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Review

Identification, characterization and biological significance of very small embryonic-like stem cells (VSELs) in regenerative medicine

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Summary. The progress of stem cell research, along with technological innovation, has brought researchers to focus on the potential role of stem cells in regenerative medicine. Ethical and technological issues have limited the applications of human embryonic stem cells (hESCs) in this field. As a promising candidate, very small embryonic-like stem cells (VSELs) express a multitude of pluripotent stem cell markers and demonstrate the ability to differentiate into three germ-layer lineages *in vitro*. 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. In this review, we will focus on the biological characteristics, as well as the potentiality and remaining challenges in clinical application of VSELs. Moreover, a comparison among VSELs and the other pluripotent stem cells will be illustrated to highlight the unique advantages of VSELs.

Key words: Very small embryonic like stem cells (VSELs), Bone marrow, Hematopoietic stem cells (HSCs), Regenerative medicine, Transdifferentiation

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Introduction

Regenerative medicine is the process of creating living, functional tissues to replace or regenerate human cells, tissues or organs (Mason and Dunnill, 2008). This field offers unique opportunities for developing new therapeutic approaches to prevent and treat a wide variety of debilitating and life-threatening diseases, as well as for expanding new ways to explore fundamental questions of biology. However, the shortage of donor organs or tissues for regenerative therapy has stimulated research into the differentiation capacity of stem cells into various cell types and the potential application of stem cells in patients.

Although considerable progress has been made with regard to differentiation, culturing, maintenance and gene manipulation of human embryonic stem cells (hESCs) and their derivatives (Passier, 2003; Li et al., 2009), there are still numerous hurdles to overcome. The most pressing difficulties are ethical issues and teratoma formation (Li et al., 2008). In this regard, adult stem cells could potentially provide a real therapeutic alternative to cells from human embryos and therapeutic cloning (Ratajczak et al., 2008a,b). Besides, stem cells isolated from adult tissues have a technical advantage, since autologous cells could be used for transplantation without causing an immune response.

Recently, a population of Sca-1$^+$lin-CD45$^-$ cells named very small embryonic-like stem cells (VSELs) was isolated from adult murine bone marrow (Kucia et al., 2006b). These small cells were demonstrated to

exhibit pluripotency by *in vitro* and *in vivo* experiments. Subsequently, VSELs were detected in several organs other than bone marrow in adult mice (Zuba-Surma et al., 2008a-d). Inspiringly, a similar population of very small cells expressing markers of pluripotent stem cells at both mRNA and protein levels has been identified in human cord blood, as well as bone marrow (Kucia et al., 2006a). These findings stirred up great interest in the scientific community and numerous investigations are currently in process to study the potential utilization of VSELs in regenerative medicine.

Bone marrow derived stem cells and VSELs

Bone marrow-derived stem cells, including hematopoietic stem cells (HSCs) and stromal cells, can potentially restore the function of diseased or damaged tissues/organs, offering significant potential for regenerative medicine (Voltarelli et al., 2007). However, the controversy on transdifferentiation or plasticity of HSCs and the role of non-hematopoietic stem cells (non-HSCs) in repair or rejuvenation of tissues and organs have drawn increasing attention (Mezey et al., 2000; Orkin and Zon, 2002; Orlic et al., 2003; Corti et al., 2004; Wagers and Weissman, 2004). These bone marrow derived non-HSCs are described as endothelial progenitor cells (EPCs) (Asahara et al., 1997; Shi et al., 1998), mesenchymal stem cells (MSCs) (Peister et al., 2004; Dominici et al., 2006; Kolf et al., 2007), multipotent adult progenitor cells (MAPCs) (Jiang et al., 2002; Schwartz et al., 2002), marrow-isolated adult multilineage inducible (MIAMI) cells (D'Ippolito et al., 2004, 2006), and multipotent adult stem cells (MASCs) (Beltrami et al., 2007). It is likely that these cells are overlapping populations of stem cells detected in bone marrow by different investigators with various experimental strategies. Continuous investigations not only bring newly defined populations of stem cells, but also progressively push the community to put a premium on the existence of these rare multipotent/pluripotent stem cells, which could alternatively interpret the phenomenon of transdifferentiation or plasticity.

Owing to advances in methods, the identification of VSELs is earning increasing attention and wider investigation. Recent research has demonstrated that HSCs and non-HSCs, including VSELs in bone marrow, are capable of maintaining homeostasis and can be mobilized from the bone marrow into peripheral blood during tissue injury and stress to contribute to the regeneration of damaged organs (Kucia, 2004; Kucia et al., 2005b, 2008; Dawn et al., 2008; Wojakowski et al., 2010). Therefore, the heterogeneity of bone marrow derived non-HSCs may be the best interpretation for plasticity or transdifferentiation. Accordingly, we propose that the presence of VSELs in mice and humans could explain some positive results in tissue/organ repair/regeneration. In this regard, the versatile non-HSCs, including VSELs, deserve further investigation to elucidate their relationship to HSCs, as well as their potential role in tissue/organ repair and rejuvenation.

Identification and purification of VSELs

Recent study has revealed that murine bone marrow contains a population of highly enriched GATA-4 and Nkx2.5/Csx nonhematopoietic Sca-1$^+$lin-CD45$^-$CXCR4$^+$ cells, which can migrate to stromal-derived factor (SDF)-1 gradient and undergo rapid mobilization into peripheral blood in experimental myocardial infarction (Kucia, 2004). Subsequently, a developmentally primitive stem cell population from murine bone marrow, named very small embryonic-like stem cells (VSELs) (Kucia et al., 2006) was purified. These rare Sca-1$^+$lin-CD45$^-$CXCR4$^+$ cells, accounting for ~0.01% of bone marrow mononuclear cells (MNCs), are morphologically small in size (<5 μm) and contain relatively large nuclei filled with a primitive type of unorganized euchromatin. Moreover, they possess a high nuclear-to-cytoplasmic (N/C) ratio, indicating their primitive characteristics. VSELs have been found to express not only cardiac and endothelial but also several developmental markers, such as Oct-4, Nanog, and stage-specific embryonic antigen-1 (SSEA-1) (Kucia et al., 2006b).

The strategy for VSEL isolation by FACS depends on gating strategy based on their small size, expression of pluripotent stem cells and absence of hematopoietic lineage markers. Actually, traditional FACS-based sorting protocols exclude events smaller than 5 μm in diameter, which primarily include erythrocytes, platelets and debris. Thus, the fact that VSELs are very small should be taken into consideration, because these cells could be lost during certain procedures such as gradient or velocity centrifugation. To preserve these small cells to maximum extent, Kucia and his coworkers employed size beads to control this novel approach (Kucia et al., 2006). Additionally, by employing a novel two-step isolation procedure, a population of human cells that are similar to murine bone marrow-derived VSELs was isolated from human cord blood (Kucia et al., 2006b). These cord blood derived VSELs are very small (3-5 μm) and highly enriched in a population of CXCR4$^+$ CD133$^+$CD34$^+$Lin$^-$CD45$^-$ mononuclear cells, possessing large nuclei containing unorganized euchromatin. They express nuclear embryonic transcription factors Oct-4, Nanog and surface embryonic antigen SSEA-4 (Kucia et al., 2006b; Zuba-Surma et al., 2008a-d). Thus, VSELs could play an important role in maintaining homeostasis of stem cell pools in mammals (Ratajczak et al., 2009).

Subsequently, Zuba-Surma and his group employed a multi-dimensional approach, novel ImageStream System (ISS) and confocal microscopy integrating with traditional flow cytometry (FCM) (Zuba-Surma et al., 2008a-d). The ISS analysis, featuring high-resolution brightfield, darkfield and fluorescence images, provides ideal access to the observation of small cellular events

and morphological parameters of VSELs. Benefiting from this novel image method, researchers not only confirmed the size characteristics, but also demonstrated the pluripotential features of VSELs. Next, the same group managed to provide further evidence in testifying the existence of these pluripotent stem cells in several organs other than bone marrow in adult mice (Zuba-Surma et al., 2008a-d). They reported that the highest total numbers of Oct-4$^+$ VSELs were found in the brain, kidneys, muscles, pancreas and bone marrow. However, the number of VSELs per organ could be overestimated because of contamination by anucleated cell debris if classical FCM analysis without nuclear staining. More recently, researchers optimized the isolation by proposing a relatively short and economical three-step protocol that allows isolation of highly enriched Oct-4$^+$ and SSEA-4$^+$ cells from a population of small Lin-/CD45$^-$/CD133$^+$ cells (Zuba-Surma et al., 2010a,b). However, this requires further study to clarify whether cord blood derived VSELs are similar to their murine bone marrow-derived counterparts and are endowed with properties of pluripotent stem cells.

As discussed above, several features, including small size, high N/C ratio, as well as expression of pluripotent stem cell markers, indicate the primitive characteristics of VSELs. In an *in vitro* culture system, this population of primitive cells has been confirmed to be able to differentiate into cells from all three germ layers, implying that these cells are an enriched pluripotent stem cell population (Kucia et al., 2006b). If plated over C2C12 murine sarcoma cell feeder layer, ~5%-10% of purified VSELs are able to form spheres that resemble embryoid bodies (Kucia et al., 2006b). Moreover, after re-plating over C2C12 cells, VSEL-derived spheres (up to 5-7 passages) may grow new spheres again or, if plated into cultures promoting tissue differentiation, expand into cells of all three germ cell layers. Similar sphere formation of VSELs from murine fetal liver, thymus and spleen was also observed. Interestingly, the formation of VSEL-derived spheres was associated with a young age in mice and VSEL-derived spheres were barely found in cells isolated from mice older than 2 years. Parallelly, a gradual decrease was noticed in the number of VSELs in bone marrow from C57/BL/6 mice between the ages of 2 months and 3 years. Thus, it is postulated that this age-dependent content of VSELs in bone marrow may explain why the regeneration process is more efficient in younger individuals (Kucia et al., 2005a,b; Ratajczak et al., 2008a,b). Further results also revealed that the concentration of VSELs is much higher in bone marrow of long-lived mouse strains as compared with short-lived ones (Kucia et al., 2006b).

More recently, Ratajczak and his colleagues reported that VSELs isolated from murine bone marrow can differentiate along the hematopoietic lineage in a similar way to embryonic stem cells (ESCs) or inducible pluripotent stem cells (iPSCs) after coculture over OP9 stromal cells (Ratajczak et al., 2011a,b). Specifically, the "OP9-primed" VSEL-derived cells were observed to express several hemato/lymphopoiesis-specific genes and markers, to give rise to hematopoietic colonies *in vitro*, and to be capable of protecting lethally irradiated mice in transplant models. Their results confirm again that VSELs are a primitive bone marrow-residing population of stem cells and suggest that VSELs may share some characteristics with the most primitive long-term repopulating hematopoietic stem cells.

Potential contribution of VSELs to regenerative medicine

Although VSELs appear to be relatively rare and are dispersed throughout the tissues, they hold great potential for clinical application. Kucia and his colleagues provided the evidence for the first time that under steady-state conditions VSELs circulate at very low levels in peripheral blood, and could be additionally mobilized during pharmacological granulocyte colony-stimulating factor (G-CSF)-induced or stress-related mobilization in the model of toxic liver or skeletal muscle damage (Kucia et al., 2008a,b). Furthermore, evidence has been provided that the level of circulating VSELs was significantly elevated after acute myocardial infarction (AMI) occurring either in mice models or in patients (Zuba-Surma et al., 2008a-d; Wojakowski et al., 2009). More recently, researchers have demonstrated that murine bone marrow-derived VSELs can be mobilized into peripheral blood after exposure to intermittent hypoxia (Gharib et al., 2010).

Parallelly, clinical trials also provided evidence that AMI, stroke and skin-burn injury could induce mobilization of VSELs expressing pluripotent stem cell markers (Oct-4, Nanog, SSEA-4), as well as early cardiac and endothelial markers (Kucia, 2004; Paczkowska et al., 2009; Drukala et al., 2012). Taken together, these results not only support the proposition that organ/tissue injury promotes recruitment of VSELs from bone marrow into circulation, but also confirm the implication that such recruitment could occur in patients with organ/tissue damage.

More recently, attention was focused on the direct contribution of VSELs to regenerative therapy. Direct intramyocardial injection of freshly isolated VSELs ($1x10^4$) can promote myocardial function recovery in mice ischemia reperfusion model, and the beneficial effects on improvement of global and regional left ventricular (LV) contractility can be observed after 35 days of follow-up. In contrast, transplantation of HSCs ($1x10^5$) failed to present any functional or structural benefits (Dawn et al., 2008). However, due to the low frequency of VSELs in bone marrow, it cannot be ignored that the limitation on acquisition may block clinical application. It is gratifying to see that expansion and pre-differentiation of VSELs in cardiopoiesis-guided medium before the injection can increase their effectiveness, leading to an increase of left ventricular ejection fraction, myocardial systolic thickening, and attenuated remodeling (Zuba-Surma et al., 2010a,b). In

an *in vitro* culture system, VSELs exhibit a wide range of differentiation potential and are capable of differentiating into cells from all three germ layers (Kucia et al., 2006b). The success of persistent benefits in left ventricular function and without tumor formation after VSEL transplantation in animal models of myocardial infarction raises the possibility that VSELs can be used in other degradation diseases.

All tissue-specific stem cells move around during embryonic development and HSCs continue to migrate via the blood stream throughout adulthood (Scadden, 2008). However, bone marrow is not the only major reservoir of stem cells. A previous report has demonstrated that the highest total numbers of Oct-4$^+$ VSELs can be found in the brain, kidneys, muscles, pancreas and bone marrow (Zuba-Surma et al., 2008a-d). Here, we hypothesize that continuous trafficking and homeostasis of VSELs among the organs and circulation likely fill empty or damaged niches and contribute to the maintenance of normal organ function and restoring degraded tissues (Fig. 1).

Although recent data indicate the promising prospects of VSELs for therapeutic utility, there remain drawbacks for the application of these pluripotent stem cells in therapy. First, the availability of VSELs is a major problem in basic research and clinical application. The regeneration of organ damage will require local delivery of a higher number of purified, isolated and expanded VSELs from adult tissues (Ratajczak et al., 2009). Even if ongoing optimized methods on isolation and expansion make it more proficient and effective to harvest VSELs, given the low frequency (x0.01% of bone marrow MNCs), it is difficult to acquire a sufficient number of VSELs from bone marrow, or other tissues. Moreover, the valid total number of VSELs at a dose for local injection in mice, varying greatly from 1104 to 1x10^6 cells (Dawn et al., 2008; Zuba-Surma et al., 2010a,b), requires further study. Accordingly, improvements in obtaining pure populations and guiding these cells to differentiate into specialized cells are essential in order to scale up the number of cells suitable for transplantation.

Secondly, the aging of VESLs requires further investigation. Previous report revealed that the number of VSELs residing in mice bone marrow presents an age-dependent phenomenon (Kucia et al., 2006b). As a result, despite the possibility that an aged cell may contribute to organ repair, another concern exists that the therapeutic effects attained by autologous transplantation could be impaired due to an insufficient number of VSELs. This means that the potential clinical use of VSELs in the older patients will probably be invalid.

Thirdly, safety evaluation should be ensured before the clinical application of VSELs. Several groups have demonstrated that bone marrow-derived stem cells may initiate tumor growth if they are mobilized at the wrong time and incorporated into the wrong place, and may acquire oncogenic mutations (Houghton et al., 2004;

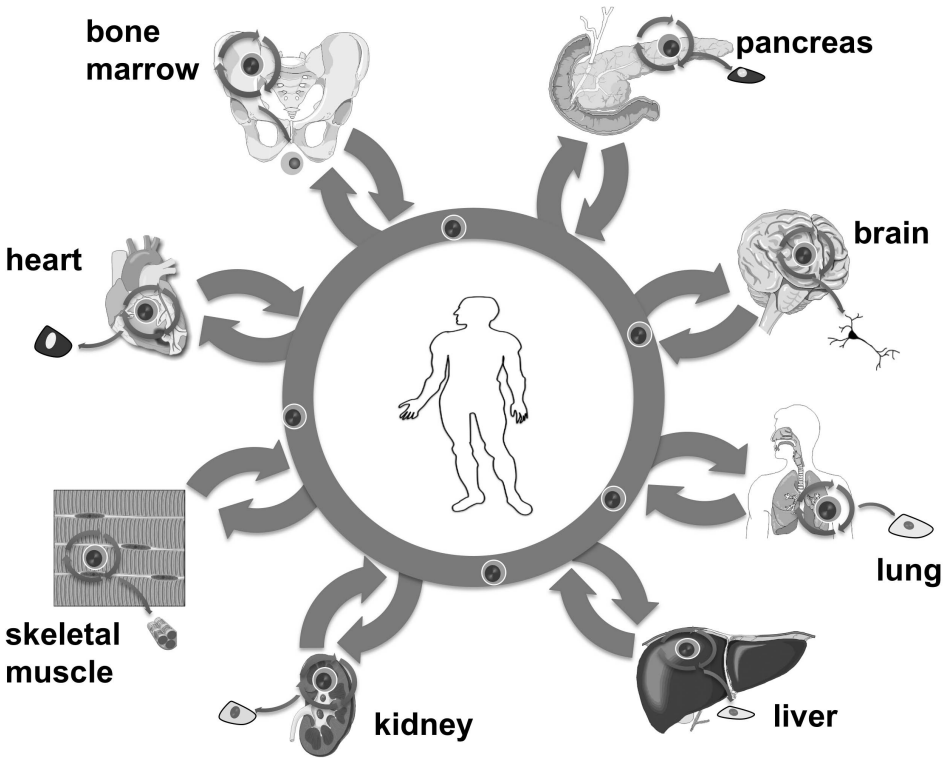

Fig. 1. Maintenance and trafficking of VSELs in various tissues and organs. Besides the initial findings that VSELs are located in adult murine bone marrow, this extremely rare population of cells is also detected in other adult tissues (e.g. brain, kidney, skeletal muscle, pancreas). VSELs could be a potential back-up source for restoring the function of diseased or damaged tissues/organs. Further studies are required to identify VSELs in multiple tissues and to explore if they are similar to their bone marrow counterpart in pluripotency.

Rizvi et al., 2006).

VSELs compare to other kinds of pluripotent stem cells

Pluripotent stem cells are defined as a cell population that can differentiate into cells from all three germ layers. Despite their availability from various potential sources, the therapeutic application of embryonic stem cells (ESCs) has been limited because of ethical and religious issues. Moreover, there is a major problem of histoincompatibility between ESCs and the recipient, which may cause immunogenic responses (Ratajczak et al., 2011a,b). Furthermore, teratoma formation is another scientific concern that hampers a broader usage of ESCs for regenerative therapy.

An alternative strategy for obtaining pluripotent stem cells by genetic modification of normal somatic cells has been proposed recently (Takahashi and Yamanaka, 2006). These pluripotent stem cells, designated as induced pluripotent stem cells (iPSCs), are artificially derived from non-pluripotent cells by inducing "forced" expression of specific genes (i.e. Oct3/4, Sox2, c-Myc, and Klf4). iPSCs are similar to ESCs in many respects, such as the expression of key pluripotency genes, chromatin methylation patterns, embryoid body formation, and doubling time. Owing to development from the patient's own somatic cells, it is possible that iPSC therapy could avoid immunological rejection. However, before moving any iPSCs into the clinic, several issues should be addressed first. The major limitation of this technology is the use of viruses that integrate into the genome which carry potential risks of insertional mutagenesis (Stadtfeld et al., 2008). The low efficiency of reprogramming (0.01 to 0.1% of input cells) also raises the possibility that insertional mutagenesis may be a prerequisite for *in vitro reprogramming*.

In the past 5 years the morphologic, functional and genetic traits of VSELs have been well defined by a variety of investigations and analyses. VSELs have similar properties to ESCs and iPSCs, including the expression of pluripotent markers, the ability to give rise to cellular derivatives of all three germ layers and form embryoid-like bodies. There are several main advantages of VSELs over ESCs and iPSCs. Firstly, VSELs are normally derived from bone marrow, so there are no ethical debates for clinical applications. Secondly, besides the expression of pluripotent markers, bone marrow-derived VSELs do not express MHC-I and MHC-II antigens, which makes them attractive for regeneration therapy (Ratajczak et al., 2008a,b). Thirdly, in contrast to ESCs and iPSCs, VSELs have previously shown their safety in animal experiments without teratoma formation (Dimomeletis et al., 2010).

Recent research demonstrated that bone marrow derived multilineage-differentiating stress-enduring (Muse) cells contribute to the primary source of iPSCs (Wakao et al., 2011). This population of cells holds the potential to self-renew and differentiate into all three germ layers, but they do not form teratomas in contrast to ESCs or iPSCs. It is likely that Muse cells are a sub- or overlapping population of VSELs, but further confirmation should be made. Given the properties of VSELs, we may not take the trouble to reprogram somatic cells to obtain iPSCs in future.

In summary, stem cell therapy provides hope for the future in treatment of a variety of refractory diseases. Current data both in humans and mice render VSELs a putative candidate with pluripotent characteristics for regenerative therapy. Although scientists have gained much insight into the biology of VSELs, many key questions remain to be addressed before the full potential of these cells can be realized.

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