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# Potentiality of Very Small Embryonic-Like Stem Cells to Repair Myocardial Infarction

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<http://dx.doi.org/10.5772/54942>

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## 1. Introduction

Heart failure (HF) after myocardial infarction (MI) or the ischemic cardiomyopathy (ICM) continues to be the most prevalent cause of morbidity and mortality worldwide. MI results in myocardial necrosis, scar formation, ventricular remodeling and eventually HF or death. Faced with the end stage of ICM, the most of present therapy protocols could only slow worsening of HF. Cardiac resynchronization therapy may be definite therapeutic effect to those cases with HF and complete left bundle-branch block. Heart transplantation can more efficiently improve the cardiac status, but, limited donor supply and organ rejection confine its widespread use. As a result, a significant proportion of survivors with ICM will still develop HF and have briefer life-span. Pathologically, HF and myocardial remodeling aggravate each other and a core pathogenic factor of ICM is loss of massive cardiomyocytes [1]. In fact, the myocardium itself possesses little capacity for self-regeneration. Although there are still considerable dispute in the clinical therapeutic effect based on stem cells (SCs), the positive results obtained in the repair of damaged myocardium indicated it has become a promising strategy [2-4]. In this regard, an array of SCs types has been identified and applied, including bone marrow-derived mononuclear cell (BM-MNCs) [5] and umbilical cord blood-derived stem cells (UCB-SCs). At the current state of SCs clinical application there is no convincing data showing the superiority of any tissue committed multipotent stem cells (TCSCs), so heterogeneous population of BM-MNCs is most often used [6]. Some recent studies have showed pluripotent stem cells (PSCs) are precursors of TCSCs during organ/tissue rejuvenation and a source of these cells in emergency situations when organs are damaged (e.g., MI or stroke). The application of PSCs has showed very encouraging results. PSCs includes induced pluripotent stem cells (iPSCs)

using gene transfer [7-8] and very small embryonic-like embryonic/epiblast-like stem cells (VSELs) isolated from the adult tissues or UCB [9]. A rare Sca1+Lin-CD45- SCs population were initially identified, isolated and named as VSELs in adult mice using fluorescence activated cell sorting (FACS) [10]. Although VSELs are currently studied in a lot of laboratories worldwide, the research series of VSELs was mainly contributed by Kucia & Ratajczak and their colleagues. VSELs possess very primitive morphology and express PSCs markers (e.g., Oct4, Nanog, and SSEA-4) as well as the surface phenotype Sca1+/ CD133+Lin-CD45- in mice / humans. As VSELs can be mobilized into PB following acute MI [11], improve heart function and alleviate cardiac remodeling [12,13], these cells seem to possibly become an optimal seed cells for cardiovascular repair. Recently, employing anti-CD133-conjugated paramagnetic beads followed by staining with Aldefluor has also been proposed for a faster large-scale VSELs isolation [14]. More recent evidences demonstrate that VSELs deposited in adults tissue share several markers with epiblast/germ line cells and play a role in rejuvenation of the TCSCs responsible for tissue regeneration/repair after organ injuries. Even, VSELs with maximum regenerative potential are recommended as the true PSCs in adult tissues, whereas the hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs) are actually progenitor SCs arised from asymmetric cell division of VSELs [15]. As a promising candidate, their unique characteristics and potentiality may have very important pathophysiological and therapeutic implications for regenerative medicine including myocardial and endothelial repair.

## 2. Discovered history of VSELs

Small cells able to differentiate into cells from all three germ layers and called “spore-like stem cells” were isolated from adult mammalian tissues, however, it was not provided for these small SCs how to be purified and for their surface markers how to be expressed in the original paper [16]. Afterwords, small SCs expressing CXC chemokine receptor 4 (CXCR4+) and markers characteristic for embryonic stem cells (ESCs), epiblast stem cells (EPSCs), and primordial stem cells (PGCs) were purified from the murine BM and several adult organs. Based on their small size, presence of PSCs markers, distinct morphology (open-type chromatin, large nucleus, narrow rim of cytoplasm with multiple mitochondria) and ability to differentiate into all three germ layers, including mesoderm-derived cardiomyocytes, these cells were named as VSELs [9].

## 3. Found source of VSELs

The rare Sca-1+Lin-CD45-SCs population was initially discovered in mice BM [9]. Phenotypically similar cells were subsequently identified and purified in murine peripheral blood (PB), fetal liver, brain, retina, kidneys, pancreas, skeletal muscles spleen, and thymus [17]. In humans, VSELs were identified in UCB, PB, BM, and cardiac tissue [18]. VSELs deposited in adult tissues seem to be a reserve pool for TCSCs [19].

## 4. Morphology of VSELs

The most common shape feature of VSELs is that they possess very primitive morphology and relatively small size. The distinctive morphology of VSELs was confirmed using confocal and transmission electron microscopy [9,10]. Comparison with other populations of cells, murine VSELs (4-6  $\mu\text{m}$ ) are smaller than HSCs, MNCs and granulocytes and erythrocytes, but, larger than platelets. Human VSELs (6-8  $\mu\text{m}$ ) are larger than murine. At the ultramicrostructural level, they show a very immature morphology, for example, possess a relatively large nucleus surrounded by a narrow rim of cytoplasm, a few mitochondria, scattered ribosomes, small profiles of endoplasmatic reticulum and a few vesicles [20]. Recently, the high resolution of ImageStream system (ISS) analysis enables the identification of objects as small as 1  $\mu\text{m}$  in diameter. Employing ISS analysis, murine VSELs are more precisely confirmed as ~3.6  $\mu\text{m}$  in diameter [21].

## 5. Molecular biology and functional features of VSELs

VSELs not only possess the primitive morphology of early developmental cells but also express typical markers for PSCs

Characteristic markers of VSELs were confirmed using several complementary research tools including flow cytometry (FCM), ISS, direct immunofluorescence staining, confocal microscopy, reverse-transcription polymerase chain reaction (RT-PCR) and etc. Early embryonic markers (Oct-4, Nanog, SSEA-1, Rex1, Dppa3, Rif-1) were demonstrated at the protein /mRNA levels using immunofluorescent staining, ISS and FACS [21,22]. VSELs express SSEA-1 antigen on their surface and Oct-4 in their nuclei. Recent study indicates that the promoters of Oct4 and Nanog contain transcriptionally active chromatin in VSELs excluding the possibility of amplification of pseudogenes [23]. CD133+Lin-CD45- VSELs identified in Human UCB like their murine counterparts, i) highly express telomerase, ii) are diploid, and iii) are viable, as shown by their ability to exclude dye (7-aminoactinomycin D). Moreover, some of the CD133+Lin-CD45- VSELs, which represent only a very small subfraction among UCB Lin-CD45-non-hematopoietic cells, may co-express other stem cell markers, including CD34, CXCR4, and SSEA-4, may contain other stem cell types, including endothelial progenitor cells (EPCs) and mesenchymal stem cells (MSCs), and may be identified based on very small size (FSClow/SSClow) and co-expression of CD133, CD34, and, CXCR4 [23].

Also, there are some differences of the VSELs phenotype between mice and humans. Human VSELs surface markers consist of lin-CD45-CXCR4+, CD133+ and CD34- as confirmed on the mRNA level by RQ-PCR and protein level by IF and ISS, whereas Murine VSELs express Sca-1 antigen [24].

VSELs express chemokine receptor CXCR4 and are absent of pan-hematopoietic marker (CD45-) and hematopoietic lineage markers (Lin-) [9,10,25]. Further study demonstrated

that VSELs can not primarily reveal hematopoietic ability immediately from isolation and expansion, but may eventually acquire hematopoietic potential following co-culture in hematopoiesis permissive environment over OP9 stroma feeder layer and reconstitute hematopoiesis in lethally irradiated mice 4–6 weeks after transplantation [26].

Freshly sorted VSELs can be expanded in coculture with C2C12 murine myoblast feeder layer. After 7 days of co-culture, approximately 5–10% of all VSELs form sphere-like clusters consisting of a few hundred cells resembling embryoid bodies (VSEL-derived spheres, VSEL-DSs). VSEL-DSs express placenta-like alkaline phosphatase. Expanded population of VSELs isolated from VSEL-DS retain the pluripotent capacity and have ability to differentiate into all three germ layers, including mesodermal cardiomyocytes, ectodermal neural cells and endodermal pancreatic cells [27].

The differentiation potency was also documented in circulating murine VSELs after injection of G-CSF. Rapidly mobilized VSELs showed up-regulation of PSC markers. These findings support not only the pluripotency of VSELs, but also their tissue repair function[10]. Study also showed that VSELs possess diploid DNA. They do not express MHC-1 and human leukocyte antigen-D related (HLA-DR) antigens and are CD90–CD105–CD29–. Moreover, if plated over a C2C12 murine sarcoma cell feeder layer, ~5–10% of purified VSELs are able to form spheres that resemble embryoid bodies [9]. Similar spheres were also formed by VSELs isolated from murine fetal liver, spleen, and thymus [17]. Interestingly, VSELs are somewhat heterogenous developmentally. Although all of VSELs express the Oct-4, some of them express genes that are more closely related to genes expressed by EP-SCs and others to genes expressed by migrating PGCs [28].

In parallel, VSELs exhibits their potential biologic function. Oct-4+ SSEA-4+ SCs harvested from BM via elutriation, has been recently shown to give rise into functional insulin-producing cells in vivo in induced diabetic mice [29]. In another report, VSELs purified from rat BM successfully repaired damaged myocardium in a model of MI [30].

Similarly, in vivo exposure to hypoxia in mice elicits chemoattractant a gradients that promote the mobilization of pluripotent very small embryonic-like stem cells from the bone marrow to peripheral blood. VSELs in the BM are the primary source of lung epithelial cells [31–33]. VSELs were also identified in neonatal retina and involved in optic nerve retinal regeneration in a rodent model [34]. VSELs may also play a major role as populations of cells that preferentially give rise to induced pluripotent stem cells (iPSCs) when BM-derived stromal cells are induced to pluripotency by genetic manipulation [35]. In particular, VSELs derived by parthenogenesis have also been identified successfully [36].

## 6. Isolation strategies of VSELs

Isolation of VSELs using FACS is dependent on gating strategy based on their small size, expression of PSC (Oct4, Nanog, and SSEA-4), surface markers (CXCR4, CD133 /Sca-1,



CD34) and absence of hematopoietic lineage markers (lin, CD45). Briefly, the first step is the lysis of red blood cells to obtain the fraction of nucleated cells. Erythrocyte lysis buffer is used instead of Ficoll centrifugation because the latter might deplete the population of very small cells [14]. Subsequently, cells are stained and sorted with antibodies against Sca-1 (murine VSELs) or CD133 (human VSELs), pan-hematopoietic antigen (CD45), hematopoietic lineages markers (lin), and CXCR4 [9]. Extended lymphocyte gate was used to include events with diameter 2–10  $\mu\text{m}$ , approximately consisting of VSELs. The width of the gate was validated by using synthetic beads of predefined size (1–15  $\mu\text{m}$ ) [14]. Several other approaches to define the population of small cells were used, including ISS. Above standard procedure employing FACS, however, is time consuming, which usually requires up to 4 days to process and isolate VSELs from UCB MNCs in one entire cord blood unit (~50–100 ml). It is not very difficult for the future clinical application to take into consideration cell viability, the time of sorter usage, and the time commitment of a sorter operator.

In order to speed up isolation of VSELs, a faster large-scale isolation protocol based on anti-CD133-conjugated paramagnetic beads followed by staining with Aldefluor were recently employed. In this novel approach (i) A UCB research unit is lysed in a hypotonic ammonium chloride solution for 15 min at room temperature to deplete erythrocytes and washed twice in phosphate-buffered saline (1st step); (ii) A single-cell suspension of total nucleated cells was treated with antibodies against CD133 antigen-coated immunomagnetic beads and separated by a MACS Separator to obtain CD133+including VSELs (2nd step); and subsequently (iii) The CD133(+)cell fraction was reacted with the Aldefluor™ Kit reagent for detecting aldehyde dehydrogenase (ALDH). Cells were incubated with phycoerythrin (PE)-conjugated murine anti-human CD235a, PE-CY7-CD45, and allophycocyanin (APC)-conjugated CD133/2. Cells were washed and resuspended in cold Aldefluor buffer and sorted by FACS to obtain populations enriched in CD45–GlyA–CD133+ALDH<sup>low</sup> VSELs. The whole isolation process takes approximately 2–3 h per UCB unit and these small Lin-CD45-CD133+cells isolated from human UCB highly express Oct-4, Nanog, and SSEA-4 at both the mRNA and protein levels [13,14].

This new isolation protocol was based on the following rationale. (i) Using erythrocytes lysis buffer was higher yield of VSELs than a Ficoll-Paque gradient centrifugation to remove erythrocytes [14]. (ii) On the other hand, CD133+ VSELs are highly enriched for PSC transcription factor expression (e.g., Oct-4 and SSEA-4) [14]. (iii) Small erythroblast GlyA+ that are present in UCB do not express CD45 antigen. Thus, selection for CD45–cells was used to enrich for these cells.

The isolation from one entire UCB unit can obtain  $\sim 10^3$ /100 ml of UCB for CD45–GlyA–CD133+ALDH<sup>low</sup> cells and  $\sim 4 \times 10^3$ /100 ml of UCB for CD45–GlyA–CD133+ALDH<sup>high</sup> cells. Freshly isolated CD45-GlyA-CD133+ALDH<sup>high</sup> VSELs express more hematopoietic transcripts (e.g., c-myb), CD45-GlyA-CD133+ALDH<sup>low</sup> VSELs exhibit higher levels of PSCs markers (e.g., Oct-4) [34,35].

## 7. Special potency and hypothesized role of VSELs

PSCs must correspond to certain *in vitro* and *in vivo* conditions. According to these criteria, PSCs should be provided with (i) giving rise to cells from all three germ layers, (ii) completing blastocyst development, and (iii) forming teratomas after inoculation into experimental animals. ESCs are generally known as PSCs. However, both VSELs and iPSCs are not different from ESCs in next two conditions. In special, There are some own unique superiority that VSELs deposited in various adult organs as a backup for primitive stem cells share several markers with epiblast/germ line cells, plays a role in rejuvenation of the pool of TCSCs involved in tissue regeneration, but, not complete blastocyst development and not form teratomas. During steady-state conditions, VSELs may be responsible for tissue rejuvenation and for processes of regeneration/repair after organ injuries. VSELs similarly as epiblast-derived PGCs change the epigenetic signature of some of the imprinted genes and therefore remain quiescent in adult tissues. This quiescence of VSELs is epigenetically regulated by DNA methylation of genomic imprinting [36]. VSELs highly express growth-repressive genes (H19, p57KIP2, Igf2R) and downregulate growth-promoting ones (Igf2, Rasgrf1). The unique genomic imprinting pattern may explain the quiescent status of VSELs. Thus, VSELs may be progeny of epiblast cells to develop tissues and a reserve pool of PSCs to repair tissue. Furthermore, the quiescent state of VSELs may also be a physiological protective mechanism of preventing uncontrolled proliferation, tumor formation [28]. Furthermore, The bone-forming activity of VSELs, exceeded the activity of other populations of BM-purified cells tested in the same assay if embedded in gelatin sponges and implanted into living mice. Even, as few as 500 UCB VSELs was capable of forming bone-like structures *in vivo* [37]. Based on these finding, VSELs have been described as at the top of the hierarchy for the mesenchymal and endothelial lineages in BM [38,39]. Interestingly, the content of VSELs from mice BM at different ages (2 months-3 years) was evaluated employing FCM. the number of these cells gradually decreases over time from  $0.052 \pm 0.018\%$  to  $0.003 \pm 0.002\%$  between age of 2-months and 3-years, respectively. In another report, the concentration of VSELs is much higher in BM of long-lived (e.g., C57Bl6) as compared to short-lived (DBA/2J) mice. Especially, not only a number of these cells in adult organs decreases with the age but also their ability to form spheres containing VSEL-DS declines with time. Whereas, a number of monopotent hemato/lymphopoiesis committed HSC increase in older animals [40,41]. This age-dependent content and ability of VSELs in adult organs may explain that these cells could play a pivotal role in the normal cell turn over and the life span control of mammals. Moreover, a significantly higher number of VSELs in long-living murine strains (e.g., Laron dwarfs and Ames dwarfs), whose longevity is explained by low levels of circulating IGF1 and a decrease in IIS. By contrast, the number of VSELs is reduced in mice with high levels of circulating IGF1 and enhanced IIS (e.g., growth hormone-overexpressing transgenic mice) compared to normally aging littermates [42,43]. There was a envision that in future, VSELs could be isolated from the patient at young age and than inject back into same recipients several years latter to regenerate damaged organs and to expand life-span, in case of major health complications (e.g., heart infarct, stroke) due to aging.

## 8. Mobilization and cardiovascular repair of VSELs

VSELs express early cardiac and endothelial lineages markers (GATA-4, Nkx2.5/Csx, VE-cadherin, and von Willebrand factor), SDF-1 chemokine receptor CXCR4. Under steady-state conditions, VSELs circulate in PB is very rare, however, undergo rapid mobilization during acute MI [44]. The processes are regulated by SDF-1, and its receptor CXCR4 as well as other important cytokine-receptor systems of regulating the stem cell mobilization and homing include leukemia inhibitory factor (LIF) – LIF receptor, hepatocyte growth factor (HGF) – c-met axis, stem cell factor-CD117 axes [45-48]. Interestingly, the number of these cells in PB is significantly higher in younger acute MI patients than in older ones. Number of VSELs was also correlated with left ventricular ejection fraction, troponin I and creatinine kinase-MB levels [44]. Further studies provided evidence that VSELs can be mobilized into PB in adult patients injected with granulocyte-colony stimulating factor (G-CSF) and their number could be of prognostic value [49]. Consistently, a protocol which VSELs differentiate into cardiomyocytes in vitro has been developed. In the first step, VSELs are co-cultured with myoblast line (C2C12) where the cells expand and form VSEL-DS. Subsequently, VSELs isolated from VSEL-DS by FACS sorting are plated on cardiac media to differentiate them into cardiomyocytes. The period over 21 days in expression of early cardiac markers and cardiac structural proteins also resembles the maturation of cardiomyocytes from ESCs [50].

Murin experiments in vivo also showed that expanded and subsequent cardiopoiesis-guided VSELs were markedly more effective than expanded and non-pre-differentiated cells. Interestingly, beneficial effects were observed despite use of only small number ( $10^4$  cells) of VSELs. At the same time a much higher number of hematopoietic cells ( $10^5$  cells) was not effective [37].

so far, a cochrane controlled trial called Myocardial Regeneration by Intracoronary Infusion of Selected Population of Stem Cells in Acute Myocardial Infarction (REGENT) study are available. The objective study was to assess the efficacy of intracoronary infusion of autologous BM-derived CD34+CXCR4+ progenitor cells in comparison to non-selected BMMNC on LVEF in patients with acute ST-segment elevation MI and reduced below 40% LVEF. CD34+CXCR4+ cells which is enriched for VSELs were isolated by two step immunomagnetic selection using the magnetic beads. The result demonstrated that the use of selected CD34+CXCR4+ cells or non-selected BMMNCs in patients with significantly reduced LV function is safe, feasible and not leads to a significant improvement of LVEF, there was however a trend in favour of cell therapy. In another, although CD34+CXCR4+ cells were not pure population of VSELs, The use of a relatively small number of selected CD34+CXCR4+ cells is associated with similar trend as the use of 100 times higher number of non-selected BMMNCs ( $1.90 \times 10^6$  vs.  $1.78 \times 10^8$ ) for improvement of LVEF. It also further showed which the activity of VSELs is more superior than that of BMMNC [52]. Based on above finding, administration of VSELs after an acute MI increases LVEF and improves left ventricular structure, and these benefits remain stable during long-term follow-up. Although the mechanisms remain under investigation, paracrine effects, regeneration of cellular constituents, and stimulation of endogenous stem/progenitors may play combinatorial roles [49], because the rare VSEL-derived cardiac myocytes expressing cardiac markers were present in the recipients myocardium [50,52].



Thus, VSELs may serve as an ideal SCs source for cardiac repair by their ability to secrete various cardioprotective growth factors/cytokines, as well as their ability to differentiate into cardiomyocytes and endothelial cells

## 9. Remaining challenges of VSELs application

However, there are also some challenges and conflict coming out from recent VSELs' studies. At first, a recent study showed that VSELs from human UCB lack SCs characteristics and fail to expand in vitro under a wide range of culture conditions [53]. We also found that it is very difficult for human VSELs to be cultured or expanded in vitro using general culture conditions. Thus, it has to be further determined whether these cells are merely developmental remnants found in the adult tissue that cannot be harnessed effectively for regeneration or whether they are real SCs population for regeneration medicine.

Subsequently, the biological characteristics and role of VSELs were studied mostly in mice and human. No information of VSELs from large animal close to human has been reported. Future clinical studies using autologous VSELs are needed to validate those promising large animal experimental data.

Furthermore, it has seldomly obtained for parallel experiments to compare several populations of putative SCs to determine the similarities and differences between these cell populations.

## 10. Summary

Overall, as mentioned above, the importance of SSEA-1+Oct-4+Sca-1+/ CD133+CXCR4+Lin<sup>-</sup>CD45<sup>-</sup> pluripotent VSELs in adult tissue or UCB is now being stressed. New data from Kucia & Ratajczak group and other groups has provided mounting evidence on the existence and potential biological role of VSELs mostly in mice. VSELs would very possibly be a promising PSCs population for cardiac repair in future clinical application of patients with ICM. Their cardiogenic potential should be confirmed in large animal similar to humans and technical issues regarding their isolation, expansion and differentiation need to further be addressed. We also look forward to share how to higher efficiently isolate and expand these rare cells as well as to know about further information on their biology and in vitro and in vivo differentiation potential.

## Acknowledgements

This study was supported by the National Natural Science Fund Committee (NSFC 81170104), Natural Science Foundation of Jiangsu Province (BK2012688) and Program for Jiangsu Province Outstanding Medical Talented Leader (JS2006038).

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