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Minireview

Molecules and Cells

Molecular Characterization of Isolated from Murine Adult Tissues Very Small Embryonic/ Epiblast like Stem Cells (VSELs)

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Pluripotent very small embryonic/epiblast derived stem cells (VSELs) as we hypothesize are deposited at begin of gastrulation in developing tissues and play an important role as backup population of pluripotent stem cells (PSCs) for tissue committed stem cells (TCSCs). We envision that during steady state conditions these cells may be involved in tissue rejuvenation and in processes of regeneration/repair after organ injuries. Molecular analysis of adult bone marrow (BM)-derived purified VSELs revealed that they i) express pluripotent stem cells markers e.g., Oct4, Nanog, Klf-4, SSEA-1 ii) share several markers characteristic for epiblast as well as migratory primordial germ cells (PGCs), and iii) possess a unique pattern of genomic imprinting (e.g., erasure of differently methylated regions at Igf2-H19 and Rasgrf1 loci and hypermethylation at KCNQ1 and Igf2R loci). This supports that VSELs are related to epiblast-derived migrating PGC-like cells and, despite their pluripotent stem cell character, changes in the epigenetic signature of imprinted genes keep these cells quiescent in adult tissues and prevent them from teratoma formation. In contrast epigenetic changes/mutations that lead to activation of imprinted genes could potentially lead to tumor formation by these cells. Mounting evidence accumulates that perturbation of expression of imprinted genes is a common phenomenon observed in developing tumors.

INTRODUCTION

The rapidly developing field of regenerative medicine is searching for safe and therapeutically efficient sources of stem cells (SCs) that should give rise to cells from all three germ layers. Such primitive SCs endowed with broad spectrum of differentiation potential are relatively well characterized during embryogenesis and are called pluripotent stem cells (PSCs) (Ratajczak et al., 2007).

An adult organism develops from the most primitive stem cell (SC) called a zygote, which is an oocyte fertilized by a sperm cell. This totipotent zygote, the "mother of all stem cells" in the developing body, first gives rise to morula that consists of PSCs and, subsequently, at blastocyst level a population of PSCs that is maintained in inner cell mass of the blastocyst will give rise to the epiblast, a part of the developing embryo, which is the origin of SCs committed to all the three germ layers (meso-, ecto-, and endoderm) (Tam and Loebel, 2007). Thus, the PSCs that form epiblast could be considered the origin for the tissue-specific/committed SCs (TCSCs) for all the organs and tissues in the developing embryo proper.

Recently, our group isolated a population of pluripotent Sca-$1^{+}Lin^{-}CD45^{-}$ very small embryonic-like stem cells (VSELs) from adult murine bone marrow (BM), murine fetal livers (FLs) and several adult murine organs including brain, liver, kidney, lungs skeletal muscles and retina (Kucia et al., 2006; Zuba-Surma et al., 2009b; 2009c). VSELs express several morphological (e.g., relatively large nuclei containing euchromatin) and molecular (e.g., expression of SSEA-1, Oct4, Nanog, Rex1) markers characteristic for embryonic SCs (ESCs) or epiblast SCs (EpiSCs). We hypothesize that VSELs are deposited during early gastrulation in developing tissues/organs, survive into adulthood, and play an important role as a back-up population of PSCs in the turnover of TCSCs (Kucia et al., 2007).

Thus the presence of pluripotent VSELs in adult tissues may reconcile previously published data stating that adult tissues may contain a population of PSCs (Ratajczak et al., 2007). Though, the existence of such cells had been postulated by several investigators, such cells were never purified and identified at the single cell level. Their presence was predicted mainly based on experiments showing that some populations of cells were enriched in adherent cell populations isolated from the BM or that certain solid organs contain some primitive cells that may differentiate into various tissues. Such cells were described as i) mesenchymal (M)SCs; ii) multipotent adult progenitor cells (MAPCs); iii) marrow-isolated adult multilineage inducible (MIAMI) cells; iv) multipotent adult (MA)SCs; and v) OmniCytes (Beltrami et al., 2007; D'Ippolito et al., 2004; Jiang et al., 2002; Pochampally et al., 2004). It is conceivable that all these cells are closely related, overlapping populations of SCs were described by different investigators and given various names according to circumstance. Furthermore, the potential relationship between these cells and VSELs is not clear. Since

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all these cells are largely derived from the adherent fraction of BM- or adult organ-derived cells, they could potentially contain some VSELs from beginning.

VSELs - morphological characteristics

VSELs are purified from BM and other adult organs by FACS sorter (Kucia et al., 2006; Zuba-Surma et al., 2009b). Murine BM-derived VSELs: i) are very rare (~0.01% of nucleated cells); ii) are small in size (~3-6 μm); iii) express pluripotent markers such as Oct4, Nanog, Rex-1, and SSEA-1; iv) possess large nuclei containing unorganized chromatin (euchromatin); and v) are capable of differentiation *in vitro* into cells from all three germ lineages. In ImageStream system (ISS) analysis, which is flow cytometry combined with high resolution fluorescence microscopy, we found that VSELs exhibit a significantly higher nuclear/cytoplasm (N/C) ratio and a lower cytoplasmic area as compared with HSCs (Zuba-Surma et al., 2008).

When purified VSELs are plated over a C2C12 myoblast feeder layer, they form spheres that resemble embryoid bodies (EBs) (Kucia et al., 2006). The VSEL-derived spheres (VSEL-DSs) contain primitive stem cells that, after replating into tissue differentiation specific media differentiate into cells from all three germ layers. Furthermore, we reported that VSELs are mobilized into peripheral blood during organ injuries (e.g., heart infarct, stroke), which suggests that these cells could participate in the regeneration of damaged organs (Kucia et al., 2005; Paczkowska et al., 2009; Wojakowski et al., 2009).

We have calculated that the number of VSELs in murine BM gradually declines with age, ranging from 0.052 ± 0.018% to 0.003 ± 0.002% between ages of 2 months and 3 years, respectively (Ratajczak et al., 2008). More importantly, the ability of VSEL-DS formation decreases with age, thus no VSEL-DSs were observed in cells isolated from older mice (> 2 years). The aging-dependent decrease of the pool and function of VSELs in BM may explain the decline of the regeneration potential during aging. This hypothesis has been further confirmed 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) as compared to short-lived (DBA/2J) mice (Kucia et al., 2006).

Developmental origin of VSELs

PSCs in the epiblast undergo a sequel of specification events, first into multipotent and subsequently into versatile TCSCs, which play a role in the formation and rejuvenation of various organs (Tam and Loebel, 2007). The most important questions emerge whether some of these primitive epiblast-forming PSCs can "escape" specification into more differentiated populations of SCs and retain their pluripotential character, thus surviving among differentiated daughter TCSCs. Conversely, would all of them undergo tissue/organ specific differentiation and then "disappear" after embryogenesis, not be found in the adult body.

We envision that VSELs are epiblast derived PSCs deposited early during embryonic development in developing organs as a potential reserve pool of precursors for TCSCs and thus this population has an important role in tissue rejuvenation and regeneration. We also hypothesize that VSELs originate or are closely related to a population of proximal epiblast migratory EpiSCs that ~ embryonic day (E)7.25 in mice, becomes specified to PGCs and egress from the epiblast into extra-embryonic tissues (extra-embryonic mesoderm) (Hayashi et al., 2007). These cells subsequently make a turn and through the primitive streak return to the embryo proper and migrate to genital ridges, where they ultimately give rise to gametes (precursors of sperm or oocytes).

Accumulating evidence also indicates that PGCs could somehow be related to hematopoietic stem cells (HSCs), another population of highly migratory SCs (Kritzenberger and Wrobel, 2004; Ohtaka et al., 1999; Rich, 1995; Saito et al., 1998). To support this notion, the first primitive HSCs appear in the extra-embryonic tissues in yolk sac blood islands at a time when proximal epiblast-specified PGCs enter the extra-embryonic mesoderm (Mikkola and Orkin, 2006). Furthermore, the appearance of definitive HSCs in the aorta-gonad-mesonephros (AGM) region in the embryo proper corresponds timewise to migration of PGCs to the genital ridges through the AGM (De Miguel et al., 2009). To support this hypothesis further, PGCs isolated from murine embryos were described as being able to grow HSC colonies and robust hematopoietic differentiation was observed in some classical germ tumors (Ohtaka et al., 1999; Rich, 1995; Saito et al., 1998). Thus all this together suggest developmental overlap between PGCs and HSCs.

Our data indicate that VSELs share several characteristics both with PGCs and HSCs. To support this notion, our recent molecular analysis data indicate that in fact VSELs share several markers characteristic for epiblast/germ line (Shin et al., 2010). Furthermore, VSELs follow developmental route of HSCs colonizing together with HSCs first FL and subsequently BM (Zuba-Surma et al., 2009c). Furthermore, VSELs in appropriate culture conditions could be also differentiated toward hematopoietic lineage (Zuba-Surma et al., 2009a). In the future, it will be important to evaluate the potential presence of VSELs in yolk sac blood islands and to determine whether VSELs are detectable in *Ncx1*$^{-/-}$ embryos that do not initiate a heart beat and thus lack definitive HSCs in embryonic tissues (Lux et al., 2008).

Thus PGCs, HSCs and VSELs all together form a unique highly migratory population of interrelated SCs that could be envisioned to be a kind of "4th highly migratory germ layer" (Fig. 1). Due to this unique developmental origin, VSELs show characteristic epigenetic reprogramming and gene expression in stemness-, germ line-, and imprinted-genes that maintain their pluripotency, but also prevent their unleashed proliferation and teratoma formation (Shin et al., 2010).

VSELs and their unique molecular characteristics

We employed several molecular strategies to evaluate molecular signature of VSELs. Highly purified Sca-1^{+}Lin^{-}CD45^{-} VSELs from murine BM or FL were evaluated for expression of i) pluripotent genes, ii) epiblast/germ line markers and iii) expression of developmentally crucial imprinted genes.

VSELs and expression of PSCs genes

We found that VSELs express at mRNA and protein level, transcription factor Oct4 that is characteristic for ESCs (Shin et al., 2009; 2010). However, recently some doubts were raised if cells isolated from adult tissues may express these embryonic genes and it has been postulated that positive PCR data showing Oct4 expression may be due to amplification of Oct4 pseudogenes (Lengner et al., 2007; Liedtke et al., 2007). Thus, to prove true expression of the Oct4 gene in VSELs we investigated the epigenetic status of Oct4 promoter in these cells. The Sca-1^{+}Lin^{-}CD45^{-} VSELs were double purified and we examined the DNA methylation status of the Oct4 promoter in these cells by employing bisulfite-sequencing. We noticed that the Oct4

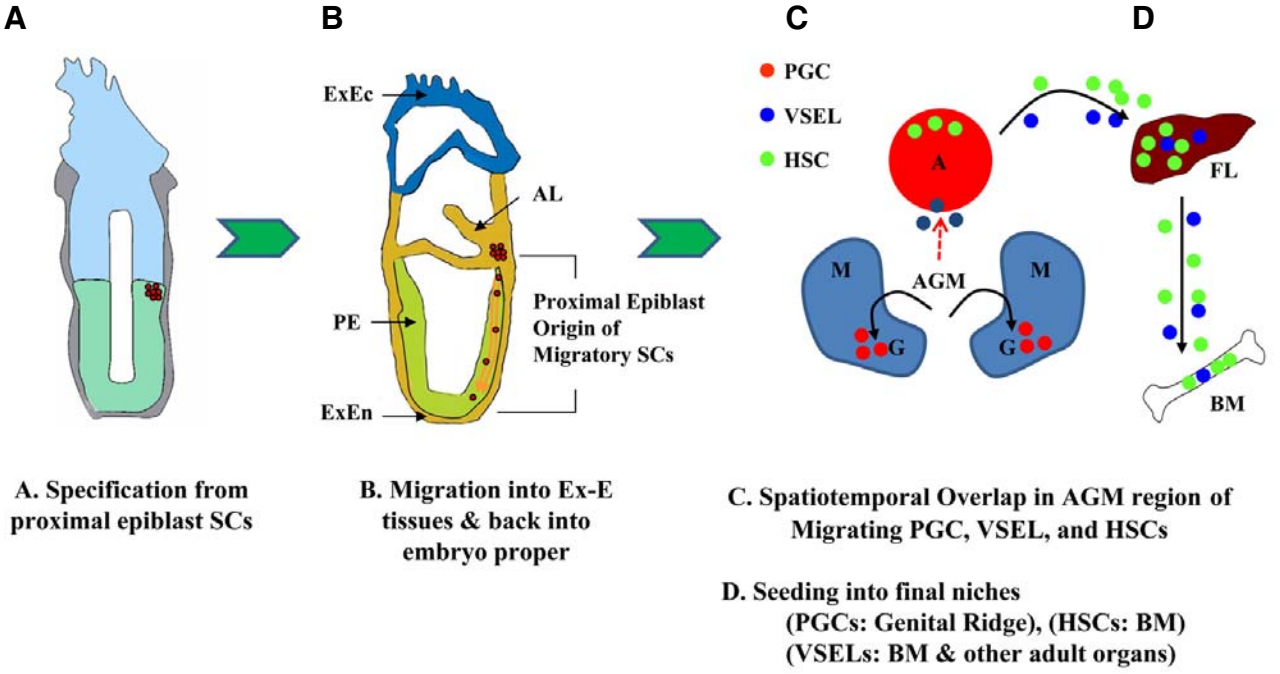

Fig. 1. Developmental origin of VSELs - presence of highly migratory 4th germ layer? Migratory PGCs become specified in proximal epiblast (A) and migrate first to extra-embryonic (Ex-E) tissues (B). Some of them could potentially initiate primitive hematopoiesis in yolk sac blood islands (?). Subsequently, these cells, known as PGCs, return via the primitive streak into the developing embryo proper and migrate toward genital ridges (B). Some of these cells (VSELs?) go astray from the main migratory route and seed around the aorta in the aorta-gonado-mesonephros (AGM) region (C). Further studies are needed to determine whether these cells may contribute to definitive HSCs. Finally, (D) we envision that these cells colonize FLs and BM by following the developmental route of HSCs and seed to adult organs via circulation. Aorta (A); Aorta-Gonad-Mesonephros region (AGM); Bone Marrow (BM); extra-embryonic ectoderm (ExEc); extra-embryonic (Ex-E); extra-embryonic endoderm (ExEn); Fetal Liver (FL); Genital ridge (G); Mesonephros (M); primitive ectoderm (PE).

promoter in VSELs, similar to cells isolated from ESCs-derived EBs, is hypomethylated (28% and 13.2%, respectively) (Shin et al., 2009). Next, to provide additional direct evidence that the Oct4 promoter in VSELs is in an active/open state, we performed the chromatin-immunoprecipitation (ChIP) assay to evaluate its association with acetylated-histone3 (H3Ac) and dimethylated-lysine-9 of histone-3 (H3K9me2), the molecular features for open- and closed-type chromatin, respectively. By employing Carrier-ChIP assay using human hematopoietic cell-line THP-1 as carrier we found that Oct4 promoter chromatin is associated with H3Ac and its association with H3K9me2 is relatively very low (Shin et al., 2009). Since VSELs also express Nanog, we evaluated the epigenetic status of the Nanog promoter in these cells as well. We found that the Nanog promoter was methylated (~50%). However, quantitative ChIP data confirmed that the H3Ac/H3K9me2 ratio supports the active status of the Nanog promoter in these cells (Shin et al., 2009). Based on these results, VSELs truly express Oct4 and Nanog. Of note we also reported that VSELs express also several other markers of PSCs such as e.g., SSEA-1 antigen as well as Sox2 and Klf4 transcription factors. While the expression levels of transcripts of Oct4 and Nanog in VSELs was around 50% and 20%, respectively, compared to ESC-D3 cells, VSELs express a similar level of *Sox2* transcript and ~3.5 times more *Klf4* as compared to ESC-D3 cells (Shin et al., 2010).

Expression of epiblast/germ line markers

Since we hypothesized that VSELs could be epiblast-derived precursors of TCSCs, we focused on expression in adult BM-derived VSELs, of genes that are characteristic for EpiSCs (Gbx2, Fgf5, and Nodal) and ESCs from inner cell mass of blastocyst (Rex1/Zfp42), It is known that Gbx2, Fgf5, and Nodal are upregulated in EpiSCs, but expressed at lower levels in ESCs isolated from the inner cell mass of blastocysts (Hayashi et al., 2008). In contrast, the level of *Rex1/Zfp42* transcripts is highly expressed in inner cell mass cells. We found that VSELs highly express Gbx2, Fgf5, and Nodal, but express less Rex1/Zfp42 transcript as compared to ESC-D3s. It suggests that VSELs are more differentiated than ICM-derived ESCs and share several markers with more differentiated EpiSCs (Shin et al., 2010).

Next since we hypothesize that VSELs could be developmentally related to epiblast-derived PGCs, we evaluated expression of genes involved in the germ line specification of the epiblast (e.g., Stella, Prdm14, Fragilis, Blimp1, Nanos3, and Dnd1) (Hayashi et al., 2007). By employing RQ-PCR, we noticed that VSELs highly expressed all the genes involved in germ line specification from the epiblast. Subsequently, we confirmed the expression of Stella, Blimp1, and Mvh in purified VSELs at the protein level by immunostaining (Shin et al., 2010). Furthermore, our ChIP results show that the Stella promoter in VSELs displays transcriptionally active histone modifications [H3Ac and trimethylated-lysine-4 of histone3 (H3K4me3)] and was less enriched for transcriptionally repressive histone markers [H3K9me2 and trimethylated-lysine-27 of histone3 (H3K27me3)] (Shin et al., 2010). Thus, overall our results demonstrate that VSELs express specific genes and display a Stella promoter chromatin structure that are characteristics for germ line specification. VSELs also highly express Dppa2, Dppa4, and Mvh, which characterize late migratory PGCs, however

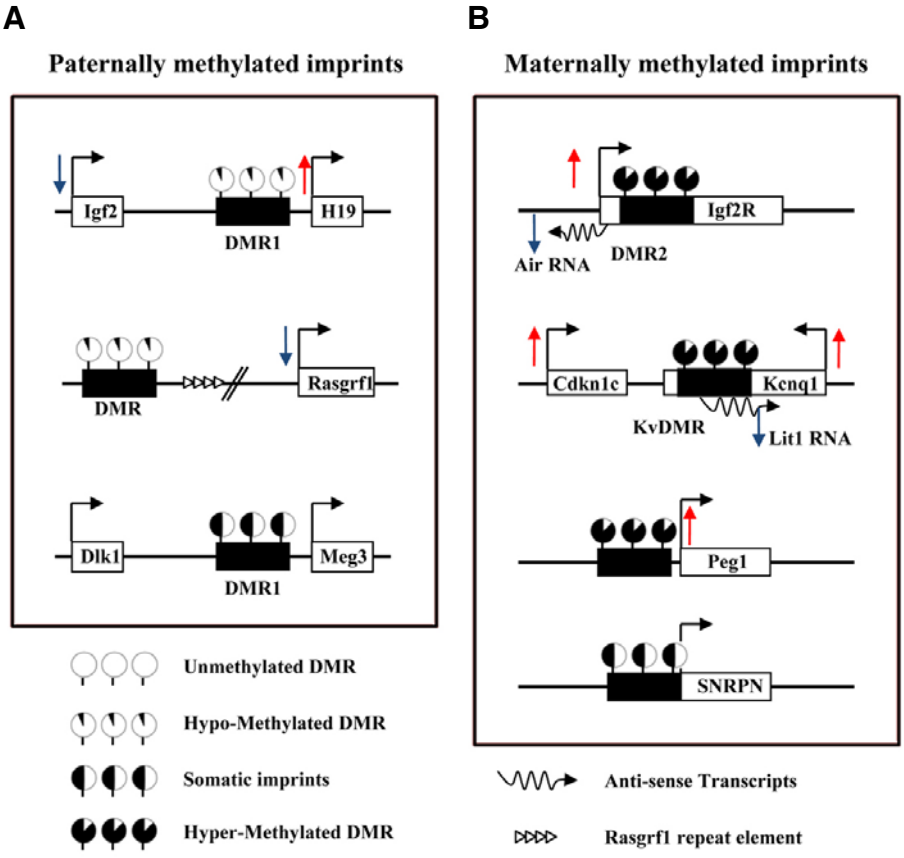

Fig. 2. Status of imprinted genes in VSELs. The unique DNA methylation pattern and expression of imprinted genes in VSELs. Panel A - Schematic diagram of paternally (Igf2-H19, Rasgrf1 and Dlk1/Meg3) methylated loci. Panel B - Schematic diagram of maternally (Igf2R, Kcnq1, Peg1 and SNRP) methylated loci. Arrow indicates the activity transcription from indicated genes (Red-up: upregulated; Blue-down: downregulated). Circles - level of methylation. Differential methylated regions (DMR); maternal chromosome (M); paternal chromosome (P); DMR for Kcnq1 locus (KvDMR).

they do not express Sycp3, Dazl, and LINE1 genes that are highly expressed in post-migratory PGCs (Shin et al., 2010). Thus, our results *in toto* support a concept that VSELs deposited into murine BM show some similarities in gene expression and epigenetic signatures to epiblast-derived migratory PGCs (~E10.5-E11.5).

Epigenetic changes of imprinted genes that regulate VSELs pluripotency

The rapidly developing field of regenerative medicine is searching for safe and therapeutically efficient sources of PSCs. By definition, PSCs should: i) give rise to cells from all three germ layers; ii) complete blastocyst development; and iii) form teratomas after inoculation into experimental animals (Ratajczak et al., 2007). Unfortunately, in contrast to immortalized embryonic ESC lines or inducible (i) PSCs, these last two criteria have not been obtained thus far with any potential PSC candidates isolated from adult tissues. There are two potential explanations for this discrepancy. The first is that PSCs isolated from adult tissues are not fully pluripotent; the second is that there are some physiological mechanisms involved in keeping these cells quiescent in adult tissues to preclude their unleashed proliferation and risk of teratoma formation.

We postulated that VSELs similarly as PGCs may modify methylation of imprinted genes that prevents them from unleashed proliferation and may explain their quiescent status in adult tissues. We noticed that Oct4$^+$ VSELs do not proliferate *in vitro* if cultured alone and that the quiescence of these cells is epigenetically regulated by DNA methylation of genomic imprinting, which is an epigenetic program that ensures the parent-of-specific monoallelic transcription of imprinted genes (Shin et al., 2009). It is well known that the imprinted genes play a crucial role in embryogenesis, fetal growth, totipotential status of the zygote, and pluripotency of developmentally early stem cells (Reik and Walter, 2001). The expression of imprinted genes is regulated by DNA methylation on differential methylated regions (DMRs), which are CpG-rich *cis*-elements in their loci. We noticed that VSELs freshly isolated from murine BM erase the paternally methylated imprints (e.g., Igf2-H19, Rasgrf1 loci); however at the same time they hypermethylate the maternally methylated ones (e.g., Igf2 receptor (Igf2R), Kcnq1-p57^{KIP2}, Peg1 loci) (Fig. 2).

Because paternally expressed imprinted-genes (Igf2, Rasgrf1) enhance the embryo growth and maternally expressed genes (H19, p57^{KIP2}, Igf2R) inhibit cell proliferation (Reik and Walter, 2001), the unique genomic imprinting pattern observed on VSELs demonstrates growth-repressive imprints in these cells. As supported, VSELs highly express growth-repressive genes (H19, p57^{KIP2}, Igf2R) and downregulate growth-promoting genes (Igf2, Rasgrf1), which explains the quiescent status of VSELs (Shin et al., 2009). Importantly, the quiescent pattern of genomic imprinting was progressively recovered during the formation of VSEL-DSs, in which stem cells proliferate and differentiate. These results suggest that epigenetic reprogramming of genomic imprinting should maintain the quiescence of the most primitive pluripotent adult stem cells (e.g., Oct4$^+$ VSELs) deposited in the adult body and protect them from premature aging and tumor formation. Therefore, it will be important to investigate whether this genomic imprinting pattern differs between VSELs isolated from young versus old mice and whether these potential epigenetic changes could contribute to the previously mentioned decrease in the pool and func-

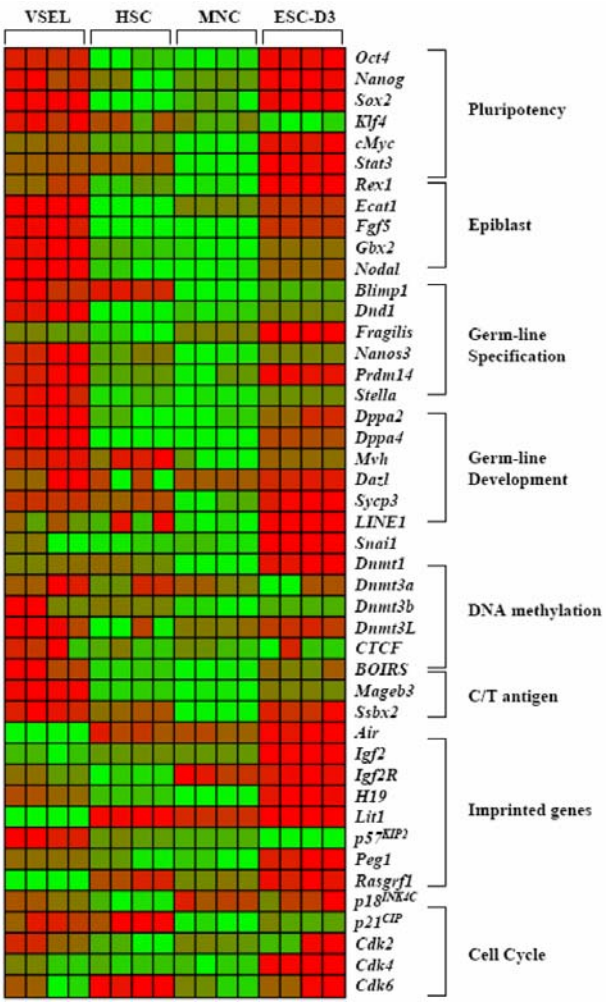

Fig. 3. Expression of pluripotent/epiblast/germ line genes. Gene expression profiles of BM-VSELs, BM-HSCs, BM-MNCs and ESC-D3. Heat-map analysis of *C*t values from RQ-PCR experiments for the indicated cells was prepared employing Heatmap Builder® software. The expression level is indicated by red (high expression) and green (low expression) colors.

tion of VSELs during aging.

CONCLUSIONS

Several attempts have been made in the past few years to purify a population of PSCs from adult tissues. VSELs isolated from adult tissues are an alternative and not ethically controversial source of SCs for regenerative medicine. However, there are several missing answers to this timely issue, especially in view of the current and widely performed clinical trials with BM-derived SCs in cardiology and neurology, before VSELs can find their potential application in regenerative medicine.

First, there is the obvious problem of isolating a sufficient number of VSELs from the BM, umbilical cord blood (UCB), or mobilized peripheral blood (mPB). The number of these cells among BM MNCs is very low. For example, VSELs represent ~1 cell in 10^5 of BM MNCs (Kucia et al., 2006). Furthermore, our data show that these cells are enriched in the BM of young mammals and their number decreases with age (Ratajczak et al., 2008). It is also likely that if VSELs that are released from the BM, even if they are able to home to the areas of tissue/organ injury, they may function only in the regeneration of minor tissue injuries. Heart infarct or stroke, on the other hand, may involve severe tissue damage beyond the effective repair capacity of these rare cells. We are also indentifying crucial factors involved in mobilization of VSELs into peripheral blood, and our data indicate a crucial role of stromal derived factor-1 (SDF-1), complement cascade cleavage fragments and sphingosine-1-phosphate (S1P) in this process (Ratajczak et al., 2010). Second, the allocation of these cells to the damaged areas depends on homing signals that may be inefficient in the presence of proteolytic enzymes released from leukocytes and macrophages associated with damaged tissue. Thus, VSEL-SCs may potentially circulate as a homeless population of SCs in PB and return to the BM or home to other organs. Third, we have to develop efficient *ex vivo* culture conditions that will allow for efficient expansion of VSEL-SCs without supportive feeder layer cells (e.g., C2C12, OP-9 cells).

Although purified adult BM-VSELs share several markers characteristic for EpiSCs as well as migratory PGCs (Fig. 3), there are some discrepancies between migratory PGCs and VSELs. First, migratory PGCs demethylate both paternally and maternally DMRs, although each imprinted locus shows a different sensitivity to demethylation (Hayashi et al., 2007). In contrast, VSELs deposited into adult BM show a different imprint pattern depending on the parental origin for DMRs (Shin et al., 2009). Furthermore, VSELs express the same genes (Klf4*, c-Myc, Stat3, Snai1, Ecat1*), which are highly expressed in ESCs but not in PGCs (Shin et al., 2010). Therefore, however VSELs are similar to migratory PGCs, they still show some differences in gene expression, which could be explained by different modulatory effects of the microenvironments in niches where they reside (genital ridge for PGCs and BM for VSELs).

Nevertheless, our data strongly indicate that VSEL-SCs could potentially 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 is ripe for exploration. The current work in our laboratory indicates that VSELs could be efficiently employed in the realm of regenerative medicine and that they are physiologically more important than merely being potential developmental remnants. Finally, we believe that the controlled modulation of somatic imprint status in VSELs such as we hypothesized, a proper *de novo* methylation of somatic imprinted genes on maternal and paternal chromosomes, could increase the regenerative power of these cells. The coming years will bring important answers to these questions.

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