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Very Small Embryonic/Epiblast-Like Stem Cells (VSELs) Residing in Adult Tissues and Their Role in Tissue Rejuvenation and Regeneration

Dong-Myung Shin[1], Janina Ratajczak[1],
Magda Kucia[1] and Mariusz Z. Ratajczak[1]
[1]*Stem Cell Institute at the James Graham Brown Cancer Center, University of Louisville, KY, USA*

1. Introduction

Embryonic development and later rejuvenation of adult tissues are regulated by a population of stem cells (SCs) that, by undergoing self-renewal, maintain their own pool and, by giving rise to differentiated progenitors, replace cells used up during life (Ratajczak et al., 2007). Thus, SCs are guardians of tissue/organ integrity and regulate the life span of an adult organism. The most important SCs, from a regenerative potential point of view, are pluripotent stem cells (PSCs). According to their definition, such cells must meet certain *in vitro* and *in vivo* criteria. PSCs must: i) give rise to cells from all three germ layers; ii) complete blastocyst development; and iii) form teratomas after inoculation into experimental animals.

The SC compartment shows a high degree of hierarchy (Hayashi & Surani, 2009). In embryonic development, the most primitive stem cells are the fertilized oocyte (zygote) and the first blastomers in the morula. These cells are called totipotent, possessing the ability to give rise to both embryo and placenta. The developing morula gives rise to the blastocyst, where PSCs are found in the inner cell mass (ICM). These cells may give rise to all three germ layers of the developing embryo; however, they have lost the ability to differentiate into placenta. The PSCs at this stage can be expanded *ex vivo* as immortalized embryonic stem cell (ESC) lines (Evans & Kaufman, 1981).

After implantation of the blastocyst, PSCs from the blastocyst ICM give rise to pluripotent epiblast stem cells (EpiSCs) that will form the entire embryo proper (Brons et al., 2007; Tesar et al., 2007). During the gastrulation process, cell lineage determination programs are initiated and EpiSCs respond to the signals from surrounding extra-embryonic tissues, which leads to their differentiation into several types of tissue-committed stem cells (TCSCs) (Ratajczak et al., 2007). TCSCs are monopotent (unipotent), which means they are restricted in their differentiation potential to cells for one tissue only (e.g., epidermis, intestinal epithelium, liver, skeletal muscles, or lympho-hematopoietic). TCSCs terminate expression of pluripotent genes and, at the same time, turn on lineage-specific molecular programs.

The first population of SCs, which at around embryonic day 7.25 (E7.25) become specified in the proximal epiblast, are primordial germ cells (PGCs), and alkaline phosphatase (AP)-

positive PGCs grow into extra-embryonic mesoderm at the base of the allantois as an appendage arising from around the posterior primitive streak (Surani et al., 2007). These cells transcribe pluripotency-related genes, such as *Oct-4, Nanog,* and *Sox2,* and are the only population of SCs that maintains expression of these genes during gastrulation. When PGCs are cultured over murine embryonic fibroblasts and exposed *ex vivo* to three growth factors (kit ligand, leukemia inhibitory factor, and basic fibroblast growth factor), they continue to proliferate and form large colonies of embryonic germ cells (EGCs), which, like ESCs, can be expanded indefinitely (Matsui et al., 1992). At around E12.5, PGCs arrive at the genital ridges, lose their markers of pluripotency, and initiate their commitment to becoming gametes (oocytes and sperm).

As mentioned above, SCs show a developmental hierarchy (Hayashi & Surani, 2009), and PSCs that emerge during embryogenesis give rise to more differentiated SC populations with the ability to self renew, but with a more limited ability for multilineage differentiation (Surani et al., 2007). Evidence is accumulating that differentiation potential is regulated by epigenetic reprogramming (Surani et al., 2007). PSCs from the ICM show global DNA demethylation, which results in i) activation of the X chromosome, ii) expression of germ-line, lineage-characteristic genes (e.g *Stella, Mvh, Dazl,* and *Sycp3*), and iii) expression of repetitive sequence families (e.g. *LINE1, SINE,* and *IAP*). After implantation of the blastocyst in the uterus, ICM-derived PSCs give rise to EpiSCs and again methylate i) the X chromosome, ii) promoters for genes characteristic of PSCs in the ICM (*Rex-1* and *Stella*), and iii) repetitive sequences (Hayashi et al., 2008). Whereas most EpiSCs undergo further differentiation into TCSCs by stable repression of promoters for pluripotent-specific genes, some EpiSCs in the proximal epiblast (precursors of PGCs) revert to a state that resembles ICM PSCs by undergoing genome-wide DNA demethylation (Hayashi & Surani, 2009). This leads to re-activation of the X chromosome and promoters for germline-lineage genes and repetitive sequences.

Unlike differentiated somatic cells, PSCs commonly express the pluripotency core transcription factors (TFs) such as Oct-4, Nanog, and Sox2 (Kim et al., 2008). These TFs form the pluripotency core circuitry by reinforcing the expression of genes involved in keeping PSCs in an undifferentiated state and, at the same time, repressing their differentiation. The biological significance of these core TFs has been experimentally proven by the generation of inducible pluripotent stem cells (iPSCs), in which fully differentiated somatic cells can be reprogrammed into ESC-like stem cells after transduction by so-called Yamanaka factors (*Oct-4, Sox2, Klf4,* and *cMyc*) (Takahashi & Yamanaka, 2006; Wernig et al., 2007).

Another hallmark of pluripotency is the presence of transcriptionally active chromatin structures at the promoters of core TFs due to methylation and histone modifications of promoter DNA (Cedar & Bergman, 2009). Thus, promoters for core TFs in PSCs are de-methylated and highly enriched for histone codes associated with active transcription, such as acetylated histones and trimethylation on lysine 4 of histone 3 (H3K4me3). In addition to the expression of pluripotency core TFs, undifferentiated PSCs also exhibit specific epigenetic marks called bivalent domains (BDs) (Azuara et al., 2006; Boyer et al., 2006; Lee et al., 2006; Stock et al., 2007). In BDs, the transcriptionally active H3K4me3 code coexists with repressive histone codes, such as trimethylated lysine 27 in histone 3 (H3K27me3). The BDs are mainly detected in the promoter regions of homeodomain-containing developmental master TFs, such as *Dlx-, Irx-, Lhx-, Pou-, Pax-,* and *Six*-family proteins. Due to the overwhelming effect of the transcription-repressive activity of H3K27me3, the transcription of BD-controlled genes is transiently repressed to prevent premature differentiation.

However, in response to developmental stimuli, BDs in promoters of these genes are switched into the monovalent type that promotes transcription. Therefore, both positive (expression of Oct-4-Nanog-Sox2) and negative (repression of differentiation-inducing TFs by bivalent domains) mechanisms are indispensable for controlling the pluripotent state of PSCs.

As mentioned before, PSCs are detected only during very early embryonic development and they disappear after differentiation into TCSCs and germline cells (Niwa, 2007). However, recent evidence has accumulated demonstrating that PSCs may reside in adult tissues and are able to differentiate into TCSCs (Ratajczak et al., 2007). These cells have been variously described in the literature as i) multipotent adult progenitor cells (MAPCs), ii) marrow-isolated adult multilineage-inducible (MIAMI) cells, iii) multipotent adult (MA) SCs, or iv) OmniCytes (Beltrami et al., 2007; D'Ippolito et al., 2004; Jiang et al., 2002; Pochampally et al., 2004). Thus, the physical presence of PSCs in adult tissues may better explain stem cell plasticity, according to which TCSCs are purportedly plastic and can trans-dedifferentiate into SCs for other tissues.

However, several questions remain to be addressed regarding these rare PSCs. First, the developmental origin of these cells is unresolved. As shown in **Figure 1,** PSCs during embryogenesis/gastrulation may become eliminated after giving rise to TCSCs, or conversely, they may survive among TCSCs and serve as a back-up/reserve source for these cells. Thus, it is important to elucidate whether PSCs found in adult tissue cells are functional under steady-state conditions or are merely remnants from developmental embryogenesis that reside in a dormant state in adult tissues. Second, is the question of

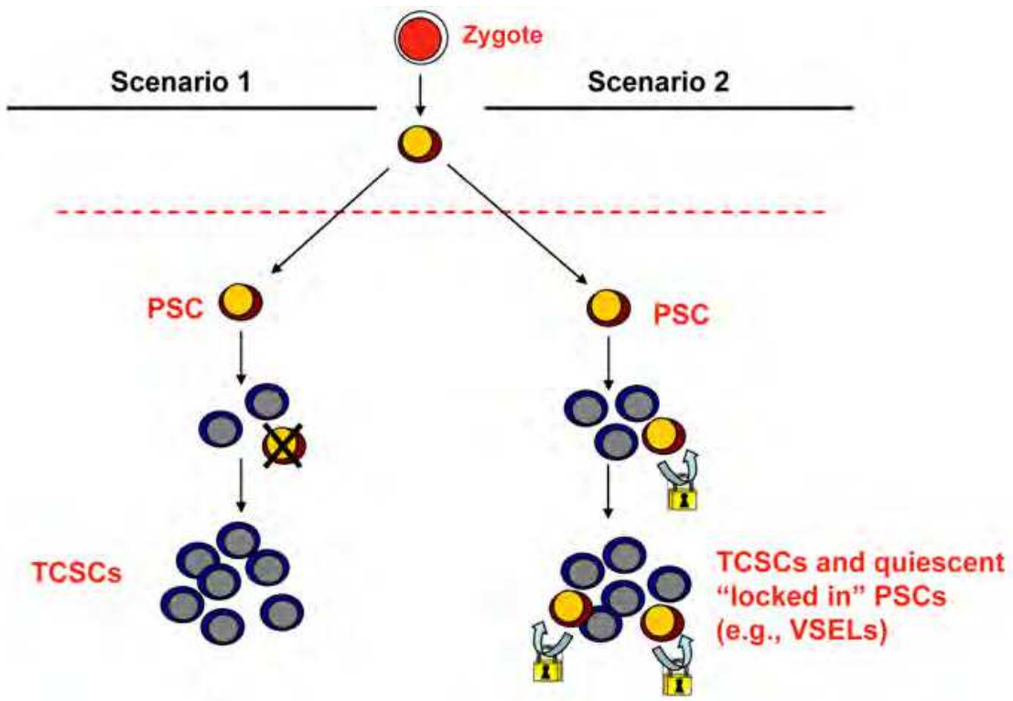

Fig. 1. **Developmental specification of PSCs into TCSCs. Scenario I:** During embryogenesis/gastrulation, PSCs are eliminated after giving rise to TCSCs and PGCs. **Scenario II:** PSCs survive and serve as a back-up/reserve source of TCSCs.

whether the dormant state of these cells is regulated by cell-intrinsic epigenetic reprogramming similar to other PSCsduring embryogenesis. Finally, there is the question of whether their dormant state is influenced by microenvironmental cues, such as their (i) location in non-physiological niches, (ii) exposure to inhibitors, or (iii) deprivation of appropriate stimulatory signals. The answer to these questions could be key to successful application of these adult-tissue-derived PSCs in the clinical setting. In this chapter, we will discuss these issues in more detail.

2. Very small embryonic-like stem cells (VSELs) residing in adult tissues

Recently, our group purified a population of very small embryonic-like stem cells (VSELs) from BM by employing a multiparameter fluorescence-activated cell sorter (FACS) (Kucia et al., 2006b). These rare Sca-1$^+$Lin$^-$CD45$^-$ cells reside in several adult murine organs (*e.g.*, brain, liver, skeletal muscles, heart, and kidney) (Zuba-Surma et al., 2008) and recently were detected also in human umbilical cord-blood and mobilized peripheral-blood (Kucia et al., 2006a; Paczkowska et al., 2009; Wojakowski et al., 2009). VSELs are very small in size (~3–6 μm) and express pluripotent markers, such as Oct-4, Nanog, Rex-1, and SSEA-1 (Kucia et al., 2006b). They are morphologically, similarly to ESCs, possessing large nuclei containing unorganized chromatin (euchromatin) and exhibiting a significantly higher nuclear/cytoplasm (N/C) ratio and a lower cytoplasmic area than hematopoietic stem cells (HSCs). The true expression of *Oct-4* and *Nanog* in BM-derived murine VSELs was recently confirmed by demonstrating transcriptionally active chromatin structures for both *Oct-4* and *Nanog* promoters (Shin et al., 2009). If cultured under a C2C12 myoblast feeder layer, freshly isolated VSELS form spheres corresponding to embryoid bodies (EBs). These VSEL-derived spheres (VSEL-DSs) contain primitive SCs that, after replating into tissue-specific differentiation media, are induced to differentiate into cells from all three germ layers (Kucia et al., 2008a). From experiments with mouse models, it has been proposed that VSELs are mobilized into peripheral blood in response to injury and circulate to the organ of injury in an attempt to enrich and regenerate damaged tissues (e.g., following heart infarct or stroke) (Kucia et al., 2008b; Paczkowska et al., 2009; Wojakowski et al., 2009). This physiological mechanism probably plays a significant role in the regeneration of some small tissue and organ injuries; however, further studies are needed to demonstrate that these cells do in fact home to the damaged organs.

3. Molecular signature of VSELs residing in BM

To investigate the relationship between VSELs and embryonic PSCs (e.g. embryonic stem cells [ESCs], epiblast stem cells [EpiSCs], primordial germ cells [PGCs], and embryonic germ cells [EGs]), we employed several molecular strategies to evaluate VSEL molecular signatures (**Figure 2**). Highly purified Sca-1$^+$Lin$^-$CD45$^-$ VSELs from murine BM were evaluated for i) expression of pluripotent genes, epiblast/germ line markers, and developmentally crucial imprinted genes; ii) the presence of BDs; and iii) reactivation of the X chromosome in female VSELs.

3.1 VSELs express PSC genes

PSCs express the essential pluripotency TF *Oct-4*. The importance of this TF is well-established by the fact that transduction with *Oct-4* is obligatory in several protocols for

generating iPSCs. We found that VSELs express Oct-4 at both the mRNA and protein level (Kucia et al., 2006a). However, recently some doubts have been raised about whether cells isolated from adult tissues express these embryonic genes, and it has been suggested that positive PCR data showing *Oct-4* expression may be due to amplification of *Oct-4* pseudogenes (Lengner et al., 2007; Liedtke et al., 2007). Thus, to prove true expression of the *Oct-4* gene in VSELs, we investigated the epigenetic state of the *Oct-4* promoter. Our DNA methylation studies of the *Oct-4* promoter using bisulfite sequencing revealed that it is hypomethylated in highly purified Sca-1$^+$Lin$^-$CD45$^-$ VSELs, similarly to cells isolated from ESC-derived EBs (28% and 13.2%, respectively) (Shin et al., 2009). Next, to evaluate the state of histone codes for the *Oct-4* promoter, we performed the chromatin-immunoprecipitation (ChIP) assay to verify its association with acetylated histone 3 (H3Ac) and dimethylated lysine 9 of histone 3 (H3K9me2), the molecular marks for open- and closed-type chromatin, respectively. By employing the carrier-ChIP assay (using the human hematopoietic cell-line THP-1 as carrier) we found that *Oct-4* promoter chromatin is associated with H3Ac and its association with H3K9me2 is relatively low (Shin et al., 2009). We also evaluated the epigenetic state of another core TF, *Nanog*, and observed that its promoter has a higher level of methylation in VSELs (~50%). However, in quantitative ChIP experiments performed in parallel, it was confirmed that the H3Ac/H3K9me2 ratio favors transcription and supports its active state (Shin et al., 2009). Based on these results, we conclude that VSELs truly express *Oct-4* and *Nanog*. Of note, we also reported that VSELs express several other markers of PSCs, such as SSEA-1 antigen, as well as *Sox2* and *Klf4* TFs (Shin et al., 2010b).

3.2 Expression of epiblast markers

As a result of the epigenetic reprogramming that occurs during implantation of the blastocyst, EpiSCs exhibit transcription profiles different from ESCs. For example, the expression of *Nanog, Sox2,* and *Stella* is reduced in EpiSCs through DNA methylation of their promoters (Surani et al., 2007). In functional assays, EpiSCs, unlike ESCs, show a highly restricted capacity to complement blastocyst development (Brons et al., 2007; Tesar et al., 2007). However, pluripotent EpiSCs may differentiate *in vivo* into TCSCs, which during embryogenesis orchestrate organogenesis and later in adult life are involved in rejuvenation of tissues and organs. Like EpiSCs, highly purified BM-derived Oct-4$^+$ VSELs do not complement blastocyst development and therefore cannot enable *in vitro* differentiation into cells from all three germ layers. Therefore, we have hypothesized that VSELs may be epiblast-derived precursors of TCSCs (Ratajczak et al., 2010). To investigate the similarity between VSELs and EpiSCs, we examined the expression of genes that are characteristic of EpiSCs (*Gbx2, Fgf5,* and *Nodal*) and ESCs from the blastocyst ICM (*Rex-1*) in adult BM-derived VSELs. It is known that *Gbx2, Fgf5,* and *Nodal* are upregulated in EpiSCs, but are expressed at lower levels in ESCs isolated from the ICM (Hayashi et al., 2008). In contrast, the level of *Rex-1* transcripts is highly expressed in ICM cells. We found that VSELs highly express *Gbx2, Fgf5,* and *Nodal*, but express the *Rex-1* transcript at a low level compared to the established murine ESC cell line, ESC-D3. This suggests that VSELs are more differentiated than ICM-derived ESCs and share several markers with more differentiated EpiSCs (Shin et al., 2010b).

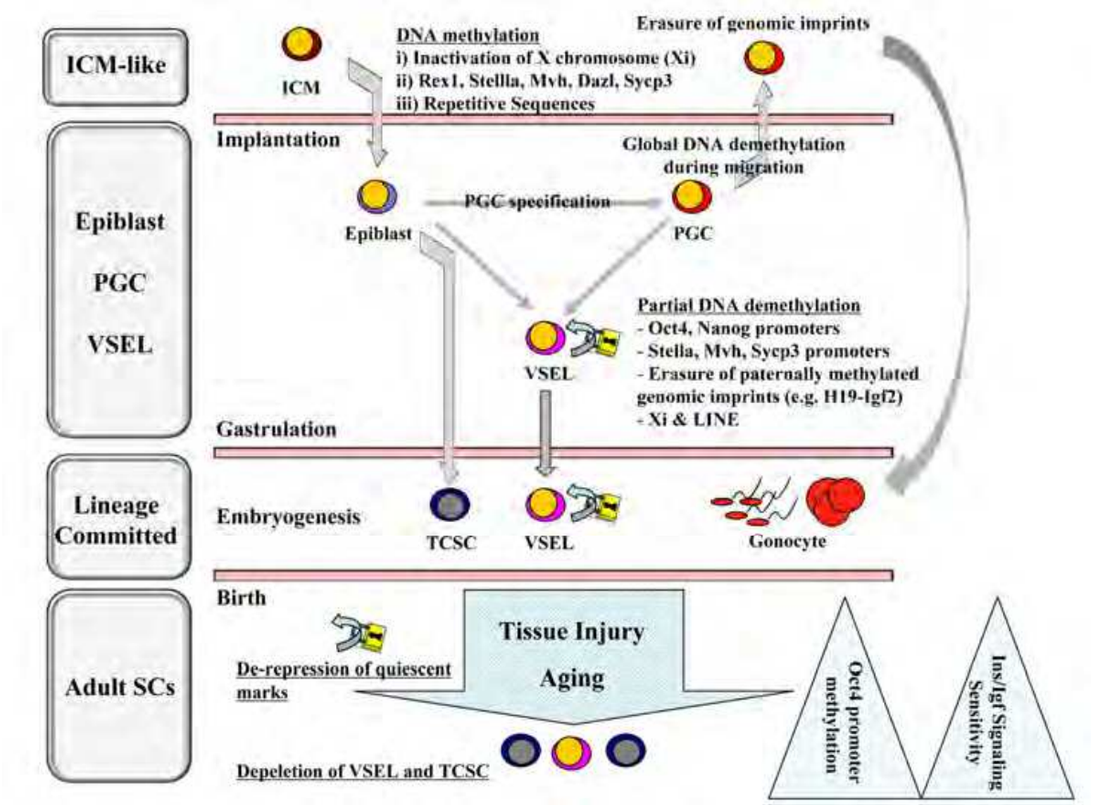

Fig. 2. **Epigenetic modification of VSELs during embryogenesis and aging.** Epigenetic modifications control the differentiation potential of SCs during embryogenesis and aging. The DNA methylation processes during development of ICM-derived epiblast SCs specify them to TCSCs. However, at the beginning of gastrulation, the proximal epiblast-specified PGCs can reset their epigenetic profile to one that characterizes ICM-derived PSCs. Subsequently, during PGC migration to the genital ridges, global DNA demethylation leads to the erasure of genomic imprints. Consistent with the hypothesis that VSELs originate from an epiblast-derived PGC population, they show a PGC-like epigenetic profile, including partial DNA demethylation of several pluripotency, germ-line, and genomic imprints. These epigenetic profiles of developing VSELs are retained after their deposition into adult tissues. This parent-specific reprogramming of the genomic imprinting of VSELs deposited in adult tissues (e.g., BM) functions as i) a "lock-in mechanism" to prevent their inappropriate proliferation and ii) a mechanism to restrict their sensitivity to Ins/Igf signaling. During the aging process, while residing in adult tissues, VSELs exposed to oxidative stress or chronic Ins/Igf signaling de-repress locked-in genomic imprints and progressively methylate DNA in the Oct-4 promoter (Ratajczak et al 2011a). As a result of these epigenetic changes, the total number and pluripotency of VSELs decreases with age (Ratajczak et al. 2008), which leads to impaired tissue regeneration and rejuvenation.

3.3 Expression of germline markers

During gastrulation, EpiSCs lose expression of core pluripotency TFs. On the other hand, during specification of proximal epiblast EpiSCs into PGCs, the expression of these early embryonic genes is re-activated by resetting epigenetic programs in these cells (Hayashi &

Surani, 2009). Thus, PGCs reset epigenetic marks to an ICM-like state, which results in re-activation of pluripotency and germline-related genes (Hayashi & Surani, 2009). The specification of PGCs is initiated at E7.25 by expression of germline master regulators, such as *Fragilis, Blimp1,* and *Stella,* in response to signals from extra-embryonic tissues (Surani et al., 2007). At this time, around 40 proximal epiblast-derived PGCs are detected. At E8.5, PGCs enter back into the embryo proper through the primitive streak and start migration through the hindgut endoderm and mesentry to the aorta-gonads-mesonephros (AGM) region, and at around E11.5 they reach the genital ridge in which PGCs differentiate into monopotent gametes (sperm and egg). Our data indicate some relationship between VSELs and PGCs (Shin et al., 2010b). Accordingly, VSELs are reported to highly express genes that are involved in germ-line specification of the epiblast (e.g., *Stella, Prdm14, Fragilis, Blimp1, Nanos3,* and *Dnd1*). The expression of Stella, Blimp1, and Mvh has been confirmed at the protein level by immunostaining. Furthermore, the *Stella* promoter in VSELs is partially demethylated and displays transcriptionally active histone modifications (H3Ac and H3K4me3) and is less enriched for transcriptionally repressive histone marks (H3K9me2 and H3K27me3) (Shin et al., 2010b). It can be concluded that VSELs express several germline-specific genes and display a *Stella* promoter chromatin structure that is characteristics of germline specification. VSELs also highly express *Dppa2, Dppa4,* and *Mvh*, which characterize late migratory PGCs; however, they do not express *Sycp3, Dazl,* and *LINE1* genes that are expressed in post-migratory PGCs (Maatouk et al., 2006; Maldonado-Saldivia et al., 2007). Thus, our results *in toto* support the conclusion that VSELs deposited into murine BM show some similarities in gene expression and epigenetic signatures to epiblast-derived migratory PGCs (at ~E10.5–E11.5).

3.4 VSELs are marked by BDs

As mentioned above, in undifferentiated ESCs, most of the homeodomain-containing developmental TFs are repressed by BDs (Bernstein et al., 2006), which are chromatin structures in which transcriptionally opposite histone codes physically co-exist in the same promoter. In undifferentiated ESCs, BD epigenetic codes at the promoters of these TFs are temporarily repressed, preventing their premature differentiation. During differentiation, the transient repressive epigenetic marks in these TFs become monovalent and thereafter activate or repress expression of the appropriate TFs. Our preliminary data indicate that murine VSELs display BDs in the promoters of several homeodomain-containing developmental TFs (*Sox21, Nkx2.2, Dlx1, Lbx1h, Hlxb9, Pax5,* and *HoxA3*). The presence of transcriptionally active histone codes, such as H3K4me3, physically coexisting with repressive histone codes, such as H3K27me3, was confirmed by employing the carrier-ChIP assay (submitted for publication).

3.5 VSELs from female mice partially activate an X chromosome

The process of X-chromosome inactivation is mediated by expression of the large noncoding RNA *Xist,* which is transcribed on the inactivated X chromosome. Coating of the X chromosome to be inactivated by spreading of Xist RNA induces the silenced chromatin structure (Payer & Lee, 2008). As already mentioned above, it is well known that female PSCs (*e.g.*, murine and human ESCs isolated from the blastocyst ICM, as well as PGCs) reactivate the X chromosome that was inactivated after fertilization, and, as a result, female PSCs display two equivalently activated X chromosomes (Surani et al., 2007). Our initial

studies in murine female VSELs show that these cells partially reactivate the inactivated X chromosome. As mentioned above, female murine VSELs partially hypermethylate the *Xist* promoter (~80%), unlike somatic cells which show 50% DNA methylation. This result strongly suggests that murine VSELs, like ESCs and PGCs, can undergo the process of X chromosome reactivation (submitted for publication).

4. Developmental origin of VSELs

Taking into consideration all the molecular signatures characteristic of VSELs, we propose that VSELs are epiblast-derived PSCs deposited early during embryogenesis in developing organs as a potential reserve pool of precursors for TCSCs. Thus, VSELs have an important role in tissue rejuvenation and regeneration (Shin et al., 2010a). Because of the gene expression profile and epigenetic state of the core TFs, expression of epiblast and germline genes suggests that VSELs deposited in adult BM originate from migratory PGCs that have gone astray from the "orthodox" migration route.

From the second trimester on, VSELs are easily found as a Sca-1^{+}Lin^{-}CD45^{-} population in murine fetal liver, which is the main embryonic hematopoietic tissue. VSELs that emerge in fetal liver (FL-VSELs) follow the developmental route of hematopoietic stem cells (HSCs) and subsequently colonize BM together with HSCs (Zuba-Surma et al., 2009). FL-VSELs and their BM-derived counterparts express a similar pattern of pluripotent and epiblast/germline genes at the mRNA and protein levels. Accordingly, the promoters for *Oct-4, Nanog,* and *Stella* show significant DNA demethylation and enriched histone modifications for an open, transcriptionally active structure in these promoters (Shin et al., 2010a).

Mounting evidence also indicates that PGCs could be related to HSCs, another population of highly migratory SCs (De Miguel et al., 2009). In support of this notion, the first primitive HSCs appear in the extra-embryonic tissues in yolk sac blood islands at the time when proximal epiblast-specified PGCs enter the extra-embryonic mesoderm (Mikkola & Orkin, 2006). In addition, the appearance of definitive HSCs in the AGM region of the embryo proper coincides with migration of PGCs to the genital ridges through the AGM (De Miguel et al., 2009). Furthermore, PGCs isolated from murine embryos have been proven to be able to grow HSC colonies while, on the other hand, robust hematopoietic differentiation has been observed in some classical germline tumors (Kritzenberger & Wrobel, 2004; Ohtaka et al., 1999; Rich, 1995; Saito et al., 1998; Woodruff et al., 1995). All this suggests developmental overlap between PGCs and HSCs.

On the other hand, VSELs share several characteristics with both PGCs and HSCs. In particular, VSELS i) share several BM- and FL-derived markers characteristic of the epiblast/germ line (Shin et al., 2010b), ii) follow the developmental route of HSCs (Zuba-Surma et al., 2009), and iii), in appropriate culture conditions, can also be differentiated toward the hematopoietic lineage. All of this suggests that VSELs are the most primitive murine BM-residing population of SCs and function as precursors for long-term repopulating HSCs (Ratajczak et al., 2011b).

Thus, PGCs, HSCs , and VSELs together form a unique highly migratory population of interrelated SCs that may be envisioned as a kind of 4th (highly migratory) germ layer. Due to this unique developmental origin, VSELs show characteristic epigenetic reprogramming and gene expression in stemness, germline, and imprinted genes (as described below) that maintain their pluripotency, but also prevent inappropriate proliferation and teratoma formation (Shin et al., 2009).

5. Epigenetic changes of imprinted genes that regulate VSEL pluripotency

Unlike ESCs, highly purified BM-derived Oct-4$^+$ VSELs do not proliferate *in vitro* if cultured alone and do not grow teratomas *in vivo*. On the other hand, cells from VSEL-DSs have restored their proliferation potential, demonstrating that their quiescent state can be modulated. This suggests that VSELs are a quiescent cell population and that some mechanisms must exist to prevent their unleashing of proliferation and teratoma formation. Like VSELs, PGCs in cultures freshly isolated from embryos proliferate only for a few days before disappearing, either because they differentiate or die (De Felici & McLaren, 1983). They also neither grow teratomas nor complement blastocyst development (Surani et al., 2007). However, when PGCs are cultured over a feeder layer supplemented by a specific combination of growth factors, they continue to proliferate and can be reprogrammed into EGCs (Shamblott et al., 1998; Turnpenny et al., 2003). Therefore, it is possible that these two SC populations employ a similar molecular mechanism to regulate their pluripotency and to prevent cell proliferation.

The hallmark of epigenetic reprogramming during PGC development is erasure of genomic imprinting (Surani et al., 2007), which is an epigenetic process ensuring paternal-specific, mono-allelic expression of imprinted genes (Reik & Walter, 2001). Around 80 imprinted genes (expressed from maternal or paternal chromosomes only) have been reported in the mouse genome and their proper mono-allelic expression regulates totipotency and pluripotency of the zygote and developmentally early SCs, respectively. Furthermore, most imprinted genes, such as insulin-like growth factor 2 (*Igf2*), H19, Igf2 receptor (*Igf2R*), and $p57^{KIP2}$ (also known as *Cdkn1c*) are directly involved in embryo development. Since the majority of imprinted genes exist as gene clusters enriched for CpG islands, their expression is coordinately regulated by the DNA methylation state of CpG-rich cis-elements known as differentially methylated regions (DMRs) (Delaval & Feil, 2004). The differential methylation state of DMRs is mediated by DNA methyltransferases (*Dnmts*), depending on the parental allele of origin. Depending on the developmental period of methylation, there are two types of DMRs: "primary DMRs" are differentially methylated during gametogenesis and "secondary DMRs" acquire allele-specific methylation after fertilization. So far, 15 primary DMRs have been identified in the mouse genome. Interestingly, most DMRs are methylated at the maternal allele and only three DMRs (at *Igf2-H19, Rasgrf1,* and *Meg3* loci) are paternally methylated (Kobayashi et al., 2006). In addition to DNA methylation of DMRs, histone modifications also contribute to monoallelic expression of imprinted genes (Fournier et al., 2002; Mager et al., 2003).

Shortly after PGC specification at E7.25, PGCs initiate epigenetic reprogramming programs, resulting in global DNA demethylation and changes in histone modifications (Seki et al., 2007). As a result, epigenetic marks for genomic imprinting in both parental chromosomes are erased during migration into the genital ridge and new genomic imprints are established during differentiation into gametes in a sex-dependent manner. The erasure of genomic imprints could be a mechanism to protect the Oct-4-expressing germline SCs from uncontrolled expansion and teratoma formation. For example, while the nuclei of early migrating PGCs at E8.5–9.5 can be successfully used as donors for nuclear transfer, nuclei from post-migratory PGCs after E11.5 are incompetent to support full-term development (Yamazaki et al., 2003).

Since VSELs, as discussed above, share similar molecular signatures as PGCs, we have proposed that VSELs, like PGCs, modify methylation of imprinted genes, which prevents

them from unleashing proliferation and may explain their quiescent state in adult tissues. Indeed, as shown in **Figure 3** and **Table 1**, VSELs freshly isolated from murine BM erase the paternally methylated imprints (e.g., *Igf2-H19* and *Rasgrf1* loci), while they hypermethylate the maternally methylated ones (e.g., *Igf2* receptor (*Igf2R*), *Kcnq1-p57^{KIP2},* and *Peg1* loci). Because paternally expressed imprinted genes (*Igf2* and *Rasgrf1*) enhance embryo growth and maternally expressed genes (*H19, p57^{KIP2},* and *Igf2R*) inhibit cell proliferation (Reik & Walter, 2001), the unique genomic imprinting pattern observed in VSELs demonstrates growth-repressive imprinting in these cells. As coordinated with genomic imprinting reprogramming programs, VSELs highly express growth-repressive imprinted gene transcripts (*H19, p57^{KIP2},* and *Igf2R*) and downregulate growth-promoting ones (*Igf2* and *Rasgrf1*), which explains the quiescent state of VSELs (Shin et al., 2009). Importantly, all the growth-repressive patterns of genomic imprinting are progressively recovered during the formation of VSEL-DSs, in which SCs proliferate and differentiate. These results suggest that epigenetic reprogramming of genomic imprinting should maintain the quiescence of Oct-4$^+$ VSELs deposited in the adult body and protect them from premature aging and tumor formation. Therefore, the modulation of mechanisms controlling genomic imprinting in VSELs is crucial for developing more powerful strategies to unleash the regenerative potential of these cells for efficient employment in the clinical setting.

6. VSELs and ageing

Tissue regeneration depends on the proper function of SCs, and we envision that aging can be partially explained by a decline in the regenerative potential of VSELs (Ratajczak et al., 2008). In support of this notion, the number of VSELs in murine BM gradually declines with age, ranging from 0.052 ± 0.018% at 2 months to 0.003 ± 0.002% at 3 years of age (Kucia et al., 2008a). Furthermore, the frequency of VSEL-DS formation decreases with age, thus little VSEL-DS formation was observed in cells isolated from older mice (>2 years). Accordingly, VSELs from older mice (2 years) also show lower expression of pluripotency master regulators, such as *Oct-4, Nanog, Sox2, Klf4,* and *cMyc,* while the *Oct-4* promoter in VSELs becomes hypermethylated with age and shows a closed chromatin structure (Ratajczak et al., 2011a). The age-dependent decrease of the pool size and function of VSELs in BM may explain the decline of regeneration potential during aging. This hypothesis has been further corroborated by looking for differences in the content of these cells among BM mononuclear cells (BMMNCs) in long- and short-lived mouse strains. The concentration of VSELs was much higher in the BM of long-lived (e.g., C57B6) compared to short-lived (DBA/2J) mice (Kucia et al., 2006b).

We have also reported that while long-lived Laron dwarf mice, with low levels of circulating Igf1, have a higher number of VSELs in BM compared to normal littermates (Ratajczak et al. 2011a), short-lived bovine-growth-hormone-expressing transgenic mice, with high circulating Igf1 levels, prematurely deplete this population of PSCs (submitted for publication). These observations suggest interesting links between high caloric uptake, increases in chronic insulin/insulin growth factor signaling, and premature depletion of VSELs.

In support of this linkage, it is well known that changes in insulin/insulin-like growth factors (Ins/Igf) signaling molecules play a crucial role in aging. In particular, insulin-like growth factor 1 (*Igf1*) signaling negatively regulates the life span of animals, from worms

and flies to mammals (Russell & Kahn, 2007), whileIgf1 and insulin level in blood is regulated positively by caloric uptake (Piper & Bartke, 2008). Overall, the genomic imprinting reprogramming in VSELs leads to impaired Ins/Ingf signaling due to i) downregulation of expression of *Igf2*, ii) upregulation of expression of *Igf2R,* which serves as a molecular sink for Igf2, and iii) a decrease in expression of *Rasgrf1,* which is a small GTP exchange factor (GEF) for Ins/Igf signal transduction. This suggests that the epigenetic mechanism governing the VSEL quiescent state regulates the sensitivity to Ins/Igf signaling and could, if overactivated, lead to premature depletion of primitive VSELs in tissues (Ratajczak et al., 2011a).

Thus, high chronic calorie uptake, followed by high plasma insulin and Igf-1 levels may, over time, prematurely deplete VSELs from adult organs and thus accelerate aging.

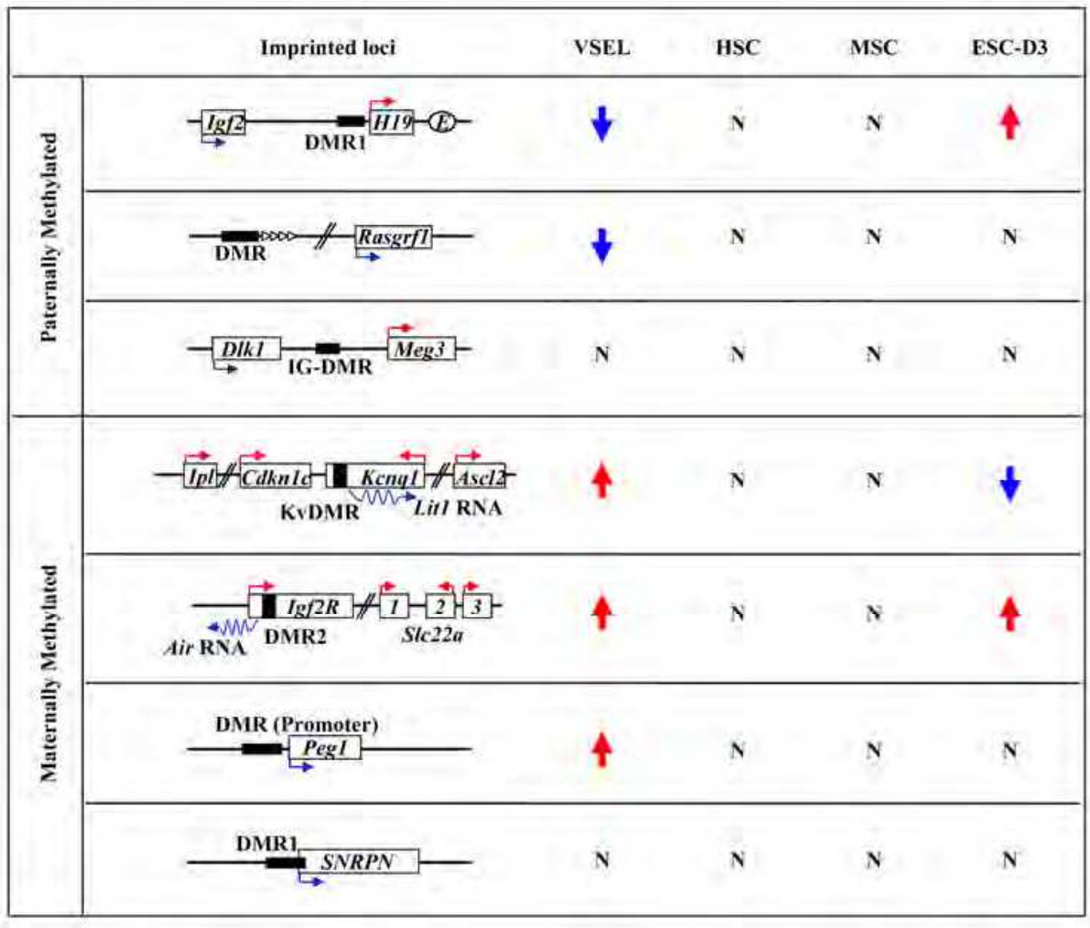

Fig. 3. **The unique DNA methylation pattern and expression of imprinted genes in VSELs.** Arrows in the schematic diagram of paternally (Igf2-H19 and Rasgrf1) or maternally (Igf2R and Kcnq1) methylated loci indicate the transcriptional activity of the indicated gene. Red (up) and blue (down) arrows indicate upregulated and downregulated gene expression, respectively. M = maternal chromosome, P = paternal chromosome. DMR1, IG-DMR, KvDMR, DMR2 = DMRs for Igf2-H19, Rasgrf1, Kcnq1, and Igf2R loci, respectively.

Imprinted gene	Expression	Proliferation	VSEL
Igf2	Pat	+	↓
Rasgrf1	Pat	+	↓
Dlk1	Pat	+	N
Air	Pat	+	↓
Lit1	Pat	+	↓
H19	Mat	–	↑
Meg3	Mat	–	N
Igf2R	Mat	–	↑
$p57^{KIP2}$/Cdkn1c	Mat	–	↑
SNRPN	Mat	–	N

Table 1 . Expression profiles of crucial imprinted genes in murine VSELs.

The expression level of paternally (pat) or maternally (mat) expressed imprinted genes in murine BM-derived VSELs is indicated by red-up (up-regulated) and blue-down (down-regulated) arrows. The effect of the indicated imprinted genes on cell proliferation is marked as '+' (proliferation promoting) or '–' (repressing).

7. Regeneration potential of VSELs *in vivo*

To address the most important question of whether these primitive VSELs could be efficiently employed in the clinic, we have tested their potential role in several *in vivo* tissue-regeneration animal models. First, VSELs can be specified *in vivo* into mesenchymal stem cells (MSCs). Accordingly, in the first study by Taichman *et al*, VSELs isolated from GFP^+ mice were implanted into SCID mice, and 4 weeks later the formation of bone-like tissues was observed (Taichman *et al*., 2010). Second, freshly isolated BM-derived VSELs from GFP^+ mice were injected into the hearts of mice that had undergone ischemia/reperfusion injury. After 35 days of follow-up, VSEL-treated mice exhibited improved global and regional left ventricular (LV) systolic function (as determined by echocardiography) and attenuated myocyte hypertrophy in surviving tissue (as determined by histology and echocardiography) when compared with vehicle-treated controls (Zuba-Surma et al., 2010). Finally, we observed that VSELs, if plated over the supportive OP9 cell line, give rise to

colonies of $CD45^+CD41^+Gr\text{-}1^-Ter119^-$ cells that, when transplanted into wild-type animals, protected them from lethal irradiation and differentiated *in vivo* into all major hematopoietic lineages (e.g., $Gr\text{-}1^+$, $B220^+$ and $CD3^+$ cells) (Ratajczak et al., 2011b). Thus, we propose that VSELs are a population of BM-residing PSCs that give rise to long-term-engrafting hematopoietic SCs.

8. Conclusion

In the past few years, several attempts have been made to purify a population of PSCs from adult tissues. We propose that the VSELs described by our team play a physiological role in rejuvenation of the pool of TCSCs under steady-state conditions. VSELs developmentally originate from epiblast-derived migrating PGCs and they could be deposited in adult organs early in development as a reserve pool of primitive SCs for tissue repair and regeneration. Therefore, VSELs share several molecular signatures with epiblast and migrating PGCs with respect to gene expression and epigenetic programs. Based on the developmental origin of VSELs, their proliferation, like PGCs, is controlled by the DNA methylation state of some of the developmentally crucial imprinted genes (e.g. *H19, Igf2,* and *Rasgrf1*). During the ageing process, proliferation-repressive epigenetic marks progressively disappear, resulting in the increased sensitivity to Ins/Igf signaling and, concomitantly, to depletion of the primitive VSEL population. The decrease in the number and pluripotency of these cells will affect pools of TCSCs and have an impact on tissue rejuvenation and life span. Furthermore, VSELs can be specified into several tissue-residing TCSCs (e.g. MSCs, HSCs, and cardiac SCs) in response to tissue/organ injury. Therefore, VSELs isolated from adult tissues are an alternative and ethically uncontroversial source of SCs for regenerative medicine. However, to successfully employ VSELs for this purpose, it is very important to establish experimental protocols for reprogramming of the growth-repressive genomic imprinted state of VSELs into the regular somatic pattern to unleash their regenerative potential.

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10. Conflicts of interest statement

The authors declare that they have no competing financial interests.

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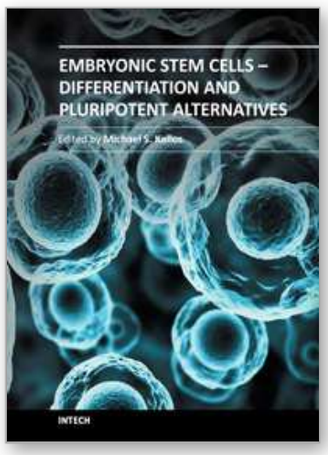

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The ultimate clinical implementation of embryonic stem cells will require methods and protocols to turn these unspecialized cells into the fully functioning cell types found in a wide variety of tissues and organs. In order to achieve this, it is necessary to clearly understand the signals and cues that direct embryonic stem cell differentiation. This book provides a snapshot of current research on the differentiation of embryonic stem cells to a wide variety of cell types, including neural, cardiac, endothelial, osteogenic, and hepatic cells. In addition, induced pluripotent stem cells and other pluripotent stem cell sources are described. The book will serve as a valuable resource for engineers, scientists, and clinicians as well as students in a wide range of disciplines.

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