A Novel View of the Adult Stem Cell Compartment From the Perspective of a Quiescent Population of Very Small Embryonic-Like Stem Cells

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Abstract: Evidence has accumulated that adult hematopoietic tissues and other organs contain a population of dormant stem cells (SCs) that are more primitive than other, already restricted, monopotent tissue-committed SCs (TCSCs). These observations raise several questions, such as the developmental origin of these cells, their true pluripotent or multipotent nature, which surface markers they express, how they can be efficiently isolated from adult tissues, and what role they play in the adult organism. The phenotype of these cells and expression of some genes characteristic of embryonic SCs, epiblast SCs, and primordial germ cells suggests their early-embryonic deposition in developing tissues as precursors of adult SCs. In this review, we will critically discuss all these questions and the concept that small dormant SCs related to migratory primordial germ cells, described as very small embryonic-like SCs, are deposited during embryogenesis in bone marrow and other organs as a backup population for adult tissue-committed SCs and are involved in several processes related to tissue or organ rejuvenation, aging, and cancerogenesis. The most recent results on successful ex vivo expansion of human very small embryonic-like SC in chemically defined media free from feeder-layer cells open up new and exciting possibilities for their application in regenerative medicine. (Circ Res. 2017;120:166-178. DOI: 10.1161/CIRCRESAHA.116.309362.)

Key Words: adult stem cells | germ layers | hematopoietic stem cells | parental imprinting | ras-GRF1 | stem cells | very small embryonic like stem cells

With the advent of better discovery tools, such as cell sorting and high-resolution microscopic imaging, it has become possible to identify and subsequently purify various populations of stem cells (SCs) from adult tissues, which possess the ability to self-renew. However, SCs are heterogeneous and follow a developmental hierarchy. Moreover, evidence has accumulated that in several organs, including bone marrow (BM), it may reside a dormant population of rare, primitive, and quiescent SCs among more numerous tissue-committed SCs (TCSCs).1-20 It has been demonstrated that some of these SCs possess transgerm layer differentiation potential.

It is widely accepted that SCs have a distinct morphology (ie, small size and with a lymphocyte-like appearance), express a distinct panel of surface markers (ie, CD133+, CD34+, CD44+, Lin-), show low accumulation of selected metabolic fluorochromes (eg, rhodamine 123, pyronin Y, or Hoe3342), and display differences in activity of certain enzymes (eg, aldehyde dehydrogenase [ALDH]). All of these traits are helpful in SC identification and in purification strategies.21-25

An intriguing question has also been raised: Do already lineage-committed TCSCs residing in adult tissues show plasticity and transdedifferentiate into cells from other lineages?26-29 This concept is based on the hypothesis that SCs already committed to a given tissue, for example, hematopoietic SCs (HSCs), can transdedifferentiate and become SCs for different types of tissues—for example, cardiac or liver SCs. However, this hypothesis has been challenged because it has been hard to reproduce some of the initially published (and perhaps optimistic) reports showing robust transdedifferentiation of one type of TCSC into SCs for other tissues.30,31 Instead, several other alternative explanations have been proposed to explain the involvement of SCs in tissue repair, including (1) cell fusion,32 (2) the involvement of SC paracrine effects by soluble factors and SC-derived extracellular microvesicles,33,34 or (3) the presence of rare pluripotent or multipotent SCs in adult tissues that are developmental precursors for various types of TCSCs.35

Examples of such published reports of SCs with broader pan-germ-layer differentiation potential include (1) mesenchymal SCs (MSCs),34,36,37 (2) multipotent adult progenitor cells,38 (3) marrow-isolated adult multilineage inducible cells,39 (4) multipotent adult SCs,40 (5) elutriation-derived (Fr25/Lin-) SCs (ELH SCs),39 (6) spore-like SCs,19 (7) pluripotent Sca-1+CD45-c-kit- cells,20 (8) multilineage-differentiating,
stress-enduring SCs,\textsuperscript{13,15,42} and (9) very small embryonic-like SCs (VSELs), described by our group.\textsuperscript{1,43–45} All of these SC types were identified by using different direct or indirect isolation protocols and identification techniques. The similarity in expression of certain early-development genes in these cells suggests that they are related to each other, that they may represent similar, overlapping populations of primitive SCs that reside in adult tissues, and that they are endowed with broader cross-germ-layer differentiation potential.

In this review, we will focus on VSELs, as these cells (\(\approx 4–5\;\mu\text{m}\) in diameter as measured in murine BM and \(\approx 5–6\;\mu\text{m}\) in human BM or umbilical cord blood (UCB)) have been highly purified and well characterized at the molecular level.\textsuperscript{46–48} We propose that VSELs represent the most primitive population of quiescent SCs residing in adult tissues and resemble some of their properties of other primitive SCs described by other investigators (eg, ELH SCs). The small size of these cells and the paucity of mitochondria are signs of their quiescence and low metabolic activity.\textsuperscript{49} These very rare cells are isolated from adult tissues (eg, BM, UCB, and mobilized peripheral blood [mPB]) by multiparameter cell sorting (Figure 1), and several groups that carefully followed the original protocol for their isolation (published by us in Current Protocols of Cytometry\textsuperscript{50} or contacted us for help) successfully identified these small cells in postnatal tissues.\textsuperscript{51–54} BM-purified VSELs have been demonstrated by other investigators to be precursors of HSCs,\textsuperscript{55,56} MSCs,\textsuperscript{57,58} endothelial SCs,\textsuperscript{59,60} lung alveolar epithelial cells,\textsuperscript{61,62} and cardiomyocytes.\textsuperscript{63,64} At the same time, VSELs isolated from gonads have been proposed to be precursors of male and female gametes.\textsuperscript{10,65–67}

What is of particular interest, we have recently developed an efficient protocol to expand these highly quiescent cells isolated from human and murine hematopoietic tissues in chemically defined media, without support of third-party feeder-layer cells or transduction by vectors encoding pluripotency-promoting factors. This newest development opens up new and exciting possibilities for VSEL application in regenerative medicine.

**Hierarchy of the SC Compartment in Embryonic and Postnatal Tissues: Do We Have a Definitive Model?**

The most accepted, but unfortunately oversimplified, view of the BM SC compartment is based on the assumption that it consists of HSCs, MSCs, and endothelial progenitor cells (EPCs). However, this old and dogmatic view has been challenged by several reports that suggest the presence of other more primitive SCs in BM tissue.\textsuperscript{2–4,6,13,14,19,20,38,42,68} SCs possess the unique property of symmetrical self-renewal or asymmetrical division, and evidence indicates that they are not all equal from a hierarchical point of view. Some of them may be endowed with broader differentiation potential across germ layers.\textsuperscript{69–75} Thus, the hierarchy of the SC compartment needs to be revisited, as many of the early-development SCs may be left unaccounted for in a simplified hierarchy.

From a developmental point of view, the most primitive SCs are the fertilized oocyte (zygote) and the first blastomeres in the morula, as these cells are able to give rise to both embryo and placenta. Such SCs are called totipotent. By contrast, embryonic SCs (ESCs) isolated from the inner cell mass (ICM) of the blastocyst differentiate into embryonic tissues only, losing the ability to form the placenta and are therefore called pluripotent SCs (PSCs). After implantation of the blastocyst into the uterus, ICM-derived PSCs of the blastocyst form the epiblast, and epiblast SCs (EpiSCs) subsequently give rise to all 3 germ layers (meso-, epto-, and endoderm), including primordial germ cells (PGCs). Pluripotent EpiSCs gradually lose their pluripotency by giving rise to multipotent SCs, which are specific for one of the germ layers, and finally monopotent TCSCs, for example, SCs for epidermis, intestinal epithelium, liver, skeletal muscle, or lymphohematopoiesis. TCSCs in adult organisms reside in SC niches (eg, in the basal layer of epidermis and hair budge [epidermal SCs], the bottom of intestinal crypts [intestinal SCs], around Herring ducts in liver [oval SCs], around muscle fibers [satellite SCs], and in endothelial and vascular niches of the BM [HSCs]). In heart, SCs are thought to be located in the atrial appendages.\textsuperscript{76–81}

Although considering the hierarchy of the SC compartment and their developmental specification, beginning with PSCs and ending with TCSCs, an important question emerges: Is the differentiation process of PSCs or multipotent PSCs during embryogenesis complete, or perhaps do some of these early-development SCs survive beyond embryogenesis into adulthood and remain as a hibernated or a quiescent backup population of TCSCs?\textsuperscript{7} This possibility is supported by the presence in adult BM, UCB, and mPB of VSELs and other rare SC populations that express embryonic, epiblast, and PGC markers that overlap with VSELs. Examples of such cells detected in adult tissues are listed in the Table.

One of the intriguing puzzles in mammalian development is the fact that PGCs are the first population of SCs specified before gastrulation in the proximal epiblast—a precursor of the entire embryo proper.\textsuperscript{82,83} These Blimp\textsuperscript{1} cells, which emerge in the proximal epiblast close to the extraembryonic
endoderm in response to BMP4 (bone morphogen protein 4) signaling, migrate out of the embryo into extraembryonic tissue, and then make a turn and reenter the embryo proper through the primitive streak at the beginning of gastrulation. On the way to the genital ridges, which is their final destination, the PGCs are amplified in number and, what is worthwhile to explore, certain cells closely related to PGCs (eg, EpiSCs) may be deposited in adult tissues as a population of dormant SCs that are endowed with broad, cross-germ-layer differentiation potential.69–75,84,85

What is also an intriguing hypothesis is that the developmental migration of PGCs from the epiblast over the extraembryonic tissues and back to the gonadal ridges may be related to the developmental origin of HSCs and EPCs. First, at the
time when PGCs migrate through the extraembryonic tissues, the first hemangioblasts, which are precursors of primitive HSCs and EPCs, emerge at the bottom of the yolk sac. Later on, when the PGCs migrate across the embryo proper to the genital ridges and pass through the aorta-gonado-mesonephros region, the first definitive HSCs emerge in the aortic endothelium. This potential link between PGC migration—the emergence first of hemangioblasts and later on definitive HSCs—suggests that the developmental origin of VSELs is from migrating PGCs.

In support of this concept, both PGCs and VSELs are reportedly able to give rise to HSCs and EPCs. Moreover, PGC-derived precursors of gametes, VSELs, and HSCs express several functional sex hormone (SexH) receptors and the erythropoietin receptor. Moreover, VSELs highly express certain hemangioblast markers (eg, Flk1) and, as reported in an elegant study, they can be specified into endothelial cells in vitro and in vivo. This concept of the origin of definitive HSCs from migrating PGCs has been recently demonstrated in elegant work by the Scaldaferrri et al and discussed in an inspiring review by Virant-Klun. Nevertheless, as always with new scientific ideas, more experimental evidence is needed to support this novel and intriguing concept.

Evidence for the Presence of Very Small SCs in Adult Tissues: A Roadmap for Discovery of Very Small Embryonic-Like SCs

SCs are characterized by a high nuclear/cytoplasmic ratio, and one may make the assumption that those that are more quiescent and dormant (eg, VSELs) compared with those that are actively proliferating (eg, HSCs or intestinal epithelium SCs) are much smaller and contain only sparse, round mitochondria as signs of low metabolic activity. In support of this claim, several types of small SCs were initially described in hematopoietic tissues and later confirmed to be present in other organs as well. The exact size of the small SCs may depend on the method of their measurement (microscopic template grids or size beads) and may also be affected by procedures such as fixation or mounting on slides. In general, we consider small cells to be up to 5 μm in diameter for mice and slightly larger in humans. We will briefly discuss these cells, keeping in mind the tempting speculations that these SCs could be precursors for multipotent adult progenitor cell, marrow-isolated adult multilineage inducible cells, multipotent adult SCs, MSCs, or multilineage-differentiating, stress-enduring SCs. It is likely that these particular SCs, which seem to be somewhat larger, from the beginning have been contaminated by populations of small SCs attached to them or internalized by a process of emperipolesis.

Below, we will briefly summarize examples of small SCs that have been isolated from murine, rat, and human tissues. These cells were identified primarily as candidates for quiescent HSCs. However, with time it became obvious that some of them are also endowed with broader differentiation potential across germ layers.

Small SCs With Primary Hematopoietic Potential

Lin−/ALDHhigh Long-Term Repopulating HSCs

The population of small, long-term repopulating HSCs, which were isolated by using elutriation followed by fluorescence-activated cell sorter and selection for high activity of ALDH (ALDHhigh) and not hematopoietic lineage markers (which these cells lacked), was described by Jones et al and Sharkis et al. Although the exact size of these cells was not published in the original publication, a recent article from this group described these SCs as <5 μm. These interesting cells, however, were not analyzed for the expression of pluripotent or multipotent SC markers. However, recent evidence suggests their broader nonhematopoietic differentiation potential (see Elutriation-Derived (Fraction 25 [Fr25]/Lin−) SCs section of this article).

BM Mononuclear Cell-Derived Progenitor-Like Cells

Small cells (4–5 μm in diameter) in BM were identified by using the electron microscopy by Matsuoka et al. This population of small cells exhibited certain morphological features

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BM indicates bone marrow; and FACS, fluorescence-activated cell sorter.
characteristic of hematopoietic progenitors. Similarly, small cells (≈5 μm) were reported by Berardi et al101 as the most primitive HSCs in human BM.

**Lin–/Rh−/Hoechst Long-Term Repopulating HSCs**

Radley et al102 described a population of small (≈4–5 μm), primitive HSCs isolated from murine BM that were capable of long-term hematopoietic reconstitution. These cells were characterized by a lack of hematopoietic lineage markers and an ability to exclude both Rhodamine 123 (Rh<sup>ull</sup>) and Hoechst 33342 (Hoechst<sup>ull</sup>) dyes.

**Small Cells With Multitissue Differentiation Potential**

**Elutriation-Derived (Fraction 25 [Fr25]/Lin–) SCs**

As mentioned above, these interesting SCs were initially described as Lin–/ALDH<sup>high</sup> long-term repopulating HSCs.25,41,99,103 More careful analysis of these cells and modification of their isolation strategy by elutriation (E, Fr25), combined with their lineage negativity (L) and transplantation into lethally irradiated mice and recovery by fluorescence-activated cell sorter after short-term homing (H), demonstrated that they contribute to multiple epithelial tissues. Recent experiments from the Krause group65 demonstrated that they are distinct from classical HSCs and may repopulate lung alveolar type II pneumocytes, producing surfactant after transplantation into surfactant-deficient mice. These particular SCs have been recently proposed to overlap with VSELs.66 Similar small Lin– SCs isolated by elutriation (Fr25) from murine BM were shown to contribute to regeneration of retinal epithelium and to differentiate into insulin-producing cells in mice.104,105

**CD45–Sca-1–c-kit– Cells With Pluripotent Characteristics**

These cells have been isolated by using fluorescence-activated cell sorter–based phenotypic analysis of single-cell suspensions from murine brain, blood, and intestinal epithelium by Howell et al106 and proposed to represent universal PSCs residing in multiple murine tissues.

**Spore-Like SCs**

The presence of small SCs (≈5 μm) in adult tissues known as spore-like SCs was demonstrated by Vacanti et al.19 Unfortunately, the isolation strategy and the exact markers for these cells were not described in the original publication.

**VSEL-Like SCs in Murine and Human Gonads**

Very small SCs with pluripotent characteristics were described in the human and murine ovarian surface epithelium and identified in murine and human testes independently by the Virant-Klun and Bhartiya groups.18,65–67,106 These small cells express embryonic markers such as SSEA, Oct-4, Sall4, Nanog, and Sox-2 and are able to form embryoid body-like structures in vitro. It was proposed that these cells are the precursors of gametes.

**VSEL-Like SCs in Rat BM**

SSEA-1<sup>+</sup>Oct-4<sup>+</sup> VSEL-like SCs were also purified from rat BM by the Yuzhen Tan group and successfully used to regenerate damaged myocardium in an experimental model of acute myocardial infarction.107

**Small UCB-Derived SCs**

McGuckin et al17 demonstrated the presence of very small SCs in UCB and estimated the size of these cells as 2 to 3 μm. As reported in the original publication, these Oct-4<sup>+</sup>, Sox2<sup>+</sup> cells exhibit pluripotent characteristics and possess neural differentiation potential.

**Small SCs Remaining in the BM Filtrate**

Finally, while isolating MSCs on a double-layer culture plate containing 3-μm pores to filter out the relatively large MSCs, Hung et al108 isolated a population of very small SCs residing in human BM that were able to migrate through the 3-μm pores.

All of these small SCs listed above, which were isolated from adult tissues, most likely represent overlapping cell populations. Although many of them were not well characterized at the molecular level, we envision that they are closely related to VSELs in the developmental hierarchy. This concept will be presented in more detail below.

**Discovery of VSELs and Their Challenge to Understanding the Adult SC Compartment**

Evidence has accumulated for a very likely scenario in which some primitive epiblast/PGC-derived SCs escape specification into TCSCs and thus retain their pluripotent character and survive as VSELs into adulthood, forming a reserve pool of precursors for TCSCs. Thus, VSELs could play an important role in tissue rejuvenation and regeneration.

VSELs were initially purified by using fluorescence-activated cell sorter–based multiparameter sorting of murine BM and several adult murine organs (eg, brain, liver, skeletal muscle, heart, gonads, and kidney).45,46,30 Murine BM-derived VSELs (1) are very rare (≈0.01% of nucleated BM cells), (2) are small in size (≈3–5 μm), (3) express several PSC markers, including Oct4, Nanog, Rex-1, and SSEA-1, (4) contain sparse, round mitochondria, and (5) have large nuclei filled with unorganized chromatin (euchromatin). Importantly, to exclude expression of Oct-4 pseudogenes in these cells, we confirmed the true expression of Oct4 by demonstrating transcriptionally active hymoplastic DNA associated with acetylated histone chromatin in the Oct4 promoter.46,47 The Oct-4 amplicon was also sequenced for accuracy.

Moreover, a corresponding population of small (≈4–7 μm) CD133<sup>+</sup>SSEA-1<sup>+</sup> Lin CD45<sup>−</sup> SCs that display embryonic-like cell morphology have been purified from UCB and mPB.31,37,109,110 Human VSELs, like murine VSELs, have large nuclei that contain unorganized euchromatin and a relatively small rim of cytoplasm with sparse, round mitochondria. These cells also express Oct4 and Nanog in their nuclei and display the SSEA-4 antigen on their surface.109

Evidence indicates that VSELs are a population of migratory cells, and their number increases both in mice and in humans in PB during stress situations related to tissue or organ injuries (eg, heart infarct, stroke, skin burns, or acute colitis).90,111–114 These cells, which are mobilized into PB where they then circulate, may play a physiologically important surveillance role in repairing minor tissue damage. The elevated number of VSELs observed in UCB may be explained as a physiological mechanism in which these cells are mobilized in
newborns, which, because of hypoxia and delivery stress, experience numerous minor tissue injuries. Thus, the mobilization of VSELs into UCB is an inborn protective mechanism, which can be considered as the original physiological SC therapy, that everybody experiences in life after delivery. The number of VSELs circulating in PB also increases after administration of certain drugs that are used on a routine basis in the clinic to mobilize HSCs into PB (eg, G-CSF [granulocyte-colony stimulating factor] or AMD3100 [Plerixafo, Mozobil]).47,115 Thus, VSELs could be harvested for potential clinical applications, like HSCs from mPB, by using similar protocols for leukopheresis. The problem of low recovery of these cells from BM, UCB, and mPB is ameliorated by a recently established ex vivo expansion protocol for these cells (see below).

As recently reported, the number of VSELs in murine BM increases in vivo in response to regular physical activity and prolonged caloric restriction.116,117 On the contrary, highly quiescent VSELs in murine BM may enter the cell cycle, as confirmed by bromodeoxyuridine accumulation in these cells after administration of SexHs (such as follicle-stimulating factor or luteinizing hormone) or erythropoietin.88,89 The responsiveness of VSELs to SexHs supports a developmental relationship of these cells to PGC progeny and has been recently used in our ex vivo-expansion protocol for these cells (see below).

**Molecular Characteristics of VSELs: Evidence for Their Pluripotency or Multipotency**

According to their definition, PSCs should, at the molecular level (1) express acknowledged markers of pluripotency, (2) have bivalent domains at promoters for homeodomain-containing transcription factors (TFs), and (3) have 2 active X chromosomes in female PSCs. VSELs have been carefully characterized according to these criteria by using several complementary techniques, including gene expression studies at the mRNA level, miRNA analysis, the creation of cDNA libraries from highly purified cells, DNA methylation studies, the analysis of histone methylation and acetylation, and direct immunostaining.47,48,118 All of these studies revealed that VSELs express several markers characteristic of PSCs. However, at the same time, our single-cell–sorted library results indicate that, despite similar morphology and expression of similar surface markers, these cells residing in adult BM are somewhat heterogeneous in the expression of certain lineage-of similar surface markers, these cells residing in adult BM are somewhat heterogeneous in the expression of certain lineage-

The protein expression of PGC markers such as *Stella, Blimp1*, and *Mvh* in purified VSELs was subsequently confirmed by immunostaining. More importantly, chromatin immunoprecipitation results revealed that the *Stella* promoter in VSELs displays transcriptionally active histone modifications (acetylated histone 3 and dimethylated lysine 4 of histone 3) and was less enriched for transcriptionally repressive histone markers (dimethylated lysine 9 of histone 3 and trimethylated lysine 27 of histone 3).47 At the same time, VSELs also highly express transcripts for *Dppa2, Dppa4*, and *Mvh*, which are characteristic of late migratory PGCs; however, they do not express the *Sycp3, Dazl*, and *LINE1* genes, which are markers of postmigratory PGCs.47 Finally, the partial DNA demethylation of repetitive DNA sequences (*LINE1* and *IAP*) and promoters of *Mvh* and *Sycp3* further supports a close relationship between VSELs and late-migratory PGCs.47 As mentioned above, a potential relationship between VSELs and PGCs is also somewhat supported by the expression of several pituitary and gonadal SexH receptors by these cells,88 as well as the presence of the receptor for erythropoietin.89

In further support of murine VSEL pluripotency, these small cells also express bivalent domains at promoters for homeodomain-containing TFs, such as *Sox21*, *Nks2.2*, *Dlx1*, *Lhx1h*, *Hlxb9*, *Pax5*, and *HoxA3*, which, as mentioned above, is one of the characteristics of PSCs.48 Bivalent domains represent the state of chromatin structure in which transcriptionally opposite histone codes physically coexist in the same promoter of homeodomain-containing TFs. Although in undifferentiated PSCs bivalent domains prevent premature differentiation, during differentiation the transient repressive epigenetic marks in promoters of homeodomain-containing TFs become monovalent to activate or repress the expression of the appropriate TFs. The presence of transcriptionally active histone codes, such as trimethylated lysine 4 of histone 3, physically coexisting with repressive histone codes, like trimethylated lysine 27 of histone 3, within bivalent domains was confirmed by using the carrier-chromatin immunoprecipitation assay.48

The phenomenon of X chromosome inactivation in female PSCs (eg, ESCs isolated from the female blastocyst ICM) is the epigenetic process for transcriptional silencing of 1 of the 2 X chromosomes in female cells to compensate for gene dosage.119 It is well known that female-derived PSCs reactivate the X chromosome that is inactivated after fertilization, and, as a result, female PSCs display 2 equivalently activated X chromosomes.120 Reactivation of the silenced X chromosome in female PSCs is one of the important features of pluripotency, and our results also indicate that VSELs purified from female mice partially activate an X chromosome, which indicates that murine VSELs, like ESCs, undergo the process of X chromosome reactivation.

**In Vivo Differentiation of VSELs Reveals Their Pluripotent or Multipotent Character**

It has been demonstrated in several elegant studies that purified VSELs differentiate into cells from different germ layers. Some of these exciting reports will be briefly discussed below. These reports demonstrate the pluripotent or multipotent character of these cells; however, to achieve robust differentiation
and tissue contribution in vivo, a greater number of injected cells would be needed. Thus, our recent exciting results showing that VSELs can be expanded ex vivo will provide more of these cells for in vivo testing.

Our group has demonstrated that BM-, mPB- or UCB-derived VSELs could be specified into HSCs. Below, we will discuss data from other groups that demonstrated specification of VSELs into other types of adult cells.

### VSELs at the Top of the Mesenchymal Lineage Hierarchy

In a very elegant study, Taichman et al. reported that VSELs isolated from GFP+ (green fluorescent protein) mice formed bone-like structures when implanted into SCID (severe combined immunodeficiency) mice. To further confirm that this effect depends on VSELs that exhibit true MSC activity (bone formation), stromal cells were harvested from Col2.3ΔTK mice and implanted into SCID mice to generate thymidine kinase-sensitive ossicles. At 1.5 months after implantation, these ossicles were injected with 2000 GFP+ VSELs. At harvest, colocalization of GFP-expressing cells with antibodies to the osteoblast-specific marker Run-2, the endothelial marker CD31, and the adipocyte marker PPARγ marker CD31, and the adipocyte marker PPARγ was observed. Based on the ability of uncultured VSELs to (1) differentiate in vivo into multiple mesenchymal lineages and (2) generate osseous tissues at low density, Taichman et al. proposed that VSELs fulfill many of the required characteristics of precursors for MSCs. Recently, a similar bone-forming potential of human VSELs has been demonstrated in vivo in an immunodeficient mouse model.

### VSELs as a Source of Endothelial Progenitors

In another elegant paper, Guerin et al. demonstrated that human VSELs are mobilized into PB in patients with critical limb ischemia, and in vivo assays human VSELs were able to differentiate into endothelial cells. Accordingly, VSEL-derived cells in vitro, like EPCs, released low levels of VEGF-A (vascular endothelial growth factor A) and a similar repertoire of inflammatory cytokines. More importantly, in vivo in immunodeficient mouse models supplemented with leukemia inhibitory factor and basic fibroblast growth factor, the authors convincingly demonstrated that VSEL-derived embryoid-like bodies in soft agarose models supplemented with leukemia inhibitory factor and basic fibroblast growth factor can differentiate into cells from the 3 germ layers, giving rise to cardiomyocytes and endothelial cells. In further support of this finding, transplantation of human VSELs derived from male rats reduced the scar area and significantly improved cardiac function in a female rat myocardium infarction model. Moreover, analyzing cells for the donor-derived Y chromosome, the authors convincingly demonstrated the presence of male VSEL-derived cardiomyocytes and endothelial cells. The authors concluded that cells from VSEL-derived embryoid-like bodies may contribute to cardiomyogenesis and angiogenesis in vivo.

### VSELs and Their Contribution to Epithelial Cells

As mentioned above, ELH SCs isolated from murine BM differentiated into several epithelial lineages after injection into mice. Because these cells share several characteristics with VSELs, Krause et al. tested whether VSELs purified from BM overlap with ELH SCs and compared the level of BM-derived epithelial cells after transplantation of (1) VSELs, (2) hematopoietic stem/progenitor cells, and (3) other nonhematopoietic cells. It turned out that VSELs clearly had the highest rate of epithelial cell formation in the lung. Furthermore, in these elegant studies using VSELs from donor mice expressing H2B–GFP under a type 2 pneumocyte-specific promoter, Krause et al. demonstrated that this engrafment occurs by differentiation of VSELs into type 2 pneumocytes, excluded the phenomenon of fusion, and concluded that ELH SCs and Oct4+ VSELs in the adult BM exhibit broad differentiation potential.

### Hepatocyte Differentiation of VSELs

In another recent study, Chen et al. reported that VSELs differentiated into hepatic colonies in the presence of hepatocyte growth factor and, if transplanted into mice with CCl4-induced liver injury, they significantly reduced serum ALT (alanine transaminase) and AST (aspartate transaminase) levels. It was therefore concluded that VSELs play a role in the repair of liver injury.

### VSELs and Their Role in Gametogenesis

Finally, the potential role of VSELs in postnatal gametogenesis has been addressed by independent groups led by Viran-Klunt and Bhartiya. These investigators identified a population of very small cells in human and animal gonads that mimic VSELs. As they proposed, these cells could be used as an alternative source of oocytes and sperm in patients with damaged gonads after high-dose chemotherapy. In support of this possibility, VSELs are localized in the ovary surface epithelium and in the basement membrane of

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In Vivo Pluripotency Criteria, or Why Do VSELs Not Contribute to Blastocyst Development and Not Grow Teratomas?
Understanding the Consequences of Epigenetic Modification of Parentally Imprinted Genes
Accumulating evidence has unequivocally demonstrated that murine VSELs exhibit several features of PSCs. However, they do not fulfill 2 “gold standard” in vivo criteria of pluripotency that are seen in the cases of ESCs and inducible PSCs (iPSCs); namely, (1) do not complete blastocyst development and (2) do not form teratomas after transplantation into immunodeficient mice. These in vivo pluripotency criteria were proposed based on research with ESCs and iPSCs. However, they do not apply to PGCs, which, despite being SCs endowed with developmental totipotency, do not comply with this definition. To explain this difference in PGCs, it has been reported that they modify the methylation of certain crucial parentally imprinted genes, and this prevents them from proliferation, complementation of the blastocyst, and teratoma formation and, at the DNA level, explains their quiescent state. Taking into consideration the similarities in gene expression and, at the DNA level, explains their quiescent state, mainly by attenuating proteins that are involved in insulin-like growth factors 1 and 2 (IGF-1 and IGF-2) and insulin signaling, including those encoded by the Igf2–H19 and Rasgrf1 loci. These erased loci are methylated during development in the gonads in PGC-derived gametes after the first meiotic division by de novo DNA methyltransferases. This step is required for normal development of the embryo after fertilization.

Based on these findings, it became obvious to us that, by methylation of erased differentially methylated regions at parentally imprinted loci by DNA methyltransferases, we would be able to reverse in vitro VSEL cultures more efficiently from the quiescent state.

Epigenetic Regulators Stimulate Remethylation of Erased Loci in Paternally Imprinted Genes and Facilitate Ex Vivo VSEL Expansion
As mentioned above, we assumed that the critical step in reversing the quiescent state of VSELs would be remethylation of the erased loci in parentally imprinted genes. We found that treatment of VSELs by the histone deacetylase (HDAC) inhibitors valproic acid (VPA) or nicotinamide remethylated the differentially methylated regions of parentally imprinted genes that were erased in VSELs. As a result, we were able to force the proliferation of VSELs and expand them ex vivo by ≈100-fold in chemically defined media in cultures supplemented with VPA and a cocktail of 2 pituitary SexHs, follicle-stimulating factor and luteinizing hormone together with BMP-4, IGF-2, and kit ligand. As mentioned above, follicle-stimulating factor and luteinizing hormone had been effective in stimulation of murine VSELs in vivo.

VSELs in these culture conditions began to proliferate, and after 1 to 2 months of expansion we can distinguish in our in vitro cultures many small cells (Figure 2). In our expansion, many of small cells still express Oct-4 and some large cells express certain markers of the germ lineage (eg, Blimp-1 and Stella) at the protein level. These observations can be explained by the fact that the follicle-stimulating factor and luteinizing hormone present in expansion cultures maintained the germline potential of VSELs.

This novel expansion system is somewhat supported by recent data on the expansion of HSCs from CD133+ or CD34+ cells purified from UCB using immunomagnetic beads. These cells were effectively expanded in the presence of the HDAC inhibitors VPA or nicotinamide. Because immunomagnetic beads isolate both large and small cells, we propose that efficient expansion of long-term repopulating HSCs (LT-HSCs) from paramagnetically purified CD133+ or CD34+ cells was the result of expansion of small CD133+ or CD34+ VSELs that contaminated the immunomagnetically purified cells.

Specifically, in the first article in which Hoffman’s group used VPA for the expansion of CD133+ cells, the authors observed an increase in the number of Oct-4 cells, and this remarkable effect on the expansion of the most primitive LT-HSCs was reversed after Oct-4 had been downregulated by shRNA during expansion. Therefore, because Oct-4 VSELs can be specified into LT-HSCs, we envision that this effect was most likely because of the increased hematopoietic expansion of small CD133+ VSELs.

However, we have to keep in mind that, by using HDAC inhibitors, not only are paternally methylated imprints
released from an inhibitory complex with HDAC1,14–16 but also the addition of these inhibitors increases the acetylation of histones in several genes. However, consistent with the crucial involvement of HDAC inhibition on the normal expression of paternally imprinted genes, we observed a similar effect when one of the HDACs was downregulated in VSELs by using an shRNA strategy (manuscript in preparation).

We are aware that this is a first step, and our expansion system is still open for optimization using different lineage-specific growth factors and other DNA modifiers—particularly HDAC inhibitors that are more potent and specific than VPA. However, what is very important, by developing this ex vivo–expansion protocol, we have demonstrated that VSELs can be woken up from their dormant/quiescent state and expanded ex vivo in cell cultures that are free of third-party feeder-layer cells or vectors for overexpressing genes involved in cell pluripotency, as proposed by Yamanaka and coworkers.137–139

Finally, our work on VSELs and imprinted genes has for the first time connected the role of caloric restriction, the beneficial effect of regular exercise, insulin, and IGF-1/2 signaling, and metformin to the number of VSELs playing a role in tissue and organ rejuvenation. In particular, Sirt1 is an important HDAC that regulates all of these biological processes, and its high expression in VSELs keeps them in a quiescent state. Overall, our results suggest that imprinted genes are involved in longevity as guardians of the insulin/insulin-like growth hormone, a stimulator of the IGF-1 level in blood.140,141

It is worthwhile mentioning that, as reported by other groups, downregulation of Sirt1 in BM cells leads to premature depletion of HSCs.142,143 Taking into consideration the possibility that VSELs play the role of precursors for LT-HSCs, these observations explain why HDAC inhibition in VSELs promotes their controlled specification into LT-HSCs. This phenomenon may play a role in VSEL specification into other types of TCSCs and explains at the molecular level the novel role of HDAC in extending longevity. In fact, Laron dwarf and Ames dwarf mice, which have an elevated number of VSELs in tissues, are long-living animals.

Conclusions

Regenerative medicine is still looking for a reliable source of PSCs that could be safely used in the clinic. One of potential promising candidates are iPSCs; however, with advent of high-throughput technologies including next-generation sequencing evidence accumulated showing genomic instability of these cells.144 In addition, the presence of genetic variations in iPSCs has raised serious safety concerns, hampering the advancement of iPSC-based novel therapies and first clinical trials in humans have been stopped. That is why it is important to emphasize that the only SCs to date that have been successfully used in the clinic are those isolated from postnatal tissues. Moreover, several recent publications support the presence of pluripotent or multipotent VSELs in adult tissues. These small cells seem to be at the top of the SC hierarchy in adult tissues and most likely play a role in tissue and organ rejuvenation as a source of adult TCSCs. Their premature depletion in adult

Figure 2. Example of expansion of human umbilical cord blood derived very small embryonic-like stem cells (VSELs). A, Freshly sorted VSELs (5×10^2) were plated in 0.2 mL of DMEM+10% FBS, supplemented with valporic acid and a cocktail of two pituitary sex hormones, follicle-stimulating factor and luteinizing hormone together with BMP-4 (bone morphogen protein 4), insulin-like growth factor 2, and kit ligand. Right inset shows enlarged image of freshly sorted VSEL. Cells were cultured for 2 mo, and half of culture medium has been changed every 7 d. B, Top, VSELs in these culture conditions began to proliferate, and after 2 mo of expansion, we can distinguish many small cells and some larger cells. Maximal expansion is achieved after 2 to 3 mo of culture. Bottom, Cells aspirated from the cultures. Left and middle, Light microscope image. Right, Hoe3342 intravitral staining of cells aspirated from the expansion.
tissues is prevented by decreasing insulin, IGF-1, and IGF-2 signaling, caloric restriction, metformin supplementation, and regular physical activity. HDACs play a crucial role in preventing remethylation of erased paternally imprinted loci, and their inhibition reverses the quiescent state of these cells in adult tissues. By inhibiting HDAC activity with VPA, we were able to successfully expand human and murine VSELs ex vivo. This latest development opens up new and exciting possibilities for application of these intriguing cells in regenerative medicine and sheds new light on the mechanisms affecting aging.

Sources of Funding
This work was supported by National Institutes of Health grants R01 DK074720, R01HL112788, the Steifa and Henry Endowment and the Harmonia NCN grant UMO-2014/14/M/NZ3/00475 to M.Z. Ratajczak and by Basic Science Research Program through the National Research Foundation of Korea (2015R1A2A1A15054754) to D.-M. Shin.

Disclosures
University of Louisville owns IP on VSELs technology.

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References


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Circ Res. 2017;120:166-178
doi: 10.1161/CIRCRESAHA.116.309362

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
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