Published in final edited form as:
Leukemia. 2011 November ; 25(11): 1674–1686. doi:10.1038/leu.2011.167.

Bone marrow stem cell mobilization in stroke: a 'bonehead' may be good after all!

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Abstract

Mobilizing bone cells to the head, astutely referred to as 'bonehead' therapeutic approach, represents a major discipline of regenerative medicine. The last decade has witnessed mounting evidence supporting the capacity of bone marrow (BM)-derived cells to mobilize from BM to peripheral blood (PB), eventually finding their way to the injured brain. This homing action is exemplified in BM stem cell mobilization following ischemic brain injury. Here, I review accumulating laboratory studies implicating the role of therapeutic mobilization of transplanted BM stem cells for brain plasticity and remodeling in stroke.

Keywords

stroke; cell therapy; migration; homing; neurogenesis; angiogenesis

Introduction

Bone marrow (BM) consists of a heterogeneous population stem and progenitor cells[1] (Figure 1), with hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs) as the two most studied BM-derived stem cells. Additionally, endothelial progenitor cells (EPCs) and very small embryonic-like stem cells (VSELs) have also been isolated from the BM. Previous reports of *in vitro* differentiation of BM-derived stem cells into neurons on exposure to various inducing regimens,[2] and their secretion of growth factors critical for neuronal survival,[3–5] have prompted interest in using BM as stem cell donor source for transplantation therapy in neurological disorders, such as stroke.

Stroke remains a primary cause of death in the United States and around the world. Over the last decade, stem cell therapy has shown promise as an experimental treatment for stroke.[6–8] Although the first clinical trial occurred in 1998,[9–11] only recently new clinical trials on cell therapy have been again initiated in stroke patients. In the clinic, a minimally invasive transplant procedure via intravascular route is thought to be practical. However, this peripheral route of cell injection requires mobilization of the cells and their secreted products proximal to the site of injury in order to afford brain plasticity and remodeling. A better understanding of mechanisms underlying the homing of cells from the periphery to the brain is likely to aid in optimizing cell therapy for stroke. Along this theme, I discuss the unique but sometimes overlapping mobilization pathways that guide the homing of HSCs,

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Conflict of interest
The author declares no conflict of interest.

MSCs, EPCs and VSELs from BM (and other stem cell niches) to blood and eventually into ischemic brain.

Hematopoietic stem cells

Repopulation of the ablated BM by HSCs has been arguably presented as the defining feature of HSC stemness[12] (Table 1). The quiescent HSCs, although low in number during homeostasis, can quickly become motile with increased migration from their resident BM to blood circulation in response to injury.[13–15] A key chemokine implicated in the quiescence of HSCs within the BM is stromal derived factor-1 (SDF-1, also termed CXCL12) acting via its major receptor CXCR4 (C-X-C chemokine receptor type 4),[13,46] which is also highly expressed in other stem cells niches, similarly playing the role of preserving the HSC primitive status.[12,46] It is originally found to be expressed by murine and human BM endothelial and endosteal bone-lining stromal cells,[47] and later also found in various tissues, including skin,[48] epithelial cells in human liver bile ducts[49] and brain endothelium.[50] In the hematopoietic system, SDF-1 is a crucial chemoattractant for CXCR4-expressing BM-derived cells, including HSCs. This SDF-1/CXCR4 chemoattractant pathway is essential for stem cell migration and seeding. When SDF-1 is activated, these stem cells migrate out from the BM reservoir to the circulation, replenishing the blood with new cells.[51]

The HSC exit from and re-entry to stem cell niches involve adhesion to the vascular wall and crossing over the endothelial blood–BM barrier.[12] The central nervous system (CNS) contributes to this HSC mobilization via cytokine production, and under stress condition (that is, stroke) can amplify the recruitment of HSCs into the brain.[12–15] This cytokine-mediated recruitment of HSCs from BM to circulation is an established clinical mobilization regimen, such as treatment with granulocyte-colony-stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF), for generation of ample supply of HSCs for transplantation.[13–15]

Neural control of migration of BM-derived HSCs

As noted above, CNS-induced cytokine cues may serve as migratory signals to HSCs. Recently, a crosstalk via the neurotransmitter catecholaminergic signaling pathway has been proposed to link the nervous system and the immune system.[12] The sympathetic system produces catecholamines, which are amplified during stress situations and secreted by activated leukocytes, including lymphocytes and lipopolysaccharide-stimulated macrophages, harnessing mobilization of BM-derived stem cells.[12] In particular, catecholamines, either through the sympathetic system secreting these neurotransmitters into the blood circulation or directly from the nerve endings in the BM acting via a paracrine fashion, can modulate the migration of BM from its residence to the injured CNS.[52,53] That this directed migration may entail a ligand-receptor mechanism is indicated by upregulated levels of catecholaminergic receptors in mobilized human CD34 HSCs compared with tissue-anchored BM human CD34 HSCs, with the catecholaminergic receptor expression further increased by repeated stimulation with the mobilizing agents G-CSF and GM-CSF. This homing process of BM-derived stem cells via the catecholaminergic neurotransmitter system involves multiple signaling pathways, including Wnt and β-catenin, as well as specific migratory molecules, such as membrane-bound enzyme membrane type 1 matrix metalloproteinase and SDF-1[53–56] that altogether contributes to the cell proliferation, increased motility and engraftment capabilities of human CD34 HSCs.

HSC regulation of neural function

The neurotransmitter-mediated interaction between CNS and BM is bidirectional, with a hematopoietic signaling mechanism equally contributing to HSC regulation of CNS

functions.[57] Accumulating scientific evidence advances the concept that human HSCs can affect the nervous system and modulate its action.[53] In the field of stroke, clinical data show that human acute stroke is followed by large and bursting mobilization of peripheral blood (PB) immature hematopoietic CD34+ cells, colony-forming cells and long-term culture-initiating cells,[58] and the extent of such mobilization is directly related to recovery of function.[59] The main postulated mechanism underlying HSC mobilization implicates the upregulation of SDF-1 within ischemic tissues of stroke individuals, which promotes CXCR4+ HSC recruitment from PB to the site of injury.[53] A multipronged neuroprotective and/or neurorestorative set of events closely precede HSC mobilization to the ischemic site, notably neoangiogenesis that parallels the therapeutic window of G-CSF treatment for stroke therapy.[60] The critical role of angiogenesis in HSC fate following ischemic injury is further supported by the observation that systemic administration of human CD34+ cells to SCID (severe combined immunodeficiency) mice exposed to stroke 48 h earlier induces neovascularization in the ischemic zone,[61] thereby creating a conducive microenvironment for survival of both exogenous grafts and endogenous stem cells, which are pivotal for neuronal regeneration. In addition to stroke, HSC mobilization to the brain, which may correspond to early host endogenous repair mechanism, is also seen in other neurological diseases; for example, elevated number of human BM CD34+ cells accompany patients with chronic spinal injury.[62] Similarly, not only BM-derived, but also cord blood (CB)-derived CD34+ cells have replicated the homing event and subsequent therapeutic benefits of HSCs in brain disorders. Human CB CD34+ cells injected to rats before[63] or during heat stress[64] significantly reduce symptoms of heatstroke and increase animal survival time by attenuating inflammatory, coagulatory and multiorgan dysfunction; transplantation of human CB CD34+ enriched cells into the injured spinal cords of rats produces a significant recovery of functional outcome and increases survival rate;[65,66] and injection of human CB mononuclear cells to mice with amyothropic lateral sclerosis delays disease progression and increases lifespan.[67] Recent studies have shown that systemic injections of human CB mononuclear cells in Alzheimer's disease animal model decrease parenchymal and cerebral vascular β-amyloid deposits, and increase microglial phagocytic activity,[68] whereas their transplantation in aged rats significantly enhances the hippocampal neurogenic niche characterized by rejuvenation of the aged neural stem/progenitor cells.[69] Taken together, the experiments above lend support to the notion that human HSCs play key regulatory roles in the maintenance of homeostasis and the repair of the nervous system. The crosstalk between the hematopoietic and the nervous system, and the resulting HSC mobilization to the site of injury and subsequent observation of therapeutic benefits, suggest their potential application for designing treatment strategies that cater to regenerative medicine. HSCs have been proposed to be an ideal donor graft source for cell therapy, especially those derived from BM, because of their safety and efficacy profile in the clinic for other disease indications such as for treatment of cardiovascular, bone, cartilage, bladder and liver dysfunctions.[70–72]

Mesenchymal stem cells

MSCs were initially reported by Friedenstein *et al.*,[73] referring to a population of plastic-adherent fibroblastic cells isolated by Percoll density centrifugation (see Table 1). Human MSCs (hMSCs) express CD105 (SH2), SH3, Stro-1 and CD13, but lack the hematopoietic surface markers CD34 and CD45. MSCs have the potential to differentiate into mesodermal cell lineages such as adipocytes, chrondroblasts, fibroblasts, osteoblasts and skeletal myoblasts both *in vitro* and *in vivo*.[20,74–77] hMSCs lack telomerase activity, with about 18 population doublings (PDs).[18] A study conducted at the single cell level by Muraglia *et al.*[20] demonstrates MSCs, with capability of differentiation into osteogenic, chondrogenic and adipogenic phenotypes up to 19 PDs, to lose their apparent proliferation potential at 22 to 23 PDs in ~80 days in culture. Strikingly similar to this study, clonal hMSCs cultured *in vitro* by Banfi *et al.*[21] are also found to gradually lose the differentiation potential when reaching

22 to 23 PDs after ~80 days of culture. The study of Kobune *et al.*[23] reveals that MSCs exhibit a reduced mitogenic activity after ~5 PDs over the course of ~6 weeks and undergo crisis at 16 PDs. Bruder *et al.*[19] demonstrate that the growth rate of MSCs decreases with passaging, in that the PDs for hMSCs before degenerating are around 38, even though the osteogenic differentiation potential is preserved. The cumulative population doubling level for hMSCs selected with serum-deprived medium is ~9 PDs at passage 10, whereas for hMSCs selected with regular medium, the cumulative population doubling level is ~7 PDs at passage 8.[22] Although evidence on the limitation of the differentiation and proliferation potential of MSCs exists, a report from Pittenger *et al.*[78] indicates that MSCs maintain telomerase activity in both early and late passage. The discrepancy here might be due to the fact that cell phenotypes isolated from different laboratories are distinct. Gene manipulation strategy has been utilized to overcome the senescent problem of MSCs. Hamada *et al.*[79–81] transfected MSCs with the human telomerase gene by retroviral infection to generate stable cell clones with high efficiency and low cell mortality. These cells, regarded as human telomerase reverse transcriptase (hTERT)-MSCs, can survive in culture for over 1 year and maintain their characteristic surface antigens as well as typical morphology. Cerebral infarction volume is significantly reduced and functional outcome is improved in hTERT-MSCs-treated stroke animal model.[80]

MSC transplantation in stroke

MSCs have been employed in experimental stroke models and shown to improve the functional recovery of neurological deficits induced by cerebral ischemia.[82–91] Clinical reports of MSC transplantation in stroke patients reveal that MSCs significantly improve the functional recovery of patients without adverse side effects.[82] The underlying mechanism remains unclear. The subsequent sections address some potential mechanisms that might mediate the therapeutic effects of MSC implantation in stroke.

MSC-mediated trophic factor secretion

The neuronal differentiation as the underlying mechanism for grafted MSCs to produce therapeutic benefits remains controversial. Moreover, transplantation of MSCs, via intravenous, intracarotid, or even intracerebral delivery, leads to very low, or at best modest, graft survival rate.[92] A far more reasonable explanation for the graft-derived beneficial effects is that MSCs secrete neurotrophic factors that may induce the host ischemic brain to activate endogenous repair mechanisms. Chopp *et al.*[93] show that MSCs produce hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF),[94] nerve growth factor,[95] brain-derived neurotrophic factor (BDNF),[93,95] basic fibroblast growth factor (bFGF, FGF-2)[96] and insulin growth factor-1 (IGF-1).[97] These neurotrophic cytokines have been implicated to play an important role in the process of neurogenesis and angiogenesis. For instance, the upregulation of VEGF and the VEGF receptor 2 (VEGFR2) at the impaired site could increase the number of enlarged and thin-walled blood vessels as well as newly formed capillaries at the ischemia border zone (IBZ).[47] Although the early increase (1 h after stroke) of VEGF could increase the blood–brain barrier (BBB) leakage and hence increase ischemia hemorrhagic transformation, and exacerbate ischemic cell damage, when administered 48 h after stroke, VEGF could enhance angiogenesis in the IBZ and significantly improve neurological recovery.[98] From hMSC administration to the secretion of VEGF by the graft cells, the time course highly exceeds 1 h after stroke; hence, VEGF here functions as the angiogenesis promoter and improves the functional recovery after stroke.

HGF has been demonstrated to ameliorate BBB destruction without exacerbating cerebral edema, decrease intracranial pressure and induce angiogenesis. Equally compelling evidence suggests that grafted MSCs promote neurogenesis. Over the years, laboratory studies have

established the subventricular zone (SVZ) and subgranular zone as remarkable brain neurogenic sites. The presence of MSCs in the brain has been shown to promote the induction and migration of new cells from these primary sources within the SVZ and the subgranular zone into the injured brain.[86,95,96] The newly differentiated cells appear as progenitor-like neurons and astrocytes. The process of neurogenesis is highly dependent upon the neurotrophic factors secreted by MSCs.[96] Upregulation of BDNF levels is proven to be capable of recruiting neural progenitors from the endogenous progenitors of the forebrain.[99] Recent reports show that bFGF is capable of exerting neuroprotective or vasodilating effects, as well as enhancing the proliferation of the neuronal progenitors.[100,101] To enhance the efficacy of MSCs, gene-modified MSCs have been used as donor cells for transplantation in stroke models. In these experiments, specific genes, such as *FGF-2/HGF/BDNF*, are transfected into the cells before they are transplanted into the brain. These grafted cells subsequently are able to express the target gene in the brain. Kurozumi *et al.*[81,102] transfected telomerized hMSCs with the *BDNF* gene using a fiber-mutant F/RGD adenovirus vector and transplanted these cells in stroke rats. BDNF production by MSC-BDNF cells is 23-fold greater than that seen in unmodified MSCs. Stroke rats that received MSC-BDNF displayed significantly better functional recovery than control stroke rats. This research group also conducted a parallel study in which they transfected MSCs with *glial cell line-derived neurotrophic factor* (*GDNF*), *ciliary neurotrophic factor* (*CNTF*) and *neurotrophin 3* (*NT3*) genes.[81] Stroke rats that received MSC-GDNF, but not those treated with CNTF and NT3, showed significantly greater functional improvement than control stroke rats. A study by Ikeda *et al.*[103] demonstrated that stroke animals that received bFGF-MSCs showed significantly better attenuations of behavioral impairments and infarction volumes than those that received nontransfected MSCs. These data suggest that bFGF enhances the beneficial effects of MSCs.

A relatively new addition to the list of growth factors exerting benefits in stroke is HGF. Ischemia animals treated both with and without HGF-modified MSCs exhibit improvement of neurological deficits, yet those that received HGF-MSCs treatment display superior therapeutic effect to the nonmodified MSC-treated group.[104] Although graft survival rate or neuronal differentiation appear to play minor roles in the MSC transplantation therapeutic effect, studies demonstrate that transplantation with MSCs that are committed to differentiate into a neuronal phenotype indicate better therapeutic effects. MSCs transfected with Notch intracellular domain and subsequently treated with bFGF, forskolin and CNTF have the potential to differentiate into neurons, termed by the authors as BM stromal cell-derived neuronal cells.[105] Transplantation of BM stromal cell-derived neuronal cells in stroke models reveals better functional improvement than those treated with MSCs.[105] Although gene-based strategies appear to improve MSC graft functional effects, the use of viral vectors poses clinical problems, especially with uncontrolled gene replications that may cause neoplasm, tumors and even death. An alternative approach to circumvent the adverse side effects associated with genetic manipulation, while still facilitating the therapeutic effects of MSCs, is to exogenously deliver neurotrophic factors. A study shows that BDNF delivered along with MSCs significantly improves the recovery of the stroke animals.[106] In an effort to further improve MSC efficacy, MSC transplantation is combined with adjunctive treatment of specific reagents. A cell-permeable inhibitor of caspases, Z-Val-Ala-DL-Asp-fluoromethylketone (Z-VAD), is found to enhance graft survival and behavioral recovery[107] when intracerebrally infused together with MSCs into the ischemic region. Nitric oxide has been proven to play an important role in cell proliferation and neurogenesis. Intravenous infusion of MSCs with a nitric oxide donor, (Z)-1-[*N*-(2-aminoethyl)-*N*-(2-ammonioethyl) aminio] diazen-1-ium-1,2-diolate (DETA/NONOate), significantly improves functional recovery in stroke animals, with accompanying enhancement of vessel perimeter and endothelial cell proliferation. In addition, such combined DETA/NONOate and MSC grafts lead to increased neurogenesis in the SVZ, as

well as VEGF and bFGF expression, in the ischemic area compared with single treatments.[108]

MSC-induced cell proliferation and axonal remodeling

Grafted MSCs could modulate glial cell proliferation and result in neuron remyelination as well as synaptogenesis.[109] Oligogenesis and astrocytogenesis are markedly enhanced in the SVZ of the ischemia animal models treated with MSCs.[92,109–111] In addition to enhancing gliogenesis, reducing apoptosis is shown to be another critical effect of MSCs. Using an anaerobic chamber to duplicate the *in vivo* ischemia scenario *in vitro*, Gao *et al.*[112] demonstrate that astrocytes, when cocultured with MSCs, suffer less cell death and apoptosis. *In vivo* studies indicate that intravenous administration of MCSs in stroke models could reduce apoptosis in the penumbral zone of the lesion.[95,96] A recent study demonstrates that MSCs significantly increase astrocytic expression of connexin-43 (Cx43)[57] and growth associated protein-43 (GAP-43).[110] This Cx43 upregulation is concomitant with altered gap junction intercellular communication with the participation of phosphatidylinositol 3-kinase signaling pathway.[113] *In vivo* data confirm that transplanted MSCs enhance bone morphogenetic protein 2/bone morphogenetic protein 4 (BMP2/4) and Cx43 expression in astrocytes.[114] Cx43 is the primary component of intercellular channels in astrocytes, whereas BMP2/4 belongs to a subgroup of the transforming growth factor-β superfamily. BMP2/4 maintains extensive gap junctional communication through Cx43 and hence mediates communication between astrocytes and increases synaptogenesis in the IBZ.[114] Compared with nontransplanted ischemic animals, areas of the corpus callosum and the numbers of white matter bundles in the striatum are larger in the IBZ in MSC-treated ischemic animals;[109,115] at the same time, the axons and myelin thickness are increased along with scar thickness being reduced.[92,110,111] In contrast, the numbers of microglia and macrophages within the scar wall are reduced.[110] Whether microglia inhibit the axonal regeneration warrants further investigation.

MSC homing via SDF-1/CXCR4 chemoattractant pathway

As discussed above, SDF-1 is a crucial chemoattractant for CXCR4-expressing BM-derived cells in the hematopoietic system (that is, HSCs). In the nonhematopoietic system, CXCR4-positive cells similarly respond to SDF-1 signals secreted by injured organs, and migrate into these areas. Such SDF-1 upregulation and cell migration are found to subsequently lead to upregulation of various trophic factors either secreted by the mobilized stem cells or by endogenous cells. In stroke rats, the chemokine SDF-1 level is significantly increased in the injured hemisphere compared with the spared site.[92] The upregulation of SDF-1 is mostly recognized in the IBZ and maintained for up to 30 days after the injury. Of note, CXCR4 is detected in the transplanted MSCs. The interaction between SDF-1 and CXCR4 serves as the chemoattractant guide for MSC migration toward the impaired site.[92] SDF-1 gene expression is regulated by the hypoxia-responsive transcription factor, hypoxia-inducible factor 1,[116] and follows a gradient pattern consistent with the hypoxia gradient in the penumbra of stroke animals. In general, the expression of SDF-1 following a brain injury serves as a significant signal for attracting the BM-derived stem cells to migrate into the damaged area, and is crucial for the functional recovery of stroke.

With MSCs being one of the most studied stem cells for therapeutic applications, although their mechanisms of action are not well understood, two recent Food and Drug Administration (FDA)-approved clinical trials have been initiated via intravenous autologous MSCs (University of Texas at Houston with Dr Sean Savitz) and stereotactic transplantation of allogeneic Notch-induced MSCs (Stanford University with Dr Gary Steinberg) in acute and chronic ischemic stroke, respectively.

Endothelial progenitor cells

EPCs, initially described by Asahara *et al.*,[24] are immature endothelial cells that circulate in PB (see Table 1). EPCs mature as endothelial cells, and are important components of the vascular system.[25–29] In their pioneering study,[24] transplanted EPCs, isolated from human umbilical cord blood (UCB), were found in the endothelium of newly formed vessels in ischemic regions, indicating that a discrete cell population within the human blood participates in the formation of new vessels after ischemia. Griese *et al.*[117] also found that grafted EPCs populated the endothelium in animals with experimentally induced endothelial damage,[118] further advancing the notion that EPCs contribute to the repair of damaged endotheliumx. Although EPCs are hematopoietic in origin and they can be found in PB of adults, a population of cells with similar characteristics can also be derived from human UCB.[117,119] Several lines of investigations from both animal and human studies indicate that EPCs principally participate in re-endothelialization during the neovascularization of ischemic organs, suggesting that EPC modulation could be directed toward treatment of cerebrovascular diseases.[25,26,29,30,117,119,120]

EPC phenotypic characterization

Immunological surface markers and functional profiling via colony-formation capacity of these cells have been employed to determine their characteristics.[30–34] EPCs were first isolated from human PB, which expressed shared markers with HSCs, angioblasts and receptors for VEGFR2/KDR.[24] Cultured EPCs could differentiate into endothelial cells and incorporate into sites of active angiogenesis in animal models of ischemia.[26] Isolation of EPCs remains controversial, in that although different markers such as CD31, VE-cadherine, E-selectin, endothelial nitric oxide synthase and von Willebrand factor among others[26,30,33,34,121] have been employed to harvest EPCs, equally solid evidence indicates that only CD34-positive EPCs isolated from BM or UCB possess the potency to differentiate into mature endothelial cells.[29,117,122] In parallel, recent studies have used the HSC-specific AC133 as surface marker to isolate EPCs.[30,31] Varying developmental stages of EPCs or the presence of residual cells derived from the mature vascular wall may influence the heterogeneity of tissue source of EPCs. The prevailing marker of choice for obtaining highly homogenous EPCs is via double labeling with CD34/VEGFR2.[30,31,35]

To further define the phenotypic feature of EPCs, another approach via functional profiling has been used that involves counting the number of EPC colonies formed after 7 days of culture. A colony consists of a central cluster of rounded cells with surrounding radiating thin, flat cells.[26,123–125] These colonies exhibit many endothelial characteristics including expression of CD31, VEGFR2 and Tie-2.[26,125] Another method is to measure the uptake of Dil-labeled acetylated low-density lipoprotein or binding to specific lectins. This method has also been useful in defining endothelial cells; however, these characteristics are also displayed by most macrophages in a culture.[26,32,126,127] These characteristics are mostly considered to be functional in nature and do not represent the number of EPCs present in the culture. Several sources of EPCs have been identified, including PB, BM and UCB.[25,26,28,29,117] Recently, it has been reported that EPCs may even originate from the area between smooth muscle and adventitial layer of the human adult vascular wall.[128] EPCs remain extremely rare in adult PB (0.01% of mononuclear cells, under-steady state conditions), which may be a contributing factor for the lack of clearly defined methods for cell isolation and definition.[32] Whereas BM-derived stem/progenitor cells have been widely considered as a source of EPCs, the BM represents heterogeneous groups of cells with at least two major progenitor populations, namely MSCs and hematopoietic EPCs, with both populations capable of inducing neovascularization through vasculogenesis, making them good candidates for cell therapy in cerebrovascular diseases.[117,129–135] Although characterization of EPCs remains a challenging cell culture protocol, the unique properties

of these cells as recommended by Finkel and co-workers[122] may suffice as a starting point in delineating this novel BM cell population. In particular, EPCs are circulating, BM-derived cells distinct from mature endothelial cells due to their ability to differentiate into endothelial cells, as assessed by expression profiles and functional characteristics; and their regenerative capacity, especially for promoting vasculogenesis and/or vascular homeostasis.[136]

EPC, angiogenesis and vasculogenesis

The dogma that existed until recently is that neovascularization, or formation of new blood vessels, results exclusively from proliferation and migration of pre-existing endothelial cells, a process referred to as angiogenesis.[137] Furthermore, vasculogenesis or vascularization, defined as *in situ* differentiation of vascular endothelial cells from endothelial precursor cells, was thought to occur only in the embryo during vascular development. However, recent evidence has now established that circulating BM-derived EPCs are capable of homing to neovascularization sites, proliferating and differentiating into endothelial cells.[138,139] EPCs have been identified mainly in the mononuclear cell fraction of PB, leukapheresis products and in UCB,[140,141] but can also be harvested from BM as presented in our preliminary data section. Over the last few years, EPCs have been studied as biomarkers to assess the risk of cardiovascular disease in human subjects. For example, a low EPC count predicts severe functional impairments in several cardiovascular pathologies such as diabetes,[142] hypercholesterolemia,[143] hypertension,[144,145] scleroderma,[146,147] aging,[142,148] cigarette smoking[142,149,150] and coronary artery disease.[151] In addition, EPCs have been examined as potent donor graft cells for transplantation therapy. Transplantation of EPC into ischemic tissues has emerged as a promising approach in the treatment of diseases with blood vessel disorders.[152–154] In mouse models of ischemic injury, EPC injection led to improved neovascularization in hind limb ischemia.[152–154]

EPCs for cell therapy

Based largely on these laboratory findings suggesting angiogenic and vasculogenic potential of EPCs, clinical studies have been initiated to reveal whether patients with lower EPC numbers are at higher risk for atherosclerotic events, and whether patients with ischemic events may benefit from EPC administration.[155] Clinical studies to date suggest the therapeutic potential of EPC transplantation, although this assumption should be approached with much caution due to these studies being open-label trials, observational and/or anecdotal accounts having a limited number of patients. *Ex vivo* expanded EPCs, isolated from PB mononuclear cells, can incorporate into the foci of myocardial neovascularization,[156,157] and intracoronary infusion of PB or BM-derived progenitors in patients with acute myocardial infarction was associated with significant benefits in post-infarction remodeling.[158–165] Still in observational studies in patients with myocardial infarction, higher numbers of EPCs correlate with better prognosis, more myocardial salvage,[166] viability and perfusion[167] and more collaterals in the ischemic zone.[168] Randomized clinical trials on autologous BM-derived cells are mixed; whereas transplanted coronary artery disease patients display improved left ventricular function at least in the short term,[169] transplanted patients with chronic ischemic heart failure exhibit modest to no effects on change in left ventricular function.[170] Similar randomized trials of autologous BM-derived cells have been carried out in patients with peripheral artery disease and have shown improved endothelium-dependent vasodilation,[171] ankle brachial index, rest pain and pain-free walking time,[172] but the degree of functional recovery was not as robust as seen in animal models. Clearly, these results are obtained from autologous BM-derived cells, which are heterogeneous with scarce number of EPCs, and thus may not closely approximate EPC end points.

For clinical application of EPCs in neurovascular disease, the available studies are much more limited, with only three observational studies in patients with stroke. In 25 patients with an ischemic stroke, CD34+ cells peaked 7 days after stroke but generally reverted to baseline after 30 days.[173] Interestingly, higher CD34+ cell levels at 30 days related to higher numbers of infarcts on magnetic resonance imaging and also to cerebrovascular function as measured with positron emission tomography scanning (cerebral metabolic rate of oxygen and cerebral blood flow). On the other hand, decreased numbers of clusters of rapidly adhering cells were seen after stroke and in 'stable cerebrovascular disease,' compared with controls free of vascular disease.[174] Higher age and the presence of cerebrovascular disease in general independently related to lower EPC numbers. The discrepancies in the results of these studies may be because of mismatched controls for age of patients and/or the lack of methodological design for testing specific hypotheses on the causal role of EPCs in cerebrovascular disease.[174]

Although the primary mitigating mechanisms underlying stroke pathogenesis and its abrogation by cell therapy are still uncertain, there is substantial evidence implicating immunological attack upon the brain and/or its vasculature; widespread inflammatory reactions in stroke may trigger a cascade of events that alter the integrity of the BBB, resulting in migration of leukocytes into the CNS. Leukocyte transmigration across the BBB during stroke-mediated immune/inflammatory processes could influence interendothelial junctional complex function, leading to vascular endothelium damage and BBB breakdown. Equally a key component to our mechanism-based hypothesis is that disruption or dysfunction of the BBB, preceding entry of harmful substances into the brain parenchyma, could be a key initial factor in stroke pathogenesis. Thus, restoration of barrier integrity may have a critical role in preventing stroke progression, highlighting the need for EPCs as transplantable cells for stroke therapy.

Very small-like embryonic stem cells

As mentioned above, stress conditions such as ischemic injury acutely increases the number of various types of stem cells in PB in both experimental animals and patients.[175–178] Stem cells, including HSCs,[179] MSCs[180] and EPCs,[181] are mobilized into PB from BM and probably other nonhematopoietic niches as well, likely contributing to an endogenous regenerative medicine.[177,178,182,183] However, as brain damage after stroke still leads to irreversible brain damage, these cells circulating in PB are not sufficient to halt the stroke damage. A hostile microenvironment ensues during stroke progression that alters chemotaxis and homing of circulating PB cells to the ischemic brain. In addition, the BBB may limit the entry of circulating stem cells from periphery to the brain, thereby further blocking the therapeutic benefits of this endogenous repair mechanism. Treatment strategies designed to increase the number of circulating stem cells in PB (that is, after administration of mobilizing agents such as G-CSF and/or CXCR4 antagonists) maintain the chemoattractant pathway to provide migratory cues for circulating stem cells to home to the site of injury, and enhance permeability of the BBB to facilitate stem cell entry into the brain, or stereotactic delivery of stem cells into the peri-infarct area may foster an effective regenerative regimen against stroke.[184–187] Along this line, the acute endogenous stem cell mobilization following ischemic injury can be enhanced in both mice and humans after G-CSF treatment during acute cardiovascular diseases, for example, myocardial infarction and stroke.[178,182] Accordingly, stem cell-based strategies aimed at regeneration of the stroke brain stands as a potent therapeutic modality. In the study of Ratajczak *et al.*,[177] the research team recently discovered a unique population of stem cells called VSELs. Of note, the number of circulating VSELs in PB increases in mice after experimental stroke[177] as well as in stroke patients,[178] suggesting that VSELs residing in adult tissues or mobilized into PB

are a potent source of adult tissue-derived stem cells that can be utilized for regenerative medicine specifically for neural repair after stroke.

In the pioneering studies by Ratajczak *et al.*,[177,188] they showed that VSELs could be mobilized into PB in patients after stroke, in a similar way as in patients after acute heart infarct in humans and mice. By employing a staining strategy, they observed an increase in mRNA for both pluripotent (Oct-4 and Nanog) and neural (GFAP, Nestin, b-IIItubulin, Olig1, Olig2, Sox2, and Musashi-1) stem cell markers in PB-borne nucleated cells circulating in stroke patients, resembling the general phenotypic patterns that they previously noted in an experimental murine model of stroke,[177] with the exception that maximally increased expression of neural stem cell markers in humans was delayed by 2 days (that is, 1 day for mice vs 3 days for humans). Further analyses using computer tomography scans revealed differences in VSEL mobilization in that patients with posterior circulation infarcts have the best chance of recovery, whereas partial anterior circulation infarcts are associated with the highest risk of early recurrence of stroke (that is, within 3 months), although not associated with high mortality and significant disability. Whether stroke morbidity or mortality correlates to location of the stroke area in relation to the neurogenic regions of SVZ and subgranular zone warrants further examination. Additionally, whether the number of these cells could be increased by administration of mobilization-promoting agents (for example, G-CSF or AMD3100) represents a logical extension of this therapy for clinical application. Nonetheless, these studies indicate that the mobilization of VSELs and tissue-committed progenitor cells expressing early neural markers into PB are closely associated with ischemic stroke and could be utilized as a prognostic tool.[189,190]

Phenotypic characterization and genomic imprinting of VSELs

VSELs express several progenitor stem cell markers[38] (see Table 1), and are present in a variety of adult organs; specifically, the brain contains a relatively high number of cells that display the VSEL phenotype.[38,39] Initially separated from murine BM,[40] VSELs are smaller than erythrocytes (3–5 μm in diameter in mice) with a corresponding human population of small cells (5–7 μm in diameter) purified from human UCB and mobilized PB.[38] In the Ratajczak laboratory, well-validated protocols for VSEL identification in human PB using flow cytometric methods have been established. Following tissue and organ injuries, human VSELs are mobilized into PB of patients and recognized as very small cells belonging to the nonhematopoietic fraction of leukocytes (Lin−/CD45− cells) expressing CD34, CD133 and CXCR4 antigens.[42,177] The absolute numbers of circulating VSELs in PB are exceptionally low (1 to 2 cells in 1 ml of blood under steady-state conditions) and thus special flow cytometric protocols have to be applied for their identification. Phenotypic markers used to identify VSELs include negative expression of CD45 (mouse and human), positive expression of Sca-1 (mouse), CXCR4, CD133 and CD34+ (mouse and human), positive for progenitor stem cell markers (that is, Oct-4, Nanog and SSEA),[38,41–43] and express several markers characteristic of epiblast/germ line stem cells.[44] Based on a multianalytical flow cytometric approach, the identity and confirmation of the presence of VSELs among blood leukocytes, in addition to a quantitative determination of the absolute numbers of these rare cells circulating in the blood of patients with various tissue/organ injuries and disorders, could be ascertained.[41,177] An independent group has successfully purified VSELs from human mobilized PB as well as BM.[45]

In deciphering the genomic imprint of VSELs, a notable feature is that most of the homeodomain-containing developmental transcription factors in VSELs are repressed by specific epigenetic marks called bivalent domains that represent a state of the DNA structure characteristic of progenitor stem cells, where transcriptionally antagonistic histone codes physically coexist within the same promoter.[190] Murine Oct-4+ VSELs do not proliferate

spontaneously *in vitro* if cultured alone and that the quiescence of these cells is regulated by genomic imprinting through DNA methylation, which is an epigenetic program that ensures the parent-specific monoallelic transcription of some developmentally important genes such as those implicated in embryogenesis, fetal growth, maintaining the totipotential state of the zygote and maintaining the pluripotency of developmentally early stem cells.[43] The expression of imprinted genes is regulated by DNA methylation with freshly isolated VSELs from murine BM deleting the paternally methylated imprints, but hypermethylating the maternally methylated imprints. Because paternally expressed imprinted genes (*Igf2* and *Rasgrf1*) enhance embryo growth, whereas those that are maternally expressed (H19, p57KIP2, and Igf2R) inhibit cell proliferation,[43] the unique genomic imprinting pattern observed in VSELs demonstrates a growth-repressive program in this developmentally early stem cell. Although the quiescent pattern of genomic imprinting is largely influenced by DNA methylation, cell culture conditions such as the formation of spheres by VSELs in cocultures with myoblastic C2C12 cells[191] could prime VSELs to proliferate and differentiate. Taken together, these findings suggest that the epigenetic reprogramming of genomic imprinting is capable of maintaining quiescence of the most primitive pluripotent adult stem cells (that is, Oct-4+ VSELs) deposited in the adult body and protect them from premature aging and tumor formation, but that specific external microenvironmental signals can influence the epigenetic state of this imprinting and force VSELs to differentiate.[191] Whether human VSELs, as well as for cells isolated from nonhematopoietic tissues (for example, the CNS) that display the VSEL phenotype, also recapitulate such DNA methylated genomic imprint remains to be determined.

VSELs for cell therapy

The notion that VSELs are epiblast-derived progenitor stem cells deposited early during embryonic development in immature organs, as a potential reserve pool of precursors for tissue-committed stem cells, suggests that this population has an important role in physiological tissue rejuvenation and regeneration. The observation that murine VSELs have the potential to differentiate into neurons, oligodendrocytes and macroglia further advances the utility of these cells as donor grafts for regeneration of a damaged CNS. In contemplating with clinical application of the cells, the ease of harvesting VSELs should be considered. With autologous transplantation in mind, the patient's own BM, stored UCB and mobilized PB appear as readily accessible sources of VSELs for regenerative medicine. For allotransplantation, VSELs could be harvested from histo-compatible-related or unrelated donors as is currently done with HSC allotransplantation.[191] A potentially limiting factor for clinical application is the small number of VSELs that could be harvested, requiring the need for efficient *ex vivo* expansion strategy, especially if the goal is to generate an ample supply of neural stem cells for stroke therapy. Another caveat to pursuing VSELs in the clinic is the observation that the number of VSELs decreases with the age, implying that enhanced regeneration potential of human patients may be correlated with higher number of these cells deposited during embryogenesis in the adult tissues.[192] Of note, calorie uptake-related increase in plasma level of insulin and insulin-like growth factors may deplete VSELs in adulthood.[193]

Isolation of VSELs from BM, UCB and PB

In designing treatment strategies for stroke, the acute and subacute stage (time 0 to 1 week post injury) seems to offer the best opportunity to initiate the therapeutic intervention in order to immediately abrogate the rapidly deteriorating ischemic brain. Because of this limited therapeutic window, it may not be feasible and practical to purify these rare cells from BM aspirates, UCB or mobilized PB by employing multiparameter staining and regular high-speed sorting only. The Ratajczak group calculated that by employing only one cell fluorescence-activated cell sorter, isolation of all VSELs present in 100 ml of UCB would

take up to 4 working days.[194,195] For efficient cell isolation, they propose a relatively short and economical three-step isolation protocol that allows recovery of ~60% of the initial number of Lin−/CD45−/CD133+ UCB− VSELs. The rationale for this novel strategy takes into account lysis of erythrocytes in a hypotonic ammonium chloride solution, CD133+ cell selection by immunomagnetic beads and sorting of Lin−/CD45−/CD133+ cells by FACS with size-marker bead controls. The entire isolation procedure takes 2 to 3 h per UCB unit (but should also be applicable using BM aspirates and mobilized PB) and isolated cells are highly enriched for an Oct-4+ and SSEA-4+ population of small, highly primitive Lin−/CD45−/CD133+ cells. The envisioned clinical product will be VSELs freshly isolated from BM, PB or UCB or VSELs precommitted to the neurological lineage in *ex vivo* cultures.[190]

Conclusions

The discovery of stem cell plasticity in adult tissues has been a major driving impetus for advancing regenerative medicine in many neurological disorders, including stroke. BM-derived HSCs, MSCs, EPCs and VSELs have been proposed as potential sources of adult stem cells for regeneration of the CNS. However, the rationale for employing these non-neuronal stem cells remains controversial, and most recently MSCs have been put into the spotlight as a possible cause of tumor formation following their deposition into the brain.[196] Whereas BM-, PB- or UCB-derived HSCs for regeneration of neural tissues have been explored, the postulated stem cell plasticity of HSCs, such that these cells may become neural stem cells,[197] has been not confirmed in recently published studies. On the contrary, the transdifferentiation may be explained as a transient change in HSC phenotype induced by neural tissue-derived, spherical membrane fragments called microvesicles (or exosomes) that may transfer neural cell-surface receptors, mRNA and microRNA to the HSCs employed for regeneration.[198] Along similar technical obstacles as HSCs, MSCs could be isolated from BM, PB or UCB by expansion of an adherent population of fibroblast-like cells,[199] and it is widely accepted that MSCs contribute to the regeneration of mesenchymal tissues (that is, bone, cartilage, muscle, ligament, tendon, adipose and stromal support). MSCs could also be obtained from several other tissues (for example, PB, UCB, adipose tissue, dental pulp and menstrual blood).[200,201]

An unexpected discovery from a few years ago suggested that MSCs are able to give rise to neuronal cells.[202] Like HSCs, this rare transdifferentiation event of MSCs was challenged, pointing to the possibility of an *in vitro* contamination in the cell culture media[203,204] that could alter the morphology of MSCs. Under these same cell culture conditions, fibroblasts could shrink, elongate and mimic neurons, but this proved to be only an *in vitro* morphological artifact.[203,204] Thus, the rationale behind applying MSCs, just like HSCs, in brain regeneration is not well supported. On other hand, there is no doubt that modest functional recovery occurs in animal models and patients after treatment with HSCs or MSCs.[184–186] The mechanism of action, however, likely does not involve differentiation of these cells into neurons, because such cells are rapidly eliminated after local delivery.[184–186] Instead, the most plausible explanation currently receiving much support from the literature is that HSCs or MSCs employed for regeneration could, however, propel a by-stander effect in that release of growth factors or cytokines facilitates neovascularization of damaged tissues leading to neurogenesis, as well as afford anti-inflammatory, antiapoptotic and antioxidative stress effects among other reparative responses.[40,205–207]

More recently, my group has taken a keen interest in repairing the BBB after stroke via EPC transplantation. Our overarching hypothesis is that BBB breakdown accompanies stroke and may be exacerbated by tissue-type plasminogen activator. To date, most stroke therapies have not considered the repair of this BBB damage after stroke. If BBB restoration via EPC transplantation alone or in combination with tissue-type plasminogen activator is proven

effective, we believe that direct clinical application of this cell therapy will be far reaching, as this treatment could help a large population of ischemic stroke patients who otherwise would have missed the limited 3-h tissue-type plasminogen activator window. In addition, we envision that this EPC transplantation can supplement other stroke therapeutics that require BBB manipulation in order to afford beneficial effects, and can be extended to other neurological disorders characterized by BBB breakdown.

Finally, the advent of VSELs is equally appealing for cell therapy in stroke. A major caveat in delivery of stem cells into the brain, whether via stereotactic local implantation or peripheral delivery (that is, intravascular or intraarterial), is the possibility of creating embolism with the high number of stem cells required to exert therapeutic effects. In this regard, the very small size of VSELs is one of the most unique properties of the cells that could avoid the potential adverse effects of embolism associated with cell therapy. That the VSEL isolation and expansion may require tedious steps and longer days to acquire ample supply of the cells can be circumvented by pursuing allogeneic overautologous transplants. As stroke episodes cannot be predicted, an off-the-shelf treatment intervention is likely optimal; thus, allogeneic transplant therapy may very well be more suitable than autotransplantation. Moreover, that aging or disease process entails less number of viable and healthy stem cells implies that the harvest of VSELs from young donors (that is, PB) represents an advantageous cell source.

In summary, the encouraging laboratory results of cell therapy using HSCs, MSCs, EPCs and VSELs as donor sources are rapidly being translated into clinical trials. Over the past 5 years, recommendations to better guide the successful entry of stem cell therapy into the clinic have received continuing critical assessments from unique collaborative meetings involving thought leaders from academe, industry, the National Institutes of Health (NIH) and the FDA.[6–8] The mechanism of action underlying cell therapy remains to be fully determined, and this persisting gap in our knowledge should be seriously considered when evaluating the risk-to-benefit ratio of this novel, experimental treatment for stroke. In the end, systematically designed translational preclinical studies need to precede initiation of clinical trials in order to allow rigorous investigations of the safety and efficacy profile of these adult BM-derived stem cells for cell therapy in stroke and other neurological disorders.

Acknowledgments

I extend appreciation to Ms Loren E Glover who provided excellent technical assistance in the final preparation of the manuscript. CVB is supported by James and Esther King Foundation for Biomedical Research Program 1KG01-33966 and NIH R01 5R01NS071956-02, and received research grant support for his projects on bone marrow stem cell therapy for stroke from SanBio Inc., Celgene Cellular Therapeutics, KMPHC and Neural- Stem Inc. Some of the stem cell therapy thematic discussions originated from NINDS UO1 5U01NS055914-04.

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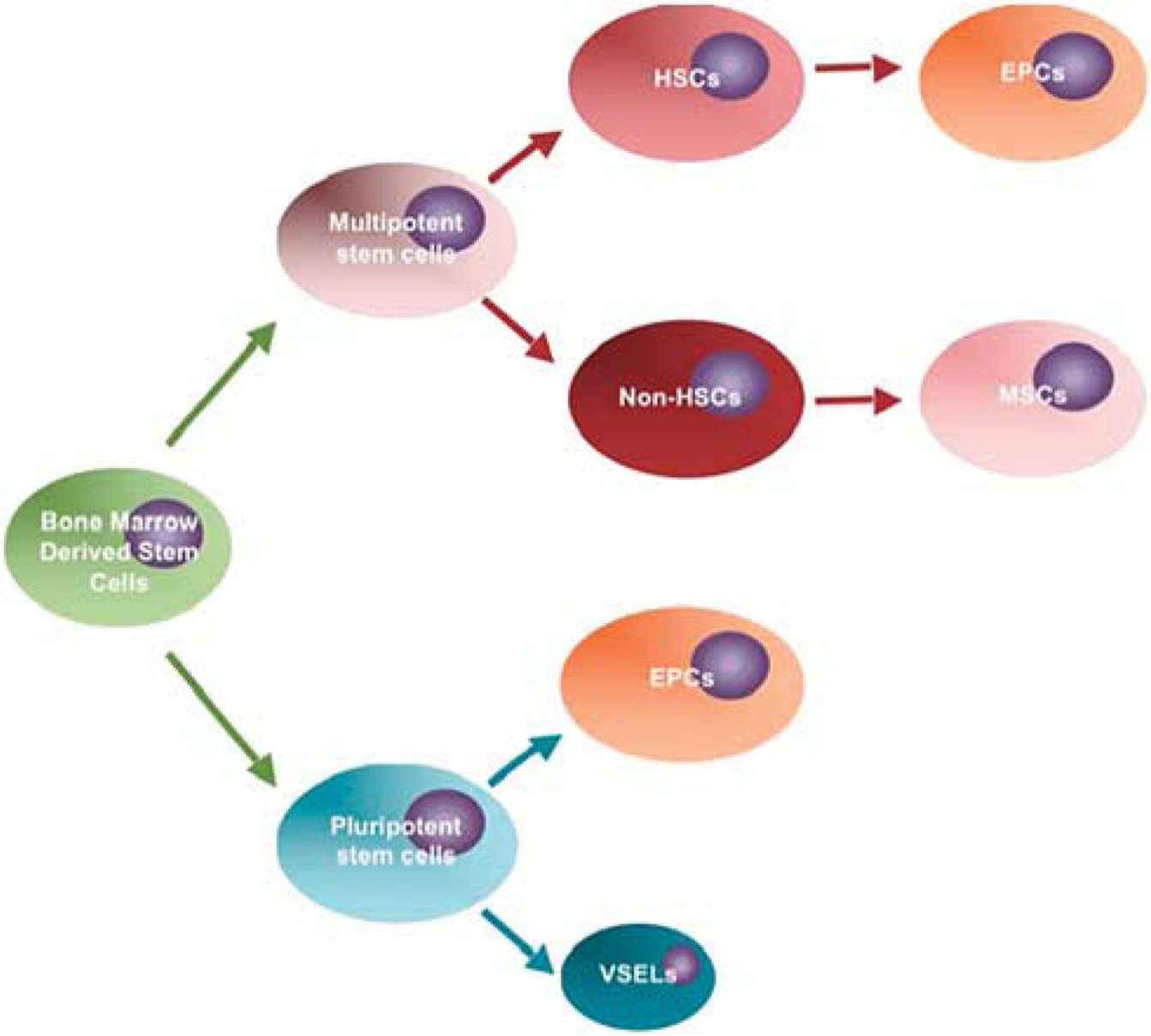

Figure 1.
Bone marrow-derived stem cells. Schematic diagram shows subsets of bone marrow-derived stem cells, including HSCs, MSCs, EPCs and VSELs, that have been examined in the laboratory and are rapidly being translated into clinical applications as efficacious stem cell sources for transplantation therapy in stroke.

Table 1

Characteristics of bone marrow-derived hematopoietic and nonhematopoietic stem cells

Bone marrow subset	Phenotype	Differentiation potential	Telomerase	Senescence tendency	Reference
HSCs	CD34+, CXCR4+	Blood cells	Positive	Up for debate, thought to be dysfunctional in aging HSCs, but even reduced telomere expression neither shorten telomere nor limit PP	12–17
MSCs	SH2+, SH3+, CD29+, CD44+, CD14−, CD34− and CD45−	OCA cells	Negative	CPDL is ~18 PDs at ~80 days	18
	Not addressed	OCA cells	Not addressed	Mean CPDL is ~38 PDs at passage 15	19
	Not addressed	OCA cells	Not addressed	Lose MDP at 19–22 PDs. CPDL is ~22 to 23 in 80 days of culture	20
	Not addressed	OCA cells	Not addressed	Lose MDP at 19–22 PDs. CPDL is ~22 to 23 in 80 days of culture	21
	Not addressed	OCA cells	Not addressed	CPDL in serum-deprived hMSCs is ~9 PDs at passage 10. CPDL for control hMSCs is ~7 PDs at passage 9	22
	Not addressed	OCA cells	Not addressed	CPDL is 16 PDs of ~40 days of culture	23
hTERT-MSCs	Not addressed	OCA cells	Not addressed	PP remains until 80 PDs of ~400 days of culture	23
EPCs	CD34+, CD31+, AC133+, VEGFR2+, Tie-2+	Endothelial cells	Positive	PDs until 7 to 8 days, with senescence augmented by estrogen or SDF-1 treatment	24–37
VSEL cells	SSEA-1+, Oct-4+, Nanog+, Rex-1+, Sca-1+, CD45−	Three germ lineages	Positive	Expand for ~7 PDs (CV Borlongan, personal communication)	38–45

Abbreviations: CPDL, cumulative population doubling level; EPC, endothelial progenitor cell; hMSC, human mesenchymal stem cell; HSC, hematopoietic stem cell; hTERT, human telomerase reverse transcriptase; MDP, multiple lineages differentiate potential; MSC, mesenchymal stem cell; OCA cell, osteogenic, chondrogenic and adipogenic cell; PD, population doubling; PP, proliferation potential; SDF-1, stromal derived factor-1; VSEL, very small embryonic-like stem cell.

Not Addressed: authors did not describe this topic in their study.
Negative/positive: the activity of telomerase is negative or positive.