Chapter 1
Potential Clinical Applications of Stem Cells in Regenerative Medicine

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

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

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

1.1 Introduction

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

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

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

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

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

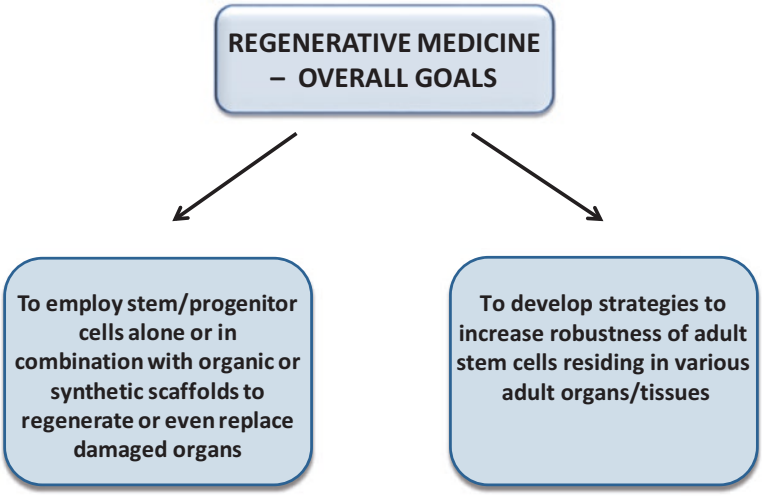

Fig. 1.1 Overall goals of regenerative medicine. There are two major goals of regenerative medicine—(i) to employ stem cells in emergency situations and (ii) to modulate in positive way adult stem cell compartment so that stem cells will retain their robustness during adult life

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

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

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

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

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

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

1.2 Stem Cells and Their Role in Tissue Development and Regeneration

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

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

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

Table 1.1 Developmental hierarchy of stem cell compartment

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

Table 1.2 Contribution of germ layers to postnatal tissues

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

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

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

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

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

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

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

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

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

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

1.3 Therapeutic Application of Stem Cells Isolated from Adult Tissues

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

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

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

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

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

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

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

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

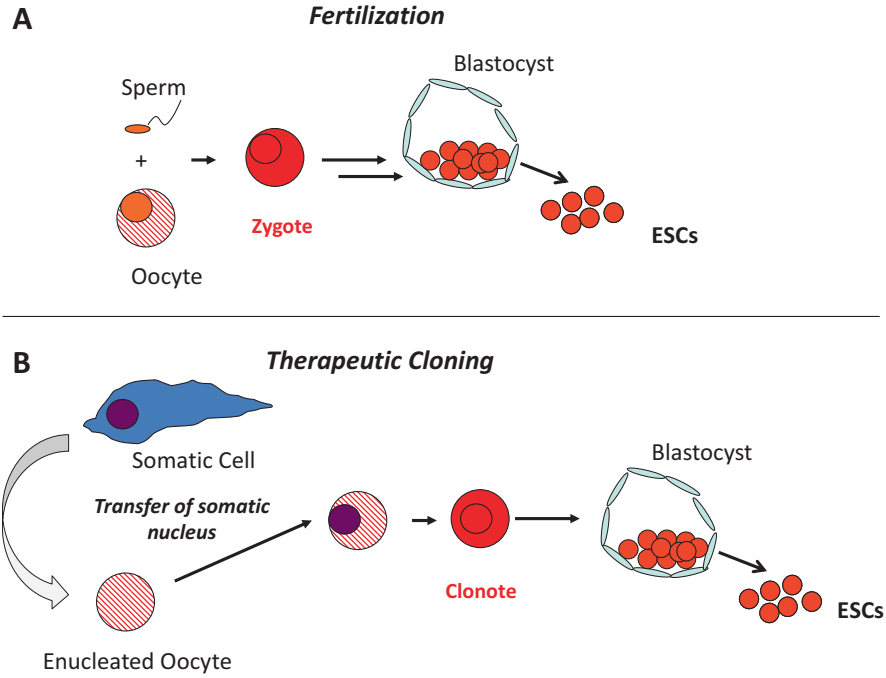

Fig. 1.2 Embryonic stem cells (ESCs) obtained from embryos by fertilization of the oocyte by sperm (**a**) or after nuclear transfer of a somatic nucleus into an enucleated oocyte (**b**). *Panel A.* ESCs isolated from blastocysts derived from an oocyte fertilized by sperm (a zygote). *Panel B.* ESCs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

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

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

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

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

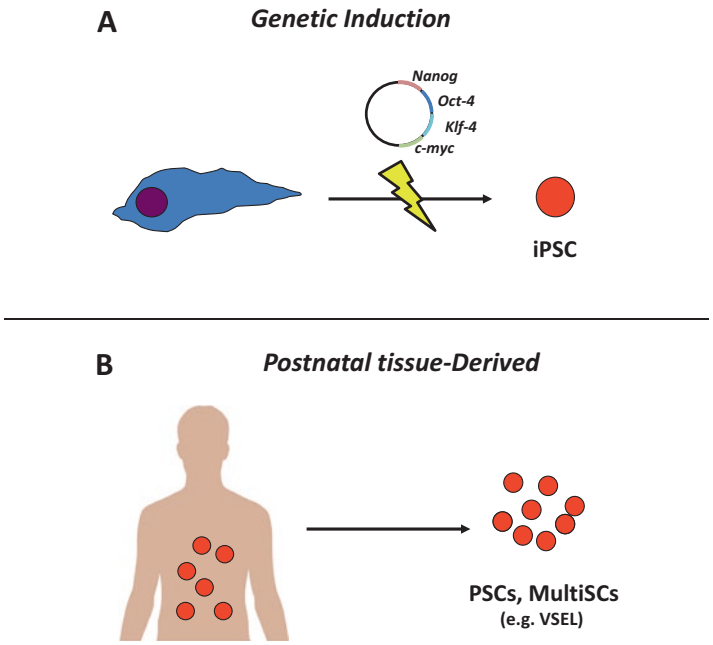

Fig. 1.3 Pluripotent stem cells obtained from postnatal tissues by genetic modification of adult cells (**a**) or pluripotent/multipotent stem cells isolated from postnatal tissues (**b**). *Panel A*. PSCs can be obtained by transforming somatic cells (e.g., fibroblasts) using genes that encode embryonic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

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

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

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

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

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

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

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

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

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

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

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

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

1.5 Conclusion

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

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

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