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Review article

Very small embryonic-like (VSEL) stem cells: recent knowledge

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Abstract

Very small embryonic-like stem cells (VSELs) are a unique and rare stem cell population, which have the same structural, genetic, biochemical, and functional characteristics as embryonic stem cells and they are able to differentiate to all three germ layers. In several emergency situations related to organ damage, VSELs can be activated and mobilized into peripheral blood, and in appropriate animal models they contribute to tissue organ/regeneration. Optimized methods for isolation and expansion of VSELs have aroused the scientific community's interest in use of this kind of cells for regenerative purposes. The properties of such pluripotent cells make them a potential candidate in regenerative medicine.

Keywords: very small embryonic-like stem cells (VSELs), primordial germ cells PGC, regeneration potential, isolation methods

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Abbreviations:

BM	Bone Marrow
FACS	Fluorescence-activated cell sorting
FSH	Follicle-stimulating hormone
iPS	Induced pluripotent stem cells
ISS	ImageStream system
LT-HSCs	Long-term repopulating hematopoietic stem cells
MAPC	Multipotent adult progenitor cells
MASC	Multipotent adult progenitor cells
MIAMI	Marrow-isolated adult multilineage inducible (cells)
MSC	Mesenchymal stem cells
OSCs	Ovarian stem cells
PB	Peripheral blood
PGC	Primordial germ cells
SSCs	Spermatogonial stem cells
VSELs	Very small embryonic-like stem cells
UCB	Umbilical cord blood

Introduction

Stem cells have long been an area of scientific intrigue and have provided lot of hope and promise owing to their potential to regenerate diseased organs. Stem cells have the capacity to self-renew as well as give rise to differentiated progeny. They have generated a lot of interest amongst the general public as well as the scientific fraternity because of their potential for regenerative medicine. Stem cell based therapy, referring to both adult and embryonic stem cells, is widely used in medicine, mainly in regenerative medicine. Adult stem cells are multipotent, while embryonic stem cells are pluripotent, they possess the potential to differentiate into derivatives of all three embryonic germ layers: endoderm, mesoderm and ectoderm.

The promising application of embryonic stem cells (ES) in various therapeutic approaches of the regenerative medicine meets even nowadays practical and also ethical barriers, which render their clinical use nearly impossible (De Wert and Mummery, 2003)(de Miguel-Beriain, 2015). In recent years, a population of very small cord blood derived stem cells which bear embryonic like features (Very small embryonic-like stem cells-VSELs) as the most primitive cord blood derived stem cell population is under study.

Very small embryonic-like stem cells-VSELs

In 2006, Professor Ratajczak and his colleagues first isolated the Very Small Embryonic-like Stem Cells (VSELs) in the University of Louisville in the adult murine Bone Marrow (BM). Later VSELs were found in various organs in adult mice and also in human cord and peripheral blood (Kucia et al., 2007, 2008; Zuba-Surma et al., 2008a, b). These are very small cells in size, that located in small numbers in adult organs, remain quiescent and have a high nuclei/cytoplasmic ratio (Kucia et al., 2006). They have been demonstrated to be able in vitro cultures to differentiate into cells from all three germ layers and were described in the literature as i) mesenchymal stem cells (MSC) (Y. Jiang, B. N. Jahagirdar, R. L. Reinhardt et al., 2003), ii) multipotent adult progenitor cells (MAPC), iii) marrow-isolated adult multilineage inducible (MIAMI) cells (G. D'Ippolito et all., 2006), iv) multipotent adult stem cells (MASC) (Y. Jiang et all., 2002) and v) very small embryonic like stem cells (VSELs).

Since first reported in 2006, various independent groups have shown the presence of VSELs, their mobilization and elevated numbers during disease conditions. However, in 2012–2013, a few groups researching stem cells were unable to isolate VSELs (Danova-Alt et al., 2012; Miyanishi et al., 2013; Szade et al., 2013), which cast doubt on the very existence of these cells (Abbott, 2013). The reasons for the differences observed in detection of VSELs and why they remain poorly studied until now include: they exist in very low numbers in adult organs; remain quiescent under homeostatic conditions; are very small; have a high nucleo-cytoplasmic ratio with minimal cytoplasm; and they do not pellet down at 250–280g (speed used universally to pellet down cells during processing for various experiments) (Fig.1). However in 2014 the technical difficulties in the previously applied protocols were studied in detail (M. Z. Ratajczak et al., 2014) with several groups to finally confirm their existence (Tsagias et al., 2015) (Kassmer et al., 2013)(Sovalat et al., 2011)(Virant-Klun et al., 2008)(Bhartiya, Kasiviswananthan, and Shaikh, 2012).

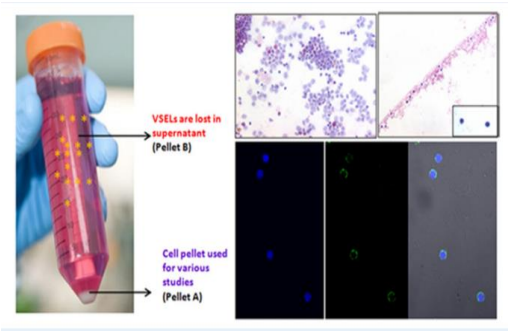


Figure 1. Cells obtained after enzymatic/mechanical digestion and processing of any adult tissue are generally spun at 250g to obtain a cell pellet for further studies (Pellet A), whereas the supernatant is invariably discarded. However, the VSELs remain suspended in the supernatant (yellow asterisk) and unknowingly get discarded. VSELs could be isolated by spinning the supernatant at 1000g to obtain Pellet B (Deepa Bhartiya et al., 2016).

Very small embryonic-like stem cells (VSELs) are present in the bone marrow, peripheral blood as well as in umbilical cord blood (UCB). They have also been identified in various adult organs in mice such as brain, kidneys, pancreas, testis, fetal liver and muscles (Zuba-Surma, Kucia, Ratajczak, et al., 2009)(Zuba-Surma, Kucia, Wu, et al., 2008)(Zuba-Surma, Kucia, Rui, et al., 2009) (Fig. 2). Compared with other source, UCB seems an attractive source of VSELs with a large accessibility and tolerance to allogenic graft, but still limited by a low number of stem cells countenance.

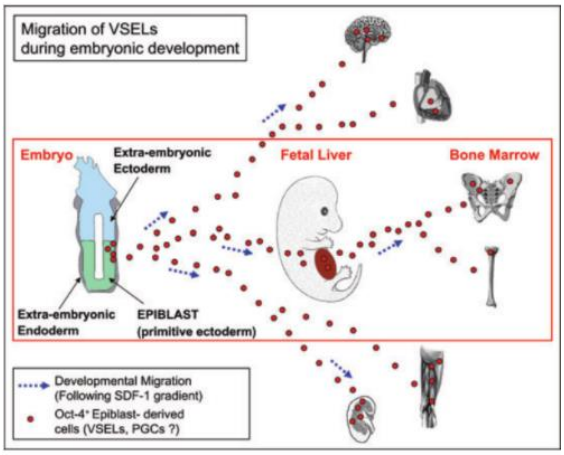
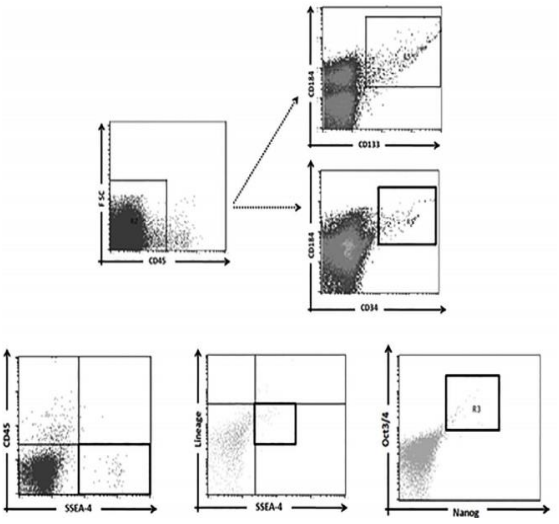


Figure 2. Hypothesis of developmental deposition of epiblast-derived embryonic stemcells in adult tissues. The presence of VSELs in the fetal liver, BM, and other tissues could be explained by the developmental deposition of CXCR4+epiblast-derived VSELs that follow an SDF-1 gradient. (Zuba-Surma, Kucia, Rui et al., 2009).

According to the origin of these cells, it is an overlapping population of primordial germ cells (PGCs), which are pluripotent stem cells present in the epiblast-stage embryo. Like PGCs, VSELs do not divide into cell cultures, nor form a teratoma, are able to differentiate into all three germ layers, express pluripotent (Oct4, Nanog, Sox2) and PGC (Stella, Fragilis) specific markers and display similarities in their epigenetic reprogramming profiles and pattern of genomic imprinting (including erasure of differently methylated regions at Igf2-H19 and Rasgrf1 loci and hypermethylation at KCNQ1 and Igf2R loci) with PGCs (Saitou and Yamaji, 2012)(D. M. Shin et al., 2009)(Shin DM et al., 2010) (Kim et al., 2014), thus fulfilling three of the six criteria that currently define

pluripotency (Pera et al., 2000). Being equivalent to PGCs, VSELs also migrate to all developing organs and survive by forming a "reservoir" of stem cells in quiescence (M. Z. Ratajczak, 2015)(M. Z. Ratajczak et al., 2014). Thus, VSELs are better endogenous, pluripotent stem cell candidates for making gametes as well as for regenerative medicine. They spontaneously differentiate into oocyte-like structures (Parte et al., 2011, 2014; Sriraman et al., 2015) and sperm (Anand et al., 2015) in vitro. They have a unique pattern of genomic imprinting and despite their pluripotent stem cell character, changes in the epigenetic signature of imprinted genes keep them quiescent in adult tissues and prevent teratoma formation (Shin et al., 2010). This is how it is ensured to avoid frequent eruption of tumors because of their inability to divide in culture or form a teratoma when injected in immuno-compromised mice.



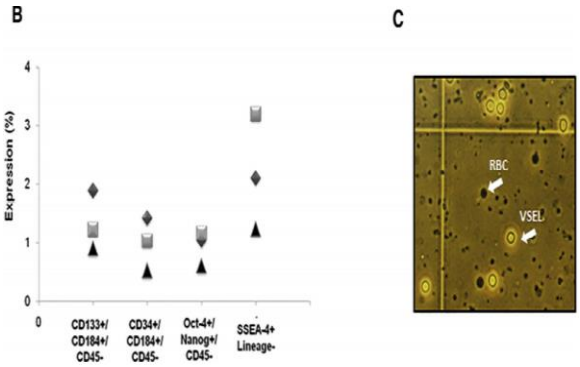


Figure 3. Immunophenotypic and morphological characterization of the VSELs via flow cytometry analysis and Turk's staining, respectively. (A) Small size CD45- cells were isolated after the final high-rotation centrifugation (upper and left). Gating this cell population, CD184+/CD133+, CD184+/CD34+ (upper, right), as well as SSEA-4+/Lineage-/Oct3/4+/Nanog+ cells (down) were detected. (B) Percentage of the characteristic for the VSEL marker expression in the isolated population following the final centrifugation. Every symbol corresponds to an individual cell population for analysis (n = 3). (C) Morphological characterization and counting of VSELs in a Neubauer chamber after RBC staining with Turk's solution (Gounari et al., 2018)

VSELs were first reported in mouse bone marrow (Kucia et al., 2006) and then in human cord blood (Kucia et al., 2007) due to their characterization as Lin-, CD34+, CD45-, CD133+ and/or CXCR4+ cells (Fig. 3). They were 3–5 μm in size (smaller than red blood cells (RBCs) and bigger than platelets), spherical in shape with high nucleo-cytoplasmic ratio and open type euchromatin (Fig.4). They are actively mobilized from the bone marrow into peripheral blood following stressful conditions such as stroke, myocardial infarction, critical leg ischemia, pulmonary diseases, cytotoxic treatments (Zuba-Surma, Kucia, Dawn, et al., 2008) (Kucia et al., 2008) (Guerin et al. 2015) (Marlicz et al., 2012) (M. Z. Ratajczak et al., 2011). Moreover, it was shown that the same cells were entrapped in the erythrocyte layer and discarded after centrifugation in density gradient solutions. Thus, these cells could constitute an additional cell population to be collected during cord blood banking (Bhartiya et al., 2012).

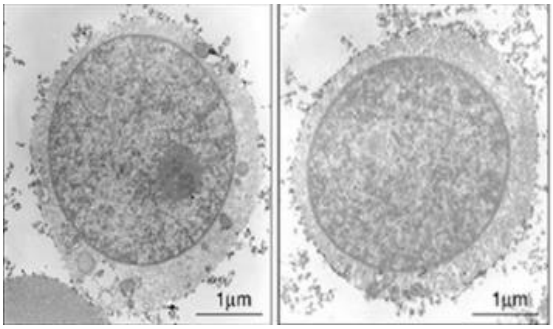


Figure 4. Transmission electron microscopy images of human cord blood VSELs. (Bhartiya at al 2016)

Thus, PGCs survive as pluripotent VSELs in various adult organs throughout the life and serve as a backup pool for tissue committed stem cells. Also VSELs circulate at a similar frequency into the SS-PB of young, middle-aged, and aged subjects. This may indicate that the pool of VSELs persists along the life as a reserve for tissue repair in case of minor injury, and that there is a continuous efflux of these cells from the BM into the PB (Hanna Sovalat et al., 2015). These primitive, endogenous, pluripotent, stem cells supposedly undergo asymmetric cell divisions to self-renew and give rise to the slightly bigger adult stem cell 'progenitors', which in turn divide rapidly, undergo clonal expansion and differentiate into tissue-specific cell types—thereby maintaining life-long homeostasis in various body organs.

Regeneration Potential of Very Small Embryonic-like Stem Cells

Kucia et al. (2006) reported that mouse bone marrow VSELs could differentiate into three EG layers in vitro. Also their regenerative potential is confirmed in vivo in preclinical models of hepatic cirrhosis, chemoablated testis, partial pancreatectomy, low density osseous tissues, myocardial infractus and others (Chen et al., 2015)(D. Bhartiya et al., 2014)(Taichman et al., 2010)(Zuba-Surma et al., 2011). When cultured on OP9 feeder layer (mouse bone marrow stromal cells), VSELs differentiated into hematopoietic colonies and cells expressing several hemato/lymphopoiesis-specific markers (Ratajczak et al., 2011). Thus VSELs, as analogs of PGCs, it is

speculated that they are precursors of Long-Term Repopulating Hematopoietic Stem Cells (LT-HSCs) as they present transcriptional similarities associated with gonadal hormone receptors and early-development markers (Sal4) (Zhang et al., 2006)(Ohtaka, Matsui, and Obinata, 1999)(Rich, 1995).

It is reported that VSELs exists in adults that undergoes hematopoiesis in bone marrow and gametogenesis in the gonads. Similar to the PGCs, VSELs are quiescent in nature, do not expand in culture like ES or iPS cells and throughout life serve as a backup pool and give rise to SSCs/OSCs (spermatogonial/ ovarian stem cells) which undergo clonal expansion, meiosis and further differentiation to produce haploid gametes (Fig.5). Ovarian VSELs respond to FSH (follicle-stimulating hormone) via FSHR3 and pontaneously differentiate into oocyte-like structures in vitro during OSE (ovarian surface epithelium) culture. Also, adult peri-menopausal ovarian VSELs express Stella and Fragilis (specific markers for PGCS) suggesting that the VSELs are indeed the PGCs that survive into adulthood (D. Bhartiya et all., 2014). Further, Sriraman et al 2015 has shown survival of VSELs in mouse ovary which otherwise undergoes premature failure due to chemotherapy. Kurkure et al. 2015 reported VSELs in otherwise azoospermic human testicular biopsies collected from adult survivors of childhood cancer. Virant-Klun et al. 2013 has also reported similar ES-like cells up to 4 mm in size expressing SSEA-4 in human testis and also in patients with Sertoli cell-only syndrome (Stimpfel M et al., 2012). Very small embryonic-like stem cells express pluripotent markers including nuclear octamer-binding transcription factor 4 (OCT-4A; POU5F1) (Pesce M et al., 2001), which is a transcription factor required for maintaining pluripotent state of cells, whereas the SSCs divide rapidly and express cytoplasmic OCT-4 in the human testis (Bhartiya D et al., 2013) (H Patel and D Bhartiya, 2016).

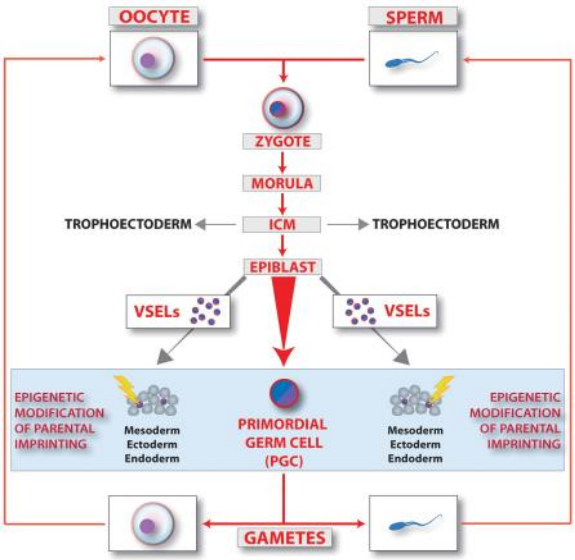


Figure 5. The cycle of life—from zygote to germ cells. From a developmental and evolutionary point of view, the germline (shown by red arrows) carries the genome (nuclear and mitochondrial DNA) from one generation to the next, and all somatic cell lines bud out during ontogenesis from the germline to help germline cells accomplish this mission effectively. The germline potential is established in the fertilized oocyte (zygote) and subsequently retained in

the morula, inner cell mass of the blastocyst (ICM), EPSC, PGCs and mature germline cells (oocytes and sperm). The first cells that bud out from the germ lineage are trophoectodermal cells, which give rise to the placenta. Subsequently, during gastrulation, EPSCs are a source of PSCs for all three germ layers (meso-, ecto- and endo-derm) and PGCs. We hypothesize that at this stage some EPSCs and PGCs are deposited as Oct-4 þ VSELs in tissues developing from meso-, ecto- and endo-derm (blue circles) (MZ Ratajczak et al., 2014).

Isolation methods

The difficulties in isolating the VSELs remain due to its special characteristics, such as their small size. Therefore, modifications of gating strategies employed in FACS and a particular focus on the fraction of small objects allowed to purify these primitive, very small cells from animal and human specimens (Zuba-Surma et al., 2010). M. Halasa et al. (2008) describes a two-step method to purify very small embryonic-like stem cells from umbilical cord blood (UCB). UCB samples were first lysed with the lysing buffer, then stained with the appropriate antibodies, and finally 07-3×108 cells were run on the FACS Aria sorter (Fig.6). Gounari et al. (2018) presented a VSELs' isolation protocol from UCB based on the chemotaxis of this population to SDF-1a factor. Moreover, very useful technology seems ISS. The ISS-based analysis involves features of classical flow cytometry combined with fluorescent microscopy and allows for statistical analysis of various cellular parameters, as well as visualization of cells in suspension during flow analysis via high resolution brightfield, darkfield, and fluorescence images. The high resolution of the instrument enables the identification of objects as small as 1-µm in diameter (Basiji et al., 2007). Thus, this novel imaging technology seems to be ideal for analyzing small cellular events such as VSELs (Zuba-Surma et al., 2010).

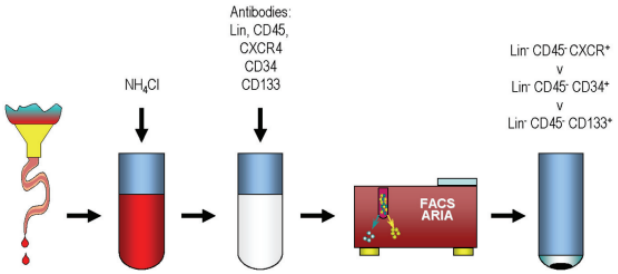


Figure 6. VSELs' isolation protocol M. Halasa et al. 2008

Conclusion

Our data indicate that VSELs could potentially provide a real therapeutic alternative to the controversial use of human ES cells obtained by therapeutic cloning, isolation of single blastomers or from parthenogenetic embryos. Hence, while the ethical debate on the application of ES cells in therapy continues, the potential of VSELs is ripe for exploration. From hematological point of view the fact that CD45− VSELs may differentiate into CD45+ HSC makes from these cells a candidate for long term repopulating HSC. Furthermore, since VSELs may

differentiate in vitro into cells from all three germ layers make these cells potential candidates in regenerative medicine. Finally, the mechanism by which VSELs could contribute to development of some malignancies could shed more light on origin of tumors. In conclusion it is of vital importance to evaluate if VSELs could be efficiently employed in the clinic or whether they are merely developmental remnants found in the BM that cannot be harnessed effectively for regeneration. The coming years will bring important answers to these questions.

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