Published in final edited form as:

Adv Exp Med Biol. 2013 ; 777: 125–141. doi:10.1007/978-1-4614-5894-4_9.

CD133 Expression Strongly Correlates with the Phenotype of Very Small Embryonic/Epiblast-like Stem Cells

Mariusz Z. Ratajczak^{1,2}, Kasia Mierzejewska², Janina Ratajczak^{1,2}, and Magda Kucia^{1,2}

¹Stem Cell Biology Program at the James Graham Brown Cancer Center, University of Louisville, Louisville, Kentucky, USA ²Department of Physiology Pomeranian Medical University, Szczecin, Poland

Abstract

CD133 antigen (prominin-1) is a useful cell surface marker of very small embryonic-like stem cells (VSELs). Antibodies against it conjugated to paramagnetic beads or fluorochromes are thus powerful biological tools for their isolation from human umbilical cord blood, mobilized peripheral blood, and bone marrow. VSELs are described with the following characteristics: i) are slightly smaller than red blood cells; ii) display a distinct morphology, typified by a high nuclear/cytoplasmic ratio and an unorganized euchromatin; iii) become mobilized during stress situations into peripheral blood; iv) are enriched in the CD133⁺Lin[−]CD45[−] cell fraction in humans; and v) express markers of pluripotent stem cells (e.g., Oct-4, Nanog, and stage-specific embryonic antigen-4). The most recent *in vivo* data from our and other laboratories demonstrated that human VSELs exhibit some characteristics of long-term repopulating hematopoietic stem cells, and are at the top of the hierarchy in the mesenchymal lineage. However, still more labor is needed to characterize better at a molecular level these rare cells.

Keywords

VSELs; CD133; cell isolation; stem cells

9.1 Introduction

One of the most challenging questions in stem cell biology is whether stem cells, which are able to differentiate into cells from multiple germ layers, can be isolated from adult tissues. From a developmental point of view, stem cells that are able to differentiate into all three germ layers (meso-, endo-, and ectoderm) are named pluripotent stem cells (PSCs), while stem cells that are already more restricted in differentiation and thus able to differentiate into cells from two or one germ layer are named multipotent stem cells. Several groups of investigators employing i) various isolation protocols, ii) detection of surface markers, and iii) experimental *in vitro* and *in vivo* models, have reported the presence of cells that possess

a pluripotent or multipotent character in adult tissues, including adult bone marrow (BM) (1-9) and umbilical cord blood (UCB) (10-15).

For example, BM and UCB have each been described as a source of various stem cells that, in experimental animals, have been shown to contribute to skeletal muscle, liver, neural, and myocardial regeneration after delivery to injured tissues (9, 16-21). Since hematopoietic stem cells (HSCs) are most abundant among the stem cells present in UCB, this unique capability of UCB-derived cells was initially (and wrongly) explained by the trans-dedifferentiation or plasticity of hematopoietic stem cells (HSCs) (22-24).

Instead, evidence has accumulated that BM and UCB contain a heterogeneous population of stem cells. For example, BM has been reported to contain multipotent adult progenitor cells (MAPCs) (25), marrow-isolated adult lineage inducible (MIAMI) cells (1), and multipotent adult stem cells (MASCs) (3), while UCB was reported to be a source of unrestricted somatic stem cells (USSCs) (15), multipotent mesenchymal cells (MSCs) (22), and omnicytes (14, 26). Moreover, we have purified a rare population of small cells from both BM (8) and UCB (10) that exhibit embryonic stem cell characteristics, and these cells were named very small embryonic-like stem cells (VSELs). The presence of similar cells that display PSC markers characteristic of UCB-VSELs was recently confirmed independently by other groups in human UCB, BM, and mobilized peripheral blood (mPB) (11-13).

VSELs isolated from UCB are phenotypically similar to VSELs initially described in adult murine BM (8). Morphologically, they are very small in size (smaller than erythrocytes) and possess large nuclei containing an unorganized euchromatin. They express the nuclear embryonic transcription factors Oct-4 and Nanog, and the stage-specific embryonic antigen-4 (SSEA-4) is present at their surfaces. They are enriched within the CD133⁺, lineage negative (Lin⁻), and CD45⁻ cell fractions (27, 28). and co-express CD34 and CXCR4. (10, 27).

Thus, evidence has accumulated that BM, mPB, and UCB could be a source of primitive VSELs, and in this chapter we will focus on strategies to purify such cells and their potential applications in the clinic. We envision that these cells are at the top of the hierarchy of stem cells and perhaps may even give rise to MAPCs, MIAMI cells, MASCs, MSCs, USSCs, and omnicytes. This, however, needs further experimental evidence.

9.2 Why do PSCs Remain in Adult Tissues and Organs after Completion of Embryogenesis?

For many years it has been accepted that adult tissues contain only tissue-committed stem cells (TCSCs), such as epidermal stem cells, HSCs, or skeletal muscle stem cells, which have a limited potential for differentiation, developing into only one type of differentiated cell (7, 20, 29, 30). Nevertheless, we have to consider two scenarios that could occur during early embryogenesis and the development of lineage-restricted TCSCs (29-32). In the first scenario, PSCs present in the inner cell mass of the blastocyst (and at later developmental stages in epiblasts), after giving rise to more differentiated lineage-restricted TCSCs, gradually disappear from the growing embryo and are not present in adult tissues. In the

second scenario, which we believe actually takes place, some PSCs give rise to TCSCs but some survive in adult tissues as a backup population of PSCs that renews the pool of TCSCs over time. In this second scenario, PSCs such as VSELs are precursors of TCSCs during organ and tissue rejuvenation and a source of these cells in emergency situations when organs are damaged (e.g., by heart infarct or stroke). Molecular analysis of murine BM-derived VSELs has revealed that these cells in fact express several genes that are characteristic of PSCs from the inner cell mass of the blastocyst (*Rex1*, also known as *Zfp42*) as well as from the epiblast (*Gbx2*, *Fgf5*, and *Nodal*) (33-35). These cells, however, have to be protected from uncontrolled proliferation that could lead to formation of teratomas. In fact, due to epigenetic changes in genes that regulate insulin/insulin-like growth factor signaling (IIS), murine VSELs deposited in adult tissues are kept under control, and in a quiescent state, which is essential for preventing their uncontrolled proliferation (33-35). This important control mechanism affects genes that show paternal imprinting (e.g., *Igf2-H19*, *Igf2R*, and *RasGRF1*) and has been reviewed elsewhere (30). In brief, by modifying the expression of paternally imprinted genes, VSELs down regulate pathways that are crucial in IIS and thus remain in a quiescent state (30, 33-35).

9.3 Data Supporting the Pluripotency of Murine VSELs

There are stringent criteria *in vitro* and *in vivo* for classifying a stem cell as a PSC. These criteria were established by embryologists working with embryonic stem cells (ESCs) isolated from embryos, established *in vitro*-immortalized ESC lines, or induced pluripotent stem cells (iPSCs). The most important *in vitro* criteria required to call a stem cell pluripotent include i) undifferentiated morphology, euchromatin, and a high nuclear/cytoplasm ratio, ii) expression PSC markers (e.g., Oct-4, Nanog, and SSEA), iii) open chromatin at the Oct-4 promoter, iv) the presence of bivalent domains, v) female PSC reactivation of the X chromosome, and finally vi) the ability for multilineage differentiation into cells from all three germ layers (meso-, ecto-, and endoderm). For *in vivo* criteria, PSCs should i) complement blastocyst development and ii) form teratomas after inoculation in immunodeficient mice. However, it is worth mentioning that these *in vivo* criteria of pluripotency are not always applicable to pluripotent epiblast-derived stem cells (36, 37).

9.3.1 In vitro Criteria that Support VSEL Pluripotency

Our morphological and molecular analysis of murine VSELs supported their pluripotent character (34, 38). At the molecular level, these cells not only truly express acknowledged markers of pluripotency (Oct-4 and Nanog) but also possess bivalent domains, while in addition, female mouse-derived VSELs reactivate the X chromosome. Moreover, the *Oct-4* and *Nanog* promoters in VSELs, like cells isolated from ESC-derived embryoid bodies, are hypomethylated and the chromatin immunoprecipitation (ChIP) assay revealed their association with histone H3Ac, which is a marker for open-type chromatin (33, 35, 38). Like undifferentiated ESCs, VSELs exhibit bivalent domains in which transcriptionally active histone codes such as H3K4me3 physically coexist with repressive histone codes such as H3K27me3 at promoters for homeodomain-containing transcription factors (*Sox21*, *Nkx2.2*, *Dlx1*, *Lbx1h*, *Hlxb9*, *Pax5*, and *HoxA3*). Finally, we also observed that VSELs derived from female animals, like female ESCs isolated from the blastocyst ICM, reactivate one of the X

chromosomes that was inactivated after fertilization, and, as a result, female PSCs display two equivalently activated X chromosomes.

9.3.2 In Vitro Data Supporting the Pluripotency of VSELs

Several co-culture conditions have been successfully developed in which murine VSELs, when plated over supportive cell lines, expand/differentiate into somatic cell types (8, 39, 40). One of these conditions is co-culture over the myoblastic cell line C2C12, which allows some of the murine VSELs to differentiate and form spherical structures (41). The cells in these spheres are immature, with large nuclei containing euchromatin and, when plated into cultures promoting tissue differentiation, expand into cells from all three germ-cell layers (e.g., neural cells, cardiomyocytes, and insulin-producing cells). Another co-culture system that allows the hematopoietic specification of VSELs employs the OP9 stromal cell support (40). Of note, this OP9-based co-culture system, which we employed for VSELs, is widely employed by other investigators for hematopoietic specification of ESCs and induced pluripotent stem cells (iPSCs) (42).

9.3.3 In vivo Multilineage Differentiation of VSELs

Our recent collaborative work indicates that VSELs may also be specified *in vivo* into mesenchymal stem cells (MSCs), cardiomyocytes, and HSCs (43-46). For example, in the first study by Taichman *and colleagues*, VSELs isolated from (green fluorescent protein (GFP)⁺ mice were implanted into severe combined immunodeficiency (SCID) mice, and 4 weeks later the formation of bone-like tissues was observed (43). Based upon the ability of uncultured VSELs to (i) differentiate *in vivo* into multiple mesenchymal lineages and (ii) generate osseous tissues at low density, they proposed that this population of cells fulfills many of the required characteristics of MSCs. Furthermore, they also recently obtained similar results with human VSELs (personal communication from Dr. Taichman; manuscript submitted).

In another collaborative study, BM-derived VSELs freshly isolated from GFP⁺ transgenic mice were injected into the hearts of mice that had undergone ischemia/reperfusion injury (44). After 35 days of follow-up, VSEL-treated mice exhibited improved global and regional left ventricular (LV) systolic function (determined by echocardiography) and attenuated myocyte hypertrophy in surviving tissue when compared with vehicle-treated controls (determined by both histology and echocardiography). Since GFP⁺ VSELs were employed for transplantation, we could track the fate of injected cells in the myocardium. Although VSEL transplantation resulted in isolated new myocytes and capillaries in the infarct region, their numbers were too small to account for all of the observed benefits. Thus, it is likely that in this particular tissue injury model, some paracrine effects by transplanted VSELs played an important additional role, and we are now analyzing the profile of growth factors and cytokines secreted by these cells. In parallel, we are also optimizing our *ex vivo* protocol to differentiate VSELs into cardiomyocytes (45, 46).

Finally, to directly test the possibility of hematopoietic differentiation of VSELs and their relationship with HSCs, we purified these cells from murine BM, primed/co-cultured them over OP9 stromal cells, and subsequently tested their hematopoietic potential by *in vitro* and

in vivo assays (39, 40). In parallel, cells derived from VSELs were evaluated for expression of hematopoietic genes and surface markers. While we observed that freshly isolated VSELs do not exhibit *in vitro* and *in vivo* hematopoietic potential, they may, upon co-culture over OP9 stromal cells, differentiate along the hematopoietic lineage (40). “OP9-primed” VSEL-derived cells acquired expression of several hemato/lymphopoiesis-specific genes and markers, gave rise to hematopoietic colonies *in vitro*, and protected lethally irradiated mice in both primary and secondary transplant models upon transplantation (40).

Recently, we also demonstrated that human UCB-derived CD133⁺ VSELs could also be specified into the hematopoietic lineage in co-cultures with OP9 stromal cells. Importantly, hematopoietic cells derived from VSELs were also able to engraft in immunodeficient mice (39). Based on these observations, we propose that VSELs are the most primitive murine BM-residing population of stem cells that has the potential to become specified into the hematopoietic lineage, and thus they may share some of the characteristics of long-term repopulating HSCs (LT-HSCs). Moreover, they are a common precursor cell for both the hematopoietic and mesenchymal lineages in BM.

9.4 The Presence of Primitive Stem Cells in UCB—VSELs as Circulating Paramedics

One could ask: why are VSELs present in UCB? To address this question we have to remember that delivery is one of the greatest stresses experienced in life, accompanied by the release of several cytokines, chemokines, and bioactive lipids, while several types of stem cells, including HSCs, VSELs, EPCs, and MSCs, are mobilized into neonatal PB and thus enriched after delivery in harvested UCB. The mobilization of these cells probably plays an important role as an endogenous mechanism to protect and repair small-scale organ and tissue damage that occurs due to hypoxia and mechanical injury during delivery. Thus, we can envision that mobilization of stem cells during delivery is a first-line stem cell therapy that we have all experienced as a neonate. It is probably an old evolutionary mechanism in mammals to increase the mobile pool of stem cells in neonatal blood to perform this important task. While HSCs are involved in immune surveillance of tissues after birth, circulating EPCs, MSCs, and, in particular, pluripotent VSELs play a role in regeneration of neonatal tissues. Moreover, several types of stem cells, including VSELs, are mobilized from adult tissues and organs into PB in patients after heart infarct, stroke, and severe hypoxia, and we envision that in these cases they play the role of “circulating paramedics” that contribute to healing and regeneration of damaged tissues (47–49).

Furthermore, given the small size of CD133⁺ VSELs, their highly migratory properties, as well as their potential to differentiate into all three germ layers, an interesting area of investigation worth pursuing is the possible role of these cells in establishing so-called fetal–maternal microchimerism. Observed following pregnancy, this phenomenon is based on bi-directional transplacental trafficking of cells present in UCB and occurs routinely during the normal course of pregnancy. While poorly understood, it is proposed to involve migration of very primitive stem cells from the fetus to the mother and from mother to fetus (26, 50).

These cells can stay in a dormant state in host tissues for many years and it is very likely that VSELs are involved in this phenomenon. This possibility, however, requires further study.

9.5 Reports by Other Groups on VSELs and Closely Related Cells Present in Adult Tissues and Neonatal UCB

One of the most common features of VSELs is their very primitive morphology, high nuclear/cytoplasmic ratio, and relatively small size (8, 10, 27, 51). Recently, there have been several published reports that support the existence of small, primitive VSELs or VSEL-like cells in adult tissues, and we will briefly discuss the most important of these (43, 52-57).

In one of the abovementioned reports, a population of murine VSELs was isolated from BM, and, in a set of elegant experiments, these cells were demonstrated to be able to give rise to mesenchymal and endothelial lineages (43). The bone-forming activity of these cells, if embedded in gelatin sponges and implanted into living mice, exceeded the activity of other populations of BM-purified cells tested in the same assay. Based on this finding, it has been proposed that Sca-1⁺Lin⁻CD45⁻ VSELs are at the top of the hierarchy for the mesenchymal and endothelial lineages in BM (43).

In another report, VSELs were purified from rat BM and successfully employed in regenerating damaged myocardium in an experimental rodent model of acute myocardial infarction (58). These cells expressed SSEA-1 antigen on their surface and Oct-4 in their nuclei. Similarly, small non-hematopoietic murine BM-derived cells that correspond in size to VSELs were also shown to give rise to type 2 pneumocytes, which produce lung surfactant protein after transplantation into surfactant-deficient mice (53). In order to determine whether surfactant-producing epithelial cells were derived from the non-hematopoietic or the hematopoietic fraction of BM cells used to treat surfactant-deficient mice, the authors employed a lineage-tracing approach in which Cre recombinase-specific, *vav*-promoter-activated GFP from a ROSA-GFP construct was used as a reporter transgene (53). Similarly small cells with VSEL markers were also identified in murine neonatal retina (59), while in another report it was demonstrated that small non-hematopoietic lineage-negative (Lin⁻) cells isolated from adult BM by elutriation (Fraction 25) were involved in retinal regeneration following the induction of anterior ischemic optic neuropathy and optic nerve crush in a rodent model (60). A similar population of non-hematopoietic CD45⁻ small stem cells harvested from BM via elutriation has recently been shown to give rise to functional insulin-producing cells *in vivo* in chemically induced diabetic mice (52). Finally, several features of VSELs are displayed in so-called multilineage differentiating, stress-enduring (Muse) cells recently isolated from murine and human BM populations (55, 61). Of note, it has been postulated that these cells play a major role as cell populations that preferentially give rise to iPSCs when BM-derived stromal cells are induced to pluripotency by genetic manipulation (55, 61).

The presence of VSELs in human tissues has also been supported by several recent publications. For example, an interesting connection has been made between VSELs and stem cells in female (54, 56) and male gonads (57). Cells similar to UCB-VSELs were reported by two independent groups to reside in an ovarian epithelial layer in

postmenopausal ovaries (54, 56). Moreover, it has been proposed that the ovary harbors two distinct populations of stem cells: VSELs that are pluripotent and quiescent with nuclear expression of Oct-4, and VSELs that give rise to slightly larger “progenitor” ovarian germ stem cells expressing Oct-4 in the cytoplasm (56).

Finally, VSELs or cells closely related to them have been successfully purified from human mPB (11) and UCB by other groups (12, 13, 62, 63). A corresponding population of primitive stem cells that resembles VSELs and was named “omnicytes” was also described as circulating in UCB and is probably capable of migrating into the maternal circulatory system (14, 26).

Thus, mounting evidence supports the conclusion that small cells described as VSELs or VSEL-like cells that express developmentally primitive markers reside in adult murine and human tissues. However, to confirm that these populations of small, primitive stem cells overlap with VSELs requires further direct comparison. Most importantly, VSELs are currently purified in several laboratories worldwide and the coming years will bring more information about their biology and *in vitro* and *in vivo* differentiation potential.

9.6 Isolation of VSELs from UCB

As mentioned before, human VSELs express CD133 as a specific cell surface antigen, and those lacking it should not consequently be referred to as VSELs. Thus, we are employing anti-CD133 monoclonal antibodies (mAbs) conjugated with fluorochromes or attached to paramagnetic immunobeads in our technical setting for the isolation of human VSEL.

9.6.1 Multiparameter Fluorescence-Activated Cell Sorting

Historically, the human UCB-derived VSELs were isolated first by our team as a minute population of Lin[−]CD45[−] cells that express CD133 as well as CD34 and CXCR4 (10). Further analysis has revealed that the most primitive VSELs, which strongly express Oct-4 and SSEA-4, are enriched within the sorted CD133⁺Lin[−]CD45[−] cell fraction and, based on these observations, we concluded that CD133 is the most reliable cell surface marker for these cells (Fig. 9.1). Most recently, we identified an anti-SSEA-4 antibody that is suitable for fluorescence-activated cell sorting (FACS) and isolation of UCB-VSELs as SSEA-4⁺Lin[−]CD45[−] cells (unpublished data).

9.6.2 Enrichment for VSELs by CD133 Paramagnetic Immunobead Selection Followed by Multiparameter Sorting

As mentioned above, VSELs from human UCB were initially purified from an erythrocyte-depleted population of nucleated cells by multiparameter sorting as a population of small CD133⁺CD45[−]Lin[−] cells (10). However, this procedure is time consuming, and the sorting time required to process an entire UCB unit (~50–100 ml) to isolate rare VSELs is up to 3–4 days (10). Therefore, it has been clear from the beginning that a more rapid and less-expensive method for cell isolation must be found. In order to establish a more efficient method for VSEL purification from UCB, we employed a three-step isolation strategy based on the removal of erythrocytes by hypotonic lysis (1st step), immunomagnetic separation of

CD133⁺ cells (2nd step), followed by FACS-based isolation of CD133⁺Lin⁻CD45⁻ cells (3rd step). The entire isolation procedure takes only 2–4 hours per 100 ml of UCB (27, 28).

9.6.3 CD133 Cell Selection Combined with Aldefluor Staining

As an alternative strategy (Fig. 9.2), we recently proposed reducing the number of lineage factors required for purification by exposure of erythrocyte-depleted, immunomagnetic bead-selected CD133⁺ cells to Aldefluor, followed by staining with anti-CD133⁺ antibodies and only two lineage-specific antibodies (anti-CD45 and anti-GlyA) for the third step (39). Aldefluor is a substrate for aldehyde dehydrogenase (ALDH), a cytosolic enzyme highly expressed in less-differentiated hematopoietic cells, and is implicated in resistance to some alkylating agents (64). In the presence of ALDH, Aldefluor becomes modified to a fluorescent molecule that may then be used to mark ALDH-expressing cells. Inclusion of an anti-GlyA antibody is based on the well-known fact that small erythroblast GlyA⁺ cells present in UCB do not express the CD45 antigen. Thus, selection for CD45⁻ cells enriches for these unwanted cells. By employing this strategy, we are able to subsequently sort CD133⁺ cells enriched for VSELs and are able to obtain $\sim 10^3$ CD133⁺/CD45⁻/GlyA⁻/ALDH^{low} and 4×10^3 CD133⁺/CD45⁻/GlyA⁻/ALDH^{high} UCB-VSELs from 100 ml of UCB (39). These numbers illustrate how rare these cells are in UCB. When we compared both fractions of VSELs by real-time polymerase chain reaction (RT-PCR) gene expression analysis, we found that CD133⁺/CD45⁻/GlyA⁻/ALDH^{low} VSELs have a higher expression of *Oct-4* than the CD133⁺/CD45⁻/GlyA⁻/ALDH^{high} fraction (39).

However, we are aware that there is yet room to improve sorting and to develop alternative strategies for purifying UCB-VSELs. To accomplish this goal, one should consider the use of metabolic fluorochromes to see whether VSELs are enriched among Rh123^{dull}, Pyronin Y^{low}, and Hoechst 33342^{low} cells. Moreover, we expect that our current proteomics data analysis with UCB-VSELs will reveal the presence of novel positive markers that could be employed for the purification of these rare cells.

9.7 Novel Characteristics of Human VSELs

In the past two years we investigated several experimental approaches to better characterize murine and human UCB-derived VSELs, both at the molecular and morphological levels. First, our molecular gene array analysis of limited numbers of highly purified VSELs revealed that murine VSELs are somewhat heterogeneous developmentally (33-35). In particular, while all of them express the pluripotency marker Oct-4, some of them express genes that are more closely related to genes expressed by epiblast-derived stem cells and others to those of migrating primordial germ cells. This suggests that VSELs resemble early stem cells developmentally; if isolated from adult BM, they may differ to some extent in their differentiation potential (33-35).

Based on well-established FACS identification protocols, we also found that human CD133⁺Lin⁻CD45⁻ VSELs identified in UCB, like their murine counterparts, i) highly express telomerase (unpublished observation), ii) are diploid, and iii) are viable, as shown by their ability to exclude the viability dye (7-aminoactinomycin D, 7-AAD) (30). Importantly, we observed that the highest number of pluripotent Oct-4⁺ VSELs reside in the population

of CD133⁺Lin⁻CD45⁻ UCB-derived cells (10, 27). Moreover, some of the CD133⁺Lin⁻CD45⁻ VSELs, which represent only a very small subfraction among UCB Lin⁻CD45⁻ non-hematopoietic cells, may co-express other stem cell markers, including CD34, CXCR4, and SSEA-4 (10, 27). Thus, VSELs represent a very rare subpopulation of stem cells among the Lin⁻CD45⁻ non-hematopoietic UCB cell population, which also contains other stem cell types, including endothelial progenitor cells (EPCs) and MSCs. VSELs may be identified by their very small size (FSC^{low}/SSC^{low}) and co-expression of CD34, CXCR4 and notably CD133 (10, 27).

9.8 Conclusion

New data from our group and others have provided additional evidence for the existence and biological role of primitive embryonic-like stem cells in murine adult tissues and their potential role in i) tissue and organ rejuvenation, ii) longevity, and iii) regeneration and repair of damaged tissues. These cells are kept however in a quiescent state in adult tissues by epigenetic changes in expression of some paternally imprinted genes (e.g., *Igf-2*, *RasGrf1*, *Igf-2R*) (Fig. 9.3), which are involved in cell proliferation in response to insulin/insulin-like growth factor signaling (IIS) (30-35). However, in the presence of chronic elevated levels of IGF1 and insulin in blood, VSELs deposited in adult tissues may proliferate in an uncontrolled manner and become depleted much faster over time. This may contribute to the accelerated aging observed in situations with high circulating IGF-1 and insulin levels (e.g., chronic high caloric uptake or obesity). At the same time, chronic exposure to IIS may also trigger uncontrolled activation of VSELs and their malignant transformation.

The identification of UCB-derived VSELs opens up new areas of investigation and new challenges. Our data indicate that these cells can be specified along the hematopoietic lineage and, in BM and UCB, are probably equivalent to a population of LT-HSCs. The fact that many of these cells could be lost in currently employed banking procedures requires the development of better VSEL-saving banking protocols. In fact, some of the commercial UCB banks have acknowledged this fact and now employ proper volume-depletion procedures. The possibility of differentiating UCB-derived VSELs into other type of cells (e.g., neurons, MSCs, or cardiomyocytes) is currently being investigated in our laboratories. However, our data already strongly indicate that VSELs could provide a therapeutic alternative to the controversial use of human ESCs and strategies based on therapeutic cloning. Hence, while the ethical debate on the application of ESCs in therapy continues, the potential of VSELs for tissue repair and regeneration is ripe for exploration, and the expression of CD133 combined with specific antibodies offer a great advance in their prospective isolation.

We are aware that more works are needed to better characterize the small UCB-isolated cells at the molecular level. If we can confirm that a similar mechanism operates for human UCB-derived and murine BM-derived VSELs, perhaps a controlled modulation of the somatic imprint state to produce proper *de novo* methylation of somatic imprinted genes on the maternal and paternal chromosomes could increase the regenerative power of these cells. The coming years will determine whether these exciting possibilities are realized.

Acknowledgments

This work was supported by NIH grant R01 DK074720, the EU Innovative Economy Operational Program POIG. 01.01.02-00-109/09-01, and the Stella and Henry Hoenig Endowment to MZR.

References

1. D'Ippolito G, Diabira S, Howard GA, Menei P, Roos BA, Schiller PC. Marrow-isolated adult multilineage inducible (MIAMI) cells, a unique population of postnatal young and old human cells with extensive expansion and differentiation potential. *Journal of Cell Science*. 2004; 117:2971–81. [PubMed: 15173316]
2. Pittenger MF, Mackay AM, Beck SC, Jaiswal RK, Douglas R, Mosca JD, et al. Multilineage potential of adult human mesenchymal stem cells. *Science*. 1999; 284:143–7. [PubMed: 10102814]
3. Beltrami AP, Cesselli D, Bergamin N, Marcon P, Rigo S, Puppato E, et al. Multipotent cells can be generated in vitro from several adult human organs (heart, liver, and bone marrow). *Blood*. 2007; 110:3438–46. [PubMed: 17525288]
4. Krause DS. Bone marrow-derived cells and stem cells in lung repair. *Proceedings of the American Thoracic Society*. 2008; 5:323–7. [PubMed: 18403327]
5. Vacanti MP, Roy A, Cortiella J, Bonassar L, Vacanti CA. Identification and initial characterization of spore-like cells in adult mammals. *Journal of Cellular Biochemistry*. 2001; 80:455–60. [PubMed: 11135375]
6. Orlic D, Kajstura J, Chimenti S, Jakoniuk I, Anderson SM, Li B, et al. Bone marrow cells regenerate infarcted myocardium. *Nature*. 2001; 410:701–5. [PubMed: 11287958]
7. Orkin SH, Zon LI. Hematopoiesis and stem cells: plasticity versus developmental heterogeneity. *Nature Immunology*. 2002; 3:323–8. [PubMed: 11919568]
8. Kucia M, Reza R, Campbell FR, Zuba-Surma E, Majka M, Ratajczak J, et al. A population of very small embryonic-like (VSEL) CXCR4(+)SSEA-1(+)Oct-4+ stem cells identified in adult bone marrow. *Leukemia*. 2006; 20:857–69. [PubMed: 16498386]
9. Mezey E, Chandross KJ, Harta G, Maki RA, McKercher SR. Turning blood into brain: cells bearing neuronal antigens generated in vivo from bone marrow. *Science*. 2000; 290:1779–82. [PubMed: 11099419]
10. Kucia M, Halasa M, Wysoczynski M, Baskiewicz-Masiuk M, Moldenhawer S, Zuba-Surma E, et al. Morphological and molecular characterization of novel population of CXCR4+ SSEA-4+ Oct-4+ very small embryonic-like cells purified from human cord blood: preliminary report. *Leukemia*. 2007; 21:297–303. [PubMed: 17136117]
11. Sovalat H, Scrofani M, Eidenschenk A, Pasquet S, Rimelen V, Henon P. Identification and isolation from either adult human bone marrow or G-CSF-mobilized peripheral blood of CD34(+)/CD133(+)/CXCR4(+)/Lin(-)CD45(-) cells, featuring morphological, molecular, and phenotypic characteristics of very small embryonic-like (VSEL) stem cells. *Experimental Hematology*. 2011; 39:495–505. [PubMed: 21238532]
12. Bhartiya D, Shaikh A, Nagvenkar P, Kasiviswanathan S, Pethe P, Pawani H, et al. Very small embryonic-like stem cells with maximum regenerative potential get discarded during cord blood banking and bone marrow processing for autologous stem cell therapy. *Stem Cells and Development*. 2012; 21:1–6. [PubMed: 21780911]
13. McGuckin C, Jurga M, Ali H, Strbad M, Forraz N. Culture of embryonic-like stem cells from human umbilical cord blood and onward differentiation to neural cells in vitro. *Nature Protocols*. 2008; 3:1046–55. [PubMed: 18536651]
14. Gordon MY, Levicar N, Pai M, Bachellier P, Dimarakis I, Al-Allaf F, et al. Characterization and clinical application of human CD34+ stem/progenitor cell populations mobilized into the blood by granulocyte colony-stimulating factor. *Stem Cells*. 2006; 24:1822–30. [PubMed: 16556705]
15. Kogler G, Sensken S, Airey JA, Trapp T, Muschen M, Feldhahn N, et al. A new human somatic stem cell from placental cord blood with intrinsic pluripotent differentiation potential. *The Journal of Experimental Medicine*. 2004; 200:123–35. [PubMed: 15263023]

16. Buzanska L, Machaj EK, Zablocka B, Pojda Z, Domanska-Janik K. Human cord blood-derived cells attain neuronal and glial features in vitro. *Journal of Cell Science*. 2002; 115:2131–8. [PubMed: 11973354]
17. Pesce M, Orlandi A, Iachininoto MG, Straino S, Torella AR, Rizzuti V, et al. Myoendothelial differentiation of human umbilical cord blood-derived stem cells in ischemic limb tissues. *Circulation Research*. 2003; 93:e51–62. [PubMed: 12919944]
18. Newsome PN, Johannessen I, Boyle S, Dalakas E, McAulay KA, Samuel K, et al. Human cord blood-derived cells can differentiate into hepatocytes in the mouse liver with no evidence of cellular fusion. *Gastroenterology*. 2003; 124:1891–900. [PubMed: 12806622]
19. Kim BO, Tian H, Prasongsukarn K, Wu J, Angoulvant D, Wnendt S, et al. Cell transplantation improves ventricular function after a myocardial infarction: a preclinical study of human unrestricted somatic stem cells in a porcine model. *Circulation*. 2005; 112:I96–104. [PubMed: 16159872]
20. Wagers AJ, Weissman IL. Plasticity of adult stem cells. *Cell*. 2004; 116:639–48. [PubMed: 15006347]
21. Di Campli C, Piscaglia AC, Pierelli L, Rutella S, Bonanno G, Alison MR, et al. A human umbilical cord stem cell rescue therapy in a murine model of toxic liver injury. *Digestive and Liver Disease*. 2004; 36:603–13. [PubMed: 15460845]
22. Castro RF, Jackson KA, Goodell MA, Robertson CS, Liu H, Shine HD. Failure of bone marrow cells to transdifferentiate into neural cells in vivo. *Science*. 2002; 297:1299. [PubMed: 12193778]
23. Murry CE, Soonpaa MH, Reinecke H, Nakajima H, Nakajima HO, Rubart M, et al. Haematopoietic stem cells do not transdifferentiate into cardiac myocytes in myocardial infarcts. *Nature*. 2004; 428:664–8. [PubMed: 15034593]
24. Nygren JM, Jovinge S, Breitbach M, Sawen P, Roll W, Hescheler J, et al. Bone marrow-derived hematopoietic cells generate cardiomyocytes at a low frequency through cell fusion, but not transdifferentiation. *Nature Medicine*. 2004; 10:494–501.
25. Sohn A, Verfaillie CM. Multipotent adult progenitor cells. *Best practice & Research Clinical Haematology*. 2011; 24:3–11. [PubMed: 21396588]
26. Mikhail MA, M'Hamdi H, Welsh J, Levicar N, Marley SB, Nicholls JP, et al. High frequency of fetal cells within a primitive stem cell population in maternal blood. *Hum Reprod*. 2008; 23:928–33. [PubMed: 18238907]
27. Zuba-Surma EK, Klich I, Greco N, Laughlin MJ, Ratajczak J, Ratajczak MZ. Optimization of isolation and further characterization of umbilical-cord-blood-derived very small embryonic/epiblast-like stem cells (VSELs). *European Journal of Haematology*. 2010; 84:34–46. [PubMed: 19758351]
28. Zuba-Surma, EK., Ratajczak, MZ. Overview of very small embryonic-like stem cells (VSELs) and methodology of their identification and isolation by flow cytometric methods. In: Robinson, J Paul, et al., editors. *Current protocols in cytometry*. Vol. Chapter 9. 2010. Unit9 29
29. Ratajczak MZ, Zuba-Surma EK, Wysoczynski M, Wan W, Ratajczak J, Wojakowski W, et al. Hunt for pluripotent stem cell -- regenerative medicine search for almighty cell. *Journal of Autoimmunity*. 2008; 30:151–62. [PubMed: 18243661]
30. Ratajczak MZ, Shin DM, Liu R, Mierzejewska K, Ratajczak J, Kucia M, et al. Very small embryonic/epiblast-like stem cells (VSELs) and their potential role in aging and organ rejuvenation--an update and comparison to other primitive small stem cells isolated from adult tissues. *Aging*. 2012; 4:235–46. [PubMed: 22498452]
31. Kucia M, Ratajczak J, Reca R, Janowska-Wieczorek A, Ratajczak MZ. Tissue-specific muscle, neural and liver stem/progenitor cells reside in the bone marrow, respond to an SDF-1 gradient and are mobilized into peripheral blood during stress and tissue injury. *Blood cells Molecules & Diseases*. 2004; 32:52–7.
32. Ratajczak MZ, Kucia M, Reca R, Majka M, Janowska-Wieczorek A, Ratajczak J. Stem cell plasticity revisited: CXCR4-positive cells expressing mRNA for early muscle, liver and neural cells 'hide out' in the bone marrow. *Leukemia*. 2004; 18:29–40. [PubMed: 14586476]

33. Shin DM, Liu R, Klich I, Ratajczak J, Kucia M, Ratajczak MZ. Molecular characterization of isolated from murine adult tissues very small embryonic/epiblast like stem cells (VSELs). *Molecules and Cells*. 2010; 29:533–8. [PubMed: 20526817]
34. Shin DM, Liu R, Klich I, Wu W, Ratajczak J, Kucia M, et al. Molecular signature of adult bone marrow-purified very small embryonic-like stem cells supports their developmental epiblast/germ line origin. *Leukemia*. 2010; 24:1450–61. [PubMed: 20508611]
35. Shin DM, Liu R, Wu W, Waigel SJ, Zacharias W, Ratajczak MZ, et al. Global Gene Expression Analysis of Very Small Embryonic-Like Stem Cells Reveals that the Ezh2-Dependent Bivalent Domain Mechanism Contributes to Their Pluripotent State. *Stem Cells and Development*. 2012; 21:1639–52. [PubMed: 22023227]
36. Muller FJ, Goldmann J, Loser P, Loring JF. A call to standardize teratoma assays used to define human pluripotent cell lines. *Cell Stem Cell*. 2010; 6:412–4. [PubMed: 20452314]
37. Smith KP, Luong MX, Stein GS. Pluripotency: toward a gold standard for human ES and iPS cells. *Journal of Cellular Physiology*. 2009; 220:21–9. [PubMed: 19326392]
38. Shin DM, Zuba-Surma EK, Wu W, Ratajczak J, Wysoczynski M, Ratajczak MZ, et al. Novel epigenetic mechanisms that control pluripotency and quiescence of adult bone marrow-derived Oct4(+) very small embryonic-like stem cells. *Leukemia*. 2009; 23:2042–51. [PubMed: 19641521]
39. Ratajczak J, Zuba-Surma E, Klich I, Liu R, Wysoczynski M, Greco N, et al. Hematopoietic differentiation of umbilical cord blood-derived very small embryonic/epiblast-like stem cells. *Leukemia*. 2011; 25:1278–85. [PubMed: 21483440]
40. Ratajczak J, Wysoczynski M, Zuba-Surma E, Wan W, Kucia M, Yoder MC, et al. Adult murine bone marrow-derived very small embryonic-like stem cells differentiate into the hematopoietic lineage after coculture over OP9 stromal cells. *Experimental Hematology*. 2011; 39:225–37. [PubMed: 21034791]
41. Kucia M, Wysoczynski M, Ratajczak J, Ratajczak MZ. Identification of very small embryonic like (VSEL) stem cells in bone marrow. *Cell and Tissue Research*. 2008; 331:125–34. [PubMed: 17828555]
42. Lohle M, Hermann A, Glass H, Kempe A, Schwarz SC, Kim JB, et al. Differentiation efficiency of induced pluripotent stem cells depends on the number of reprogramming factors. *Stem Cells*. 2012; 30:570–9. [PubMed: 22213586]
43. Taichman RS, Wang Z, Shiozawa Y, Jung Y, Song J, Balduino A, et al. Prospective identification and skeletal localization of cells capable of multilineage differentiation in vivo. *Stem Cells and Development*. 2010; 19:1557–70. [PubMed: 20446812]
44. Dawn B, Tiwari S, Kucia MJ, Zuba-Surma EK, Guo Y, Sanganalmath SK, et al. Transplantation of bone marrow-derived very small embryonic-like stem cells attenuates left ventricular dysfunction and remodeling after myocardial infarction. *Stem Cells*. 2008; 26:1646–55. [PubMed: 18420834]
45. Zuba-Surma EK, Guo Y, Taher H, Sanganalmath SK, Hunt G, Vincent RJ, et al. Transplantation of expanded bone marrow-derived very small embryonic-like stem cells (VSEL-SCs) improves left ventricular function and remodelling after myocardial infarction. *Journal of Cellular and Molecular Medicine*. 2011; 15:1319–28. [PubMed: 20629987]
46. Wojakowski W, Tendera M, Kucia M, Zuba-Surma E, Milewski K, Wallace-Bradley D, et al. Cardiomyocyte differentiation of bone marrow-derived Oct-4+CXCR4+SSEA-1+ very small embryonic-like stem cells. *International Journal of Oncology*. 2010; 37:237–47. [PubMed: 20596650]
47. Wojakowski W, Tendera M, Kucia M, Zuba-Surma E, Paczkowska E, Ciosek J, et al. Mobilization of bone marrow-derived Oct-4+ SSEA-4+ very small embryonic-like stem cells in patients with acute myocardial infarction. *Journal of the American College of Cardiology*. 2009; 53:1–9. [PubMed: 19118716]
48. Paczkowska E, Kucia M, Koziarska D, Halasa M, Safranow K, Masiuk M, et al. Clinical evidence that very small embryonic-like stem cells are mobilized into peripheral blood in patients after stroke. *Stroke*. 2009; 40:1237–44. [PubMed: 19246697]
49. Kucia M, Zhang YP, Reca R, Wysoczynski M, Machalinski B, Majka M, et al. Cells enriched in markers of neural tissue-committed stem cells reside in the bone marrow and are mobilized into the peripheral blood following stroke. *Leukemia*. 2006; 20:18–28. [PubMed: 16270036]

50. Gammill HS, Nelson JL. Naturally acquired microchimerism. *The International Journal of Developmental Biology*. 2010; 54:531–43. [PubMed: 19924635]
51. Zuba-Surma EK, Kucia M, Abdel-Latif A, Dawn B, Hall B, Singh R, et al. Morphological characterization of very small embryonic-like stem cells (VSELs) by ImageStream system analysis. *Journal of Cellular and Molecular Medicine*. 2008; 12:292–303. [PubMed: 18031297]
52. Iskovich S, Goldenberg-Cohen N, Stein J, Yaniv I, Fabian I, Askenasy N. Elutriated stem cells derived from the adult bone marrow differentiate into insulin-producing cells in vivo and reverse chemical diabetes. *Stem Cells and Development*. 2012; 21:86–96. [PubMed: 21457125]
53. Kassmer SH, Bruscia EM, Zhang PX, Krause DS. Nonhematopoietic cells are the primary source of bone marrow-derived lung epithelial cells. *Stem Cells*. 2012; 30:491–9. [PubMed: 22162244]
54. Virant-Klun I, Skutella T. Stem cells in aged mammalian ovaries. *Aging*. 2010; 2:3–6. [PubMed: 20228938]
55. Kuroda Y, Kitada M, Wakao S, Nishikawa K, Tanimura Y, Makinoshima H, et al. Unique multipotent cells in adult human mesenchymal cell populations. *Proceedings of the National Academy of Sciences of the United States of America*. 2010; 107:8639–43. [PubMed: 20421459]
56. Parte S, Bhartiya D, Telang J, Daithankar V, Salvi V, Zaveri K, et al. Detection, characterization, and spontaneous differentiation in vitro of very small embryonic-like putative stem cells in adult mammalian ovary. *Stem Cells and Development*. 2011; 20:1451–64. [PubMed: 21291304]
57. Bhartiya D, Kasiviswanathan S, Unni SK, Pethe P, Dhabalia JV, Patwardhan S, et al. Newer insights into premeiotic development of germ cells in adult human testis using Oct-4 as a stem cell marker. *The Journal of Histochemistry and Cytochemistry*. 2010; 58:1093–106. [PubMed: 20805580]
58. Wu JH, Wang HJ, Tan YZ, Li ZH. Characterization of rat very small embryonic-like stem cells and cardiac repair after cell transplantation for myocardial infarction. *Stem Cells and Development*. 2012; 21:1367–79. [PubMed: 22032240]
59. Liu Y, Gao L, Zuba-Surma EK, Peng X, Kucia M, Ratajczak MZ, et al. Identification of small Sca-1(+), Lin(-), CD45(-) multipotential cells in the neonatal murine retina. *Experimental Hematology*. 2009; 37:1096–107. [PubMed: 19539690]
60. Goldenberg-Cohen N, Avraham-Lubin BC, Sadikov T, Goldstein RS, Askenasy N. Primitive stem cells derived from bone marrow express glial and neuronal markers and support revascularization in injured retina exposed to ischemic and mechanical damage. *Stem Cells and Development*. 2012; 21:1488–500. [PubMed: 21905921]
61. Wakao S, Kitada M, Kuroda Y, Shigemoto T, Matsuse D, Akashi H, et al. Multilineage-differentiating stress-enduring (Muse) cells are a primary source of induced pluripotent stem cells in human fibroblasts. *Proceedings of the National Academy of Sciences of the United States of America*. 2011; 108:9875–80. [PubMed: 21628574]
62. McGuckin CP, Forraz N, Baradez MO, Navran S, Zhao J, Urban R, et al. Production of stem cells with embryonic characteristics from human umbilical cord blood. *Cell Proliferation*. 2005; 38:245–55. [PubMed: 16098183]
63. Seo KW, Lee SR, Bhandari DR, Roh KH, Park SB, So AY, et al. OCT4A contributes to the stemness and multi-potency of human umbilical cord blood-derived multipotent stem cells (hUCB-MSCs). *Biochemical and Biophysical Research Communications*. 2009; 384:120–5. [PubMed: 19394308]
64. Hess DA, Wirthlin L, Craft TP, Herrbrich PE, Hohm SA, Lahey R, et al. Selection based on CD133 and high aldehyde dehydrogenase activity isolates long-term reconstituting human hematopoietic stem cells. *Blood*. 2006; 107:2162–9. [PubMed: 16269619]

Fig. 9.1.

Gating strategy for multiparameter sorting of human UCB-VSELs and UCB-HSCs by using CD133 antibody. UCB-derived cells were isolated by FACS by employing the following gating criteria. Panel 1: All events $>2\text{-}\mu\text{m}$ are included in gate R1 after comparison with bead particles at standard diameters of 1, 2, 4, 6, 10, or 15 μm . Panel 2: UCB-derived total nucleated cells are visualized on a dot plot based on forward scatter (FS; x-axis) vs side scatter (SS; y-axis) signals. Panel 3: Cells from region R1 (panel 2) are further analyzed for Lin and CD133. Events for cells expressing Lin⁻ and CD133⁺ are included in region R3. Panel 4: The Lin⁻/CD133⁺ population from region R3 is subsequently analyzed based on

CD45 antigen expression, and the CD45⁻ and CD45⁺ subpopulations were visualized with a dot plot showing CD133⁺Lin⁻CD45⁻ (VSELs, region R4) and CD133⁺Lin⁻CD45⁺ (HSCs, region R5) populations.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 9.2.

Gating strategy for FACS analysis of UCB-VSELs and UCB-HSCs based on CD133 and CD45 expression and ALDH activity. UCB-derived CD133⁺ VSELs were isolated by FACS by employing the following gating criteria. Panel 1: UCB nucleated cell populations are stained using mAbs against human CD235a (GlyA), CD45, and CD133 and exposed to Aldefluor. UCB-derived total nucleated cells are visualized on a dot plot based on forward scatter (FS; x-axis) vs side scatter (SS; y-axis) signals. Panel 2: Cells from region R1 (panel 1) are further analyzed for CD45⁻/GlyA⁻ (region R2) and CD45⁺/Gly⁻ (region R7) populations. Panel 3: Cells from region R2 (panel 2) are sorted as CD45⁻/GlyA⁻/CD133⁺/

ALDH^{low} (region R3) and CD45⁻/GlyA⁻/CD133⁺/ALDH^{high} (region R4) subfractions of VSELs. Panel 4: Cells from region R7 (panel 2) are sorted as CD45⁺/GlyA⁻/CD133⁺/ALDH^{low} (region R8) and CD45⁻/GlyA⁻/CD133⁺/ALDH^{high} (region R9) HSC subpopulations.

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript

Fig. 9.3.

Murine VSELs deposited in adult tissues are protected from excessive insulin/insulin-like growth factor signaling (IIS). (A) VSELs (green) are deposited in adult tissues as a backup population for tissue-committed stem cells, and express non-signaling insulin-like growth factor-2 receptor (IGF2R) and two signaling receptors, insulin like-growth factor-1 receptor (IGF1R) and insulin receptor (InsR). Thus, they can be stimulated by an autocrine IGF2 loop (left panel) and by paracrine/endocrine IGF-1 and insulin (right panel). (B) Due to changes in expression of paternally imprinted genes (e.g., *Igf2R*, *Igf2*, and *RasGrf1*), VSELs

are protected from IIS, which would otherwise lead to their premature depletion from adult tissues as well as trigger uncontrolled proliferation and teratoma formation. Left panel: VSELs are protected from autocrine IIS by changes in expression of imprinted genes that are important in IIS. Down regulation of autocrine expression of IGF2, up regulation of IGF2R (a non-signaling receptor that binds IGF2 and prevents its binding to signaling IGF1R), and down regulation of RasGRF1 (which is involved in signal transduction from IGF2R), make VSELs less sensitive to autocrine/paracrine IGF2 signaling. Right panel: Down regulation of RasGRF1, which is involved in signaling from activated IGF1R and InsR in VSELs, also plays an important role in attenuation of IIS signaling by paracrine circulating levels of IGF1 and insulin. For the sake of simplicity, other imprinted genes (H19 and p57^{Kip2}) that are not involved in IIS and negatively affect VSEL proliferation are not depicted.