Chapter 2
Types and Origin of Stem Cells

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2.1 Introduction

Stem cells are undifferentiated cells with both self-renewal capacity and the potential to differentiate into specialized cell types according to the microenvironment. They may be referred to as embryonic or adult stem cells according to their presence either in the inner cell mass of the embryo or in specific tissues throughout the fetal and postnatal life, respectively. They may also be distinguished according to their developmental potency, which refers to the range of their potential fates, i.e., their varying ability to give rise to different cell types. In this case, they can be classified as totipotent (a term restricted to the zygote and the two-cell stage blastomeres with the capacity to generate both embryonic and extraembryonic tissues), pluripotent (the stem cells that are capable of forming all specialized tissues that originate from the embryo germ layers), and multipotent or unipotent cells (that are tissue-restricted stem cells and give rise to specific cell types). Besides, they may be obtained from their natural niche (the specific microenvironment in which they reside) or may be engineered in a laboratory by reprogramming somatic cells, as will be discussed later in this chapter.

At all stages of potency, stem cells play essential roles in development as well as in homeostasis and disease pathogenesis. Based on the concept that stem cells are the organizing principle for tissue formation and homeostasis, their use in clinical applications in the field of regenerative medicine, including cell transplantation therapy and tissue engineering, was a matter of time and great hope and expectation

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has been placed in it. In fact, much effort has been made to identify and test different sources of cells to treat and cure a wide range of diseases, including cardiovascular diseases. In that way, among promising therapies for vascular diseases, the stem cell-based ones are in progress and demand for stem cell specialists.

Virtually, all stem cell types could be used for regenerative purposes. Nevertheless, it is important to keep in mind that, although stem cells have the capability of differentiating into specialized cell types, they may themselves, without the need of differentiating, act as biofactories for producing a wide range of molecules that modulate cells around them by paracrine signaling and likewise are significant for inducing processes central to tissue healing and regeneration.

2.2 Autologous, Syngeneic, Allogeneic, and Xenotransplantation

The procedure in which stem cells are introduced into patients with regenerative and medical purposes is generally referred to as stem cell transplantation. The primary goal of stem cell transplantation is to repopulate injured areas with specific cells so that tissues become functional again. The transplantation procedure may occur by different strategies, including autologous, syngeneic, allogeneic, or xenograft transplants. The type of transplant chosen will depend on the recipient's medical conditions and the availability of a matching donor. The assurance of a sufficient number of donor stem cells must also be offered [36].

2.2.1 Autologous Transplantation

In autologous transplantation, also called autotransplant or autograft, stem cells are extracted from the patient themselves, for example, from the peripheral blood, bone marrow, or adipose tissue, and transplanted back to the patient. This modality of transplantation is readily available, and there is no need for human leukocyte antigen (HLA, i.e., markers used for the immune cells to recognize what is self and nonself) typing and matching. Autologous transplants have a lower risk of rejection, and there is no need for immunosuppressive therapy to prevent graft rejection.

2.2.2 Syngeneic Transplantation

Also known as syngeneic graft or isograft. In this modality of transplantation, cells come from a different but genetically identical donor, such as the identical twin. In this case, individuals must be sufficiently identical and immunologically

compatible. Similarly to autologous transplantation, the syngeneic graft has a lower risk of being rejected.

2.2.3 Allogeneic Transplantation

In the allogeneic transplantation, also called allotransplant, allograft, or homograft, stem cells are extracted, for example, from peripheral blood, bone marrow, umbilical cord, or adipose tissue, from a compatible donor and transplanted to the recipient. Therefore, the primary condition for donor selection is HLA compatibility. A close match between HLA markers between patients and donors is essential for a successful transplant; however, less than 30% of patients can find an HLA-matched sibling. To circumvent this, alternative sources, such as HLA-matched adult unrelated donors, umbilical cord blood stem cells, and partially HLA-mismatched (also known as HLA-haploidentical) related donors, are in continuous advance, especially in the hematopoietic stem cells (HSC) transplantation segment [94]. Besides, regenerative medicine researchers have put much effort into studying stem cells either with intrinsic immunomodulatory effects, such as the mesenchymal stromal cells (MSC) [103], or by HLA engineering of donor cells [63, 88, 105, 125]. Nevertheless, although this modality strengthens the possibility of finding a donor, allotransplant shows a higher risk of potentially fatal complications associated with organ toxicity, graft failure, and graft-versus-host disease (GVHD).

2.2.4 Xenotransplantation

In this type of transplant, the donor belongs to a different species than the recipient. This modality has emerged as an alternative to human transplants due to the scarcity of donor cells, tissues, and organs in contrast to rising numbers of potential recipients. Sheep, pigs, and nonhuman primates have been studied as potential sources of human stem cells and organs. Mesenchymal stem cells (MSC) obtained from different animals are of significant potential due to their immunosuppressive effects [108]. Xenograft practices such as blood transfusion from nonhuman species to patients were customary between the seventeenth and twentieth centuries. The first chimpanzee to human organ transplantation was attempted in the early 1960s [44, 69, 144, 153]. However, the high mortality due to vigorous immunogenicity and donor organ failure, combined with concern over viral transmission, has halted xenotransplantation for a time. To circumvent these issues, scientists have recently introduced gene editing (CRISPR/Cas9) and human pluripotent stem cells to create genetically “humanized” animals owning human organs [68, 112]. The idea is to create animals that possess organs, like the heart, lungs, kidney, liver, or even vessels, made up entirely of human cells. Attempts to genetically engineer cells to

modify the immune-related genes making xenotransplantable organs compatible with the human immune system have also been studied.

2.3 Embryonic and Induced Pluripotent Stem Cells

When talking about stem cells, very often one either refers to embryonic stem cells (ESC) or induced pluripotent stem cells (iPSC). Both cell types share many similarities: they possess the ability to self-renew indefinitely and can produce cells from all three primal germ layers (endo-, ecto-, and mesoderm). Furthermore, the cells express "stemness" proteins that support distinct stem cell properties (e.g., fast cell division, telomere elongation). Thus, in many aspects, these cells are comparable and may be used interchangeably for many applications. However, their history and the respective isolation procedures vary considerably as described later in the chapter.

In 1981, the first embryonic stem cells were isolated from a mouse embryo [59]. Followed in 1998 by the first established human embryonic stem cell line [175]. Since then, ESC technology was faced with many ethical concerns leading to strict legal regulation in numerous countries.

In 2006, Yamanaka et al. made the breakthrough discovery that through overexpression of four transcription factors (Oct-4, Sox2, Klf4, and c-Myc) in mouse somatic cells the cells could be reprogrammed to an embryonic-like state [172]. These cells were aptly called induced pluripotent stem cells (iPSC). In 2007, the same group successfully applied the reprogramming procedure also to human somatic cells [171].

2.4 Adult Stem Cells: Bone Marrow, Peripheral Blood, Umbilical Cord, and Adipose Tissue

Adult stem cells, the so-called somatic stem cells or tissue-specific stem cells, are undifferentiated cell populations present during both fetal development and postnatal life. They are multipotent, i.e., they can differentiate into a limited number of different cell types of their tissue of origin and according to the microenvironment they are located in. In physiological conditions, these cells are maintained in a quiescent state (a way to avoid the accumulation of genetic damage) and, in response to specific stimuli, they may be activated and proliferate. They may keep up tissue homeostasis and contribute to cell self-renewal, but also support tissue repair. Which way the cell chooses in any situation depends on the information about the state of the tissue the cell receives, i.e., the microenvironmental cues. Although virtually every organ harbors stem cell niches, we will focus on the most ideal and commonly used sources for the therapeutic application of adult stem cells in vascular diseases,

that is, the bone marrow, peripheral blood, umbilical cord, and adipose tissue. They have in common the facility of cell harvesting and the higher number of stem cells when compared to other sources.

2.4.1 Bone Marrow

The bone marrow (BM) is a spongy tissue found inside some bones in the body, including the hip and thigh bones. The primary function of the BM is providing signals to support hematopoiesis (i.e., the production of the blood cells) and the quiescence and self-renewability of the resident stem cells. Stem cells contained in the bone marrow mostly belong to two types: hemopoietic (giving rise to blood cells) and stromal (supporting the hematopoietic development and differentiating into other cell types) [114].

The bone marrow stromal cells consist of several populations, including osteolineage cells, endothelial cells, perivascular CXCL12-expressing cells, and mesenchymal stem cells (MSC) [7]. The MSC are of particular interest in vascular regenerative medicine and have been widely explored in clinical trials. The term was first coined by Arnold Caplan in 1991 to describe a perivascular BM stromal cell population able to differentiate into cartilage, bone, and fat [30]. After that, there was a plethora of reports not only alleging the presence of those cells in other tissues but also indicating they would be capable of differentiation in several lineages such as adipocytes, chondrocytes, and osteoblasts, cardiomyocytes, skeletal myocytes, fibroblasts, myofibroblasts, endothelial cells, neural cells, hepatic and tubular renal cells, particularly in vitro [74]. However, the term "stem cells" should be restricted to the populations of cells that demonstrate multipotency and self-renewal in vivo. Thus, it has been appealed that the term "Mesenchymal Stem Cells" should be abandoned as MSC can be induced to differentiate in many cell types in vitro, but they do not seem to do it in vivo. In fact, the therapeutic functionality presented by MSC is suggested to be achieved due to paracrine effects [24, 31, 160]. Therefore, despite still lacking a consensus of which term should be used and based on the recommendation of the International Society for Cellular Therapy (ISCT) for its re-designation as "stromal," instead of "stem," [52], the MSC initials will be used throughout this chapter.

The hematopoietic stem cells (HSC) microenvironment of the BM is responsible for controlling the self-renewal, proliferation, differentiation, and migration of HSC and progenitor cells under determined stimuli. HSC give rise to blood cells, including white and red blood cells, as well as platelets. Based on mouse studies (and pieces of evidence that adult human BM is highly similar), the BM is composed of, at least, three hematopoietic niches: endosteal, periarteriolar, and perisinusoidal [34]. Although the prevailing hypothesis used to be that long-time repopulating HSC are maintained in a hypoxic niche in the BM, it has now become more evident that the majority of HSC are located in the perivascular well-oxygenated regions. More precisely, an imaging-based study shows that BM nondividing stem cells are

mainly perisinusoidal [3]. The endosteal microenvironment seems to harbor only a subset of early lymphoid progenitors, while the bona fide HSC are found in the perivascular niches [51]. Worth mentioning, it has been shown that differences among the endothelium of the perivascular regions regulate the metabolism and reactive oxygen species (ROS) production in HSC in order to keep them quiescent (periarteriolar) or cycling (perisinusoidal) [170]. This characteristic is relevant to regenerative medicine seeing as it is known that the BM-HSC pool expands during aging, but its regenerative potential is reduced, maybe because of an altered capacity of the BM endothelium in regulating HSC metabolism and ROS production.

Of note, the transplantation of BM-derived mononuclear cells (BM-MNC) expressing $CD34^+$ and/or $CD133^+$, usually used in hematological diseases, has also been considered for treating ischemic diseases in humans [5, 84, 121, 155, 166, 189, 192]. The initial rationale was that those cell fractions would be enriched for the envisioned stem cells believed to be the best option for vascular regenerative purposes, the endothelial stem/progenitor cells (EPC) [11]. This premise was based on the close developmental association between hematopoietic and endothelial cell lineages during embryogenesis. However, the origin and identity of truly adult EPC is still a matter of intense debate in the scientific community, and many studies indicate that the BM is not the source of these stem/progenitors in adults. Instead, the BM-MNC fraction is known to be enriched for proangiogenic hematopoietic cells [37, 66, 197, 198].

2.4.2 Peripheral Blood

Peripheral blood stem cells (PBSC) are present in a very limited number under physiological conditions but may be mobilized from BM under certain conditions and with distinct stimuli, such as ischemia. Furthermore, circulating stem cells may also be derived from the vessel wall, especially during endothelial damage. The identification of such a minimally invasive stem cell source has made PBSC interesting for regenerative medicine and clinical applications. Indeed, peripheral blood (PB) has largely replaced BM in autologous stem cell transplants.

Usually, the stem cells used for transplantation in several hematological and neoplastic diseases are isolated from the bloodstream by their expression of the CD34 surface marker. The average percentage of $CD34^+$ cells among total circulating cells is 0.06% in the bloodstream, while in BM, this percentage reaches 1.1% in healthy donors [102]. Strategies such as cytokine treatment are used to mobilize stem cells from BM to the bloodstream, including recombinant human granulocyte colony-stimulating factor (rhG-CSF) administration, which increases $CD34^+$ cell concentration in the peripheral blood by 50–100-fold over baseline, and CXCR4 antagonists, which, in combination with G-CSF, contribute a further two- to threefold increase [90].

The cell composition of unmanipulated PBSC differs significantly from bone marrow stem cells (BMSC). It has been demonstrated, for example, that T cells,

monocytes, and natural killer cells contaminants in a PBSC allograft were more than ten times higher than in a BM one [102]. In practice, cells isolated from the bloodstream intended to be used in cellular therapy correspond to the fraction of the mononuclear cells. The so-called peripheral blood mononuclear cells (PBMC) are a mixture of leukocytes and stem/progenitor cells that, when cytokine-mobilized, are enriched for $CD34^+$ cells that also express the VEGF receptor 2 (VEGFR2/KDR) and the leukocyte marker CD45, although only a few of them express CD133 [90, 198]. Besides, some researchers have tried to isolate MSC from PBMC, but their existence in the bloodstream remains controversial [56, 110, 118].

Regarding EPC, the presence of a hierarchy of resident endothelial progenitor cells in the endothelium of blood vessels has been recognized that could account for the presence of EPC in the bloodstream replacing the earlier theory that these cells would come from BM [86, 186]. In the 2010s, there was further progress in understanding the origin and identity of the true EPC in adults and the paradigm shift began to strengthen. It was initially demonstrated that CD117/c-Kit^+ cells present in the endothelium, the so-called vascular endothelium-resident stem cells (VESC), display high-clonogenic capacity and can differentiate into endothelial cells [60]. More recently, it was shown that these quiescent endothelial stem/progenitor cells are activated in response to injury [126, 188]. Overall, these studies, along with other recent findings, provide strong support not only for the existence of vascular endothelial stem/progenitor cells but also for the hierarchy of endothelial cell types within the endothelium [80], suggesting they can be used for therapeutic purposes. Further studies, however, are still necessary to better understand the identity of these vessel-derived endothelial stem/progenitor cells and their projected application in vascular regeneration.

2.4.3 Umbilical Cord

The ISBT 128 standard terminology for medical products of human origin [87] classifies the umbilical cord-derived cells into two categories: cord blood (CB)- and umbilical cord tissue (UCT)-derived cells.

The CB is a rich source of HSC and can be used as an alternative to BM- or PB-derived HSC in allogeneic transplants, especially when HLA-matched sibling and unrelated donors are unavailable. Although the CB yields lower numbers of HSC when compared to BM or cytokine-induced PB, it holds the advantage of being less immunogenic, thus requiring less stringent HLA-matching criteria. When compared to BM and PB, the CB-HSC presents a lower risk of graft-versus-host disease, a fatal complication of HSC transplantation. Besides, the CB-$CD34^+$ cells exhibit higher hematopoietic repopulating ability than those from BM and PB. However, with the advent of the transplantation of haploidentical HSC, in addition to the high cost of allogenic CB transplantation, there was a decline in the use of CB for HSC transplantation purposes. On the other hand, the existence of cord blood banks allows its correct frozen maintenance for future use, making CB-derived

cells available for, at least, 20 years without loss of their viability and engraftment potential. Noteworthy, beyond its primary use in hematological disorders, recent evidence points toward the potential use of CB cells in nonhematopoietic conditions as a source for regenerative cell therapy and immune modulation, amplifying the perspective for the use of CB-derived cells [48, 152].

UCT is a rich source of MSC and can be used as an alternative to BM- or adipose tissue (AT)-derived MSC. UCT-MSC may be isolated from the placenta, the perivascular space, and the Wharton's jelly present in the umbilical cord stroma and yields higher numbers of MSC when compared to BM or AT. Besides, UCT-MSC have higher proliferative potential than BM- and AT-MSC and express higher amounts of cytokines and hematopoietic growth factors, such as G-CSF, GM-CSF, LIF, IL-1α, IL-6, and IL-8. Of note, its proangiogenic capacity is independent of VEGF-A. As a source of perinatal cells, UCT-MSC express markers of pluripotency higher than postnatal tissue, but lesser than ESC, and are not known to induce tumorigenesis. Its therapeutic effects in preclinical and clinical studies, including vascular diseases, encourage further studies to pursue the clinical use of UCT-MSC [10].

2.4.4 Adipose Tissue

The white adipose tissue is one of the most important adult sources for therapies based on MSC, the so-called adipose-derived MSC (ASC). They are more readily available and yield a higher amount of stem/stromal cells when compared to bone marrow-derived MSC. Therefore, ASC are of great interest for cell therapy and tissue engineering [38]. These cells are obtained from the Stromal Vascular Fraction (SVF) that, besides ASC, typically contains various cell types, such as endothelial cells, smooth muscle cells, pericytes, leukocytes, fibroblasts, and preadipocytes. Of note, similar to ASC the freshly isolated SVF can induce new blood vessel formation and may be used directly for therapeutic neovascularization without the need for cell isolation [99, 206]. Compared to BM-MSC, ASC are less osteogenic and more effective in producing collagen. Besides, ASC are more stable in long-term culture with reduced senescence and higher proliferation capacity [165].

The $CD34^+$ ASC subpopulation has been associated with the formation of nonhematopoietic colonies in vitro. They may show endothelial characteristics, such as the expression of the surface markers CD146 and CD31, depending on culture conditions. Pericyte-like subpopulations expressing CD146 but that are $CD31^-$ and may be CD34+ or $CD34^-$, may also be present among ASC. Moreover, the $CD146^-CD31^-CD34^+$ subpopulation shows a higher potential to form adipocytes [209].

The use of ASC for cellular therapy has advantages regarding its easy access by subcutaneous lipoaspiration, which is a less painful procedure when compared to BMSC collection [70, 130]. In fact, ASC have been employed in many clinical trials

for treating conditions that require tissue regeneration, diabetes mellitus, liver disease, corneal lesions, articular, and cutaneous lesions [19, 50, 101, 194, 196, 210, 211].

2.5 Isolation and Cell Culture

2.5.1 Isolation of Embryonic Stem Cells

Embryonic stem cells are obtained by isolation of cells from the inner cell mass (ICM) of a mammalian embryo in the blastocyst stage (Fig. 2.1). This is usually done by the destruction of the trophoblast or by the isolation of ICM cells from the embryo. The cells are then cultured either on a layer of murine or human feeder cells or on culture plates treated with extracellular matrix proteins. After cell culture has been established, adequate quality control is tantamount. First, cells are continuously cultured for at least 6 months to ascertain indefinite proliferation capacity. Furthermore, cells need to be able to form cells from all three primal germ layers: endoderm, ectoderm, and mesoderm. Cells are then tested for NANOG expression, an essential protein for upholding pluripotency.

2.5.2 Isolation of Induced Pluripotent Stem Cells

Induced pluripotent stem cells (iPSC) are obtained through cellular reprogramming of somatic cells. Most commonly, skin fibroblasts, peripheral blood monocytes, or urine-derived cells are used. However, iPSC lines have been established from numerous other cell types. Following the isolation of somatic cells, the overexpression of Yamanaka factors is induced by the use of various vectors (Fig. 2.2).

Early approaches used retroviral vectors [171, 172], while subsequent approaches used adeno-, RNA-virus, or plasmid vectors [137, 158, 207, 208]. In recent years, reprogramming through mRNA vectors has gained traction [33]. This method has the advantage that it lacks the potential for mutagenic DNA insertions and mRNA

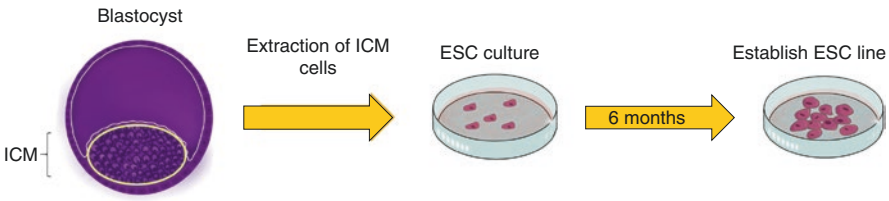

Fig. 2.1 Schematic of embryonic stem cell derivation, ICM = inner cell mass

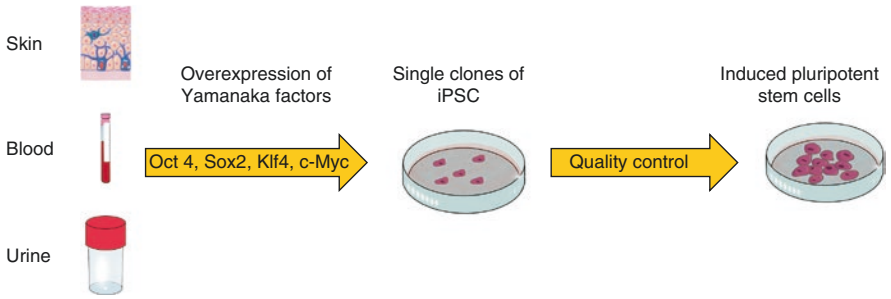

Fig. 2.2 Schematic-induced pluripotent stem cell derivation from somatic cells of different tissue origin

is quickly degraded; however, not all somatic cell types are susceptible to mRNA vectors and in animal experiments mRNA induced iPSC-derived somatic cells demonstrated increased immunogenicity.

About two weeks after induction of overexpression of Yamanaka factors, small colonies appear in the culture dish. These colonies each represent a distinct clone of emerging iPSC. Several of these clones are then picked and propagated separately. Afterward, cells need to undergo quality control and are tested for their pluripotency, ability for indefinite self-renewal as well as expression of at least two of the species-specific stem cell proteins. For human iPSC, these are Oct-4, SSEA-4, NANOG, Sox-2, TRA-1-60, and TRA-1-81 [168]. The gold standard for assessment of pluripotency is the implantation into immunodeficient mice where pluripotent stem cells will form teratomata (tumors consisting of tissues from all three germ layers).

2.5.3 Culture of ESC and iPSC

As previously stated, ESC and iPSC are very similar in many regards and there are few differences in their respective culture methods; thus, they are described together in the following. In the beginning, ESC and iPSC were commonly cultured on a layer of mitomycin-treated or irradiated (to stop any further proliferation) mouse embryonal fibroblasts. This feeder layer not only helps the stem cells attach to the culture dish but also produces essential growth factors. However, in recent years the trend has been going toward GMP (good manufacturing practice) and GTP (good tissue practice) conform culture methods. This includes the use of chemically defined, xeno- and serum-free culture media sans the use of either mouse or human feeder cells. Culture media are widely available from numerous companies, but all contain either bFGF (basic fibroblast growth factor) for human or LIF (leukocyte inhibitory factor) for mouse stem cells to keep the cells in their pluripotent state. Nevertheless, significant variations exist in the composition and

concentration of the respective growth factor mixtures between different culture media. This represents a significant hurdle for the broad application of standardized culture conditions and hampers the reproducibility of differentiation protocols between labs.

2.5.4 Isolation and Culture of Adult Stem Cells

2.5.4.1 Bone Marrow Stem Cells (BMSC)

Hematopoietic stem/progenitor cells (HSPC) and BM-MSC are the two main cell populations that can be isolated from the BM. Human BM can be obtained by aspiration from the iliac crest [132], the femoral head [45], and the vertebral body [146].

Regarding the MSC, they are present at a shallow frequency in the BM. Although they can grow in culture and their number is expanded [12], there is still some challenge to use them in cell-based therapies due to the high variability in the culture conditions used for their isolation and expansion. As a result, a range of protocols has been published. Besides, xenogeneic substances for cell expansion may affect the cells properties compromising clinical application. Except for their tissue source, there are no standardized culture methods yet available for culturing and expanding MSC for transplantation purposes. Therefore, although very promising, its approval for clinical use as a feasible treatment modality is still a matter of intense study to define optimal conditions and enable standardization.

BM-MSC show important characteristics that permit their isolation and purification process, such as their physical adherence to plastic cell culture plates [178]. The main techniques used for isolation and BM-MSC enrichment include an antibody-based cell sorting, low- and high-density culture techniques, positive and negative selection methods, frequent medium changes, and enzymatic digestion procedures [13, 57, 58, 159, 162, 185]. Following the ISCT proposed minimal criteria for human MSC identification [52], these cells exhibit (a) plastic adherence, (b) the ability to differentiate in vitro into adipocyte, chondrocyte, and osteoblasts, and (c) can be immunophenotypically characterized by the expression of CD73, CD90, CD105, and the lack of expression of CD14, CD34, CD45, and human leukocyte antigen-DR (HLA-DR). Moreover, the expression of the cell markers CD29 and CD71, as well as the absence of endothelial cell markers, such as CD31, VEGFR2, CD62E, and vWF, is also very often used to characterize these cells better [14].

For MSC isolation, the procedure involves the isolation of the mononuclear (MNC) fraction by density centrifugation [15, 77–79]. The resultant buffy coat is then seeded in culture flasks containing the medium of choice supplemented with 10% of fetal bovine serum (FBS) and maintained at 37 °C in a humidified atmosphere. The MSC-like cells form a single layer and the adherent cells are maintained in culture for 8–12 days. The resultant cells show heterogeneous fibroblastic appearance, distinct colony formation, and high proliferation [14].

In order to perform the functional characterization, known as the ability of the cultured cells to differentiate in osteoblastic- and adipocytic-like cells, alizarin staining is used to show the formation of calcium oxalates for characterizing osteoblastic activity and, for adipocytic activity, the oil red staining is commonly employed [14, 141].

BM-MNC positive for CD34 are selected for HSPC isolation and transplantation. However, it is worth mentioning that this fraction contains cells that vary either in their metabolic or mitotic activities and the $CD34^+$ expression alone does not provide an accurate measure of HSPC in BM. Therefore, it is advised to use cocktails of antibodies to deplete or exclude hematopoietic lineage positive cells to better characterize the BM-HSPC [107, 193]. The isolation methods used to separate these cells include immunomagnetic and fluorescence-activated cell sorting (FACS).

Similar to BM-MSC, BM-HSPC can be stimulated to proliferate in culture for expansion. Different methods have been used, and the composition and biological characteristics depend on the procedure adopted. $CD34^+$ cells may be cultured for several weeks in a medium supplemented with some cytokines such as IL-3, IL-6, SCF, Flt3 ligand, TPO, GM-CSF, and G-CSF [22, 41, 131]. Moreover, HSC may also be co-cultured with MSC that are shown to support their survival and self-renewal [62].

Among human hematopoietic stem/progenitor cells, the CD34 surface marker is known for its unique expression, although recent findings suggest the existence of a population of cells that do not express CD34 ($CD34^-$) but become $CD34^+$ before cell division [2, 53]. Differently, the HSPC expression profile of naïve mice includes $Lin^-Sca\text{-}1^+cKit^+$ (LSK) [8, 167]. Besides, other markers may also be used to identify HSC isolated from BM, such as the absence of expression of CD41 and CD48 and the expression of CD150 [96].

2.5.4.2 Peripheral Blood Stem Cells (PBSC)

Peripheral blood is an alternative and highly envisioned source of stem cells since it can be easily acquired with minimal invasiveness. PBSC includes HSPC and, possibly, endothelial progenitor cells (EPC) and MSC. All of them are present in the mononuclear cell (MNC) fraction [49, 75, 140]. The culture protocols and expansion conditions of PB-MSC, however, are not well defined, and even their presence in the bloodstream has been challenged. Additionally, for PB-EPC, due to the new body of evidence about vessel-derived stem/progenitor endothelial cells, thus far there are no protocols available that ensure their cultivation and expansion in vitro. Nevertheless, what exists are well-defined cultivation protocols of the PB-MNC fraction to enrich for proangiogenic cells, as will be described at the end of this subtopic. Therefore, the use of PBSC cells for transplantation is currently only approved for hematopoietic stem cell transplantation.

Two primary techniques are used to isolate PBMC from the bloodstream: density gradient centrifugation or leukapheresis. In the first one, the PBMC fraction corresponds to the thin white layer at the interface between the plasma and the density

medium. The leukapheresis method uses an automated machine to separate the whole blood from the target PBMC fraction through high-speed centrifugation.

The isolated PBMC comprise a heterogeneous mixture of cells. The so-called peripheral blood mononuclear cells (PBMC) fraction contains circulating cells that include lymphocytes – $CD3^+$ T cells (45–70%) and $CD19^+$ B cells (5–15%) – monocytes (10–30%), natural killer cells (5–10%), dendritic cells (1–2%), and HSC (0.1–0.2%). Nevertheless, as mentioned earlier in this chapter, it is possible to increase the percentage of circulating HSPC by the administration of G-CSF to the donor.

After that, readily transplantable cells, mainly used for treating hematological and neoplastic diseases, are isolated based on their positivity for the CD34 surface marker. The cell profile of the PBMC subset may be analyzed by flow cytometry which allows differentiating cell populations based on complexity – side scatter (SSC) and forward scatter proprieties (FSC) – and biomarkers expression [16, 136].

As mentioned above, the PBMC fraction may also be used for the culture-based isolation of proangiogenic cells. The reported assays rely on the adhesion of MNC to specific substrates, such as fibronectin and collagen I, in endothelial-specific culture media. At least two types of circulating angiogenic cells, formerly known as "putative EPC", may be isolated in culture dishes: (1) the so-called early outgrowth endothelial progenitor cells (eEPC or EOC) and (2) the late outgrowth endothelial progenitor cells possessing clonal endothelial colony-forming cells (ECFC) ability [127, 145, 199].

The first one (EOC) appears after 4–7 days in culture, can be spherical or spindle-shaped, and has low proliferative potential (if any). The second one (ECFC) appears after 2–4 weeks in culture, forms a cobblestone-shaped cell monolayer, and has high proliferative potential. Both cell types bind to isolectin, endocyte acetylated LDL, and express CD31. However, only ECFC express the endothelial cell marker von Willebrand Factor (vWF), besides to express more VEGFR2, CD105, and CD146 than EOC. The progenitor cell markers CD34 and CD117/c-Kit are more expressed in ECFC than EOC. In contrast, hematopoietic markers are present in EOC, but not in ECFC, suggesting ECFC are more committed to the endothelial lineage. Finally, in functional assays, ECFC, but not EOC, are much more prone to integrate into endothelial cells network in vitro and in vivo. Overall, although both cell types are interesting candidates for inducing therapeutic angiogenesis, while EOC are undoubtedly hematopoietic cells with paracrine angiogenic capacity, ECFC appear to be programmed to differentiate into endothelium [18].

2.5.4.3 Umbilical Cord Stem Cells (UCSC)

Cord Blood (CB) Collection and Processing for Cell Isolation

The procedure of CB collection is noninvasive, not painful, and relies on a venipuncture and blood drainage to a sterile container, usually with a citrate-based anticoagulant. The most critical steps at the moment of the collection are to avoid contamination during the venipuncture procedure and to clamp and extract the cord blood within a

precise timing before umbilical vessels collapse and blood entrapping. Usually, the red blood cells (RBC)-depleted fraction is used for cryopreservation, as it allows volume reduction and lesser RBC-related thawing cytotoxicity. Several methods may be used to isolate CB cells, including density gradient separation of mononuclear (MNC) cells, sedimentation by gelatin, rouleaux formation induced by hydroxyethyl starch and centrifugation, and differential centrifugation with separation of RBC and plasma.

The optimization of recovery procedures for cells from CB is a vital step for its clinical use and, according to FDA recommendations, must achieve a recovery of, at least, 85% of viable nucleated cells after volume reduction and before cryopreservation. Each banked cord blood unit must contain, at least, 5×10^8 total nucleated cells (TNC). The minimum dose acceptable for consistent engraftment of CB is 2.5×10^7 TNC per kilogram of body weight of the prospective recipient. This dose is considered to reflect the MNC content that meets the required $CD34^+$ count of, at least, 0.25% of viable TNC before cryopreservation (approximately 2×10^6 $CD34^+$ cells). A suboptimal dose may result in delayed hematological recovery, graft failure, and a higher risk of infection. Of note, this minimum dose limits the CB transplantation to children and adults of low body weight. For weightier individuals, the double-unit of CB transplantation augments the graft doses, but its clinical benefits are still controversial [61, 152, 190]. Although controversial, the TNC count is mostly used in hematopoietic cell transplantation. However, its advantages (especially when considering the reduction in time of isolation and costs) have been challenged. It has been demonstrated that storing UCB units as MNC fractions instead of TNC fractions would provide more accurate and reliable results concerning the quality and potency of the UCB unit [139].

Although the CB-HSPC isolation for transplantation purposes is primarily based on the CD34 expression in the MNC fraction, further characterization of CB cells is a challenge since it involves the maturation degree of these cells in the UCB. It is known that the phenotype $CD34^+CD38^-$ is more primitive than the $CD34^+CD38^+$ [176]. Besides, other surface markers can be used to determine the maturity degree of CB cells, among them CD90, CD117 (c-Kit), CD135, CD75 [82, 154].

In an attempt to increase the number of cells, the expansion of CB-HSC in culture is possible as long as supplemental factors are present in the medium. The main factors used for the CB-HSC enrichment and culture are thrombopoietin, Flt3 ligand, IL-6, fibroblast growth factor (FGF), angiopoietin, insulin-like growth factor binding protein 2 (IGFBP2), Notch ligands, Wnts, and insulin-like growth factor 2 (IGF-2) [142, 201, 203, 204]. Besides, co-culture with MSC enhances the process of HSC expansion, as adhesion, proliferation, and differentiation of HSC depend on the production of soluble factors produced by MSC and matrix molecules [147].

Umbilical Cord Tissue (UCT) Isolation and Culture

Two main techniques are used to isolate cells from the umbilical cord tissue: enzymatic digestion and explants [29, 85]. Different lab-made protocols for isolation and expansion have been used. In the method of enzymatic digestion, collagenase and hyaluronidase are used to digest UCT and release cells from the extracellular matrix. The disadvantage of this method is the long duration of tissue incubation with

enzymes, which may compromise the biology and the process of cell adhesion. The explant method is cheaper and more straightforward and does not require enzymatic incubation. However, this method depends on the ability of cells to migrate and adhere to the culture vessel [73, 76, 200].

Although there is no standardized protocol for isolation, expansion, or cryopreservation, UCT-MSC have mainly been isolated by enzymatic digestion of Wharton's jelly and cultured in medium supplemented with human or fetal calf serum. The culture medium may also contain specific growth factors such as bFGF, EGF, PDGF, and VEGF. Besides, the UCT-MSC may be isolated by a nonenzymatic explant culture method. However, some studies show the isolated cells obtained by each method display different characteristics as the cells isolated by the explant culture present a higher proliferative potential (although cells reach arrest earlier) and higher variation of phenotypes, being, therefore, a more heterogeneous population. Overall, independent of enzymatic or nonenzymatic isolation, the UCT-MSC phenotyping follows the minimum criteria of ISCT. Besides, they display higher proliferative potential in culture than MSC from other postnatal or neonatal sources and, under specific conditions, may display pluripotent specific markers that are not present in other postnatal sources. Of note, culture conditions influence the immunomodulatory properties of UCT-MSC as xeno-free or serum-free media and allow a more effective suppression of T-cell proliferation [10].

2.5.4.4 Adipose Tissue Stem Cells (ASC)

Subcutaneous fat deposits are present in high quantities in the human body, and liposuction surgeries are an excellent choice for harvesting adipose tissue. Adipose tissue stem cells (ASC) are isolated from the stromal vascular fraction (SVF). SVF is heterogeneous and contains ASC, endothelial cells, fibroblasts, leukocytes, pericytes, and pre-adipocytes, among others [156, 202].

Despite the high volume of publications in recent years, the procedure for collecting and isolating ASC requires better standardization of the forms of manipulation of the collected tissue in order to optimize and unify the processes [40, 182]. For isolating cells from liposuction, enzymatic and nonenzymatic methods may be used. After centrifugation, the SVF corresponds to the pellet present in the aqueous fraction of the enzymatically digested lipoaspirate or to the nontumescent non-oily fat fraction of the nonenzymatic mechanical disrupted lipoaspirate [47]. The choice of the method may interfere with the phenotype and biology of the isolated cells.

The enzymatic digestion is the method adopted by most research groups and represents a modification of the methods initially described by Rodbell and colleagues [150]. In brief, adipose tissue is minced, digested with collagenase and fractionated by differential centrifugation, and the pelleted SVF cells placed in culture [133, 148–150]. This method is particularly indicated for extracellular matrix (ECM) disruption and separation from binding adipocytes and other cells. Although effective, this method is complex, expensive, time-consuming, and yet more restricted to experimental approaches since the high manipulation of these cells makes their clinical use difficult [156].

As an alternative procedure, nonenzymatic methods that use mechanical (manual or in automated commercially available closed systems) forces appear to be promising for the therapeutic use of cells. The resulting product is a suspension composed of adipose tissue micro fragments, suspended cells, growth factors, and ECM components of the original adipose tissue. The nonenzymatic methods are faster and easier for handling than enzymatic digestion and can be performed inside the operating room, besides guaranteeing the maintenance of structural integrity of the cells [9, 23, 47, 169, 181].

Similar to BM-MSC, ASC are characterized by the expression of a range of surface markers, such as CD29, CD90, CD105, CD73, and CD44, and the absence of the expression of CD45 and CD31 [65]. Besides, according to the joint statement of the International Federation for Adipose Therapeutics and Science (IFATS) and the International Society for Cellular Therapy (ISCT) to establish minimal definitions of stromal cells and avoid confusion in the use of the terms ASC, the culture adherent stromal/stem cells population, and the heterogeneous uncultured SVF (that cannot be called stem cells), ASC can be distinguished from BM-MSC by their positivity for CD36 and negativity for CD106. Moreover, the IFATS/ISCT joint statement recommends the immunophenotype characterization of SVF and ASC as follows: CD13, CD29, CD44, CD73, CD90 (>40%), and CD34 (>20%) as primary positive markers for SVF and CD13, CD29, CD44, CD73, CD90, and CD105 (>80%) for ASC. CD34 is also considered a primary, although unstable, positive marker for ASC, as its expression can be found in freshly isolated cells but disappears when they are expanded in culture. As negative markers CD31 (<20%), and CD45 (<50%) are considered for SVF and, CD31, CD45, and CD235a (<2%) for ASC. Other secondary positive, such as CD36, or negative, such as CD3, CD11b, and CD106, markers may also be considered for ASC [28].

Regarding the cell culture protocols, the employment of commercial media (e.g., DMEM and aMEM), routinely used for ASC expansion, and two supplements, fetal bovine serum (FBS) and human platelet lysate, results in satisfactory conditions. Of note, platelet lysate provides the highest isolation and proliferation rates and commitment for osteogenic lineage. The hematopoietic support is performed through a constant secretion of G-CSF and SCF [134].

Nevertheless, it is important to highlight that most of the described culture settings are not actually xeno-free and are instead research-grade conditions. Therefore, they need to be adapted to attend good manufacturing practice (GMP) or good tissue practice (GTP) conditions following regulations and standards for quality and safety clinical applications.

2.6 Advantages and Disadvantages

The biggest hurdles for stem cell research before the discovery of iPSC have been ethical concerns and legal restrictions for working with stem cells of embryonic origin. Worldwide, the work with ESC is heavily regulated and sometimes excluded

from national funding programs. In most cases, it is not allowed to generate embryos solely for research purposes and ESCs have to be obtained from surplus embryos from in vitro fertilization procedures with the consent of the donors. Nonetheless, many ethical concerns remain. The discovery of iPSC has relieved many of these aforementioned problems. Seeing as donors for iPSC can give their informed consent for the generation of iPSC, and there is no longer any need for the destruction of human embryos for the generation of stem cells.

Furthermore, using donor-specific somatic tissue for stem cell manufacturing allows for the application of autologous stem cell-derived tissues and therapies, minimizing the need for post-intervention immunosuppression. One of the major advantages of ESC and iPSC is simultaneously a chief disadvantage of stem cell technology: the inherent self-renewal capacity. By culturing cell lines over long periods, sometimes for multiple decades, not only may cell characteristics change, but there is also the risk of acquired genetic abnormalities and the resulting potential for tumorigenesis in the recipient. Moreover, certain induction vectors carry the risk of genome integration further increasing the risk for genetic aberrations in resulting cells. This is best addressed by building a cell bank with a low passage number as well as regular quality checks of cultured stem cells (e.g., karyotyping, genotyping).

The main advantages of adult stem cells are their ready availability for therapeutic applications and the possibility of autologous transplantation, in addition to lower ethical concerns and risk of oncogenesis. Also, they exert significant paracrine effects that allow their widespread use in several different medical conditions, besides the possibility to be used as biofactories. Most of them are also easily expanded in vitro, besides to be possible to improve their functionality ex vivo before transplantation. This, however, adds a drawback to the cost and increases the possibility of contamination with other products. One significant disadvantage, when compared to ESC or iPSC, is the restricted differentiation potential of adult stem cells. Furthermore, the possibility of impaired cell function, especially concerning donor morbidity, and even disease transmission, represents a hurdle to be overcome as new methods and technologies for cell improvement are being generated.

Finally, despite the considerable advances in our understanding of stem cell biology and the harnessing of their potential as cell therapy, much progress still has to be made in order to standardize protocols for isolation, manipulation, and identification of cells suitable for therapeutical purposes.

2.7 Clinical Applications and Future Perspectives

While ESC- and iPSC-based therapies hold great promise, only a minimal number of clinical trials have been undertaken. Most were targeted toward the treatment of retinal degenerative diseases due to the easy accessibility of the retina and its immunologically privileged status [120, 163]. Some progress has also been made toward cardiovascular stem cell treatments. One animal study in mini pigs tested the

survival of human iPSC-derived cardiomyocytes in combination with an omentum flap and could show improved survival of the transplant as well as increased vascular density [93]. After iPSC-CM/omentum transplantation, mini pigs received a triple immunosuppressive therapy consisting of tacrolimus, mycophenolate mofetil, and corticosteroid, due to the procedure constituting xenotransplantation. In another study, mice received a stem cell treatment 48 h after myocardial infarction and were intramyocardially injected either with iPSC or iPSC-derived extracellular vesicles (iPSC-EV) [4]. While both groups demonstrated improved left ventricular function, iPSC-EV was more effective than iPSC alone. This highlights the importance of contributing paracrine effects in stem cell treatments, especially for regenerative therapies. Another study showed similar results using an ESC-based approach [95]. However, ESC-derived cells and ESC-derived extracellular vesicles displayed comparable improvements in left ventricular function. Moreover, in 2018, the first human trial for cardiovascular disease took place. Patients with severe ischemic heart disease undergoing bypass surgery received an epicardial ESC-cardiomyocyte patch with the primary endpoints being feasibility and safety, which were both successfully reached [128].

Even as there have been many fears about the safety profile of ESC- and iPSC-based therapies, no serious adverse reactions have been observed in clinical trials so far. Still, safety concerns need to be taken seriously as showed by the discontinuation of a Japanese iPSC-based clinical trial conducted at the RIKEN institute due to irregularities in the iPSC-derived retinal cells intended for transplantation. This reinforces the need for rigorous and standardized quality control.

While the number of performed trials is still small, they have proven the feasibility of both ESC- and iPSC-derived therapies in multiple fields of medicine. Recently a novel iPSC line has been established which lacks major histocompatibility complex I and II and is thus hypoimmunogenic. Although this is a promising approach, the potential for tumorigenesis is increased and this issue needs to be addressed, e.g., by adding a “suicide switch” to the cells.

Regarding adult stem cells, most studies in which those cells are used as strategies for therapies focused on vascular diseases use the fraction of MNC in autologous transplants from BM and PB after stimulation of G-CSF mobilization [143]. Currently, new sources of SCs have been proposed, in addition to cell populations with different immunophenotypic characteristics, including $CD34^+$ cells, tissue repair-associated monocytes ($CD14^+CD45^+$), high aldehyde dehydrogenase (ALDH)-activity progenitors, and expanded MSC [143]. However, the challenges in this area hinder advances related to vascular therapy focused on vascular diseases, among them lack of standardization of isolation and culture protocols that prevent comparisons between research groups, and the necessity of an effective immunophenotypic characterization of cells used in therapies.

In this section, we will discuss the advances associated with the use of stem cells for the clinical treatment of vascular diseases, as well as the challenges and perspectives for this field. We hope that the information will foster discussions aimed at improving processes that allow advances in the field of vascular regenerative medicine associated with better understanding and use of stem cells.

2.7.1 Peripheral Arterial Disease (PAD)

Peripheral arterial disease (PAD) is a term often used to refer to the lower extremity arterial disease secondary to atherosclerotic narrowing or occlusion of the arteries, which results in a decline in blood supply to the limbs. This disease is the third leading cause of cardiovascular morbidity related to atherosclerotic disease after coronary diseases and stroke and is associated with life and limb-threatening complications. Patients with PAD may be asymptomatic or may have symptoms such as intermittent claudication (pain in the lower limbs during walking) or pain at rest with or without ulcers or gangrene.

In the worst scenario, when available therapies (whether pharmacological, endovascular or surgical) are not effective or are not indicated, amputations of digits and limbs are common. Angiogenic therapies haves been considered as novel attempts to direct stimulate the revascularization of the affected limb. Among the possible approaches, cell therapy has been evaluated experimentally [25].

MNC derived either from BM or PB have been used in models of hindlimb ischemia and in patients with PAD. Although the injection of autologous BM-MNC in the gastrocnemius of patients with ischemic limbs have reduced rest pain and increased transcutaneous oxygen pressure, there was no significant effect of the cell therapy on amputation rate [173]. The selection of MNC subsets with angiogenic or cytoprotective properties could be an interesting approach for further development of cell therapy strategies in PAD patients [64].

In a double-blind study, autologous BM-derived aldehyde dehydrogenase bright ($ALDH^{br}$) cells were injected in the leg of patients with symptom-limiting intermittent claudication. $ALDH^{br}$ administration did not improve peak walking time (PWT) or capillary perfusion measured by magnetic resonance imaging (MRI) outcomes. The benefits of cell therapy were more apparent in patients with completely occluded femoral arteries, which showed an increased number of collateral arteries [138]. The selection of higher doses or treatment of patients with different clinical characteristics remains to be investigated.

Despite some benefits of cell therapy in PAD treatment, the efficacy of these cells on all endpoints was no longer significant in placebo-controlled studies, and the current knowledge does not support the effectiveness of cell therapy in patients with PAD. However, treatments that bring improvement in symptoms and that benefit, even temporarily, patients with PAD should be encouraged. For this, refinement of isolation techniques, administration routes, parameters to be evaluated, testing different doses, and selection of the source and phenotypic profile of the cells to be used may help in the improvement and response of the treatment.

Chronic limb-threatening ischemia (CLTI) is the end stage of PAD and shows higher rates of limb amputation, mortality, and impaired quality of life. CLTI is a clinical syndrome characterized by rest pain, gangrene, and/or lower limb ulcerations longer than 2 weeks. Venous, traumatic, embolic, and nonatherosclerotic etiologies are excluded. Since 2013, by the time of the launch of the Global Vascular Guidelines initiative, CLTI is considered the first priority disease area of focus for vascular specialists [42].

Treatment options for patients with CLTI are limited to surgical procedures that include arterial reconstruction, endovascular therapy, and limb amputation. Moreover, patients with severe comorbidities and limb gangrene/sepsis are often not eligible for surgical revascularization procedures. Stem cell-based therapies for limb amputation prevention in these patients have been encouraged [6, 46]. Indeed, in the above mentioned guidelines [42], stem cell therapy was identified as one of the key research priorities to advances the management of CLTI.

Although promising clinical results based on cell therapy still show more discreet results when compared to preclinical studies, a meta-analysis indicates that cell therapy in CLTI is associated with a reduction of the risk of a major amputation, identifying it as a promising strategy in the management of the disease [39, 43, 109]. Strategies such as the search for new sources of stem cells and the possibility of allogeneic transplants and cell enrichment may lead to the increase and success of cell therapy aimed at treating patients with CLTI. Besides, the ability of pluripotent stem cells in differentiating into endothelial cells should be assessed in the clinic since neovascularization processes are essential to the CLTI prognostic [115].

The intramuscular transplantation of autologous BM-MNC or G-CSF-mobilized PB-MNC in CLTI patients has shown promising results associated with the improvement of the ankle-brachial index (ABI) and tissue oxygenation compared to cells administrated in the contralateral leg [83, 173]. Besides, pain and ulcer reduction in the follow-up of intramuscular BM-MNC transplantation has been reported [124]. Adverse effects related to intramuscular injection include the only transient presence of the graft after intramuscular injection, with poor survival and retention of the cells in the ischemic tissue and poor integration into the host vasculature [32, 183].

Intra-arterial administration of BM-MNC arose on the premise that stem cell delivery with hematopoietic potential would bring benefits to ischemic tissue since cell delivery would occur more effectively through well-vascularized areas potentiating their angiogenic effects [143]. Studies using this approache have shown improvement in clinical parameters related to resting pain and ulcer pattern compared to the placebo group [184, 189]. However, cell therapy did not reduce the incidence of limb amputation when compared to control groups in two independently performed studies [184]. Strategies that could optimize the efficacy of cell delivery are required to improve the efficiency of cell therapy in vascular disease. Alternatives combining intramuscular and intra-arterial modes of delivery could improve cell graft survival along with more refined techniques with more specific and guided microinjections.

The transplantation of a more homogenous cell population has been suggested to improve cell survival. Additionally, a better definition and characterization of transplanted cells should be encouraged for comparisons and replicability in other studies. Furthermore, the development of strategies seeking to minimize and treating comorbidities to CLTI should be emphasized and is central to improving the success of cell therapy [143].

Another question to be considered concerns the so-called stem cell exhaustion in case of autologous transplantation. Meaning that in end-stage CLTI patients, the functions of stem cells and their progenitors could be compromised or in some

cases, these cells could be barely present. Thus, the continuous search for new methods of cell enrichment and enhancement of functionality, for alternative stem cells sources and for improvements in allogeneic and xenotransplantation are crucial [89, 174].

One example of a promising alternative to cell therapy is the use of extracellular vesicles (EV) containing potent proangiogenic agents, exosomes, and microvesicles in the management of CLTI. These strategies aim to promote a microenvironment where cell-to-cell communication is most effective in promoting the development, growth, and maturation of new blood vessels [177]. The injection of $CD34^+$ cell-derived exosomes into the ischemic hindlimb in a preclinical model showed enhanced limb perfusion via the upregulation of important proangiogenic molecules such as VEGF, ANGF1, ANG2 and MMP9 [123]. Also, MSC-derived EV increased blood reperfusion and stimulated the formation of new blood vessels in a preclinical murine model of hindlimb ischemia via overexpression of VEGFR1 and VEGFR2 in endothelial cells [67].

Other shortcomings to be addressed concern the delivery systems and stem cell survival after administration. Both intramuscular and intra-arterial injections may be inefficient because they do not deliver cells to the target site due to inefficient host vasculature [39]. Moreover, studies on the microenvironment at the injection site, as well as dosage and frequency of cell therapy, should be conducted. Complementary approaches using biocompatible scaffolds may also represent interesting experimental strategies.

2.7.2 Diabetic Foot Ulcers (DFU)

Diabetic foot ulcers develop as a result of the progressive and cumulative effects of longstanding diabetes that significantly disturbs the wound healing process [26]. The pathophysiology of DFU is complex and multifactorial and requires interventions able to accelerate and improve healing. In this sense, cell therapy emerges as a promising strategy to ameliorate the severity of foot ulcers in diabetic patients.

Many authors have demonstrated the benefits of stem cell therapy to ischemic and wounded tissues by secretion of growth factors and chemokines that promoting neovascularization and tissue remodeling [91, 195]. In a study comparing the effects of BM-MNC and PB-derived progenitor cell therapies in patients with diabetic foot disease and CLTI unresponsive to revascularization, for example, a lower rate of amputation and improved oxygenation and blood flow at the ulcer site have been observed [54]. These results are comparable to percutaneous transluminal angioplasty (PTA) and prove that cell therapy is safe and may constitute a new therapeutic approach for the treatment of diabetic ulcers [55]. In another study, autologous $CD90^+$ BM-derived cells were used in the treatment of diabetic ulcers. Parameters such as ABI, $TcPO_2$, reactive hyperemia, and angiographic imaging before and after therapy were taken. Improved microvascularization and complete wound closure in most of the patients who received cell therapy were observed [98].

A meta-analysis conducted by Zhang et al. [205], including randomized controlled clinical trials, suggested that cellular therapy could accelerate the healing of DFU, associated with a higher ankle-branchial index, higher transcutaneous oxygen pressure, higher ulcer healing rate, the lower reported pain levels and higher amputation-free survival. However, the authors draw attention to the limitations of the study that includes only a small number of papers and a lack of standardization in the execution and evaluation in the underlying trials, possibly limiting the conclusions drawn by the meta-analysis.

Therefore, although preclinical and clinical studies indicate the benefits of cell therapy on DFU treatment, the meta-analysis highlights the lack of a consensus regarding the optimal type of stem cell to be used, therapy regimen, and protocols to deliver cells properly. More effective delivery methods would improve the rates of success [113].

2.7.3 Venous Leg Ulcer (VLU)

Venous leg ulcers (VLU) arise from chronic venous insufficiency in the lower limbs and are widespread and debilitating, with high morbidity and associated costs, straining healthcare budgets and negatively impacting quality of life. The treatment of VLU can be conservative or surgical, but they are quite resistant to healing with standard care compression therapy and have high recurrence rates often leading to chronicity [92, 164].

The use of stem cells in VLU has been based on their capacity to stimulate wound healing through two mechanisms: attenuating the general inflammatory response and differentiating into cells involved in tissue repair, such as fibroblasts, myofibroblasts, and antigen-presenting cells [157].

In recent years, the use of adipose tissue as a source for cell therapy has been explored. The variety of cell types found in SVF represents a potential advantage for wound healing compared to cultured stem cells. Although the current clinical trials are significantly different in terms of study design and included subjects, most of them demonstrate the safety of ASC and SVF, as well as the improvement of chronic ulcers and reduction of pain [81]. A study involving 31 patients who had undergone surgery for an underlying venous pathology where venous ulcers had not healed post-surgery were treated with adipose-derived autologous stem cell injection at the ulcer site. Cell injection induced ulcer contraction and epithelization, even though no full closure was observed. In the follow-up, only three patients exhibited a recurrent ulcer. No adverse events were reported [92].

The ability of autologous SVF in treating chronic ulcers of venous (VLU) and arterial-venous (AVLU) origin were studied by Konstantinow et al. [100]. The patients received a single topical treatment with noncultured 9–15 $\times$ 10^6 cells isolated from abdominal lipoaspirates by enzymatic digestion. All VLU and four of nine AVLU patients who received the cells showed complete epithelization of the ulcers within 71–174 days. A considerable reduction in the intensity of pain and no severe side effects

were observed. The authors emphasize, however, that one-time application may not be sufficient in patients with larger predominantly ischemic AVLU and comorbidities.

2.7.4 Lymphedema

Lymphedema represents a debilitating condition manifesting as an excess of lymphatic fluid and swelling of subcutaneous tissue due to obstruction, destruction, or hypoplasia of lymphatic vessels [179]. Besides, lymphedema is a common complication with breast cancer treatment and does not have a definitive cure despite several microsurgical techniques and conservative management used in clinical practice [117].

Lymphedema treatment requires restoration lymphatic vessels from the capillary to the collector level and stem cell therapy could be an effective strategy to induce a complex regenerative response in patients affected by disorders of the lymphatic system. The transplantation of BM-derived cells in a murine skin flap wound model, for example, promoted the growth of blood vessels and lymphatic capillaries at the injury site and restored lymphatic drainage. The xenotransplantation of human cells into mice was also able to improve survival and functional reconnection of lymph nodes transplanted to the host lymphatic network, enhancing lymphatic vascular supply [20]. Given this evidence, cell therapy may be promising for improving lymphatic circulation and treating lymphedema in the clinical setting.

In another example, autologous G-CSF-mobilized $CD34^+$ cells were used for the treatment of lymphedema secondary to mastectomy and axillary lymphadenectomy. The cells were administered via microinjections in the affected arm, followed by a 12-week follow-up. Volume reduction of lymphedema compared to standard therapy (compression sleeves) was observed as well as pain reduction and improved sensitivity [117]. In contrast, a more recent study using autologous adipose-derived regenerative cells for treating breast cancer-related lymphedema reported no improvement in lymphoscintigraphy, despite patients requiring less conservative management after transplantation. No serious adverse effects were observed [180]. The authors conclude that more refined, randomized studies should be conducted to confirm findings and propose improvements in cell therapy applicable to lymphedema.

2.7.5 Thromboangiitis Obliterans (TAO): Buerger Disease

Thromboangiitis obliterans (TAO) or Buerger's disease is a nonatherosclerotic, segmental inflammatory disease affecting small- and medium-sized arteries and veins in the upper and lower extremities [187]. The pharmacological treatment of TAO focuses on anticoagulation, vasodilators, systemic anti-inflammatory drugs, and analgesics. Besides, surgical options are limited in efficacy and the absence of distal vascular targets makes surgical revascularization complicated. Cellular therapy

represents a potential alternative treatment for TAO patients since benefits associated with this modality include more rapid angiogenesis, reduced inflammation, increased temperature and perfusion of ischemic limbs and healing rates of wounds size [122].

The use of adult human BM-derived, cultured, pooled, allogeneic MSC is safe when injected via intramuscular (i.m.) route in TAO patients [71]. In a phase II, prospective, nonrandomized, open-label, multicentric, dose-ranging study, the same authors also tested the efficacy and safety of i.m. injection of adult human BM-derived MSC as a treatment of TAO disease. Reduction in rest pain and improved ulcer healing were demonstrated in the group receiving cell therapy compared to the control group. Few adverse effects were reported, indicating the possible use of cell therapy in TAO treatment [72].

When G-CSF-mobilized PB cells were subcutaneously injected close to the tibia bone, 26 out of 34 treated TAO patients showed a moderately improved outcome. Among them, 13 out of 17 limb ulcers healed. The development of new collaterals was also observed, indicating that autologous cells could be used safely and effectively for therapeutic angiogenesis in patients with TAO [97]. Corroborating these findings, a study involving 67 patients with symptomatic TAO that received autologous whole BM cells into the limb by intramuscular injections, showed clinical and angiographic improvements in almost half of the patients evaluated. Reduction in the amputation rate in symptomatic TAO patients was also a critical prognostic factor considered in this study [106].

Intravenous allogeneic MSC administration has also been explored since it could exert a systemic anti-inflammatory effect in the vasculature and modulate the immune response in TAO. In the case of a single male patient at risk of amputation, four sequential intravenous infusions of BM-MSC from a healthy donor induced significant regression of foot ulcers and improvements in rest pain, walking impairment, and quality of life. 16 months after infusion, the patient had no requirement for further amputation, indicating a potential for sequential infusions as an effective schedule treatment for TAO [122].

2.7.6 Myocardial Infarction (MI)

Cardiovascular events as a consequence of ischemic heart disease represent the leading cause of morbidity and mortality worldwide. Although medical and surgical treatments can improve patient outcomes, no treatment currently available can generate new contractile tissue or reverse ischemia in the myocardium [116]. In this context, the use of stem cells has emerged as a promising and potential therapeutic strategy to regenerate damaged heart tissue as an option for myocardial infarction (MI) treatment.

The use of BM-derived cells, adipose tissue-derived stem cells, skeletal myoblasts, as well as embryonic stem cell-derived cardiomyocytes had been proposed

as cardiac cell therapy substrates [104]. An ideal cell therapy applicable to MI should regenerate the vascular network and stimulate the formation of new contractile tissue that can align and synchronize with the existing heart tissue. Also, stem cells should be able to differentiate into other cardiac cell types such as myocytes and vascular endothelial cells or, at least, act via paracrine effects to promote the regenerative process [116].

Pericardial adipose-derived stem cells, for example, were shown to be superior in inducing reparative activities, including myogenesis, vasculogenesis, and expression of cardiogenic transcription factors compared to subcutaneous stem cells, indicating that cell origin is also essential to the outcome in cell therapy applicable to MI [191].

On the other hand, early clinical trials using BM-derived cells only show modest or marginal benefits when cellular therapy was used in acute or chronic MI patients [1, 111]. However, more recent studies have shown promising results with patients experiencing beneficial cardiac effects such as enhanced perfusion, improved left ventricular ejection fraction, and reduced left ventricular end-systolic volume [135]. The differences in outcomes from studies using BM-MNC could be explained by the inter-individual heterogeneity of this cell population. Of note, a clinical trial using an intramyocardial injection of cardiopoietic BM-MSC in post-MI ischemic heart failure patients showed a favorable effect on left ventricular ejection fraction (LVEF), remodeling and overall patient wellness compared to unstimulated BM-MSC or standard clinical care. The cardiopoietic BM-MSC were generated by priming BM-MSC using a combination of cardiogenic factors to transform these cells into cardiac progenitors able to differentiate in functional cardiomyocytes [17, 21].

Cardiac stem cells (CSC) might be more appropriate to promote heart tissue repair since these cells are residing in the heart itself. They are considered a heterogeneous cell population isolated from atrial appendages, pericardial adipose tissue, or epi-/endomyocardial biopsies that represent a purer source of cells with the capacity to differentiate into cardiomyocytes [27]. CSC have been shown to more efficiently express cardiac markers and more effectively differentiate into cardiomyocytes in vitro and in MI murine in vivo models, emerging as the most effective cell source for cell therapy in MI [151]. The challenges of using these cells, however, concern their low availability, the invasive isolation, and the need for costly ex vivo expansion to obtain adequate cell numbers for injection.

Likewise, cardiosphere-derived cells (CDC) constitute a cardiac progenitor cell population isolated from atrial or ventricular biopsy specimens of patients undergoing heart surgery. After tissue processing and culturing, a fibroblast-like cell layer forms and can be further purified and cultured to form cardiospheres. Most of the expanded cells are $CD105^{+}$, and also express CD117/c-Kit, CD90, CD34, and CD31. These cells were also negative for the MDR1, CD133, and CD45 [129, 161]. Autologous and allogeneic intracoronary CDC transplantation induces myocardial regeneration with a decrease in scar size and an increase in viable and

functional tissue in patients with MI, and ischemic left ventricular dysfunction [35, 119]. Despite promising findings, the challenge of obtaining cardiac stem cells should be considered and standardization of isolation, and culturing protocols should be addressed.

2.8 Final Remarks

Although no ideal stem cell source is yet established for treating vascular diseases, there is an expected interest in clinical research using the transplantation of endothelial progenitor cells (EPC) as a potential approach to regenerate endothelial cells and blood vessels and to induce therapeutic angiogenesis. Even though very promising, the clinical trials have shown moderate to low improvements in humans when compared to animal preclinical studies. The clinical effectiveness may vary among patients, in part, due to different genetic and physiologic status and medical conditions. It is also important to highlight that what has been termed as "putative EPC" and used in clinical trials are, actually, a heterogeneous population of hematopoietic cells that may take part in neovascularization processes by paracrine supportive mechanisms. Nonetheless, the scientific community is getting closer to reaching a consensus on the bonafide endothelial stem/progenitor cells' identity. Additionaly, many efforts are being made using the differentiation of induced pluripotent cells into vascular progenitors for therapeutic purposes.

Beyond the efforts to identify actual EPC, the majority of clinical trials on cell-based transplantation for vascular diseases concentrates on using MSC with paracrine activity derived mainly from BM and AT. As discussed earlier in this chapter, MSC is a generic term for a variety of cell types derived from bone marrow and connective tissue that meet the minimum criteria formulated by the ISCT. The high interest in using these cells for therapeutic purposes relies on their angiogenic and immunosuppressive properties and tissue repair capabilities, besides the easy accessibility from BM, AT, or even UC. This makes them not only an attractive therapeutical option in autologous but also in allogeneic transplants. Furthermore, the already completed trials have shown that MSC are safe and seem to cause no significant side effects to the patients.

Finally, it is also important to keep in mind that even if the best cells are transplanted into a patient, arriving in a hostile environment, they are susceptible to fail. Therefore, the parallel modulation of the host environments also appears to be important in order to achieve long-term effects. Taken together, the fast-growing field of regenerative medicine is progressively paving the road for the future use of cell-based bioproducts for vascular diseases. These bioproducts may originate not only from cells and known modalities of transplantation as discussed in this chapter but also from bioengineering of cells and tissues and their use as an "off-the-shelf" medical product. Given the significant advances made in adult stem cell research in conjunction with the, as of now, tentative entry of both ESC- and iPSC-based therapies into clinical practice, the future seems very promising. The ground is fertile, and much remains to come.

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