Chapter 8
Stem Cell Technology in Medical Biotechnology

Shahnawaz Wani, Tahir Dar, Swanand Koli, Willayat Yousuf Wani, Mumtaz Anwar, and Zeenat Farooq

Abstract Stem cells are small, unspecialized, and undifferentiated cells with a chromatin conformation that is not characteristic of any particular cell type and can be programmed, upon appropriate stimulation, into different cell types. These cells provide base material for formation of many different body cells for therapeutic and research applications. There has been a revolution in the therapeutic applications of stem cell technology during the past decade and the revolutionary introduction of CRISPR-Cas9 has further increased the possibilities of their use. This chapter describes stem cell technology, its types, applications in various established pathological conditions, and ethical concerns revolving their use. It also provides insightful details about the culture conditions required for propagating and differentiating stem cells, tissue engineering, establishment of organ cultures, and limitations in establishing stem cell cultures.

Keywords Stem cell technology and Applications · Stem cell therapy · Organ culture and tissue engineering

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Shahnawaz Wani and Tahir Dar contributed equally to this work and they are co-first authors.

S. Wani
Department of Medicine, SKIMS, Srinagar, Jammu & Kashmir, India

T. Dar
Department of Radiation Oncology, Cedars-Sinai Medical Center, Los Angeles, CA, USA

S. Koli
Department of Ophthalmology, School of Medicine, University of California, San Francisco, San Francisco, CA, USA

W. Y. Wani
Department Neurology, Feinberg School of Medicine, Northwestern University, Chicago, IL, USA

M. Anwar · Z. Farooq (✉)
Department of Pharmacology and Regenerative Medicine, University of Illinois at Chicago, Chicago, IL, USA

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8.1 Introduction, Classification, and Significance of Stem Cells, Isolation and Identification of Stem Cells, and Differentiation of Stem Cells

8.1.1 Introduction

Stem cells are small, specialized, undifferentiated cells in the body with a huge potential for cell division and growth. The term is basically derived from the "*stem cells*" of plants, cells which can divide and re-divide and contribute to unabated growth. In addition to their potential for division and self-renewal, these cells have the potential for differentiating into all the cell types of the organism from which they are derived. For example, a stem cell from a mouse embryo can be cultured and differentiated into an endothelial cell, hematopoietic cell, or muscle cell under correct culture conditions with the help of agents that assist in the differentiation process toward a particular lineage [1].

Stem cells can be of different types (Fig. 8.1):

1. **Totipotent stem cells.** Cells which possess the capability or potential of forming an entire organism through cell division are known as totipotent stem cells (*totipotent = total potential*). These cells can differentiate into all the cell types contained within the organism and lead to formation of an entire organism. Cells of plants remain totipotent throughout their development. It is possible to use plant tissue for re-growing an entire plant of its kind. In case of animals, only the zygote is considered totipotent because it divides, differentiates, and leads to

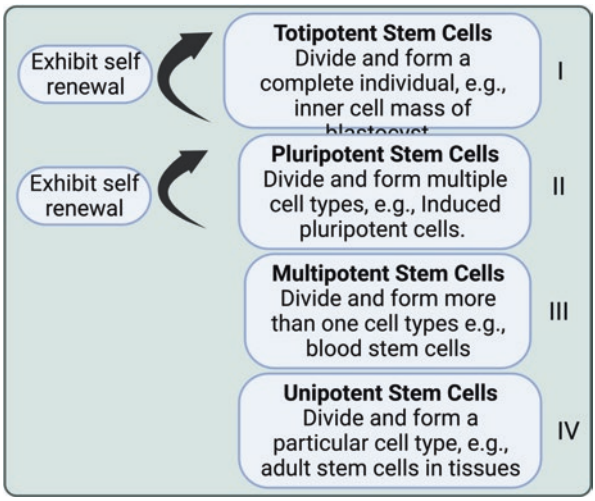

Fig. 8.1 Schematic of the hierarchical representation of different types of stem cells and their characteristic features

formation of an entire organism. Stem cells post-zygotic stage (embryoid stem cells) and tissue-specific stem cells contain varying levels of "*stemness*" and can be classified into different sub-groups.

2. **Pluripotent stem cells.** Cells which have the capability of differentiating into multiple cell types. For example, cells isolated from an embryo (embryoid cells) are pluripotent. Most of the stem cells derived from embryos of mammals are pluripotent. In fact, the term originates from the word *plural* = *pluri*, implying *different choices* for differentiation.
3. **Multipotent stem cells.** Cells which have the capability of differentiating into a set of closely related cells in a particular microenvironment. For example, a hematopoietic stem cell can form a red blood cell, white blood cell, macrophage, or any other cell type of the hematopoietic lineage.
4. **Unipotent stem cells.** Inside the body of humans and other animals, reserves of stem cells are found in different tissues which replenish the cells that are lost after completing their life span. These cells are specific to the parent tissues and differentiate only into the tissue cells in which they reside. These cells are called as unipotent stem cells (*uni* = *one*).

Based on the source of stem cells, these can be classified into adult stem cells and embryonic stem cells. As the name suggests, adult stem cells are derived from adult tissue, whereas embryonic stem cells are derived from inner cell mass of a developing embryo. There are a lot of ethical concerns surrounding the use of embryonic stem cells. While research on human embryos per se is completely banned, studies involving animal and also human embryonic cells are being carried out and are strictly regulated by institutional ethical committees and animal care committees. Oversight from federal and international agencies is also ensured for such research.

8.1.2 Isolation of Stem Cells

Cells that maintain the stemness for a specific cell type while undergoing division and replication in a controlled cultured environment are referred to as stem cell lines. To culture and propagate these stem cell lines, it is important to identify the source and extract the desired specimen from adult tissue or from an embryo. The cells isolated and enriched from the tissue source are placed in a controlled culture medium which allows the cells to undergo division and propagation but preventing them from further lineage-specific specialization. Scientists preserve the stem cell lines for long-term storage for a variety of uses and also often share them with other researchers in the field. The stem cells can be stimulated using different growth factors and modulators to induce specialization in the desired cell lineage. This process is referred to as direct differentiation. It is much easier to grow and propagate embryonic stem cells as compared to adult stem cells but with the recent advancements made in the field, scientists have made significant progress toward the establishment of both stem cell types.

8.1.3 Isolation of Embryonic Stem Cell (ESC) Lines

Embryonic stem cells (ESCs) are pluripotent stem cells that are derived from early-stage embryonic tissue. ESCs from the mouse are well characterized and most studied, although the basic protocol for the ESCs isolation and culture remains more or less similar within all species. Generally, the ESCs are harvested at the blastocyst stage (Day 5) post-fertilization from the inner cell mass referred to as embryoblast. The cells from this embryoblast are further isolated for culture and expanded to obtain viable cells in culture conditions (Fig. 8.2). Generally, the process of ESCs procurement and maintenance in vitro is highly inefficient due to the failure of primary cells to adapt and proliferate in culture conditions. The survival rate for the freshly isolated primary ESCs is variable and depends on the medium and culture environment.

8.1.4 Somatic Stem Cells

Somatic stem cells sometimes also referred to as adult stem cells are primarily located in major body organs and tissues. The protocol used for the isolation and culture of adult stem cells depends upon the tissue source and lineage of the cells from where the stem cells are procured. Currently, most of the protocols used for the isolation of somatic stem cells involve the use of fluorescent associated cell sorting (FACS) or magnetic associated cells sorting (MACS) systems. Depending on the

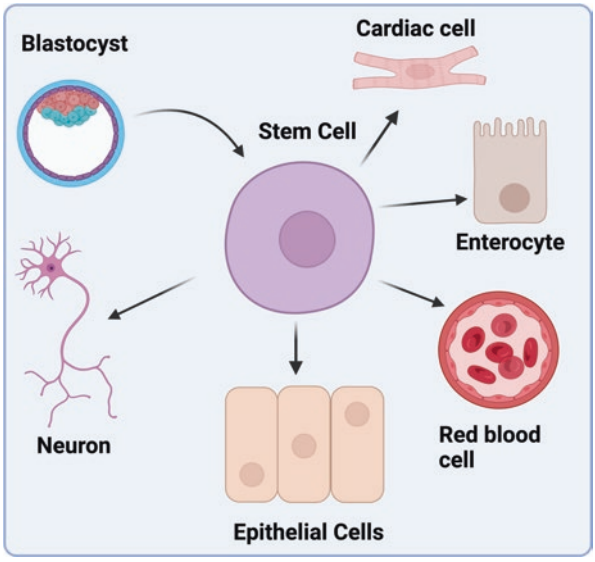

Fig. 8.2 Schematic representation of a totipotent stem cell, derived from the inner cell mass of blastocyst. The figure depicts how the same stem cell can lead to formation of all the different cell types

cell surface marker chosen, these stem cells can be further processed for the enrichment of specific cell types by either positive or negative sorting mechanisms.

The cell count and population of specific somatic stem cells enriched after isolation highly depend on the tissue or organ of origin. For example, spermatogonial stem cells are very rare and comprise about 0.01–1% of the total testis cell population depending on the species, whereas hematopoietic stem cells (HSCs) are considerably available in sizable populations hence they are easily procured, isolated, and enriched for routine bone marrow transplantation. The bone marrow transplant started in the late 1950s. After depleting the recipient's bone marrow stem cell pool, the patient receives new stem cell infusion either from his own (autologous) or retrieved from other donor patients (heterologous) whose human leukocyte antigen (HLA) type matches the recipient HLA type. HSCs used for bone marrow transplantation are either enriched directly from bone marrow or enriched via apheresis (a process of removal of white blood cells from peripheral blood supply). To mobilize the stem cells from the donor's bone marrow, granulocyte-colony stimulating factor is used.

Mesenchymal stem cells (MSCs) are adult stem cells that were isolated originally from bone marrow but later discovered to be present in various other tissue types such as adipose tissue, periodontal jaw ligaments, skin, and cord blood. The mesenchymal stem cells of bone marrow do not contribute to the formation of blood cells and do not express CD34 hematopoietic stem cell marker and are hence referred to as bone marrow stromal stem cells. The major and important source for MSCs is adipose tissue because of its accessibility and the relatively large amount present in the body. Roughly, a yield of 5000 MSCs per gram of adipose tissue has been reported in the literature. The most primitive MSCs can be obtained from umbilical cord blood or tissue (Wharton's jelly). The concentration of MSCs is higher in Wharton's jelly as compared to cord blood.

8.1.5 Culture of Stem Cells

There are specific types of media and culture protocols for stem cell culture that depend on the stem cell belonging to that specific cell lineage. Depending on the protocol used, the stem cell line can be maintained in an undifferentiated form or induced into specific cell linage and cell type using growth factors, inhibitors, and other metabolites. The stem cells are usually cultured using a feeder layer or feeder-free layer.

8.1.6 Feeder Cell Layers

Feeder layer cells are generally adherent monolayered growth-arrested, but viable and supporting cells. These cells are mostly used as a basement substratum to provide support and condition the medium used to grow the target stem cells (usually

plated at low density). Feeder layer cells are irradiated or chemically treated to limit their cell division and growth. Of the many new methods that have been reported in past years to arrest the growth of feeder cells, γ-irradiation (GI) and mitomycin C (MMC) treatment remain the preferred choice to prevent feeder cells expansion. Essentially most of the ESCs cultures are maintained on feeder cell layers. For ESCs expansion and growth, inactivated mouse fibroblasts (MEFs) were used which provide suitable substratum and necessary factors. MEFs can be freshly made in laboratories as well as are commercially available from the vendors. Briefly, embryonic Day 15 (E15) mice are retrieved from the embryo sac and the fibroblast cells are isolated which are further expanded in suitable culture for 3–5 days. These MEF cultures are subsequently mitotically inactivated using GI or MMC treatment. These MEFs can be cryopreserved either before or after treatment. Even though both treatments seem to be equally effective, some studies suggest that GI is more suitable and efficient than MMC treatment. The study by Roy et al. showed that MMC-treated feeder cells were metabolically altered, thus subsequently less efficient at maintaining target cell expansion as compared to the GI feeder layer. Alternatively, chemically fixed feeder cells are shown to support the growth and maintenance of hematopoietic stem cells, ESC, and MSCs. Mild treatment with glutaraldehyde (GA) or formaldehyde (FA) causes significant growth arrest and further immobilization of cell surface proteins of the feeder cells. The main advantage of using chemically fixed cells is that after detaching target stem cells, fixed feeder cells remain immobilized on the plate surface, hence these chemically fixed feeder cells can be reused multiple times without altering or modifying their functions. The other benefit of chemically fixed feeder cells is they barely detach from plates and do not contaminate stem cell cultures after detachment.

8.1.7 Feeder-Free Culture

There has been booming clinical interest in the use of human embryonic stem cells (hESCs) after the establishment of ex vivo culture conditions for hESCs and embryonic germ cells. However, the limitation that hESCs require to be co-cultured with the mouse or human-derived feeder cells has hindered the clinical applications for the use of hESCs. This is because there is a possibility that feeder cells might deliver or transfer animal or human viruses to hESCs. Therefore, there is a need to identify and develop a feeder-free culture system that essentially provides the same critical factors secreted by the feeder and some other cellular factors or activators supporting the signaling pathways. There are basically two types of feeder cell-free media: defined media and conditioned media. The growth factors of the media and supplements vary depending on the type of stem cell and species. A defined media is essentially a serum-free media that has been supplemented with recombinant growth factors such as leukemia inhibitory factor (LIF), bone morphogenetic protein (BMP), bovine pituitary extract (BPE), and other molecules necessary for the growth and pluripotency of stem cells. Rho-associated protein kinase (ROCK)

inhibitors such as Y-27632 and thiazovivin have been shown to increase the viability of stem cells. There are reports which suggest that long-term culture in serum-free media causes epigenetic changes in target cells to adapt to the culture environment. Therefore, the use of condition media to support the target stem cells is highly advantageous. Basically, cells in culture secrete several factors into the media that support cell growth. After the cells have grown and divided for a long time, the spent media are removed. This spent media is termed as conditioned media which can then be used as a supplement to fresh media. Even though there is a possibility and concern about the inclusion of viruses while using the conditioned media, it is far less as compared to using cross-species feeder cells. One advantage of using conditioned media is that it contains more factors than defined media.

8.2 Introduction to Stem Cell Technology: Transdifferentiation Potential of Stem Cells, Induced Pluripotent Stem Cells, Factors Involved in Pluripotency

8.2.1 Transdifferentiation

The process of direct reprogramming of one somatic cell type into another cell type, bypassing the transitional stage of induced pluripotency is referred to as transdifferentiation. Transdifferentiation is an alternative method used to generate tissue-specific terminal differentiated cells. Utilizing this process without going into the pluripotent stage, adult differentiated cells are directly programmed to induce and commit into another specific terminal cells [2]. In contrast to the ESCs and iPSCs reprogramming methodology where cells' epigenetic signatures are erased to achieve what is known as the pluripotent ground state, transdifferentiating is primarily focused on rewriting the epigenetic codes selected for the desired terminal adult cell type, thereby achieving the direct conversion between two unrelated cell phenotypes. Therefore, direct cell reprogramming is gaining popularity toward developing newer tissue engineering methods required for the treatment of tissue injuries and diseases where a limited number of cells hinder the tissue repair or tissue healing process. In some tissue damages, the proliferation rate of terminally differentiated adult cells decline which further deteriorates the injury due to the inability of tissue to heal itself. The notable examples include neurodegenerative diseases and myocardial infarctions. The transdifferentiation process takes the advantage of direct reprogramming of body cells which are in abundance and easily available, into the desired cell phenotypes which have the potential to heal the damaged body part and restore the tissue function. Hence transdifferentiation has tremendous potential and holds a promising future direction in the field of regenerative medicine.

8.2.1.1 Transdifferentiation Techniques and Mechanisms

The cellular reprogramming process can be carried out through protocols readily available in the works of literature having their pros and cons. The basis of reprogramming generally follows upregulating or transducing reprogramming factors which initiate and support the terminal cell identity, function, and phenotype. Generally, somatic cells are used for direct reprogramming without the introduction of a pluripotency state which considerably cut down the likelihood of tumorigenesis in this conversion process. The process of direct differentiation can be achieved in three different ways. (a) Few select key transcription factors can be exogenously transduced as transgenes which can overexpress and initiate the transdifferentiation process [3–6]. (b) Using targeted manipulation techniques that can directly manipulate DNA or the epigenetic signatures such as CRISPR/Cas9 can be utilized to either silence or upregulate the endogenous genes vital for transdifferentiation [7–10] (c). Several transcriptional pathways can be directly targeted using pharmacological agents activating cellular immunological response which in turn leads to a cascade of epigenetic signature remodeling or epigenetic cellular environment [11]. Currently, use of plasmids and viral vectors are popular methods used for introducing transgenes into cells although its efficiency reported is often too less. Conversely, upregulation of endogenous genes involved in transdifferentiation results in much higher direct conversion efficiencies, hence they have more potential for upscaling the transdifferentiation to a large-scale environment [7].

Transdifferentiation Through Exogenous Transgene Overexpression

Viral mediated delivery of foreign genetic material is a commonly used method to deliver transgene into target cells and initiate the process of transdifferentiation [12]. Lentiviruses, Adenoviruses, and other retroviruses are often used to induce cell transdifferentiation. The advantage of using lentiviruses and retroviruses is that they can effectively deliver the exogenous DNA into the target host genome [13]. Other viruses such as non-integrating viruses are less frequently used to initiate the transdifferentiation process due to lower efficiencies as compared to lentiviruses and it may take a longer time to achieve the same yield and number of reprogrammed viable cells. Using adenovirus, the transgene is expressed transiently, one report shows that 2.7% efficiency was observed in transdifferentiation of fibroblast to neurons [5].

The major hurdle in inducing transdifferentiation is selecting precise transcription factors (TFs). TFs modulate gene expression by regulating the gene transcription rate by upregulating or downregulating it. The TFs expression results in a change in cellular fate such as division, growth, differentiation, activation, and migration. Thus by modulating the expression levels of TFs it is possible to change the cell identity. The TFs can be used individually or in conjugation because studies have shown that TFs can work in an orchestrated manner to transdifferentiate target cells quickly and efficiently. Margariti et al. in 2012 first used OSKM (Oct4, Sox2,

KLF4, and c-Myc) to prime and initiate reprogramming of target cells for transdifferentiation process that are commonly referred to as a partial-iPSC (PiPSC) state [14]. Using this approach, the efficiency of transdifferentiation was improved by roughly 34% as compared to other studies that did not make PiPSCs but used viral-directed transdifferentiation [3, 15].

Transdifferentiation Through Endogenous Gene Regulation

Endogenous Gene Silencing with CRISPR/Cas9

Transdifferentiation can be achieved by endogenously silencing certain genes in the target cell with the help of CRISPR/Cas9 system. Target gene-specific gRNA when delivered to the cell along with CRISPR/Cas9 enzyme complex can induce DNA double-strand break and introduces mutation either by insertion or deletion caused due to error in proofreading activity, thus disrupting the gene function. Using CRISPR/Cas9 [16] disrupted Myod1 gene which drives the transdifferentiation of mouse myoblast into adipose cells [16]. CRISPR/Cas9 can also be used to enhance the normal transdifferentiation process which was shown by Rubio et al. [9] by utilizing CRISPR/Cas9 to transdifferentiate fibroblast into neuronal cells [9]. This was achieved by silencing TSC2 gene in fibroblast, loss of function of TSC2 is involved in the onset of tuberous sclerosis. The fibroblast cells were further transduced with Ascl1, Lmx1a, and Nurr1 for lentiviral mediated overexpression of these genes that convert fibroblasts to neuronal cells [9]. Thus CRISPR/Cas9 system can be used for transdifferentiation either by targeting specific gene silencing or through conjugation with other techniques to create specialized lineage-specific cells.

Endogenous Genes Upregulation by dCas9

With CRISPR/Cas9 system target gene can be disrupted, alternatively, a mutant version of Cas9 also known as dCas9 which is a nuclease-deactivated version of CRISPR/Cas9 which does not cause a double-strand break in DNA can be used to perform a different function. The dCas9 can be fused with master transactivator proteins which in turn can recruit other transcriptional machinery complexes and cause changes in chromatin structure, thereby upregulating normally silenced genes. This strategy was performed by Chakraborthy et al. [7], where they used dCas9 fused with VP64 a transactivator protein to induce transdifferentiation of fibroblast to skeletal myocytes by upregulation of Myod1 gene [7]. There are many ongoing studies toward the utilization of dCas9 in the transdifferentiation process in different cell types. Overall, several transactivators and/or repressor proteins domains can be fused with dCas9 and used to enhance or repress the target gene function. The most common fusion proteins studied so far include Vp64, VP64-p65-Rta9 (VPR), histone acetyltransferases (HATs), synergistic activation mediators (SAMs), and SunTag [7, 10, 17–19].

Transdifferentiation Through Pharmacological Agents

Viruses like lentiviruses can activate innate immune signaling pathways through Toll-like receptor 3 (TLR3) which in turn cause changes in epigenetic signatures, thus affecting the gene expression [14]. Some of these genes are an important part of the maintenance of the pluripotency network in the cell. Margariti A et al. [14] showed that treating fibroblasts cells with polyinosinic: polycytidylic acid (Poly I:C) stimulates TLR3 in human foreskin fibroblasts which transdifferentiate into endothelial-like cells expressing CD31, a key endothelial protein marker required for adhesion and monolayer formation [14]. Although the transdifferentiation efficiency was low, the cells were mimicking endothelial cell functions such as nitric oxide production and forming a "cobblestone" morphology that is a characteristic of endothelial cells. Using 5-azacytidine, a DNA methyltransferase inhibitor, [20] reprogrammed fibroblast cells into skeletal myocytes [20].

Cells metabolize 5-azacytidine which is a chemical analog of cytidine that leads to a cascade of reactions, finally incorporating it into DNA by binding it to guanine molecule. Due to differences in molecular structure azacytidine is not methylated, thus inhibiting DNA methylation which further leads to a change in the epigenetic environment, modulating target gene expression [21]. 5-azacytidine treatment to cardiac cells causes upregulation of Myod1, a skeletal myocyte-specific marker, and multinucleated myotubes typical skeletal myocyte properties [20]. Dexamethasone, a glucocorticoid capable of activating certain transcription factors, is another pharmacological agent used for promoting direct differentiation of several cell types. The mode of action of dexamethasone is through binding to glucocorticoid receptors which lead to modulation of gene expression [22].

8.2.2 Pluripotency Factors Involved in Stem Cells

In embryonic stem cells (ECSs) their pluripotency identity is attributed to the expression of trio core transcription factors Oct4, Sox2, and Nanog [23]. The pluripotent stem cells undergoing specification during the mouse embryonic development requires the genome to express Oct4 and Nanog but not necessarily Sox2, because of maternal Sox2 protein which can live long in the embryo [24, 25]. These pluripotency transcription factors regulate stem cell pluripotency and specification through their expression, colocalization, orchestrated regulation through polycomb repressive complexes (PRC), and microRNAs in the transcriptional and epigenetic modulation of genes involved in stem cells [26].

One of the Pit-Oct-Unc (POU) family of homeodomain proteins, Pou5f1 gene encodes Oct4 protein. Oct4 nuclear localization can be observed in primordial germ cells (PGCs), totipotent blastomere cells, and also in the pluripotent epiblast cells [27, 28]. In Oct4 knockout mice, the embryos fail to form pluripotent inner cell mass (ICM) but rather show differentiated trophectodermal tissue; therefore, Oct4 expression is critical for the establishment and preservation of pluripotency [29].

Precise control over the Oct4 expression is required for the maintenance of ESCs in undifferentiated form. Silencing of Oct4 by 50% can cause stem cells to enter trophectodermal differentiation [30]. Whereas overexpression of Oct4 by more than 50% could induce stem cells to mesodermal and endodermal differentiation [30].

SRY-box 2 also known as SOX2 protein is highly expressed within the inner cell mass, extraembryonic ectoderm of blastocyst before implantation [25]. Similar to Oct4 null mice, blastocyst in Sox2-knockout mice fails to develop pluripotent inner cell mass and mouse ESCs lacking Sox2 differentiate into trophectoderm [25]. This phenotypic pattern of similarity observed due to loss of either Oct4 or Sox2 can be attributed to the cooperative/synergistic mechanisms of Oct4/Sox2 required in modulating pluripotent gene regulation in several ESCs [31–35]. The differentiating phenotype observed in Sox2 knockout mESCs can be reversed through ectopic Oct4 overexpression [36].

Homeobox protein NANOG is a transcriptional factor required by ESCs to maintain a pluripotency state by suppressing cell differentiation factors. Nanog is the third transcription factor of the core ESCs pluripotent transcription factors which was identified by screening pluripotency factors that can maintain the self-renewal of mouse ESCs in the absence of leukemia inhibitor factor (LIF) [37, 38]. Similar to Oct4 and Sox2 knockout mice, Nanog null mice embryos lack pluripotent inner cell mass [37, 39]. However, Nanog knockout mouse ESCs can be sustained and established in vitro even with the loss of both Nanog alleles [37, 38]. These Nanog deficient mESCs that can be maintained in the pluripotent state in culture conditions suggest that although Nanog is required to reach a pluripotent state, it becomes dispensable once that pluripotency is achieved [38].

The fourth factor known as c-Myc is indirectly involved in the maintenance of pluripotency of ESCs. Myc is a family of regulator genes and proto-oncogenes encode for several transcription factors. The Myc module which consists of c-Myc, n-Myc, Rex1, Zfx, and E2f1 is known to be involved in self-renewal and cell metabolism [40–42]. The c-Myc gene acts as a “master regulator” of cellular metabolism and proliferation. About one-third of ESCs genes participating in the maintenance of pluripotency are bound by both the trio core transcription factors (Oct4, SOX2, and Nanog) and also with c-Myc [43]. But the mode of action for maintaining ESC pluripotency identity differs for both trio core factors and c-Myc. The trio core factors Oct4, Sox2, Nanog acting synergistically along with mediator complex can recruit RNA polymerase II (RNA Pol II) to initiate the gene transcription [44]. While c-Myc with the help of p-TEFb cyclin-dependent kinase regulates the transcriptional pause release of RNA Poly II [42]. Thus, it is considered that the core trio factors can regulate the ESCs pluripotent active genes expression via recruitment of RNA Pol II, whereas c-Myc participate in the pluripotent gene expression by aiding the release of transcriptional pause [23].

Since the trio core ESC transcription factors play an important role in establishing and maintenance of pluripotent identity of the stem cells, several molecular techniques such as chromatin immunoprecipitation have been utilized to map the genome-wide binding sites of these trio core ESC factors in both mouse and human ESCs. Many studies have shown that these core factors act synergistically and

therefore show co-binding or binding at near vicinity relative to each other at several active genomic sites [45, 46]. Overall, it is believed that these trio core transcription factors help to maintain the pluripotency identity of a stem cell (a) through activation and expression of a multitude of other pluripotency factors or genes and subsequently downregulating genes that are involved in stem cell differentiation and specification [23]. (b) Also, a feedback mechanism that regulates the expression of self-genes (Oct4, Sox2, and Nanog) and also each other, which is how the pluripotent stem cells can undergo self-renewal process but simultaneously holds differentiation potential when the need arises.

8.3 Stem Cell Technology and Therapy

Stem Cell Technology is a rapidly growing field at the intersection of biology, chemistry, and biomedical engineering. The field involves use of stem cells for correcting various health problems of individuals due to poor function or loss of function of tissues/organs. For example, patients suffering from type I diabetes mellitus experience destruction of pancreatic β-cells due to generation of auto-antibodies against them which results in loss of insulin synthesis, rise in blood sugar levels and a plethora of other problems. With the help of stem cell technology, stem cell of the individual can be isolated, cultured under in vitro conditions, and appropriately stimulated (with the help of growth and differentiation factors) to form pancreatic β-cells for re-introduction into the individual's body to correct the dysfunction. This specific discipline of stem cell technology is known as "transplantation technology or regenerative medicine." Although transplantation technology can rely on use of "donor or non-self" tissues or organs for treatment, regenerative medicine generally relies on the "regeneration potential of self-stem cells to form a particular tissue/organoid for therapeutic applications." Use of stem cell technology for therapeutic applications is also known as stem cell therapy. The field of stem cell technology is rapidly growing and has already been applied in clinical practice under various situations. The next section describes some of the most exciting applications of stem cell therapy [1, 47].

8.3.1 Applications of Stem Cell Therapy

Amyotrophic lateral sclerosis (ALS) , also known as Lou-Gehrig's disease is a neurodegenerative disorder characterized by progressive degeneration of motor neurons. The most common symptoms of this disease include muscle atrophy, weakness, spasticity [48]. ALS represents the most common motor neuron disease throughout the world, with an incidence of 2–3 per 100,000 individuals worldwide. The average time from diagnosis to death for ALS is typically 3–5 years and, in most cases, death ensues because of progressive loss of motor neurons and weak-

ness of skeletal muscles, especially those responsible for breathing [49]. ALS can be familial or sporadic, with a multifactorial representation in patients [50, 51]. Adding to this, ALS can be extremely heterogenic in its presentation that makes it extremely difficult to underpin the exact cause and makes treatment difficult [52, 53]. Stem cell therapy can offer an excellent potential treatment option for ALS since research into stem cells' plasticity and differentiation into various neural cell types has been well established [54, 55]. Several types of stem cells have been studied to test their utility in treating ALS, including embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), neural stem cells (NSCs), and mesenchymal stem cells (MSCs). Current research on the applicability of these stem cell types in ALS and the different ways of differentiating and introducing them has been described elsewhere in detail [56].

Orthopedics and Bone Regeneration Applications of stem cell technology in orthopedics involve bone regeneration, usually required after severe accidents that lead to bone damage. The technique also has immense clinical potential in autoimmune and genetic or hereditary disorders which result in compromised formation, function, or progressive degeneration of bone or cartilage tissue. Bone regeneration technology has seen immense growth, particularly due to the boost in clinical research and practice in adult mesenchymal stem cells (MSCs) and bone marrow stromal cells (BMSCs). The adult MSCs can be typically defined as the cells having potential of self-renewal and multilineage differentiation into osteoblasts, chondrocytes, and adipocytes. Also, according to the criteria of "Mesenchymal and Tissue Stem Cell Committee of the International Society for Cellular Therapy," these cells must also possess the capacity of plastic adherence when cultured in vitro, express CD73, CD90, and CD105 biomarkers but should not express CD14, CD11b, CD34, CD45, CD19, and CD79 biomarkers [57]. BMSCs are one of the kinds of stromal cells isolated from bone marrow which, after purification, fulfill the criteria of being classified as mesenchymal cells, that is, they are able to differentiate into chondrocytes, osteocytes, and adipocytes [58] and also express the osteoblast biomarker *Runx* expression [59]. Various animal studies have shown that BMSCs are capable of healing appendicular defects [60], maxillofacial regeneration and spinal fusion [61], long one defect repair [62]. BMSCs have also been successfully demonstrated to induce posterolateral spinal fusion in humans [63]. However, despite all the successfully demonstrated applications, the field of Bone Regeneration is still in its infancy owing to drawbacks like low yield at procurement, requirement of growth factors for in vitro expansion, and increased donor site morbidity due to requirement of higher initial amounts for successful culture and regeneration [64]. Current progress and applications in the field of bone regeneration technology have been reviewed elsewhere [65].

Blood Malignancies Stem cell transplantation in case of blood cell malignancies like leukemia is given to patients for replenishing their stem cells for healthy blood formation [66]. Diagnosis of blood related malignancies in a patient is usually followed by chemotherapy to kill the "malignant precursor blood cells or stem cells"

formed inside the bone marrow. In some cases (if a patient is young, relatively healthy, and able to tolerate the radiation), chemotherapy can be combined with radiotherapy. Also, a "full body irradiation" is also carried out in some patients to destroy as many cancer cells as possible for treatment purposes. These interventions damage the bone marrow cells of the patient and hence make it necessary to replace the damaged cells with healthy ones. Stem cell transplantation is carried out after chemo- and radiotherapy. It can either be autogenic or allogenic. In an autogenic transplant, blood borne stem cells of the patient are extracted before beginning chemotherapy, are purified, expanded in vitro and later re-injected into the patient to form new bone marrow cells [67]. This method can be potentially risky because there is a probability of the "self-cells" used for re-injection to carry some mutations to become malignant in the future. However, it is more common in clinical practice and is used due to non-availability of tissue matched donor. Allogenic transplant involves re-injection of donor stem cells into a patient. This offers the advantage of having healthy, non-cancerous cells in the patient's body to form blood and other cells of the hematopoietic lineage. The clinicians usually administer immunosuppressants to the patient upon receiving donor cells in order to minimize the risk of graft versus host disease (GVHD), a form of immune condition. This application of stem technology in blood malignancies is more common in clinical practice compared to others because blood borne stem cells have a huge potential of blood cell formation upon entering the host system and populating the bone marrow. Also, administration of stem cells is carried out intravenously and the injected cells reach bone marrow through bloodstream. Upon entering bone marrow, these cells grow and divide to replace malignant marrow cells with a very high success rate. These advantages make this application highly useful and popular among clinicians and patients alike. Despite these advantages, new malignancies have been found in a large number of patients after previously suffering from blood malignancy and receiving stem cell transplantation [68]. More research into the field is required to develop better insights into the potential of this application with no to minimal adverse effects [69].

Cardiovascular Disease Cardiovascular disease remains one of the leading causes of mortality due to non-infectious causes, accounting to 30% cases. Myocardial infarction combined with low regenerative potential of cardiomyocytes is responsible for a huge number of these cases. This condition therefore calls for novel therapeutic approaches of treatment like regenerative medicine [70]. Skeletal myoblasts (SM) have been used in stem cell therapy for cardiovascular diseases. SM cells are derived from satellite cell derivatives residing in skeletal muscle fibers and therefore hold the flexibility of sharing embryonic and morphological features with cardiac muscle cells [71]. This makes availability of SM cells from autologous muscle biopsies effortlessly easy. Easy availability combined with the potential of rapid in vitro expansion, ischemic tolerability, and low risk of tumorigenesis has fueled a huge wealth of pre-clinical research into the utilization of SM in cardiac regeneration in many animal studies [71, 72] and clinical trials [73, 74] with demonstrated improved outcomes like reduction in myocardial fibrosis and infarct size. However,

this therapeutic model also suffered from limitations due to lack of electrochemical coupling between transplanted SM cells and resident cardiomyocytes because of failure to form gap junctions [75, 76]. Bone marrow (BM) derived macrophages have also been utilized for stem cell replacement therapy for cardiomyopathies [77]. The effectiveness of this model has also been demonstrated in several clinical studies [78]. Recently, research has been carried out into the utilization of cardiac progenitor cells for cardiac stem cell therapy. These are cells residing in cardiac microenvironment with the potential of regeneration upon in vitro stimulation. Research on these cells is exciting since it offers the advantage of using cardiac cells for regenerative therapy and thereby might overcome the limitation of failure of gap junction formation. It also challenges the long-standing notion that cardiac cells are post-mitotic and have almost no regenerative potential. In fact, advanced research into understanding and harnessing the research potential of cardiac progenitor cells could lead to similar research into other tissue progenitor cells [79, 80]. More current research and applications of stem cell technology in cardiovascular problems have been reviewed elsewhere [81].

8.3.2 Research in the Stem Cell Field

In addition to the various clinical applications, research in stem cell field has seen robust growth over the last decade, especially with advances in the field of genome-wide sequencing technologies. Research in stem cell field can help recapitulate the events that occur during in vitro differentiation and can hence lead to a better understanding of various physiological processes. It can also help us understand various pathological conditions in detail, by comparing normal versus abnormal development. This can be immensely helpful in developing deeper understanding of the biological processes and their mis-regulations at a molecular level which can serve as a key factor for development of effective therapeutic strategies.

Another exciting aspect of application of stem cell technology is drug testing. A vast number of drug testing modules have been developed over the years wherein stem cells are directed to develop into particular tissues to test the drug before testing it in human individuals. This form of efficacy and safety testing benefits from use of human tissues which can mimic the pathophysiological conditions in a better way than animal models. Remarkably though, it offers the advantage of avoiding any side-effects (short and long term) by excluding use of human volunteers. Many researchers across the globe agree that culture-based models should be used for testing a greater number of drugs before the drugs can be tested in human volunteers. It provides an additional blanket of safety for humans participating in the trial study. This approach is especially beneficial for testing chemotherapeutic agents.

8.4 Stem Cell Technology and Infertility: In Vitro Fertilization and Embryo Transfer

Basic and classical definition for stem cells is that these are progenitor cells that are capable of self-renewal and differentiation into many different lineages of cells. Since their discovery as pluripotent stem cells (PSCs) from mouse bone marrow cells, many other types of stem cells have been discovered and generated from other tissues and organisms. Liu et al. mention five types of stem cells in the last few decades. These include embryonic stem cells (ESCs), very small embryonic-like stem cells (VSELs), nuclear transfer stem cells (NTSCs), reprogrammed stem cells (RSCs), and adult stem cells (ASCs) [82]. Last decade has witnessed bloom of basic, translational, and clinical advances in the field of stem cell technology. ESCs and iPSCs have shown great application potential in regenerative and transplant medicine [83, 84], disease modeling, drug discovery screening, and human developmental biology [85, 86]. ASCs are seeking their future in treatment of infertility. Stem cell technology and infertility treatment have a two-way connection. On the one hand, stem cells are sought for treatment of infertility and on the other hand infertility treatment procedures turn out to be source of stem cells. Application of stem cells for infertility treatment is a diverse area wherein different types of stem cells are being harnessed to treat different causes and aspects of infertility. Infertility is defined as a condition when a couple of reproductive age cannot achieve pregnancy after having regular unprotected sex for a period of 1 year or more. It is a complex pathophysiological medical condition with either male inability (20–30%) or female inability (20–35%) affecting millions of people of reproductive age worldwide and impacting their families. Estimates suggest that between 48 million couples and 186 million individuals live with infertility globally [87]. Male infertility issues arise due to problems in the ejection of semen due to obstruction of the reproductive tract, absence or low levels of sperm due to hormonal disorders, or abnormal shape (morphology) and movement (motility) of the sperms. In females infertility may be attributed to abnormalities of the ovaries such as polycystic ovarian syndrome and other follicular disorders; uterus dysfunctionalities such as endometriosis, fibroids, septate uterus; fallopian tubes such as blocked fallopian and the endocrine system disorders causing imbalances of reproductive hormones. A breakthrough in major treatment for infertility came with birth of Louise Brown in 1978 when assisted reproductive technologies (ART) came into being and first in vitro fertilization (IVF) got successful [88]. This was the process of bringing healthy sperm and egg together in dish in a laboratory and developing embryos which would be transferred to a healthy uterus for further development into fetus. Basic technique of IVF was successful partially as infertile couples with problem in production of healthy gametes and women with unhealthy uterus had no option of having their biological babies. Stem cell technology is developing to give solutions to such IVF issues. Main steps at which stem cells resolve IVF issues are:

- Potential of generating eggs from stem cells: Several laboratory studies and clinical trials are investigating stem cells as a strategy for generating healthy gametes. Different types of stem cells differentiate into embryonic germ cells and

precursor cells, respectively [89]. Oocyte-like cells have been successfully derived from embryonic stem cells. Murine female embryonic stem cells have been shown to differentiate into oocyte-like cells that give rise to functional ovaries. Mesenchymal stem cells (MSCs) have been used as an experimental approach to restore egg production by ovaries and improve ovarian physiology in terms of follicular density in experimental models. There have also been some mechanism-based reports related to use of MSC in infertility treatment. MSCs derived from human Wharton's jelly derived express oocyte developmental genes when co-cultured with placental cells; adipose-derived mesenchymal stem cells have the ability to differentiate into granulosa, Takehara et al. reported differentiation of MSC into primordial follicles density [90]. Stem cells derived from human umbilical cord of first trimester have the potential to develop into oocyte-like structures with zona pellucida like layer. Stem cell technology has boosted infertility treatment by a technique autologous germline mitochondrial energy transfer (AUGMENT) wherein mitochondria from Ovarian germline stems cells (OGSCs) are injected into oocytes of women with poor ovarian function. This has fetched almost 18% success rate in birth of healthy babies from infertile women [91].

- Treatment of female reproductive system diseases using stem cell technology is also being studied: Gynecological disorders are associated with abnormalities in one or more of the reproductive organs: ovaries, uterus, fallopian tubes, and cervix causing premature ovarian failure (POF), polycystic ovary syndrome (PCOS), endometriosis, Asherman syndrome, and preeclampsia. Stem cell technology paves new ways for treatment of these disorders. In addition to self-renewal and differentiation, stem cells have many other beneficial characteristics which enhance their potency for infertility treatments. Bone marrow mesenchymal stem cells (BMSCs) have bioactive factor rich secretome consisting of insulin-like growth factor (IGF), vascular endothelial growth factor (VEGF), and other growth factors to induce cell growth, differentiation, and immunoregulation to restore and support ovarian function. MSCs are also known for their paracrine activity and immunomodulatory effects to prevent ovarian dysfunction [92]. Placenta mesenchymal stem cells (PMSCs) have very high differentiation and proliferation potential, making them an attraction for transplantation and regenerative medicine. PMSCs reduce the levels of estradiol, FSH, and luteinizing hormone (LH) and induce the expression of FSH receptor (FSHR) and anti-Müllerian hormone (AMH) in POF mice, hence contributes to restoration of ovarian function [93, 94]. Upon transplantation, PMSCs improve ovarian function in ovariectomized rats by inducing the production of estrogen and the expression of folliculogenesis-related genes [95]. Many studies have examined the functional nature and the differentiation capacity of BMSCs. In addition to differentiating into chondroblasts, osteoblasts, and adipocytes, BMSCs also have the ability to differentiate into endometrial endothelial and granulosa cells [96]. A distressing consequence of cancer chemotherapy is ovarian dysfunction and infertility. BMCs have shown the protective effects on ovarian function and reduced ovarian failure in mice after chemotherapy. A study has reported multi-facts and mecha-

nistic revelations about BMSCs potency to improve ovarian function in cyclophosphamide-induced POF; by inducing VEGF expression, increasing estradiol levels, restoring ovarian structure, and decreasing expression of the apoptotic factor Caspase-3 [97]. Like PMSCs, remarkable effects of BMSCs might be attributed to their angiogenic and growth factor rich secretome. Their secretome is also reported to contain exosomes, a subset of membrane-bound extracellular vesicles, which enclose various proteins, lipids, and non-coding RNAs such as miRNAs. The small non-coding RNAs may play role in regulating the physiological and pathological mechanisms of stem cells and outcomes related to the ovarian treatment. Two independent studies have demonstrated a role of two different miRNAs miR-644-5p and miR-144-5p carried by BMSCs-derived exosomes in treatment of POF animal models [98, 99]. Various experts speculate that miRNA-regulated gene expression underlies BMSC-based therapy outcomes. Transplanting characteristic is another attractive feature that makes BMSCs more efficient to treat gynecological dysfunctions. BMCs migrate to the uterus and induce endometrial repair in various experimental models including even humans. Asherman syndrome treatment with BMCs has also been demonstrated in a menstruation model as well as in patients of Asherman syndrome [98]. BMSCs have also been reported to play a crucial role in improving endometrial functions by improving endometrial stromal and epithelial compartment. Zhao et al. and others have conducted studies showing increase in endometrium thickness upon infusion of BMSCs into uterus in a rat model [99, 100].

- Male reproductive dysfunction in majority of the cases is attributed to impaired spermatogenesis. Spermatogenesis is a process of sperm formation from male germline stem cells, called spermatogonial stem cells (SSCs). Reproducing these events in vitro has not been successful yet and there is no cellular replacement therapy available for men who suffer from azoospermia. Transplantation of mouse SSCs from an infertile donor to a favorable testicular environment could restore fertility of the infertile male donor [87]. This represented case of azoospermia due to missing stem cell factor on Sertoli cells. Similar treatment of defective testicular environment in men has not yet been studied. The main issue for Azoospermic males is defect in germ cells. Intensive efforts are being made to develop male gametes from SSCs. Optimal isolation and purification of SSCs is an important first step for the downstream applications. SSCs are isolated using two step enzymatic digestion of testis tissue from non-human primates and humans [101]. Enrichment of germ cells is attained with different methods like use of antibodies followed by FACS or MACS in human and donkey testis. MACS has also been successfully employed for the enrichment of human spermatogonia using antibodies against GPR125 and SSEA4. Other studies report obtaining 87% purity of human SSCs using OCT4-antibody. Property to adhere to the culture plate or to extracellular matrices (ECM) is alternative method to enrich SSCs; differences in velocity sedimentation or density gradient centrifugation can be used to separate somatic and germ cells. Percoll density gradient for SSCs enrichment in human testis cells led to ~87% pure population of SSCs [102]. Establishment of an efficient in vitro culture system to maintain both the

self-renewal and proliferation capacity of human SSCs is crucial for their potential clinical applications and has been achieved for 2 months in case of human SSCs. Efficient in vitro culture systems replicating the process of male germ cell development and spermatogenesis have several important applications and is being termed as in vitro spermatogenesis (IVS). IVS would also allow experimentations as genome editing of germ cells or correction of genetic causes of infertility to serve benefits to research and ethical issues.

- Generation of stem cells from healthy embryos during IVF and healthy implantation via stem cell technology: The ability to successfully derive human embryonic stem cells (hESC) lines from human embryos following in vitro fertilization (IVF) opened up a plethora of potential applications of this technique. The main source for human embryos has been "discarded" or "spare" fresh or frozen human embryos following IVF. It is a common practice to stimulate the ovaries of women undergoing any of the assisted reproductive technologies (ART) and retrieve multiple oocytes which subsequently lead to multiple embryos. Of these, only two or maximum of three embryos are transferred, while the rest are cryopreserved as per the decision of the couple. In case a couple does not desire to "cryopreserve" their embryos, then all the embryos remaining following embryo transfer can be considered "spare" or if a couple is no longer in need of the "cryopreserved" embryos, then these also can be considered as "spare." Improving implantation rates in IVF has been the center of focus as failure rates are high due to implantation failures. Since stem cell technology has been used in many pathologies as myocardial infarction and spinal cord injuries, endometrial receptivity, using stem cells can be enhanced. Rate of implantation failures are so high that "recurrent implantation failure (RIF)" term was coined for couples who failed to achieve pregnancy in three consecutive IVFs from good embryos. Almost 10% couples in Europe and the USA are affected by RIF, and it is estimated that RIF has a prevalence of 15–20% in IVF [103]. Very crucial aspect of implantation failure is appropriate endometrial thickness (Eth) and a thin endometrium (<7 mm) is associated with low pregnancy rates [104]. Unfortunately, 0.6–0.8% of patients do not reach minimum thickness for embryo transfer [105] due to various issues like inflammatory causes (acute or chronic endometritis/CE); iatrogenic (repeated curettage, polypectomy); hysteroscopic (myomectomy or laparoscopic) where the cavity is opened and the irrational use of clomiphene citrate or individual uterine structural pattern. Endometrial stem cells can provide therapeutic resources in endometrial atrophy, thinned endometrium, and Asherman syndrome. Their properties to maintain normal chromosomal number after several passages, the ability to differentiate into multiple cell lines under standard culture and immunosuppressive properties (inhibits LT, LB and NK make endometrial mesenchymal cells (enMSCs) a source of excellence in certain regenerative therapies). These immunomodulatory properties are explained by the release of inflammatory cytokines in the tissue [106]. Transplantation of EnMSCs to uterus has been studied. EnMSCs, for their properties of high clonality, multipotentiality, regenerative capacity, immunomodulatory, angiogenic and low immunogenicity are an alternative in severe endometrial

lesions. There was a highly significant increase in endometrial thickness after the inoculation of enMSCs, expressing the high regenerative capacity of the intervention. Finally, the endometrium thickness and the standardization of histopathology and immunohistochemistry in post-treatment with enMSCs resulted in higher clinical pregnancy rates in a population with repeated implantation failures, representing a reliable strategy in assisted reproduction.

Stem cells have been used in animal experiments to repair and improve injured endometrium. Though understanding of adipose-derived stem cells (ADSCs) in endometrial injury repair and their further therapeutic mechanisms is incomplete. Benefits of ADSCs in restoration of injured endometrium were demonstrated by utilising a rat endometrial injury model. It was shown that 30 days after ADSCs transplantation, injured endometrium was significantly improved, with increased microvessel density, endometrial thickness and glands when compared with the model group. Furthermore, the fertility of rats with injured endometrium in ADSCs group was also improved and had a higher conception rate [107].

Stem cells are initially undifferentiated cells that display a wide range of differentiation potential with no distinct morphological features. Stem cell therapy method recently has become a novel procedure for treatment of tissue injury and fibrosis in response to damage. Currently, there is massive interest in stem cells as a novel treatment method for regenerative medicine and more specifically for the regeneration of human endometrium disorder like Asherman syndrome (AS) and thin endometrium. AS also known as intrauterine adhesion (IUA) is a uterine disorder with the aberrant creation of adhesions within the uterus and/or cervix. Patients with IUA are significantly associated with menstrual abnormalities and suffer from pelvic pain. In addition, IUA might prevent implantation of the blastocyst, impair the blood supply to the uterus and early fetus, and finally result in the recurrent miscarriage or infertility in the AS patients. It has been evidenced that the transplantation of different stem cells with a diverse source in the endometrial zone had effects on endometrium such as decline in the fibrotic area, elevated number of glands, stimulated angiogenesis, enhanced thickness of the endometrium, better formed tissue construction, protected gestation, and improved pregnancy rate. This study presents a summary of the investigations that indicate the key role of stem cell therapy in regeneration and renovation of defective parts [108]. However, there are still issues as regards the efficacy and safety of SC related infertility treatment as no clinical proofs are available in humans.

8.5 Limitations and Ethical Considerations of Stem Cell Technology

Multiple abilities of self-renewal and differentiating caliber into any gametic or somatic cell without losing standards of normal cell characteristics, stem cells have come to become single point of focus and hope for medical professionals, scientists,

and patients. Stem cell therapies are being developed for genetic disorders, and biomaterials including human tissues are being developed for efficient treatment of common and rare diseases [109, 110]. However, the endless endeavors of stem cell researchers have landed this technology in certain ethical concerns and safety issues. Ethical issues are born due to the need for balance between concepts of saving life or respecting life. Human embryonic stem cells (hESC) represent the worst form of imbalance between the two concepts and hence are center of ethical controversies in stem cell technology. hESCs are derived from the spare pre-implantation embryos which either could be cryopreserved to develop into fetus or implanted to become humans [111, 112]. But to yield stem cells from pluripotent inner cell mass of the would-be humans (embryos), these need to be dismantled. This is where ethical concern regarding hESCs rises and hESC technology is not allowed to grow at its pace. This ethical dilemma has seeked legal intervention in different legislations throughout the world. Some countries including UK allow use of hESCs for research but not for therapeutic applications. While other countries like Italy exhibit more stringent stances, as it prohibits all hESC-based research [113]. The USA banned production of any hESCs line that requires the destruction of an embryo and research using hESCs lines is limited on usage of lines created prior to August 9, 2001. Such legalities and their diverse executions have hampered progress of hESCs technology internationally and development of cell-based clinical therapies globally. Giving a pause to enthusiastic caliber of hESCs, the realistic approach emphasizes to safety issues regarding hESC-based therapy for their clinical use. The pluripotency of hESCs turns out to be a double-edged sword as tumors can generate from these cells upon implantation in vivo [114–116]. Besides scientific ethics and safety concerns, hESCs have few non-medical impacts related to social and psychological aspects of life. Allowing destruction of embryos might lead to de-sensitization of human values. If fear of taking other life disappears, we might end up in increased crime against our life and security of other lives. Embryos might be used to grow tissues only and misuse of organs for commercial benefits will create lacuna of morality in our society. Further, use of hESCs for only research purpose instead of reproductive purpose faces challenges. However, Dworkin's views seem to be a balanced approach toward hESCs technology which states that "Embryos shall not be considered as humans but are valuable enough to begin or extend a human life", condemning creation of embryos for research purposes. As the philosopher John A. Robertson says, "In taking such a stance, persons define or constitute themselves as highly protective of human life" [117]. Robertson notes, however, that this same symbolic respect for life can be expressed through allowing embryos to be created so that others' lives can be prolonged, or deaths averted. The discovery of iPSCs overcame this concern. Safe autologous generation of iPSCs and storage in tissue repository and stem cell banks gave an edge to safer generation of stem cells. But other ethical controversy of human cloning came into being for iPSCs technology. Therapeutic use of iPSCs has certain safety issues as well.

Stem cell characteristic features, such as longer life span, apoptosis resistance and growth regulators and control mechanisms resemble cancer cells. The potency for malignant transformation is a key obstacle to the safety of stem cell based

therapeutics. The risk of tumor formation is further enhanced by other intrinsic and extrinsic risk factors. The site of administration (i.e., the local environment of the stem cell in the recipient) and the need for in vitro culturing contribute to the tumorigenic potential.

8.5.1 Genetic Modification

Genetic modification/reprogramming is required for manufacturing certain types of some stem cells (e.g., iPSC) prior to their clinical application. Genetically modified retroviruses and lentiviruses have been used for such modifications to generate mouse or human iPSCs. The use of viruses raises safety issues of cancer occurrence due to integration of therapeutic vectors activating oncogenes [118, 119].

8.5.2 Bystander Tumor Formation

Stem cells might act as activators of the existing tumor cells.

8.5.3 Immune Responses

Administration of stem cells may affect the host immune system. The administered cells may directly induce an immune response or may have a modulating effect on the immune system. Both ESC-derived cells and especially MSCs have been reported to be immune-privileged and have a low immunogenic potential. An immune suppressive effect of MSC has also been observed in an animal model of rheumatoid arthritis In addition, MSCs have been shown to suppress lymphocyte proliferation to allogenic or xenogenic antigens leading to acceptation of allo/xenotransplants in animal models. In clinical studies MSCs have been used to facilitate the engraftment of HSC and decrease GVHD [120–122].

8.5.4 Biodistribution

Biodistribution of the administered stem cells is a matter of concern. Preferred distribution of MSCs is known for specific tissues, e.g., the bone marrow, muscle, or spleen and tissues facing pathophysiological stress like ischemia or cancer. The mechanism underlying the migration of MSC remains to be clarified [120].

8.5.5 Unwanted (De)differentiation

For clinical use, iPSCs or ESCs must undergo in vitro differentiation prior to administration. However, what if dedifferentiation of stem cells occurs post administration? Dedifferentiation or redifferentiation into another cell type has been already described [123] but the clinical consequences remain unclear. MSC differentiation into unwanted mesenchymal cell types such as osteocytes and adipocytes has been described [124]. Encapsulated structures containing calcification and/or ossifications in the heart have been seen in animals treated with BM-derived MSC for (induced) myocardial infarction [124]. Thus, unwanted differentiation is a theoretical risk; however, the factors contributing to this risk are unknown.

8.5.6 Purity and Identity

Another critical issue is purity of the desired stem cell population. Contamination with other types of cells or undifferentiated cells could cause undesirable effects. Cross contamination of HT1080 human fibrosarcoma cells in MSC led to non-reproducible results on spontaneous transformation events of MSC and publications were retracted since the reported observations could not be reproduced [125–127]. These examples illustrate that even relatively simple risks should be considered. The primary concern being unwanted differentiation upon transplantation in vivo. Stem cells might differentiate into undesired tissues after being implanted for a specifically desired tissue. Safety concerning tumor tissue generation is another unwanted threat of stem cell technology. Earlier, reports show that adipose tissue stem cell-based therapy developed loss of vision in patients treated for macula degeneration promoted metastasis upon implantation [128, 129]. However certain regulatory guidelines laid by the Food and Drug Administration (FDA) define safe and effective protocols of stem cell-based therapies. These guidelines state that minimal laboratory manipulation shall be done to stem cells desired for treatment purposes and shall be intended for homogeneous use without requiring premarket approval to come into action and shall only be subjected to regulatory guidelines against disease transmission. In 2014, a radical regulatory reform in Japan passed two new laws that permitted conditional approval of cell-based treatments following early phase clinical trials preconditional to submission of safety data from at least ten patients. These laws deny earlier "fast track approvals" where treatments were classified according to risk [130]. To date, the treatments that acquired conditional approval include those targeting spinal cord injury, cardiac disease, and limb ischemia [131]. Now, the regulatory authorities demand "Good Manufacturing Practice," use of Xeno-free culture media, recombinant growth factors for safety protocols for cellular products. The balanced approach has led to many clinical trials to study application of hESCs. Exploitation of hESC-based therapy for the treatment of diabetes mellitus has begun in 2014 [117, 132], subretinal transplantation of

hESC-derived retinal pigment epithelial cells (hESC-RPE) for treatment of macular degeneration, dental pulp regeneration, periodontal tissue regeneration, Parkinson's disease [133].

8.6 Organ Culture: Agar Gel, Grid Method, Plasma Clot: Tissue Engineering

Organ culture refers to the explantation of organs or part of organs in vitro, so as to grow a new organ same as the parent organ. In newly developed organ, the parent in vivo characteristics of various tissue components with their anatomical relationship and function are preserved within the culture, in vitro [134]. In tissues lined with squamous epithelium, such as skin or esophagus, or in bladder lined with transitional epithelium, the epithelium follows a similar pattern of differentiation as in the organs in vivo. Hormone-dependent tissues remain hormone sensitive and responsive, and endocrine organs continue to secrete specific hormones. Finally, in fetal tissues, morphogenesis in vitro closely resembles that seen in vivo. Applications of organ culture are diverse in research and medicine. Animal studies cannot mimic state of human physiology to the extent that valid conclusions can be drawn for different experiments. Moreover, animal ethics restrict use of certain drugs or procedures and also limit number of animals for studies. Organ culture thus seems to be a better approach [135]. Modern culture approaches, such as three-dimensional (3D) cultures or organoids or organs-on-a-chip have been designed to better replicate the tissue microenvironment resembling natural tissue histology, physiology and responses to different stimuli. These are grown in a defined three-dimensional (3D) environment in vitro as mini-clusters of cells that self-organize and differentiate into functional cell types, mimicking the structure and functionality of an organ in vivo (hence, also called "mini-organs"). Organoids can be derived from either embryonic stem cells (ESCs), induced pluripotent stem cells (iPSCs), neonatal/adult stem cells (ASCs), or explants from human tissues obtained upon biopsy or surgery. Self-organization within the organoid occurs through spatially restricted lineage commitment and cell sorting, which requires activation of various signaling pathways mediated by intrinsic cellular components or extrinsic environments such as extracellular matrix (ECM) and media [136].

Organ culture techniques are described below:

1. **Clotted Plasma Substrate.** It is a watch glass technique introduced by Fell and Robison to grow organ rudiments or whole organs. Organs are grown on surface of a clot consisting of chick plasma and chick embryo extract, kept in a watch glass. This was the classical standard technique for morphogenetic studies of embryonic organ rudiments. The method has been used to study the action of hormones, vitamins, and carcinogens in adult mammalian tissues. A modification of this method was introduced by Rudnick and later adopted by Gaillard. It consisted of culture vessel containing an embryological watch glass, a plasma

clot and closed with a glass lid sealed on with paraffin wax. Clot consists of two parts of human plasma, one part of human placental serum, and one part of human baby brain extract mixed with six parts of a saline solution. The plasma clot method had several disadvantages. Liquefaction of media led explants to lie in a pool of medium. Due to the complexity of the medium used, biochemical investigation could not be made possible.

2. **Agar Substrate.** To address the problems of plasma clot technique, agar gels were introduced by Spratt. The agar method has been successfully used for developmental and morphogenetic studies like the watch glass technique. The medium used for this method is composed of a salt solution, serum as well as the embryo extract or a mixture of various amino acids and vitamin with 1% agar. The explant has to be subcultured every 5–7 days. The method is largely used for the study of developmental aspects of normal organs and tumors. Although the agar does not liquefy, it requires transplanting the cultures for any study purposing. This disadvantage was overcome by the use of fluid media combined with a support which prevented the cultures being immersed.
3. **Grid Method.** The difficulty of immersing the cultures was overcome by Trowell's grid technique using metal grids, made of tantalum wire gauze. This has been replaced by more rigid continuous sheet of stainless steel or titanium. The dimensions of grids are 25 × 25 mm, with the edges bent over to form four legs, and height about 4 mm. The grids are ideal to grow harder tissues like skeletal tissues. For softer tissues, such as glands or skin, explants need to be placed on strips of lens paper and then deposited on the grids. Finally, the grids with their explants are placed in the culture chamber filled with medium up to the level of the grid. Grid technique was originally developed to maintain adult mammalian tissues having higher requirement for oxygen than fetal organs. Therefore, culture chambers are enclosed in containers which are perfused with a mixture of carbon dioxide and oxygen. This method has proved efficient for preserving the viability and histological structure of the adult tissues, such as prostate glands, kidney, thyroid, and pituitary.
4. **Tissue engineering.** Tissue engineering is an interdisciplinary field that merges engineering and life sciences for the development of organ or tissue substitutes to either restore or replace the lost function. It involves implantation of suitable cells isolated from donor tissue and biocompatible scaffold materials to construct bioartificial tissues in vitro. Combinations of cells and biomaterials must have the ability to reorganize themselves based on the nature of biomaterial and implanted cells. Optimum strength of adhesion between cells and substrate, controlled surface chemistry, porosity, and biodegradability of scaffolding biomaterial are required to aid migration and deposition of extracellular matrix materials by the implanted cells. There are two main methods to produce engineered tissue: First, cells are seeded on scaffolding matrix in vitro and thereafter cells are allowed to lay down on matrix to produce the foundations of a tissue for transplantation. The second approach involves using scaffold only to deliver growth factors or drugs, which upon implantation help cells from the patient body to get recruited to the scaffold site and form tissue upon and throughout the matrices.

These two approaches can be combined as well. To switch cells between growth and differentiation, strategies are required to allow interaction and integration with tissue and cells through incorporation of appropriate physical and cellular signals. For this, biologically active proteins and DNA are involved. After successful generation of the constructs, they must be intimately integrated into the host's vascular system for efficient nutrient supply and waste removal. This need is fulfilled by scaffold matrices to fill the tissue void, provide structural support, and deliver growth factors and/or cells that have the ability to form tissues within the body upon transplantation. The source of cells is also an important choice for success of tissues implanted [137]. The production of an engineered tissue in vitro requires the use of cells to populate matrices and produce matrix resembling that of the native tissue. The most favorable choice for such purpose comes from the use of cells taken from the patient. However, the patient cells are likely to be in a diseased state. Therefore, the use of stem cells, including embryonic stem (ES) cells, bone marrow mesenchymal stem cells (BM-MSCs), and umbilical cord-derived mesenchymal stem cells (UC-MSCs) has been focused upon. Previously, certain procedures have been successful using primary chondrocytes for the replacement of damaged cartilage [138, 139] skin cell sheets for damaged skin [140]. Certain larger and more complex tissue reconstructions, notably the bladder, have also been successfully performed [141], offering hope for more complex tissue engineered procedures in the future.

8.7 Applications of Organ Culture and Tissue Engineering in Medical Biotechnology

Tissue engineering has emerged as a chimera of apparently unrelated disciplines, i.e., biotechnology, engineering, and bedside medicine, with a common goal to solve pathological issues through artificially facilitated tissue regenerative processes. Tissue replacement, generation of prosthesis for lost extremities, biomechanical targeted muscle prosthesis, iron lung and heart pumps are few examples of artificial organs developed by tissue engineers to serve humanity. The discovery of human stem cells (SCs) has been proven the basic foundation for onset of tissue engineering era for the creation of biological substitutes in order to restore, maintain, or improve tissue and organ functioning. Currently, the trachea and the main bronchus replacement are promising in clinical phase trials. Biotechnologically developed artificial esophagus, intrathoracic organ, is in wet-lab phase [142]. Although self-regenerative capabilities of various organs have been exploited in medicine for decades, medical sciences have been working hard to accelerate the search for novel ways to direct tissue regeneration. Progress is often slow, as regenerative potentials, structural and functional requirements vary from organ to organ and range from the highly regenerative liver to the ominously resistant central nervous system [143]. Clinical research in tissue engineering is steadily advancing toward applications in operating theaters. Development of tissue engineered heart valves, reconstruction of functional intact distal airways, [144]. mesh chest-wall

prosthesis, interposition of artificially made vascular grafts, synthesis and functional implantation of tubular structures, like trachea and blood vessels, are the widening scope of tissue engineering in clinical applications. Besides, SCs find greater applications of tissue engineering in medical sciences. Injection of myocardial SCs, for myocardial infarction therapy, bone marrow or lymphatic SC replacement in hemopoietic malignancies offer good examples [145]. Three-dimensional (3D) and four-dimensional (4D) printing is a recent shot of tissue engineering which enables the use of intelligent materials to construct patient specific scaffolds and improve the extent and rate of targeted tissue regeneration. Bone tissue engineering is an important application of tissue engineering with printed polymers which increase strength to heal the bone tissue [146, 147] and then printed [148]. Poly(ε-caprolactone) (PCL) is the most commonly used polymer for 3D printing of scaffold for bone tissue [149] to improve the properties of the printed constructs. Besides, various types of SCs are reported for bone tissue engineering applications. Based on their potential to differentiate into bone cells, mesenchymal stem cells (MSCs) isolated from bone marrow or adipose tissue are the most frequently used for bone engineering [150, 151]. Combined with human umbilical vein endothelial cells (HUVECs), 3D printed scaffolds improve vascularization at the injury site and generate a tissue engineered bone tissue. This technology was updated to printing the cells and the scaffolds together [152]. These printed tissue engineered products have been successfully implanted in rabbit femurs at the defect site in order to study their effect on bone regeneration [153]. MSCs are incorporated with the PCL based scaffolds and are reported to improve the bone regeneration when applied to rabbit femurs [148].

Microtechnology is another shoot of tissue engineering with promising applications for liver system development. It is developed to mimic the complex in vivo microenvironment and microlevel ultrastructure of the organ using two-dimensional (2D) and three-dimensional (3D) culture conditions. Microtechnology based liver tissue engineering uses 3D culture methods, to maintain liver functions and recapitulate native liver [154]. Three-dimensional cell culture models when combined with bioengineered constructs lead to generation of tissue architecture which isolated 3D cell cultures are unable to generate. Lone organoids with self-renewal capacity can develop into early structures and mimic early development, but full-fledged tissues are yielded with implantation of bioengineered constructs. Human forebrain tissue with self-organizing capacity has been generated using floating microfilaments comprising of poly(lactide-co-glycolide) copolymer (PLGA) to generate elongated embryoid bodies. Likewise, microfluidic chip technology combined with natural alginate hydrogels has been developed to construct 3D liver tissues mimicking hepatic plates. These 3D cultures are capable enough to change in the bile secretion pathway via effector mechanisms associated with various receptors and efflux transporters [155]. Thus, tissue engineering gives a firm hope of developing physiologically relevant and active bionic organs and such systems of developing organs and tissues may have further applications, including drug development and disease exploration.

8.8 Summary

Stem cell technology (SCT) is a multifaceted technology and rapidly evolving to offer a utility in various scientific fields especially biomedicine. It involves combined efforts from cell biologists, geneticists, and clinicians and presents immense potential to help us treat various conditions including but not limited to malignant and non-malignant diseases. Since stems cells are multipotent with excellent ability for self-renewal and differentiation into multiple lineages, they become ideal targets for manipulation in vitro. Stems cells can be cultured in a controlled manner to offer utility in different fields. Stem cell technology has a diverse range of applications, which makes this cutting-edge technology most valuable in advancing healthcare and medicine, offering tremendous promise to treat, besides others, difficult diseases like Parkinson's disease, diabetes, and spinal cord injury. In the absence of substantiated research, however, the potential for harm to patients—as well as to the field of stem cell research in general—may outweigh the potential benefits.

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