Chapter 2
Novel Therapeutic Approaches in Regenerative Medicine—Adult Tissue-Derived Very Small Embryonic-like Stem Cells and Harnessing Paracrine Signals of Adult Stem Cells

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2.1 Introduction

Regenerative medicine is searching for a reliable source of pluripotent stem cells (PSCs) with potential to give rise to cells from all three germ layers that could be employed for therapy. For almost 20 years, there have been attempts to harness embryonic stem cells (ESCs) isolated from embryos generated by in vitro fertilization [1–4] or by therapeutic cloning [5, 6] in a variety of preclinical animal models. This strategy, however, encounters some ethical considerations [7–9]. A novel promising source of PSCs are induced pluripotent stem cells (iPSCs) generated by genetic modification from adult cells, however again, this strategy is still under development and bares a risk of teratoma formation by injected cells [10, 11] as well as their rejection by a host immune system [12].

Therefore, in the meantime, the various types of adult stem and progenitor cells isolated from bone marrow (BM), mobilized peripheral blood (mPB), umbilical cord blood (UCB), or derived from expanded in vitro cultures of adherent cells such as mesenchymal stem cells (MSCs) or multipotent adult stem cells (MAPCs) are employed in clinical trials to regenerate damaged organs (e.g., heart, kidney, or neural tissues) [13–15]. It is striking that, for a variety of these adult tissues-derived cells, the currently observed final clinical outcomes of cellular therapies are often similar. This fact and the lack of convincing documentation for a robust donor-recipient chimerism in treated tissues in most of the patient studies indicates that a mechanism other than transdifferentiation of cells infused systemically into peripheral blood or injected directly into damaged organs as therapeutics may play an important role [16–18].

Overall, the rare cases of low level of chimerism observed after infusion of donor BM, UCB, or mPB cells reported by some investigators could be explained by cell fusion [19] or presence of rare populations of stem cells present in adult organs that

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are endowed with multi-tissue differentiation [20–23]. Therefore, one of the most intriguing questions in stem cell biology is whether pluripotent stem cells (PSCs) able to differentiate into cells for all three germ layers or multipotent stem cells (MultSCs) able to differentiate into cells for two different germ layers exist in adult tissues. Several groups of investigators employing (1) various isolation protocols, (2) detection of surface markers, and (3) experimental in vitro and in vivo models have reported the presence of cells that possess a pluripotent/multipotent character in adult tissues. Such cells were assigned various operational abbreviations and names in the literature (e.g., multipotent adult progenitor cells (MAPCs) [22], multipotent adult stem cells (MASCs) [21], unrestricted somatic stem cells (USSCs) [24], or marrow-isolated adult multilineage-inducible (MIAMI) cells [25]) and raised the basic question of whether these are truly distinct or overlapping populations of the same primitive stem cells. In fact, taking into consideration their common features described in the literature, it is very likely that various investigators have described overlapping populations of developmentally early stem cells that are closely related. Unfortunately, these cells were never characterized side-by-side to address this important issue. Moreover, the rare and quiescent population of so called very small embryonic-like stem cells (VSELs) isolated from murine tissues and human UCB initially by our group [26, 27] and subsequently isolated in other laboratories [28–31] expresses several markers of PSCs and shares some of the characteristics with above mentioned cell populations. As shown in Fig. 2.1, we envision that VSELs may contribute to donor-recipient chimerism and in this review we will discuss their potential role in regenerative medicine.

On the other hand, it is striking that, for a variety of stem cells such as hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs), or even unpurified mononuclear cells (MNCs), the observed final outcomes of cellular therapies are often similar and there is a lack of convincing documentation for robust donor-recipient chimerism in treated tissues in most of the patient studies. This indicates that a mechanism other than transdifferentiation of cells infused systemically into peripheral blood or injected directly into damaged organs may play an important role. Thus, we will also discuss the involvement of (1) growth factors, cytokines, chemokines, and bioactive lipids and (2) microvesicles (MVs) and exosomes released from cells employed as cellular therapeutics in regenerative medicine (Fig. 2.1). In particular, stem cells are a rich source of these soluble factors and in addition MVs that released from their surface may deliver various species of ribonucleic acid (RNA) and microRNA (miRNA) that are important for cell proliferation and survival into damaged organs [32–34]. Based on these phenomena, paracrine effects probably make major contributions in most of the currently reported positive results in clinical trials employing adult stem cells [35–37]. In this review, we will also discuss different strategies how these paracrine mechanisms could be exploited in regenerative medicine to achieve better therapeutic outcomes. This may yield critical improvements in current cell therapies before true pluripotent stem cells such as VSELs isolated in sufficient quantities from adult tissues and successfully expanded ex vivo will be employed in the clinic.

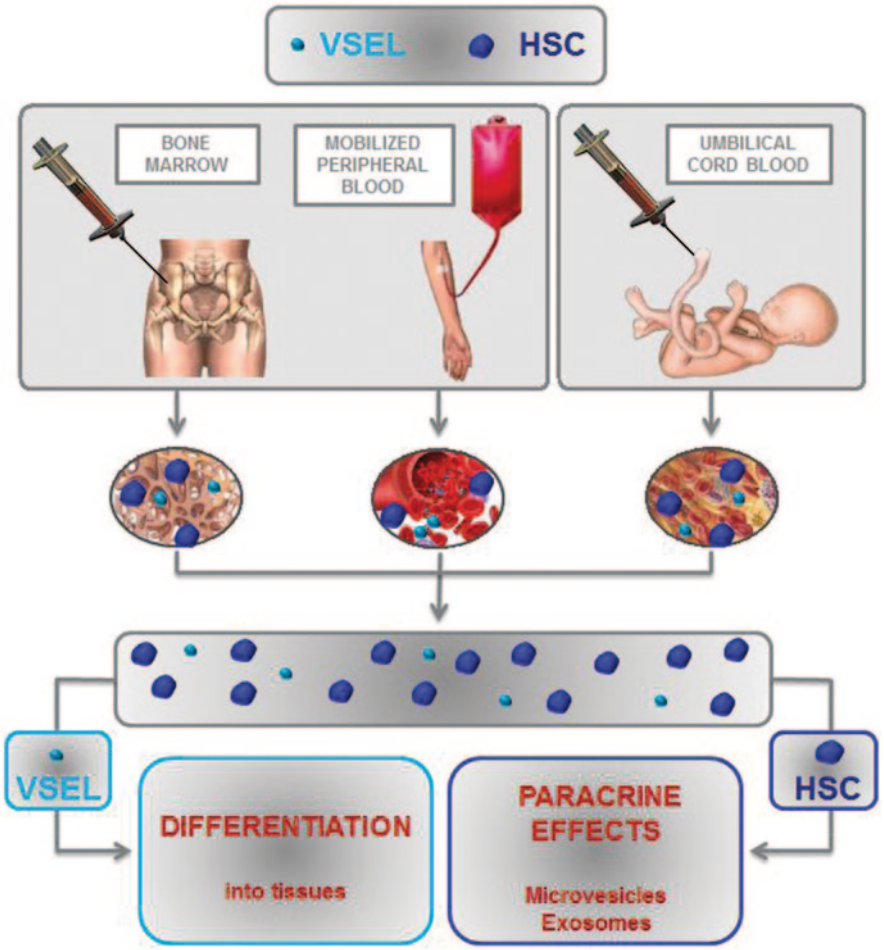

Fig. 2.1 The potential role of cells isolated from bone marrow, mobilized peripheral blood, and umbilical cord blood in regeneration of non-hematopoietic organs. The hematopoietic tissues are a relatively accessible source of cells that can be employed in regenerative medicine (e.g., hematopoietic stem cells (*HSCs*) or MSCs). We propose that the major beneficial effects of these cells, in regeneration of damaged organs, is based mostly on paracrine signals (e.g., secretion of growth factors, cytokines, chemokines, bioactive lipids, and miRNA and RNA transfer by MVs; *right panel*). In addition, cells isolated from adult tissues may contain a small admixture of true pluripotent stem cells (e.g., very small embryonic-like stem cells (*VSELs*)) that can differentiate into organ-specific cells. However, because of epigenetic changes in some of the imprinted genes, these cells have a locked cell cycle and remain quiescent (*left panel*). Without appropriate modulation of the expression of imprinted genes, their contribution to cells in damaged organs is not effective. PSCs must first be purified in therapeutically significant quantities from adult tissues and efficiently expanded ex vivo before they can be successfully employed in the clinic

2.2 Presence of Potential Pluripotent Stem Cells in Cell Preparations Derived from Adult Tissues

From a developmental point of view, an important question is why should PSCs may reside in adult organs? For many years, it has been accepted that adult tissues contain only tissue-committed stem cells (TCSCs), such as epidermal stem cells, hematopoietic stem cells, or skeletal muscle stem cells, that have a limited potential for differentiation. To address this question, we consider two scenarios that could occur during early embryogenesis and the development of lineage-restricted TCSCs [38]. In the first scenario, PSCs present in the inner cell mass of the blastocyst/epiblast, after giving rise to more differentiated lineage-restricted TCSCs, gradually disappear from the growing embryo and are not present in adult tissues. In the second scenario, which we believe is more likely to take place during embryogenesis, some PSCs give rise to TCSCs but some survive in adult tissues as a backup population of PSCs that renews the pool of TCSCs over time. In this second scenario, PSCs are precursors of TCSCs during organ/tissue rejuvenation and a source of these cells in emergency situations when organs are damaged (e.g., heart infarct or stroke). This scenario, however, requires such PSCs population deposited in adult tissues to be kept under control and in a quiescent state, which is essential to preventing uncontrolled proliferation leading to teratoma formation.

We envision that VSELs could be such population of PSCs that play a role as a backup population for more differentiated TCSCs [26]. These rare cells, which are slightly smaller than red blood cells, (1) become mobilized during stress situations into peripheral blood, (2) are enriched in the $Sca1^{+}Lin^{-}CD45^{-}$ cell fraction in mice and the $CD133^{+}Lin^{-}CD45^{-}$ cell fraction in humans, (3) express markers of pluripotent stem cells (e.g., Oct4, Nanog, and Stage Specific Embryonic Antigen (SSEA)), and (4) display a distinct morphology characterized by a high nuclear/cytoplasmic ratio and undifferentiated chromatin [26] called euchromatin. Furthermore, the recent evidence indicates that murine VSELs are kept quiescent in adult tissues and protected from teratoma formation by epigenetic modification of imprinted genes (e.g., erasure of differently methylated regions at Igf2-H19 and Rasgrf1 loci and hypermethylation at KCNQ1 and Igf2R loci) that regulate insulin/insulin-like growth factor signaling (IIS) [39]. As discussed later on in this review, the successful reversal of these epigenetic changes in VSELs that render them quiescent will be crucial for efficient ex vivo expansion of these cells. Moreover, the molecular analysis of adult BM-derived purified VSELs revealed that they share several markers characteristic for epiblast as well as migratory primordial germ cells (PGCs) [40]. In support of this, molecular analysis of murine BM-derived VSELs has revealed that these cells express several genes that are characteristic of epiblast SCs (*Gbx2, Fgf5,* and *Nodal*) and germ line specification (*Stella, Prdm14, Fragilis, Blimp1, Nanos3,* and *Dnd1*) [40].

Based on this, pluripotent VSELs that share epiblast/PGC markers as we hypothesize are deposited at the beginning of gastrulation in developing tissues and play an important role as a backup population for TCSCs [41, 42]. We hypothesize that VSELs play an important role in tissue/organ rejuvenation, and their proliferation

Table 2.1 In vitro and in vivo criteria expected from pluripotent stem cells and pluripotent status of murine VSELs

In vitro criteria for PSCs	*VSELs*
1. Undifferentiated morphology, euchromatin, and high nuclear/cytoplasm ratio	Yes
2. PSC markers (e.g., Oct-4, Nanog, and SSEA), open chromatin at the Oct-4 promoter, bivalent domains, and reactivation of the X chromosome in female PSCs	Yes Yes
3. Broad multilineage differentiation into cells from all three germ layers (meso-, ecto-, and endoderm)	
In vivo criteria for PSCs	*VSELs*
1. Complementation of blastocyst development	No
2. Teratoma formation in immunodeficient mice	No

VSELs very small embryonic-like stem cells, *PSCs* pluripotent stem cells

and potentially premature depletion is negatively controlled by epigenetic changes of imprinted genes that regulate IIS [39, 43]. On the other hand, the same epigenetic changes of imprinted genes keep these cells quiescent in adult tissues unfortunately prevent them from efficient ex vivo expansion in vitro.

The most recent data in vivo from our and other laboratories demonstrated that both murine [44] and human VSELs [45] exhibit some characteristics of long-term repopulating hematopoietic stem cells (LT-HSCs), are at the top of the hierarchy in the mesenchymal lineage [28, 31], may differentiate into organ-specific cells (e.g., cardiomyocytes and lung alveolar epithelium cells) [29, 46, 47], and in gonads are precursors of gamets [48, 49]. Moreover, as recently demonstrated, the number of these cells positively correlates in several murine models with longevity [43, 50, 51].

2.3 VSELs as Potential Pluripotent Stem Cells

There are stringent in vitro and in vivo criteria for classifying a stem cell as a PSC (Table 2.1). These criteria were established by embryologists who are working with ESCs or iPSCs [10, 52, 53]. However, some of these stringent criteria listed as in vivo criteria of pluripotency, such as complementation of blastocyst development and teratoma formation, are not always visible for example in pluripotent epiblast-derived stem cells [54, 55].

Our most recent experimental data support that murine VSELs fulfill all the in vitro criteria listed in Table 1. In particular, as mentioned above murine VSELs not only possess the primitive morphology of early developmental cells and express typical markers for PSCs (e.g., *Oct-4, Nanog,* and *Rex-1*) [26], but we recently confirmed the true expression of *Oct-4* in murine VSELs by the presence of an open-type chromatin in the *Oct-4* promoter by direct analysis of its methylation state [39] and association with transcription-permissive histones [39]. Specifically, we observed that the *Oct-4* promoter in murine VSELs is hypomethylated, and by employing the carrier-ChIP assay, we found that the chromatin in the *Oct-4* promoter

is associated with the gene transcription-promoting histone H3Ac while its association with the transcription-restricting histone H3K9me2 is relatively low [39]. Finally, amplified by reverse transcription polymerase chain reaction (RT-PCR) Oct-4 mitochondrial RNA (mRNA) has been cloned and sequenced to demonstrate that Oct-4 but not any of the Oct-4 pseudogenes has been amplified.

We also evaluated the epigenetic state of another core transcription factor, *Nanog*, and observed that its promoter has a higher level of methylation in VSELs (~50%). In quantitative ChIP experiments performed in parallel, we also observed that the H3Ac/H3K9me2 ratio favors transcription and supports an active state [39]. Based on these results, we conclude that murine VSELs truly express both of the embryonic transcription factors *Oct-4* and *Nanog* [39].

With respect to the other in vitro criteria of pluripotency (Table 1), murine VSELs also possess bivalent domains in promoters that encode developmentally important homeobox-containing transcription factors (*Sox21, Nkx2.2, Dlx1, Lbx1h, Hlxb9, Pax5,* and *HoxA3*) [56]. Furthermore, VSELs derived from female mice reactivate the X-chromosome. Finally, VSELs can differentiate in vitro into cells from all three germ layers [56]. Data published in our and other laboratories demonstrated that in appropriate experimental models murine BM-derived VSELs can be specified in vivo into HSCs [44, 45], mesenchymal stem cells (MSCs) [28, 31], cardiomyocytes [47], pneumocytes [29], and ovary-derived VSELs may give rise to oocytes [49].

However, on other hand taking into consideration the in vivo criteria expected from PSCs (Table 1), murine VSELs do not complete blastocyst development and do not grow teratomas. This discrepancy between in vitro and in vivo criteria of PSCs for VSELs has recently been explained by epigenetic changes in expression of some paternally imprinted genes [39]. Accordingly, we observed that VSELs, in a similar manner as late migratory PGCs, modify the methylation of imprinted genes, preventing them from uncontrolled proliferation and explaining their quiescent state in adult tissues [39].

It is well known that imprinted genes play a crucial role in embryogenesis, fetal growth, the totipotential state of the zygote, and the pluripotency of developmentally early stem cells [57, 58]. It has been demonstrated that VSELs freshly isolated from murine BM erase the paternally methylated imprints at regulatory differentially methylated regions (DMRs) within the *Igf2-H19* and *Rasgrf1* loci; however, they also hypermethylate the maternally methylated imprints at DMRs for the Igf2 receptor (*Igf2R), Kcnq1-p57^{KIP2},* and *Peg1* loci [39]. Because paternally expressed imprinted genes (*Igf2* and *Rasgrf1*) enhance embryonic growth and maternally expressed genes (*H19, p57^{KIP2},* and *Igf2R*) inhibit cell proliferation [59, 60], the unique genomic imprinting pattern observed in VSELs demonstrates the growth-repressive influence of imprinted genes on these cells [39] and may explain their quiescent state.

Thus, these results suggest that epigenetic reprogramming of genomic imprinting maintains the quiescence of Oct4^{+}epiblast/germ line-derived VSELs deposited in the adult body and protects them from premature aging and uncontrolled proliferation (e.g., teratoma formation). On the other hand, reversal of this mechanism

will be crucial to employing VSELs as a population of PSCs in regenerative medicine. Currently, we are testing whether downregulation of the expression of H19 enhances VSEL expansion, as has recently been demonstrated for PSCs derived by parthenogenesis [61].

Taking everything into consideration, we have to pinpoint that while murine BM-derived VSELs have been extensively characterized more work is needed to better characterize these small cells at the molecular level in humans. Nevertheless, similar morphology, pattern of expression of some genes regulating pluripotency (e.g., Oct-4, Nanog, Sal-4) in both murine and human VSELs, as well as differentiation of both murine and human VSELs into HSCs [44, 45] and MSCs [28, 31] suggests that these are corresponding populations of stem cells.

2.4 Paracrine Effects of Cells Employed in Regenerative Medicine

It is known that cells and in particular stem cells secrete a variety of growth factors, cytokines, chemokines, and bioactive lipids that regulate their biology in an autocrine/paracrine-manner and orchestrate interactions with the surrounding microenvironment [33, 62–64]. These factors are secreted, in particular, from activated cells removed from their physiological niches (e.g., aspirated from the BM or mobilized into peripheral blood, PB). Therefore, different types of cells, including HSCs, MSCs, skeletal muscle myoblasts (SMMs), adipose tissue stem cells (ASCs), neural stem cells (NSCs), or cardiac stem cells (CSCs) that are employed in regenerative medicine to rescue damaged organs are potential sources of paracrine factors. These factors may (1) inhibit apoptosis of cells residing in the damaged organs, (2) stimulate proliferation, and (3) promote vascularization of affected tissues to improve oxygen delivery and metabolic exchange. As reported the most important factors secreted from stem cells include vascular endothelial growth factor (VEGF), stem cell factor (SCF), hepatocyte growth factor (HGF), insulin-like growth factor-1 and −2 (IGF-1, -2), and stromal derived factor-1 (SDF-1) [33, 62, 65, 66].

In addition to soluble factors, activated stem cells also secrete MVs, which are small, spherical membrane fragments shed from the cell surface or secreted from the endosomal compartment and seem to play an important and underappreciated role in improving the function of damaged organs [34, 67–71]. A growing body of evidence suggests that MVs secreted, for example, from HSPCs, MSCs, ASCs, NSCs, or CSCs employed in various treatment strategies efficiently inhibit apoptosis of cells residing in the damaged tissues, stimulate their proliferation, and promote vascularization [66, 67, 72, 73]. These pro-regenerative effects mediated by MVs can be explained by the fact that these small, spherical membrane fragments (1) are enriched in bioactive lipids (e.g., sphingosine-1-phosphate [S1P], ceramide-1-phosphate [C1P]), (2) display several anti-apoptoic and pro-stimulatory growth factors or cytokines (e.g., VEGF or SCF) on their surface, and (3) deliver mRNA, regulatory miRNA, and proteins that improve overall cell function [32, 33, 65]. In

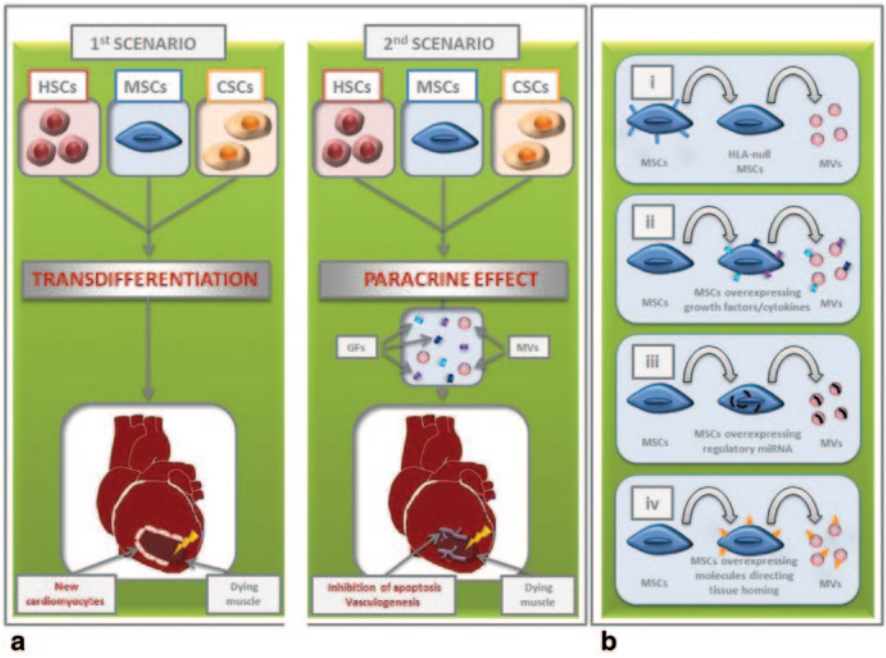

Fig. 2.2 Beneficial effects of hematopoietic stem cells (*HSCs*), mesenchymal stem cells (*MSCs*), or cardiac stem cells (*CSCs*) in regenerative medicine using heart infarct as a model. **a** *Left part, 1st scenario:* Cells employed for therapy may theoretically transdifferentiate into cardiomyocytes. However, if this occurs at all, it is a very rare and random phenomenon and is not well substantiated by current experimental data. *Right part, 2nd scenario:* Cells employed for therapy do not transdifferentiate into cardiomyocytes, but secrete several paracrine factors and shed microvesicles (*MVs*) that inhibit apoptosis in damaged cardiomyocytes, promoting their proliferation and stimulating angiogenesis. Evidence is accumulating that this is a major effect in currently employed stem cell therapies. **b** Different approaches to generating ex vivo more efficient pro-regenerative MVs. MVs could be harvested from large-scale in vitro cultures of MV-producing cell lines. Such cell lines may be modified to obtain MVs that *i* are less immune and do not express human leukocyte antigens (*HLAs*), *ii* are enriched in growth factors, cytokines, and chemokines that promote regeneration of damaged organs, *iii* are enriched in mitochondrial ribonucleic acid (*mRNA*) and regulatory microRNA (*miRNA*) facilitating regeneration of damaged tissues and/or promoting angiogenesis, and *iv* display molecules that direct them to, and subsequently retain them in, damaged tissues

support of this notion, several species of miRNA that regulate cell survival and angiogenesis (e.g., mir126 and mir130) have been identified in $CD34^+$HSPCs-derived MVs [66].

Such cell-derived paracrine signals may explain why, after applying various types of stem cells, the final therapeutic benefits are similar (Fig. 2.2a). For example, coronary infusion or local delivery of $CD34^+$or $CD133^+$HSPCs, MSCs, SMMs, or CSCs following heart infarct often yield a similar improvement in left ventricular ejection fraction [35–37]. This argues against significant transdifferentiation of cells employed for therapy into cardiomyocytes and strongly supports a role for paracrine effects [74].

These observations should prompt investigators to develop novel therapeutic strategies that harness these mechanisms to ensure more efficient outcomes than current therapies. This could be achieved, for example, by modification of cells employed in regenerative medicine to become better sources of paracrine signals. On the other hand, the therapeutic application of large-scale, modified MVs, instead of whole cells, is emerging as an exciting new concept in regenerative medicine. This could be achieved as will be discussed latter in this review by ex vivo manipulation of cells to enhance secretion of pro-regenerative factors and the development of novel therapeutic strategies in which large-scale, ex vivo-generated, modified MVs replace intact cells.

2.4.1 Increase in Release of Pro-regenerative Factors from Cells Employed in Regenerative Medicine

Since autocrine secretion of various soluble factors, as well as MVs, by stem cells can be increased during the stress response, one can envision different approaches to augmenting this phenomenon. Theoretically, this could be accomplished by (1) exposure of cells to hypoxia prior to infusion and delivery to the injured organ, (2) transduction of these cells by expression vectors that increase secretion of pro-angiopoietic factors (e.g., VEGF or FGF-2), (3) modulation of expression of miRNAs that regulate transcription of anti-apoptotic or pro-angiopoietic genes, and (4) exposure of these cells to complement cascade cleavage fragments or cytokines that promote autocrine release of appropriate paracrine peptides or bioactive lipids.

2.4.2 Development of Engineered MVs for Regenerative Medicine Therapies

Based on the fact that MVs have similar beneficial effects in regenerative therapy as the intact cells from which they are derived [67, 68, 72], it should be possible to produce MVs on a large scale and even to modify their composition. Several possibilities for how to modify MVs are shown in Fig. 2.2b**.** Overall, MVs for application in regenerative medicine could be isolated from appropriate generator cells (e.g., MSCs) expanded ex vivo.

First, as depicted in Fig. 2.2b, it should be possible to expand MV-producing cell lines that lack genes encoding histocompatibility antigens. This approach would minimalize the possibility of cross-immunization with donor human leukocyte antigens (HLAs). Second, MV producer cell lines could be transduced with genes that overexpress on the cell surface (1) peptides that protect target cells in damaged organs from apoptosis and stimulate proliferation of residual remaining cell populations (e.g., SCF or Notch ligands) or (2) factors that effectively induce angiogenesis (e.g., VEGF, FGF-2, or SDF-1). Third, we speculate that MVs derived from cells

in hypoxic conditions would be enriched in mRNAs and miRNAs that promote angiogenesis. Furthermore, producer cell lines could be enriched for mRNA and regulatory miRNA species that, after delivery to the damaged tissues, would promote regeneration. Finally, we envision that MV producer cell lines could be enriched for molecules that facilitate their tropism to the damaged organ and subsequently promote retention of MVs in the damaged tissues.

Conclusions

Current therapeutic strategies in regenerative medicine employing adult stem cells for damaged solid organs are mainly based on utilization of their paracrine effects by secreted soluble factors and MVs [72, 73, 75, 76]. Overall, paracrine effects and MV-based therapies also open up new possibilities for clinical applications of iPSCs. Since in vivo application of iPSCs is limited by the high risk of teratoma formation by these cells, MVs from patient-derived iPSCs could be employed as a novel generation of therapeutics to rescue damaged organs and tissues. Based on this possibility, we envision that patient-derived iPSCs could be employed as MV-producing cells. Moreover, taking advantage of the recently proposed epigenetic memory of cells employed for generation of iPSCs [77], one can also envision that, for example, MVs from keratinocyte-derived iPSCs would be preferentially enriched for mRNA and miRNA for epidermis stem cells and thus affect regeneration of damaged skin (e.g., after burns), or similarly MVs isolated from supernatants of cardiomyocyte-derived iPSCs would have by similar mechanisms advantages in regeneration of damaged myocardium.

However, beside paracrine effects of more differentiated adult stem/progenitor cells it is also a hope for clinical application of pluripotent/multipotent isolated from the adult tissues and new data from our group [26, 44, 45] and other groups [28, 47, 49] has provided more evidence on the existence of primitive embryonic-like stem cells in murine adult tissues and their potential role in (1) tissue organ rejuvenation, (2) longevity, and (3) regeneration/repair of damaged tissues. Nevertheless, while murine BM-derived VSELs have been extensively characterized, we are aware that more work is needed to better characterize small $CD133^{+}Lin^{-}$ $CD45^{-}$ cells at the molecular level in humans. We need to determine whether human VSELs have the same molecular signature (e.g., an open chromatin structure at the *Oct4* promoter, modification of somatic imprinting, and the presence of bivalent domains) as their murine counterparts. If we can confirm that a similar imprinting-related mechanism operates for human VSELs, perhaps a controlled modulation of the somatic imprinted state to produce proper de novo methylation of somatic imprinted genes on the maternal and paternal chromosomes could increase the regenerative power of these cells [39] and lead to their broader application in the clinic. Of note, recently National Institutes of Health (NIH) sponsored a first clinical trial to employ human BM-derived VSELs for treatment of periodontitis lesions in patients [http://www.neostem.com/news/vsel1mgrant.html].

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