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Very small embryonic-like (VSEL) stem cells in adult organs and their potential role in rejuvenation of tissues and longevity

Mariusz Z. Ratajczak, **Ewa K. Zuba-Surma**, **Dong-Myung Shin**, **Janina Ratajczak**, and **Magda Kucia**
Stem Cell Institute at James Graham Brown Cancer Center, University of Louisville, Louisville, KY 40202, USA and Department of Physiopathology, Pomeranian Medical University, Szczecin, Poland

Abstract

Recently, we purified rare CXC chemokine receptor 4 expressing ($CXCR4^+$) small stem cells (SCs) from the murine bone marrow (BM) that express markers characteristic for embryonic (E)SCs, epiblast (EP)SCs, and primordial germ cells (PGCs). We named these primitive cells very small embryonic-like (VSEL) SCs (VSELs). Our data indicate that VSELs are also present in many other organs in mice and that they may differentiate into cells from all three germ layers. Similar SCs were also isolated from human cord blood (CB) and mobilized peripheral blood (mPB). We hypothesize that VSELs are deposited during gastrulation and organogenesis in developing organs/tissues of mammals as a population of pluripotent stem cells (PSCs) that give rise to tissue committed monopotent SCs and that their number decreases with age. Therefore VSELs could play a pivotal role in normal rejuvenation of adult tissues as well as involvement in regeneration of damaged organs. Thus, these cells are potential SCs candidates for regenerative medicine and we envision that the regenerative potential of these cells could be harnessed to decelerate the aging processes.

Keywords

VSEL; CXCR4; Oct-4; Nanog; SSEA

Introduction

Growing evidence indicates that adult organs contain, in addition to tissue committed monopotent stem cells (SCs), some rare more primitive pluripotent stem cells (PSCs). These cells are located in specific stem cell niches. Isolation of these cells from several adult organs is usually difficult and requires homogenization of tissue fragments. One of the exceptions from this rule is the bone marrow (BM) which is a rich source of SCs that are relatively easily accessible by aspiration of bone marrow cavities. Evidence accumulated that BM contains heterogeneous populations of hematopoietic (H)SCs and non-HSCs. These non-HSCs are described in the literature as: endothelial progenitor cells (EPCs) (Asahara et

Address for correspondence: Mariusz Z. Ratajczak, M.D., Ph.D., Hoenig Endowed Chair in Cancer Biology, Professor and Director of Stem Cell Institute, University of Louisville, 500 South Floyd Street, Louisville, KY 40202, Kentucky, USA, Tel: (502) 852-1788, Fax: (502) 852-3032, mzrata01@louisville.edu.

al., 1997; Shi et al., 1998); mesenchymal (M)SCs (Dominici et al., 2006, Peister et al., 2004; Prockop, 1997); multipotent adult progenitor cells (MAPCs) (Jiang et al., 2002); marrow-isolated adult multilineage inducible (MIAMI) cells (D'Ippolito et al., 2004); and multipotent adult (MA)SCs (Beltrami et al., 2007). Moreover, BM could also be postulated as a potential source of precursors of germ cells (GCs) (oocytes and spermatogonial cells) (Johnson et al., 2005; Nayernia et al., 2006). It is likely that similar or overlapping populations of primitive SCs in the BM detected using various experimental strategies were assigned different names.

Recently, we purified rare, small CXC chemokine receptor 4 expressing ($CXCR4^+$) SCs from the murine BM and several adult organs in mice that express markers characteristic for embryonic (E)SCs, epiblast (EP)SCs, and primordial (P)GCs (Kucia et al., 2006a). We named these primitive cells very small embryonic-like (VSEL) SCs (VSELs). Our data indicate that VSELs may differentiate into cells from all three germ layers (Kucia et al., 2006a). The number of these cells is high in young mice and decreases with the age. Thus, VSELs could play a pivotal role in normal cell turn over and rejuvenation of adult tissues as well as they could be involved in regeneration of damaged organs due to aging.

It is widely accepted that aging is related to decrease in rejuvenation/regeneration potential of several vital organs and tissues and could be explained by i) age related stem cell dysfunction, ii) accumulation of mutations in stem cell pool over a life-span and iii) shortening of the telomeres as result of decrease in telomerase activity. In this review we will present a hypothesis that aging could be also at least partially explained by an age-related decrease in number of pluripotent VSELs in adult tissues. We believe that this decrease in number of VSELs residing in adult tissues directly affects/impairs regenerative potential of organs as observed in advanced age.

VSELs as we hypothesized are derived from epiblast, part of embryo from which develop all there germ layers (meso-, ecto- and endoderm). Since epiblast derived SCs (EPSCs) give rise to the all organs in developing organism, they are a precursors of tissue committed (monopotent) SCs for particular tissues. As presented in Figure 1 panel A we hypothesize, that EPSCs after giving rise to tissue committed monopotent SCs may survive among them and play a role as a backup pool for tissue committed SCs. Based on our data we also envision that VSELs are a mobile pool of SCs that could be mobilized during tissue/organ injuries in an attempt to regenerate damaged tissues. Relatively high number of VSELs is observed in BM, and thus BM plays an important role in redistribution and shuttling of these cells among various organs/tissues (Figure 1 panel B). This concept and data that support it will be presented in this review.

BM-derived stem cells and their potential role in regeneration – does stem cell plasticity really exist?

BM is rich source of SCs that are relatively easily accessible by aspiration of bone marrow cavities and over the past decade several investigators demonstrated that BM-derived cells can contribute to the regeneration of various organs and tissues (Di Campli et al., 2004; Lagasse et al., 2000; Orlic et al., 2001). These observations were mainly explained by the hypothesis that BM-derived HSCs are “plastic” and thus could trans-dedifferentiate into SCs committed for various non-hematopoietic organs and tissues(Mezey et al., 2000). This hypothetical possibility that HSCs are fully plastic and able to trans-dedifferentiate raised much hope that HSCs isolated from BM, mobilized peripheral blood (mPB), or cord blood (CB) could become a universal source of SCs for tissue/organ repair. This excitement was bolstered by several reports demonstrating the remarkable regenerative potential of HSCs in animal models, e.g., after heart infarct (Orlic et al., 2001), stroke (Hess et al., 2004), spinal cord injury (Corti et al., 2002), and liver damage (Petersen et al., 1999). However, despite

such promise, the role of BM SCs in the repair of damaged organs has become controversial (Orkin and Zon, 2002; Wagers et al., 2002). Further experiments with highly purified populations of HSCs showed their ineffectiveness for regenerating damaged heart (Murry et al., 2004) or brain (Castro et al., 2002) tissue. Following these unexpected results, the scientific community became polarized in its view of SC plasticity.

These obvious discrepancies in published results could be explained by differences in the tissue injury models employed and/or problems in detection of tissue chimerism. However, several other possibilities such as i) cell fusion, ii) microvesicles-induced modification of phenotype, iii) epigenetic changes, iv) paracrine stimulation and v) presence of heterologous population of stem cells in BM have been proposed to explain these discrepancies that are summarized in Table I.

The last possibility listed in Table I postulates that during all of these deliberations concerning SC plasticity and the potential contribution of BM-derived cells to organ regeneration, the concept that BM may contain heterogeneous populations of SCs was not carefully considered (Orkin and Zon, 2002; Ratajczak et al., 2004). We assert that regeneration studies demonstrating a contribution of donor-derived BM, mPB, or CB cells to non-hematopoietic tissues without addressing this possibility (by including the appropriate controls) could mislead interpretations. It is reasonable to assume that the presence of heterogeneous populations of SCs in BM, mPB, or CB before experimental evidence is interpreted would be considered as plasticity or trans-dedifferentiation of HSCs (Kucia et al., 2005). Hence, the presence of non-HSCs in BM rather than “trans-dedifferentiation” of HSCs could explain some of the positive results of tissue and organ regeneration as witnessed by several investigators using BM-derived cells (Kucia et al., 2005; Petersen et al., 1999). On the other hand, when highly purified HSCs were employed for regeneration experiments, non-HSCs were likely excluded from these cell preparations. In the current state of knowledge, the phenomenon of trans-dedifferentiation of HSCs and their contribution to damaged tissue regeneration remains questionable. Thus, we believe that the allegedly positive data supporting SC plasticity can be re-interpreted under the assumption that BM-derived SCs are heterogeneous and that BM tissue contains different types of SCs, including a rare population of PSCs.

Accumulating evidence that BM-derived SCs are in fact heterogeneous

The presence of versatile SCs populations in the BM is hypothesized to be the result of SCs developmental migration during ontogenesis as well as the permissive and attractive BM environment. Generally, HSCs and other non-HSCs are actively chemoattracted by factors secreted by BM stroma cells and osteoblasts [e.g., stromal derived factor-1 (SDF-1), hepatocyte growth factor (HGF), and/or vascular endothelial growth factor (VEGF)] and colonize marrow by the end of the second and the beginning of the third trimester of gestation (Nagasawa, 2000). We envision that all these non-HSCs residing in the BM (e.g., VSELs) have some role in the homeostasis and turnover of peripheral tissues and, if needed, could be mobilized from the BM into circulation during tissue injury and stress (Figure 1 panel B), thereby facilitating the regeneration of damaged organs (Kollet et al., 2003; Kucia et al., 2004; Kucia et al., 2006a; Kucia et al., 2006b; Kucia et al., 2006c, Kucia et al 2008).

Table II lists different types of non-HSCs that have been postulated to reside in BM tissue. It is quite likely that several investigators using different isolation strategies have described the similar/overlapping populations of SCs but named them differently according to circumstance. Some of these cells were described as expressing transcription factors characteristic for ESCs such as Octamer-4 (Oct-4). However, since the normal genome contains several Oct-4 pseudogenes, the real Oct-4 expression and its potential role in adult somatic cells requires further study (Lengner et al., 2007).

Identification, purification, and biological potential of VSELs

Recently, our team isolated a population of rare (~0.01%) Sca-1$^+$/Lin$^-$/CD45$^-$ cells from BM MNCs. VSEL SCs as determined by RQ-PCR by employing specific primers and by immunohistochemistry markers of PSCs such as SSEA-1, Oct-4, Nanog, and Rex-1 as well as Rif-1 telomerase protein (Kucia et al., 2006a).

- VSELs are smaller than HSC—Our preliminary data indicated that VSELs will be very small in size and reside in extended to the left lymph gate area of cytogram (Kucia et al., 2006a). Thus to isolate VSELs from the BM by FACS, we employed a novel size-based approach controlled by size bead markers. The overall sorting strategy was to gate in regions containing small events (2–10 μm). This region mostly contains cell debris, but also has some rare nucleated cell events. Since it is well known that most of the sorting protocols exclude events smaller than 6 μm in diameter that contain cell debris, erythrocytes, and platelets, small VSELs are usually excluded from the sorted cell populations.

The events enclosed in region R1 (Figure 2), which include an average of ~50% of total events, are further analyzed for the expression of Sca-1 and lineage markers (Lin). The Sca-1$^+$/Lin$^-$ events shown in region R2 (Figure 2) consist of 0.30 ± 0.05% of total analyzed BM nucleated cells on average. Cells from region R2 are subsequently sorted according to the expression of CD45 antigen as Sca-1$^+$/Lin$^-$/CD45$^-$ (region R3) and Sca-1$^+$/Lin$^-$/CD45$^+$ (region R4) subpopulations (Figure 2) that contain VSELs and HSCs, respectively (Kucia et al., 2006a; Zuba-Surma et al., 2008a). We found that VSELs comprise ~0.03% while HSCs are ~0.35% of total BM nucleated cells (Figure 2). We found that 95% of Sca-1$^+$/Lin$^-$/CD45$^-$ (VSELs) are located within the 2–6 μm size range, while 86% of Sca-1$^+$/Lin$^-$/CD45$^+$ (HSCs) are found in the 6–10 μm size range. Thus, by employing flow cytometry and the size marker beads, we have confirmed that the majority of Sca-1$^+$/Lin$^-$/CD45$^-$ cells isolated from adult BM is unusually small (<6 μm) (Zuba-Surma et al., 2008a). Thus, VSELs are larger than peripheral blood platelets and smaller than erythrocytes.

It is worthwhile to mention, that from a developmental point of view such very small immature cells, which correspond in size to VSELs, are present during development in pre-implantation blastocyst in an area called epiblast, that gives rise to the stem cells belonging to all three germ layers (ecto-, endo- and mesoderm).

- Confocal microscope and transmission electron microscope (TEM) analysis of VSELs—We have also confirmed the very small size of VSELs, when compared to hematopoietic stem cells by confocal (Zuba-Surma et al., 2008a) as well transmission electron microscopy (TEM) (Kucia et al., 2006a). Confocal microscopic analysis showed that sorted Sca-1$^+$/Lin$^-$/CD45$^+$ HSCs are relatively larger (>6μm) and stain negative for Oct-4 In contrast, VSELs are small nucleated cells (<6μm), do not express CD45 antigen on the surface, and express the ESC transcription factor Oct-4 in the nucleus (Zuba-Surma et al., 2008a). Direct TEM analysis revealed that these cells display several features typical for ESCs such as small size, a large nucleus surrounded by a narrow rim of cytoplasm, and open-type chromatin (euchromatin) (Kucia et al., 2006a).

- Image Stream analysis of VSELs to calculate size and nuclear/cytoplasmic ratio—We also employed ImageStream system (ISS) analysis to better evaluate the VSEL SCs. The IS-based analysis employs flow cytometry combined with microscopy and allows for statistical analysis of various cellular parameters as well as visualization of cells in suspension during flow analysis via high resolution brightfield, darkfield, and fluorescence images (Basiji et al., 2007; Zuba-Surma et al., 2007; Zuba-Surma et al., 2008b). The high resolution of ISS imaging enables the identification of objects as small as 1 μm in diameter

(Ortyn et al., 2006). Employing ISS analysis, we confirmed with greater precision that murine VSELs are ~3.6 μm in diameter, while Sca-1$^+$/Lin$^-$/CD45$^+$ HSCs are larger at ~6.5 μm in diameter (Zuba-Surma et al., 2008a). Finally, we investigated the N/C ratio as well as the cytoplasmic area of VSELs in comparison with HSCs. We noticed that VSELs have significantly higher ($P \leq 0.05$) N/C ratio as compared with HSCs (1.47 ± 0.17 and 0.82 ± 0.03, respectively). Furthermore, VSELs had significantly lower ($P \leq 0.05$) cytoplasmic area as compared with HSCs (5.41 ± 0.58 and 33.78 ± 1.68, respectively) (Zuba-Surma et al., 2008a). Similar population of cells that correspond to murine VSELs we recently identified in human cord blood (Kucia et al., 2007). These cells as it is shown in Figure 3 are smaller than HSC and erythrocytes yeat larger than blood platelets.

- Biological characteristics of VSELs—Despite their small size, VSELs possess diploid DNA. They do not express MHC-1 and human leukocyte antigen-D related (HLA-DR) antigens and are CD90$^-$ CD105$^-$ CD29$^-$. Our data indicate that VSELs may be released from BM and circulate in blood during tissue and organ injury (e.g., heart infarct and stroke). Interestingly, if plated over a C2C12 murine sarcoma cell feeder layer, ~5–10% of purified VSELs are able to form spheres that resemble embryoid bodies. Cells from these VSEL-derived spheres (VSEL-DSs) are composed of immature cells with large nuclei containing euchromatin and are CXCR4$^+$SSEA-1$^+$Oct-4$^+$, just like purified VSELs (Kucia et al., 2006a).

Furthermore, after re-plating over C2C12 cells, VSEL-DSs may again (up to 5–7 passages) grow new spheres or, if plated into cultures promoting tissue differentiation, may expand into cells from all three germ-cell layers. Since VSELs isolated from (green fluorescence protein) GFP$^+$ mice grew GFP$^+$ VSEL-DSs showing a diploid content of DNA, this confirms that VSEL-DSs are derived from VSELs and not from the supportive C2C12 cell line. Furthermore, it also excludes the possibility of cell fusion. Similar spheres were also formed by VSELs isolated from murine fetal liver, spleen, and thymus. Interestingly, formation of VSEL-DSs was associated with a young age in mice and no VSEL-DSs were observed in cells isolated from older mice (> 2 years) (Kucia et al., 2006a; Kucia et al., 2005). This age-dependent content of VSELs in BM may explain why the regeneration processes is more efficient in younger individuals. There are also differences in the content of these cells among BM MNCs between long- and short-lived mouse strains. The concentration of these cells is much higher in BM of long-lived (e.g., C57Bl6) as compared to short-lived (DBA/2J) mice (Kucia et al., 2005). It would be interesting to identify the genes responsible for tissue distribution and expansion of these cells, as they could be involved in controlling the life span of mammals.

Since VSELs express several markers of PGCs [fetal-type alkaline phosphatase (AP), Oct-4, SSEA-1, CXCR4, Mvh, Stella, Fragilis, Nobox, Hdac6], they could be closely related to a population of epiblast-derived PGCs. VSELs are also highly mobile and respond robustly to an SDF-1 gradient, adhere to fibronectin and fibrinogen, and may interact with BM-derived stromal fibroblasts. Confocal microscopy and time-lapse studies revealed that these cells attach rapidly to, migrate beneath, and undergo emperipolesis in marrow-derived fibroblasts (Kucia et al., 2005). Since fibroblasts secrete SDF-1 and other chemottractants, they may create a homing environment for small CXCR4$^+$ VSELs. This robust interaction of VSELs with BM-derived fibroblasts has an important implication, namely that isolated BM stromal cells may be contaminated by these tiny cells from the beginning. This observation may somehow explain the unexpected “plasticity” of marrow-derived fibroblast-like cells, e.g., MSCs or MAPCs. Moreover, since VSELs are pluripotent they may also give rise to MSCs, and thus could potentially reside at the tip of MSCs hierarchy. To support this notion, a population of primitive cells with some of the VSELs markers was recently described by another group as the most primitive population of MSCs (Anjos-Afonso and Bonnet 2007).

Recently, a very similar population of cells showing similar morphology and markers to murine BM-derived VSELs was purified from human CB (Kucia et al., 2007). Evidence has also mounted which suggests that similar cells are also present in the human BM, particularly in young patients. It is anticipated that VSELs could become an important source of PSCs for regeneration. At this point, however, it is not clear whether VSELs contribute to the blastocyst development.

VSEL number decreases with aging

Employing flow cytometry, we evaluated the content of VSELs among small BM- derived Sca-1^{+}Lin^{-} nucleated cells isolated from mice at different ages (2 months – 3 years) (Figure 4). Cells that display VSEL phenotype were also identified in several other adult organs (Figure 4). However, we noticed that the number of these cells (Sca-1^{+}Lin^{-}CD45^{-}) in BM from C57BL/6 mice gradually decreases over time from 0.052±0.018% to 0.003±0.002% between age of 2-months and 3-years, respectively (Figure 4). More importantly, not only a number of these cells in adult organs decreases with the age but also their ability to form spheres containing primitive stem cells (VSEL-DS) declaims with time. This age-dependent content of VSELs in BM may explain why the regeneration processes are more efficient in younger individuals, when compared to older organisms. Further studies require the possibility that age-dependent decrease in number of pluripotent VSELs may be connected with age-related expansion of some tissue committed stem cells (TCSCs) – as seen in adult murine BM, where a number of monopotent hemato/lymphopoiesis committed HSC increase in older animals (Chambers and Goodell 2007, Oakley and van Zant 2007).

Moreover, we have also established the differences in the content of VSELs among BM-derived nucleated cells isolated from long- and short-lived murine strains. We reported that the number of these cells is much higher in BM of long-lived (e.g., C57BL/6) as compared to short-lived (DBA/2J) mice (Kucia et al., 2006a). In our opinion, it would be interesting to identify genes that are responsible for tissue distribution/expansion of these cells as they could be involved in controlling the life span of mammals.

However, there are more scientific challenges coming out from recent VSELs' studies. Based on our animal data it would be interesting to study whether the number of VSELs depends also on human age. It would be also important to evaluate if there are some differences in VSELs number in BM and other tissues as function of senescence between members of long- and short-living families.

Does fully functional PSC really exist in BM?

Table II shows that several types of non-HSCs that have been identified so far in the BM, i.e., VSELs, MSCs, MAPCs, MIAMIs, and MASCs. An important question remains, however whether a PSC, a founder cell for cells forming all three germ layers, resides in the adult BM. Several lines of evidence support the presence of PSCs in BM tissue. First, expressions of typical PSC markers such as SSEA-1, Oct-4, and Nanog were reported in BM-derived SCs isolated using various strategies. These early embryonic transcription factors that are characteristic for ES cells and epiblast-derived cells were demonstrated at the protein and/or mRNA levels in VSELs, MAPCs, MSCs (in particular the SD fraction), and MIAMI cells (D'Ippolito et al., 2004; Jiang et al., 2002; Kucia et al., 2006a; Lamoury et al., 2006; Pochampally et al., 2004). Second, the contribution of BM-derived cells for regenerating multiple non-hematopoietic organs and tissues indirectly suggests the existence of pluripotent or multipotent SCs in BM. Illustrating this are experiments performed at the single cell level with BM-derived SCs (Fr25 Sca-1^{+}Kit^{+}Lin^{-} or Fr25SKL cell) that contributed to multi-organ, multi-lineage engraftment in lethally irradiated mice (Krause et al., 2001). These cells were first fractionated by elutriation at a flow rate of 25 ml/min

(Fr25), then lineage depleted (lin^-), labeled with cell membrane-marking fluorochrome (PKH26), and injected intravenously into lethally irradiated animals. Two days post-transplant, $PKH26^+$ cells were recovered by flow cytometric sorting of recipient BM. These single adult BM-derived Fr25SKL cells have a robust capacity to differentiate into epithelial cells of the liver, lung, gastrointestinal tract (endoderm), and skin (ectoderm) (Krause et al., 2001).

Nevertheless, several questions relating to the presence of putative PSCs in BM as well as other tissue/organs remain. First, it is important to elucidate whether this cell is merely a remnant from developmental embryogenesis that resides in a dormant state in the adult organism or if it is a rare but mitotically active cell that contributes to the renewal of the pool of other tissue committed stem cells SCs e.g., HSCs in BM, satellite muscle SCs in muscles, neural stem cells in brain. Second, the relationship of this cell must be determined as related to the cells recently described and purified from adult organs, including: $SSEA\text{-}1^+$ $Oct\text{-}4^+$ $Nanog^+$ VSELs; small $Oct\text{-}4^+$ MAPCs; $Oct\text{-}4^+$ SD-fraction of MSCs, $Oct\text{-}4^+$ MIAMI cells and $Oct\text{-}4^+$ $Nanog^+$ MASC. As previously mentioned, it is likely that all of these versatile $Oct\text{-}4^+$ SCs were given different operational names but are indeed very closely related to the same type of PSCs.

As shown in Figure 5, it is possible that these BM-residing PSCs, as remnants of embryonic development (e.g., derivatives from the epiblast), reside in a dormant state in ectotopic BM niches. The dormant status of these cells could be the result of being located in a non-physiological niche, exposed to inhibitors, deprived of some appropriate stimulatory signals, and/or limited in pluripotency because of the erasure of the somatic imprint on some of the crucial somatically-imprinted genes [e.g., H19 and insulin-like growth factor 2 (IGF2)]. These cells, however, could be activated if they are exposed to appropriate activation signals (e.g., upregulated during organ/tissue injury, oncogenesis) or undergo epigenetic changes that alter the methylation status of their DNA and acetylation of histones. Finally, they may be reactivated if a proper somatic imprint is reestablished (Ratajczak et al., 2007a).

Finally, we envision that there is also a potential “dark side” of a presence of VSELs in adult tissues. If these cells don arise somatic imprint or accumulate mutations that may give rise to several tumors including teratomas/teratocarcinomas (persistent somatic imprint), germinal tumors (explained by germ line commitment of VSELs), “small round blue cell” pediatric sarcomas (mutations) or even some types of solid cancers (explained by VSELs fusogeneic potential, mutations).

In our opinion, there is mounting evidence that BM as well as other adult tissues does in fact contain PSCs (e.g., VSELs). These cells display a very primitive morphology and, as shown at the single cell level, are able in *in vitro* cultures to differentiate into cells from all three germ layers. The most convincing evidence for the full pluripotency of BM-derived SCs would be the demonstration that these cells can complement blastocyst development after injection into a developing blastocyst. Unfortunately, such evidence for pluripotency has so far not been achieved in a reproducible way with any of the BM-derived SCs.

Conclusions

Humanity continually searches for an end to the suffering caused by illness and a better quality of life in advancing years. Medical science is now looking to SCs to advance this important quest. Adult SCs could potentially provide a real therapeutic alternative to the controversial use of cells from human embryos and therapeutic cloning. Data obtained in our laboratory indicate that VSELs could potentially provide a real therapeutic alternative. Hence, because of this ethical debate on a proper usage of SCs (embryonic vs. isolated from adult tissues) the potential of VSELs is ripe for exploration. Further studies are also required

on a role of VSELs in aging. Would it be for example possible to control safely a pool of these cells in adult organism, expand their survival time and harness them effectively to rejuvenate senescent organs? Would it be also possible to regenerate damaged organs and to expand life span by transplantation of VSELs? We can envision a possibility that VSELs could be isolated from the patient at young age and than inject back into same recipients several years latter, in case of major health complications (e.g., heart infarct, stroke). Such strategies of course should be first tested in animal models.

Therefore, it has to be determined whether these cells could be efficiently employed in the clinic or whether they are merely developmental remnants found in the BM that cannot be harnessed effectively for regeneration. The coming years will bring important answers to these questions.

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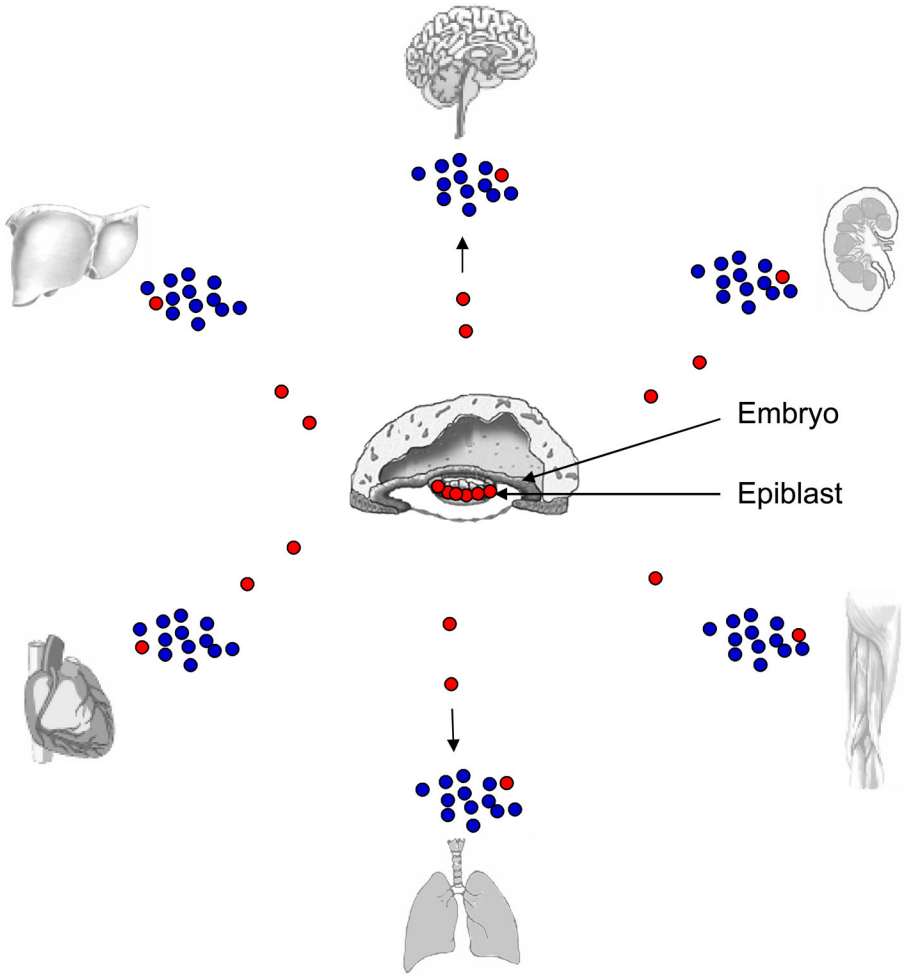

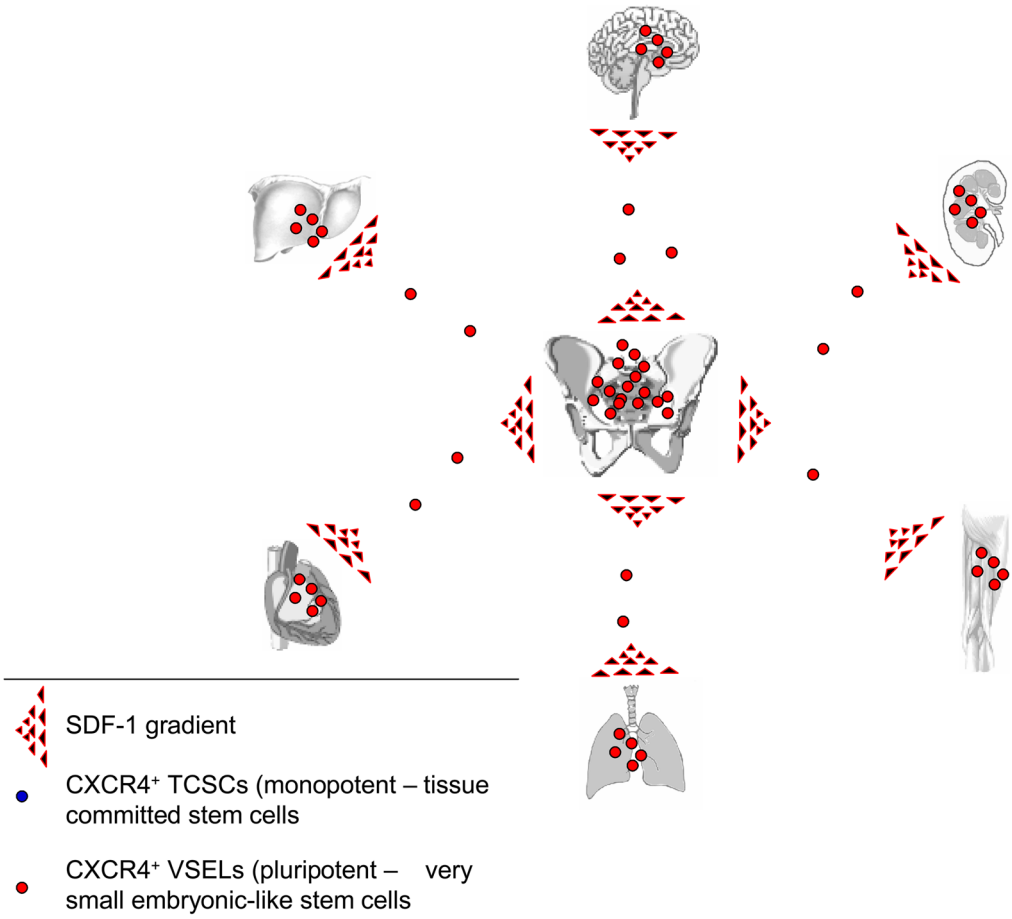

Figure 1. Developmental origin of VSELs and their potential role in tissue homeostasis

Panel A – Epiblast derived stem cells (EPSCs) (red circles) become stem cells for all developing tissues/organs. We hypothesized that after they generate tissue committed/ monopotent stem cells (TCSC) shown as blue circles - some of them may survive into adulthood in various organs (red circles). **Panel B -** BM-derived VSELs circulate in peripheral blood. The concept is presented based on the assumption that $CXCR4^+$ VSELs circulate in the PB. Although the percentage of these cells circulating in PB is much lower than that of early hematopoietic stem/progenitor cells, these mobilized non-HSCs may have an important role in tissue repair following injury. The mobilization of these cells occurs during tissue damage or injury (e.g., heart infarct, stroke, toxic liver damage). It is postulated that after mobilization into PB, $CXCR4^+$ VSELs may subsequently be chemoattracted by an SDF-1 gradient to the damaged tissues. In this context, BM tissue becomes a “hiding place” for both HSCs and circulating $CXCR4^+$ VSELs. We hypothesize that they could serve an important role in tissue/organ repair as a mobile reserve pool of PSCs. Besides the SDF-1-CXCR4 axis, the trafficking of these cells involves several other motomorphogens such as HGF, VEGF, and leukemia inhibitory factor (LIF).

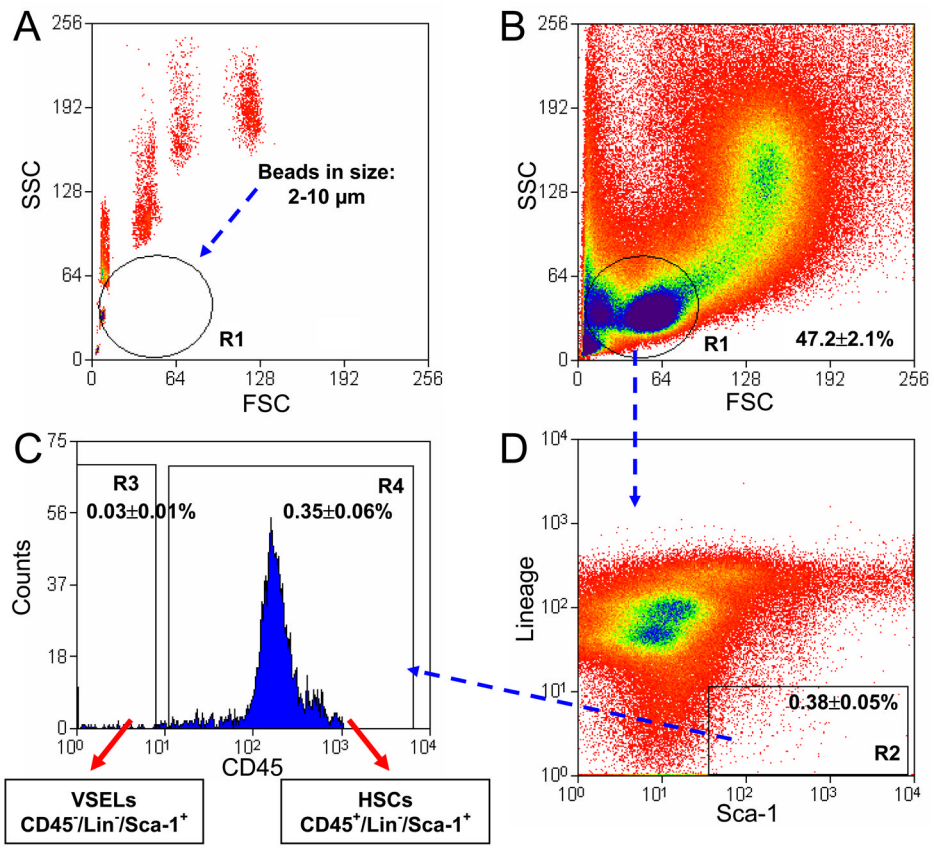

Figure 2. Gating strategy for sorting VSELs by FACS

BM-derived VSELs were isolated from immunofluorescence stained murine BM nucleated cells by FACS. **Panel A:** Agranular, small events ranging from 2 – 10 µm were included into gate R1 after comparison with six differently sized bead particles with standard diameters of 1, 2, 4, 6, 10, and 15 µm (Flow Cytometry Size beads, Invitrogen; Molecular Probes, Carlsbad, Ca, USA). **Panel B:** BM nucleated cells were visualized by dot plots showing forward scatter (FSC) vs. side scatter (SSC) signals, which are related to the size and granularity/complexity of the cell, respectively. **Panel D:** Cells from region R1 were further analyzed for Sca-1 and Lin expression and only Sca-1^{+}/Lin^{-} events were included into region R2. Population from region R2 was subsequently sorted based on CD45 marker expression into $CD45^{-}$ and $CD45^{+}$ subpopulations visualized on histogram (**Panel C**, regions R3 and R4, respectively). Sca-1^{+}/Lin^{-}/$CD45^{-}$ cells (VSELs) were sorted as events enclosed in logical gate including regions R1, R2, and R3, while Sca-1^{+}/Lin^{-} /$CD45^{+}$ cells (HSCs) from gate including regions R1, R2, and R4. Percentages show the average content of each cellular subpopulation (± SEM) in total BM nucleated cells.

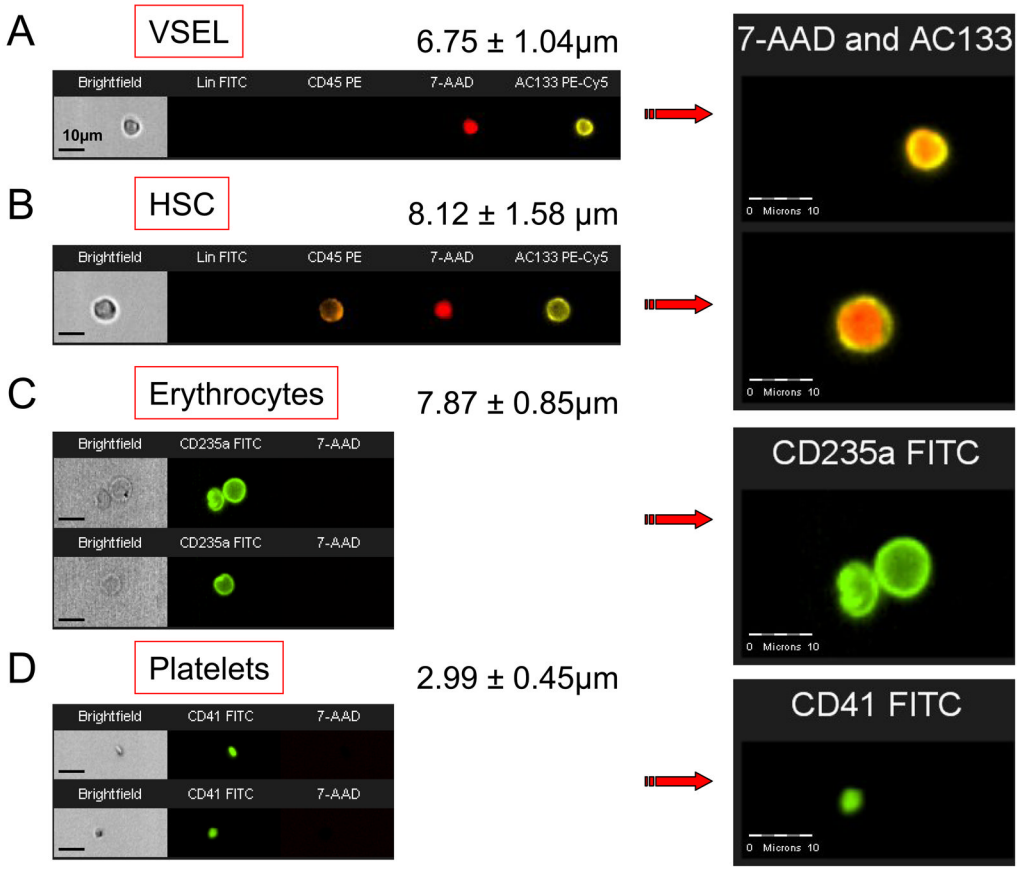

Figure 3. ImageStream images of VSELs and other cells derived from human cord blood

CB- derived VSELs (**Panel A**) and CB-derived HSCs (**Panel B**) were stained for hematopoietic lineages markers (Lin) [fluoresce in isothiocynate (FITC), green], CD45 [phycoerythrin (PE), orange] and AC133 [PE-Cy5, yellow]. Cells were subsequently fixed, permeabilized and nuclei were stained with 7-aminoactinomycin D [7-AAD, red]. **Panel C** shows erythrocytes derived from CB and stained with glycophorin A (CD235a) [FITC, green], while **Panel D** presents CB-derived platelets stained against CD41 [FITC, green]. Both mentioned populations were further stained with 7-AAD following fixation. Erythrocytes and platelets do not possess nuclei, while VSELs show cellular structure containing nuclei. which confirmed lack of nuclei. Average size of each population was calculated by IDEAS software and is presented as Mean ± SD. Scale bars show 10μm.

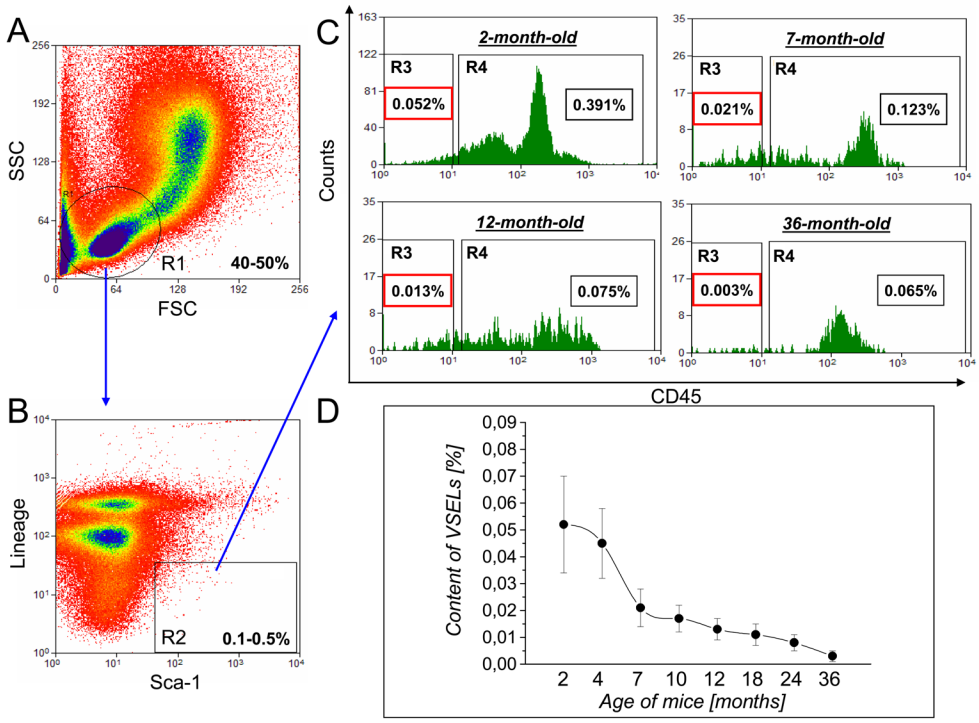

Figure 4. Age dependent decrease in the content of VSELs in murine bone marrow

As shown on Panels A-C, VSEL content was analyzed as described on Figure 2. Total nucleated bone marrow cells were stained for Sca-1, CD45 and lineage markers and subsequently analyzed by MoFlo. The graph on Panel D shows the average contents of Sca-1^{+}/Lin^{-}/CD45^{-} VSELs according to the age of animals (Mean ± SEM).

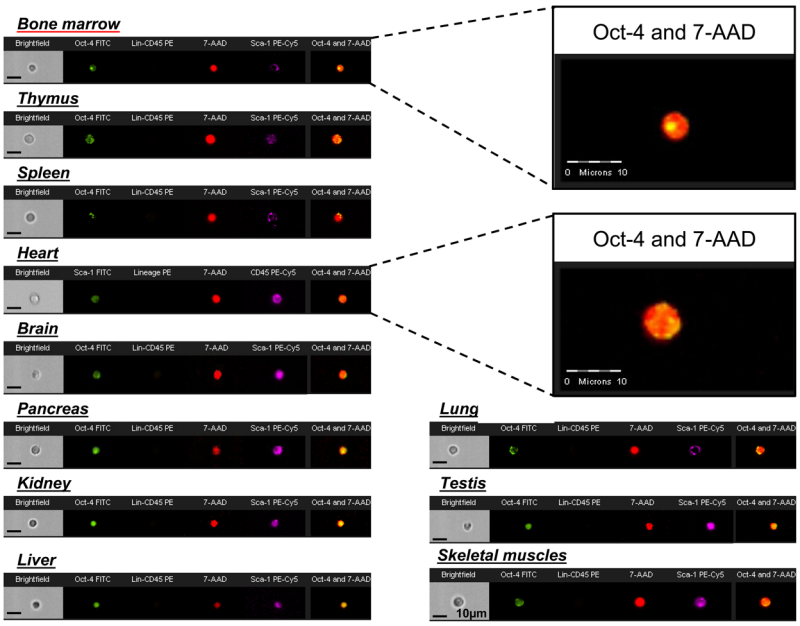

Figure 5. Examples of Oct-4^{+} VSELs isolated from the organs from adult mice

Figure shows representative images of Oct-4^{+} cells exhibiting VSELs phenotype (Sca-1^{+}/Lin^{-}/CD45^{-}) obtained by ImageStream system. Cells isolated from various murine tissues after enzymatic digestion were fixed, permeabilized and stained for transcription factor Oct-4 (FITC, green) as well as hematopoietic lineages markers (Lin), CD45 (PE, orange) and Sca-1 (PE-Cy5, magenta). Nuclei were visualized by staining with 7-aminoactinomycin D (7-AAD, red) and cells were analyzed by ImageStream System 100. Scale bars show 10µm.

Table I

Mechanisms that may alternatively explain the “phenomenon of trans-dedifferentiation or plasticity” of hematopoietic stem cells (HSC)

Potential Mechanism	Explanation of phenomenon at molecular/cellular level	References supporting or refuting concept
Cell fusion	The relatively rare phenomenon by which infused HSC may fuse with cells in damaged tissues and form heterokaryons. Heterokaryons created this way express markers of both donor and recipient cells (pseudochimerism).	Terada et al., 2002, Harris et al., 2004
Microvesicles- induced change of phenotype (e.g., transfer of mRNA and proteins)	Some of the plasticity data could be explained by a transient modification of cell phenotype by the transfer of receptors, proteins and mRNA between HSC and damaged cells by membrane- derived microvesicles.	Aliotta et al., 2007, Deregibus et al., 2007, Ratajczak et al., 2007b.
Epigenetic changes	Factors present in the environment of damaged organs induce epigenetic changes in genes that regulate pluripotency of HSC (involvement of changes in DNA methylation, acetylation of histones). More evidence needed to support that it is a robust and reproducible phenomenon.	Jaenisch and Young 2008.
Paracrine stimulation	HSC are a source of different trophic and angiopoietic factors that may promote tissue/organ repair.	Majka et al. 2001.
Heterologous population of stem cells in BM	In addition to HSC, BM contains other stem cell populations. Regeneration could be explained by the presence of endothelial progenitors that promote neo-vasculogenesis and also by the presence of other stem cells including PSC (e.g., VSELs). This possibility could also explain the loss of contribution of BM cells to organ regeneration with use of highly purified populations of HSC.	Orkin and Zon 2002, Ratajczak et al., 2004, Wagers et al., 2002

Table II

Versatile non-HSCs described in BM

Stem Cells	Phenotype	Differentiation potential	References
Mesenchymal Stem Cells (MSCs) *	**International Society for Cellular Therapy criteria:** CD105$^+$, CD73$^+$, CD90$^+$, CD45$^-$, CD34$^-$, CD14$^-$, CD11b$^-$, CD79a$^-$, CD19$^-$, HLA− DR$^-$ **Other additional markers:** Stro-1$^+$, SB-10$^+$ (CD166), SH-2$^+$ (epitope on CD105), SH-3$^+$ (epitope on CD73), SH- 4$^+$ (epitope on CD73), CD44$^+$, CD29$^+$, CD31$^-$, vWF $^-$ **Markers of most primitive MSC:** CXCR4, CD133, CD34 (?), p75LNGFR	Osteoblasts, chondrocytes, fibroblasts, adipocytes, neural cells?, spermatogonia?, cardiomyocytes?	Dominici et al., 2006
Multipotent Adult Progenitor Cells (MAPCs) *	SSEA-1$^+$, CD13$^+$, Flk- 1^{low}, Thy-1^{low}, CD34$^-$, CD44$^-$, CD45$^-$, CD117(c-kit)$^-$, MHC I$^-$, MHC II$^-$	Endothelium, neural cells, hepatocytes, intestinal epithelium, retina, kidney, lung, hematopoietic cells	Jiang et al., 2002
Marrow-isolated Adult Multilineage Inducible (MIAMI) Cells *	CD29$^+$, CD63$^+$, CD81$^+$, CD122$^+$, CD164$^+$, c- Met$^+$, BMPR1B$^+$, NTRK3$^+$, CD34$^-$, CD36$^-$, CD45$^-$, CD117 (c-kit)$^-$, HLA-DR$^-$	osteoblasts, chondrocytes, adipocytes, neural cells, pancreatic islets.	D'Ippolito et al., 2004
Multipotent Adult Stem Cells (MASCs) *	CD13$^+$, CD49b$^+$, CD90$^+$, CD73$^+$, CD44$^+$, CD29$^+$, CD49a$^+$, CD105$^+$, MHC I$^+$, HLA- DR$^-$, CD14$^-$, CD34$^-$, CD45$^-$, CD38$^-$, CD133$^-$, c-kit (CD117)$^-$	Neural cells, osteoblasts, skeletal muscle cells, endothelial cells, hepatocytes.	Beltrami et al., 2007
Very Small Embryonic Like (VSEL) Stem Cells	CXCR4$^+$, AC133$^+$, CD34$^+$, SSEA-1$^+$ (mouse), SSEA-4$^+$ (human), AP$^+$, c-Met$^+$, LIF-R$^+$, CD45$^-$, Lin$^-$, HLA-DR$^-$, MHC I $^-$, CD90$^-$, CD29$^-$, CD105$^-$	Cardiomyocytes, neural cells, insulin producing cells, hematopoietic cells.	Kucia et al., 2006a

* (phenotype of expanded/cultured adherent cells)

Abbreviations: fetal alkaline phosphatase (AP), bone morphogenetic protein receptor 1B (BMPR1B), receptor of hepatocyte growth-factor (c-Met), receptor for leukemia inhibitory factor (LIF-R), neurotropic tyrosine kinase receptor 3 (NTRK3), von Willebrand factor (vWF)