144]. These factors lead to differentiation of more CAFs, increased tumor stemness, and tumor progression [145]. Several studies have showed that CAFs have a mesenchymal origin as opposed to normal fibroblasts. In vitro direct co-culture of colon cancer cells or breast cancer cells with bone marrow MSCs showed that the latter to adopt a CAF phenotype and exhibit typical CAF characteristics [146, 147]. Cancer cells promote the production of several molecules that facilitate the transformation of fibroblasts and MSCs into CAFs. These include basic fibroblast growth factor (bFGF), TGF-β, platelet-derived growth factor (PDGF), and interleukin-6 (IL-6) [148–153]. Recent studies showed that transformation of MSCs and fibroblast is not the only source of CAFs. Interestingly, it was reported that CSCs can

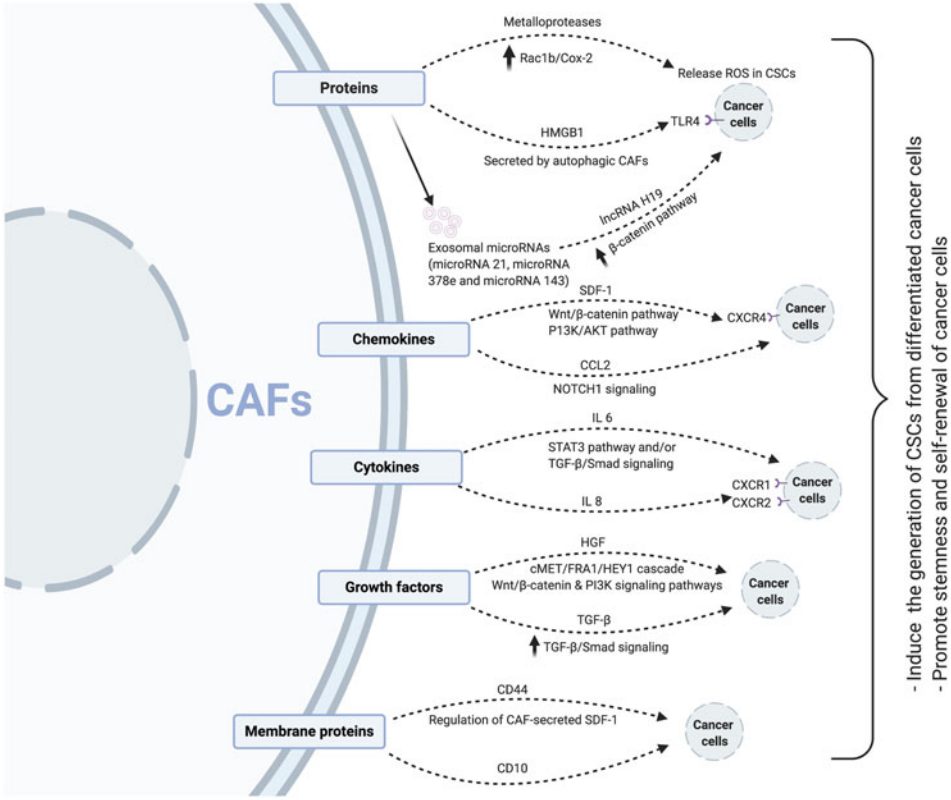

Fig. 5.3 The crosstalk between CAFs and CSCs: Showing the different factors secreted by CAFs promoting the stemness of differentiated cancer cells, thus inducing generation of CSCs

differentiate into CAFs via secretion of transforming growth factor-β (TGF-β) and fibroblast growth factor 5 (FGF5), for the purpose of metastasis [154–156]. In addition, CAFs can contribute to the de-differentiation of cancer cells into CSCs via activation of the Wnt signaling pathway in cancer cells, mediated by hepatocyte growth factor (HGF) secretion from CAFs [157]. In a nutshell, CAFs can enhance the generation of CSCs, as CAFs are able to induce the expression of stemness markers (e.g., Sox2, Bmi-1, and CD44), to promote self-renewal and expansion of CSCs. These processes are also maintained through secretion of several factors, such as proteins (including chemokines, cytokines, and growth factors) and exosomes or the direct cell–cell contact with tumor cells. [140]. The different factors and pathways integrated in this transformation are illustrated in Fig. 5.3.

We can conclude that there is a bi-directional loop between cancer cells and CAFs with incorporation of TGF-β in both directions and other signaling pathways, thus sustaining the self-renewal and stemness of CSCs as shown in Fig. 5.4.

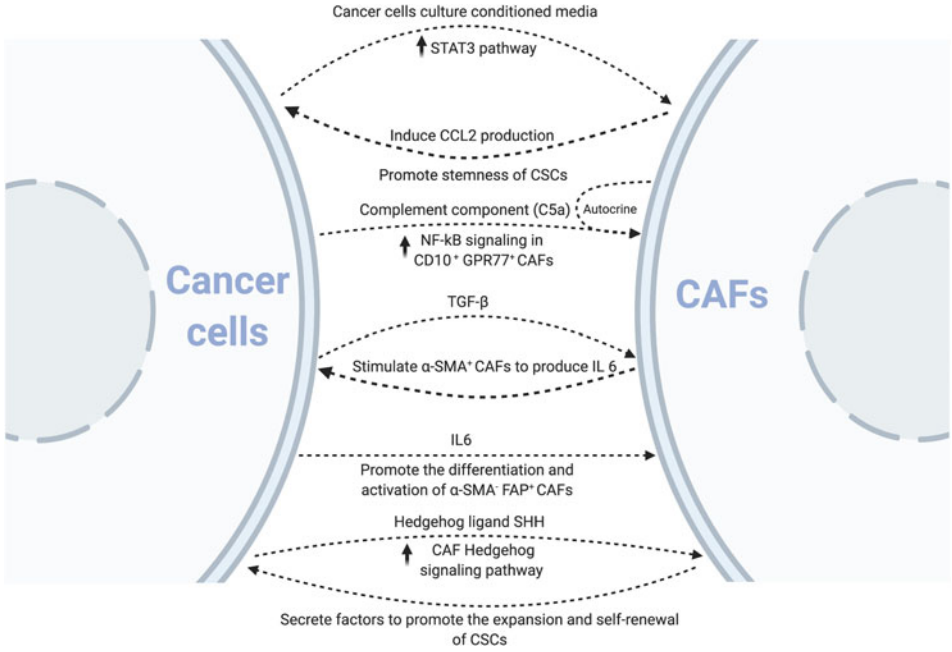

Fig. 5.4 Bi-directional feedback loop between cancer cells and CAFs enhancing generation of CSCs

The plasticity and stemness of CSCs are modulated by the paracrine secretions of TGF-β, stromal-derived factor-1 (SDF-1), and matrix metalloproteinase 9 (MMP9) from CAFs, resulting in the induction of EMT [144, 145, 149].

Although pathological data are still lacking, a number of studies have suggested that CSCs originate from transformation of tissue-resident stem cells, such as hematopoietic stem cells (HSCs) and MSCs [152, 158–161]. MSCs in particular have a dual role in the spread of cancer, not only via their own transformation into cancerous cells but also via supporting the development of CSCs. IL-6 secreted by CSCs attracts MSCs that stimulate the production of key cytokines, such as CXCL7, which suppresses the immune system and enhances the tumor growth [162]. Moreover, CXCL12 secreted from MSCs activates the NF-κB signaling pathway and enhances production of more interleukins, such as IL-6 and IL-8. These molecules were shown to play a role in CSC stemness [163, 164]. It has been shown that IL-6 regulates CSC-associated pluripotent OCT-4 gene expression through the IL-6-JAK1-STAT3 signal transduction pathway to transform non-CSCs into CSCs [165, 166]. It was also reported that IL-8 regulates the expression of CSC markers through the IL-8/CXCR1 axis [167–169]. MSCs upregulate the expression of microRNAs related to CSC maintenance and survival, such as miR-199a [170]. MiR-199a prevents CSC differentiation and senescence to maintain stem-like properties [170]. Furthermore, it was shown that CSC-associated MSCs downregulate Forkhead box protein P2 (FOXP2), a

transcription factor that plays a role in tumor suppression, to vigorously increase tumor proliferation [171, 172].

The crosstalk between CSCs and MSCs is also mediated by extra-vesicular molecules (EVs) called exosomes. CSC exosomes deliver proteins and miRNAs that drive the MSCs to enhance angiogenesis and invasiveness and even to activate dormant cancer cells [3, 173]. However, EVs derived from MSCs were shown to have a dual effect on tumors by either promoting growth or triggering apoptosis [3, 173]. EVs secreted by MSCs were found to activate ERK1/2 signaling pathways responsible for maintaining cell proliferation and survival in renal carcinoma. ERK1/2 pathways are reported to enhance the progression of the cell cycle from G0/G1 to S phase [174]. Also, the metastatic phenotype of tumors and enrichment of CSC characteristics were shown to be achieved through activating ERK1/2 and Wnt/β-catenin pathways [175, 176]. However, microvesicles derived from MSC exosomes can trigger apoptosis in cancer cells via blockage of cell cycle progression, resulting in cell cycle arrest at the G0/G1 phase. It is noteworthy that there is little evidence in the literature to support a direct effect of MSC-derived microvesicles on CSCs [177, 178].

Chronic inflammation provides an ideal microenvironment for tumor initiation, progression, and immune evasion [135]. CSCs secrete IL-13 and IL-34, which recruit tumor-associated macrophages (TAMs), tumor-associated neutrophils (TANs), and myeloid-derived suppressor cells (MDSCs). These immune cells are strongly involved in tumor progression and immune suppression [135, 136]. In glioblastoma, CSCs were shown to secrete periostin (POSTN) to activate M2 macrophages to become pro-tumor cells. The inhibition of POSTN leads to the inhibition of the tumor-supportive TAMs in xenografts [179]. TAMs produce specific chemo-attractants such as CCL24, CCL17, CCL20, and CCL22 in order to increase the angiogenesis, thereby promoting tumor growth [180, 181]. TAMs also upregulate EMT-associated TFs, such as *Slug*, *Snail*, and *Twist*, via TNFα and NF-κB signaling pathways. These TFs stimulate the TGF-β signaling pathway, promoting self-renewal, migration, and invasion of CSCs [182, 183]. The TGF-β signaling pathway has a complex role in tumor pathogenesis, as it is associated with cancer cell stemness, tumor chemoresistance, and metastasis via regulation of EMT [184–187].

Hypoxia is a main feature in the cancer microenvironment. Cancer transformation activates hypoxia-inducible factors (HIFs), including HIF1α and HIF2α, which increase survival, proliferation, metabolism, EMT, angiogenesis, and metastasis of the tumor [188, 189]. CSCs rely on HIF2α [190], which is responsible for the response to chronic hypoxia [191]. The activation of HIFs by CSCs plays a role in activating TGF-β, Wnt/β-catenin, TNFα, and NF-κB signaling [192–194] to induce EMT as well as associated TFs such as SNAIL, TWIST, ZEB1, SLUG, and TCF3 [195, 196].

Both tumor-associated endothelial cells and pericytes secrete proangiogenic factors and activate juxtracrine (contact) signaling, to maintain CSC survival and phenotype [197–200]. The Notch and Sonic Hedgehog pathways are activated in CSCs via Jagged-1 and Shh ligands secreted by endothelial cells to maintain CSC self-renewal [201]. Lymphatic

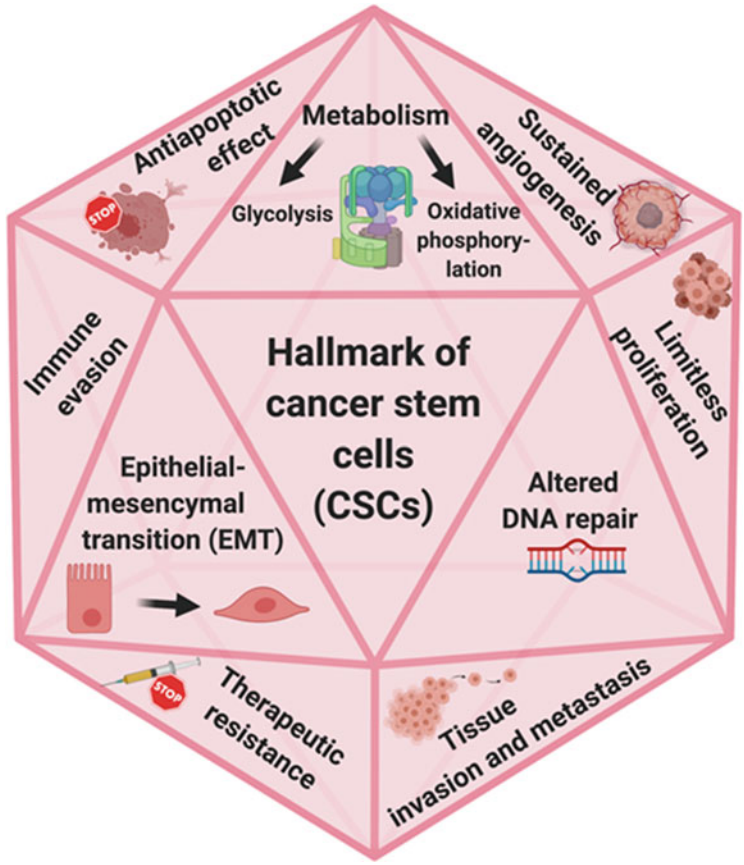

Fig. 5.5 Hallmarks of cancer stem cells (CSCs)

endothelial cells secrete CXCL1 to promote angiogenesis and increase the severity of metastasis. They also express CCL21 that enhances vascular permeability and tumor metastatic potential. CCL21 also mediates recruitment of immature dendritic cells, which play a central role in tumor immune evasion [202, 203].

In conclusion, CSCs play a critical role in tumor initiation, growth, and metastasis. Distinct characteristics that define the aggressiveness of CSCs and their tumor initiation and proliferation ability are shown in Fig. 5.5.

5.8 CSCs and Chemoresistance

The mechanisms underlying tumor recurrence are still largely unknown. Recurrent tumors are typically more aggressive and resistant to therapies. CSCs may play an essential role in chemoresistance, both intrinsic (de novo) and acquired [204]. Chemoresistance may be

achieved by many mechanisms that support the inherent ability of CSCs to resist various traditional treatments. For example, CSCs express proteins such as ATP-binding cassette (ABC) transporter proteins [204–206], enhance aldehyde dehydrogenase (ALDH) activity [207, 208], and increase expression of anti-apoptotic proteins such as Bcl-2 and Bcl-XL [209]. CSCs promote DNA repair in cancer cells to resist the chemotherapeutic agents causing DNA damage-induced cancer cell death. Thus, CSCs activate DNA damage checkpoints such as CHK1 and CHK2, which maintain CSC survival and drug resistance [210]. CD133-positive CSCs in neuroblastoma, ovarian cancer, and colorectal cancer were found to exhibit chemoresistance and were associated with poor clinical prognosis [211–213]. The Wnt signaling pathway was also found to play a role in CD133-induced chemoresistance [214, 215]. Metastatic cancers highly express CXCR, which promotes invasion, migration, recurrence, and therapeutic resistance [216, 217]. CSCs are mostly metabolically reprogrammed to depend on the glycolytic pathway more than oxidative phosphorylation (OXPHOS) to maintain their energy resources, survival, and proliferation. Metabolic reprogramming determines the fate of CSCs that eventually contribute to chemoresistance. During hypoxia, glycolysis-associated genes in CSCs, such as GLUT1 transporters, are upregulated, in addition to the resetting of their mitochondrial activity [218–220].

EMT is a key process in increasing cancer proliferation, angiogenesis, and metastasis, as well as maintenance of stemness [221]. EMT regulators, such as ZEB1, promote chemoresistance via miR-200c and c-MYB, which upregulate O-6-methylguanine DNA methyltransferase (MGMT), resulting in increased tumor aggressiveness [222]. Tumor side population (SP) cells possessing cancer stem-like characteristics can express various drug resistance proteins, including MDR1, ABCG2, and ABCB1. These proteins expel cytotoxic therapeutics outside cancer cells, contributing to chemoresistance and survival, as well as tumor recurrence [223–225]. The PI3K/Akt pathway, which is known to be activated in CSCs, was shown to control ABCG2 activity by localizing this protein to the plasma membrane [226]. Also, the mutations in phosphatase and tensin homolog (PTEN) that inhibit the PI3K/Akt pathway also promoted the SP phenotype, thus enhancing drug resistance and metastasis [227]. The functional activity of ALDH, characterizing an intracellular, metabolic marker for most CSCs [228], has been widely used to identify and isolate CSCs in a number of malignancies, such as pancreatic, ovarian, and breast cancer [229–235]. ALDH was shown to be expressed in $CD34^{+}/CD38^{-}$ leukemic stem cells and contributed to drug resistance [236, 237]. In addition, $ALDH1A1^{+}$ CSCs were shown to have greatly enhanced the resistance to various anti-cancer chemotherapeutics, such as EGFR-TKI (gefitinib), cisplatin, etoposide, and fluorouracil [238, 239]. A balanced level of ROS is a key mediator for CSC survival and tumorigenicity [240]. ALDH1 was reported to reduce ROS stress and protect the cells from its toxic effects. Also, the CD44 surface marker expressed on CSCs interacts with glutamate–cystine transporter and upregulates its expression in response to ROS [241]. This mechanism keeps the ROS levels low in CSCs and is mainly associated with activation of radical scavenging systems leading to more resistance to chemo- and radiotherapy [242].

CSC dormancy is another key contributor to chemoresistance. Chemotherapeutic drugs induce a damage in the cancer cells. As a consequence to the damage in the tumor mass, the dormant CSCs will be recruited for tumor repair and repopulation of the tumor [243] and hence, more tumor proliferation and drug resistance [244, 245].

In addition to the previously named factors, the tumor microenvironment plays an important role in cancer resistance to therapy. For example, the hypoxic microenvironment in tumors induces the expression of hypoxia-inducible factor-α (HIFα). HIF, especially HIF1α, binds to the hypoxia responsive element (HRE) sequence and activates the target genes, which enhances the cancer aggressiveness and drug resistance [246]. This binding enhances the expression of VEGF, IL-6, and CSC genes, such as Nanog, Oct4, and EZH2, leading to angiogenesis, migration, and stemness promotion, respectively [247]. CAFs, however, contribute to chemoresistance via activation of the IGF-1/ERβ signaling axis, which promotes the expression of anti-apoptotic genes such as Bcl-2 [248]. The profile of exosomal contents secreted by TAMs has not yet been identified, although it was found that these exosomes contain miRNA-21, which promotes drug resistance of CSCs [249–252]. In colorectal cancer, it was reported that microRNA-21 induces chemoresistance via repression of human DNA MutS homolog 2 (hMSH2), which is involved in DNA damage recognition and repair [251].

In addition, epigenetic modifications further contribute to drug resistance. Histone modification and DNA trimethylation of histone H3 at the residue Lys4 (H3K4Me3) were shown to contribute to Lsd1-regulated chemoresistance [253]. Furthermore, the epigenetic silencing of E3 ubiquitin ligases TRIM17 and NOXA upregulated MCL-1 to enhance the immune evasion and chemoresistance of CSCs [254]. Wnt/β-catenin pathway is activated in the resistance to combination therapy of IFN-α/5-FU [255], and the Notch signaling pathway is also highly activated in CSCs and known to promote the multidrug resistance in $CD133^+$ cells [256].

ABC transporters

Are pore like transmembrane found in all living organisms.

1. It is over-expressed in CSCs acting as efflux pump to get rid out of chemotherapy from CSCs.
2. Protect CSCs against xenobiotics.
3. Maintain low ROS in CSCs by transporting antioxidants such as Glutathione (GSH).

Aldehyde dehydrogenases (ALDHs)

Are a family of mitochondrial enzymes that catalyzes the detoxification of aldehydes into carboxylic acids via oxidation.

ALDH contributes to:
- Chemoresistance in CSCs.
- Reduction of oxidative stress in CSCs.

5.9 Epigenetic Regulation of Cancer Stem Cells

Epigenetic regulation mechanisms, including histone modifications, DNA methylation, chromatin remodeling, and changes in microRNA (miRNA) [257–259], all greatly influence CSC development, differentiation, and characteristics [260].

5.9.1 Methylation Patterns in CSCs

5.9.1.1 CpG Island Methylation of DNA

DNA methylation entails adding a methyl group at the 5′ position of the cytosine residues of CpG dinucleotides, which are highly concentrated in the CpG islands over the length of the genome, by DNA methyltransferases (DNMTs) [261]. DNMT1 transfers a methyl group onto hemimethylated DNA during cell division, while DNMT3 (DNMT3A and DNMT3B) targets the unmethylated DNA during cellular development [262]. The demethylation process occurs through a series of chemical reactions. One important mechanism is controlled by methylcytosine dioxygenases, known as ten-eleven translocases (TET1 and TET2), which initiate methylation by converting 5-methylcytosine (5-MC) into 5-hydroxymethylcytosine (5-HMC)[263]. The methylation pattern of CpG islands in normal somatic cells differs from those in ESCs, induced pluripotent stem cells (iPSCs), and CSCs [260]. For instance, the CpG islands in promoter regions of differentiation-specific genes, such as the TERT gene [264], are hypermethylated in pluripotent cells, while those promoters would be hypomethylated in somatic cells. In this exceptional case, the hypermethylation in iPSCs enhanced the expression of TERT, and vice versa in somatic cells.

DNMTs are responsible for reparation of DNA damage, especially the DNMT3A [265]. Mutations in one of these genes would lead to tumorigenesis [266, 267]. In AML patients, the mutation of DNMT3A leads to the expansion of leukemic stem cells (LSCs) [268–273]. Also, the loss of function in TET proteins due to mutation can lead to the expansion of LSCs [266, 268]. Mutations in DNMTs during the early tumorigenesis have been observed in both leukemia and solid tumors [274]. Regardless of the type of mutation in epigenetic machinery, a common outcome may be a disturbance of the differentiation program. This disturbance also manifests via altering the balance between the expression of tumor suppressor genes and proto-oncogenes.

The Wnt/β-catenin signaling pathway was shown to contribute to the maintenance and tumor formation abilities of CSCs. Atypical DNA methylation activates the Wnt/β-catenin signaling pathway via facilitation of promoter methylation of Wnt/β-catenin inhibitors, such as Wnt inhibitory factor 1 (WIF-1), AXIN2, secreted frizzled-related protein 1 (SFRP-1), and Dickkopf-related protein 1 (DKK1) [275–277].

5.9.1.2 Histone Methylation and Acetylation

Histone methylation refers to the addition of a methyl group on lysine (K) and/or arginine (R) residues of histone proteins. Methylation status can be associated with gene activation or suppression [278, 279]. For example, methylation of histone H3 lysine 4 (H3K4), histone H3 lysine 36 (H3K36), and histone H3 lysine 79 (H3K79) is representative of the activation of gene expression activation. In contrast, methylation of histone H3 lysine 9 (H3K9) and histone H3 lysine 27 (H3K27) is involved in gene expression suppression. Any modifications or mutations in histone methylation mechanisms can thus lead to disease and malignancies [279]. ESCs sustain a "bivalent chromatin state" by having active chromatin mark H3K4me3 (H3K4 trimethylation) and inactive chromatin mark H3K27me3 (H3K27 trimethylation).

A disruption in epigenetic regulation machinery, especially histone methyltransferases (HMT), may lead to genomic instability. As a result, some stem cells gain enhanced properties such as stemness and self-renewal ability, thus generating CSCs.

The methylation state of histones is controlled by multiple histone modifiers, for example, enhancer of zest homolog 2 (EZH2), which trimethylates H3K27 (H3K27me3) and promotes the inhibition of transcription machinery [280]. EZH2 is linked to CSCs and, doing several functions that maintain CSC properties. For example, in colorectal cancer, EZH2 was found to induce some stem cell-associated genes such as Nanog and Sox2 in a $CD133^+/CD44^+$ subpopulation of SW480 cells [281]. Furthermore, EZH2 enhances the trimethylation of H3K27 leading to the turning off of transcriptional machinery via chromatin compaction in CSCs [282]. Chromatin compaction is also mediated via direct binding of EZH2 to different DNA methyltransferases (DNMTs), such as DNMT3A, DNMT3B, and DNMT1, to methylate CpG sites [282, 283]. Moreover, EZH2 showed the ability to initiate, maintain, and enhance the survival of CML[284]. This was achieved via H3K27me3 targets in CML stem cells, leading to PRC2 dysregulation in CSCs [285]. In addition, the bone morphogenetic protein (BMP) signaling pathway, which regulates differentiation, was shown to be inhibited by EZH2 via inhibiting expression of BMPR1B in CSCs, thus enhancing stemness and inhibiting differentiation [286].

Earlier studies have reported that, in both breast cancer and large B cell lymphomas, EZH2 is hyperactivated. This hyperactivity promotes the trimethylation of H3K27me3, switching the transcriptional machinery toward the repressed state at the promoters of differentiation-related genes (e.g., p16, p19, and anti-metastatic genes such as E-cadherin) [287]. The hyperactivating mutation in EZH2 and subsequent trimethylation H3K27 contribute to human mammary epithelial cell transformation in early lymphomagenesis [288–290]. Conversely, in other types of cancer, EZH2 loses its activity, due to histone variant mutations, which reduces the levels of H3K27me3. This mutation led to reprogramming of the cells to a more primitive stem-like state [291, 292]. These data indicate that dysregulation of H3K27me3 is the driving force for CSC induction. However, KDM1A, a flavin adenine dinucleotide (FAD)-dependent lysine-specific demethylase, silences genes by functioning as a histone demethylase. KDM1A is considered a regulator of MLL-AF9 LSCs, where its oncogenic properties block their ability to differentiate [293].

Acetylation also plays a role in CSC-related drug resistance. Nanog was reported to upregulate histone deacetylase 1 (HDAC1) by binding to its promoter and decreasing K14 and K27 histone H3 acetylation. Accordingly, stem-like features were achieved through epigenetic repression of cell cycle inhibitors CDKN2D and CDKN1B [254]. Furthermore, HDAC1 and HDAC2 are associated with maintained expression of pluripotent TFs such as Oct4, Nanog, Esrrb, and Rex1 in CSCs as well as self-renewal [294]. HDAC3 expression in CSCs is associated with sphere and clone formation [295]. Sirt1, a nicotinamide adenosine dinucleotide (NAD)-dependent deacetylase, is also expressed in CSCs and plays an important role in inhibiting P53 (a cancer suppressor gene) via deacetylating the C-terminal Lys120, Lys164, and Lys382 residues [295–298].

Histone acetyltransferases (HATs) in CSCs were shown to maintain expression of stemness-related core TFs including Nanog, Oct4, and Sox2, which are methylated in adult and differentiated cells [299, 300]. Colony stimulating factor 1 receptor (CSF1R) is activated in CSCs by monocytic leukemia zinc finger MOZ, a MYST family histone acetyltransferase (HAT), leading to maintenance of LSCs [301]. In addition, the interaction of MOZ with transcriptional intermediary factor 2 (MOZ-TIF2) is shown to enhance transformation of HSCs causing an increase in leukemic stem cells via the STAT5 signaling pathway [301, 302].

5.9.2 Chromatin Remodeling

Mammalian cells have developed several mechanisms to keep DNA compacted into the chromatin nucleosomes. To regulate expression of desired genes, the chromatin must be modified to allow TFs to recognize the gene promoters on the DNA. Chromatin modifiers must thus keep the transcriptional machinery of the cell under strict control. Chromatin modifiers also play a critical role in the maintenance of stem cell proliferation and differentiation into a specific type of cells. Since CSCs may originate from normal stem cells, any mutation in the chromatin modifiers may induce CSCs formation [260]. To change the chromatin structure, a group of chromatin remodeling factors restructure the nucleosome in ATP-dependent manner. Four common families of nucleosome remodelers, ISWI, SWI/SNF, INO80, and CHD, are involved in controlling the chromatin structure of the DNA [303]. Mutations mostly affect the SWI/SNF complex, and studies have shown that, this mutation is associated with some pediatric tumors. For example, a mutation of SMARCB1 subunit of the SWI/SNF complex could lead to rhabdoid tumors by blocking the differentiation and activating tumorigenic signaling [304–306]. This is caused by the involvement of SWI/SNF complex in the oncogenic reprogramming of CSC self-renewal [307]. Mutation in the ARID1A subunit of the SWI/SNF complex was reported to be associated with tumor invasion and metastasis-promoting colon cancer [308]. Zinc finger and SCAN domain containing 4 (ZSCAN4) causes histone 3 hyperacetylation at the promoters of OCT3/4 and Nanog, leading to an increase in these pluripotency factors and maintaining CSC stemness [309]. ZSCAN4 also maintains the function of telomeres

[310]. The NURF (nucleosome remodeling factor) complex is highly expressed in liver CSCs. It promotes the self-renewal of CSCs via OCT4 induction [307]. In addition, some histone variant modifications such as acetylation of H2A.Z (acH2A.Z) contribute to CSC self-renewal [307].

5.10 Immune Evasion of CSCs

The enhanced ability of CSCs to give rise to new tumors suggests that these cells may also have an advantage in evading immune detection and elimination. In the early 1900s, Paul Ehrlich introduced his magic bullet theory hypothesizing that cancer would occur at high rates if it was not seen by the surveillance exerted by the immune system [311]. Immune evasion is a crucial trait for cancer sustainability, and CSCs are important players in this process. The four main discovered mechanisms in immune evasion include alteration in expression of immune cell-activating molecules, activating inhibitory immune cells, manipulation of macrophages into TAMs, and inhibition of the immune checkpoints.

5.10.1 Altering the Expression of Immune Cell-Activating Molecules

CSCs can alter the expression of some immune cell-activating molecules. For example, natural killer surface receptor (NKG2D) enhances the cytotoxic response to cancer cells that express NK cell-activating receptor ligands (NKG2DLs) [312]. In acute myeloid leukemia, immune evasion was established by inhibiting the expression of NKG2DLs or secreting soluble NKG2DLs. This soluble form has the potential to downregulate NKG2D receptor binding on NK cells, resulting in the deactivation of their immune response [313]. It was shown that the chemotherapy-resistant leukemia stem cells (LSCs) have low expression of NKG2DLs, which was associated with the expression of poly-ADP-ribose polymerase 1 (PARP1). Genetic or pharmacologic inhibition of PARP1 was accompanied by induction of NKG2DLs on the LSC surface [314]. However, other compounds that have previously been reported to induce NKG2DLs, such as retinoic acid, valproic acid, or 5-azacytidine, did not show a similar effect [315–317].

Interleukin (IL) secretion by CSCs plays an important role in immune evasion. Interleukins can exhibit a pro- or anti-tumor effect [318]. A specific polypeptide in the IL27p28 subunit, known as IL-30, expressed in prostate cancer, was reported to suppress the differentiation of anti-tumor type 1 helper cells (Th1). IL-30 was also shown to limit the function of IL-27 in stimulating natural killer (NK) cells, Th1, and cytotoxic T lymphocytes (CTLs) leading to their impairment [318–320]. IL-4 also interferes with the cytotoxic T lymphocytes, resulting in the promotion of tumor progression [321].

Some other antigen-presenting cells such as DCs are also key players that facilitate chemoresistance and tumorigenesis of CSCs [322]. This is achieved in follicular lymphoma by binding of DCs to CSCs through CXCL2/CXCR4 signaling axis [323]. CSCs inhibit the

antigen-presenting pathways of antigen-presenting cells (APCs), including DCs, by lowering the expression of transporter associated with antigen processing (TAP) heterodimer. TAP is responsible for transporting endocytosed and digested peptides from endoplasmic reticulum to be presented on immune cells [324, 325]. Moreover, CSCs expressing CD44 were shown to downregulate the expression of MHC-I and TAP2, thus escaping antigen presentation and propagating tumor formation [326]. CSCs not only manipulate APCs but also change immune cells into pro-tumor cells, for example by tolerization of infiltrating antigen-presenting DCs [327]. Neutrophils recruited by CSCs promote cancer invasion via secretion of osteopontin that enhances neutrophils infiltration in the cancer niche. Neutrophils secretes neutrophil elastase and matrix metalloproteinase 9 (MMP-9) leading to stromal-derived factor-1α (SDF1α/CXCL12) degradation. The latter retains the cancer in its niche when binding to CXCR4, and its degradation results in the release of cancer cells from their microenvironment allowing invasion.

5.10.2 Activation of Inhibitory Cells

The expansion of NK cells in response to tumors limits the proliferation of regulatory T cells (Tregs) [328]. CSCs enhance the release of IL-4, IL-6, and TGF-β to trigger the generation of Tregs and myeloid-derived suppressor T lymphocytes (MDSCs). These cells normally regulate immunity by inhibiting NKs, DCs, and T cells. CSCs interact with MDSCs to secrete microRNA-101 (mir-101) that promotes stemness of CSCs [329]. β-galactoside-binding protein is one mechanism by which $CCR4^+$ Tregs can kill NK cells and directly promote metastasis [330]. Overexpression of the Sox-2 transcriptional factor in CSCs recruits Tregs by secreting higher levels of chemokine CCL1. In return, the recruited Tregs were shown to increase the cancer stemness marker, ALDH, on breast cancer cells in unknown pathways [331]. ALDH was shown to be overexpressed in CSCs resistant to several anti-cancer drugs, such as cyclophosphamides and paclitaxel [332]. IL-30 and IL-27 share some pro-tumor effects, by promoting Tregs expansion. Propagated Tregs secrete IL-35 leading to immune evasion through T-cell exhaustion, anti-tumor CTL inhibition, as well as Th1 and Th17 differentiation suppression. In a positive feedback loop, the suppression of Th1 and Th17 allows more expansion of Tregs, leading to more secretion of IL-35, and more aggressiveness of the cancer. [318, 333]. Based on the foregoing, Tregs are considered a key regulator in promoting chemoresistance in CSCs, either directly by inhibiting other immune cells, such as Th1, or indirectly via enhancing expression of other inhibitory molecules.

5.10.3 The Generation of Tumor-Associated Macrophages

Macrophages are important heterogeneous and multifunctional immune cells. They are also the main antigen-presenting cells and are key players at the site of inflammation.

Macrophages can be classified into classical (M1) and alternative (M2) cells. M1 cells are activated by bacteria and function in inflammation initiation, while M2 cells function as anti-inflammatory and repairing cells. The difference between the two cell types lies in their mechanism of metabolizing arginine [334, 335]. IL-4 is essential in M2 proliferation and in producing an anti-inflammatory effect [336]. CSCs in the tumor microenvironment secrete IL-4, IL10, and IL 13 that enhance the proliferation of M2 over M1 cells. Tumor-associated macrophages (TAMs) are characterized as M2 cells within the tumor tissue and their accumulation is associated with higher tumor proliferation, angiogenesis, and malignancy [337]. Infiltration of monocytes and macrophages in the tumor is stimulated by different CSC-associated chemokines, including the chemokine (C-C motif) ligands CCL2, CCL5, CCL7, chemokine (C-X3-C motif) ligand CX3CL1, and cytokines, such as macrophage colony-stimulating factor (M-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) secreted by the tumors [338, 339]. The mechanism by which macrophages enhance cancer cell proliferation is still poorly understood. However, it is thought that epidermal growth factor receptor (EGFR) family ligands, activators of signal transducer, and activator of transcription 3 (STAT3), oncostatin M, IL-6, and IL-10, are all involved in the tumor-promoting mechanism of TAMs [340]. EGFR activation is shown to have effect on maintaining CSC characteristics via AKT signaling pathway. In glioma, activation of the STAT3 pathway by M2 cells led to the proliferation and progression of tumors [341]. STAT3 was found to play a role in induction of CSC markers, increasing their viability and tumorsphere formation [342–345]. Besides, STAT3 signaling pathway is involved in telomerase enzyme activation leading to promotion of CSC traits and survival [346]. Direct interaction between tumor cells and macrophages is a crucial factor in STAT3 activation and the production of immunosuppressors [347]. The polarization of macrophages into M2 TAMs results in the release of HLA-G, IL-10, and TGF-β, leading to efficient immune suppression [348].

5.10.4 Immune Checkpoints

Immune checkpoints are responsible for regulating the immune response and inhibiting the auto-immune reactions. They are either co-stimulatory, such as CD28, ICOS, and CD137 or co-inhibitory, such as PD-1, CTLA-4, and VISTA [349]. Cancerous tumors express the co-inhibitory immune checkpoints in order to suppress immune cells that fight cancer cells, so that the cancer growth can take over normal cells aggressively [350]. Cancer expresses two main co-inhibitory checkpoints for evasion: cytotoxic T-lymphocyte-associated antigen 4 (CTLA4, also known as CD152) and programmed cell death protein 1 (PD-1 or CD279). Overexpression of PD-1 by CSCs induces cytotoxic and T-helper cell apoptosis [351]. CSCs stimulate EMT, β-catenin, and STAT3 signaling pathways, which lead to overexpression of PD-1 [352]. CD200 is an immune checkpoint protein expressed in many immune cells such as macrophages, dendritic cells, B cells, and activated T lymphocytes

[353]. Enhanced expression of CD200 by CSCs further suppresses immunity by interfering with IL-2, IL-13, IL-17, tumor necrosis factor α (TNFα), and interferon α (IFNα), thus promoting tumor aggressiveness [354, 355].

5.11 CSCs as Therapeutic Targets

Based on their role in tumor progression, aggressiveness, and recurrence, many approaches have been recently developed to target CSCs for better cancer therapy. In this regard, genetically engineered stem cells and their derivatives have been proven to be effective in targeting CSCs [356, 357]. Recently, we have reported that hTERT plays role in CSC chemoresistance, proliferation, migration, and tumorsphere formation [208]. Hence, targeting hTERT could be one of the potential therapeutic strategies to eliminate CSCs [346]. Moreover, we could decrease the proliferation of CSCs specifically but not adult stem cells by using novel-formulated platinum nanoparticles (Pt-NPs) supported on polybenzimidazole (PBI)-functionalized polymers and multiwalled carbon nanotubes (MWCNTs) [358].

Treatment with antibodies against CSC surface markers (CD20, CD52, and CD44v6, EpCAM, etc.) already shown promising outcomes and good survival rates as a safe therapy for some blood malignancies, head, and neck cancer, and hormone-resistant prostate cancer [359–361]. These therapies were also successful in amplifying the anti-tumor effect when accompanied by radiotherapy, especially for patients who fail to respond to chemotherapy [362]. Other strategies targeted CSCs signaling pathways (i.e., TGF-β, JAK-STAT, PI3K, and NF-κB, Notch, Wnt, and Hh signaling pathways). Promising results have been reported in the treatment of leukemia, glioblastoma, breast cancer, lung cancer, ovarian cancer, pancreatic cancer, and colon cancer [363, 364].

Targeting the CSC microenvironment is an essential approach for the comprehensive treatment of hematological malignancies [365, 366]. Promising results supported the use of immunotherapeutic agents, such as anti-immune checkpoint inhibitors (i.e., anti-PD-1, PD-L1, and anti-CTLA-4) and CAR-T cells against many CSC markers (i.e., CD19, CD20, CD22, CD123, EpCAM, and ALDH) [367–369]. Many of these protocols entered into different phases of clinical trials, and some have already been FDA approved. Combination therapies are recommended as a promising strategy against CSC evasion to any single approach [364]. Because of their heterogeneity, understanding of the CSC surface markers, surviving mechanisms, and crosstalk with microenvironment in different cancers and in different patients may improve the CSCs' therapeutic targeting efficacy. Moreover, since most studies are conducted on hematological cancers, more experiments are needed to target the various solid tumors via targeting CSCs.

Take Home Message

- Cancer development is multifactorial.
- Cancer stem cells contribute to cancer progression, dormancy, metastasis, and chemotherapy resistance through different pathways. These pathways are interconnected, and by understanding the interplay between those pathways, efficient anti-cancer therapy could be achieved.
- The tumor microenvironment is highly heterogeneous, containing various cell types, which interact and influence CSCs.
- Recent studies have exemplified the critical role of cancer microenvironment in regulating CSCs and their role in tumor progression.

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Stem Cell Applications in Metabolic Disorders: Diabetes Mellitus

6

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Abbreviations

ASCs	Adipose stem cells
DM	Diabetes Mellitus
BMMNCs	Bone marrow mononuclear cells
BMMSCs	Bone marrow MSCs
COVID-19	The 2019 novel coronavirus disease
CSII	Continuous subcutaneous insulin infusion
CVD	Hematopoietic stem cells (HSCs), Diabetes Mellitus (DM), Cardiovascular disease
ESCs	Embryonic stem cells
GAD	Glutamic acid decarboxylase
GBC	The antidiabetic agent glibenclamide
GDM	Gestational diabetes mellitus
GSIS	Glucose-stimulated insulin secretion
HbA1c	Hemoglobin A1c
IA-2	Insulinoma-associated protein 2
IDF	International Diabetes Federation
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
Ins-CM-DESs	Insulin Choline chloride-Deep eutectic solvents
iPSCs	Induced pluripotent stem cells
LADA	Latent Autoimmune Diabetes in Adults
MODY	Maturity-Onset Diabetes of the Young
MSCs	Mesenchymal stem cells
NOD mice	Non-obese diabetic mice
OGTT	Oral glucose tolerance test
PPARs	Peroxisome proliferator-activated receptors
ROS	Reactive oxygen species
TZDs	Thiazolidinediones
WHO	World Health Organization
ZnT8	Zinc transporter 8

What You Will Learn in This Chapter

In this chapter we will discuss the definition of the metabolic disorders and the role of stem cells in their treatment. We will review the pathogenesis and current treatment of diabetes mellitus as an example of metabolic disorders. We will examine the regenerative therapy approaches to treat both Type I and Type II diabetes, and the different types of stem cells used for experimental transplantation and clinical trials. We will conclude with challenges in cell replacement therapy for diabetes, and new approaches to address these challenges.

6.1 What Are Metabolic Disorders?

The Greek word Metabole, meaning "to change," refers to metabolism, an array of intricate biochemical reactions that are necessary for sustaining life by maintaining vital cellular activities [1]. The disruption of this dynamic process causes metabolic disorders [1, 2], which may be congenital or acquired. Congenital metabolic disorders, or inborn errors of metabolism, may result from defect in the structure and function of an enzyme, or defects in a set of genes that control metabolic pathways. These disorders are mostly autosomal-inherited but are rarely autosomal dominant [3, 4], and cause defects in carbohydrate and protein metabolism, and in fatty acid oxidation [5–7]. Defects may be in the intermediary metabolic pathways leading to the accumulation of metabolic intermediates, such as urea cycle defects and amino acid disorders [8–11]. Phenylketonuria (PKU) is an autosomal recessive amino acid disorder, in which the PKU gene encodes a mutant variant of phenylalanine hydroxylase (PAH) enzyme that normally converts the amino acid phenylalanine (PHE) into tyrosine is mutated. Deterioration of PHE function is manifested in the accumulation of phenylalanine (hyperphenylalaninemia) along with reduction in tyrosine levels. The disease causes developmental deterioration of the nervous system, intellectual disability, epilepsy, motor disturbances, and psychiatric disorders. Following a low-PHE diet at an early age is the optimal treatment; interestingly, an IQ reduction was observed in adults with PKU who did not adhere to the restricted low-PHE diet [9–11].

There is a diverse array of inherited metabolic disorders. Galactosemia is an example of a defect in carbohydrate metabolism. It is a recessively inherited disorder in galactose metabolism stemming from a deficiency of galactose-1-phosphate uridylyltransferase (GALT), leading to the accumulation of galactose-1-phosphate (GAL1P). Dietary intake of galactose leads to the development of jaundice, hepatic failure, Escherichia coli (E. coli) sepsis, and renal tubular dysfunction in newborn. This condition is associated with long-term complications including cognitive function and memory impairment, tremors, and speech difficulty [12, 13]. Other defects may involve energy production, such as deficiencies in fatty acid oxidation and glycogen metabolism defects, or defects in organelles such as the Golgi apparatus and lysosomes [14]. Medium-chain acyl-CoA dehydrogenase deficiency (MCADD), the most common genetic fatty acid oxidation disorder, is an autosomal recessively inherited disease. It resulted in a high mortality rate of approximately 30% before the development of methods of newborn screening and early treatment. The clinical symptoms of this disorder include reduction in oral intake accompanied by vomiting, hypoketosis, and hypoglycemia that mostly led to death. Postmortem analysis revealed fatty liver and cerebral edema. Early detection and treatment of this disorder accompanied by prohibiting prolonged fasting dramatically decreased its mortality rate [15, 16]. Mucopolysaccharidosis type I is a lysosomal storage disorder in which there is accumulation of mucopolysaccharides or glycosaminoglycan in the lysosomes [17]. Mucopolysaccharidosis type I is caused by a defect in the *IDUA* gene, which is responsible for the production of for alpha-L-iduronidase, leading to impaired

breakdown of heparan and dermatan sulfate [17]. This causes the accumulation of glycosaminoglycan in different organs leading to their progressive dysfunction. Organ dysfunction may present clinically as intellectual disabilities, gastrointestinal symptoms, and skeletal deformities [18].

Acquired metabolic disorders are frequently accompanied by predisposing factors, including metabolic syndrome, obesity, and diabetes [19]. The affected organs may include the endocrine glands, liver, pancreas, kidneys, and the cardiovascular system [19]. The clinical manifestations of metabolic disorders range from relatively mild hyperglycemia, hypercholesterolemia, and small changes in liver function to debilitating and complicated pathologies [19]. The obesity epidemic and modern sedentary lifestyle have increased the incidence of metabolic diseases. For example, the incidence of Type II diabetes mellitus (DM), an acquired metabolic disorder has alarmingly increased, affecting almost 9% of the human population [20].

The pathology of acquired metabolic diseases follows a prolonged state of chronic inflammation. Treatments primarily aim at relieving the symptoms. Organ transplantation and cell replacement therapy have emerged as promising approaches for cures by replacing the diseased organ with a new functioning one [21–23]. For example, Gaucher's syndrome, an autosomal disorder in which lipids and glucosylceramide are deposited in the hepatic, splenic, and bone marrow cells due to a defect in β-glucosidase, responds effectively to bone marrow stem cell transplantation intended to restore the enzymatic function in monocytes [24].

6.2 Stem Cells Therapy for Metabolic Diseases

6.2.1 Stem Cell Therapy for Inherited Metabolic Disorders

Treatments for inherited metabolic disorders include enzyme replacement therapy and palliative measurements in advanced cases. Organ transplantation and cell therapy have been considered possible approaches to replace damaged cells, and substitute defective organs [21, 22]. However, organ transplantation carries many drawbacks such as the complications arising from immunosuppression, surgical complications, organ unavailability, prolonged recovery, and high operation costs [25–29]. Cell therapy, such as hepatocyte replacement therapy, has been used as an aid in liver transplantation in an effort to overcome such drawbacks [30, 31]. However, the feasibility of cell therapy depends on many factors that have not been fully investigated in the clinic. The proliferation and metabolic function of hepatocytes are greatly diminished in vitro, and hepatic infusion carries the risk of portal hypertension and portal vein thrombosis [32]. Additionally, when using cells that have not been fully tested, the number of defective cells may in fact outnumber the donor cells, and the level of functioning enzyme remains insufficient [33, 34]. Furthermore, finding a matched hepatocyte donor is a challenge. Cell replacement therapy thus seems effective, but only with the proper number of cells and a validated

standardized protocol that takes into consideration the efficacy of the enzyme replacement after manipulating the cells in vitro [35]. Hepatic stem cells and oval cells thus present an attractive alternative to liver transplantation and hepatocyte cell therapy [36]. Unlike hepatocytes, oval cells constitute a homogenous population which can be easily propagated and maintain hepatic cell identity, morphology, and hepatic makers such as α-fetoprotein, albumin, and CK19 in vitro, and additionally have a robust and replicative potential [37, 38]. However, oval cells are rare cells that can only be obtained after chronic liver injury in humans, and their isolation from a healthy donor in sufficient quantities for clinical use may be impossible [36]. Hence, it became necessary to look for another source for cell therapy.

Petersen et al. showed that bone marrow-derived stem cells could differentiate into hepatocyte-like cells after injection into a rat model of liver injury [39]. The Y-chromosome positive hepatocytes were detected in the livers of female rats that had been injected with the bone marrow stem cells of a male donor and were shown to express oval cell markers [40, 41]. In an animal experiment, bone marrow cells were administered to rats after hepatic injury in rats using either 2-acetylaminofluorene and or a 70% partial hepatectomy. The oval cell population in the recipients expressed hepatic markers such as α-fetoprotein, confirming that the bone marrow may be a source for hepatic progenitor cells [36, 39, 42–44].

Hematopoietic stem cells (HSCs) were used as a vehicle to deliver enzymes in inherited metabolic disorders, in both the allogeneic and the autologous setting, but in the case of the latter only after genetic modifications of the defective gene were made [45]. Hurler syndrome is the most severe form of mucopolysaccharidosis Type I. The disease results from the accumulation of glycosaminoglycans, leading to neurological defects, corneal clouding, bone deformities, organomegaly, and respiratory and cardiac defects [46]. The high mortality rate of Hurler syndrome results from cardiac or respiratory failure. HSC transplantation was shown to be an effective therapy for Hurler syndrome, although not all the manifestations were corrected; however, the magnitude of organomegaly and corneal clouding were reduced. The amount of glycosaminoglycans in the cardiac tissues was also reduced [47]. The mortality and cardiac complication were studied in 54 children following HSC transplantation. Out of 54 patients, 9 died after transplantation and 18 had graft rejection, 17 had another HSC transplantation and one patient did not continue the follow up [47]. Total survival was 73.7% at 1- and 20-year endpoints. In addition, 27.3% had normal cardiac assessment with 60% had mild to moderate aortic defects [47]. Thus the mortality rate of children with Hurler syndrome was greatly reduced after HSC transplantation [47]. Furthermore, patients receiving HSC transplantation experienced great improvement in musculoskeletal symptoms such as joint movement [48]. HSCs act as an enzyme delivery system that is then acquired by recipient cells [49]. HSCs can cross the blood–brain barrier, transforming into brain macrophages or microglia, and then secrete the deficient enzyme in the brain and enhance the neurocognitive function of the brain [50, 51]. The advantage of HSC enzyme replacement therapy is the capability of these cells to cross the blood–brain barrier, which is unique among enzyme replacement

therapies. Furthermore, long-term enzyme replacement therapy may induce anti-enzyme antibodies that reduce the efficiency of these drugs [52].

6.2.2 Stem Cell Therapy for Insulin-Dependent Diabetes

In insulin-dependent diabetes, the administration of insulin to reduce blood glucose levels does not always ensure glucose homeostasis, since patients' responsiveness to the administered insulin is different regarding either dose or activity [53]. Islet transplantation was seen as a possible alternative for the proper control of blood glucose [54]. However, the scarcity of donor pancreata and the high number of islets needed to achieve glucose hemostasis presented two major drawbacks of islet transplantation [54, 55]. Finding another source for islets, such as beta cells, was hence investigated. Unfortunately, beta cells do not expand well in culture, even with the addition of growth factors that cause the senescence of these cells in vitro [56]. Stem cells may be repurposed as insulin-producing machines, constituting a replacement for deficient beta cells in Type I diabetes mellitus (DM).

6.3 The Pathology of Diabetes

Diabetes mellitus is a heterogeneous metabolic disorder. It is characterized by hyperglycemia, or excessive blood glucose level, due to the impairment of insulin secretion, defective insulin action, or a combination of both, and accompanied by disturbed metabolism of carbohydrate, fat, and protein [57]. This chronic hyperglycemia is accompanied by particular long-term microvascular and macrovascular complications, elevating the patients' risk for developing cardiovascular disease (CVD), and pathology may be present in the eyes, nerves, and the kidneys [58]. Individuals with diabetes are also more susceptible to infectious diseases like latent tuberculosis infections [59]. Additionally, in cases of the 2019 novel coronavirus disease (COVID-19), diabetic patients experienced increased severity of the disease and higher mortality rates [58, 60]. Prediabetes is a condition characterized by impaired fasting glucose (IFG), impaired glucose tolerance (IGT), or a blood level of a glycated (HbA1c) of 6.0–6.4%. Prediabetic markers predict the development of overt diabetes and/or its complications [61].

6.3.1 Classification of Diabetes Mellitus (DM)

DM has classically been categorized into two types, childhood-onset diabetes Type I (insulin-dependent DM), and adult onset Type II DM, which is insulin-independent. Hyperglycemia is the common feature between all types of DM (Box 6.1). According to the International Diabetes Federation (IDF), around 1.1 million people are diagnosed with

Type I DM in childhood and early adulthood [62]. Although it is commonly diagnosed in childhood, a percentage of adults are diagnosed with type I DM [63]. The pathogenesis of the disease is defined by gradual auto-destruction of the pancreatic β-cells in the islets of Langerhans, resulting in insufficient insulin secretion, and consequently hyperglycemia. However, recent reports though demonstrated that a number of patients with Type I DM still produce endogenous insulin in response to a meal, albeit at low levels [64]. Preceding disease onset, there is a preclinical period of autoimmunity against islet antigens [65, 66]. Islet-autoantibodies are directed against insulin, glutamic acid decarboxylase (GAD), insulinoma-associated protein 2 (IA-2), and zinc transporter 8 (ZnT8) [67].

Box 6.1 Types of Diabetes

The most common types of diabetes include:

Type 1 (insulin-dependent DM): is an autoimmune disease caused by gradual destruction of pancreatic β-cells, leading to insulin deficiency and hyperglycemia. It is usually diagnosed in childhood and early adulthood [68].

Type II (insulin-independent diabetes DM): is characterized by decreased insulin sensitivity, due to insulin resistance, and frequently predisposed by obesity, and genetic factors. It is mostly diagnosed at adulthood [68].

Gestational diabetes: is also known as pregnancy diabetes, as it is usually temporary and restricted to pregnancy period. Insulin resistance, obesity, and family history of diabetes are among the factors contributing to this type of diabetes [69].

Type II DM is the most common type of diabetes, accounting for 90–95% of diabetics. Although it is mainly diagnosed in adulthood, there is recent evidence of cases diagnosed in children and adolescents [70]. It is characterized by a decline in insulin sensitivity due to insulin resistance, and is usually associated with obesity and a mild reduction in insulin secretion [20, 71].

Gestational diabetes mellitus (GDM) is a condition of abnormal glucose metabolism that initially occurs during pregnancy and is usually temporary. According to a 2013 estimate by the IDF, there is a 14.2% incidence rate of GDM in pregnant women between the ages of 20 and 49 [72]. The progress of GDM is enhanced by insulin resistance, obesity, genetic factors, and the age of menarche [73]. Recently, the WHO proposed a modern classification system for DM that includes a category of hybrid forms of diabetes, such as slowly evolving immune-mediated diabetes, previously known as Latent Autoimmune Diabetes in Adults (LADA). Another category is designated for diseases of the exocrine pancreas, including pancreatitis, and monogenic defects of β-cell function, such as Maturity-Onset Diabetes of the Young (MODY) [58].

LADA is a form of Type I DM that typically develops during late adulthood. LADA and Type I DM patients were reported to both have elevated B-cell subsets, including marginal zone B (MZB) cells. This suggests that B lymphocytes play a role in the β-cell destruction that is observed in both types of diabetes [74]. However, the mortality rate and

cardiovascular complications in LADA cases are minor compared with cases of Type I and Type II DM [75]. Despite the presence of islet antibodies at the onset of diagnosis, LADA has a characteristically slow progression of autoimmune β-cell failure. Consequently, LADA patients are insulin-independent for only the first 6 months after diagnosis [76]. The use of oral antidiabetic dipeptidyl peptidase 4 (DPP-4) inhibitors showed a protective effect on β-cell function. This makes it a potential agent to stop the gradual autoimmune β-cell destruction in LADA patients [77].

MODY is caused by the absence of one of the autosomal genes responsible for insulin secretion [78]. Its autosomal mode of inheritance leads to a primary defect in the function of pancreatic β-cells. It is characterized by a genetic heterogeneity where a defect in a single gene of 13 different genes can cause this disorder. The most common types of MODY include those caused by a mutant variant of the glucokinase gene (*GCK MODY*) and the hepato-nuclear factor gene (*HNF1A MODY*). Glucokinase is an enzyme encoded by the GCK gene that catalyzes glucose phosphorylation in a rate-limiting pattern. Patients with GCK MODY show mild fasting hyperglycemia, but are not vulnerable to developing vascular complications and rarely require pharmacological treatment. However, HNF1A MODY patients have a high risk of developing both microvascular and macrovascular complications. Defects in HNF1A genes induce a reduction in pancreatic insulin production leading to hyperglycemia [79, 80].

Due to the common features between Alzheimer's Disease (AD) and DM, the term Type III DM was proposed term to describe AD as a type of neuro-endocrine degenerative disorder. This categorization was further confirmed by the downregulation of insulin-like growth factor Type I and II (IGF-I and IGF-II) in the neurons of AD patients' central nervous system (CNS). Additionally, a reduction in insulin receptor substrate (IRS) mRNA, tau mRNA, and IRS-associated phosphatidylinositol 3-kinase were reported [81]. The imbalance in glucose homeostasis as a result of the reduction in insulin sensitivity (elevated insulin resistance) was accompanied by hippocampal β-amyloid accumulation [82]. Antidiabetic medications were thus proposed as a potential treatment for AD. Glibenclamide (GBC), an antidiabetic drug, showed amelioration of cognitive function impairments in rats, and an improvement in memory function along with a decrease in hippocampal inflammation [83, 84].

6.3.2 Diabetes Complications and Their Pathophysiology

There is an inexorable increase in the prevalence of worldwide [85]. In 2017, the International Diabetes Federation stated that about 451 million people in the age range of 18–99 years were diagnosed with diabetes [86]. With the absence of an intervention to stop the increasing incidence of diabetes, the WHO estimates that there will be at least 693 million people with diabetes by 2045 [86]. Diabetes complications observed in multiple tissues, including the vascular system, eyes, kidneys, and peripheral nerves. The development of DM complications follows the chronic hyperglycemia, an imbalanced breakdown of lipids, excessive generation of Reactive Oxygen Species (ROS), and reduced

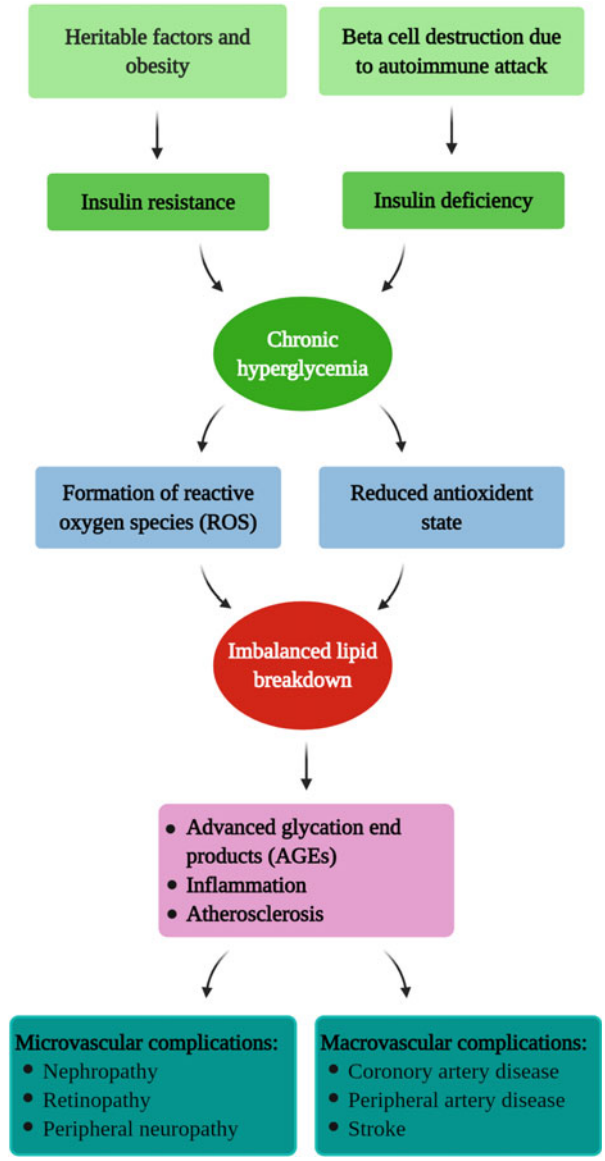

Fig. 6.1 The pathophysiology of diabetes

antioxidant status, resulting in sustained micro- and macrovascular damage [87]. This hyperglycemic state causes an increase in glycosylation, inflammation, and injury to the arterial walls, promoting changes in vascular tissue, and leading to atherosclerosis. Atherosclerosis is the main culprit in "macrovascular complications," including coronary artery diseases, stroke, and diabetic foot. Atherosclerotic damage to smaller blood vessels can lead to microvascular complications including diabetic peripheral neuropathy and retinopathy, which is the main cause of blindness in adulthood [88] (Fig. 6.1) (Box 6.2).

Box 6.2 Pathogenesis of Diabetes

- Pathogenesis of insulin-dependent diabetes is caused by an autoimmune attack on pancreatic β-cells. Histological analysis of Type I DM pancreata demonstrated an invasion of the islets of Langerhans by T and B lymphocytes, macrophages, natural killer cells, dendritic cells, in addition to islet-reactive autoantibodies and islet-reactive T-cells [89]. The initial β-cells damage promotes the release of autoantigens, which are presented by the antigen-presenting cell (APC) to T-helper cells. This process is followed by migration of APCs to the pancreatic lymph node. Those APCs activate autoantibodies and auto-reactive T-cells that attack β-cells causing their destruction [90]. T-cells and macrophages produce cytokines, interleukin-22 (IL-22), interferon-α (IFN-α), and tumor necrosis factor-β (TNF-β) that participates in Type I diabetes development [91].
- *Pathogenesis of insulin-independent diabetes is caused by* poor diet and sedentary life style, leading to obesity that in turn causes insulin resistance [92]. Insulin gene expression is downregulated in the high fatty acid environment and hyperglycemic conditions [92]. Sterols accumulate in the blood due to cholesterol transporter impaired function, and islet inflammation leads to β-cells destruction [93].

6.4 Current Therapeutic Approaches for Diabetes

The ultimate goal of diabetes management is to achieve normal glycemic control through an integrated approach of medical care and proper nutrition. Based on the type of the disease, the primary approach for managing of DM encompasses following a healthy diet and getting physical exercise. If necessary, this is followed by the administration of an oral hypoglycemic, such as metformin, sulfonylureas, and thiazolidinediones, and finally, insulin injections for some patients. The etiology of diabetes is a key factor in determining the course of treatment. Oral hypoglycemics aim at increasing insulin sensitivity in patients suffering from insulin resistance, thus relieving the symptoms of Type II DM [94]. Insulin replacement therapy by route of subcutaneous injection is usually prescribed to insulin-dependent patients Type I, and to Type II DM patients who have insufficient insulin secretion.

6.4.1 Oral Hypoglycemic Drugs

Metformin is widely used as an oral drug that induces hypoglycemia by decreasing hepatic glucose production and increasing the utilization of glucose by boosting insulin sensitivity. The appetite suppressing effect helps with weight control of the frequently obese patients [95]. Thiazolidinediones (TZDs) are another class of oral hypoglycemic agents that act as

agonists of Peroxisome Proliferator-Activated Receptors (PPARs) or glitazone receptors. This family of receptors is responsible for the expression of genes that play a vital role in the metabolism of carbohydrates, lipids, and proteins. These proteins also play a role in adipocyte differentiation, increase insulin sensitivity, and prevent oxidative stress. TZDs act as insulin sensitizers, either directly by enhancing the uptake and storage of fatty acids in the adipose tissues, or indirectly by the alteration of the release of adipocytokines, which are signaling molecules secreted by adipose tissue and play a role in metabolic homeostasis. Some adipocytokines also contribute to the development of insulin resistance and Type I Type II DM [96].

6.4.2 Insulin

Insulin is mainly prescribed to patients with insulin-dependent diabetes as a substitute for endogenous insulin secretion deficiency due to the destruction of β-cells. It is also used by insulin-independent diabetics when oral hypoglycemic drugs fail to achieve the optimum glycemic control. One of the drawbacks of insulin therapy is its route of administration, which is through subcutaneous self-injection, or intravenous administration by health care providers to hospitalized patients. Recently, continuous subcutaneous insulin infusion (CSII) by insulin pumps is being adopted instead of repeatedly injecting insulin. Since repeated injections are burdensome and require nuance, new forms of insulin are being developed for administration through the buccal cavity; transdermal, rectal, ocular, and intranasal insulin are also under development [97].

Under normal physiological conditions, the portal vein has a threefold higher insulin concentration than the systemic circulation. Subcutaneous insulin injection disrupts the portal-systemic insulin gradient by elevating the concentration of systemic insulin. This disturbs glycogen storage and glucose output, leading to hyperglycemia. Oral delivery of insulin has the advantage of mimicking the physiological path of pancreatic insulin. However, orally delivered insulin is vulnerable to degradation due to the acidic environment of the stomach and the enzymatic activity in the gastrointestinal tract leading to low insulin bioavailability. Enzymes such as pepsin in the stomach and pancreatic enzymes such as trypsin, chymotrypsin, and carboxypeptidases in the small intestinal play a physiological role in digesting polypeptides into their amino acids subunits. Current efforts to enhance oral insulin delivery encompass insulin encapsulation using nano particle carriers, such as chitosan-based nanoparticles. Proteolytic inhibitors such as sodium glycocholate and aprotinin are used to improve the bioavailability of orally administered insulin [97]. The use of absorption enhancers including bile salts, surfactants, fatty acids, calcium ions, and chelating agents can increase oral insulin bioavailability by either transcellular uptake through modulating the cell membrane structure of the intestinal epithelium or through paracellular transport via the cell tight junctions [98].

Intranasal delivery takes advantage of the large surface area of the nasal mucosal (150 cm^2), which is covered with microvilli, and the high permeability of the nasal

epithelium. Additionally, compared to the gastrointestinal tract, the nasal mucosa has lower activity of enzymes. Biocompatible enhancers with high efficiency are being developed to overcome the low permeability of intranasal administered insulin compared to the subcutaneous injection form. Various enhancers have been tested on enhancing insulin nasal absorption including dimethyl-β-cyclodextrin, surfactants, and chitosan. However, they showed some side effects such as irritation of the nasal mucosa. Recently, Yang Li et al. developed choline chloride-deep eutectic solvents (DESs) to enhance insulin delivery. The compound showed an improvement in the bioavailability of intranasal administered insulin. Insulin choline chloride-deep eutectic solvents (Ins-CM-DESs) at 25 IU/kg showed equivalent hypoglycemic effect of subcutaneous insulin (1 IU/kg). It was also shown to be non-toxic to rat nasal epithelia, providing evidence for a possible replacement for insulin injections. However, as shown by its release profile, Ins-CM-DESs have a fast release for the first 1 h that declines later which can negatively affect the sustainability of effective glycemic control [98, 99].

6.4.3 Islet Transplantation

Uncontrolled insulin therapy may lead to dangerous hypoglycemia [100]. Fluctuation of insulin levels without synchronization of the drug dosage and timing with the level of carbohydrate consumption and physical activity requires strict glucose monitoring. Islet transplantation has been proposed as a potentially radical solution to restore insulin regulation by normal physiological glucose-stimulated insulin secretion (GSIS) in patients with type I DM. The limitations of islet transplantation therapy include the lifelong dependence on immunosuppression to prevent cell rejection and the scarce availability of organ donors; two to three pancreatic islets donors are required for a 68 kg patient (revised in [101]). Additionally, islets obtained must be purified which leads to further loss of cells in the procedure.

6.4.3.1 The Edmonton Protocol

The Edmonton protocol for islet transplantation was developed by Shapiro and colleagues at the University of Alberta Hospital and the Surgical-Medical Research Institute [102]. This protocol involves multiple steps, starting with the obtaining and preservation of the pancreas from cadaveric donors. The pancreatic tissue is then chemically digested and dissociated and the islets are purified and cultured. Islet transplantation occurs through injection of cell suspension in the hepatic portal vein. Transplanted islets are vulnerable to both alloimmunity and islets autoimmunity which requires the employment of immunosuppression. However, most available immunosuppressive agents, especially corticosteroids, are islet-toxic. The Edmonton protocol has the privilege of not using immunosuppressive glucocorticoid. Rather, it involves the use of sirolimus to enhance the survival of transplanted cells. The combination of low-dose tacrolimus and a monoclonal antibody against the interleukin-2 receptor (daclizumab) provide protection against

rejection and autoimmunity. This procedure resulted in insulin independence for 1 year in 8% of the treated patients, who were originally diagnosed with insulin-dependent DM [103]. This technique still has some drawbacks including the neurotoxicity associated with the use of tacrolimus [104]. The survival and function of islet β-cells are dependent on intercellular contacts and extracellular matrix (ECM)-integrin interactions. To overcome the drawbacks of the chemicals used in the Edmonton protocol, research is ongoing on the use of encapsulation to protect the transplanted islets from immune reaction, and scaffolds to modify the islet-ECM environment to improve the efficiency of the transplanted islets and overcome the drawbacks of the Edmonton protocol [102, 103, 105] (Box 6.3). A new approach for diabetes therapy aims to directly employ gene therapy. Gene therapy could be achieved by substituting malfunctioning genes with functional ones, introduction of a new gene into the body, or by deactivating disease-causing genes [106]. Because of the extensive cell manipulation for purposes of gene therapy, the most reported applications in regenerative medicine for diabetes employ transplantation of undifferentiated stem cells in the clinical setting, while manipulated cells remain experimental.

Box 6.3 The Edmonton Protocol

- Islets transplantation is a successful therapeutic approach to treat insulin-dependent diabetes.
- Limitations for this procedure include lifelong immunosuppression dependence, limited availability of organ donors, and the peripheral insulin resistance associated with the use of many drugs and chemicals [102, 103, 105].

6.5 Types of Stem Cells Used in Diabetes Therapy

6.5.1 Embryonic Stem Cells (ESCs)

The process of developing β-cells from ESCs in culture mimics the embryonic development of islet cells [107, 108]. ESCs are first isolated from the inner cell mass of the embryo, and then allowed to differentiate into endoderm cells through a chain of intermediates, eventually giving rise to β-cells [109, 110]. This series of differentiation steps are conducted by the timely activation or inhibition of certain transcription factors that control intracellular differentiation signaling [111, 112]. Certain transcription factors such as *PDX1, Isl1*, and *Foxa2* define the commitment to β-cell lineage, but the exact combination of factors needed for fully differentiated, functioning β-cells is still under investigation [111–113]. The level of C-peptide, a byproduct of insulin generation, and the sensitivity to glucose are the parameters that were used as an indicators for the generation of mature insulin-producing cells [107, 113].

The generation of functioning mature β-cells is still challenging. Kroon et al. were successful in differentiating ESCs into definitive endoderm and then into insulin-producing cells. However, these cells showed no response to glucose [110]. In another study, ESCs were differentiated into endodermal intermediates mimicking fetal pancreatic differentiation at 6–9 weeks, and then implanted into the fat pad of immunocompromised mice [108]. After 3 months, the level of C-peptide rose after glucose stimulation to a level that was similar to that of C-peptide in mice transplanted with normal pancreatic islets [108]. The glucose level was regulated for up to 200 days, indicating that the implanted ESCs were differentiated into functioning β-cells [108]. Another group confirmed the same results within 6 weeks of study [114].

Despite these promising data, the clinical applications of ESCs are still limited. At the time of writing, only one clinical trial is running, in which researchers are investigating the safety of an implant containing pancreatic progenitors obtained from ESCs shielded under the renal capsule. This prevents the immune system from attacking the progenitors while they mature in vivo into functional β-cells [108]. The rare use of ESCs in clinical trials reflects the paucity of reliable, reproducible studies, and the difficulty in generating β-cells in sufficient quantities to control blood glucose [107, 115]. Additionally, the ethical considerations regarding the use of ESCs hinder their use in clinical trials [116] (Box 6.4).

Box 6.4 Melton's Protocol: From Stem Cells to Functional Beta Cells

Douglas A. Melton, of the Harvard Stem Cell Institute reported a differentiation protocol for ESCs that could generate millions of β cells that are sensitive to glucose in vitro. Similar to adult β cells, the generated cells express mature β cell markers, secrete insulin in proper quantities relative to glucose stimulation, and could reverse hyperglycemia in mice [109]. First, the group use cell sequencing to isolate islet cells based on their hormonal release. Encapsulation of the generated β cells with CXCL12 enhanced their engraftment and function [117].

6.5.2 Induced Pluripotent Stem Cells (iPSCs)

The generation of functional β-cells from iPSCs results in the production of insulin-producing cells co-expressing other islet hormones such as glucagon and somatostatin. These cells showed no expression of the mature β-cell markers NKX6-1 and PDX1. They also failed to respond to glucose and secreted only small amounts of insulin [118]. Pagliuca et al. modified iPSC's differentiation protocol and used a 3D-culture system to generate β-cells that are sensitive to glucose and have an insulin-secreting capacity comparable to cadaveric islet cells. Although not very similar to cadaveric β-cells, these cells expressed mature β-cell markers including NKX6-1 and PDX1 [109]. The generated β-cells could secrete insulin and stabilize the glucose level to below 200 mg/dl when implanted under the renal capsule of immunocompromised mice, similar to mice implanted with islet cells, for

up to 18 weeks post-transplantation. The protocol of Pagliuca et al. is considered the most successful to date for generating β-cells from [109]. The protocol of Pagliuca et al. is considered the most successful up to date on generating β-cells from iPSCs [109].

Several factors limit to use iPSC-derived β-cells in the clinic. The use of retroviruses to generate iPSCs carries the risk of aneuploidy, reprogramming inefficiency, and unreliable function of the resultant iPSCs [119]. Additionally, immune rejection is still considered a major obstacle for the clinical application of iPSC-derived β-cells. The transplanted cells are considered allogeneic, even if they are autologous, due to the autoimmunity in type I diabetes. Applying immunosuppression for long periods to reduce autoimmunity also carries the dual risks of infection and tumorigenesis [120].

6.5.3 Mesenchymal Stem Cells (MSCs)

Adipose stem cells (ASCs) were isolated from mouse epididymal fat and used to control diabetes in non-obese diabetic (NOD) mice, which serve as type I diabetes model [121]. Hyperglycemia was controlled in 78% of cases. Additionally, the level of other islet hormones such as glucagon and amylin increased, indicating at least the partial rescue of pancreatic islet function [122]. The researchers reported a decrease in the infiltration of inflammatory cells, the suppression of $CD4^+$ T-helper cells, and an increase in T regulatory cells, accompanied by the anti-inflammatory TGF-β1 cytokine in the pancreatic islets of mice. When cultured with T regulatory cells and TGF-β1, the ASCs showed increased secretion of TGF-β1, indicating the role of cell-cell signaling in the immunomodulatory effect of ASCs. These results were reproduced by another group; however, the effect of injected cells was short-lived, as the glucose level in test mice increased at 9 weeks injection [123].

Kono et al. showed that co-culturing ASCs with islet cells resulted in an increased level of TIMP1, a factor that prevents cytokine-mediated cell death [124]. The group then investigated whether the increase in TIMP1 was due to the generation of β-cells from ASCs. They labeled ASCs with green fluorescent protein to track them in vivo after injection in immunodeficient diabetic mice. Although labeled cells were found around pancreatic islets, they did not show signs of mitosis. This indicated that a paracrine effect of ASCs increased the proliferation of β-cells and decreased their apoptosis. Unfortunately, the reversal of hyperglycemia was short-lived, lasting only 35 days. The same experiment was repeated with the use of bone marrow MSCs (BM-MSCs) with nearly the same results; an increase in β-cell proliferation was initially observed, but the reversal of hyperglycemia only lasted up to 42 days [125]. Conditioned media with BM-MSCs was injected in immunodeficient diabetic mice and produced the same results. Hence, the previous studies confirmed the results of Kono et al. [124, 125].

Another group reported that the co-culture of human cord blood MSCs with T-lymphocytes would render these cells less reactive to the patients' own islet cells,

which is an approach known as "stem cell education therapy" [126]. Human cord blood MSCs showed increased expression of the autoimmune regulator (AIRE). AIRE mediated the deletion of auto-reactive T-cells, whereas the knockdown of AIRE reduced the level of T regulatory cells in culture. The test group of patients showed increased C-peptide levels, an enhanced response to a glucose tolerance test, and normal HbA1c 2 weeks after injection, but the effects only lasted 24 weeks [126]. However, no negative side effects were noticed following the procedure, which is encouraging. These data are promising since this was a pioneering clinical trial aimed at modulating the immune pathology in the pancreatic islets.

Among the 26 available clinical trials on the use of MSCs for treating DM, only two were completed and only one is active, although not recruiting (clinicaltrials.gov). One of the completed studies examined the safety and efficacy of BM-MSCs in treatment of type I DM, while the other is examining the efficacy of a PROCHYMAL®, an MSC-containing drug, and the first stem cell-containing drug to be approved in Canada (clinicaltrial.gov) [127].

6.5.4 HSCs

Preclinical studies showed the efficacy of allogeneic HSCs for the reversal of type I diabetes [128, 129]. The first clinical trials used autologous HSCs in patients at the onset of type I diabetes [128, 130]. HSCs were mobilized from 23 patients and then re-injected after immunosuppressive therapy. After 29 months, only one patient needed insulin therapy. No mortality was reported following HSC transplantation. Similar findings were found in another study with a similar protocol for eight patients [129, 131]. Another study conducted on 12 patients following the same protocol showed that insulin-dependence was reduced in 11 out of 13 patients, and HbA1c was normalized in seven out of eight patients over a period of 2 years [132]. Gue et al. reported in a phase 2 clinical trial that autologous HSC transplantation was much more efficient in cases of patients with no history of ketoacidosis at the onset of type I diabetes [133]. Gue et al. conducted a study to investigate the safety of autologous HSC transplantation in 42 children. 14 patients received HSCs, and 28 patients in the control group received regular insulin. The test group showed no ketoacidosis, and higher HbA1c levels were observed, but there was no difference in the insulin requirements or C-peptide levels between the two groups [134]. Hence, these data are not in favor of the use of HSCs in type I diabetes.

6.6 Challenges in the Generation of Insulin-Producing Cells from Stem Cells

Several factors control the generation of insulin-producing cells from stem cells. The major limitation is how to produce functional β-cells that display GSIS, meaning that they secrete insulin in proportion to the rise in the glucose level [111, 135–137]. A possible explanation for inadequate insulin secretion maybe that generated β-cells lose important transcription factors such as PDX1, NKX6.1, and MAFA [108, 110, 138] during the differentiation process. Additionally, it was found that the expression of ESC markers such as DPPA4, LIN28A, and LIN28B is elevated in insulin-producing cells obtained from stem cells [139]. It was thus considered that it is important to implant these cells in vivo to mature into functioning β-cells. Further investigation into the signaling interactions and the improvement of the differentiation protocol are necessary to overcome this limitation.

6.7 Stem Cell Therapy for Type II Diabetes and Its Complications

6.7.1 Bone Marrow Mononuclear Cells (BM-MNCs)

BM-MNCs are a heterogenous group of immature cells and progenitors that are isolated from the bone marrow [140]. BM-MNCs include immature lymphocytes and monocytes, in addition to HSCs and MSCs, and are thus considered to have great regenerative potential [140]. In a study by Wang et al., HbA1c decreased over a 30-day period, and the C-peptide levels increased after a 3-month period following autologous bone marrow transplantation in 31 patients [141]. Additionally, the use of medication was significantly reduced [141]. In a study by Bhansali et al., 9 out of 11 patients receiving BM-MNCs showed an approximately 50% decrease in insulin-dependence, and in 10 patients, HbA1c reached levels below 7% [142]. Wu et al. injected BM-MNCs either alone or together with hyperbaric oxygen in 80 patients [143]. However, no effect of hyperbaric oxygen was reported on BMMNC efficacy. Hue et al. showed improved C-peptide levels and a decrease in HbA1c after BM-MNC therapy compared to insulin therapy [144].

6.7.2 MSCs

Both autologous and allogeneic MSCs showed great capacity to reverse hyperglycemia in diabetic mice [125, 145]. This was due to MSC paracrine action, which promotes endothelial and islet cell proliferation and healing. Vascular endothelial growth factor alpha, platelet-derived growth factor, angiopoietin-1 (ANG-1), and insulin-like growth factor (IGF-1) have been implicated in this process. Additionally, MSCs induced autophagy in the local cells that promote damage of the affected cells, thus promoting healing [146, 147]. MSCs have an immunomodulatory function in suppressing the immune

response through inhibiting the proliferation of T and B lymphocytes, which decreases antibody production by B lymphocytes and the cytotoxic action of T-cells and natural killer cells, while enhancing the proliferation of regulatory T-cells [147]. This results in decreased inflammation in the pancreas and aids in pancreatic healing.

MSCs were used to decrease insulin resistance in Type II diabetic patients. Bone marrow MSCs (BM-MSCs) at a dose of 3.8×10^8 cells were infused through pancreas feeding arteries in 10 Type II diabetic patients. The need for insulin was reduced in all patients, and three patients were able to stop insulin intake [148]. In three clinical trials, BM-MSC infusion decreased HbA1c levels; this promising outcome was maintained for an average of 1–2 years [149–151]. While the reversal of hyperglycemia and HbA1c levels appeared early following MSC infusion, there was a lag in the correction of C-peptide levels for approximately 6 months on average and lasted for 1 year from the start of the recovery [149, 152]. A multi-center clinical trial used allogeneic BM-MSCs infusion of $0.3–2 \times 10^6$ cells/kg in arteries that supplied the pancreas [153]. The HbA1c level was reduced below 7% in about half of the patients, and there were no significant adverse effects except mild gastric disturbance with nausea and vomiting [153].

The regenerative capacity of MSCs makes them a potential therapy for diabetic complications in which adverse vascular effects and inflammation underlie the pathology. Diabetic rats infused with BM-MSC-conditioned medium after an induced stroke showed improved vascular diabetic rats [154]. Intravitreal infusion of neural stem cells derived from umbilical cord MSCs improved diabetic retinopathy in diabetic rats [154]. In a study by Cao et al., the infusion of BM-MSCs improved the healing of diabetic foot ulcers [155]. Additionally, allogeneic MSC infusion in patients with neuropathy improved symptoms and was safe [156]. In patients with limb ischemia, BM-MSCs injected locally into the lesion led to an improvement of limb perfusion, eventually healing the associated ulcer [155].

6.8 New Approaches to Enhance Stem Cells Therapy for Diabetes

Immune rejection of the transplanted cells presents the most formidable barrier to many cell therapy applications. Macroencapsulation systems are devices that contain a large number of transplanted cells. The device has a semi-permeable membrane to permit the passage of fluids, while keeping the cells protected from the immune system [120]. Macroencapsulation was used to allow the diffusion of insulin, glucose, and nutrients for optimum cell survival [120]. However, encapsulation devices were shown to foil the passage of nutrients. Furthermore, with the passage of time, the immune system recognized them as foreign bodies, and they were eventually surrounded with fibrous tissues, forming scars formation [120]. Incorporating stem cells with immune system modulators has been proposed as an alternative approach for direct stem cell injection [120]. Alginate microcapsules allow for the passage of nutrients through their selectively permeable membrane. Transplantation of iPSCs with MSCs, regulatory T-cells, and Sertoli

cells in alginate microcapsules were used to reduce graft rejection [120]. Incorporation of immunosuppressive agents such as ursodeoxycholic acid, which is known to inhibit the phagocytosis of donor cells, has been shown to increase the efficiency of the engrafted cells [157]. Coating microcapsules with CXCL12, a chemokine that attracts regulatory T-lymphocytes, reduced the rejection of transplanted cells and enhanced their function [120].

Using retroviruses to generate iPSCs carries a high risk of tumorigenesis [119]. Currently, the use of episomal plasmids combats the disadvantages of retroviral use [158, 159]. Reprogramming using the RNA-based Sendai virus was efficient and had more genetic integrity compared to retroviruses, since the former does not integrate into host DNA [119]. However, the lack of commercially available virus limits its clinical application. RNA reprogramming in general produces low numbers of iPSCs from fibroblasts. MicroRNAs may enhance RNA reprogramming but are still under investigation [119]. Teratoma formation from undifferentiated cells following transplantation of either ESCs or iPSCs presents a great challenge in stem cell therapy. Hence, the use of a microencapsulation system may reduce the overgrowth of undifferentiated cells through enhancing their differentiation in vivo [160, 161].

Take Home Messages

- Stem cells therapy is applied as an effective therapy for inherited metabolic disorders.
- Cell therapy for diabetes mellitus is considered a viable alternative for islet transplantation, because of the insufficient pancreatic donors, and the lifelong requirement for immune suppression.
- Both ESCs and iPSCs have shown promise in producing insulin-secreting cells, however, their use still suffers sufficient clinical trials and complications in experimental animals.
- BM-MNCs and MSCs showed promising results in treatment of diabetes and its complications.
- Macroencapsulation is tried in several laboratories to avoid immune rejection, and functions to protect the donor stem cells from lack of oxygen and nutrition.

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Epigenetics in Stem Cell Biology

7

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What You Will Learn in This Chapter

Epigenetics is the field of study concerned with alterations in gene expression which occur without changes to an organism's DNA sequence. Epigenetic modifications include histone modifications, DNA methylation, and interactions with non-coding RNAs. In this chapter, you will learn how epigenetic modifications control the development, proliferation, and self-renewal of stem cells, and how these modifications also play important roles in cell fate decisions such as differentiation, de-differentiation, and transdifferentiation. The chapter covers epigenetic control of reprogramming of stem cells, in the generation of induced pluripotent cells, and in understanding the origins of Cancer Stem Cells (CSCs). The chapter covers the environmental factors that influence stem cell biology and aging and interact strongly with epigenetic control mechanisms. The chapter concludes with understanding these control mechanisms, and the impact of epigenetics on stem cell development, senescence, and regenerative capacity and their role in developing epigenetic-based therapeutics.

7.1 Definition of Epigenetics

Epigenetics is defined as the branch of science that investigates the heritable changes in chromatin structure and gene expression levels which do not originate from changes at the level of the nucleotide sequence [1, 2]. Epigenetic alterations can be chemical tags that are added to or removed from DNA or its associated proteins, without altering the DNA sequence itself, resulting in stable, heritable phenotypes [3].

7.2 Levels of Epigenetic Control Mechanisms

Epigenetic control is achieved via a set of epigenetic machines, including writers, readers, and erasers, that can deposit, recognize, or remove epigenetic marks. These epigenetic marks are covalent chemical tags or modifications that are added to DNA and histone proteins. The modifications include DNA methylation, histone modification, and the use of non-coding RNAs. These control mechanisms work together or separately to regulate the

functions of genes in a long-lasting and reversible manner, and can be passed from one generation to another [3, 4].

7.2.1 DNA Methylation

DNA methylation is the process of adding a methyl group (CH3) to the fifth carbon of the cytosine ring of a CpG dinucleotide [5–7]. DNA methylation patterns are unique for each cell type. In embryonic cells and neurons, methylation of CpG dinucleotides is most common. In somatic cells, however, methylation primarily occurs on the cytosine residues of CpG, except for the CpGs in promoters, which usually remain unmethylated. DNA methylation acts as a gene repression signal [8]. Gene silencing can result from the methylation of gene promoter regions [3, 8].

DNA methylation is carried out by a specific enzyme family, known as DNA methyltransferases (DNMTs), that work by making stable chemical covalent adjustments to specific cytosine bases. Mammalian cells have several types of DNMTs, including DNMT1, DNMT2, DNMT3A, DNMT3B, and DNMT3L. Each DNMT has its function; for example, DNMT1 is essential for DNA methylation pattern maintenance during DNA replication, to ensure the replication of the pattern of the parent cell [9]; while DNMT3A and DNMT3B establish de novo DNA methylation during early developmental stages [10], contributing to directing the developing cell toward specific cell lineages. Both DNMT3A and DNMT3B are regulated by DNMT3L [11–13]. Although it was previously called DNA methyltransferase 2, DNMT2 actually methylates aspartic acid tRNA at the 38th cytosine in the anticodon loop, and was therefore renamed tRNA aspartic acid methyltransferase 1 (TRDMT1) [14]. Demethylation is also vital for the regulation of chromatin states. Demethylation can be achieved actively or passively. Some active demethylation works via a base excision DNA repair mechanism [15]. Another mechanism by which active demethylation occurs is Ten-Eleven Translocation (TET) enzymes, which initiate a cascade of biochemical reactions starting with the hydroxylation of a methyl group before its removal [16]. Passive demethylation involves the inhibition and interruption of DNMT1 enzyme function during DNA replication, leading to a failure of 5-methyl cytosine formation [17].

7.2.2 Histone Modifications

Nucleosomes are the basic chromatin units in eukaryotic cells. Each nucleosome is composed of about two turns of DNA wrapped tightly around an eight-histone protein core. The nucleosome structure does not expose the DNA to the biochemical machinery, such as the transcriptional machinery [18]. Histone modifications are established by enzymes that target specific amino acids, mostly lysine residues, at the start and end of H3 and H4 histone N-terminal tails. These modifications contribute to the control of

chromatin structure, and can be either chromatin opening modifications, which make DNA more readable and enhance transcription and the expression of genes, or chromatin closing modifications, which make DNA more condensed [19]. Other forms of histone modifications include methylation, acetylation, phosphorylation, ubiquitylation, biotinylation, and sumoylation. However, acetylation and methylation have been most widely investigated. Some of these histone modifications will be discussed in the following sections.

7.2.2.1 Histone Acetylation

Histone acetylation is associated with an open chromatin conformation and is therefore usually accompanied by increased gene expression. This process is controlled by two sets of enzymes: histone acetyl transferases (HATs) and histone deacetylases (HDACs) [20]. The former are responsible for the acetylation process, in which they move acetyl groups from their substrate acetyl Co-A and add them to lysine residues on histone tails [21, 22]. This acetyl group addition interrupts the electrical charge interactions between the positively charged histone tail residues and the negatively charged DNA wrapped around theses histones, resulting in weakened DNA-histone interactions [23, 24]. These weakened DNA-histone interactions are enhanced by the presence of histone assembly protein 1 (Nap1), resulting in chromatin opening, which increases the chance of DNA exposure to different machinery [25]. HDACs work in the opposite direction by removing an acetyl group and reversing the effects of HATs.

7.2.2.2 Histone Methylation

Histone methylation involves the recruitment of different regulatory factors and the catalysis of their binding to chromatin, which in turn controls the status of chromatin activation, a process reviewed by Greer and Yang [26]. This process is modulated by histone methyl transferases (HMTs) and histone demethylases (HDMs). HMTs transfer a methyl group from the methyl donor, S-adenosyl-L-methionine cofactor (SAM), to lysine or arginine residues [27, 28]. In contrast to DNA methylation, histone methylation can involve the addition of more than one methyl group, resulting in mono-, di-, or tri-methylation. The functional outcome of histone methylation differs depending upon which residue is modified. For instance, methylation of H3K79, H3K36, and H3K4 activates gene transcription, while methylation of H3K27 and H3K9 results in repression of transcription [29–32]. Lysine-specific demethylases (LSD) and Jumonji C (JMJC) demethylases are the two main HDMs families, and counteract the action of HMTs through the removal of histone methyl groups, as reviewed by Kooistra and Helin [33]. They, therefore, have an opposite transcriptional regulatory role to that of HMTs.

7.2.2.3 Other Histone Modifications

Other modifications include histone phosphorylation, which is carried out by kinases and phosphatases, which add or remove phosphate groups, respectively. Histone phosphorylation is implicated in cellular processes such as transcription regulation, DNA damage

detection, and chromatin remodeling [34]. Histone phosphorylation does not work in isolation, but interacts with other histone modifications. For example, H3S10ph was found to be linked to H3K14ac, and together promote gene transcription [35]. Among the well-known histone modifications is histone ubiquitylation, which is controlled by the action of histone ubiquitin ligase, which can add single or multiple ubiquitin molecules, and deubiquitinating enzymes, which can remove them. Histone ubiquitylation is involved in processes including DNA damage and transcription regulation. H2A and H2B histones are the most commonly involved, as reviewed by Cao and Yan [36]. Histone SUMOylation, the addition of Small Ubiquitin-related Modifiers (SUMOs) which are ubiquitin-like proteins, leads to transcription repression, with H4 being most heavily involved in this histone modification [37]. Although each histone modification has its distinct function and output, different modifications coexist. A combination of different histone modifications constitutes what is known as a histone code. Each histone code is composed of a unique set of histone modifications that together produce a specific epigenetic regulatory function.

7.2.3 Epigenetics and Non-Coding RNAs

Non-coding RNAs (ncRNAs) are functional RNAs which are not translated into proteins. ncRNAs can be categorized according to their size, which ranges from 20 to more than 200 nucleotides (nt). Short ncRNAs, ranging from 19 to 31 nt, include small interfering RNAs (siRNAs), tRNA-derived stress-induced RNAs (tiRNAs), micro RNAs (miRNAs), and PIWI-interacting RNAs (piRNAs), while medium-sized ncRNAs ranging from 20 to 300 nucleotides include transfer RNA (tRNA), small nucleolar RNAs (snoRNA), promoter-associated RNAs (PROMPTs), promoter-associated small RNAs (PASRs), small nuclear RNA (snRNA), and transcription start site associated RNAs (TSSa-RNAs). Long ncRNAs, with a length more than 200 nt, include transcribed ultraconserved non-coding RNAs (T-UCR), ribosomal RNA (rRNA), and long intervening non-coding RNAs (lincRNAs), reviewed by Esteller [38]. ncRNAs have been implicated in cellular processes including transcription regulation, post-transcriptional gene silencing, RNA-dependent DNA methylation, the maintenance of genome stability by silencing of transposable elements, and unpaired DNA silencing during meiosis, acting either in cis or in trans [38, 39]. The recruitment of epigenetic mechanisms to their target site is aided by the presence of transcription factors, which have sequence specificity. This sequence specificity is significantly enhanced by ncRNAs guiding the epigenetic machinery in a sequence-specific manner by hybridizing to their target complementary sequences. This enhancement is attributed to the fact that their recognition sequences are much longer than those of transcription factors. This is called a guide ncRNA mechanism. Examples of guide ncRNAs include the cis-acting X-Inactive Specific Transcript (XIST) lncRNA, which is involved in X chromosome inactivation in women. XIST recruits the protein Polycomb Repressive Complex 2 (PRC2) to deposit H3K27me marks, identifying heterochromatin

transcribed from the same X chromosome. HOX Antisense Intergenic RNA (HOTAIR) lncRNA acts in trans by recruiting PRC2 and LSD1 to, respectively, establish H3K27me domains and demethylate H3k4me marks, which also results in the repression of transcription of HOXD genes that show sequence homology to its encoding locus, HOXC. In addition to their guide functions, lncRNAs can also function as signaling molecules, scaffolds, and decoy lncRNAs [40].

7.3 Early Epigenetic Studies in Stem Cells

Conrad Waddington was the first researcher to coin the term "epigenetics," and define it as "the branch of biology which studies the causal interactions between genes and their products, which bring the phenotype into being" [41]. Waddington was interested in developmental biology and how phenotypic changes are related to genetics. In the mid-twentieth century, Waddington described cell fate decisions during development as epigenetic events, referring to the "epigenetic landscape" (Fig. 7.1) [42]. The epigenetic landscape may be considered to be the first attempt at describing the role of epigenetics in embryonic stem cell development, as this description was specific for early embryonic development.

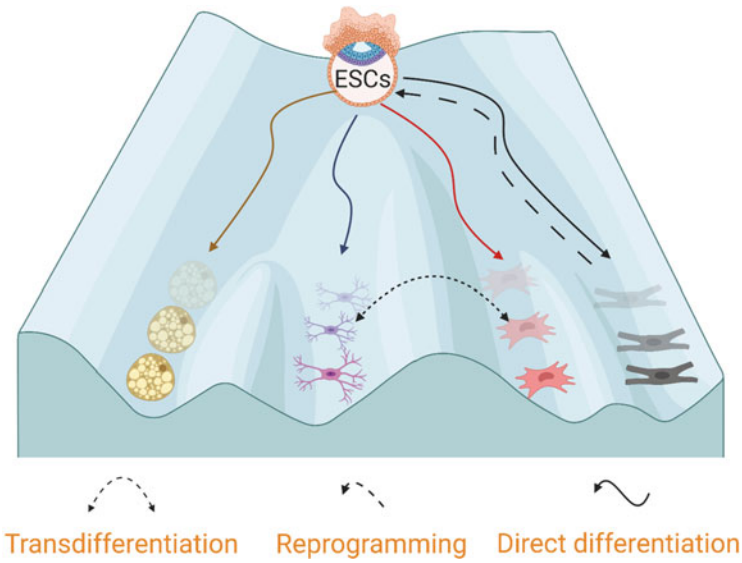

Fig. 7.1 Waddington's epigenetic landscape, depicting cell fate decisions

7.4 Epigenetics of Stem Cell Development and Reprogramming, and the Generation of Induced Pluripotent Stem Cells (iPSCs)

7.4.1 Epigenetics of Development and Reprogramming

At very early developmental stages, stem cells are not committed to specific fates, but they become committed over time. One factor contributing to this change is epigenetic interactions, which affect chromatin structure, transcription, and cellular responses to the environment. The earliest forms of stem cells are Embryonic Stem Cells (ESCs), pluripotent stem cells that can differentiate into any kind of cell, and which are isolated from the blastocyst Inner Cell Mass (ICM). The development and fate of ESCs are significantly affected by epigenetic changes. Early development involves a phenomenon known as epigenetic "reprogramming waves." These waves are responsible for the development and fate determination of ESCs, and later on for lineage commitment. The different reprogramming waves are temporally orchestrated. The first wave takes place after fertilization and zygote formation, while the second takes place during blastocyst formation, and the last wave follows after implantation [43]. The combination of these epigenetic reprogramming waves are responsible for different ESC pluripotency states. ESCs include cells with different pluripotency capacities and features. These different capacities and features are acquired after each epigenetic reprograming wave. The first wave, after fertilization, results in a loss of epigenetic marks and global demethylation, which produces an open, accessible chromatin structure. At this stage, totipotent stem cells, which can give rise to all types of cells, including the placenta, can be obtained. These totipotent stem cells form the trophoblast layer of the blastocyst, which later forms the placenta, and also form the ICM, which contains the ESCs. This process results in loss of totipotency and the beginning of differentiation. Before implantation, the blastocyst itself undergoes a wave of epigenetic reprograming. This wave results in the gain or loss of histone modifications, X chromosome reactivation in female cells, and further DNA demethylation. These epigenetic changes, which occur in the final stages of blastocyst formation, prepare the ICM to further differentiate into the hypoblast layer and ESCs, which will themselves later differentiate and give rise to all cell lineages. After implantation of the blastocyst, another epigenetic reprogramming wave takes place, and new epigenetic marks are created. The reprogramming events caused by DNA demethylation can have serious consequences if mono allelic imprinted genes—those which are expressed from one parental allele while the other allele is silenced by methylation—became demethylated, resulting in Loss Of Imprinting (LOI). LOI has been shown to result in several diseases, and there should therefore be protective mechanisms by which imprinted genes can withstand the reprogramming caused by demethylation [44].

The ZFP75- KAP1 complex has been found to recruit de novo and maintenance DNMTs, including DNMT1, DNMT3A, and DNMT3B, in addition to the HMTase and SETDB1, to the Differentially Methylated Regions (DMR) of imprinted genes. The recruitment of such epigenetic machineries maintains the imprint signature of the genes

throughout subsequent developmental reprogramming demethylation events [45]. After the last reprogramming wave, ESCs in the ICM start to restore DNA methylation, producing less accessible chromatin. Epigenetic histone repressive marks are deposited, and X chromosome inactivation is restored in female cells. Pre-implantation ESCs are called naïve ESCs, and differ from those post-implantations, which are called primed ESCs. Throughout ESCs development, the epigenetic signatures change. Pre-implantation naïve ESCs have an open chromatin structure, associated with DNA hypomethylation status, decreased numbers of repressive histone modification markers such as H3K27me3, and X chromosome reactivation. Post-implantation primed ESCs have a smaller amount of dynamic chromatin, which is associated with DNA hypermethylation, increased numbers of repressive histone modification markers such as H3K27me3, and X chromosome inactivation [43, 46].

7.4.2 Epigenetics of Somatic Cell Reprogramming into iPSCs

In 2007, Shinya Yamanaka successfully reprogrammed terminally differentiated adult human dermal fibroblasts into iPSCs using transduction with four factors: Sox2, Klf4, Oct3/4, and c-Myc [47]. iPSCs are valuable for a myriad of clinical applications, since they are autologous cells that can differentiate into any of the three germ layers in vitro. Reprogramming somatic cells into iPSCs involves increases in histone acetylation, histone methylation permissive marks (H3k4me3), and demethylation of pluripotency genes, and decreases in histone methylation repressive marks. Lineage-specific genes undergo increased deacetylation, increased DNA methylation, decreased H3k4me3, and increases in numbers of histone methylation repressive marks. In mouse iPSCs, the Oct3/4 promoter shows increased H3 acetylation, while H3K9me2 levels are decreased, leading to increased Oct3/4 expression. The Oct3/4 promoter CpGs, however, are partially methylated [48]. IPSC reprogramming includes a reduced amount of the transcription-repressive marks H3K27me3 and H3K9me3 on developmental genes. This, in turn, leads to increased transcription of developmental genes, enhancing the reprogramming of somatic cells into iPSCs [49]. Reduced H3K9me3 levels have been attributed to the depletion of different HMTases, including Suv39h1/2, setdb1, and G9a [50]. During reprogramming, the DNMT3A promoter becomes demethylated, while those of DNMT3B and DNTM3L show low methylation. The DNMT3B promoter becomes enriched in H3K4me3 histone modifications [51]. Although expression of these de novo methyltransferases improves reprogramming, it has been found to be dispensable [52]. Depletion of DNMT1 results in enhanced reprogramming of cells into iPSCs [53]. The TET1 and TET2 demethylases get activated during reprogramming, resulting in conversion of 5-methylcytosine (5-mc) into 5′ hydroxymethylcytosine (5-hmc), reducing global 5-mc in iPSCs [54, 55].

Another mechanism by which epigenetics contributes to the reprogramming of iPSCs is via the action of miRNAs. The miRNAs miR-291-3p, miR-294, and miR-295, which are downstream effectors of c-Myc, have been found to enhance reprogramming in the

presence of Sox2, Oct3/4, Klf4, and in the absence of c-Myc, which controls their expression [56]. Induction of miR-106b/25, miR-106a/363, and miR-17/92 clusters has been associated with early reprogramming changes [57]. Other ncRNAs such as linc RNA-RoR have also been implicated in the reprogramming of somatic cells into iPSCs [58]. For example, during reprogramming, XIST lncRNA levels decrease due to the action of Tsix and pgk1, leading to X chromosome reactivation [59]. Histone variants have also been shown to be involved in reprogramming. The macroH2A histone variant has been found to hinder the reprogramming process, while its deletion enhances iPSCs reprogramming [60]. This highly coordinated epigenetic regulation of iPSC reprogramming provides insights into how gene expression can be altered and suggests new ways of enhancing the generation of iPSCs.

7.5 Epigenetics of Cell Fate Determination

7.5.1 Differentiation

Epigenetic regulation continues after implantation, as ESCs continue to develop and differentiate. In ESCs, transcription start sites and promoters have been found to have both transcription-repressive and transcription-permissive histone modifications: H3K27me3 and H3K4me3, respectively [61]. The simultaneous presence of these histone marks constitutes what is known as bivalent domains. Although bivalent domains are not transcriptionally active, they are poised for rapid cell fate determination and differentiation decisions. Consider a situation in which ESCs can differentiate into two different cell types based on the expression of a bivalent domain-containing gene (x). In one cell, gene x must be active, while in the other, this gene must be silenced. In this case, ESCs will be ready to commit to any of these 2 cell types simply by losing the unneeded histone modification from the bivalent domain of their gene x- poised promoters (Fig. 7.2).

ESCs undergoing differentiation acquire specific chromatin K9 signatures, called large organized chromatin K9-modifications (LOCKs). Differentiating cells have been found to show H3K9me2-enriched LOCKs, mediated by G9a HMT [62]. There are several epigenetic differences between differentiated cells and ESCs, including bivalent domains that are reduced upon ESC differentiation. Compared to differentiated cells, ESCs have a more dynamic chromatin structure and organization, and more dispersed heterochromatic markers than the localized heterochromatic markers observed in differentiated cells [63]. ESCs also show global increases in H3K4me3 and histone acetylation, reflecting their euchromatic organization [64]. Nevertheless, differentiated cells have globally reduced H3 and H4 acetylation levels with increased H3K9me3, reflecting the heterochromatic nature of the chromatin of differentiated cells [65]. Epigenetic control of stem cell differentiation is not limited to the lineage commitment of ESCs. One well-studied example is epigenetic control over trilineage differentiation of Mesenchymal Stem Cells (MSCs). Several diverse histone-modifying mechanisms have been observed to direct

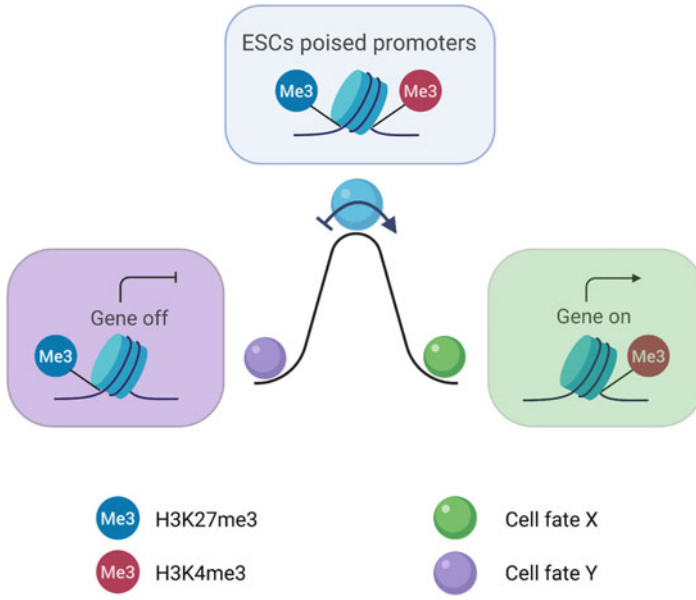

Fig. 7.2 Bivalent domains of ESCs poised promoters

differentiation into specific lineages. Tip60, mixed-lineage leukemia (MLL), SET8, EZH2, LSD1, phf2, and HDAC6 have been shown to promote MSC adipogenic differentiation by targeting the regulatory genes PPARγ, Adipsin, and Fabp4. KDM2A, KDM2B, KDM4B, KDM6A, KDM6B, and ESET are involved in osteogenic differentiation in MSCs by affecting Runx2, bone sialoprotein, and osteopontin regulatory genes. Promotion of chondrogenic differentiation in MSCs is modulated by the action of Gcn5, p300, HDAC1, and HDAC2, which target the regulatory proteins Sox9, Aggrecan, and Col2A1 [66].

7.5.2 Transdifferentiation

Transdifferentiation is the process through which terminally differentiated cells switch their lineage to another lineage without going into de-differentiation [67]. In pre-adipocytes and fibroblasts, Bmp2 and Alp genes are unresponsive to Wnt3a, which induces osteoblast differentiation. Bmp2 and Alp were found to have increased heterochromatic DNA and chromatin modifications, including increased H3K9 methylation, increased CpG methylation, and reduced acetylation. Upon treatment with 5-aza-deoxycytidine, a DNMT inhibitor, or trichostatin A, an HDAC inhibitor, pre-adipocytes and fibroblasts were successfully transdifferentiated into osteoblasts [68]. Another example of the importance of epigenetic mechanisms in controlling transdifferentiation is the conversion of pancreatic β cells to α

cells due to DNMT1 deficiency. DNMT1 methylates Arx, which is the master regulator responsible for the maintenance of the identity of α cells, and its deficiency allows the expression of Arx, promoting the conversion of β cells to α cells [69]. The transdifferentiation of MSCs into myocardial, neuronal, and endothelial lineages is regulated by a variety of histone modifiers. G9a inhibits MSC conversion to endothelial cells by targeting the regulatory proteins VCAM1 and PECAM1, while EZH2 and HDACs inhibit the conversion of MSCs to neural cells by targeting the regulatory proteins Nestin and Musashi. Transdifferentiation of MSCs to myocardial cells is inhibited by HDAC1 and HDAC2, and promoted by Gcn5 HAT, which targets the regulatory proteins GATA4 and NKx2.5, as reviewed by Huang et al. [66].

7.6 Epigenetics in Stem Cell Aging

The aging of stem cells has a significant impact on their capacity for regeneration, and on the development of degenerative diseases. Different factors contribute to stem cell aging, including the accumulation of toxic metabolites, niche degeneration, DNA damage, declines in mitochondrial efficiency, extracellular factors, and epigenetic alterations [70, 71]. The modulation of the epigenetic mechanisms that are associated with aging has been well studied in several models, of which we will focus on Hematopoietic Stem Cells (HSCs), Skeletal Muscle Stem Cells (MuSCs), and MSCs.

7.6.1 Models of Epigenetic Contributions to Stem Cell Aging

7.6.1.1 HSC Aging

Aging of HSCs is associated with several pathological conditions, of which inflammation is a hallmark. Aged HSCs have elevated levels of H3K4me3, which is involved in the regulation of the expression of self-renewal genes. Elevated levels of H3K27me3, a transcription-repressing histone, have also been reported, leading to repression of the genes involved in cell fate determination, lineage commitment, and differentiation. Among these repressed genes is Flt3, which is involved in HSC lymphoid differentiation. Consistent with these histone modification marks, hypermethylation of the DMRs associated with differentiation-promoting genes was observed, while DMRs associated with self-renewal were hypomethylated [72]. In brief, these epigenetics changes enhance HSC self-renewal, while limiting their differentiation into a lymphoid lineage. This limited lymphoid differentiation results in a differentiation capacity skewed toward the myeloid lineage. Together with the previously described increased self-renewal capacity, this phenomenon leads to clones of the myeloid lineage dominating the HSCs clones and limiting clone diversity. This phenomenon, known as clonal collapse, is among the well-known hallmarks of HSC aging [73, 74], further confirming the importance of epigenetics in HSC aging.

7.6.1.2 MuSC Aging

MuSCs, also known as satellite cells (SCs), are quiescent cells that reside in the G0 phase until they are activated by skeletal muscle injury. Aging of SCs can adversely affect the regenerative capacity of skeletal muscles. Aged SCs have been shown to acquire epigenetic alterations at the chromatin level that affect the activation and response of SCs to skeletal muscle damage. These epigenetic alterations, caused by increased H3K27me3 levels, result in the repression of chromatin domains, which are associated with reduced expression of histone genes. These alterations cause aged SCs, activated by skeletal muscle damage, to delay cell cycle entry [75]. The downregulation of expression of histone genes associated with H3K27me3 repressed chromatin domains may be a link between H3K27me3 repressed domains and cell cycle entry delay, but this link needs further investigation. The delay in cell cycle entry can be explained by data revealing other age-associated epigenetic signature alterations to be involved in delay in entry to the cell cycle. For example, H3K4me3 levels were found to be increased for genes encoding Cyclin Dependent Kinase Inhibitors (CDKIs) such as p16 and p21, increasing the expression of these genes [76]. Upregulation of p16 and p21 expression can delay cell cycle entry in aged SCs. The function of aged SCs is further influenced by other epigenetic changes. Aged SCs show increased expression of the Hoxa9 gene, which adversely affects SC function. This upregulated expression was found to be the result of increased deposition of H3K4me3 at the Hoxa9 promoter, which is associated with aging [77].

7.6.1.3 MSC Aging

The self-renewal, proliferation, and differentiation capacities of MSCs are all compromised with aging. Histone 3 (H3) acetylation levels are substantially altered in aged MSCs. H3K9 and H3K14 acetylation levels associated with TERT, Oct4, and Sox2 genes have also been found to be decreased, resulting in compromised capacity for proliferation and self-renewal. H3K9 and H3K14 acetylation levels associated with Runx2 and ALP, however, are increased, resulting in an enhanced commitment to an osteogenic lineage [78].

7.7 Metabolic Regulation of Stem Cell Epigenetics

Metabolic changes exert variable levels of control on epigenetic alterations, depending upon the cell type [79]. Metabolites such as threonine dehydrogenase (TDH) control the utilization of threonine in SAM production by controlling the catabolism of threonine into glycine, which is used for SAM production. SAM is considered to be the primary methyl donor for several methylation mechanisms. Concomitant with ESC differentiation, TDH levels have been shown to drop. This drop contributes to the reduction of SAM levels, leading to decreased H3K4me3 levels, declines in ESC proliferation, and increased differentiation [80, 81]. The metabolic contribution to epigenetic changes is modulated by metabolites acting as cofactors or chemical group donors. For example, reduced glycolysis concomitant with early ESCs differentiation results in the deacetylation of H3K27 and

H3K9. When glycolysis is reduced concomitant with the differentiation of ESCs, acetyl-CoA production, which involves the donation of an acyl group for histone acetylation, is reduced, resulting in increased H3K27 and H3K9 deacetylation [82]. Another example of how metabolites affect the epigenetic signatures of stem cells is the maintenance of murine ESC pluripotency by glutamine levels. An increased ratio of intracellular α-ketoglutarate (α-KG), a product of glutamine catabolism, to succinate increases DNA, and histone demethylation, resulting in increased expression of pluripotency genes. This modulation of demethylation has been attributed to α-KG, which serves as a cofactor for ten DNA and histone demethylases, including enzymes of the TET and Jumonji families [83]. The metabolic control of stem cell epigenetics has also been documented in other adult stem cells, including MuSCs and neural stem cells [79]. Stem cells from patients with metabolic disorders are thus expected to show altered epigenetic signatures, leading to compromised overall functionality.

7.8 Environmental Interactions and the Altered Epigenetics of Stem Cells

Environmental factors, such as malnutrition, microbiota, hypoxic conditions, and lifestyle factors all alter the epigenetic signature of stem cells. For example, butyrate produced by human gut microbiota has been shown to enhance pluripotency via the promotion of DNA demethylation and H3 acetylation of genes known to be associated with pluripotency [84]. Propionate and butyrate have been shown to block marrow stem cell generation of dendritic cells via inhibition of HDACs [85].

The stem cell niche is of particular importance in regulating the epigenetic-biophysical axis. Biophysical stimuli from the extracellular niche signal to the cells via extracellular matrix components. Murine iPSCs seeded on microgrooves were found to have enhanced reprogramming when compared to those seeded on flat surfaces. This effect was mediated by an increase in H3K4me2, H3K4me3, and histone acetylation. Such increases are controlled by an increase in the expression of WD repeat-containing protein 5 (WDR5), an H3K4 methyltransferase complex core subunit, and a decrease in the expression of HDAC2. This altered epigenetic expression was found to be modulated by cytoskeletal reorganization because of changes in the cell shape in response to the microgroove topography [86]. Laminar shear stress has been shown to induce a cardiovascular lineage commitment in murine ESCs by increasing H3K14 acetylation, H3K79 methylation, and H3S10 phosphorylation [87].

7.9 Epigenetics of CSCs

CSCs are a small subset of cells in tumors, and display both cancer and stem cell properties. CSCs are known to have an enhanced capacity for self-renewal and drug resistance. They are also involved in tumor initiation and progression. They are quiescent cells that divide asymmetrically, giving rise to both differentiated tumor cells and new CSCs [88]. Recent studies show that as CSCs become more malignant , they start shifting toward symmetric division [89]. It has been proposed that CSCs originate from cancer cells which acquire stem cell characteristics, a hypothesis that has been reviewed by Plaks et al. [90]. Another model proposes that CSCs originate from stem cells that acquire cancer characteristics [91, 92], (Fig. 7.3). Experimental work supporting this hierarchical theory of CSCs in human acute myeloid leukemia has been carried out by Bonnet and Dick [93].

Both models agree that the origin of CSCs is caused by reprogramming events, including epigenetic reprogramming. Such highly orchestrated epigenetic modulations are controlled by a variety of signaling pathways, as reviewed by Toh et al. [94]. These pathways regulate CSC properties including self-renewal, epithelial to mesenchymal transition (EMT), drug resistance, and CSC maintenance. The Wnt/β-catenin, hedgehog (Hh), and Notch signaling pathways have been shown to increase CSC self-renewal and maintenance [95–98]. As in other cancers, activation of the Wnt/β-catenin pathway is

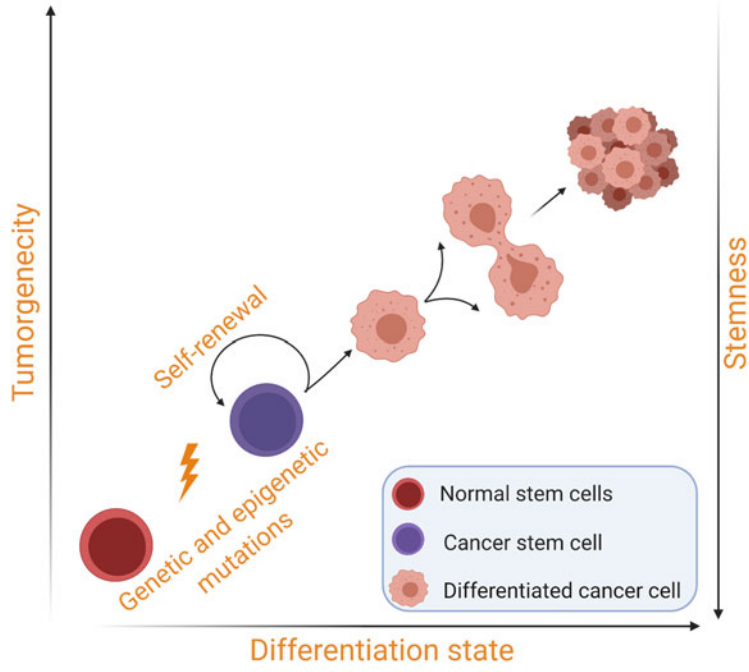

Fig. 7.3 Model of the origin of CSCs from normal stem cells that acquire cancer cell characteristics

mediated by the methylation of the promoters of its inhibitors, especially DKK1, which shows decreased H3k16 acetylation and increased H3K27me3 [99–101]. Regulation of Hh pathway in CSCs is activated by downregulation of the chromatin remodeler SNF5. SNF5 inhibits the expression of Gli proteins, required for Hh activation. Downregulation of SNF5 in CSCs thus leads to Hh activation [102–104]. Another way in which Hh is activated in CSCs is via deletion of REN (Ubiquitin ligase complex). This deletion allows the expression of the REN target HDAC1, the expression of which brings about an increase in the Gli proteins needed for Hh activation [105, 106].

The Notch pathway is important in CSCs [96, 97, 107]. Notch pathway activation increases the expression of its targets JAGGED2, HES1, and HES5. In CSCs, HDACs that are recruited to JAGGED2 are reduced, resulting in an increase in JAGGED2 expression [108]. HES1 and HES5 are occupied by the PRC2 members EZH2 and SUZ12, leading to increased H3k27 methylation. In CSCs, EZH2, and SUZ12 are sequestered by binding to the STRAP protein. This leads to a reduction in H3K27 methylation and increases in HES1 and HES5, ultimately activating the Notch signaling pathway [107].

EMT is known to be highly coordinated in CSCs [109]. CSCs showed increased methylation of E-cadherin, an epithelial marker whose loss promotes the production of EMT and increased methylation of the miR-205 and miR-200 families. These microRNAs target ZEB1 and ZEB2 proteins, E-cadherin repressors, which promote EMT [110–113]. One way in which drug resistance in CSCs is mediated is by increasing permissive epigenetic marks such as H3K4me3, H3S10ph, and H3 acetylation of the ATP-binding cassette (ABCG2), responsible for drug efflux outside the cell [114, 115]. Other examples of epigenetic control mechanisms specific to certain types of CSCs include translocation-derived fusion of MLL, an H3K4 methyltransferase, and AF4 protein. This MLL-AF4 fusion, along with Bmi1, a polycomb complex group protein, produces specific and efficient generation of Leukemia Stem Cells (LSCs) [116, 117]. Patterns of DNA methylation have also been shown to be disrupted in different types of CSCs due to mutations in epigenetic DNA methylation regulators, including DNMT3A, TET, and IDH [118].

7.10 Therapeutic Applications of Stem Cell Epigenetics

Epigenetic therapeutics have been developed to take advantage of the broad regulatory spectrum of epigenetic mechanisms. Most epigenetic therapeutics are based on establishing, maintaining, or removing specific histone modifications or DNA methylation patterns. Inhibitors, including HDAC, HMT, HDM, and DNMT inhibitors, have been used as cancer therapeutics, as summarized by Toh et al. [94]. Such inhibitors and small molecules can be used to reverse the altered epigenetic signatures of aged stem cells, CSCs, and niche-affected stem cells. If the hypermethylation of certain stem cell genes leads to stem cell aging, then DNMT inhibitors can be used to restore the original methylation state. This approach also applies to the dysregulated epigenetic signatures of CSCs and niche-affected stem cells (Fig. 7.4). For example, ovarian CSCs treated with

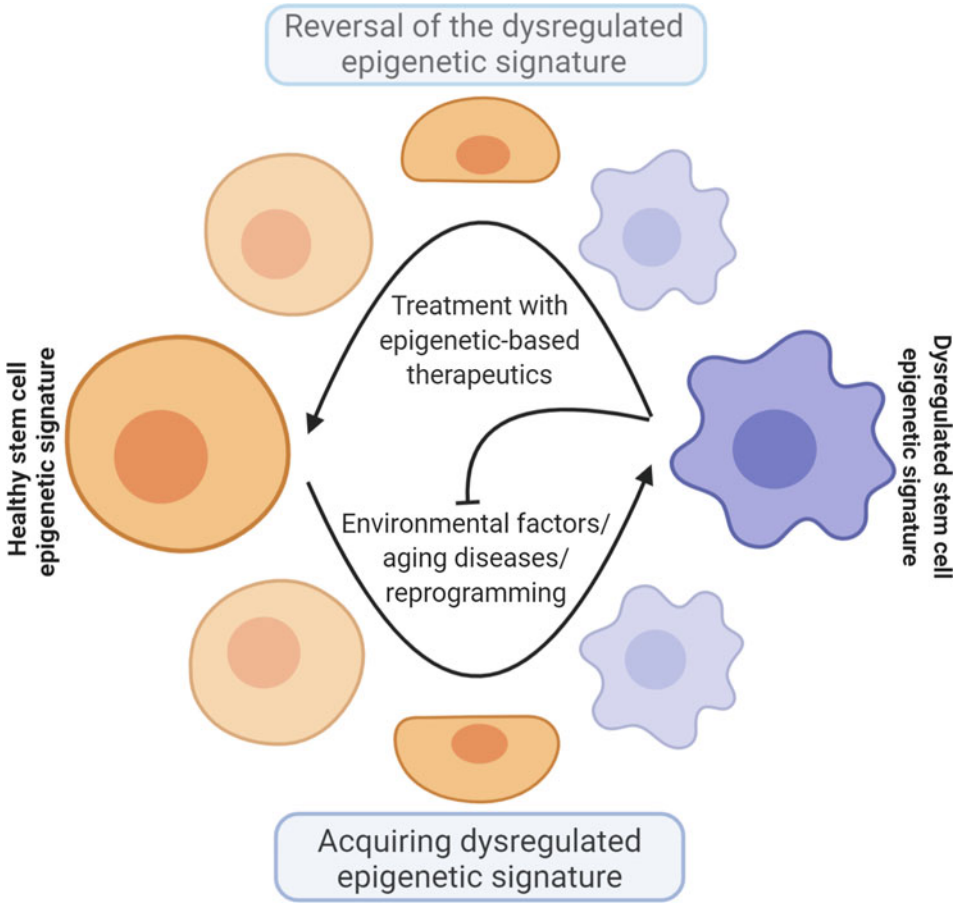

Fig. 7.4 Epigenetic-based therapeutics work by reversing newly acquired epigenetic dysregulated signatures

SGI-110, a DNMT inhibitor, showed reduced drug resistance, tumor initiation capacity, and gradual loss of stemness [119]. Knockdown of DNMT1 in lung CSCs resulted in decreased proliferation and stem cell characteristics, suggesting a promising therapeutic role for DNMT inhibitors [120].

Take Home Message

- Epigenetic regulation of stem cell is essential for understanding their development, reprogramming, and cellular differentiation.
- Each epigenetically modulated process has specific epigenetic signature that differs between cell types.
- Understanding the epigenetic control of stem cell aging and cancer stem cell origination provides insight into their development and progress, and a window for developing epigenetic-based therapeutics.
- Altered stem cell epigenetic signature due to environmental factors provides valuable knowledge on lifestyle modifications and dietary habits that impact disease development.

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Isolation of Bone Marrow and Adipose-Derived Mesenchymal Stromal Cells

8

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What You Will Learn in This Chapter

This chapter provides a brief introduction to the history and methods of isolation of mesenchymal stromal cells (MSCs) and their usage in the laboratory. The chapter provides a common protocol for isolation of two important types of MSCs, collected from the adipose tissue and bone marrow. The protocol for isolation of stem cells and primary cell culture of bone marrow and adipose tissue from Sprague-Dawley rats will concurrently cover common cell culture techniques.

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8.1 Introduction

Bone marrow (BM) and adipose tissue (AT) are considered the main sources of multipotent mesenchymal stromal cells [1, 2]. Mesenchymal stromal cells (MSCs) are characterized by a capacity for self-renewal and multilineage differentiation potential. MSCs are a rare population of adult resident cells that differ from other somatic cells in their capacity for regenerating damaged and lost cells within an organ or a tissue. MSCs are readily isolated and propagated in the lab for research purposes. MSCs have been used in various clinical applications in regenerative medicine and cell-based therapy, due to their ease of their expansion, relative safety, and lack of associated ethical concerns unlike embryonic stem cells [3–5].

The notion of stem cells was conceived at the end of the nineteenth century [6–8]. The term "stem cell" dates back to the German biologist Ernst Haeckel who adopted Darwin's theory of evolution, depicting several phylogenetic trees to represent the evolution of organisms by descent from common ancestors [9]. These trees were termed "stammbäume" (German for family trees or "stem trees"), and the German term for stem cells "stammzelle" was since used to describe the ancestor unicellular organism of all multicellular organisms [9–11]. In the 1970s, the renowned scientist Alexander Friedenstein observed that several plastic-adherent cells grew from BM cultures [12, 13]. He reported the seminal findings on what are now known as MSCs, and their distinctive traits that granted them a "stem cell characteristic" capability to form colonies, and the ability to differentiate into cells of the osteogenic lineage after transplantation into animals [12, 14–18].The plastic-adherent cells were obtained after the long-term culture of BM and other blood-forming organs, and displayed a colony-forming capacity and osteogenic differentiation characteristics both in vitro and in vivo upon re-transplantation [12, 16].

The "MSCs" term was coined by Arnold Caplan in 1991 [19–21]. MSCs are multipotent cells that have the potential to differentiate into multiple lineages and different cell types, such as chondrocytes and osteocytes [22–25], making them valuable for tissue engineering. Because MSCs produce high levels of bioactive agents [26] that are both immunomodulatory [27] and trophic [27], they are used for various clinical purposes [28]. Caplan proposed to change the name of MSCs to "medicinal signaling cells, also MSCs" based on their therapeutic role that was independent of their multipotent properties [20].

Furthermore, Caplan observed that MSCs could be isolated from almost every tissue in the human body. He attributed the vast distribution of MSCs in diverse tissues and organs to the fact all the tissues are vascularized and that every blood vessel in the body has mesenchymal cells in abluminal locations (outer surface of the blood vessels). These perivascular cells were named pericytes, the specific markers of which co-localize with MSCs markers [29, 30].

According to the International Society for Cellular Therapy (ISCT), MSCs must, first, display plastic-adherence when maintained in standard culture conditions. Secondly, 95% or more of the MSCs population must express CD73, CD90, and CD105, and only a minimal proportion (2% or less) express or loss of expression of the hematopoietic and immune markers, such as CD11b, CD79α, CD14, CD34, CD45 or CD19 and HLA-DR, respectively. Third, MSCs must differentiate in vitro into adipocytes, chondroblasts, and osteoblasts [31, 32].

The mesenchymal stromal cells (MSCs) are located as an important population of cells in the mesenchymal stroma with stem cell-like characteristics including self-renewal and differentiation capacities. MSCs can be derived from different tissue sources. These multipotent MSCs can be found in nearly all tissues such as adipose tissue and bone marrow, MSCs are mostly located in perivascular niches [33, 34].

Hematopoietic stem cells (HSCs) and bone marrow mesenchymal stromal cells (BM-MSCs) are the most common stem cell populations isolated from the BM. Pittenger et al. [22] described plastic-adherent BM-MSCs that displayed the minimal stem cell characteristics, including a stable phenotype, and remaining as a monolayer in vitro.

Adipose-derived mesenchymal stromal cells (AD-MSCs) are a stem cells population within the adipose tissues [35]. AD-MSCs are of mesodermal origin and possess the same phenotypic and differential potentials as MSCs isolated from other sources, including BM [1, 36. In 2001, Zuk et al. successfully isolated AD-MSCs population from the stromal vascular fraction (SVF) of processed lipoaspirates and exploited their plastic adherence potentials using enzymatic digestion [9, 36]. AD-MSCs were later characterized [12] morphologically (spindle-shaped, adherent cells) [15] and phenotypically according to self-renewal and tri-lineage differentiation potential (into adipogenic, osteogenic, and chondrogenic lineages) [16]. Subsequently, several studies reported methods to isolate AD-MSCs with high purity and viability [37–39]. The SVF, and especially the AD-MSCs, appear to have substantial clinical importance and play a major role in wound healing, anti-inflammatory, and immune-modulating responses associated with injury and diseases [17–19, 40–43].

There are several approaches for the isolation and purification of MSCs such as enzymatic digestion, antibody based selection methods, and explant culture techniques [44–46]. Regardless of the method, isolated MSCs should fulfill the minimal criteria of the International Society for Cellular Therapy (ISCT) to be identified as multipotent stem cells [47].

The two main methods involved in isolation of MSCs: the enzymatic dissociation of tissues (digestion method) and primary explant culture (non-enzymatic method). AD-MSCs with similar biological properties can be obtained from the adipose tissue using either methods [48]. The typical protocol for isolating AD-MSCs from adipose tissue is the digestion method by using collagenase type I treatment for 1 h. The digestion process can be harsh and damage the stem cells. Additional factors, such as the length of the digestion time and enzyme concentration, may affect the cell yield, viability, phenotype, and differentiation potential of the isolated cells [49, 50].

8.2 Mesenchymal Stromal Cell Isolation and Culture

In this section, we focus on the isolation of MSCs from BM aspirates and from AT.

8.2.1 Materials and Methods

8.2.1.1 Materials

(a) Supplies
- 60 mm diameter sterile tissue culture dishes.
- 25 cm^2 (T25) and 75 cm^2 (T75) tissue culture flasks.
- Micropipettes tips (P1000, P200, P10).
- Glass disposable pipettes 10 and 25 mL.
- Polypropylene conical centrifuge tubes, 15 mL.
- Polypropylene conical centrifuge tubes, 50 mL.
- Pasteur pipettes.
- Gloves.
- 5 mL syringes with a 21-gauge needle.
- Racks suitable for holding any 50- and 15-mm falcon tubes.
- Dissecting board.
- Dissection kit (Sterile scissors, forceps and sclapel with blades).
- 70-μm filter.
- 25-gauge needle.
- 100-μm nylon mesh.
- Stainless steel mesh.
- 0.2-μm syringe filters.
- Sterile guaze.

(b) Reagents and buffers
- Dulbecco's Modified Eagle's Medium—Low Glucose (DMEM/LG).
- Phosphate-buffered saline (PBS).
- Trypan Blue dye.
- 70% Ethanol.
- Fetal bovine serum (FBS).
- Penicillin/streptomycin solution (10,000 U/mL).
- 0.25% Trypsin–EDTA solution.
- 0.2% Collagenase type 1.
- L-glutamine.
- 2% gelatin or type 1 collagen.

(c) Equipment
- Cooling centrifuge.
- CO_2/humid incubator.
- Laminar Flow class II, type B (Biosafety cabinet).
- Inverted microscope.
- Hemocytometer.
- Water bath.

8.2.1.2 Methods

Isolation of Adipose-Derived Mesenchymal Stromal Cells (AD-MSCs)

Sample Collection and Preparation

All procedures are performed in a biosafety cabinet. AT is collected from the subcutaneous inguinal AT from (10–14 weeks old) Sprague-Dawley rats [48, 51]. AD-MSCs isolation is initiated within 20 min of AT collection.

1. Sacrifice the animal using anesthesia or CO_2 asphyxiation according to recent recommendations by the American Veterinary Medical Association (AVMA) [52].
2. Saturate the fur with 70% ethanol and place the animal in dorsal recumbency (ventral side up) with the fore- and hind-limbs abducted on a dissection board.
3. Using sterile tissue forceps, lift the skin and cut the skin and musculature of the abdomen with a scalpel blade vertically toward the head along the midline at the abdominal level. Reflect the skin to expose the peritoneal cavity.
4. Using sterile scissors and forceps, harvest the AT from the subcutaneous fat pads in the inguinal region [53].
5. Preserve the tissue in a culture dish or conical centrifuge tube containing DMEM/LG supplemented with 1% penicillin/streptomycin solution, thus the tissue is completely immersed, until starting the isolation procedure.

   N.B. Warm all reagents used in tissue culture in a water bath at 37 °C before the start.

Primary explant culture (non-enzymatic method)

Explant culture is an in vitro technique that allows small tissue fragments to adhere to the growth surface, which will usually gives rise to an outgrowth of cells. Explant culture may be preferable when only small tissue fragments are available [48].

1. Wash the AT twice with PBS supplemented with 1% penicillin/streptomycin.
2. Transfer the AT to a dry sterile 60 mm tissue culture dish and then mince into 1–2 mm^3 pieces using sterile scissors under aseptic conditions.
3. Place the tissue explants in a cell culture vessel (Culture dish or flask), allowing 5 mm of space between explant fragments.

N.B. culture dishes and flasks can be coated with 2% gelatin or type I collagen to obtain better adhesion of the explants [54].

4. Leave the explant on the tissue culture plate for 5–15 min to adhere.
5. After adhesion, gently add fresh complete culture medium (CCM) (DMEM/LG supplemented with 10% FBS, 1% L-glutamine and 1% penicillin/streptomycin) gently to the culture dish/flask without disturbing the explants.
6. Change the medium every 2–3 days according to the cell culture protocol described below.
7. After 5 to 6 days, gently remove the tissue explants from the cell culture dish/flask, leaving the adherent AD-MSCs .
8. Wash the AD-MSCs with PBS and change the medium before visualizing the cells under an inverted microscope.
9. At 80–90% confluence (80– 90% of the surface of the cell culture vessel is covered by adherent cells), the cells are either passaged by splitting them over two or more cell culture vessels to avoid over-confluence (according to the manufacture's recommendation regarding the minimal seeding density of the used cell culture vessels) as passage one (P1), or cryopreserved as passage zero (P0) [55].

- Troubleshooting of the explant culture method

1. During explant culture, tissue fragments must tightly adhere to the culture dish. Adherence of the cultured fragments was found to be essential for the migration of AD-MSCs, as they fail to migrate from floating fragments [56].
2. Stainless steel mesh can be used to help fragments to adhere to the plate and prevent their floating. Proper adherence of the explant fragment yields a more efficient AD-MSCs population [56].
3. Small tissue fragments are preferred during explant cultures to increase the surface area exposed of the explant and avoid central necrosis due to insufficient oxygen supply and nutrients [57].
4. The seeding density affects the migration and growth of cells from tissue fragments. High density seeding may inhibit the outgrowth of cells from the tissue fragments. Different substrates have been reported to enhance AD-MSCs outgrowth from tissue fragments (i.e., collagen, basement membrane proteins, or fibronectin) [58].
5. The outgrowth of AD-MSCs in explant cultures usually takes 1–3 days. The explant culture would be considered to have failed if no outgrowth was observed after 4–5 days in culture.
6. Longer maintenance of the explant (4–7 days) might induce adipogenic differentiation of AD-MSCs around the tissue fragments due to the secretion of specific adipogenic-inducible factors by the explant [59].
7. The explant culture may contain a mixture of adherent cells that are not stem cells, but the limited proliferation and self-renewal of these cells allow their exclusion during the first subcultures [60].

8. After 7 days of the explant culturing, cells are maintained in culture for an additional 10–14 days until confluence. The AD-MSCs yield obtained from the explant culture method is approximately 5–8 $\times$ 10^5 cells/g tissue [57].

- Advantages of explant culture

1. Explant culture method was found to give a higher yield of cells than the digestion method after primary culture [48, 60].
2. The presence of primary tissues in the explant provides the outgrowing cells with some of the required cytokines and growth factors.
3. The isolation of cells using explant culture methods without the involvement of enzymes benefits supports their use in therapeutic applications and limits the safety concerns associated with the use of enzymes (cellular stress and chemical contamination).
4. The explant culture method is more cost-effective and more time-efficient than the enzymatic digestion technique [60].

Enzymatic Digestion Method

The most commonly used protocol for AD-MSCs isolation from fat is the enzymatic digestion of the extracellular matrix (ECM) to obtain different cell types, including adipocytes, AD-MSCs, fibroblasts, endothelial cells, hematopoietic cells, and immune cells [1, 42, 61, 62]. AD-MSCs isolation by enzymatic degradation is considered the most conventional protocol despite the variation in cell yield and modest reproducibility [37, 47], (Fig. 8.1).

1. Collect the inguinal fat pads, and mince into fine pieces of ~2 mm in size, using sterile scissors as described previously.
2. Wash the sample with PBS supplemented with 1% penicillin/streptomycin to remove contamintaing blood and debris.
3. Transfer the minced pieces into a 50 mL tube, and add collagenase solution (0.2% collagenase type I dissolved in PBS and filtered using a 0.2-μm syringe filter), following the manufacturer's instructions, to enzymatically digest the ECM. The collagenase solution should completely cover the minced AT.

   N.B. If digesting less than 2.0 grams AT, use a minimum of 10 mL collagenase solution to ensure complete digestion. If digesting $\geq$2 grams tissue, add 5 mL collagenase solution/gram AT to each centrifuge tube [63]. Collagenase solution should be freshly prrepared.
4. Incubate the tube containing the AT and colleagenase in a water bath for 1 hr. at 37 °C with agitation [64]. Carefully observe the tissue digestion process, as longer incubations may result in damage to the cells of interest [64].

   N.B. (It is important to adjust the temperature to 37 °C for the optimal activity of collagenase).

5. After proper digestion, the tissue will be completely liquefied, with no visible solid tissue. Add an equal volume of complete culture medium (CCM) to the heterogeneous cell mixture.
   N.B. CCM is essential to neutralize collagenase activity.
6. Filter the cell suspension using 100 μm nylon mesh to remove undigested tissues.
   Centrifuge the cell filtrate at 1200 xg for 10 min at 37 °C [65]. After centrifugation, the solution is separated into two layers with the cell pellet at the bottom of the tube.
   N.B. adipocytes may be found floating as a fatty yellow layer above the aqueous supernatant.
7. Discard the fatty layer and liquid supernatant to obtain the cell pellet containing the SVF, and then resuspend in PBS.
8. Transfer the cell suspenion into a 50-mL tube, wash the cells with PBS by gently pipetting up and down and re-centrifuge at speed 1200 xg for 10 mins at 37 °C.
   N.B. washing removes any traces of red blood cells, adipocytes, and other contaminants [64].
9. Repeat step (9) if required (cell pellet appears as red colored mass).
10. Resuspend the pellet that now contains the AD-MSCs in 10 mL of CCM for primary culture.

Recent studies showed that combining both the enzymatic and mechanical methods could improve the viability, reproducibility, and yield of the AD-MSCs, as mincing the AT into small pieces facilitates the action of the digesting enzyme [37, 54, 66, 67].

- The stromal vascular fraction

AT is composed of different cell types. In the late 90s, scientists isolated what is known as the stromal vascular fraction (SVF) form AT for fat tissue engineering purposes [68]. The AT was dissociated either by enzymatic or non-enzymatic methods, centrifuged and differentiated adipocytes (the floating portion on the aqueous layer) were removed leaving behind a heterogeneous mixture of cells, known as the SVF [36, 37]. Morphologic and phenotypic studies of the heterogeneous SVF population [69] showed the presence of different cell types within the mixture including AD-MSCs [70], fibroblasts, endothelial cells, pericytes, erythrocytes [61], and immune cells [62] (i.e. monocytes/macrophages and lymphocytes).

Selected Methods: Advanced Non-enzymatic Methods of Isolating AD-MSCs

Despite the common use of the enzymatic method for the isolation of AD-MSCs, this approach has several limitations because of the variation in the efficacy of AD-MSCs isolated from different ATs and their heterogeneity [71]. In addition to the safety concerns regarding their clinical use, the enzymes used to isolate AD-MSCs can cause cell damage or incomplete dissociation from the connective tissue, in addition to the safety concerns

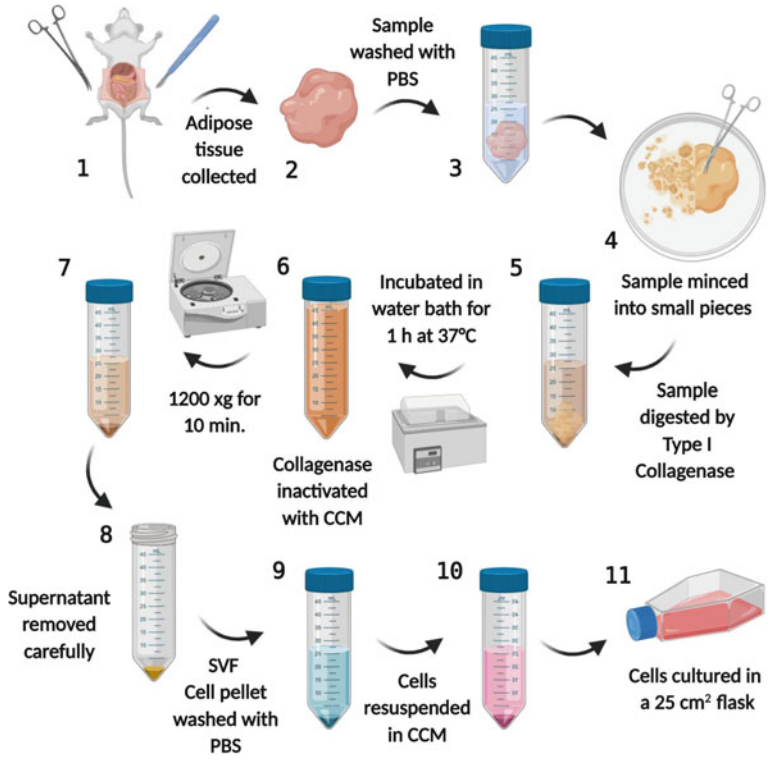

Fig. 8.1 Procedure for isolation of adipose stem cells

about their clinical use [72]. Therefore, many researchers have adopted alternative non-enzymatic approaches to isolate AD-MSCs based on mechanical forces such as centrifugation, shear force, and pressure, besides, radiation to avoid the use of enzymes and facilitate the separation of AD-MSCs aggregates from tissue samples [73]. Non-enzymatic separation techniques range from simple techniques to more advanced ones. For example, the plating of the aspirate, without any enzymes, was found to produce a high yield of AD-MSCs [74]. A considerable number of AD-MSCs were separated in lipoaspirate fluid during liposuction due to mechanical forces [75]. Another study found that vigorous shaking and washing of the adipose lipoaspirate with PBS yielded a substantial amount of AD-MSCs from the lipoaspirate floating portion [76]. Furthermore, an alternative method using mechanical dissociation yielded large quantities of adherent AD-MSCs, by the centrifugation of lipoaspirates at 900 g for 15 mins at room temperature [37]. These results indicated that advanced non-enzymatic methods could save time and provide a rather safe AD-MSCs isolation method for therapeutic applications.

Isolation of Bone Marrow Mesenchymal Stromal Cells (BM-MSCs)

Sample Collection and Isolation

To collect BM, female Sprague–Dawley rats (6–8 weeks old) are euthanized and BM-MSCs will be collected as described in (Fig. 8.2).

1. Disinfect animal skin using 70% ethanol.
2. Make an incision around the perimeter of the hind limbs where they attach to the trunk and remove the skin by pulling it toward the foot, which is cut at the ankle bone.
3. Dissect out the hind limbs from the trunk of the body by carefully cutting along the vertebral column, avoid damaging the femur and tibia (long bones).
4. Wash the hind limbs with PBS supplemented with 1% penicillin/streptomycin.
5. Preserve the limbs in ice-cold DMEM medium supplemented with 1% penicillin/streptomycin.
6. Hemisect the hind limb at the knee joint.
7. Remove the muscle and tendons from the femur and tibia by pulling the tissue toward the end of the bone, starting from the hip, or the ankle toward the knee joint, and then dislocate the joints at the knee if possible.
8. Use sterile gauze to wipe the remaining tissue, clean the bones, and place them in ice cold DMEM medium supplemented with 1% penicillin/streptomycin until the marrow extraction (preferably performed immediately).
9. Minimize the time between the dissection and BM extraction.
10. Harvest the BM using proper sterile techniques.
11. Cut the ends of the femurs and tibia just below the end of the marrow cavity using a bone cutter to expose the marrow inside.
12. Flush each bone with complete culture medium (CCM) using a 5-mL syringe with 21-gauge needle into a 50 mL conical centrifuge tube. Flush all of the marrow until the bones appear white [77].
13. Resuspend the marrow using a 25-gauge needle. Pull the cell pellets up and down slowly to break up clumps to obtain a single cell suspension.
14. Filter the cell suspension using a 70 μm filter placed on top of a 50 mL conical centrifuge tube to remove any bone fragments.
15. Centrifuge the cell suspension at 2000 rpm for 10 mins at room temperature (18ºC–25ºC) and discard the supernatant.
16. Resuspend the pellet in CCM, transfer to 25-cm^2 tissue culture flasks, and place the flasks in a 5% CO_2/humid incubator.

Cell Culture and Propagation

MSCs are cultured in low glucose Dulbecco's Modified Eagle's Medium (DMEM/LG) supplemented with 10% FBS, 1% penicillin/streptomycin, and 1% L-glutamine

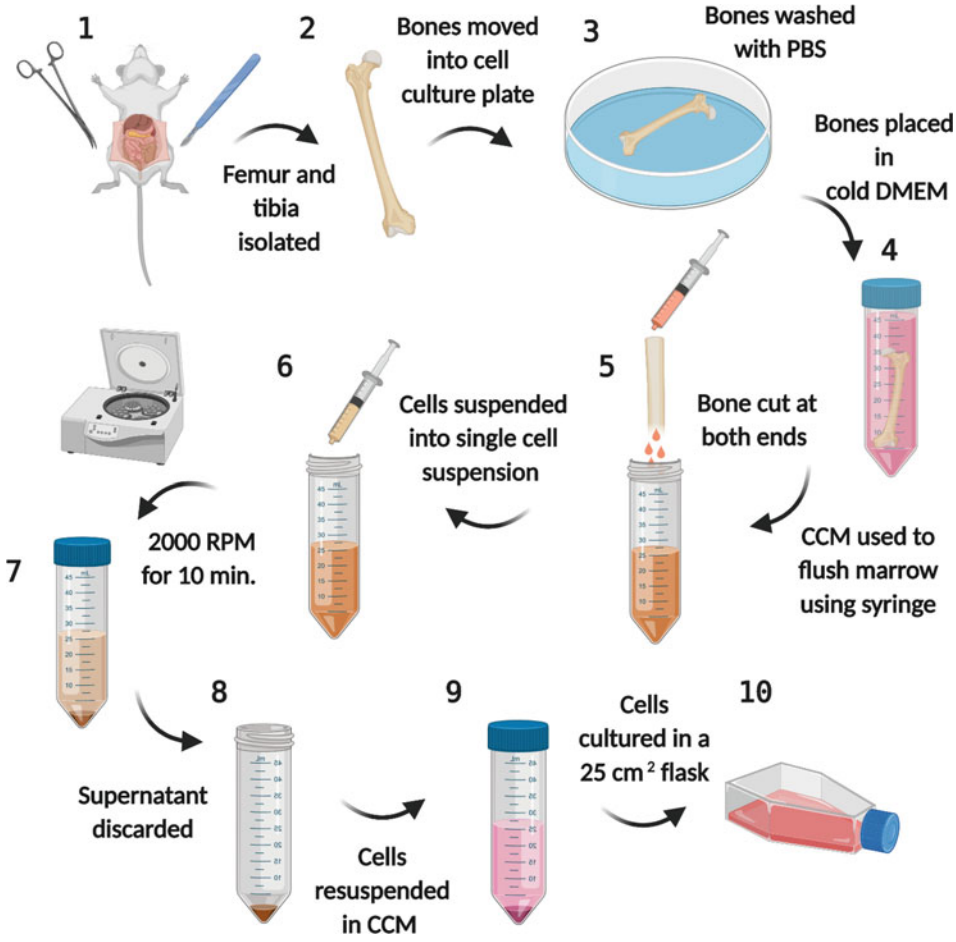

Fig. 8.2 Procedure for isolation of bone marrow mesenchymal stromal cells (BM-MSCs)

[65]. Unlike cell culture in high glucose medium, MSCs expand rapidly for up to 10 passages in low glucose medium without losing their morphology [52], and FBS provides the required growth factors to support cell growth and proliferation [78]. L-glutamine is an amino acid that serves as a source of nitrogen for high energy-demand processes. In vitro, cells can use L-glutamine to synthesize nucleotides (DNA and RNA), vitamins, and proteins needed for different metabolic processes during growth and propagation [79]. Together DMEM, FBS, and L-glutamine constitute the formation the CCM used in in vitro cell culture. Antibiotic/ antimycotic reagents such as penicillin/ streptomycin/amphotericin B solution are used at low concentrations (1%) to prevent bacterial and fungal contamination during primary cell culture, although the use of antibiotics/antimycotics may affect the experimental results [80].

1. Seed the cells at a density of 2.1 × 10^6 cells in 75-cm^2 tissue culture flasks and incubate them in 5% CO_2 and proper humidity (95%) for 72 h to allow them to adhere to the plastic surface [14].
2. After 72 h, wash the adherent cells with PBS (PBS is added gently on the walls to avoid cells detachment, loss or death by harsh pipetting), and add fresh CCM is added.
3. Incubate the adherent cells for 7 days and change the culture medium every 2–3 days by discarding the old medium and adding new medium with fresh nutrients until the cells reach confluence (80%–90%).

   N.B. There are various sizes of the cell culture vessels. The seeding density varies according to the surface area (according to the manufacture's recommendation in the product sheet).
4. Remove the CCM, wash the cells with PBS to remove any traces of the serum (avoiding enzyme inhibition), preparing the cells for passaging using trypsin enzyme.

   N.B. There are other cell dissociation reagents other than trypsin, that can be used in passaging the cells such as TrypLE [81].
5. Add 1–3 mL of trypsin to the adherent cells and incubate the cells at 37 °C for 2–3 minutes. It is important to monitor and adjust the time of cell trypsinization to prevent over digestion of the cell proteins, which can compromise cell survival [82]. Trypsin is a protease that breaks down polypeptide chains [83]. It is used in cell culture to breakdown the ECM proteins between adjacent cells and adhesion proteins that bind cells to the plastic, thereby enabling cell collection for further culture or use [83].
6. Observe the trypsinized cells under an inverted microscope to assure complete dissociation of the cells floating in the trypsin.
7. Add an equal volume of serum-containing CCM to neutralize the trypsin [82].
8. Suspend and transfer the cells into a 15-mL conical centrifuge tube for centrifugation at 300 × g for 10 min to obtain the cell pellet.
9. Resuspend the cell pellet in 10 mL CCM.
10. Count the cells using the Trypan blue exclusion assay using hemocytometer, and culture the cells in cell culture vessels of interest in proper seeding density for propagation [84].

    N.B. In Trypan blue exclusion assay, Trypan penetrates and stains dead cells that appear in blue color, while viable cells remain unstained.
11. To obtain a pure population of cells, sorting by cell surface markers can be used.

The differences between AD-MSCs and BM-MSCs are described in Table 8.1 [2, 94, 95].

Table 8.1 Differences between adipose-derived mesenchymal stromal cells (AD-MSCs) and bone marrow mesenchymal stromal cells (BM-MSCs)

	Adipose- derived mesenchymal stem cells (AD-MSCs)	Bone Marrow Mesenchymal Stem Cells (BM-MSCs)
Amount	Abundant cells AD-MSCs yield is approximately 500-fold greater when isolated from an equivalent amount of AT [34]	Low yield They constitute about 0.001–0.01% of the total bone marrow nucleated cells [85]
Accessibility	Easy access for collection during liposuction [2]	Difficult as bone marrow harvesting is an invasive procedure [2]
Gene expression	Express CD34 in early in vitro culture passages [86, 87]	Do not express CD34 [86]
Proliferation capacity	High [88]	Low [88]
Differentiation potential	High potential for both angiogenic [89] and adipogenic differentiation [90, 91]	High potential for osteogenic differentiation [92]
Stability in long term culture	More genetically and morphologically stable [93]	Less genetically and morphologically stable [93]
Senescence ratio	Low [88]	High [88]
Resistance to hypoxia and oxidative stress	High [94]	Low [94]
Telomerase activity	High [94]	Low [94]

8.3 Characterization of Mesenchymal Stromal Cells

After isolation, it is important to maintain a uniformly pure MSCs population for proper experimental design and reproducible data (Fig. 8.3).

8.3.1 Plastic Adherence and Morphology

Plastic adherence and a distinctive spindle-shaped morpology are distinguishing features for cultured MSCs. Surface receptors, mainly integrins, promote cell-matrix adherence properties via downstream gene regulation that occurs between the plastic surface, ECM, and integrin receptors [96]. Upon attachment, cells display a characterstic fibroblast-like spindle shape [14, 22]. MSCs from different tissue origins all display similar adherence and morphological characteristics with minimal noticeable differences [97]. Variabilities among MSCs of different origins reveal atypical gene expression patterns, exosome secretion, and differentiation capacity [97]. AD-MSCs isolated from the SVF appear to

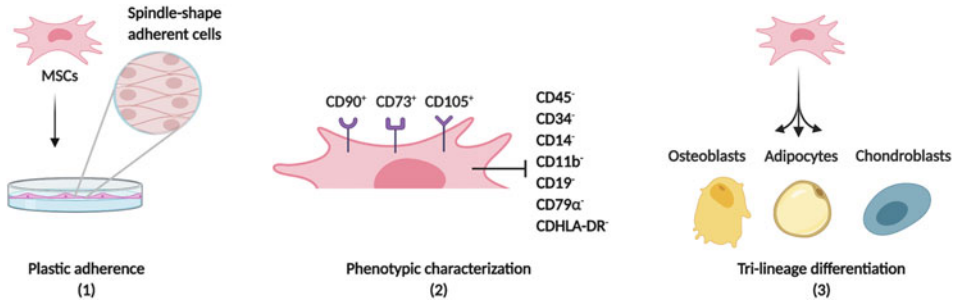

Fig. 8.3 Characterization of mesenchymal stromal cells (MSCs)

consist of different subpopulations of cells with variable adherence abilities. Late adherent cells show more proliferative and self-renewal capabilities than early adherent cells [98]. In contrast, BM-MSCs cultured at a low density in vitro show the ability to form colonies. Colony-forming unit fibroblast (CFU-F) was adopted as a standard assay to potentially determine the proliferation capacity of MSCs from a single precursor cell [12].

8.3.2 Phenotypic Characterization

Different cells express various surface markers according to their origin, lineage, differentiation state, and function. MSCs can be identified by the presence of a group of clusters of differentiation (CD) surface markers (CD90, CD105, and CD73). In addition, MSCs do not express CD14, CD11b, CD45, CD34, CD19, or human leukocyte antigen (HLA)-DR surface markers [31, 99, 100]. The surface markers included in Table 8.2 are the most common surface markers used to identify MSCs according to the minimum criteria stated by the ISCT [31, 101–103].

8.3.3 Tri-Lineage Differentiation

MSCs differentiate into adipocytes [27, 104], osteocytes [105], and chondrocytes [106] upon spontaneous or induced differentiation in vitro. Multilineage differentiation of MSCs depends on specific culture conditions [22].

8.3.3.1 Adipogenic Differentiation

Adipocytes (fat cells) are one of the cell derivatives of MSCs upon culture used to push the cells toward the adipogenic lineage and avoid unspecific cell differentiation [22, 107]. MSCs are maintained in CCM (DMEM medium containing low glucose concentration and supplemented with 10% FBS, 1% L-glutamine and 1% penicillin/streptomycin). To drive adipogenic differentiation, the medium is supplemented with

Table 8.2 MSCs Surface Markers

Surface Marker	Alternative Name	Expressionon MSCs	Notes
CD 90	Thy-1	$CD90^+$	Glycosylphosphatidylinositol (GPI)-anchored glycoprotein
CD105	Endoglin	$CD105^+$	SH2
CD73	Ecto-5′-nucleotidase	$CD73^+$	SH3, SH4
CD45		$CD45^-$	Pan-leukocyte marker
CD34	Mucosialin	$CD34^-$	Primitive hematopoietic progenitor and endothelial cell marker N.B. CD34 can show expression in BM-MSC, however, the expression declines with culture
CD14	LPS receptor	$CD14^-$	Monocyte and macrophage marker
CD11b	Integrin αM chain	$CD11b^-$	Monocyte and macrophage marker
CD19		$CD19^-$	B cell marker
CD79α	Ig-α	$CD79\alpha^-$	B cell marker
HLA-DR		$HLA\text{-}DR^-$	Appear only on MSCs during stimulation

100 μM indomethacin, 0.5 mM 3-isobutyl-1-methylxanthine, and 0.1 μM dexamethasone, in addition to, and 10 μg/mL human recombinant insulin powder [22, 108, 109]. Adipogenic differentiation of MSCs is aachieved by the release and intracellular deposition of oil droplets that can later be stained and visualized (e.g., Oil Red O staining) [21, 31, 96, 97].

8.3.3.2 Osteogenic Differentiation

Osteogenic differentiation potential is another important functional characteristic of MSCs [107, 110, 111]. Osteogenic culture medium consists of 0.05 mM ascorbic acid, 1 μM dexamethasone, and 10 mM glycerol-3-phosphate in DMEM/LG [108, 112]. Recent studies used bone morphogenetic proteins (BMPs) [105, 112, 113] and insulin-like growth factor-1 (IGF-1) [105, 114] to induce or enhance osteogenic differentiation. Alizarin Red or von Kossa staining is used to detect osteoblast differentiation by staining the extracellular calcium deposits (mineralization) [31, 108, 114].

8.3.3.3 Chondrogenic Differentiation

MSCs can differentiate in vitro into chondrocytes in the presence of chondrogenic inducing factors. The culture medium used for chondrogenic differentiation consists of DMEM (high glucose) supplemented with 1%–2% FBS [115, 116], 40 μg/mL L-proline, 100 nM dexamethasone, 100 μM ascorbic acid, 5.4 μg/mL linoleic acid [115], and a mixture of 10 μg/mL insulin −5.5 μg/mL transferrin, and 6.7 ng/mL selenium [116, 117]. Recent

studies used TGF-β3 to enhance chondrogenic differentiation [115, 116]. Chondrocyte differentiation can be demonstrated by staining acid mucins with Alcian Blue stain, and immunohistochemistry analysis of cell aggregate sections to confirm collagen type II (Col II) formation [31].

Take Home Message

- Adult MSCs were first isolated from bone marrow by Alexander Friedenstein in 1976.
- MSCs could be collected from the stroma of nearly all tissues.
- Bone marrow and adipose tissue MSCs are the most used cells in research and experimental transplantation, due to their ease of culture and expansion.
- Methods for isolation of MSCs depend on their plastic adhesion ability and their phenotype.
- Tissue explants and non enzymatic methods for isolation of AD-MSCs are gentler to the cells, and more applicable for clinical use.

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In Vitro Methods for Generating Induced Pluripotent Stem Cells

9

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Abbreviations

AMSCs	Adipose mesenchymal stem cells
BAC	Bacterial artificial chromosome
bFGF	Recombinant basic fibroblast growth factor
BJ	Human BJ fibroblasts
CCM	Complete culture medium
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide

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EBV	Epstein–Barr virus
ESCs	Embryonic stem cells
FBS	Fetal bovine serum
HDF	Human dermal fibroblast
HFF	Human foreskin fibroblasts
HUVEC	Human umbilical vein endothelial cells
iPSCs	Induced pluripotent stem cells
Klf4	Kruppel-like factor 4
KSR	Knockout serum replacement
MEF-CM	MEF culture medium
MEFs	Mouse embryonic fibroblasts
MHC	Major histocompatibility complex
MNCs	Mononuclear cells
mod-mRNA	mRNAs with modified nucleobases
Myod1	Myogenic differentiation 1
NEAAs	Nonessential amino acids
NPCs	Neural precursor cells
NSCs	Neural stem cells
Oct3/4	Octamer-binding transcription factor 3/4
Opti-MEM	Opti-minimum essential medium
OSKM	Oct3/4, Sox2, Klf4, and c-Myc factors
OSLN	Oct4, Sox2, Nanog, and Lin28
PBS	Phosphate-buffered saline
PCs	Pericytes
PPE	Personal protective equipment
SCNT	Somatic cell nuclear transfer
SeV	Sendai virus
Sox2	Sex-determining region Y-box 2
β-Me	β-mercaptoethanol

What You Will Learn in This Chapter

In this chapter, you will get a brief overview of induced pluripotent stem cells (iPSCs) and the advances in cell reprogramming. The generation of pluripotent cells with higher differentiation potential from somatic cells has opened the door for substantial advances in personalized medicine and regenerative medicine applications. You will also learn the basics of inducing pluripotent stem cells from somatic cells and the different factors affecting the reprogramming efficiency. A step-by-step protocol will guide you to different approaches to generate iPSCs from different types of somatic cells and the advantages and disadvantages of each protocol.

9.1 Introduction

Embryonic stem cells (ESCs) hold great promise for regenerative medicine and cell therapy due to their potential for unlimited propagation and generation of all varieties of somatic cells. However, the use of ESCs for clinical applications has met with significant controversy due to ethical issues associated with the manipulation of human preimplantation embryos, problems with inadequate tissue matching, and high tumorigenic potential [1–3]. By contrast, induced pluripotent stem cells (iPSCs) are generated from somatic cells from an individual patient (i.e., from an autologous source); as such, iPSC therapy may overcome some of these limitations. Autologous iPSCs could be applied as part of a personalized medicine approach and might add a new and individualized dimension to drug discovery, disease modeling, and targeted therapy [4].

IPSCs have been generated from somatic cells of both fetal and adult tissues. Somatic cell reprogramming into a pluripotent, embryonic-like state was induced by the introducing of the Oct4, Sox2, Klf4, c-Myc (OSKM factors), and Nanog transcription factors [5, 6]. Integrative and non-integrative methods were used to generate iPSCs [3, 7–11]. Effective somatic cell reprogramming was initially accomplished by integrating the genetic material via retroviral- or lentiviral-mediated gene transfer; however, this method increased the risk of mutagenesis [3, 7, 12]. Reprogramming of adult cells into iPSCs can be achieved using non-integrative methods, including gene transfer with bacterial episomal vectors and Sendai virus (SeV), which is an RNA virus that does not integrate into genomic DNA; these methods have a higher safety profile and reduce the risk of genotoxicity and mutagenesis [8, 13–15]. Several modifications of Yamanaka's original protocol have been applied in order to improve the efficiency of somatic cell reprogramming using the OSKM factors [16–20].

To enhance reprogramming efficiency, synthetic capped mRNAs with modified nucleobases (mod-mRNA) have been introduced; this has increased the reprogramming efficiency by as much as 4.4% [21, 22]. Unfortunately, this modification was only functional when applied to long-lived fibroblast cell lines such as BJs; no significant enhancement was observed when this modification was applied to freshly isolated cells [16]. To overcome this limitation, Kogut et al. improved the reprogramming efficiency up to 90.7% using a combination of miRNA-367/302s and synthetically-modified specific mRNAs that synergistically enhance reprogramming efficiency and conversion of neonatal fibroblasts into iPSCs [9].

9.2 History of Induced Pluripotent Stem Cells

IPSCs are generated by the reprogramming of adult somatic cells (e.g., epithelial cells, fibroblasts, or multipotent stem cells) into cells with pluripotent capabilities [23–25]. The concept of cellular reprogramming and nuclear transfer dates back to experiments performed nearly a century ago (Fig. 9.1) [26–28]. Early, albeit unsuccessful experiments

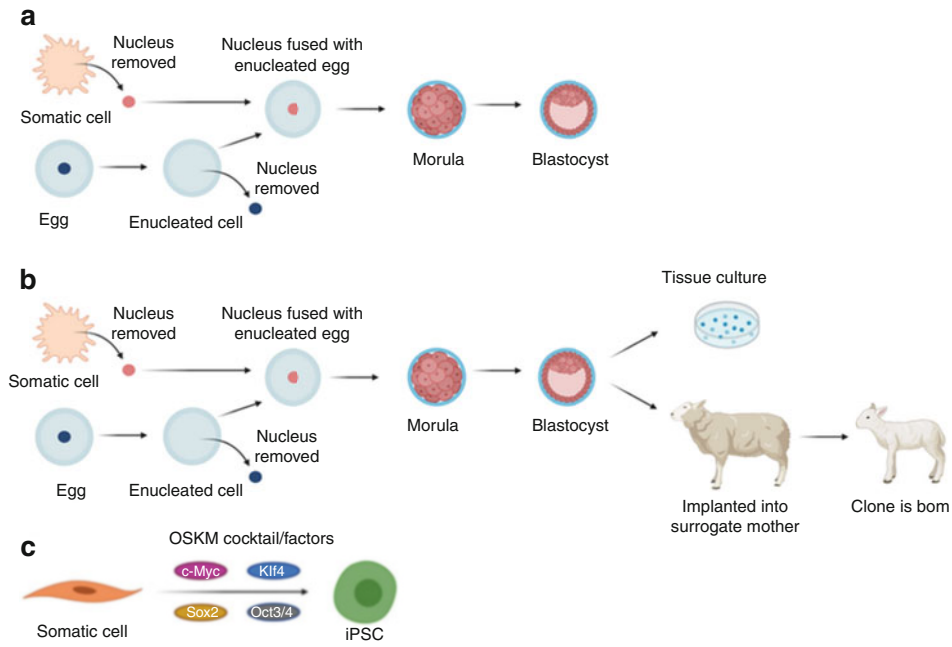

Fig. 9.1 History of somatic cell reprogramming and generation of iPSCs

carried out by Spemann (1938) who was attempting to perform nuclear transfer in mouse models nonetheless introduced the concept of cellular reprogramming in order to induce a more embryonic-like state [28, 29]. In 1952, Briggs and King pioneered the efforts in somatic cell reprogramming in their experiments that focused on transplantation of blastula cell nuclei into enucleated frog eggs. Unfortunately, they were unable to reproduce their initial findings when targeting other specialized cells [30]. In 1962, John Gurdon successfully generated cloned tadpoles from cells containing the nuclei of the frog's intestinal cells [31]. In this set of experiments, he demonstrated that differentiated nuclei revert to an undifferentiated state when transplanted into frog's eggs. Before this breakthrough, differentiation was believed to be uniformly unidirectional, with somatic cells generated from progenitor or immature cells, and not vice versa. "Epigenetic landscape" was a term coined by Conrad Waddington [32] in his efforts to elucidate that factors contributing to somatic cell reprogramming. The concept of the epigenetic landscape provides an explanation of the specific biological paths that can be undertaken by a given cell, including those leading to different developmental stages, including both progenitor and differentiated states. These biological paths have been quantified via construction probability landscapes that define cell developmental and differentiation pathways. This type of analysis works on the principle that the developmental process, the conversion of the cells from an undifferentiated to a differentiated state, as well as the stability of various cell types can be determined by the escape time, a factor that correlates with barrier heights between the differentiated

and undifferentiated states. The epigenetic landscape assumes that, as fluctuations increase, the barrier height between these states decreases and the escape time is reduced; these alterations serve to increase the chance of conversion from the undifferentiated to a differentiated state. Consequently, the possibility for deviation from the original developmental paths likewise increases. Accordingly, small fluctuations enhance the process of development and limit the opportunities for deviation from the original paths [33, 34].

A key discovery in the field of reprogramming was reported in a landmark set of experiments carried out by Davis and colleagues [35] in 1987, in which it was shown that cell fate could be directed and defined by a transcription factor. In this set of experiments, complementary DNA (cDNA) subtraction probing was performed and three genes were identified, which were expressed primarily in proliferative myoblasts. One of these genes was myogenic differentiation 1 (*Myod1*) which, when subjected to forced expression in mouse fibroblasts, resulted in their conversion into myosin-expressing myoblasts. These experiments were later considered "the dawn of direct reprogramming." Another major breakthrough was achieved by Wilmut et al. [36] who generated the cloned sheep, Dolly, from an enucleated oocyte. The researchers postulated that the nuclei of somatic cells and the enucleated egg were capable of generating a complete organism, as the nuclei contain all the genetic information and factors necessary to promote genetic reprogramming. They fused the nucleus from the cell of a mammary gland into an enucleated, unfertilized egg. Dolly was the first cloned animal that was born following this protocol from a total of 13 recipients undergoing embryo implantation into surrogate ewes. In 1997, Tada et al. [27] fused mouse ESCs with female mouse thymocytes, which were reprogrammed into pluripotent hybrids.

IPSC technology was pioneered by Shinya Yamanaka in Kyoto, Japan, who demonstrated in 2006 that adult cells can be reprogrammed into iPSCs via the introduction of four specific transcription factors [3]. In November 2007, the first human iPSCs were created from adult cells by two independent research teams. The first report was from the group of James Thomson at the University of Wisconsin in Madison, WI, USA [23], and the second from Shinya Yamanaka and colleagues at the Kyoto University, Japan [7]. Yamanaka has successfully transformed human fibroblasts into pluripotent stem cells by retroviral transduction with four pluripotency genes, including *OCT3/4*, *SOX2*, *KLF4*, and *c-Myc* [7]. In 2011, the same team generated integration-free human iPSCs via the use of bacterial episomal vectors [8]. Yamanaka was awarded the 2012 Nobel Prize in Physiology or Medicine jointly with John Gurdon for discovering that mature specialized cells could be reprogrammed into immature cells for the purpose of generating differentiated tissue cells [37].

IPSCs generated from adults can overcome many of the limitations of the ESCs; they not only bypass the need for embryos, they can be specifically engineered to match a given patient's genetic makeup [2, 38]. However, reprogramming of adult cells into iPSCs still carries significant risks that could limit their use in clinical settings. For example, viruses used in reprogramming may genetically alter the cells and may enhance the expression of cancer-associated genes [39]. This challenge has resulted in efforts to replace viral vectors

with new techniques, including those that focus on deletion of one or more oncogenes after the induction of pluripotency. However, Zhou et al. [40] demonstrated somewhat later that generation of iPSCs was possible with no detectable genetic alteration of the adult cell. In 2007, Hanna et al. reported that mice that were genetically engineered to model sickle-cell anemia were been cured by transplantation with mutant donor fibroblast-derived iPSCs that had been genetically corrected by homologous recombination to produce healthy hematopoietic progenitors [41].

Autologous iPSC-based therapy is more advantageous than allografts due to a decreased risk of immune rejection [42, 43]. However, more than 3 months are required to generate autologous iPSCs de novo; this feature is extremely disadvantageous if the iPSCs are to be used to treat critical acute disorders, for example, spinal cord injury [4]. The prolonged time required for the generation of autologous iPSC could be costly in terms of patient care and similarly suffers drawbacks associated with extensive cell manipulation. For these reasons, allogeneic iPSCs are currently the target of choice in regenerative medicine, although their use must remain highly regulated in order to avoid their undesirable effects [4]. Prior to the generation of clinical-grade iPSC clones, different criteria need to be considered, including the overall health of the donor and the degree of MHC (major histocompatibility complex) matching [44, 45]. Over the past few years, iPSC transplantation has shown promising results when used to treat various intractable diseases [46]. At this time, iPSCs can be differentiated into neural precursor cells (NPCs), astrocytes, neural cells, neurons, and oligodendrocytes; these differentiated iPSCs can be used to promote functional recovery when they are used to replace cells that were lost or damaged [47, 48].

9.3 Cell Reprogramming Techniques

Delivery of plasmid DNA into mammalian cells can be accomplished via a number of different methods. In the 1980s, transfection methods were developed using lipofection, which involved a mix of positively-charged lipids and negatively-charged plasmid DNA [49] and RNA [50]. During lipofection, the lipids enter the cells by endocytosis or fuse with the plasma membrane to facilitate the delivery of nucleic acids into the mammalian cells [51, 52]. Electroporation is another method used for transfection. This method does not involve virus vectors; instead, a short electrical pulse is used to promote electro-permeabilization of the cell membrane. Two types of pores result from electroporation, including transient and long-lived pores; both types of pores play a role in transport [53]. The cell membrane exists in a permeable state for up to several minutes after application of the electrical pulse. This facilitates the diffusion of various molecules into the cell [53, 54]. Transient membrane permeabilization facilitates recovery after the electrical charge is removed [54]. However, if the electrical pulse applied to the cells was too strong, the cell membrane will be permanently disrupted, leading to cell death. Negatively charged DNA diffuses through the permeabilized membrane during the time when the pulse is applied [54, 55]. Moreover, if long-lived pores remain open after the

removal of the electrical pulse, DNA can continue to pass through the permeabilized membrane. Increasing the electrical charge also increases the permeabilized surface area of the cell membrane [55]. The methods used to generate iPSCs can be selected to suit the needs of the specific research goals while considering the advantages and disadvantages of each approach (Table 9.1).

9.4 Practicum

9.4.1 Protocol for Generation of iPSCs

In this practicum, we will describe several approaches used to generate iPSCs reprogrammed from adult human dermal fibroblasts (HDFs) or adult adipose-derived multipotent stem cells (i.e., pericytes [PCs]). These protocols involve using integrative (retroviral and lentiviral) or non-integrative methods (bacterial episomal vectors). Different delivery methods will also be described including lipofection as a means to facilitate the integrative methods and both lipofection and electroporation for non-integrative methods.

9.4.1.1 Generation of iPSCs from HDFs Using Viral Integrative Methods Including Lentiviral and Retroviral Transduction

The following original protocol was developed by Shinya Yamanaka and colleagues at the Kyoto University, Japan. This method results in the reprogramming of mouse fibroblasts into iPSCs via the introduction of the pluripotency-inducing embryonic transcription factors Oct4, Sox2, KLF4, and c-Myc via retroviral transduction [3]. Reprogramming of HDFs by the same reprogramming factors was also achieved using a lentiviral transduction method [7].

Reagents

- pMXs including cDNAs encoding of c-MYC, KLF4, SOX2, OCT3/4, pCMV-VSV-G and psPAX2 (all available from Addgene, USA)
- Human dermal fibroblasts (HDFs)
- 293 T cell line
- Mouse embryonic fibroblasts (MEFs)
- Dulbecco's modified Eagle's medium (DMEM); low glucose
- DMEM/F12
- Phosphate-buffered saline (PBS)
- Knockout serum replacement (KSR)
- L-glutamine
- Nonessential amino acids (NEAAs)
- 2-Mercaptoethanol
- Penicillin–streptomycin–amphotericin B
- Recombinant basic fibroblast growth factor, human (bFGF)

Table 9.1 The advantages and disadvantages of retroviral, lentiviral, nonintegrating episomal DNA plasmid, synthetic self-replicating RNA, and negative sense nonintegrating RNA virus (Sendai virus) techniques

	Integrative methods [7]		Nonintegrative methods [8, 15, 56]		
Type	Retroviral [3, 7, 57, 58]	Lentiviral [23, 59–61]	Nonintegrating episomal DNA plasmid [8, 62, 63]	Negative sense nonintegrating RNA virus (Sendai virus) [13, 15, 64, 65]	Synthetic self-replicating RNA [56]
Donor cell	Mouse embryonic fibroblasts (MEFs), sensitized fibroblasts, and mouse gingival fibroblasts [57]	Primary human melanocytes, murine melanocytes, rat cells, pig cells, β cells, cells from chimeric mice [61, 66–68]	HLA matched iPSCs derived from human cord blood, human CD34+ cells, human neural stem cells (NSCs), bone marrow mononuclear cells (MNCs) [69], human fibroblasts	Human T cells, human dermal fibroblasts (HDFs), human CD34 + cells, BJ fibroblasts, MEFs [70]	Primary human foreskin fibroblasts (HFFs), human foreskin BJ fibroblasts [56]
Reprogramming cocktail	OSK/OSKM; OSNL [3]	OSK/OSKM or additional factor [23]	OCT3/4, LIN28, L-MYC, c-MYC, SOX2, NANOG, and KLF4 [8, 71]	Sendai virus cytokine cocktail (SCF, G-CSF, Flt3L, IGF-2, TPO, and VEGF) [13, 56]	(OCT4, SOX2, and KLF4, with c-MYC or GLIS1 [56]
Advantages	Used to study -Pluripotency and differentiation -Efficient methods for disease modeling and drug screening -Highly efficient reprogramming systems -Means to avoid genome integration	To understand -Reprogramming mechanisms -Highly efficient reprogramming systems -Methods used to avoid genome integration -How lentiviruses can integrate into both dividing and nondividing cells [3]	-Transgene free -Efficient method for cell therapy -Inexpensive -No virus preparation -Low immunogenicity [72, 73]	-Produces integration-free iPSCs -No premature silencing [14]	-Highest reprogramming efficiency when compared with other nonintegrative delivery systems -Single transfection -Highly immunogenic -Used in clinical treatment [56]

Disadvantages	-Lacks the necessary control over host genome integration -Retroviral and lentiviral reactivated transgenes leading to increase risk of mutagenesis [74]		Lacks high reprogramming efficiency [8, 75]	-Difficult to clear from cells due to constitutive replication -Immunogenic [76]	Integrative methods have higher reprogramming efficiency, but RNA delivery methods are safer [8]
Efficiency	High [3]	High [7]	Low [75]	Very high [14]	Low [56]

- Accutase
- 0.1% Gelatin
- ROCK Inhibitor
- Fugene HD transfection reagent
- Opti-MEM
- Hexadimethrine bromide (Polybrene)
- Lipofectamine 2000
- Fetal bovine serum (FBS)
- Distilled water

Equipment

- Biological Safety Cabinets Class II (BSL-2)
- Water bath
- Centrifuge
- Inverted microscope
- CO_2 incubator
- Liquid nitrogen storage
- Automatic cell counter
- Stereo microscope
- 4°C refrigerator
- −20°C and −80°C freezers

Materials

- Cell culture flasks (25 and 75 cm^2)
- Petri dishes (60 mm)
- Disposable sterile tubes (e.g., Falcon tubes), 50 and 15 ml
- Serological pipettes
- Cryovials
- Sterile Eppendorfs
- Sterile filtered tips (10, 200 and 1000 μl)
- Six-well plates
- Twenty-four-well plates
- Ninety-six-well plates
- Personal protective equipment (PPE), including double gloves, safety glasses, waterproof covers, and face shields
- Nitrile gloves
- Pipetman (P2, P10, and P1000)
- 0.22 μm filter
- 0.45 μm pore size cellulose acetate filter

Reagents to Prepare Hexadimethrine Bromide (Polybrene)

- Dissolve 0.8 g of polybrene in 10 ml of distilled water to reach a final concentration of 80 mg/ml (10× stock).
- To prepare the working solution, dilute 1 ml of 10x solution into 9 ml distilled water, and filter using 0.22 μm filter.
- Store at 4°C.

Complete Culture Medium (CCM)

- 5 ml FBS
- 0.5 ml penicillin–streptomycin–amphotericin
- 0.5 ml L-glutamine
- DMEM low glucose to a 50 ml final volume

Human ESC Culture Medium

- ESC medium contains DMEM/F12 with 20% KSR, 4 ng/ml bFGF, 2 mM glutamine, 0.1 mM NEAAs, 50 units/ml penicillin and 50 μg/ml streptomycin, and 0.1 mM β-mercaptoethanol.
- 50 ml of ESC medium includes 40 ml DMEM/F12, 8 ml KSR, 2 μl bFGF, 0.5 ml glutamine, 0.5 ml NEAAs, 0.5 ml penicillin, and 0.3 μl β-mercaptoethanol; add DMEM/F12 to a 50 ml final volume.

ROCK Inhibitor (Y27632)

- Dilute 10 mg of the ROCK Inhibitor Y27632 with 3 ml distilled water and mix thoroughly.
- Aliquot the stock solution into working volumes based on routine use.
- *Note*: The ROCK Inhibitor Y27632 is added to cell culture medium at a final concentration of 10 μM (1:1000 dilution).
- Freeze the aliquots at −20°C to −80°C; avoid repeated freezing and thawing. Thawed aliquots can be stored for 2 weeks at 2–8 °C.

Mouse Embryonic Fibroblast (MEF) Culture Medium

- MEF medium is DMEM supplemented with 10% FBS, 1% NEAAs, 1% penicillin–streptomycin–amphotericin B, and 1% L-glutamine.
- To prepare 50 ml medium: 5 ml FBS, 0.5 ml penicillin–streptomycin–amphotericin B, 0.5 ml L-glutamine, 0.5 ml of NEAAs, and DMEM low glucose to a volume of 50 ml.

Procedures

Culture of Human Dermal Fibroblasts (HDFs)

- Carefully remove cell vials with frozen HDFs from the liquid nitrogen storage and thaw them rapidly in a 37°C water bath until the contents are thawed nearly to completion.

- Add 1 ml of complete culture medium (CCM) to the vial and centrifuge at 1800 rpm for 10 min at room temperature.
- Discard the supernatant and resuspend the pellet in 1 ml CCM.
- Culture the thawed cells in two 100 cm^2 Petri dishes.
- Incubate the cells at 37°C in a 5% CO_2 incubator for 2 days until they reach 60–70% confluency.

Note: Cells are regularly checked under an inverted microscope for characteristic fusiform morphology and adherence. When the cells reach 80% confluency, they are removed from the tissue culture plate using trypsin as follows:

Trypsinization
- Aspirate and discard the culture medium.
- Wash the cells twice with sterile PBS.
- Cover the cells with 0.25% trypsin–EDTA and incubate for 2–3 min at 37°C.
- Shake the flask gently to detach the cells; check for cell detachment under the inverted microscope.
- After the cells are completely detached, neutralize the trypsin with an equal volume of CCM and collect the cell suspension.
- Centrifuge at 1800 rpm for 10 min at room temperature.
- Discard the supernatant and resuspend the cell pellet in 1 ml CCM.

Culture of 293 T Cells
- Carefully remove 293 T cell vial from liquid nitrogen tank and thaw them rapidly in a 37°C water bath until the contents are thawed nearly to completion. Add 1 ml of CCM to the vial and centrifuge at 1800 rpm for 10 min.
- Discard the supernatant and resuspend the pellet in 1 ml CCM.
- Culture the cells in *six* 25 cm^2 cell culture flasks at a cell density of 5×10^5 per flask.

Preparing 293T Cells for Transfection

Caution *All work with retroviruses should be conducted in a class II biological safety cabinet. All individuals handling this material should be wearing appropriate PPE.*

- Aspirate medium from the 293T cells.
- Wash the cells twice in sterile PBS.
- Add CCM *without antibiotics.*
- Use a 1.5 ml centrifuge tube for each plasmid and label them accordingly (i.e., c-MYC, KLF4, SOX2, OCT3/4). Transfer 60 μl of opti-MEM medium into each tube.
- Add 1.5–2 μg of each plasmid to each tube followed by 0.75 μg pCMV-VSV-G and 0.25 μg psPAX2; the total amount of DNA in each tube should be 3 μg.

- Add 9 μl of the Fugene HD transfection reagent to each tube and incubate at room temperature for 15 min.

Note: Each plasmid should be introduced separately into one cell culture flask. Introduction of two or more plasmids into the same flask could reduce the transfection efficiency.

- Add the plasmid/Fugene HD complex dropwise to each culture of 293T cells' and incubate overnight at 37°C at 5% CO_2.
- After 24 h, aspirate the transfection medium from each flask with 293T cells. Replace the medium with 5 ml CCM with antibiotics.
- On the following day, collect 20 ml CCM containing the virions produce; mix and filter the medium through a 0.45 μm pore size cellulose acetate filter; and transfer into a 15 ml sterile tube.
- Add Polybrene to the 20 ml medium containing the virus to a final concentration of 4 μg/ml. Mix gently by pipetting up and down.

Critical Step *Retroviruses and lentiviruses should be used immediately. Do not freeze, as this will result in loss of potency and transfection may fail.*

- Aspirate the medium from cell culture plates and add 10 ml of polybrene/virus-containing medium to each plate.
- Incubate the cells overnight at 37°C at 5% CO_2.
- After overnight culture, aspirate the medium from the transduced fibroblasts and add 10 ml fresh CCM medium per plate.

Optional: A second round of transfections can be carried out to increase the transduction efficiency.

9.4.1.2 Generation of iPSCs from HDFs and PCs (Fig. 9.2) Using Non-integrative Methods (Bacterial Episomal Vectors)

The following protocol follows the same steps as those used by Yamanaka and colleagues to reprogram HDFs with bacterial episomal vectors. We will then describe the same protocol with minor modifications that has been used to generate iPSCs from human adipose tissue-derived PCs (Fig. 9.3) [8, 25, 77].

Reagents

- Episomal vectors: cDNA of hOCT3/4-shp53-F, hSK, hUL, and eGFP (available from Addgene, USA)
- Human dermal fibroblasts (HDFs)
- Mouse embryonic fibroblasts (MEFs)
- PureYield™ Plasmid Miniprep System
- DMEM, low glucose

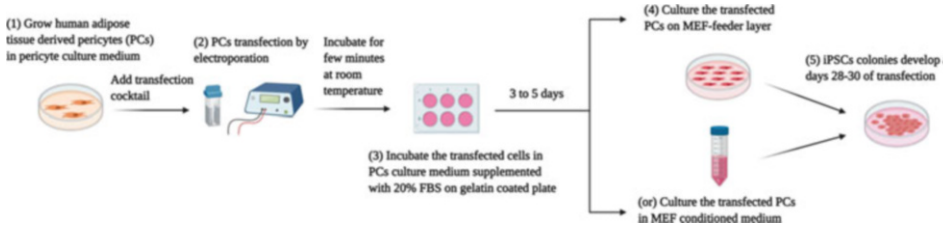

Fig. 9.2 Procedure used for PCs reprogramming with electroporation methods

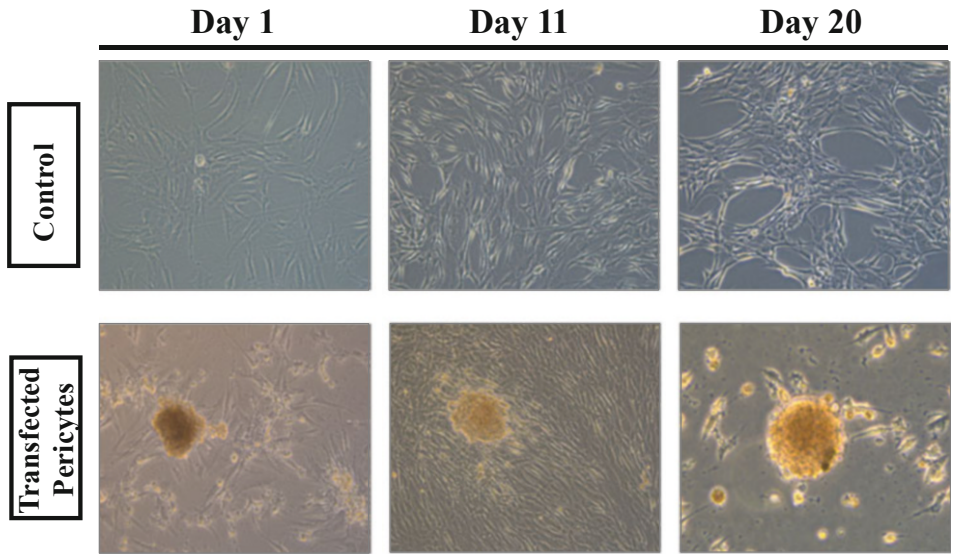

Fig. 9.3 Microscopic images of reprogrammed PCs following transfection by electroporation with episomal vectors (day 1, 11, and 20)

- DMEM/F12
- PBS
- Dulbecco's modified Eagle's medium (DMEM), mega-cell
- Collagen type IA
- Penicillin–streptomycin–amphotericin B
- Knockout serum replacement (KSR)
- L-glutamine
- Nonessential amino acids (NEAAs)
- 2-Mercaptoethanol
- Penicillin/streptomycin
- Recombinant basic fibroblast growth factor, human (bFGF)
- Accutase
- 2% Gelatin

- Puromycin
- Fugene HD transfection reagent
- Opti-MEM medium
- Luria broth (LB)
- Hexadimethrine bromide (Polybrene)
- Lipofectamine 2000
- Fetal bovine serum (FBS)
- Dimethyl sulfoxide (DMSO)
- Trypan blue dye
- Distilled water

Equipment
- Class II Biological Safety Cabinets (BSL-2)
- Water bath
- Centrifuge
- Spectrophotometer
- Flow cytometer (different types and models can be used)
- Inverted microscope
- CO_2 incubator
- Liquid nitrogen storage
- Stereo microscope
- Automatic cell counter
- 4°C refrigerator
- −20°C and − 80°C freezers

Materials
- Cell culture flasks (25 and 75 cm^2)
- Petri dishes (60 mm)
- Disposable sterile tubes, 50 and 15 ml
- Serological pipettes
- Cryovials
- Sterile Eppendorfs
- Sterile filtered pipette tips)10, 200, and 1000 μl)
- Six-well plates
- Twenty-four-well plates
- Ninety-six-well plates
- Personal protective equipment (PPE): double gloves, safety glasses, and face shields
- Pipetman (P2, P10, and P1000)
- Nitrile gloves

Reagents to Prepare

Puromycin

- Dissolve puromycin in distilled water at a final concentration of 10 mg/ml. Sterilize using a 0.22 μm filter.
- Aliquot and store at −20°C.

Complete Culture Medium (CCM) As described above.

Human ESC Medium As described above.

ROCK Inhibitor (Y27632) As described above.

MEF Medium As described above.

PCs Medium PCs medium is (DMEM)-mega-cell supplemented with 10% FBS, 1% NEAAs, 2% penicillin–streptomycin–amphotericin B, 2% L-glutamine, 0.1 mM β-mercaptoethanol, and 5 ng/ml BFGF.

- To prepare 50 ml medium: 5 ml FBS, 1 ml penicillin–streptomycin–amphotericin B, 1 ml L-glutamine, 0.5 ml of NEAAs, 03 μl β-mercaptoethanol, 2.5 μl BFGF, and DMEM mega cell to a volume of 50 ml.

Procedures HDF culture is as described above.

Isolation of Human Adipose Tissue Derived PCs PCs were isolated from human abdominal adipose tissue as previously described with minor modifications [25, 78]. Cells were stored in liquid nitrogen until needed.

Culture of Human Adipose Tissue-Derived PCs

- Remove a vial of PCs from the liquid nitrogen and thaw them rapidly in a 37°C water bath until the contents are thawed nearly to completion.
- Add 1 ml of PC culture medium to the vial and centrifuge at 1800 rpm for 10 min at room temperature.
- Discard the supernatant and resuspend the pellet in 1 ml of PC culture medium.
- Culture these cells in two 100 cm^2 Petri dishes.
- Incubate cells at 37°C in a 5% CO_2 incubator for 2 days until the cells reach 60–70% confluence.

NOTE: Cells are regularly checked under inverted microscope. When cells reach 80% confluence, they should be removed from the plates with trypsin as described above.

Recovering Plasmids from Bacterial Stock Stored in −80 °C Retrieve the bacterial glycerol stocks from −80 °C and open the tubes on ice as quickly as possible.

- Prepare five tubes containing 10 ml each of sterile LB medium with 10 μl puromycin per tube. Label each tube with the name of each plasmid (hOCT3/4-shp53-F, hSK, hUL, and eGFP). The fifth tube will serve as a negative control for bacterial growth.
- Use a sterile pipette tip to scrape some of the frozen bacterial plasmid stock off of the top of the frozen contents. Place each tip in the corresponding tube.

  Do not let the glycerol stock thaw!

- Grow the bacterial in LB medium containing puromycin at 37°C overnight in a water bath.

Plasmid Purification

- After overnight growth in LB with puromycin, plasmids can be isolated from the bacterial culture using the PureYield™ Plasmid Miniprep System according to the manufacturer's instructions.
- Quantify the amount of plasmid DNA isolated using the Qubit 3.0 fluorometer with a double-strand DNA kit or with a spectrophotometer (e.g., Nano Drop).
- Similar to instructions in the previous section, label 1.5 ml centrifuge tubes as hOCT3/4-shp53-F, hSK, hUL, or eGFP. Add 100 μl of opti-MEM medium to each tube.

Somatic Cell Transfection

1. Electroporation
   - Cells should be removed from each plate with trypsin as previously described and counted by Trypan blue dye exclusion prior to electroporation.
   - Cells were added to the purified plasmids in opti-MEM medium and electroporated according to the manufacturer's instructions.

     Notes
   - Electroporation requires a large number of the cells in order to achieve a significant response to the electrical pulse (~1×10^7 cells per tube).
   - Cells should be washed thoroughly with opti-MEM medium before combining with the plasmids. This is necessary in order to remove any traces of cell culture medium and to avoid cell damage during electroporation.
   - The voltage and time of the electrical pulse should be adjusted according to the cell type. The goal is to achieve transient membrane permeabilization followed by resealing once the current is removed.
   - If the electric pulses are too strong, the cell membranes will be disrupted beyond repair, which will result in an unacceptable level of cell death.
   - After electroporation, cells should stand at room temperature for several minutes to facilitate pore closure.

- After electroporation, cells should be cultured on 0.1% gelatin-coated six-well plates in appropriate cell culture medium supplemented with 20% FBS for 2 days to promote recovery after the shock associated with the electrical pulse.

2. Lipofection
   - Somatic cell transfection using episomal vectors can also be achieved by adding 9 μl of Fugene HD or lipofectamine 2000 to each of the four tubes followed by incubation at room temperature for 15 min. This mixture is then added to the cells undergoing transfection.
   - Culture the transfected cells in opti-MEM medium for 24 h and then replace with specific cell culture medium for 2 days to follow.

For Both Viral and Non-viral Transfection

Preparation of Mitomycin C-Inactivated MEFs This is done to prevent proliferation of the cell feeder layer.

- Treat the MEFs with 10 μg/ml mitomycin C in CCM for 2 h at 37°C at 5% CO_2.
- Aspirate the CCM and discard.
- Wash twice with sterile PBS.
- Add the appropriate amount of 0.25% trypsin–EDTA to each flask and incubate at 37 °C for 2–3 min.
- Check the cells under the inverted microscope to ensure detachment.
- After cells are completely detached, neutralize the trypsin with an equal volume of CCM. Collect the cell suspension and centrifuge at 1800 rpm for 10 min.
- Discard the supernatant and culture the cell pellet into a 60 mm dish precoated with gelatin. The MEFs will be ready for use on the following day.

Optional: Preparing MEF-Conditioned Medium

- Culture MEFs in ESC medium without bFGF for 1 day.
- Collect the MEF-conditioned medium. Add fresh bFGF immediately prior to use.

Re-plating Transfected Cells onto a Mitomycin C-Treated MEF Feeder Layer *The feeder layer is essential for growth and proliferation of the target cells. The MEFs secrete critical growth factors, act as a substrate for the cultured cells, promote antitoxic effects, and facilitate synthesis of critical extracellular matrix proteins* [79, 80].

- At 3–5 days after transfection, aspirate the medium of the transduced cells and wash twice with sterile PBS.
- Passage the cells with Accutase. Count the cells and add 5×10^5 cells per each plate onto mitomycin C-treated MEF cells in CCM. Incubate the plates overnight at 37°C at 5% CO_2.

- After 24 h of culture on feeder layer, replace the medium with 10 ml of human ESC medium.
- Change the medium every other day until the ESC-like colonies (i.e., iPSC colonies derived from reprogrammed somatic cells) reach a size that can be picked up; this is typically near or at day 15 after transfection.

Optional: Culture of the Transduced Cells with MEF-Conditioned Medium

- After 3–5 days, pass the transfected cells using Accutase onto gelatin-coated plates and culture with CCM until cells adhere to the plate.
- After cell adhesion (at 24 h), replace the CCM with MEF-conditioned medium supplemented with bFGF; bFGF is an important growth factor that maintains iPSCs in a pluripotent state.

Picking up iPSC Colonies at Days 28–30 After Transfection

- Add 20 μl of human ESC medium to each well of a 96-well plate.
- Remove the medium from iPS colonies and add 10 ml sterile PBS to each dish.
- Aspirate PBS and replace with another 5 ml PBS for each plate.
- Pick the iPSC colonies from the plate under a stereomicroscope using a P2 or P10 Pipetman under in a laminar flow hood. Each colony is transferred to a well of the aforementioned 96-well plate.
- Once all colonies have been transferred to the 96-well plate, add 180 μl of human ESC medium to each well and carefully pipette up and down to break up the colony into tiny clumps of 20–30 cells. This can be ascertained under the stereomicroscope.
- Transfer the cell suspension into 24-well plates containing mitomycin C-treated MEF feeder cells. Add 300 μl of fresh human ESC medium to each well and incubate at 37°C and 5% CO_2.
- Continue incubation until the cells reach 80–90% confluence (typically ~7 days). The colonies are then transferred into 6-well plates and ultimately to 60-mm Petri dishes with mitomycin C-treated MEF feeder cells (as needed, according to the number and size of colonies).

Passaging of iPSCs and Colony Expansion

- Aspirate culture medium and wash the cells with 0.5 ml sterile PBS.
- Remove PBS very well using P1000 Pipetman. Add 100 μl Accutase to each well and incubate at 37°C for 3–10 min; colonies should be observed continuously and should be maintained in Accutase until the edges of each of the individual colonies begin to loosen and fold back. The time of incubation may vary depending on the nature of the cell line, the colony size, and nature of the cells undergoing reprogramming.
- Aspirate and discard the Accutase solution. Wash cells with DMEM/F12 taking to leave the cells undisturbed during aspiration.

- Add 2 mL of human ESC medium per each plate. Detach the cells by pipetting up and down several times with a 1 mL tip.

Note: It is critical to avoid overpipetting; single cells in suspension will not establish colonies after seeding.

- Transfer the cell aggregates to a 15-mL sterile disposable tube.
- To collect any remaining cells, add an additional 4 mL of CCM to each plate. Transfer the remaining cells in suspension to the 15-mL disposable cell culture tube.
- Centrifuge the cell aggregates for 5 min at 200 × g. Aspirate and discard the supernatant.
- Add 2 ml of CCM in the presence of 10 μM of the ROCK Inhibitor, Y27632.
- Gently pipette the pellet up and down 2–3 times with a P1000 Pipetman, making certain to maintain small cell aggregates.
- Cells will adhere to plates containing mitomycin C-treated-MEF feeder cells throughout the first day after the passage.
- Culture the cells in the presence of 10 μM ROCK Inhibitor Y27632 in a 1:4 split ratio.
- Immediately agitate the cells in the dishes with forward to backward, then left to right movement to promote gentle dispersion evenly across the plate surface. Incubate the dishes overnight at 37 °C and in 5% CO_2.
- Change medium *daily* until the colonies are large enough to be passaged (typically after 4–5 days).

Critical Step: To ensure the quality of the iPSCs, it is important to change the culture medium with fresh medium supplemented with bFGF every other day at an absolute minimum.

Optional: Culture of iPSCs in MEF-Conditioned Medium

1. Plate 4 × 10^6 mitomycin C-treated MEFs in a T75 flask coated with 0.5% gelatin in CCM.
2. On the following day, replace CCM with 37.5-ml ESC medium with 20% KSR containing 4 ng/ml bFGF. Cells are incubated for 24 h at 37°C in 5% CO_2.
3. Collect MEF-CM from the flasks after 24 h and filter sterilize with a 0.22 μm filter. MEF-CM can be used fresh or can be stored frozen at −20°C.
4. Add fresh ESC medium with 20% KSR and 4 ng/ml bFGF to the flask.
5. Collect MEF-CM for up to 7 days using this procedure.
6. Depletion of L-Glutamine and bFGF from the MEF-CM is assumed. As such, MEF-CM needs to be supplemented with L-glutamine (to 2 mM), and bFGF (to 4 ng/ml) before use in iPSC culture. Freshly thawed β-mercaptoethanol (β-Me) is added to a final 0.1 mM concentration on each day of use.

Take Home Massage

- Induced pluripotent stem cells are generated by reprogramming of adult somatic cells terminally differentiated cells such as fibroblasts, or multipotent cells as adipose-derived MSCs.
- Reprogramming of somatic cells to acquire embryonic characteristics was achieved by exposure to a cocktail of pluripotency genes including (Oct3/4, Sox2, Klf4, and c-Myc) (OSKM factors).
- Generation of iPSCs can be achieved using viral or non-viral episomal integration methods.
- Reprogramming factors can be incorporated into the somatic cells by lipofection or by electroporation.

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Tissue Engineering Modalities and Nanotechnology

10

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What You Will Learn in This Chapter

In this chapter, you will learn the significant advances in tissue engineering, and the techniques used to generate tissues that mimic the natural structure of the native tissues and organs. You will learn the most suitable cell type or a combination of cells that can build up the tissue and incorporate them into natural or synthetic scaffolds. The chapter will cover current advances in 3D printing technology and nanomaterials, and the important role they play in the generation of scaffolds that match the extracellular matrix of almost any tissue. The difference between the mechanical method of the extrusion-based bioprinting and stereolithography, and other bioprinting techniques will be discussed. The chapter will also examine the factors involved in the scaffold synthesis and how they act synergistically to generate high-quality tissues. Finally, it will cover the recent development in organoid technology, and their application in regenerative and personalized medicine.

10.1 Tissue Healing, Regeneration, and Engineering

Tissue healing was defined by Krafts as the "restoration of tissue architecture and function after an injury," with the aim of tissue replacement or regeneration [1]. Healing may be achieved by simple closure of the defect, which bonds the two edges of the wound together after a series of biological processes, resulting in the formation of fibrous scar tissue [1, 2]. Conversely, tissue regeneration refers to a repair process in which the tissue defect is replaced with physically and mechanically functional tissue that is similar to the native tissues [1].

Some animals, such as reptiles, have high regenerative capacities. The salamander, for example, represents a distinctively superior model for tissue regeneration. After losing a limb, the salamander regenerates an entire new limb comprised of many tissues, including muscle, bone, cartilage, nerve, blood vessels, and skin [3]. This high regenerative capacity is correlated with the ability of different organ progenitor cells to migrate and form a zone of undifferentiated progenitors known as a blastema [4]. The blastema first appears as a bud-like structure at the limb stump [5]. These dedifferentiated cells are capable of re-differentiating into more specialized cells and contribute to the generation of different

organ tissues. It has recently been demonstrated that the CXCR-1/2 signaling pathway plays an important role in cell recruitment and initiation of blastema formation [6].

In higher animals, including humans, tissue regeneration is an ongoing process that occurs at varying rates in different organs. This regeneration is partially maintained by a rare population of cells, known as stem cells [7, 8]. While some organs are rich in stem cells, such as bone marrow and the gut, other organs have a limited stem cell pool, and subsequently, limited regenerative capacities. The failure of some organs to function, such as the heart, brain, or the kidneys, is a life-threatening condition. One traditional therapy for organ failure is organ transplantation from a living donor, as is the case with liver transplants, or from a recently deceased cadaver, as in heart transplants [9, 10]. Although lifesaving, organ transplantation faces the challenges of finding suitably matched donors, organ shortages, a long waiting list, and risks of graft rejection [11].

Tissue engineering, defined as the production of tissues outside the body, has recently developed as an alternative to organ transplantation. Engineering living tissues requires cells, growth factors, an extracellular matrix (ECM), and scaffolding; all of which aim to emulate the tissue of origin. By using this integrated approach, tissues and organs can be engineered to replace a large degenerated segment of tissue or failing organ with a new functional one [12]. For example, cartilage tissue degeneration in the knee joint results in pain and has a drastic effect on daily activity and movement ability. Tissue engineering technology was used to develop a collagen scaffold loaded with chondrocytes that can repair this defect. This technique is now used in a commercially available product known as NeoCart®, which has shown significant improvement in cartilage regeneration in clinical trials [13]. Skin damaged by extensive burns was also the target of a successful tissue engineering approach. A natural amniotic membrane scaffold loaded with fetal fibroblasts resulted in significantly greater reepithelization with reduced inflammation and pain [14]. Vascular tissue generated using a polyglycolic acid and ε-caprolactone/l-lactide scaffold with mononuclear cells was applied in patients with congenital heart disease [15]. The results of this study were promising because the graft was patent, intact, and did not result in calcification or infection. However, further studies are warranted because there were reports of stenosis and thrombosis in some patients. These side effects were targeted by loading antithrombotic agents in the implanted scaffold [16]. These data show the high promise of using engineered tissues to replace diseased ones. They also demonstrate the need for modifications, quality enhancement, and optimization of the scaffolds to fit certain organs and avoid relevant complications.

In addition to generating tissues or organs to replace damaged ones, tissue engineering aims to generate models for studying diseases in vitro. Organoid cultures incorporate multiple cellular components to mimic the complex organ structure. These models aim to test disease pathogenesis and drug efficacy, as well as provide therapeutic strategies to compensate for degenerated tissues [17, 18]. An important milestone in tissue engineering was achieved by Bell's group, who demonstrated that collagen gel combined with fibroblasts could successfully generate engineered skin [19]. Later, Vacanti's group successfully generated a human auricle using a three-dimensional (3D) polymer scaffold

loaded with bovine chondrocytes [20]. This was followed by other studies that integrated scaffolds with endothelial cells to generate blood vessels [21], chondrocytes to produce a trachea [22], and uroepithelial plus smooth muscle cells to form urinary bladder tissues [23]. Atala and his group transitioned the experimental work to the bedside by treating seven patients in need of cystoplasty using engineered urinary bladders made from scaffolds (a combination of collagen and polyglycolic acid) seeded with autologous somatic cells [24]. Commercially available scaffolds made of fibrinogen and hyaluronic acid (Biocart™II) seeded with autologous chondrocytes have also produced efficient cartilage regeneration in clinical trials [25].

Another milestone is tissue engineering is the using organs that have been stripped of their cellular content as scaffolds, known as decellularization of the organs. Decellularization followed by reseeding the scaffolds with recipient cells evolved as a promising approach to minimize immune rejection of transplanted organs. Decellularized organs were used as scaffolds to bioengineer heart valve [26], liver [27, 28], lung [29], and kidney [30]. Newer 3D printing technology has provided high-quality scaffolds. Using 3D printers paired with recent diagnostic modalities, like computed tomography, and ink tanks that deliver viable cells, scaffolds can be customized to faithfully mimic the organ of origin and precisely fit the defect dimensions.

10.2 The Tissue Engineering Pyramid

Tissue engineering is a multidisciplinary science in which biologists, physicians, engineers, and physicists work together to engineer an in vitro functional tissue or organ. Tissue engineering can be visualized as a tissue engineering pyramid (TEP) that is based on the integration of the scaffold, cell, and physiological microenvironment components (Fig. 10.1). Scaffolds form the base of the pyramid. Scaffold implantation alone, without additional cellular components, can generate repair and fill tissue defect gaps. For example, collagen scaffolds derived from bovine Achilles tendon effectively regenerated fibrocartilaginous meniscus tissue after being implanted in dog joints [31].

Cells are the second step of the pyramid. Scaffold implantation paired with the desired cells can improve tissue repair. For example, implantation of a β-tricalcium phosphate scaffold enriched with stem cells in patients with maxillary bone defects resulted in a higher quality engineered bone compared to patients who received scaffold alone [32]. Similarly, stem cell implantation could augment the regenerative capacity of the collagen scaffold to produce functional meniscus tissue that mimics the native one [33].

In addition to scaffolds and cells, the physiological microenvironment represented by nutrients, growth factors, and vascularization is essential for viable tissue engineering. Growth factors are critical for directed cell differentiation into specific lineages and to maintain the somatic cell phenotype. For example, combining bone morphogenic protein (BMP)-7 and transforming growth (TGF)-β3 factors can enhance chondrogenic

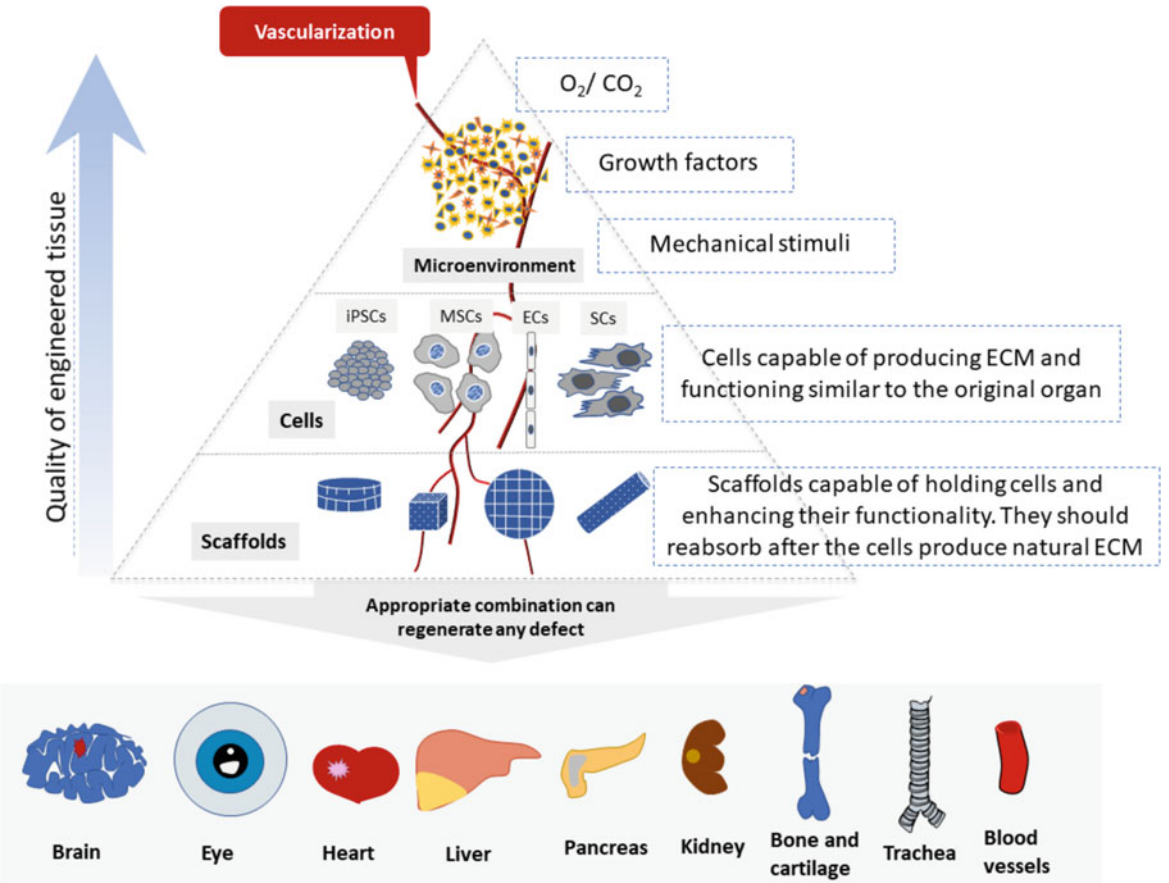

Fig. 10.1 Tissue engineering tools include cells, growth factors, and scaffolds. The optimal selection of each compartment will lead to a successful combination able to regenerate multiple tissues as neutral tissues, corneal, myocardium, liver, pancreas, renal tissue, bone/cartilage, teaches, and blood vessels

differentiation of stem cells [34]. Thus, microenvironment control and optimization is necessary to refine engineered tissue quality.

It is worth mentioning that the three TEP components can be optimized to achieve sufficient vascularization. For instance, scaffold porosity plays an essential role in vascularization [35, 36]. Co-culturing stem cells with human umbilical vein endothelial cells effectively enhanced vascularization [37]. Furthermore, including vascular endothelial growth factor in the scaffold improved cell viability and capability to penetrate the scaffold core and produce the ECM [38]. Scaffolds, cells, and microenvironmental factors all impact the engineered tissue outcome, and each must fulfill several criteria for successful results.

10.2.1 Scaffold Types and Characterization

Scaffolds represent the most important TEP component. They hold the cells at the defect site until the wound heals and is covered by the newly formed tissues. The scaffold provides structural support, improves cellular interactions, and enhances cellular growth and differentiation by mimicking the natural 3D structure of the tissues and organs [39–41].

Scaffolds can be synthesized from natural or synthetic materials. Bioscaffolds are scaffolds that can be safely implanted inside the body to fill damaged tissue gaps. A variety of materials are used for bioscaffolds. The following sections detail different types of scaffolds.

10.2.1.1 Polymeric Scaffolds

Polymeric scaffolds can be divided into natural and synthetic scaffolds, as shown in Fig. 10.2.

Polymeric scaffolds have many beneficial characteristics, such as appropriate mechanical properties, biodegradation, high porosity, adjustable pore size, and large surface area [42]. Natural polymers are typically derived from natural renewable resources, including plants, animals, algae, and micro-organisms [43]. They provide an ECM and structural support for the engineered tissue and enhance immune responses to ameliorate inflammation and toxicity [44]. Conversely, synthetic polymers are chemically engineered. A common synthetic polymer is polylactic acid, a biodegradable plastic [45, 46]. Synthetic polymers possess relatively greater mechanical properties compared to natural polymers [47]. Lynch-Aird and Woodhouse [48] compared the mechanical properties of natural catgut and synthetic nylon as suturing material for surgical wounds. Nylon was superior for suturing tissues under tension because it can maintain its tensile strength for an extended period of time. The observed differences in mechanical properties were attributed to catgut's sensitivity to humidity. It should be mentioned that some synthetic polymers may exhibit toxicity or stimulate immune interactions upon in vivo implantation [49]. Integrating natural and synthetic scaffolds may promote a synergistic effect because they possess different properties. For example, coating polyglycolic acid (PGA) with

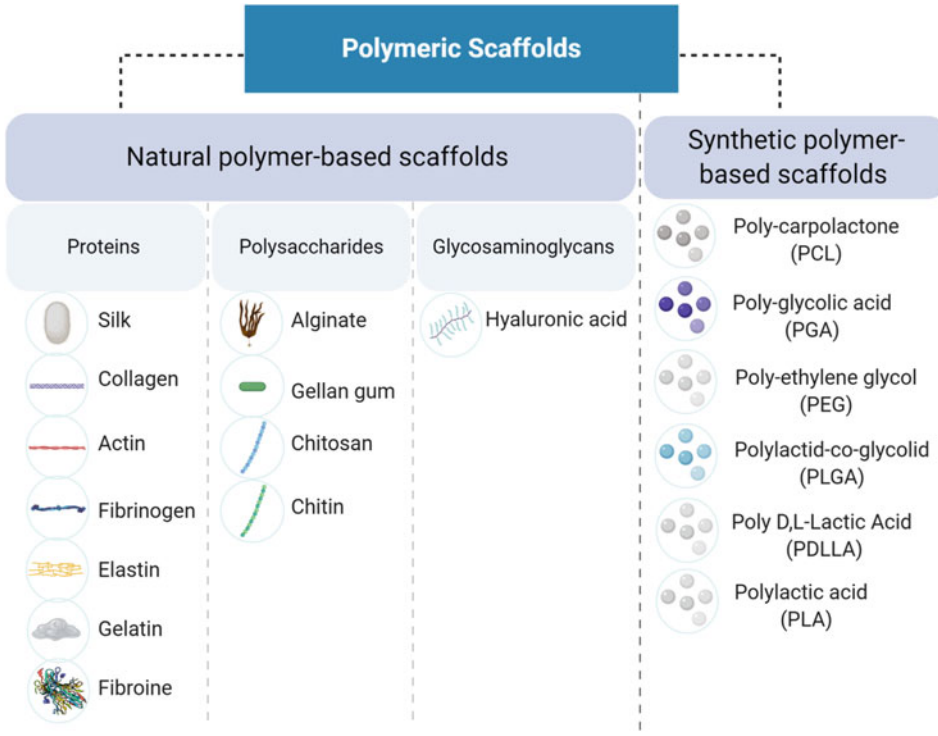

Fig. 10.2 Classification of polymeric scaffolds used in tissue engineering and regenerative medicine

hyaluronic acid resulted in high-quality cartilage tissues, as indicated by high collagen and glycosaminoglycan content, and a lower inflammatory reaction [50].

Various polymers have been used as substitutes for diseased or damaged parts of blood vessels, bone, skin, and cartilage. For example, vascular grafts, such as small-sized blood vessel substitutes and collagen tubular structures derived from small intestinal mucosa, can maintain a patent graft for up to 8 weeks following implantation in animal models [51]. Huynh, Abraham [52] showed that integrating small intestinal mucosa collagen with bovine collagen type I provided a more effective tissue engineering approach, evidenced by the tubular composite remaining patent for up to 13 weeks following implantation in a rabbit arterial bypass model. The commercially available collagen type I scaffold NeoCart® effectively regenerates cartilaginous tissue in patients suffering from microfractures [53, 54]. Moreover, polymer scaffolds are currently used for drug delivery and designing functional tissues [43]. Rai et al. reported delivery of BMP-2 for bone regeneration using biodegradable, honeycomb-shaped scaffolds synthesized from polycaprolactone (PCL) and PCL mixed with tricalcium phosphate [55]. Similarly, TGF-β has been encapsulated in chondroitin sulfate NPs and loaded in PCL and chitin polymer scaffolds to support the long-term release of TGF-β at the site of implantation. This approach enhances cartilage regeneration [56]. Similarly, an engineered membrane

composed of PCL and polyurethane, chemically treated with the antithrombotic agent conjugated linoleic acid, efficiently constructed tiny blood vessels with robust antithrombotic effect [16].

10.2.1.2 Bio-Inorganic Material-Based Scaffolds

Inorganic materials are divided into metals and bio-ceramics according to their structure. They may be further classified as bioinert, bioactive, and bioresorbable material, according to their interaction with host cells and tissues.

Metallic biomaterials have low elastic modulus, low density, and high strength, whereas bio-ceramics possess high osteoconductivity and biocompatibility. Ceramic material is considered biocompatible because it can gradually degrade into non-toxic products [57–59]. Inorganic biomaterials, such as porous calcium phosphate ceramics, titanium, niobium, and zirconium alloys, have been extensively used in bone grafting, dental implants, and bone cement. They have also been used in femoral heads and periodontal grafts [59]. Interestingly, ceramic granules enhanced chondrogenic differentiation of the stromal vascular fraction derived from adipose tissue in vitro and promoted functional osteochondral tissue in vivo [60].

10.2.1.3 Organic-Inorganic Hybrid Scaffolds

Composite scaffolds are designed to provide multi-functional materials with improved structural, mechanical, and thermal properties [61]. Hybrid scaffolds include natural polymer composites, such as gelatin, chitosan, silk, and collagen, and synthetic polymers, such as PCL, PGA, PLA, poly (lactic-co-glycolic acid) (PLGA), and polyethylene glycol. Most of these materials are FDA approved for skin regeneration products.

Hybrid scaffolds also include bio-ceramics, such as carbon nanotubes, bioactive glasses, and silicates [62–65]. Different nanoparticles have been used as fillers to upgrade the scaffold's mechanical properties and maintain osteoconductivity and biocompatibility. For example, hydroxyapatite increases polyethylene mechanical properties to suit bone regeneration [66]. Similarly, in order to enhance chitosan's biological and mechanical properties for bone regeneration, an organic/inorganic network structure was fabricated using propylene oxide. This structure formed hydroxypropylated, which was linked later with ethylene glycol functionalized nanohydroxyapatite (f-nHA) [67]. In this study, f-nHA ensured covalent linking with the hydroxypropylated chitosan scaffold to form a network structure and enhanced the scaffold's mechanical properties.

10.2.1.4 Others

Decellularized natural scaffolds derived from diverse tissues and organs have revolutionized therapeutic approaches for replacing damaged tissues [68, 69]. Table 10.1 summarizes recent advances in decellularized scaffolds derived from various tissues and organs.Decellularized scaffolds, including decellularized amniotic membrane (DAM), are paired with seeding of the desired cells and aim to produce tissues with the same

Table 10.1 Summarized recent advances in decellularized scaffolds derived from various tissues and body organs both in vitro and in vivo

Organ/ tissue	In vitro	In vivo	Reference
Liver	Induction of primary liver cells into hepatocytes	Partial function recovery by the formed vascularized network	[70, 71]
Kidney	Promotion of cell proliferation and differentiation	Renal regeneration and urine production via an engineered kidney	[72, 73]
Amniotic membrane	Allowed the development of urinary bladder urothelium	Effective substitute for pericardium lesions	[74, 75]
Heart	Induction of precursor cells into the beating cardiomyocytes	Myocardium regeneration and ischemia infarction treatment	[76, 77]
Skin	Engineered dermis which promoted endothelial cell adhesion and proliferation	Burned wound healing and breast reconstruction and transplantation	[78, 79]
Pancreas	Proliferation of pancreatic acinar cells	Increase in insulin expression and regulation of blood glucose	[80]

architecture as the native tissue upon in vivo implantation. We have previously shown that a 3D DAM scaffold is biocompatible and enhances stem cell proliferation [81, 82].

Scaffolds for biomedical applications must meet certain requirements, summarized as follows [83, 84]:

1. *Porosity*: Porosity controls ECM colonization and allows cell infiltration. Interconnected pores within a critical size range enhance cell growth, proliferation, and migration. The lower pore size limit is determined by the cell size and the upper limit is determined by the required surface area. The pores should be large enough to provide a space for blood vessel development, neural growth, and to enable drug and growth factor diffusion [85]. Sahmani et al. reported that a porous scaffold consisting of titanium oxide nanoparticles and nanoclay with pore sizes ranging between 65–100 nm exhibited improved capillary formation [86]. Conversely, a 150 μm pore size in a 3D beta-tricalcium phosphate scaffold promoted vascularization more efficiently than 100 and 120 μm pore sizes in the same scaffold [36]. Deeper investigation showed that the higher vascularization potential of the 150 μm porous scaffold was achieved via activation of the PI3K/Akt pathway. Chen et al. reported that a 3D-printed macroporous hydroxyapatite scaffold with pore sizes of ~600 μm enhanced bone repair and regeneration [87]. Porosity can be analyzed using computer software, the liquid displacement method, scanning electron microscopy, and microcomputed tomography imaging [88].

2. *Biodegradability*: Biodegradability is defined as the scaffold's capability to degrade after being replaced by tissues [89, 90]. Degradability can be controlled to match the expected time for complete tissue replacement. For example, skin regeneration takes place within one month in healthy patients [91], but takes at least six months in diabetic patients [92]. Spinal cord regeneration requires long-term structural stability for more than a year. Thus, scaffold degradation should be optimized accordingly [93]. Decreasing degradability of some scaffolds, like the amniotic membrane, can be achieved by collagen cross-linking using UV light [94] or treatment with chemicals like carbodiimide [95]. Degradation can be measured using many methods, such as the collagenase buffer method, near-infrared fluorescence imaging to quantify degradation in real-time, using magnetic resonance imaging to track tissue ingrowth through vascularization, and ultrasound elasticity imaging. This latter technique can reveal the internal structural and functional variation of implanted scaffolds with high resolution [96, 97].
3. *Biocompatibility*: Biocompatibility is defined as the material's ability to perform its desired function without inducing any undesirable local or systemic effects on the host [98]. The ideal biocompatible scaffold lacks cytotoxicity, genotoxicity, immunogenicity, and carcinogenicity [99, 100]. Our laboratory has reported that a PCL nanofibrous scaffold was biocompatible and non-toxic for stem cells derived from bone marrow or adipose tissue [101].
4. *Safety*: Scaffolds should not induce deleterious immune responses or exhibit cytotoxicity [102]. For instance, implanting metallic scaffolds, like cobalt and titanium, can induce inflammatory and allergic reactions in orthopedic surgeries due to metal ions released during degradation. Hence, the material's ability to resist corrosion should be thoroughly investigated.
5. *Stability*: Scaffold stability is strongly correlated with its degradation rate. Highly stable scaffolds display a relatively low degradation rate, lasting over 2–4 years [103]. Transplanted grafts must establish complete connectivity with the body tissues [104].
6. *Surface roughness and architecture*: The scaffold's surface topography controls cell attachment, proliferation, and influences cell differentiation potential. Surface roughness can modulate the biological tissue response [105, 106]. For example, we demonstrated that the rough surface provided by graphene nanoparticles enhanced stem cell differentiation into osteoblasts without requiring external stimulators or growth factors [107]. The degree of roughness of polystyrene also influenced pluripotent stem cell (iPSC) differentiation into dopaminergic neurons [108]. Surface properties can be measured using atomic force microscopy or microcomputed tomography imaging [109].
7. *Mechanical properties*: The scaffold's mechanical properties influence cell attachment and differentiation. An appropriate scaffold should mimic the mechanical strength of the surrounding tissues. Scaffolds should also have sufficient mechanical integrity to be handled during the surgical operation. For example, hydroxyapatite composite scaffolds showed a high compressive strength of about 14.3 MPa, which is comparable with

cancellous bone and is very promising for bone regeneration [87]. Mechanical properties can be measured using Young's modulus of elasticity [110].

8. *Cost efficiency*: One consideration when designing scaffolds is the cost of the raw materials. Natural polymers are relatively expensive compared to synthetic ones. Similarly, precious elements like platinum cost more than bio-ceramics.

10.2.2 Cells

Cells form the core of the engineered tissue. They are the physiological units of tissue building that produce the required ECM when paired with an appropriate scaffold and growth factors [111, 112]. Adult somatic cells, mesenchymal stem cells (MSCs), and iPSCs may be used for tissue engineering [113–115].

Adult somatic cells are specialized differentiated cells that form different tissues and organs. Differentiated cells provide some advantages for tissue engineering because they save time and do not require growth factors for differentiation, making them more practical and cost effective [116, 117]. Somatic cell limitations include isolation from the donor, which may be invasive, and limited in vitro proliferation rates. They may also be rejected in allogenic settings [118–120]. However, adult somatic cells have been extensively tested in vitro, in vivo in animal models, and in clinical trials. For instance, cells derived from chondrocytes, meniscus, synovium, and adipose tissue have been tested for their efficacy in regenerating cartilage [42, 121, 122]. Engineered cartilage tissue produced by autologous chondrocytes with a porcine collagen type I/III scaffold was FDA approved in 2016 [123]. Replacing degenerated cartilage with this scaffold successfully promoted hyaline cartilage formation after 6 months in a study of 56 patients [113]. Furthermore, endothelial cells have been proposed as a potential cell source to enhance vascularization inside the scaffold. Co-culturing MSCs and human umbilical vein endothelial cells on a PCL/gelatin scaffold enhanced neovascularization [37]. Similarly, endothelial progenitor cells (EPCs) may improve vascularization of engineered bone tissue generated from printed bioactive glass-ceramics loaded with bone marrow-derived MSCs [124]. Furthermore, induced endothelial cells (endothelial differentiated MSCs) can effectively enhance osteogenic differentiation of MSCs seeded on silk fibroin scaffolds and increase endothelial markers [125].

MSCs are adult stem cells with self-renewal capacities and a robust capability to proliferate and differentiate into specialized cells [126]. These criteria make MSCs one of the best choices for tissue engineering [120]. MSCs are primarily isolated from bone marrow [7] and adipose tissue [127]. Seeding adipose-derived MSCs (ADMSCs) onto a hydroxylapatite-collagen hybrid scaffold increased bone tissue regeneration in a clinical trial including 50 patients [128]. Similarly, seeding ADMSCs onto a graphene-agarose scaffold resulted in significantly greater mineralized bone than the scaffold alone [129]. Gene expression and examining tissue structure confirmed that seeding bone

marrow MSCs onto a PLGA/fibrin hybrid scaffold successfully generated high-quality cartilaginous tissue [114].

iPSCs are somatic cells that are genetically reprogrammed to become embryonic stem cell (ESC)-like cells. They may be a promising cell source for tissue engineering due to their high differentiation capacity that resembles ESCs, but involves fewer ethical concerns [130]. iPSCs have been tested for their ability to generate functional tissues. They show a high potential for bone tissue formation when seeded onto PCL/polyvinylidene fluoride nanocomposite [115], graphene oxide nanofibers [131], and bioactive glass [132]. Culturing iPSCs derived from hepatocyte-like cells into a 3D DAM scaffold was recently shown to enhance hepatic differentiation, offering an emerging model for liver tissue engineering [133].

Cell source is an essential factor in tissue regeneration. Cells from different sources have variable phenotypic and genotypic characteristics that impact clinical translation [134]. For example, stem cells isolated from bone marrow exhibited a greater osteogenic differentiation potential than those isolated from adipose tissue [135]. Furthermore, cell seeding density can affect the quality and mechanical properties of the produced tissue. For instance, high quantity cell seeding increased ECM stiffness [136, 137], which is a crucial factor in bone regeneration.

10.2.3 The Microenvironment

The third tier of the TEP represents the microenvironment, which includes growth factors and vascularization. Engineered tissue can suffer from necrotic cores due to deficient vascularization, an issue that has been intensely researched [138, 139]. Growth factors maintain cell viability, proliferation, and direct differentiation into specialized cells. A specific and customized growth factor cocktail is required to orchestrate stem cell differentiation into more specialized cells or to maintain their phenotypic characteristics. For example, a combination of stromal cell-derived factor-1 and basic fibroblast growth factor successfully induced stem cell differentiation into periodontal ligament-like fibroblasts for periodontal ligament regeneration [140]. Another example is BMPs, specifically BMP-2 and BMP-7, which are common growth factors for bone regeneration that have been approved for clinical use in bone defects [141, 142]. These growth factors can be supplemented in the growth medium in vitro or can be incorporated into the scaffold to maintain a continuous supply. The rate of growth factor release and their homogenous distribution can be controlled using nanoreservoir technology, which encases the bioactive molecule inside a degradable nanomaterial [143–145]. For instance, Strub et al. delivered BMP-2 at the nanoscale by using chitosan to increase the PCL scaffold's functionality in bone regeneration [146]. This technology also appears to protect the bioactive molecules. Furthermore, incorporating natural herbal extracts like propolis, curcumin, and bambusa tulda potentiates the proliferation, migration, and regenerative capacity of stem cells [147–150].

Other environmental factors that impact produced tissue quality include gases, mechanical stimuli, and microgravity. Understanding the effects of these elements could help to refine the generated tissues and improve their functionality upon implantation. For instance, oxygen and carbon dioxide levels are essential factors that must be considered for tissue culture. Chondrogenic stem cell differentiation is increased under hypoxic conditions [151], likely because the native niche of the joint cartilage is usually hypoxic. Conversely, supplying oxygen pressure to a greater degree than atmospheric oxygen (hyperbaric oxygen) increases stem cell proliferation and angiogenic potential [152, 153]. Culturing cells under mechanical stimuli affects the produced ECM. Applying appropriate mechanical stimuli to scaffolds loaded with MSCs enhanced scaffold strength when engineering esophageal tissues [154]. Similarly, mechanical tension-compression stimuli and an ECM similar to the native meniscus tissue promoted MSCs differentiation into fibrochondrocytes (meniscus chondrocytes) [155]. Microgravity also affects stem cell behavior [156, 157]. The different microgravity of space results in physiological changes in astronauts and laboratory animals sent to space [158, 159]. This finding inspired many researchers to study the effect of microgravity on stem cells. Simulated microgravity (SMG) potentiates stem cell proliferation [156], with SMG duration regulating stem cell fate [157]. For instance, short SMG exposure promoted stem cell differentiation into endothelial, neuronal, and adipogenic lineages. However, prolonged exposure enhanced osteogenic differentiation. Another study evaluating SMG in 3D scaffolds seeded with MSCs and chondrocytes showed that SMG increased chondrogenic differentiation. Thus, using these techniques during culture may enhance the therapeutic potential of stem cells [160].

10.3 3D Bioprinting Cells and Materials: Building Blocks

The articulation of synthetic materials to form three-dimensional objects in a printing format became useable in the late 1980s [161, 162]. The operational system of taking a computer and controlling a printer was not a new development, but making a physical dimensional object out to be used in many applications would change science and the market of production as a whole. Manufacturing in the traditional format is called subtractive manufacturing, where materials are made and shipped to stamping mills or a fabricating center. When using a computer with design software and a 3D printer, the technique is noted as additive manufacturing [163]. Over the last few decades, this has progressed into a crucial area of biomedicine. In biomedicine, additive manufacturing is considered one of the most attractive strategies for engineering 3D tissues and organs in the laboratory, which can subsequently be implemented in some regenerative medicine applications. Engineering with the use of computer-aided development software has allowed the fabrication and biofabrication of very complex 3D structures to meet the niche of a specific patient or medical device for a delicate procedure.

10.3.1 3D Bioprinting Cells and Materials

Tissue engineering using stem cells requires the appropriate niche for proper proliferation and differentiation. Technically, engineering the stem cell niche is considered the most challenging aspect of tissue engineering. 3D printing provides the three-dimensional environment for the cells and helps them to maintain their cell–cell contact and thus, their function. In conventional 2D cultures, primary cells rapidly lose their function, largely due to perturbed cell–cell communication, further emphasizing the importance of 3D culture. As a result, 3D printing of material alone provides a structure for endogenous cells to function appropriately. But, it can be also combined with exogenous cell populations to design highly sophisticated constructs that mimic the natural tissue and be adapted for the use of living material. The approach of 3D bioprinting of either material alone or of constructs consisting of material and living cells has the potential to reconstruct tissue from various regions of the body. This technology can also potentially applied to bone, skin, cartilage, and muscle tissue.

In 3D printing, several technical issues have to be considered before any cellular component can be included. These include the selection of printing technology, choice of the biomatrix, printing parameters, and considerations of the interaction between the material and cells. The scaffold is designed in a computer-aid design (CAD) program, then coded to the 3D printer for a structure formation in a layer-by-layer format. The 3D bioprinter is a multi-tool printer allowing for multiple fabrication methods and printing cells and biological materials in programed patterns and gradients. Microextrusion is the common choice of printing and is essentially the same as used in thermal inkjet printing, which can attain a spatial resolution of hundreds of micrometers. Microjet extruder bioprinting is the process in which designed droplets are deposited onto the scaffolds in a layer-by-layer preprogrammed design. The choice of the materials that can be printed is endless. In biomedicine, the choice of the material is highly dependent on the applications and the cells that will be either printed or manually added onto the printed scaffold [164].

Various cell types have been printed using a 3D bioprinter. One of the impediments of engineering any scaffold is the ineffectiveness to biomimic the extracellular matrix of healthy tissue in the body when multiple cell types are integrated [165]. With the ability to design a structured pattern, providing an optimal environment for cells can prove to be very advantageous in regenerative medicine. Printers are adjustable, easily reprogrammed with a new CAD template, and provided with interchangeable stainless-steel blunt tip needles for injection to accommodate different biomaterials and/or multiple cell types. Recent thrust has been released to print scaffolds, which can serve as biomimetic components that can orchestrate tissue regeneration, provide tissue support, direct tissue regeneration, and integration within the host tissue. As a result, some of the basic material elements that are considered during the printing process include percent porosity with ranging dimensions, internal geometric and projection modeling, biodegradation dynamics, mechanical properties, and cell biocompatibility. Much research is required to find an optimal material for a particular application [166]. With the recent advances in cell-based

therapies, 3D printing is becoming an increasingly common technique to generate scaffolds and medical devices for tissue engineering applications; some features of printing a tissue scaffold are discussed below.

10.3.2 Bioink

The extracellular matrix (ECM) is the backbone of tissue regeneration for cell proliferation, adhesion, and differentiation. The ECM is generated either by the cells that are implanted exogenously or by the endogenous cells when they are exposed to 3D printed scaffolds. As a result, it is essential to mimic the 3D tissue environment in which the scaffolds are implanted with or without cells by the 3D printing process. Hence, the choice of the "bioinks" is important. Bioinks are biomaterials that can be extruded by a printing nozzle or a needle, which maintains a biofabricated matrix for cells to produce the ECM for tissue regeneration. Alternatively, the ECM can be generated in vitro and used as a bioink. Bioinks are characterized as structural, functional, or supportive [167, 168]. There are specific biomaterial and biological criteria that are taken into consideration in the choice of bioinks for printing, based on the goal of the project. Structural biofabrication takes into consideration the mechanical support, the degradation rate, and cell proliferation in the construct. The functionality of the bioink is to provide proper cues or growth factors for cell differentiation into the needed tissue construct. The bioinks should also have sufficient mechanical strength and provide the appropriate frame, to support the physiological signaling pathways responsible for cell survival and tissue development [169]. The development of these materials needs to be studied in a stepwise process for purification, material modification, and the most challenging sterilization to be utilized as a regenerative medicine application.

10.3.3 Acellular Scaffold Bioprinting

Acellular scaffolds are those that mimic the ECM biochemical and mechanical properties to stimulate a regenerative response. Acellular scaffolds must be porous and have the potential to generate the biochemical, biomechanical, and biophysical cues for cell migration [170]. Additionally, acellular scaffolds can be bioprinted without cellular support and implanted into patients for structural and functional support of the regenerative process. In a recent study, a 3D printed acellular scaffold made of polyurethane has been designed to fill the tracheal defect. This scaffold was tested in vivo through its implantation in an induced tracheal defect in a rabbit model. Histological analysis showed that the connective tissues were infiltrated inside the scaffold after 4 weeks [171].

10.3.4 Cellular Scaffold Bioprinting

The bioprinting of a 3D cellular scaffold implements living cells in the design procedure. Assorted emulsions have been developed to generate a 3D matrix of living tissue with each iteration having different strengths and limitations. Bioinks incorporating cells have additional requirements, and thus, pose significant challenges. The printing process must preserve the cell integrity and viability during resuspension and passage through the bioprinter nozzle, and prepare the appropriate niche for cell growth and function within the printed biofabrication [172]. The deposition of the bioink, depending on the printing mechanism, can be categorized into three methods: extrusion-based (pneumatic-, mechanical-, and solenoid-based), stereolithography, and droplet-based [173].

10.3.4.1 Inkjet Bioprinting

Inkjet bioprinting is based on the usage of cell-laden bioink droplets, generated and deposited to pre-defined scaffold regions (Fig. 10.3). An advantage to droplet bioprinting is the ability to allow for concentration gradients of cells, materials, or growth factors throughout the 3D scaffold by altering droplet densities or proportions [174]. Recently, droplet bioprinting is applied for "scaffold-free" print design whereby layers of preset concentrations of cells are deposited in an approximate scaffold mold.

10.3.4.2 Extrusion-Based Bioprinting

As mentioned, extrusion bioprinting is classified into pneumatic, mechanical, and solenoid. Each of the methods can be utilized for fluid dispensing of cells, bioinks, or developed materials depending on the research needs. Pneumatic systems use secondary pressurized air for extrusion and, in some systems, contain multiple syringe functions for allowing materials in one unit and cell source in the other. Pneumatic systems enable researchers to work with various levels of viscosity for scaffold development. Mechanical extrusion is a more simplistic approach and allows for direct control of the bioinks and low viscosity

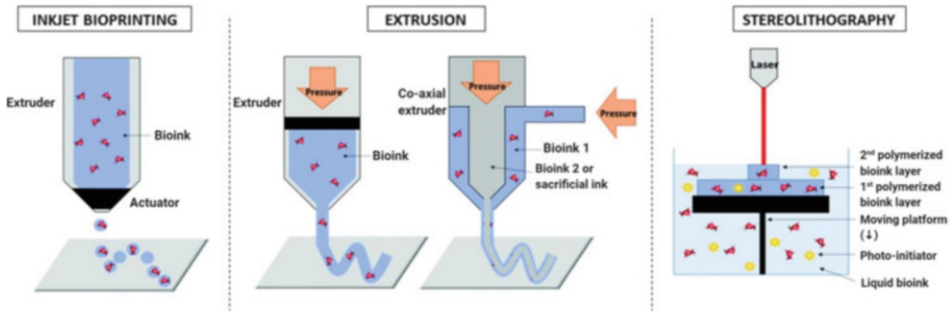

Fig. 10.3 Schematic illustration of the common methods of printing such as inkjet bioprinting, extrusion, and stereolithography

dispensing (Fig. 10.3). Mechanical extrusion, however, can potentially harm laden cells, and hence, can prove challenging [175].

10.3.4.3 Stereolithography Bioprinting (SLA)

These printers were built to meet the need for high resolution and accuracy [176]. The printer is designed to utilize a particular highly controlled radiation of laser or light to solidify the geometrical 2D pattern via photo-polymerization. Although SLA have limited design options, modifications in the polymer design can allow for more options in material usage. Using SLA, scientists can achieve 40–80% cell viability depending on the power of the unit, laser wavelength, exposure time, and toxicity of photoinitiator (Fig. 10.3) [177]. Overall, the main advantages of SLA-based bioprinting are the ability to fabricate multiplex scaffold designs with high resolution, and rapidly print constructs without support material [176, 177].

10.3.5 Sterile Conditions

One of the key drivers in 3D bioprinting for regenerative medicine is to form the foundation of the ECM scaffold to reproduce human organs and build a foundation for use in clinical transplantation. The printing procedure and the final printed tissue or replacement bioscaffolds must, therefore, be sterile to eliminate the risk of infection. If the 3D bioprinting process is prolonged, the possibility of contamination is significantly increased. Achieving sterile conditions is a continuous challenge. Approaches to reduce contamination include chemical sterilization as Ethylene oxide (EtO), which possess the least destructive effect on hydrogels. Also, decreasing the printing time of the scaffold could be beneficial in decreasing the contamination possibility [178]. However, filtration of hydrogels was demonstrated to reduce the physicomechanical properties of the bioink [178]. Lorson et al. reported that filtration followed by lyophilization is the best sterilization method of alginate and had no negative effect on its physicochemical properties [179]. Moreover, they have reported that sterilization by ultraviolet irradiation (UV) resulted in a deleterious effect on the physicochemical properties of the bioink [179].

10.4 Organoid Technology

10.4.1 Organoid Definition

Organoids are simplified micro-, multicellular, and heterogeneous 3D assemblies in which cells have a micro-anatomy arrangement that more realistically reflects their native origin [180]. The 3D structure of the organoid recapitulates the structure, heterogeneity, and development of the corresponding organ [181] and allows cells to self-assemble and organize into multicellular structures that mimic the original tissues [182].

Organoids are used in disease modeling to study the relevant pathology and mechanisms of development. They provide a representative biomimetic structure of the original organ that potentially mimics its phenotypes and cellular responses [17]. Moreover, they facilitate testing for drug sensitivity and developing personalized therapies to improve drug efficacy [183].

10.4.2 Historical View

The term "organoid" was first used in the early twentieth century to describe cell organelles, which are cellular substructures. Later, "organoid" was used to refer to complex cancerous structures, such as teratoma [184]. Today, the term "organoid" describes 3D multicellular self-organized structures that mirror the structure and function of the corresponding organ [185].

Historically, the concept of organoids was always associated with progress in culture systems. In 1906, Harrison [186] cultured tissue fragments from different organs in hanging drop tissue cultures [187]. A year later, Wilson broke the siliceous sponge to single cells, allowing them to reaggregate in a sponge-like structure. This finding raised important questions regarding whether cells can memorize their respective organ shape and whether mammalian cells show the same ability to reaggregate into their original structure [187, 188].

In 1958, Auerbach and Grobstein disaggregated metanephric mesenchymal cells and allowed them to reaggregate using an embryonic spinal cord as an inductor [189]. This is known as Grobstein assay, and kept the cells alive for a few days following reaggregation, during which time they maintained their early developmental stage [189]. This assay was followed by several studies on organ aggregation and dissociation [190, 191] that paved the way for identifying cell sorting and cell fate specificity during organogenesis and the powerful innate ability of cells to spontaneously organize into complex structures.

10.4.3 Organoid Culture Systems

Organoids can be generated from several types of somatic and embryonic cells, such as primary tissue-derived adult stem cells [192–194] and pluripotent stem cells [195, 196]. Organoids derived from pluripotent stem cell reprogramming usually give rise to heterogeneous populations that are advantageous for mimicking tissue complexity [197]. However, this may also be considered as a shortcoming, especially if the unidentified cell ratio is increased. The unknown mixed populations can result in uncontrolled and undesirable signaling pathways that affect the organoid's physiology and reproducibility [182]. Conversely, organoids derived from adult stem cells display limited unidentified populations compared to their pluripotent counterparts [198–200].

The subsequent organoid generation steps are similar regardless of the selected cell source. The cells are kept in homogenate-like matrices that are either natural, like Matrigel, or synthetic with well-defined properties and composition, like synthetic hydrogel. The primary goal is to keep the cells free from attachment in the 3D system to enable proliferation, differentiation, and ECM remodeling [201]. Human-derived ECMs, such as hydrogels derived from decellularized tissues, are preferable to those derived from animals or disease conditions that may limit potential clinical translational of the generated organoids [201].

10.4.4 Organoid Technology Applications

Organoid applications include modeling systems, such as healthy tissues, to understand their physiology and development and modeling different genetic and non-genetic diseases to study their pathogenesis and identify possible treatments [202, 203]. Examples of organoid technology applications in different tissues are detailed in the following sections.

(a) *Brain Organoids*

Studying human brain tissues and disorders is quite challenging because of the restricted availability of live brain tissues. Most studies in general have been conducted using postmortem or surgically removed samples. Preclinical models, like rodent brains, are substantially different from humans in terms of function, development, and complexity. Moreover, inconsistencies in processing and preservation methods, restricted availability of human samples, and variations in genetic backgrounds support the need for an alternative in vitro model [204]. Promising studies have shown that brain organoids mimic the epigenetic signature and neocortical development of the fetal brain [205, 206]. Lancaster et al. developed a 3D cerebral organoid using iPSCs that mimic human cortical development. They used patient-derived pluripotent stem cells to generate a brain organoid model of microcephaly, a disease without a sufficient animal model [207]. Organoid-specific human brain regions other than the cortex have been generated, including the hippocampus [208], hypothalamus [209], midbrain [209], and cerebellum [210]. Whole and partial brain organoids have been used to model neurological and neurodegenerative diseases like microcephaly [211], macrocephaly [212], Rett syndrome [213], and Alzheimer's disease [214]. Loss of vascularization is one significant limitation of organoid generation in general. To overcome this challenge, Pham et al. [215] used iPSC-derived endothelial cells co-cultured with brain organoids to promote vascularization after five weeks of culture [215].

(b) *Cardiac Organoids*

Organoid technology has provided an alternative in vitro platform to study the development, physiology, and pathology of cardiac tissues. One of the main challenges facing cardiac organoids is the heterogeneity of heart tissues and the association of different diseases with certain cell types. Keung et al. generated human ventricular-like cardiac organoid chambers from the hESC line hES2 [216]. A study by Schulze et al. showed that iPSC-derived cardiomyocyte embryoid bodies are potentially transplantable biological pacemakers [217]. Further, myocardial infarction has been modeled by applying cryoinjury in human cardiac organoids [218].

(c) *Liver Organoids*

The liver is a rich and heterogeneous tissue, primarily composed of hepatic, hepatic stellate, liver sinusoidal, and Kupffer cells. Generating liver organoids requires hepatic cells differentiated from human iPSCs or human ESCs [219, 220]. This process occurs in the presence of a suitable ECM with a rich cocktail of small molecules and growth factors, including epidermal growth factor (EGF), hepatic growth factor (HGF), and fibroblast growth factor (FGF). The Wnt and BMP signaling pathways regulate liver development and organogenesis in early stages [221]. TGF-β signaling pathway inhibition is also associated with organoid generation. The resultant organoids mimic the main phenotypic and genotypic characteristics of mature tissues and express corresponding specific markers, including ALB, CK18, and CK19 [222]. Huch et al. reported that organoids can also be generated from adult stem cells [223]. The latter group generated liver organoids from mouse $Lgr5^+$ liver cells isolated from carbon tetrachloride-injured liver. Human liver organoids were also generated from long-term cultured $Lgr5^+$ progenitor cells from the bile duct [224]. These organoids were able to differentiate into functional biliary and hepatic cells, but maintained the developmental and physiological features of fetal liver cells [225]. In 2018, Hu et al. established a long-term liver organoid model from mouse and human primary hepatocytes. Hepatocytes were incubated for 14 days with Matrigel and cultured in hepatic media that included many micronutrients [226].

Modeling liver diseases using liver organoids is an excellent way to study disease pathogenesis and screen potential medications for metabolism and cytotoxicity. Modeling metabolic disorders with genetic and non-genetic origins can also be investigated. For example, non-alcoholic steatohepatitis has been successfully modeled from hepatic progenitor cells to study metabolic disorders [227]. Also, liver organoid has been used to investigate the pathogenesis of hepatitis C viral infection [228].

(d) *Organoid Cancer Models*

Patient-derived organoids represent a powerful tool that mimics the phenotypic and genotypic features of their derivative tissue. This helps to create a precise treatment strategy that is individually optimized for each patient after testing multiple drugs for toxicity and

potential resistance [229]. Hepatocellular carcinoma (HCC) organoids have been generated from the HCC of patients' biopsies and collagenocarcinoma [230]. Huang et al. generated a pancreatic ductal adenocarcinoma model by inducing several mutations in pancreatic ductal organoids [231]. Breast cancer organoids were developed by Li and colleagues from surgical specimens of breast papillary carcinoma. The generated organoids matched the histological characteristics of the original tumor and maintained expression of the breast cancer biomarkers, including the estrogen receptor, progesterone receptor, human EGF receptor, and antigen Ki-67 [232]. Similarly, an organoid model of one rare prostate cancer was generated from collected needle biopsies of metastatic lesions [233]. The resultant organoids were successfully used to investigate the role of the epigenetic modifier EZH2 in driving molecular programs associated with neuroendocrine prostate cancer progression [233].

10.4.5 Limitations of Organoid Technology

Limitations of organoid technology include the unaddressed ethical concerns regarding organoid research and how mature these complex structures may become. This concern is not limited to organoids, but includes all starting tissues, cells, and human biomaterials [234]. On the technical level, organoid technology faces challenges of reproducibility, which could be attributed to variations in the methods adopted for tissue generation that are subject to continuous change and optimization according to the experimental aims [235]. Another challenge in organoid culture systems is the lack of vascularization in these complex structures, which subjects organoids to necrosis and a short life span if they are not sub-cultured upon reaching a specific size. Moreover, poor vascularization is associated with poor differentiation because of reduced blood circulation, a key factor in organoid maturation that was observed in kidney capsules transplanted in vivo [236]. Further, immune and endocrine systems are not represented in organoid systems, resulting in a loss of hormonal signals that are critical for tissue maturation and function [237]. Another limitation is that organoids derived from iPCSs and ESCs do not fully mature. These organoids more closely resemble fetal tissues than adult tissues [238].

Take Home Message

- Tissue engineering using the appropriate stem cells requires a special scaffold to assure cell–cell contact and cell–matrix adhesion. It also requires an optimal microenvironment of growth factors and oxygen exchange for maintaining stem cell viability and potentiating their differentiation capacity.
- Scaffolds in tissue engineering could be generated from either natural or synthetic biomaterials. The appropriate scaffold should be checked for properties such as

(continued)

porosity, biodegradability, biocompatibility, safety, stability, and mechanical suitability before its proposal for clinical application.
- The mechanical method of the extrusion-based bioprinting can potentially harm the cells. Using other bioprinting techniques such as stereolithography can provide higher cell viability, depending on the power of the unit, laser wavelength, exposure time, and toxicity of photoinitiator.
- Organoids can be generated from pluripotent stem cells (ESCs, iPSCs) or more differentiated cells. Organoids mimic the complexity found in the human body to a certain extent, which gives it an advantage over the 2D culture system.

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Scaffold Engineering Using the Amniotic Membrane

11

Radwa Ayman Salah, Hoda Elkhenany, and Nagwa El-Badri

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List of Abbreviations

°C	Celsius degree
3D	Three dimensional
AE	Amniotic epithelium
AM	Amniotic membrane
BM	Basement membrane
CD	Cluster of differentiation
CS	Chondroitin sulfate
CT	Connective tissue
DNAse	Deoxyribonuclease
EC	Epithelial cell
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
hAECs	human amniotic epithelial cells
hAM	human amniotic membrane
hAMCs	human amniotic mesenchymal stem cells
hBM-MSCs	human bone marrow mesenchymal stem cells
HBSS	Hank's balanced salt solution
HLA-DR	Human leukocyte antigen-DR isotype
IL-1ra	Interleukin-1 receptor antagonist
IRB	Institutional research board
KS	Keratan sulfate
MEM	Minimum essential medium
MMPs	Matrix metallopeptidases
MNCs	Mononuclear cells
MSCs	Mesenchymal stem cells
NaOH	Sodium hydroxide
PBMCs	Peripheral blood mononuclear cells
PBS	Phosphate-buffered saline
PDGF	Platelet-derived growth factor
SCs	Stem cells
SDS	Sodium dodecyl sulfate
SEM	Scanning electron microscopy
SER	Smooth endoplasmic reticulum
SLPI	Secretory leukocyte proteinase inhibitor
TE	Tissue engineering
TEM	Transmission electron microscopy
TGF-β2	Transforming growth factor-β2
TIMP-1	Tissue inhibitor of metalloproteinase-1

TSP-1	Thrombospondin-1
UV	Ultraviolet
VEGF	Vascular endothelial growth factor

What Will You Learn in This Chapter?
In this chapter, you will learn the structure of the human amniotic membrane (hAM) and its key functional characteristics, including the different types of hAM stem cells and their characteristics and functions. Furthermore, the chapter illustrates the differences between using an intact or decellularized hAM as a natural bioscaffold for tissue engineering. The development of the hAM as a three-dimensional (3D) cultural system is then explained as compared to the 2D cell culture system. Moreover, different methods to decellularize the hAM for bioscaffold applications will be demonstrated, including detailed sodium hydroxide (NaOH) treatment method to remove the enclosed epithelial and mesenchymal stem cells (MSCs) content.

11.1 Introduction

Tissue Engineering (TE) is described as the development of biological substitutes to enhance the function of tissues by using different chemical and biochemical factors. It is an interdisciplinary field that draws methods used in engineering and the biological sciences [1]. Scaffolds are used in TE to hold the cells in an anatomical and physiological environment that is similar to that of their parental tissue in order to support cell proliferation and differentiation [2]. Three-dimensional cultures that are made up of the scaffolds, the desired cells, relevant growth factors, and the extracellular matrix (ECM) provide an optimum biomimetic environment for successful tissue engineering [3–5]. Scaffolds can include both synthetic and natural materials [6].

There is considerable interest in the hAM, as it has potential applications in skin transplantation, burn management, surgical dressing, and corneal grafting. The hAM has a rich ECM and a variety of biological characteristics that make it highly useful in the medical field. Due to its antimicrobial and anti-inflammatory properties, the amniotic membrane (AM) has been used to decrease scarring and inflammation and to enhance wound healing [7]. The AM is also an excellent biomaterial, and a useful native scaffold for TE, due to the unique composition of its ECM and the fact that it is easy to obtain, transport, and process [8].

11.2 Scaffold Engineering Using Human Amniotic Membrane

11.2.1 The Human Amniotic Membrane as a Bioscaffold in Tissue Engineering

The use of stem cells (SCs) in TE takes advantage of both the capacity of stem cells to survive long term in culture, and their multi-lineage differentiation potential. Applying SCs-based TE techniques involves designing an in vitro culture system that precisely emulates the physiological system and the complex biological, architectural, and biophysical factors that together describe the native cells' environment [9, 10]. Traditionally, in vitro SC cultures are performed on flat, rigid, two-dimensional platforms. However, when cultured SCs are transplanted in vivo, the in vitro culture conditions can have different effects, including effects on the homing, engraftment, and function of cells inside their respective natural microenvironment [11–13]. These challenges are compounded by the inability to generate a clinically valuable quantity of cells at the site of injury [14]. This has led to attempts to develop a complicated ecosystem that can mimic the SC niche and define the natural environment of the cell [15, 16]. Recapitulating the topographic and mechanical characteristics of the niche that are crucial for the maintenance of the 3D configuration and orientation of the cells in space is of great interest. These characteristics enable effective cell-to-cell interaction, which is crucial for the determination of the SC fate and critical cell behavior [16–19].

Most of the biomaterials used to support cell cultures are either made up from synthetic polymers, or naturally obtained from either matrix proteins or adhesion molecules, such as laminin, fibronectin, collagen, or Matrigel [20–22]. 3D nanofiber networks or micropatterned arrays of the ECM components are other biomaterials that have been used to create cell cultures [23, 24]. However, these methods of creating the cell microenvironment fail to mimic the true complexity of the niche. It would be both impractical and economically infeasible to manufacture all of the native biomolecules in one culture [25]. As a result, polystyrene culture plates are medium used most frequently to mimic these biological microenvironments [26]. A natural substrate, such as hAM, represents a convenient material that can be used as a bioscaffold to enrich the biomolecular constituent of the niche [27].

The hAM is an easily available by-product of delivery in maternity hospitals, which is often discarded after clamp cutting of the umbilical cord [28]. The hAM has gained a lot of interest due to its use as a graft in treating skin burns, as well as its use in surgeries of the head, neck, oral cavity, genitourinary tract, larynx, and stomach [29, 30]. The hAM has both anti-inflammatory and antimicrobial properties, which are mediated by reduced expression of transforming growth factor (TGF-β) [31, 32], suppression of the pro-inflammatory cytokines IL-1α and IL-1β [33], and inhibition of matrix metallopeptidases (MMPs) secreted by macrophages and polymorphonuclear cells [34, 35]. The hAM has also been reported to produce compounds that have both antimicrobial and anti-inflammatory properties, including β-defensins, secretory leukocyte

proteinase inhibitor (SLPI), and elafin [36, 37]. Several studies also suggest that the hAM has antiangiogenic properties, and others have reported its angiogenic potential [38, 39]. The hAM includes two types of SCs, human amniotic epithelial cells (hAECs) and human amniotic mesenchymal SCs (hAMSCs) [40, 41]. Following transplantation of the hAM, factors secreted by hAECs and hAM SCs can potentially exert growth promoting, anti-inflammatory, antimicrobial, nontumorigenic, and antifibrotic effects on the surrounding tissue [35, 37, 42, 43]. These findings suggest that the hAM may be an excellent candidate for scaffold and tissue engineering techniques [44, 45].

One of the applications for the use of the hAM is in ocular surface reconstruction and TE [46, 47]. The antifibrotic and antiscarring properties of the hAM may be attributed to its rich laminin, collagen, and fibronectin content, as well as the additional proteoglycan components of the ECM [34]. Both hAECs and hAMCs are characterized as having a low major histocompatibility complex antigen (HLA) expression [48], which contributes to the low immunogenicity of the membrane [48–50]. hAECs and hAMSCs were shown to constitutively express HLA-ABC [51–53] as well as having limited expression of HLA class II (HLA-DR), further contributing to a lower possibility of immune rejection [54–56], making the hAM an attractive choice in the allotransplant setting.

The creation of biodegradable 3D scaffolds with multipotent SCs holds great promise for tissue repair. Many studies have focused on culturing different SC types on both natural and synthetic scaffolds. A hyaluronan-based scaffold was shown to promote rat MSC adhesion, migration, and proliferation [57] and to support the synthesis of autologous ECM components, without chemical interference and under stable culture conditions [57]. In addition to the positive cell–substrate interaction, the seeded cells were reported to be highly viable, suggesting that such a scaffold may be useful for a variety of tissue defects [57]. However, other studies reported failure of cellular attachment to the ECM, leading to anoikis, a form of apoptosis that occurs in cells that have inadequate contact with the ECM [58]. Many studies have since been conducted in order to integrate novel, natural scaffolds, such as the hAM, into a multifaceted, biomimetic cell culture that takes the respective key niche factors into consideration [27].

Bone tissue regeneration is one recent example that demonstrates the application of the hAM as a successful bioscaffold. The attachment and proliferation of MSCs on scaffolds were found to be required for their subsequent differentiation and integration into the surrounding tissues [59]. The choice of scaffold for the MSCs is critical when treating bone defects, which has led to the introduction of the hAM as a unique and valuable bioscaffold for bone regeneration [59].

11.2.2 Development of Human Amniotic Membrane

The hAM is the thin, layered, innermost protective membrane that surrounds the embryo/fetus [60]. Its development begins as a closed cavity in the embryoblast on the eighth day after fertilization [61]. The roof of this cavity is comprised of a single layer of flattened

cells, known as the amnioblast or amniotic ectoderm, while the floor of the cavity is made up of the epiblast of the embryonic disc [62]. There is a thin layer of extra-embryonic mesoderm [63] outside the amniotic ectoderm, and the amnion is connected to the margins of the embryonic disc [64]. As the embryonic disc expands and folds along its margins, the amnion and the amniotic cavity enlarge to entirely surround the embryo [65]. From the ventral surface of the embryo, the amnion is reflected on the connecting stalk, forming the outer covering of what will soon become the umbilical cord [65]. The amnion continues to expand as long as the amniotic fluid is secreted, becoming adherent to the inside surface of the chorion and leading to the removal of the chorionic cavity [65]. During early embryonic development, the inner cell mass gives rise to the epiblast and hypoblast layers [66]. The amniotic cavity begins as a tiny cavity between these two layers. The amnioblast layer is mostly derived from epiblast cells that surround the cytotrophoblast and differentiates into the epithelial layer that surrounds the primitive amniotic cavity. As the implantation progresses, a small pit in the embryoblast appears in an area near the uterine wall [62]. Amnioblasts, which are derived from the epiblast layer, separate and line the AM. Simultaneously, there are morphological changes in the embryoblast, which give rise to an almost bilaminar embryonic disc. This disc consists of a thicker, epiblast layer, columnar cells, and a thinner, hypoblastic, cuboidal cell layer, which is continuous with the extra coelomic cavity, which will later give rise to the yolk sac. The presence of two cavities that surround the embryo enables the morphogenetic changes of the growing embryonic disc [67]. A pouch that forms out from the extra embryonic mesoderm of the yolk sac gives rise to the connecting stalk, which later gains its lining and gives rise to the umbilical cord. The yolk sac then disappears, and the placenta takes over the nutrition of the embryo [68] (Fig. 11.1). The fusion of both the amnion and the chorion forms the chorio-amniotic membrane [69]. Progressive enlargement of the chorio-amniotic membrane obliterates the uterine cavity leading to the fusion of the membrane. This is covered by the decidua capsularis, with the rest of endometrium covered by the parietalis decidua. Typically, the AM ruptures right before birth [70]. A schematic structure of the hAM showing a full-term fetus with the surrounding structures, cavities, and membranes is shown in Fig. 11.2.

11.2.3 Ultrastructural Characteristics of the Human Amniotic Membrane

The AM is the innermost layer of the fetal membrane and is an avascular tissue that is composed of two main layers, the inner, greasy, smooth, amnion layer, and the outer, rough, bloody chorion layer (Fig. 11.3) [71]. Following its separation from the chorion, the hAM consists of a layer of polygonal epithelial with distinct borders and a homogeneous cytoplasm that is deficient in nuclear details. There is also a thick, acellular basement membrane (BM) that is rich in type III, IV, and V collagen beneath the hAECs. The BM is followed by the amniotic stroma, which is differentiated into two zones, a dense, reflective,

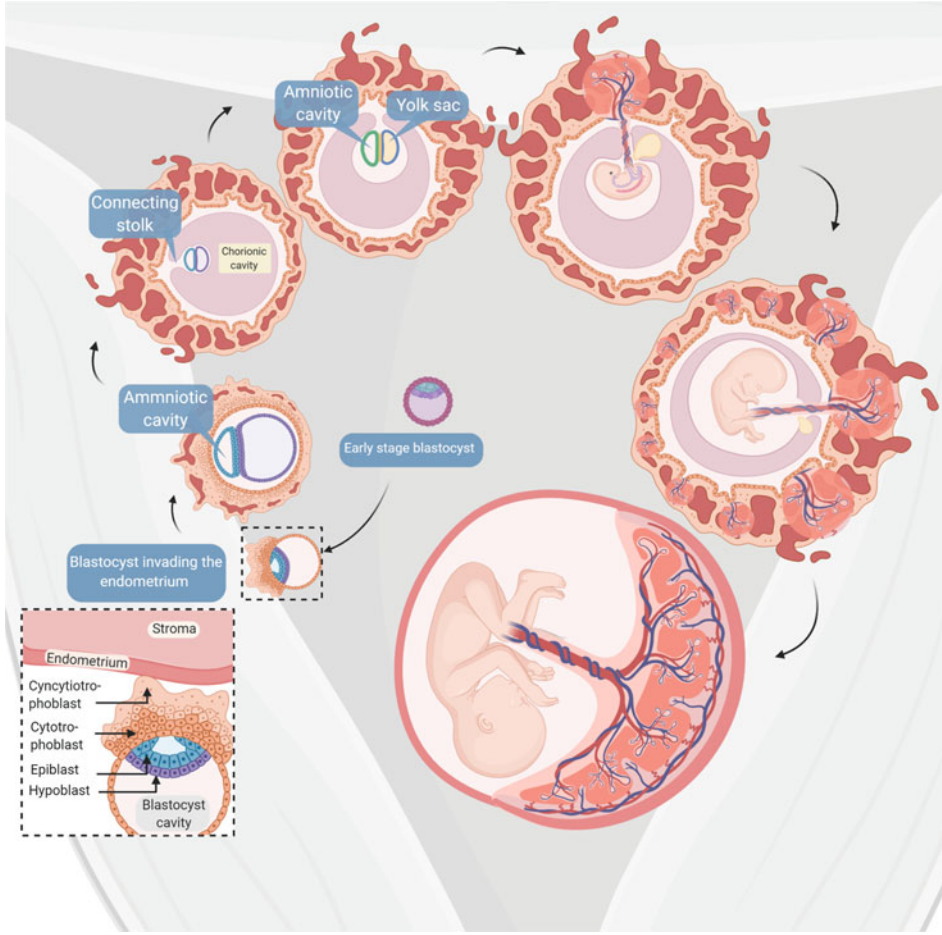

Fig. 11.1 The different stages of embryonic development and the development of the placental and fetal membranes

acellular, superficial, fibrous layer, and a deeper, thicker, less reflective layer fiber that contains a few cells arranged in a reticular network [72].

Transverse transmission electron microscopy (TEM) revealed that the hAM consists of a top layer of epithelial cells, attached to the BM that lies directly underneath [73]. The BM consists of an upper lamina densa and the basal lamina. The upper lamina densa is 10–20 nm thick and contains most of the fibronectin and laminin adhesive proteins that have binding sites for the epithelial cells and ECM components [73]. The lamina densa contains a high concentration of heparin sulfate, which is thought to be a part of the proteoglycan perlecan that interacts with type IV collagen. Interestingly, klaminin has

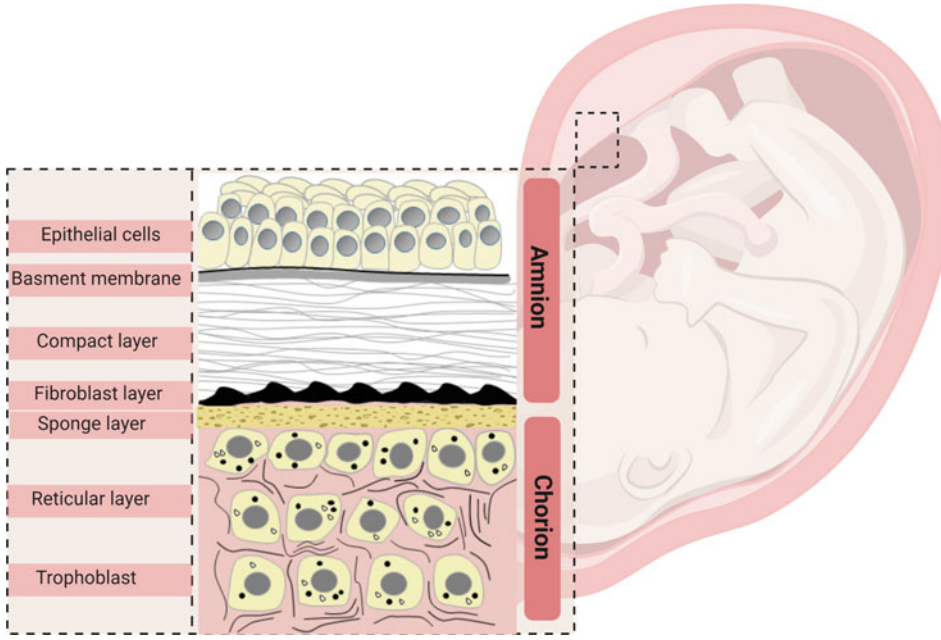

Fig. 11.2 A schematic of the structure of the hAM, showing a full-term fetus with the surrounding membranes. This figure illustrates the final stage of hAM development and the makeup of its two layers, the amnion and the chorion. The amnion consists of an epithelial layer, a basement membrane, a compact layer, a fibroblast layer, and a spongy layer. The chorion consists of a reticular layer, a basement membrane, and a trophoblast layer.

binding sites for both perlecan and type IV collagen, supporting the binding and subsequent interactions that stabilize the entire BM [73]. The lower part of the BM, which is 200–300 nm thick, is called the basal lamina and contains type IV collagen, heparin sulfate, and laminin [73]. The hAECs are attached to the BM by a number of hemidesmosomal contacts [73]. Underneath the BM is the stroma, which is a vast network of collagen fibrils (each measuring 30–40 nm in diameter) that have strong type I collagen staining [73]. Keratan sulfate (KS) and chondroitin sulfate (CS), also labeled in the stroma, are often localized on the collagen fibrils [73].

TEM and scanning electron microscopy (SEM) have been used to determine the 3D ultrastructural differences between intact nondecellularized and decellularized hAMs. No notable differences between the stromas of these two types have been reported. Electron micrographs of the intact, nondecellularized hAM showed an epithelial lining with rounded nuclei, a thick basement membrane, and underlying connective tissue (CT) that contains bundles of ECM proteins and scattered elastic fibers. A layer of cubical amniotic epithelial cells with rounded apexes and vacuolated cytoplasm containing large, rounded, or oval-

Fig. 11.3 The hAM contains two layers: the inner, smooth, greasy amnion that surrounds the fetus, and the outer, bloody, rough chorion that surrounds the placenta. Image *Courtesy: Center of Excellence for Stem Cells and Regenerative Medicine, Zewail City of Science and Technology*

shaped nuclei with irregular nuclear envelops, large euchromatin, and peripheral clumps of heterochromatin was observed [74]. Removal of this epithelial layer was confirmed after decellularization of the hAM, using NaOH (Fig. 11.4) [74].

11.3 Amniotic Membrane Stem Cells

The hAM is comprised of two types of SCs, the hAECs that rest on the BM, and the hAMCs that are present in the deeper spongy layer of the membrane [40, 41]. Both hAECs and hAMCs are typically epithelial in nature and are both are developed before the delineation of the three primary germ layers that occurs during the pregastrulation stages of embryogenesis [61]. hAECs, on the one hand, are derived from the ectoderm, before the start of organogenesis and are formed on the eighth day of fertilization. They are specialized, fetal epithelial cells that die within ten months of conception [75]. hAECs

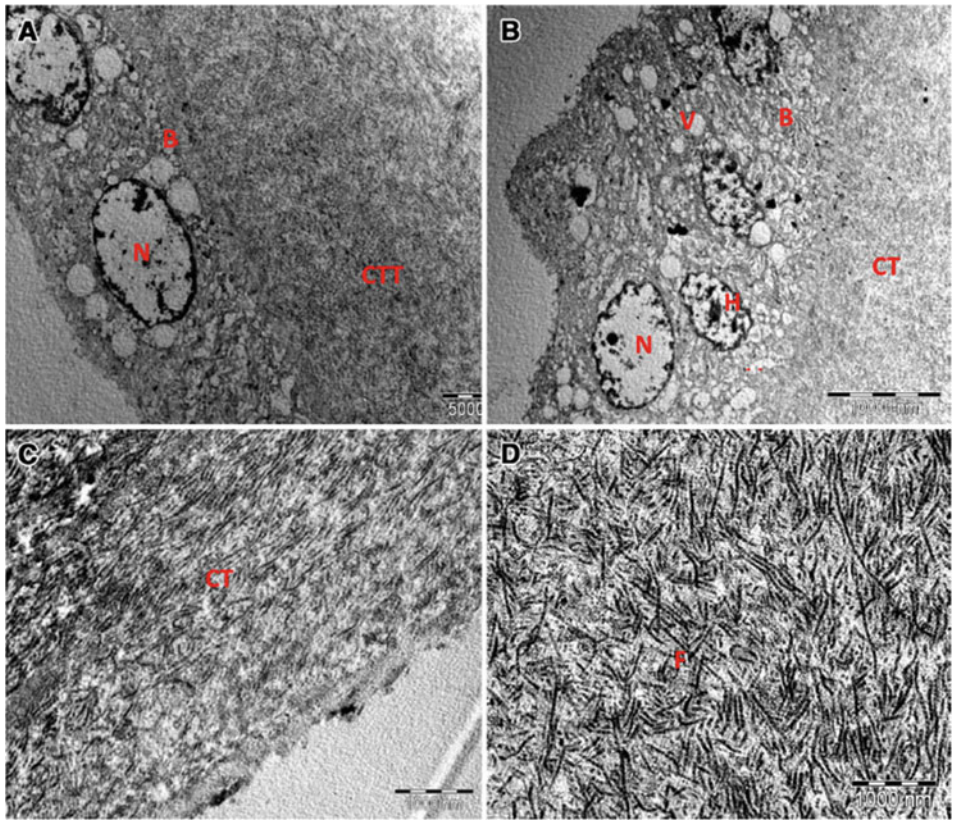

Fig. 11.4 Panels A and B show an electron micrograph of the intact, nondecellularized hAM. It contains the epithelial lining with rounded nuclei (N), a thick basement membrane (B), and the underlying connective tissue (CT) that contains bundles of ECM proteins and scattered elastic fibers. Panel B shows one layer of cuboidal amniotic epithelial cells displaying a rounded apex. The cells show the following: vacuolated cytoplasm (V) that appears to have taken a round or oval shape, large nuclei with irregular nuclear envelops (N), and large euchromatic and peripheral clumps of heterochromatin (H). Panel C is an electron micrograph of the d-hAM, showing the thick basement membrane and the underlying CT. Panel D shows a magnified section of Panel C, displaying bundles of ECM proteins and scattered elastic fibers (F). It is important to note the disappearance of the epithelial lining in these panels. *Reprinted by permission from Springer Nature Service Center GmbH: Springer: Journal of Molecular Histology* [74], *Copyright (2018)*

are cuboidal to columnar cells that form a monolayer that lines the AM. This layer is in direct contact with the amniotic fluid [74]. On the other hand, the hAMCs are derived from the extra-embryonic mesoderm of the primitive streak [76] and are dispersed throughout the ECM. AM SCs (hAECs and hAMCs) can differentiate into multiple, mature cell types, including adipocytes, osteocytes, chondrocytes, myocytes, cardiomyocytes, hepatocytes, neurocytes, and vascular endothelial cells [75].

hAECs have a high level of expression of cluster of differentiation (CD)73, CD90 and to some extent, CD105 [77]. hAECs have also been shown to express SC markers including KLF4 and c-MYC, keratinocyte markers, including K19, β1 integrin, K5, and K8, and the keratinocyte proliferation antigen K14 [78]. However, hAECs do not express HLA-DR or CD34. hAMCs have been shown to express the typical mesenchymal surface markers including, CD73, CD90, and CD105, stem cell markers, including NANOG and c-MYC, and keratinocyte markers, including K19, β1 integrin, and K8. Similar to hAECs, hAMCs do not express the hematopoietic marker CD34 or the major histocompatibility complex antigen HLA-DR [77, 79, 80].

Several protocols have been established for the isolation of epithelial and mesenchymal SCs from the placenta and AM. A key factor in the isolation protocols depends on the difficulty of separating the AM from the chorionic membrane. Enzymatic digestion is then performed to achieve complete isolation [81]. The membrane is then cultured leading to the release of hAMSCs and hAECs. hAMSCs can be recognized by their fibroblast-like structure and adherence to plastic surfaces, while hAECs are characterized by their typical cobblestone, epithelial phenotype [82].

11.3.1 Ultrastructural Characteristics of Amniotic Stem Cells

Morphologically, cultured hAECs have a cobblestone appearance, while hAMCs display a fibroblast-like phenotype [83]. Ultrastructural analysis has shown unique features of hAECs, including that they attach to the BM via many hemidesmosomal contacts [73]. hAECs contain a fairly large number of intracytoplasmic organelles, microvilli on the apical surface, loose intercellular connections, and abundant cytoplasmic processes that extend to both the lateral and basal sides [84]. SEM analysis has shown that the thickness of the BM is formed primarily from collagen fibers, while the CT stroma contains collagen fibers and cells that run in different directions. TEM has identified that hAECs are cuboidal with apical microvilli and convoluted lateral borders with desmosomes and no obvious tight junctions. The basal surfaces of hAECs are filled with hemidesmosomes at the distal termini of the cell processes, and wavy filament bundles that can be seen in the adjacent cytoplasm [85]. Mitochondria are also observed in the cytoplasm [75]. The CT stroma contains fibers and numerous cells of varying shapes and sizes. It was previously reported that these cells are mesenchymal SCs [75]. Another electron micrograph study of the amnion epithelium demonstrated that the Golgi bodies contained numerous free ribosomes, small strands of dilated smooth endoplasmic reticulum (SER), small rounded secretory vesicles, and mitochondria [74]. The lateral cell membrane also showed complex interdigitation [74].

Light microscopy revealed that hAMCs are round in shape, with an average diameter of 15 μm, and contain an abundant, multivacuolated, intensely basophilic cytoplasm [86]. TEM analysis of hAMCs showed that they have a hybrid epithelial–mesenchymal ultrastructural phenotype. Epithelial features, including nonintestinal-type surface

microvilli, intracytoplasmic lumina lined with microvilli, and intercellular properties were observed. The hAMSCs also exhibited a number of mesenchymal characteristics, including the presence of a rough endoplasmic reticulum, lipid droplets, and well-developed foci of contractile filaments containing junctions of dense bodies [86, 87]. These features were consistent with the notion that hAMCs have the potential for pluripotency [88–90].

11.3.2 Marker Expression of Amniotic Stem Cells

Amniotic SCs release cytokines that are essential for the promotion of cell proliferation, reducing inflammation, and regulating various processes involved in the healing of acute and chronic wounds [91]. These cytokines include platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), angiogenin, transforming growth factor-β 2 (TGF-β2), tissue inhibitor of metalloproteinase-1 (TIMP-1), and TIMP-2, which are thought to be produced from amnion-derived multipotent progenitor cells [91, 92]. Amniotic cells also inhibit the proliferation of peripheral blood mononuclear cells (PBMCs) after activation by phytohemagglutinin [93]. hAMCs and hAECs have been reported to cause significant reduction in PBMC proliferation (34% and 23%, respectively) in mixed lymphocyte reaction experiments [93]. However, with activation of PBMCs, comparable levels of inhibition were observed for both hAMCs and hAECs (33% and 28%, respectively) [93]. The immunoinhibitory properties of AM cells did not seem to be altered due to subcultivation; however, their immunomodulatory potential was significantly inhibited by cryopreservation [56, 93].

In addition to the features listed above, amniotic SCs have also been shown to actively inhibit lymphocyte responsiveness and to not induce allogeneic or xenogeneic lymphocytic, proliferative reactions [93, 94]. Studies have shown that in intracorneal transplantation, all grafted AM were accepted and reported as clear, with no host cell infiltration. However, skin grafts were rejected. The response to limbal transplantation was mild, although some CD4 and CD8 T lymphocytes were attracted to the site of the amniotic graft [95].

Several reports have indicated that amniotic fibroblasts express class II antigens, in which case an enforced PBMC reaction may occur due to the presence of hAMCs [93, 95]. The allogeneic amniotic epithelium (AE) has been reported to be particularly susceptible to immune rejection, especially in sensitive recipients [96]. This is supported by a study in which the transplantation of intact AEs from mice with enhanced green fluorescent protein (C57BL/6 background) and wild-type mice with the same background was applied to the cornea, conjunctiva, or anterior chambers of three different groups of mice. Normal BALB/c mice, C57BL/6 mice, or BALB/c mice had been presensitized with donor antigens. Graft survival was much shorter in both recipients that experienced recurrent implantation of the AE (where the AE was grafted in the other eye seven days after the first grafting). This was also noted in the presensitized recipients. Two weeks after transplantation, delayed hypersensitivity was provoked in the mice, but not in normal mice

[97]. These findings indicate that although the AM and its cellular elements are thought to be immunoprivileged, their respective immunogenic properties should be taken into consideration [97].

11.3.3 Immunosuppressive and Anti-Inflammatory Mechanisms of Amniotic Stem Cells

As mentioned previously, the immunosuppressive effects of amniotic SCs have been frequently reported. However, the mechanisms underlying this process are not clear. Co-culture of hAMCs and PBMCs in trans-well systems did not lead to significant inhibition PBMCs [93]. Thus, PBMC proliferation was reported to be suppressed by hAMCs via cell contact, and not by soluble factors. The anti-inflammatory response of hAM cells is mediated by various factors. Both hAMCs and hAECs express the interleukin-1 receptor antagonist (IL-1ra), TIMPs, collagen XVIII, IL-10, and thrombospondin-1 (TSP-1) [34]. IL-1ra is structurally similar to IL-1β, but it lacks agonist activity. IL-1ra competes with IL-1 when binding to its receptor and thus blocks IL-1-initiated inflammatory responses. TIMPs are a family of proteins present in many human tissues and play a diverse role in the regulation of the metabolism of the ECM. TIMPs also play an essential role in the inhibition of angiogenesis, growth, invasiveness, and metastasis of tumors. Collagen XVIII is a potent antiangiogenic factor that can inhibit endothelial cell proliferation, angiogenesis, and tumor growth. IL-10 is a broad-spectrum, anti-inflammatory cytokine that inhibits the production of IL-1, TNF-β, and other pro-inflammatory factors. IL-10 has been reported to increase the production of TIMP, as well as to inhibit the expression of matrix metalloproteinase. TSP-1 is a multifunctional matrix protein that is secreted by many cell types, and it has been also been shown to have antiangiogenic activity. All of these findings may help explain the antiangiogenic and anti-inflammatory effects of the AM and its SCs [34].

11.4 Decellularization of the Human Amniotic Membrane

In a decellularized hAM (d-hAM), the epithelial cell layer of the AM is removed, leaving the AM basal layer exposed. This process is sometimes referred to as AM denudation [98]. The d-hAM has been widely used in both research and clinical applications, and there are multiple different methods of decellularization. These include the use of urea, EDTA, thermolysin, sodium dodecyl sulfate (SDS), or by mechanical scraping [99]. For these methods, the membrane must be soaked in the reagents, which may lead to loss of the hAM structure, making it fragile and difficult to handle while being used as a scaffold. These decellularizing reagents may also cause dysfunction of the hAM matrix proteins, leading to damage of the integrity of the stroma [100]. Another method of decellularization is known as the alkaline method. In this method, the amnion is carefully pulled apart from the

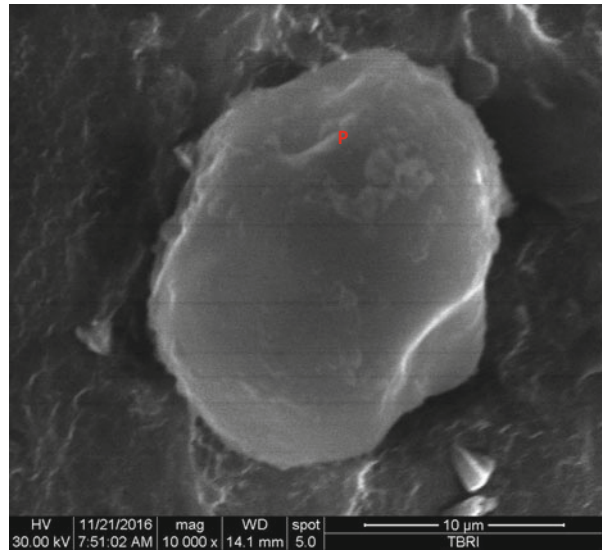

Fig. 11.5 An electron micrograph showing that hMSCs have a rough and nonuniform surface with distinct protrusions (P)

chorion. The epithelial side of the amnion membrane is spread facing upward to allow the decellularization of the hAECs. This decellularization takes place via the homogenous distribution of a solution of 40 mg/ml NaOH, dissolved in distilled water, which is then spread all over the epithelial surface of the membrane [27].

D-hAM provides 3D bioscaffolding that enhances the proliferation and differentiation of human bone marrow mesenchymal stem cells (hBM-MSCs) into adipogenic and osteogenic lineages [27]. We have previously established the unique interaction between the hBM-MSCs and d-hAM, in which the d-hAM appears to envelop segments of the hMSCs that lay on the surrounding membrane (Fig. 11.5) [74]. Umbilical cord blood mononuclear cells (MNCs) cultured on d-hAM-coated plates exhibited excellent survival and robust proliferation on the 4th day, but not the 7th day, of culture as compared to those cultured on no-hAM-coated plates [27]. In addition, hAM enhanced the survival, attachment, and proliferation of dermal fibroblasts and microvascular endothelial cells without inducing any cytotoxic effects [101]. The hAM also supported the proliferation and migration of keratinocytes via modulation of TGF-β, making hAM useful for wound healing [102, 103]. D-hAM has also shown promising results for its use as a wound dressing [104], and system to deliver SCs in the human body [105]. D-hAM has also been recently shown to support the attachment and proliferation of dermal fibroblasts, keratinocytes, and microvascular endothelial cells [34]. Therefore, hAM is considered a potential source of well-tolerated scaffolding material and has gained much interest for its use in the field of regenerative medicine [106, 107].

11.5 Material and Methods for hAM Decellularization Using Alkaline Method

11.5.1 Material

Reagents

- NaOH (solid)
- Alpha MEM
- Fetal bovine serum (FBS)
- Phosphate-buffered saline (PBS)-1×, (w/o Ca^{++}, Mg^{++})
- 0.9% Normal Saline
- L-glutamine
- Penicillin–Streptomycin-Amphetrocin
- 70% ethanol
- Trypan blue dye (Trypan blue 0.4% solution in 0.85% NaCl)

Equipment

- Laminar air flow
- MilliQ water
- Analytical balance
- Inverted microscope and bright field microscope
- CO_2 incubator

Supplies

- Falcon conical tubes 50/15 ml
- Twenty-four-well plate
- Scissors
- Forceps
- Gauze membrane
- Homemade cell crown, composed of a decapped container
- Beaker 1000 ml
- Micropipette (25 ml, 10 ml, and 2 ml)

11.5.2 Methods

11.5.2.1 Collection and Preparation of the hAM

1. hAM samples are collected from healthy subjects after delivery of the fetus and clamp cutting of the umbilical cord, following an institutional research board (IRB) protocol,

for collecting the routinely disposed AM for the study purpose, and signed informed consents. All subjects are screened and shown to be negative for blood-borne infections. Using fresh noncryopreserved hAM, samples are placed in a sterile saline solution and immediately transported to the laboratory [74].

2. The amnion layer of the hAM is separated and pulled apart manually from the chorion under sterile conditions using laminar airflow hood or by blunt dissection from the chorion layer. For consistency, it is essential to distinguish the glistening epithelial surface of the AM from the outer connective tissue interface.
3. The chorion is discarded, and the amnion is washed several times carefully in a beaker using a sterile PBS-1×, (w/o Ca^{++}, Mg^{++}) containing 0.1% antibiotic antimycotic. Washing is continued until all blood and blood clots are removed, and the light pink color of the membrane becomes vividly apparent.
4. The membrane is then cut into smaller pieces, with each piece being approximately10 cm in diameter.
5. Each piece is spread over a homemade cell crown, composed of a decapped container and a gauze membrane to allow homogenous spread of reagents over the epithelial surface of the membrane. The homemade crown is closed to tightly seal the amnion for it to stretch to its maximum (Fig. 11.6) [74].

11.5.2.2 Alkaline Decellularization of hAM Protocol

1. The floor of the laminar airflow hood is sterilized using 70% ethanol, wiping in a unidirectional manner. All glass and plastic ware are then put inside for sterlization. The protecting hood is then closed, and the ultraviolet (UV) sterilization is switched on, for 20 to 30 min before use.
2. The shinier, epithelial, amnionic side of the membrane is spread facing upwards over the homemade crown to allow the decellularization of the hAECs to take place. After identifying the amnionic side of the hAM, make sure that the inner side of the amnion, the side facing the chorion, should always be facing down during any procedure. It is important to know which side of the amnion we are working with, as it affects the outcome of following experiments [27].
3. A working concentration of 40 mg/ml NaOH is prepared by dissolving 2 g of NaOH powder (after weighing using a calibrated analytical sensitive balance in 50 ml of distilled water in a sterile disposable 50 ml tube and shake vigorously till all powder dissolves). NaOH is then applied to the membrane for 30–60 s with the help of a cotton tip or a piece of gauze soaked in the prepared NaOH solution (making sure to cover the whole surface that is exposed of the amnion).
4. Finally, the NaOH and cell debris are washed thoroughly using sterile PBS or saline for 5–10 min with the aid of a Pasteur pipette or syringe (Fig. 11.6).

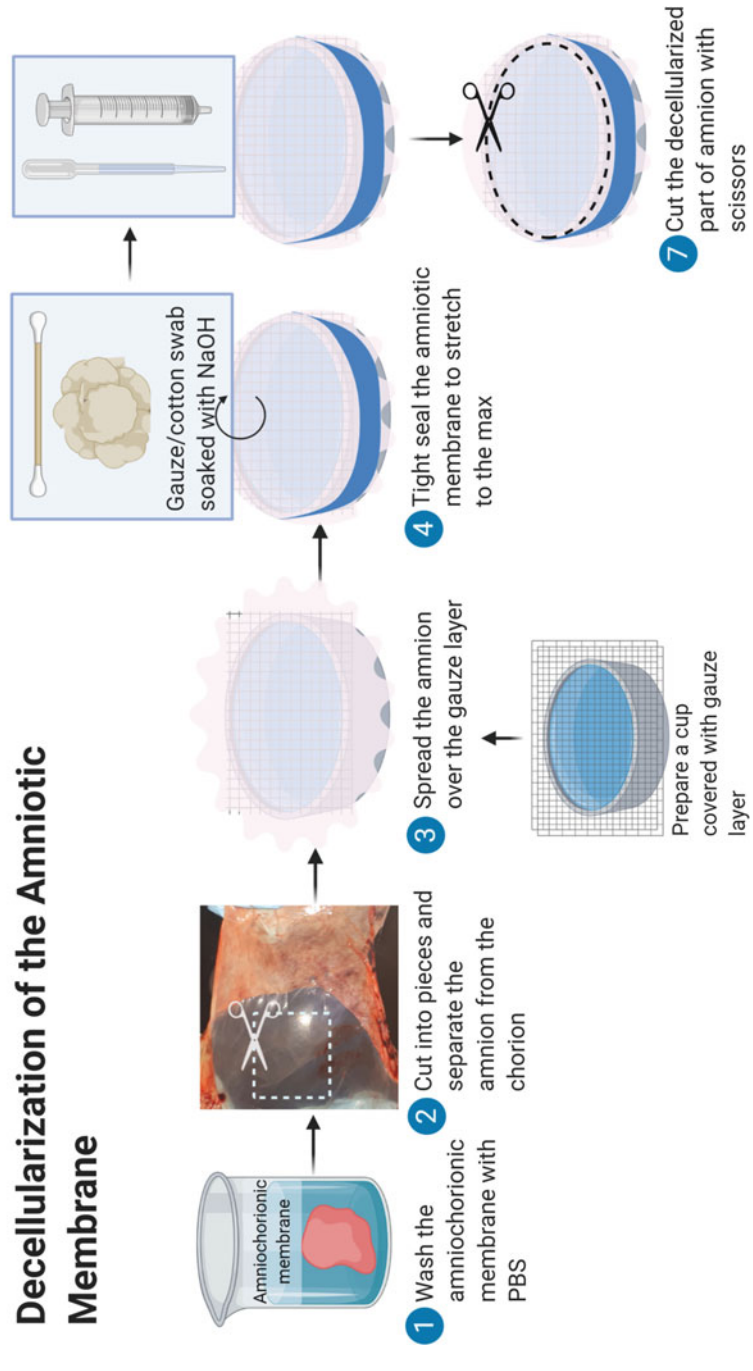

Fig. 11.6 Preparation of the hAM and surface modification of the amnion for alkaline decellularization using NaOH

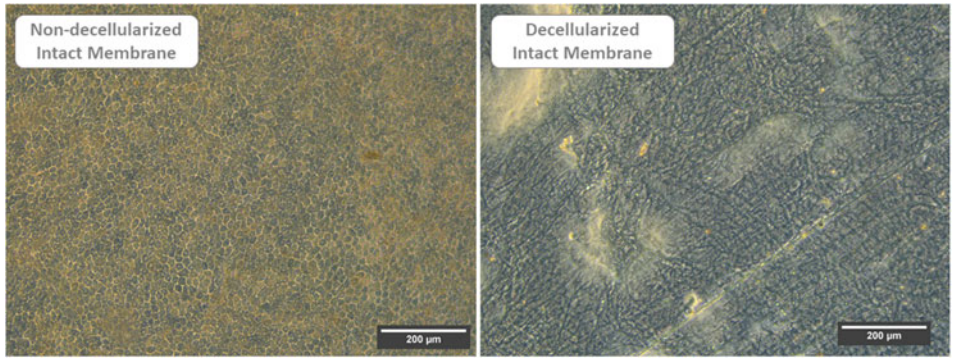

Fig. 11.7 This figure shows the hAM before and after decellularization. The left image is the nondecellularized amnion in which the regular polygonal epithelial cells are prominent and easily detectable. On the right side, the image shows the amniotic membrane after decellularization using 4% NaOH (alkaline decellularization). Note the presence of a dark blue mesh and absence of viable polygonal epithelial cells. *Image courtesy: Center of Excellence for Stem Cells and Regenerative Medicine*

5. The adequacy of cell removal is then verified via examination using methylene blue dye (0.05% solution) or trypan blue dye. The stained samples are then examined by bright field microscopy. Nonstained membranes are also examined using inverted microscope [27, 108].
6. Scissors are used to cut out the amnion pieces (according to the size of the well plate being used), and forceps are then used to separate the amnion from the gauze.
7. The amnion is placed on the well plate. Using forceps, the amnion is stretched to cover the well's surface. Special precautions have to be taken to avoid air bubbles entrapment beneath the membrane. A syringe with a 25-gauge needle should be used to create negative pressure by gentle suction of these bubbles.
8. Using a 200 µl micropipette, each well is carefully filled with 3 ml complete culture medium consisting of Alpha MEM that contains 10% inactivated FBS (by allowing frozen FBS to thaw overnight at 4 °C, immerse the FBS bottle inside a water bath with its temperature set at 56 °C, leaving it for 10 min, and then shake vigorously for any aggregates to dissolve. The immersion and shaking process is then repeated for a total heating time of 30 min) and supplemented with 2 mM L-glutamine, 100 U/ml penicillin, and 0.1 mg/ml streptomycin.
9. Alpha MEM media should be added slowly on the walls of the wells, so that the membrane adherence to the plastic surface of the well plate is not interrupted. Plates are then incubated in a humidified incubator at 37 °C and 5% CO_2 for 72 h, and the amniotic scaffold is then ready for use [27].

11.5.2.3 Safety Precautions

- The hAM is a biohazardous material, as it contains human blood with possible harmful pathogens. Therefore, tests should be confirmed negative for any possible pathogens before handling.

- FBS is a xenogeneic biomaterial. It should be handled with care to avoid any allergic reactions.
- NaOH is a corrosive material. It should be handled with care in both its solid and dissolved form.

Expected Results Before decellularization, the amnion exhibits regular polygonal epithelial cells. After alkaline decellularization and treatment of the amnion with 4% NaOH, a dark blue mesh was observed with no viable regular polygonal epithelial cells (Fig. 11.7). The cells can be imaged using an inverted fluorescent microscope (LEICA DMi8).

Take Home Message

- The AM is rich in ECM, and displays numerous biological characteristics that render it highly desirable for biomedical applications. Because of its anti-inflammatory and antimicrobial properties, the AM has been useful for its ability to reduce inflammation and scarring and enhance wound healing.
- Because of its low immunogenicity and tolerance by the host, the hAM has been used as a grafting material and bioscaffold in tissue regeneration, such as liver, bone, heart, and neurological repair.
- The hAM is rich in ECM and stem cells. Application for use as a bioscaffold necessitates decellularization to avoid immune reactions. Protocol for preparation includes alkaline decellularization of epithelial cells using NaOH.
- The hAM stem cell components of epithelial and MSCs make it also a rich source for stem cell extraction for purposes of regenerative medicine.

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Application of the Scientific Method in Stem Cell Research

12

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What You Will Learn in This Chapter

The scientific method aims to discover new reliable knowledge. It limits biases and subjective tendencies, by following a stepwise process of investigation based on rationalism, empiricism and/or skepticism. The basic steps of the scientific method include observation, questioning, hypothesis statement, hypothesis testing, generating and evaluating data, reaching conclusions, and eventually developing theories. You will learn in this chapter the basics of the scientific method, and the role of ethics in the process. The chapter will conclude with important milestones in stem cell research, and how they relate to the scientific method.

12.1 The Scientific Method

The scientific method is defined as a systematic way of developing certain steps to examine ideas and build knowledge by making observations, asking questions, formulating and testing hypotheses, collecting and analyzing data, and developing theories [1, 2]. Following the scientific method excludes bias risk and subjective tendencies, and the obtained information is highly probable to be true. When scientific research is performed using justified reliable methodology, the outcome is considered reliable knowledge, which is highly distinguishable from the false or unjustified beliefs [3]. People have different conventions, beliefs, and accumulated bodies of knowledge, and frequently, these convections are untested and unjustified. The scientific method provides a standardized process to test these beliefs and prove or disprove them [4]. Scientific thinking is the basis for the scientific method and is rooted in three essential concepts: the use of empirical evidence (empiricism), practicing rational logic (rationalism), and skepticism [5]. The latter is defined as holding a skeptical attitude towards previously known knowledge that leads to self-inquiry, hold provisional conclusions, and free thinking (willingness to change one's beliefs) [5].

- **Empiricism:** Empirical evidence is a type of evidence that relies on physical senses as smelling, hearing or touch. Empirical evidence is considered reliable because other people can experience and repeat it in the same way. Consequently, it can be replicated several times by different research groups with the same outcomes each time [5]. Scientists rely on empirical evidence as the main, and only, type of evidence to make claims about nature and phenomena due to its objectivity and the ability to be repeated and tested [5].

- **Rationalism:** Science is based on practice of the rules of logical reasoning. Scientists use logical reasoning while investigating and understanding nature. Most individuals do not think logically. Instead, they use emotional and hopeful thinking because it is far easier to believe something is true; we feel it is true or wish it was true, rather than deny our emotions and investigate phenomena in a logical and systematic way to find out what is true [5].
- **Skepticism:** Skepticism involves developing a skeptical attitude towards supposed knowledge or beliefs. Because deception is common and close to human nature compromising the ability to obtain reliable knowledge, scientists must examine the basis for holding their beliefs in a continuous manner [5]. In addition, scientists have to ensure the reliability of the surrounding knowledge. If this knowledge matches the logical consequences of one's assumption and objective reality, as measured by empirical evidence, it is then considered safe to conclude that one's beliefs and assumptions are true and justified (in other words, reliable knowledge) [5].

12.2 The Scientific Method in Action

The basic components of the scientific method include observation, asking a question, literature searching, hypothesis formulation and testing, obtaining and analyzing results, deriving conclusions, and developing theory. These components are not necessarily followed in that order (Fig. 12.1).

- **Observation:** Observation is the active acquisition of information by employing the senses [6]. It can take many forms, such as watching a natural phenomenon like an apple falling from a tree, an observation that prompted Isaac Newton to ponder his famous law of gravity [7]. It may also be the result of immersing oneself in an experiment, or in the form of cumulative evidence observed from the literature [6].
- **Developing a Question:** A research question is designed to solve a particular knowledge gap [8]. It is essential for the researcher to know how to formulate a good research question, as a good question is the corner stone of empirical research. Research questions identify, clarify, focus, and pinpoint the research problem. They help in building up a good hypothesis. When applying the scientific method, determining a good question is an important starting point that will significantly impact the outcome of the investigation [9]. When pondering a research question, it should fulfill certain criteria as described by Hulley and colleagues [10]. It should be,
- **Feasible:** affordable in cost and time, adequate logistics and technical expertise, and manageable.
- **Interesting:** answers interest the investigators and the community.
- **Novel:** rejects, confirms, improves, and/or extends previous research.

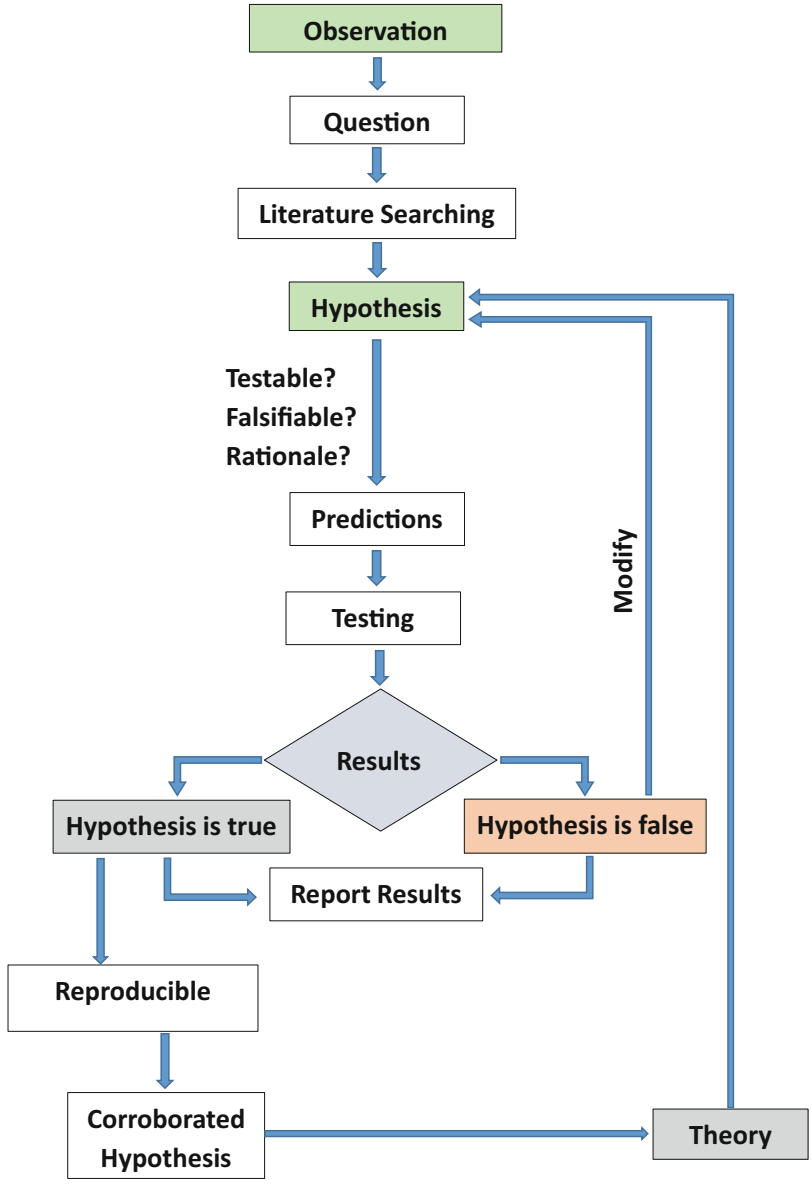

Fig. 12.1 Steps of scientific method process. (Research cycle)

- **Ethical:** the research is conducted with minimal risk of harm and passable to be approved by ethics boards.
- **Relevant:** impacts the scientific knowledge and future research.

Asking a question is followed by reviewing the literature, learning from previous research and accumulated knowledge in the particular field of specialization [11].

- **Formulating a Hypothesis:** A hypothesis is the researcher's argument to guess or assume a predictive solution or explanation for a research problem [12]. The word "hypothesis" consists of "hypo" which means "tentative" or "subject to verification," and "thesis," which refers to a statement about problem solution [13]. A hypothesis is a tentative statement for problem solution [14]. The complete hypothesis must have three components: variables, population, or elements to be tested, and a relationship between the variables. The main criteria of a good hypothesis is that it must be empirically testable, falsifiable, precise, and realistic; otherwise, it will not serve for further investigations [14]. A hypothesis helps researchers find suggested explanations or solutions to their problems, drawing meaningful conclusions based on the empirical data [14]. A hypothesis not only investigates research properly but also contributes in developing new theories, and linking theories to investigations [14].
- **Testing the Hypothesis**: Empirical research depends mainly on testing hypothesis. A hypothesis should be tested empirically to determine whether it is true or not [15]. Testing the hypothesis, in empirical research, is usually achieved by conducting experiments that help to diminish biases and obtain the most reliable knowledge. When a hypothesis is empirically verified, it supports drawing meaningful conclusion with reliable information and empirical data [14]. A hypothesis is not always completely true, and it is possible to have results which contradict it [15]. If the hypothesis fails the test, it can be either modified or changed into other hypothesis based on the results [14, 16]. If the hypothesis passed, it has to undergo further tests to be corroborated [15]. In testing a hypothesis, it is crucial that scientists avoid controversial practices that could cloud their findings. HARKing (Hypothesizing After the Results are Known), which is a post hoc hypothesis that uses known results to place a hypothesis [17] is frequently used by scientists for several reasons. HARKing depends on already known results, and rejects a true null hypothesis (type I errors), propounding hypotheses that would not pass otherwise, and using post hoc explanations as a priori explanation [17, 18]. There are various positions on the ethical use of this form of hypothesis. Some positions view the practice as completely unethical [17], as it is predetermined and averts honest communication of research. Others view it to be more or less ethical according to circumstances [19, 20]. Still, some researchers find HARKing to be acceptable provided that hypotheses are explicitly inferred from prior evidence and theories, and the reader has the ability to access the research data [18].
- **Results and Data Analysis**: Tests usually provide scientists with raw data (observations, descriptions, or measurements) that is necessary to be analyzed and interpreted to become evidence. Analyzing the data and discussing the results might initiate further options and assumptions that have to be investigated in further studies [14]. After publishing, other scientists may carry out different tests to verify the

hypothesis. If it passes subsequent tests, it becomes highly corroborated and is now considered to be reliable knowledge or a scientific fact that can build a theory [15].

- **Theory:** A hypothesis, when tested, helps to support or reject an existing theory. But also, a hypothesis that is successfully tested implies certain facts and helps in developing new theories based on the empirically tested data [14]. Thus, a theory is a buildup of reliable knowledge deduced from a process that follows the scientific method about observed phenomena, and provides explanations and predictions that can be tested [15].

12.3 Scientific Ethics in Research

New technologies and discoveries are generated daily to fulfill the evolving human needs and aspirations. These novel discoveries must be controlled by a set of guidelines to ensure a safe process without violating human beliefs on what is right and what is wrong. Scientific research ethics thus controls scientific conduct within the framework of the local and natural laws. The word "ethics" is derived from the Greek word "ethos" which means custom or habit [21]. According to the Research Excellence Framework, research is "a multi-stage process of investigation leading to new insights, which has to be regulated by ethical standers" [22]. Indeed, the importance of ethics appears when the community traditions are being challenged by the new developments [23].

12.3.1 Ethical Responsibilities of Scientists

Scientists have the main responsibility to apply the ethical regulations while conducting their own research. They have to verify ethics on different levels, including the responsibility towards their peers and the community, and to conduct research honestly and objectively [24]. In clinical research, the safety of research subjects, the research participants' information, and the validity of data present the main ethical concerns. Researchers—similar to physicians—must do no harm; the efficacy of the new products must be scrutinized and proven superior to the current best practices. Periodically, scientists have the responsibility to ask themselves about the importance of the ongoing research and its benefit to the community [25]. Safety of the community and its engagement are essential to achieve cultural conversion and to accept and understand the ramifications of new technologies [26, 27]. Despite these common beliefs, the situations arise that may push scientists to violate some of these ethical standards, for example, the urgent need of patients to test the effectiveness of therapies, and the pressures of funding agencies and research institutes for productivity and publications [28–30].

Ethical principles in human research are naturally different from those in other disciplines such as engineering and physics [31]. While the laws of physics and engineering are almost fixed, humans are variable in their physical and psychological makeup; even within the same population, variability, and the complexities arising from biological and

social differences [31, 32]. Dealing with humans in biomedical research or social studies is a complex process that requires the input of many entities of scientists, physicians, patients, and various members of the community [25].

12.3.2 New Technologies and Ethics in Stem Cell Research

"Ethics always says no to the new technologies," this was one of the most critical responses that Wolpe received from a group of scientists when asked about the rationale for not effectively advocating their own work to their communities [24]. In fact, most of new technologies seem subversive, dependent on challenging tradition and beliefs, and breaking the boundaries of the current knowledge. Ethical principles could be applied to prevent new technologies from causing any kind of harm including physical harm, personal privacy violation, and environmental damage. Scientists and ethicists are thus expected to collate their efforts to accomplish valuable scientific research without ethical violations. In addition, they should work simultaneously to prepare the community to accept new useful technologies. The acceptance of the public for the research funded from their taxes is clearly important. However, the majority of the public do not seem to have sufficient scientific knowledge to make informed decisions [24]. An important example which reflects the failure to prepare the community to new discoveries was the cloning of Dolly in 1996 [24, 33]. After announcing this scientific breakthrough of the first mammalian cloning, public surveys reported that more than 90% of Americans rejected the concept of animal cloning [24]. Not only the specialized scientific outlets but also the media played a pivotal role in science communication and spread of scientific knowledge, and in forming the public opinion. Sometimes scientific news is exported to the public without adequate clarifications or without preparation for new discoveries, and without educating the public about the scientific basis or possible applications of the new research. Many discoveries in the stem cell research field have suffered this lack of preparation. In case of the first mammalian cloning (see Milestones, 31) the "new" technique challenged many traditions and beliefs, and perpetuated a series of public reactions that were frequently not based on understanding of the new methodology, and its ramifications. After cloning of Dolly, the spotlight was focused on human cloning for reproductive purposes and as a source for human organs. Although this application was too early and still unexplored, the media reporting did not provide the opportunity to the scientific community to illustrate to the public the concept of cloning and all its expansive and valuable applications.

Similar debate followed the publications on embryonic stem cells (ESC) research, both in animals and humans [34, 35]. ESCs research has become a subject of controversy after the first publications on the differentiation potential and possible vast clinical benefits of ESCs. ESCs research was heavily debated, with questions on whether the life of an embryo was more valuable than the life of an adult patient or a child. Public debate over this technology started as soon as discoveries were announced without even much understanding of the accuracy of the scientific details, for example, the proper sources of ESCs, and

the possible applications of the new technologies to treat many ill-fated diseases. In both cases, there was no true public education or understanding of the difference between embryonic cells and fetal tissues, which face much more restrictions by the scientific community. There was also no difference in the public eye between reproductive cloning and therapeutic cloning, and its true potential to save lives. Research on fetal tissue is now banned in most of the world, and ESC research has also been hampered [34, 35]. Although, new alternative sources for stem cell research led to the Noble winning work of the induced pluripotent stem cells (iPSCs) [36–38].

12.4 Application of the Scientific Method in Stem Cell Research: Milestones

12.4.1 1745: Parthenogenesis

In 1745, Charles Bonnet noticed that the female aphids produced offspring without fertilization by the male. His observation led to the discovery of "parthenogenesis" [39], a type of asexual reproduction in which an ovum grows to a new individual without fertilization. This was followed by several experimentations. In 1899, Jacques Loeb reported the first case of induced parthenogenesis by artificial fertilization of sea urchin eggs [40]. Thereafter, Gregory Pincus produced a baby rabbit by inducing parthenogenesis in a rabbit ovum cultured in a mix of estrone and saline, then implanted in the mother rabbit [41]. This revolutionary observation led later to animal cloning, and the progress of stem cell research into induced pluripotent stem cells [41]. Research then evolved to include the derivations of human parthenogenetic stem cells, due to their ability of unlimited division and the ability to differentiate into all cell types [42–44].

12.4.2 1957: Intravenous Infusion of Bone Marrow in Patients Receiving Radiation and Chemotherapy

The basis of the Noble work of Donnel Thomas was to test the hypothesis that bone marrow transplantation could rescue lethally irradiated patients and restore their lymphohematopoietic system. Thomas pioneered the intravenous infusion of bone marrow cells in patients receiving radiation and chemotherapy. In the study reported in The New England Journal of Medicine, six patients whose bone marrow was ablated by radiation and chemotherapy received intravenous marrow infusion from healthy donors. The patients survived, and the donor marrow could reconstitute their hematopoietic cell population with mature functioning blood and lymphoid cells from donor origin [45].

12.4.3 1958–1959: Testicular Teratoma in 129 Mouse Strain

Another example of experimentation following observation is when Leroy Stevens observed that mouse strain 129 develops spontaneous teratoma during the early stages of gonadal differentiation [46]. These studies were the basis for the gold standard testing of embryonic stem cells [46, 47].

12.4.4 1978: The First Successful In Vitro Fertilization (IVF)

Louise Brown was the first baby to be born after in vitro fertilization of human eggs outside the body, in Manchester, England. Gynecologist Patrick Steptoe and scientist Robert Edwards removed a mature egg from the mother and combined it with the father's sperms in vitro, where fertilization and normal cleavage proceeded to embryonic development. The 8-cell embryo was then implanted into the uterus after 2.5 days. Few months later, the first IVF baby was born [48].

12.4.5 1981: The First Cultivation of Embryonic Stem Cells (ESCs)

After observing that mouse strain 129 develops teratoma [46], followed by the development of embryonal carcinoma cell lines, it was proposed that early embryos contain cells which are pluripotent unless they receive differentiation signals for embryogenesis [49]. Evans and Kaufman were the first to discover and identify mouse embryonic stem cells (ESCs) [50]. They isolated the ESCs from the inner cell mass of a mammalian embryo in early embryogenesis (embryoblasts) and grew them in cell cultures [50]. In the same year, Gail Martin reported similar findings [51]. Evans and Kaufman pointed out in their paper the possibility of using ESCs as a vehicle for gene modification and gene targeting.

12.4.6 1986: Bone Marrow Transplant After the Chernobyl Nuclear Accident

In 1986, the Chernobyl nuclear power station accident exposed nearly 200 people to high doses of total body radiation. After the accident, the hypothesis which links radiation damage to bone marrow stem cells was used to transplant bone marrow from allogeneic donors into victims. The results, however, were poor due to the effect of extensive burns, trauma, and other radiation-related organ toxicity [52].

12.4.7 1987: Developing Technology for Mutagenesis by Gene Targeting in Mouse ESCs

Thomas and Capecchi reported the first homologous recombination technology to mouse-derived ESCs for mutagenesis by gene targeting. The researchers isolated and cultured ESCs, introduced a mutation by a vector containing sequence similar to the gene to be modified, and replaced the target gene in the chromosome [53].

12.4.8 1992: Development of Methods for In Vitro Culture of Embryonic Germ Cells (EGCs)

In 1992, studies reported the isolation and culture of embryonic germ cells (EGCs) from primordial germ cells in mice [54, 55]. These EGCs had similar characteristics and differentiation potential to ESCs, and the pluripotent cells derived from preimplantation embryos [54].

12.4.9 1996: Mammalian Cloning (Somatic Nuclear Transfer)

Ian Wilmut and colleagues from the Roslin Institute in Scotland reported that they produced the first cloned mammal (Dolly) [33]. A nucleus of an adult sheep's mammary gland cell was successfully fused, using electric stimulation, with an enucleated egg from another sheep. The produced cell was transplanted into the uterus of a surrogate mother ewe. The newborn was an identical copy of the sheep from which the somatic cell nucleus was obtained, and the cloning technique was named somatic cell nuclear transfer (SCNT) [33]. The production of Dolly opened new horizons in stem cell research, and was the foundation for many experiments afterwards, as well as for animal cloning.

12.4.10 1998: Isolation of Human ESCs

In 1998 James Thomson and colleagues reported the isolation and culture of human ESCs derived from human blastocysts [56], and established the first human ESC line [56]. The ESCs were derived from donated embryos, after in vitro culture to the blastocyst stage [56]. Blastocysts become pluripotent upon division. Pluripotent cells of inner cell masses were isolated, and Thomson's team cultured five ESC lines that were used in research for several years [56]. The efficiency of these lines to form derivatives of embryonic germ layers was verified by the production of the three germ layers: endoderm, mesoderm, and ectoderm [56].

12.4.11 2002: Differentiation and Transdifferentiation of Adult Stem Cells

It was believed that adult stem cells have restricted potential to differentiate into their specified organ of origin. As such neural stem cells could only differentiate into cells of nervous system origin, similarly (hematopoietic stem cells) HSCs would differentiate into blood cells. New data showed that postnatal stem cells have higher differentiation capacity than previously thought, and they can differentiate into multi-lineage and unrelated cell types, resulting in plethora of publications on turning blood into brain and vice versa [57]. Many efforts have then focused on developing differentiation cocktails to facilitate the generation of cells that are most challenging to replace in the heart, lungs, nervous system, etc.

12.4.12 2006: Generations of iPSCs

In 2006, Yamanaka and colleagues reported the ability to generate ESC-like cells from adult somatic cells. These induced pluripotent stem cells (iPSCs) could differentiate into almost all other cell types [37]. The extensive experimentation that preceded this milestone took over a decade to examine over 20 different possible factors/combination of factors to reprogram fully differentiated somatic fibroblast into a more embryonic, less mature phenotype [37]. The first reported factors upregulated the pluripotency genes: Oct3/4, Sox2, c-Myc, and Klf4 [58]. The generation of iPSCs paved the way to wide applications of stem cell therapy in regenerative medicine, while overcoming the restrictions that hampered ESC research and its use in the clinic. By availing autologous stem cells, after reprogramming the patient's blood, skin, or other cells into iPSCs, growing autologous neurons, pancreatic cells, and other tissue cells for personalized medicine became possible, while avoiding much of the risk associated with immune rejection [59].

12.4.13 2016: CRISPR/Cas9 Genome Editing

CRISPR/Cas9 is the abbreviation of Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR associated protein 9. Scientists at Stanford University developed a method to correct the sickle cell disease mutations in human HSCs using CRISPR/Cas9-mediated genome editing technology, followed by autologous transplantation [60]. This technique works based mainly on two molecules: case 9 and guide RNA (gRNA). Cas 9 is an enzyme that helps to cut the two strands of the DNA, while gRNA molecules find and bind a particular sequence in the DNA.

12.4.14 2017: Mechanoresponsive Cell Systems

Scientists at UC Irvine devised a technique called mechanoresponsive cell system (MRCS) [61], which selectively identifies and destroys breast cancer cells that have metastasized in mouse lungs by sensing the matrix stiffness in the tumor niche in vivo [61]. MRCS targets the breast cancer metastases through mechanoenvironmental cues, specifically matrix stiffness, to deliver cytosine deaminase (CD) that converts the prodrug 5-fluorocytosine (5-FC) to the active anti-metabolite 5-fluorouracil (5-FU), to kill the cancer cells [61].

Take Home Message

- The scientific method follows a stepwise process of scientific thinking that aims to build reliable knowledge based on observation and experimentation.
- Achieving reliable knowledge is based on rationalism, empiricism, and/or skepticism.
- The basic steps of the scientific method include observation, questioning, hypothesis statement, hypothesis testing, generating and evaluating data, reaching conclusions, and eventually developing theories.
- Scientific ethics aim to conduct research with integrity and respect for human values and natural laws. They are governed by institutional review boards that constitute diverse members of the scientific community and other communities.
- Important milestones in stem cell research have followed the scientific method, and paved the way to current and future applications.

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