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Very Small Embryonic-Like Stem Cells: Biology and Therapeutic Potential for Heart Repair

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Abstract

Very small embryonic-like stem cells (VSELs) represent a population of extremely small nonhematopoietic pluripotent cells that are negative for lineage markers and express Sca-1 in mice and CD133 in humans. Their embryonic-like characteristics include the expression of markers of pluripotency; the ability to give rise to cellular derivatives of all three germ-layers; and the ability to form embryoid-like bodies. Indeed, quiescent VSELs may represent the remnants of epiblast-derived cells in adult organs. After tissue injury, including acute myocardial infarction (MI), bone marrow–derived VSELs are mobilized into the peripheral blood and home to the damaged organ. Given the ability of VSELs to differentiate into cardiomyocytes and endothelial cells, and their ability to secrete various cardioprotective growth factors/cytokines, VSELs may serve as an ideal cellular source for cardiac repair. Consistently, transplantation of VSELs after an acute MI improves left ventricular (LV) structure and function, and these benefits remain stable during long-term follow-up. Although the mechanisms remain under investigation, effects of secreted factors, regeneration of cellular constituents, and stimulation of endogenous stem/progenitors may play combinatorial roles. The purpose of this review is to summarize the current evidence regarding the biologic features of VSELs, and to discuss their potential as cellular substrates for therapeutic cardiac repair. *Antioxid. Redox Signal.* 15, 1821–1834.

Introduction

DURING THE PAST DECADE, the attention of biomedical researchers has increasingly been directed to stem cells as potential mediators of effective tissue repair in injured organs. Although various cell types have been used for the repair of infarcted myocardium (1, 15, 19, 77), cells exhibiting "multipotent" or "pluripotent" behaviors have proven to be especially efficacious for regenerative purposes (7, 16, 77, 78). Despite their pluripotent nature, the therapeutic applicability of embryonic stem cells (ESCs) derived from developing blastocyst or by somatic nuclear transfer has been limited because of their known propensity to form tumors and because of ethical issues (18, 40, 62). As a result, pluripotent stem cells from adult tissues that are capable of differentiating into derivatives of all three germ layers have become a major focus of interest in regenerative medicine. These cells may potentially fulfill the growing need for a reliable and noncontroversial resource for stem cells for effective regenerative therapies in humans.

Initially described in the bone marrow of adult mice as a very rare population characterized by unusually small size, very small embryonic-like stem cells (VSELs) are pluripotent cells with several embryonic-like features, albeit without tumorigenic activity (38, 54, 85). Over the past several years, the morphologic, genetic, and functional characteristics of VSELs have been established through extensive and systematic analyses (38, 57, 65, 85, 89). This body of work indicates that VSELs are able to differentiate into cells from all three germ layers; are recruited to peripheral blood during tissue injury, including myocardial infarction (MI); and participate in the repair of infarcted myocardium (3, 36, 38, 60, 74, 87). The purpose of this review is to summarize the current evidence with regard to the biologic features and therapeutic potential of VSELs for repair of the infarcted myocardium.

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Biological Features of VSELs

Phenotypic characteristics and antigenic profile of VSELs

VSELs were identified in the nonhematopoietic compartment of adult murine bone marrow (BM) as a rare population of primitive cells that were positive for stem cell antigen-1 (Sca-1) and negative for both hematopoietic lineage markers (Lin) and the panleukocytic marker CD45 ($Sca\text{-}1^+/Lin^-/CD45^-$) (38). It also was shown that the purified VSEL fraction consists of primitive cells expressing markers characteristic of multiple tissues, including neurons, endothelial cells, pancreatic cells, skeletal muscle cells, and cardiomyocytes. VSELs are enriched in mRNA for cardiac-specific antigens (Nkx2.5/Csx, GATA-4, MEF-2C) and acquire a cardiomyocytic phenotype *in vitro* (38, 54).

Subsequent analyses using the novel imaging cytometry (ImageStream System; ISS) technique have characterized and quantified the morphologic features of VSELs related to their primitive stage, including very small size, cytoplasmic area, and nuclear to cytoplasmic ratio (N/C). The ISS combines classic flow cytometry with fluorescent microscopy in one platform and allows the visualization of cells in suspension during flow acquisition through high-resolution bright-field, dark-field, and fluorescence images, as well as statistical analysis of several morphologic features of cells based on collected images (6, 86, 90). This technique allows the identification of objects as small as 1 μm in diameter (49), which is helpful for identification of "very small" VSELs in multiple tissues. By using ISS, we were able to describe in adult tissues, for the first time, the presence of the cells, which are smaller than erythrocytes (as small as 3.63 ± 0.09 μm) and possess the normal diploid number of chromosomes (53, 59, 85). The very high N/C of VSELs, greater than that of other primitive and more mature cells, confirmed our previous transmission electron microscopic (TEM) observations, which indicated the presence of relatively large nuclei surrounded by a narrow rim of cytoplasm inside these cells (38, 85).

Isolation of VSELs from murine and human tissues: sorting strategy

The existence of rare nonhematopoietic stem cells, which are committed to various nonhematopoietic tissues, was suggested by Ratajczak and colleagues (54) several years ago. For the identification and purification of these cells from the adult murine BM and human specimens, we applied novel criteria. We assumed that these cells (a) are mobile and migrate to areas of tissue injury and thus should express CXCR4, the receptor for SDF-1 chemokine; (b) express markers of stem cells including Sca-1 (in mice) and CD133 (in humans); (c) belong to the nonhematopoietic compartment and do not express CD45 antigen; and (d) most likely exhibit a very small size (54, 59, 93). The last feature was predicted based on the very small size of ESCs present in the inner mass of developing blastocysts. We expected that if pluripotent stem cells exist and are "hidden" in adult tissues, they should have a similar small appearance.

We used fluorescence-activated cell sorting (FACS) for the isolation of VSELs from murine BM (85, 93). However, because most of the standard sorting protocols exclude events smaller than 6 μm in diameter that include cell debris, erythrocytes, and platelets, small VSELs are usually excluded from sorted cell populations. The standard sorting protocols therefore needed to be modified to include all objects as small as 2 μm in diameter. To achieve this goal, we used a mixture of beads with predefined sizes and set the sorting morphologic gate to include all nongranular/lymphocyte-like cells in the size range from 2 to 10 μm (85, 93). This region mostly contains cellular debris, but also rare nucleated cell events . These small objects were further analyzed for Sca-1 and hematopoietic lineage marker (Lin) expression, and only $Sca\text{-}1^+/Lin^-$ cells were included for further analysis. Among these $Sca\text{-}1^+/Lin^-$ cells, we could subsequently identify a predominant subfraction of $CD45^+$ HSPCs and a very rare $CD45^-$ population of VSELs. We found that VSELs comprise approximately 0.03%, whereas HSPCs comprise about 0.30% of the total BM nucleated cells (85, 93) (Fig. 1).

The human CB- and BM-derived VSELs have been isolated with FACS by using modified protocols that strongly consider cellular size (84, 93). We included all objects larger than 2 μm in diameter for sorting of human VSELs and further gated for Lin^- cells. In the next step, two fractions of human HSPCs and VSELs were distinguished among Lin^- cells as $CD133^+/CD45^+$ and $CD133^+/CD45^-$ phenotypes, respectively. A similar strategy may effectively be used when the CD133 antigen is replaced with either CD34 or CXCR4 markers (84, 93) (Fig. 2).

Although the above sorting strategies have been successful for the isolation of pluripotent VSELs, they also resulted in large quantities of cellular debris, which are in the small size range. With the advent of imaging cytometry, ISS enabled us, for the first time, to distinguish between nucleated VSELs from cellular debris, to quantify the true content of VSELs, and to confirm their existence in sorted material (84, 85, 90, 92). Moreover, as in murine VSELs, we could analyze several morphologic features of human CB-derived VSELs, including their average size (6.6 to 6.8 μm) and confirm that they are smaller than human erythrocytes, which are about 7.9 μm in diameter (84) (Fig. 3). Thus, this technique is currently one of our major tools for VSEL identification in different types of specimens from animals and humans.

We believe that the very small size of both murine and human VSELs precluded the discovery of these cells earlier. Today, the protocols for VSEL identification and isolation are very well described and validated and may be used for most of the currently available flow-cytometry equipment for cell isolation (84, 93).

Embryonic-like features of VSELs and pluripotency

VSELs were termed "Very Small Embryonic-Like stem cells" based on the observations that these cells express several markers associated with a pluripotent state, including Oct-4, Nanog, SSEA-1, Rex-1, Rif-1, and give rise into cells from all three germ layers. They also exhibit several other features of embryonic cells at the ultrastructural level (38, 56, 85). Consistently, TEM analyses of purified BM-derived VSELs nuclei indicate the presence of a primitive form of open-structure euchromatin, which has been described as a feature of embryonic stem cells (68).

Besides the expression of pluripotent markers, murine BM-derived VSELs barely express markers of other stem cells,

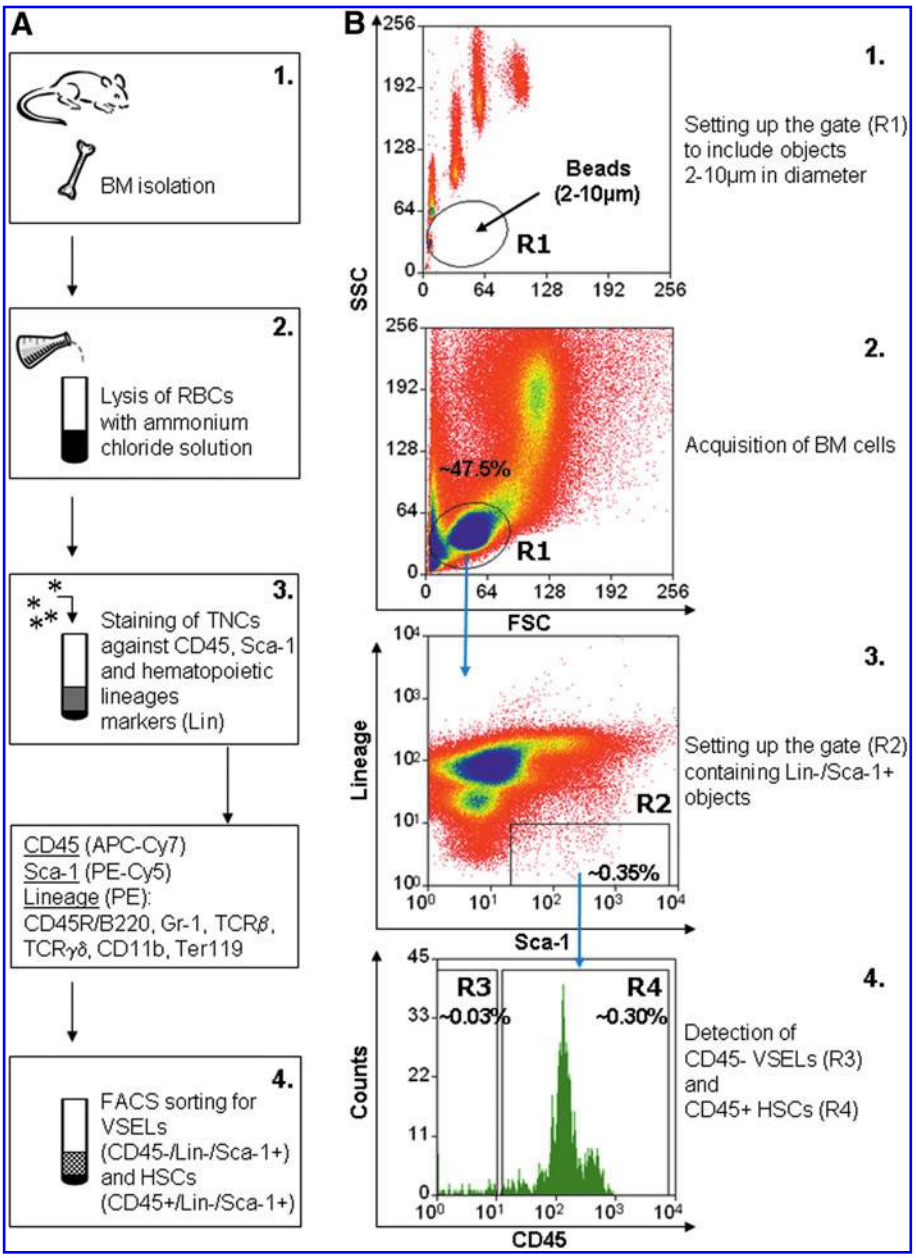


FIG. 1. Isolation of murine bone marrow (BM)-derived VSELs by flow cytometry. (A) Experimental protocol: BM-VSELs were isolated from murine BM total nucleated cells (TNCs) harvested from murine tibias and femurs after lysis of red blood cells (RBCs) with ammonium chloride followed by staining for CD45, Sca-1, and hematopoietic lineage markers (Lin). **(B)** Gating strategy for murine BM-VSEL sorting by FACS. Agranular, small events ranging from 2 to 10 μm are included into gate R1 after comparison with size-predefined bead particles with standard diameters of 1, 2, 4, 6, 10, and 15 μm. The BM nucleated cells are visualized by dot-plot showing forward-scatter (FSC) versus side-scatter (SSC) signals, which are related to the size and granularity/complexity of the cell, respectively. Cells from region R1 are analyzed for Sca-1 and Lin expression, and only Sca-1$^+$/Lin$^-$ events are included in region R2. The population from region R2 is subsequently distinguished based on CD45 expression into Sca-1$^+$/Lin$^-$/CD45$^-$ VSELs (region R3) and Sca-1$^+$/Lin$^-$/CD45$^+$ HSCs (region R4). Percentages represent the average content of each cellular subpopulation in total BM nucleated cells. (To see this illustration in color, the reader is referred to the web version of this article at www.liebertonline.com/ars).

such as mesenchymal stem/stromal cells (MSCs) (CD29, CD105) and do not express MHC-I and MHC-II antigens, which make these cells attractive substrates for transplantation (58, 59). These features, as well as the strong adhesive capacities of these cells, suggest their close relation to the MSC compartment within the bone marrow. Recent *in vivo* studies published by Taichman and colleagues (69) suggest that VSELs may be a pluripotent fraction of cells atop the MSC compartment and may be responsible for the multipotent capacities of MSCs. These data may be discussed well in the context of previous findings that showed that VSELs fulfill the criteria for pluripotency and are capable of differentiating into cells from all three germ layers, including neurons (ectodermal), pancreatic cells (endodermal), and cardiomyocytes (mesodermal) (57, 58). One may therefore postulate that VSELs can potentially give rise to fractions of more-mature and tissue-committed MSCs. The observation that a fraction of BM-derived MSCs express Oct-4 antigen may support such a close relation between VSELs and cells within the mesenchymal compartment (5, 41).

Interestingly, it has also been observed that VSELs cultured in the presence of feeder-layer cells that support hematopoietic differentiation of stem cells (OP-9 cell line) give rise to "cobble-stone"-forming cells that resemble long-term repopulating hematopoietic stem cells (LT-HSCs) (59, 88). These VSEL-derived LT-HSCs not only gave rise to all types of hematopoietic colonies *in vitro*, but also were capable of fully reconstituting all hematopoietic lineages in myeloablated mice after lethal gamma irradiation *in vivo* (59, 88).

This evidence supports that VSELs are pluripotent stem cells that most likely serve as precursors of both mesenchymal and hematopoietic compartments of stem and progenitor cells. Future investigations will elucidate this complex relation between VSELs and other well-described populations of

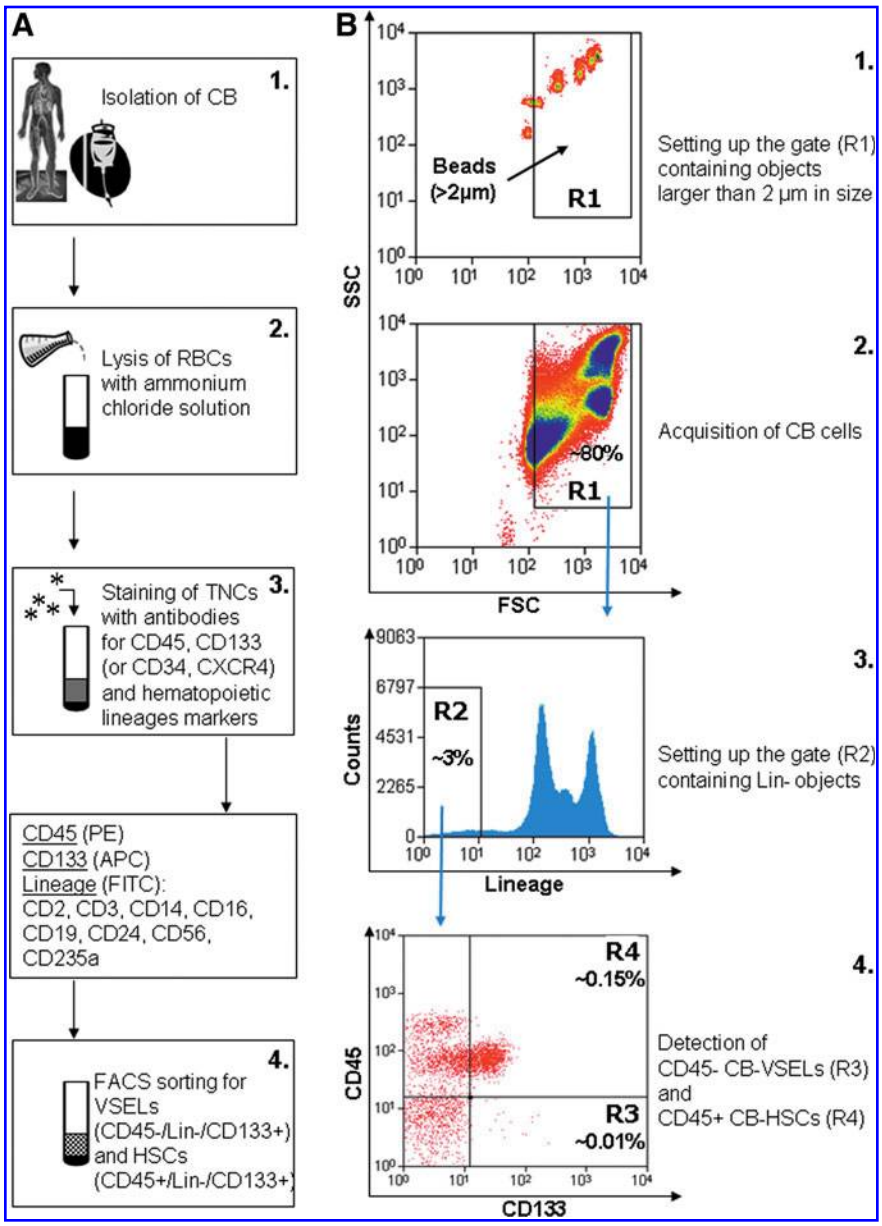


FIG. 2. Isolation of human cord blood (CB)-derived VSELs with flow cytometry. (A) Experimental protocol: CB-VSELs were isolated from the total population of human CB nucleated cells (TNCs) harvested after the lysis of red blood cells (RBCs) with ammonium chloride. TNCs were stained for CD45, hematopoietic lineage markers (Lin), as well as for one of the following stem cell antigens: CD133, CD34, or CXCR4. **(B)** Gating strategy for human CB-VSEL sorting by FACS: All events larger than 2 μm are included into gate R1 after comparison with size-predefined bead particles with standard diameters of 1, 2, 4, 6, 10, and 15 μm. The CB-derived TNCs are visualized with dot-plot, presenting forward-scatter (FSC) versus side-scatter (SSC) signals, and all cells from region R1 are further analyzed for hematopoietic lineage markers (Lin). The Lin^- subpopulation included into region R2 is subsequently analyzed based on CD133 and CD45 expression, and the two fractions of $CD133^+$ cells are distinguished based on CD45 appearance: $CD133^+/Lin^-/CD45^-$ cells (VSELs; region R3) and $CD133^+/Lin^-/CD45^+$ cells (HSCs; region R4). Percentages show the average content of each cellular subpopulation in total CB nucleated cells. (To see this illustration in color, the reader is referred to the web version of this article at www.liebertonline.com/ars).

stem/progenitor cells in the BM and other organs, including cardiac stem cells (CSCs) in the heart.

Epigenetic mechanisms that maintain pluripotency of VSELs

One of the first important observations about VSEL ultrastructure was the presence of open euchromatin in their nuclei (37, 38). This significant finding led us to extensive studies elucidating the epigenetic status of VSELs, including chromatin methylations and histone modifications regulating the expression of several genes related to VSEL pluripotency, proliferation status, and somatic imprint, such as *Oct-4, Nanog, Igf-2,* and *H19*. The results from these studies have shown that, similar to ESCs (ESC-D3 cell line), freshly isolated VSELs exhibit the hypomethylated open chromatin structure of the *Oct-4* promoter, leading to active transcription of this gene and maintenance of pluripotency (64, 65). Moreover, the results reported by Shin *et al*. (64, 65) explained fundamental concerns regarding the quiescence of VSELs, the lack of teratoma formation, and blastocyst complementation based on the unique DNA methylation pattern at some developmentally crucial imprinted genes.

Furthermore, a unique genomic imprinting pattern in VSELs described in this study showed the tendency for erasure in paternally hypomethylated genes but hypermethylation of the maternally methylated ones. It has been described that although paternally expressed imprinted genes (Igf2, Rasgrf1) enhance the growth of the embryo, maternally expressed genes (*H19, p57KIP2, Igf2R*) inhibit cell proliferation (61). Therefore, the differences observed on VSELs show growth-repressive imprints in these cells. Described epigenetic characteristics of VSELs leading to upregulation of growth-repressive genes [*H19* and *p57KIP2 (Cdkn1c)*] and repression of growth-promoting genes (*Igf2* and *Rasgrf1*), may explain the VSEL quiescent status. Moreover, because Igf2 has

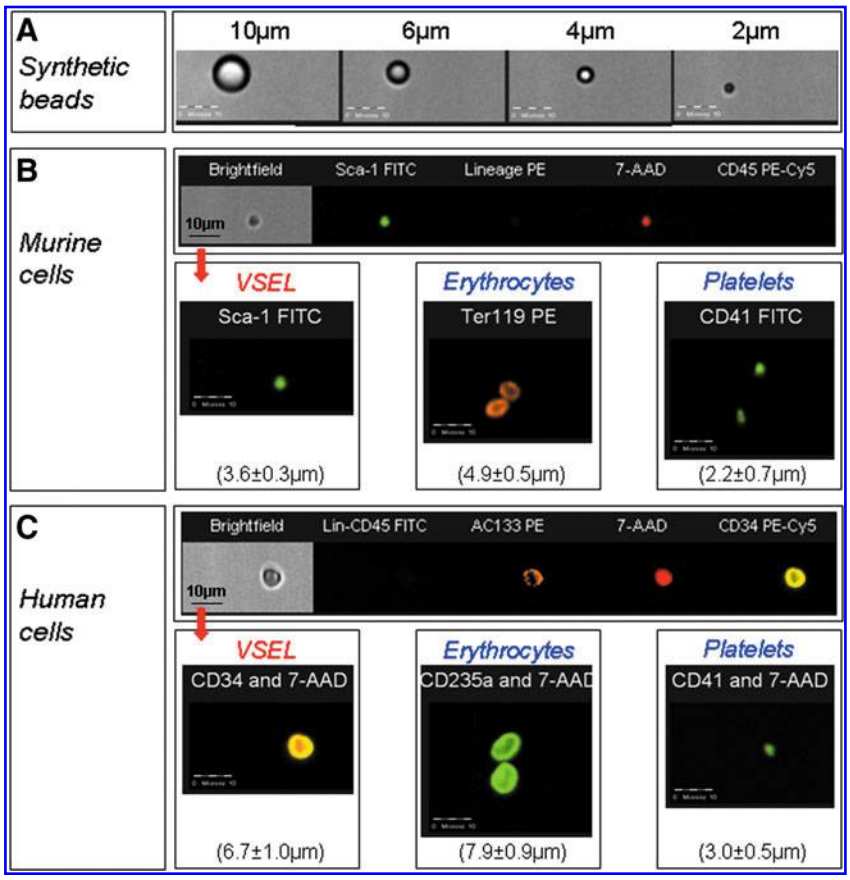


FIG. 3. Representative images illustrating the morphology of murine and human VSELs with imaging cytometry (ImageStream System). (A) Brightfield images of beads with predefined sizes serving as size standards. **(B)** Murine BM-derived Sca-1$^+$/Lin$^-$/CD45$^-$ nucleated VSELs [Sca-1 (FITC, green), Lin (PE, orange), CD45 (PE-Cy5, yellow), nucleus (7-aminoactionomycin D, red] compared with murine erythrocytes [Ter119$^+$ (PE, orange)] and platelets [CD41$^+$ (FITC, green)]. **(C)** Human cord blood–derived CD133$^+$/CD34$^+$/Lin$^-$/CD45$^-$ nucleated VSELs [Lin and CD45 (FITC, green), CD133 (PE, orange), CD34 (PE-Cy5, yellow), nucleus (7-AAD, red)]. All of the images are shown at the same magnification. Scale bar = 10 (m. The average sizes of murine and human cells are provided under the respective images. VSELs are distinguished from erythrocytes and platelets based not only on distinct surface markers, but also on the presence of nuclei in VSELs. (To see this illustration in color, the reader is referred to the web version of this article at www.liebertonline.com/ars).

been described as an important autocrine growth factor that promotes the expansion of several cell types (25), and, in contrast, H19 regulatory mRNA has been noted to inhibit cell proliferation (28), the changes in expression of these two genes may be responsible for the quiescent status of VSELs.

Supposedly, BM-residing VSELs, as remnants of embryonic development (*e.g.*, derivatives from the epiblast), reside in a dormant state in ectopic BM niches. The quiescent status of these cells could be the potential result of (a) non-physiologic location, (b) exposure to inhibitors, (c) deprivation of pivotal stimulatory signals, and (d) perhaps most important, the limitation in pluripotency because of the erasure of the somatic imprint on the crucial somatically imprinted genes (*H19, Igf2, Rasgrf1*, and *p57KIP2*) (64, 65). VSELs, however, can be activated if they are exposed to appropriate activation signals (*e.g.*, upregulated during organ/tissue injury, oncogenesis) or undergo epigenetic changes that alter the methylation status of their DNA and acetylation of histones. Finally, they may be reactivated and stimulated for proliferation when a proper somatic imprint is reestablished (55, 65). This hypothesis is supported by the recent observation of a reverted pattern in imprinted gene methylation in VSELs cocultured with C2C12 cells as well as during formation of embryoid-like bodies (ELBs), the process that enlarges the pool of proliferating VSELs able to differentiate into all three germ layers (65). Therefore, the potential modulation of mechanisms that control genomic imprinting in VSELs would be crucial for developing more-powerful strategies to expand these cells and unleash their regenerative potential for efficient clinical applications.

VSELs may represent epiblast-derived remnants in various adult organs

Subsequent to the initial discovery of VSELs in the BM, we have identified Oct-4$^+$/Sca-1$^+$/Lin$^-$/CD45$^-$ small cells that phenotypically resemble VSELs in other adult murine organs, including brain, kidney, pancreas, muscle, and gonads (53, 89, 92). Interestingly, we found the highest number of small nucleated Oct-4$^+$/Sca-1$^+$/Lin$^-$/CD45$^-$ cells in the brain, followed by kidney, skeletal muscle, pancreas, and bone marrow (43.97 ± 12.38, 19.87 ± 2.03, 15.18 ± 6.79, 9.41 ± 4.71, and $8.39 \pm 2.00 \times 10^3$ cells, respectively) (92). The biologic role of VSELs in these organs remains to be elucidated in future experiments. The potential presence of similar very small primitive cells in adult organs, including bone marrow, has also been reported by other investigators (89). However, such a possibility has not been systematically explored, and these other cell types have not been fully characterized at a single-cell level (89).

We have also established that similar to BM-derived VSELs, Oct-4$^+$/Sca-1$^+$/Lin$^-$/CD45$^-$ cells from different organs are enriched in markers of pluripotency (Oct-4, Nanog, Rex-1, Dppa-1) at both mRNA and protein levels (53, 92). Moreover, we have established that VSELs share phenotypic and genetic similarities with primordial germ cells (PGCs), the population of epiblast-derived cells migrating to genital ridges during gestation, giving rise to germline cells (59, 64).

Importantly, VSELs share similarities in the unique methylation pattern with PGCs, which are responsible for the quiescent status of PGCs and make them ineffective in blastocyst complementation and somatic nuclear-transfer assays (65). In mice, PGCs gradually reprogram and erase their genomic imprinting during migration to genital ridges between 8.5 and 12.5 days *post coitum* (dpc) (27), and we suspect that similar genomic changes may take place in migrating VSELs during similar stages of embryonic development.

The vigorous migratory capacity of VSELs, the expression of genes characteristic of PGCs (*PLAP, Oct-4, SSEA-1, CXCR4, Mvh, Stella, Fragilis, Nobox, Hdac6*), and a similar pattern of genomic imprint, as well as other embryonic-like features of VSELs, lead us to hypothesize that VSELs represent a remnant population of epiblast-derived PSCs deposited in different organs during developmental migration in early stages of embryogenesis (55, 64, 65). In adulthood, such cells could potentially be responsible for cellular turnover and restoration of pools of progenitors in different organs participating in endogenous tissue repair. Although it has now been well established that neither the brain nor the heart is a postmitotic organ made of a finite number of mature cells, our data provide strong evidence that, similar to other organs, both brain and heart constantly undergo tissue renewal relying on the activity of progenitors and other residual stem cells. Thus, we envision that VSELs, at the top of the hierarchy of progenitor cells in each organ, are the rare quiescent population of pluripotent cells in adult tissues.

Importantly, we established that murine VSELs may already be detected in embryonic tissues at 8 dpc of development indicating their embryonic, but not extraembryonic origin (95). By using the transgenic model of NCX-1–knockout mice, which do not develop a circulation system necessary for stem cells to migrate from the extraembryonic yolk sac to the embryonic tissues, we confirmed that VSELs, in contrast to hematopoietic stem/progenitor cells (HSPCs), do not migrate from extraembryonic tissues, but are already present in developing embryo (95). At 12 dpc, VSELs also have been found in rapidly developing fetal liver (91). We established that VSELs leave this organ along with HSPCs and migrate to developing bone marrow tissue between 12 and 15 dpc (91).

In fact, in addition to those in BM (5, 41, 57), populations of stem cells expressing markers of epiblast cells have recently been described in several nonhematopoietic organs, such as epidermis (24, 80), bronchial epithelium (42), myocardium (8), pancreas (17, 35), testis (31), dental pulp (32), retina (34), and amniotic fluid (22). The morphology of these cells and their sizes vary slightly, depending on the tissue/organ in which they are located. However, the presence of epiblast markers in these cells generally supports a concept of developmental deposition of Oct-4$^+$ epiblast-derived cells/VSELs in developing organs (55). We believe that the vigorous process of cell migration during early stages of embryogenesis creates the opportunity for epiblast-derived VSELs to infiltrate and remain in the developing organs until adulthood.

Cells analogous to VSELs are present in cord blood and adult human tissues

Similar populations of very small cells enriched in fractions expressing markers of human pluripotent stem cells (Oct-4, Nanog, SSEA-4) at both mRNA and protein levels have been identified in human specimens, including umbilical cord blood (CB) and BM (37, 84). Human CB has been previously described as a source of various stem/primitive cells (10, 44, 72) that may potentially contribute to endothelial (52), hepatic (23, 47), neural (11, 12), and myocardial (79) regeneration when transplanted after tissue injury (29). Although this unique capability of CB-derived cells was initially explained by the trans-dedifferentiation or plasticity of CB-derived HSPCs (4, 45), several reports challenged this concept of plasticity and trans-dedifferentiation of HSPCs (13, 46, 48). Moreover, growing evidence indicates the presence of nonhematopoietic primitive cells in the CB, which can potentially contribute to the organ/tissue regeneration (11, 54). The CB has also been reported to contain several pluripotent nonhematopoietic stem cell populations, including the unrestricted somatic stem cells (USSCs) (33).

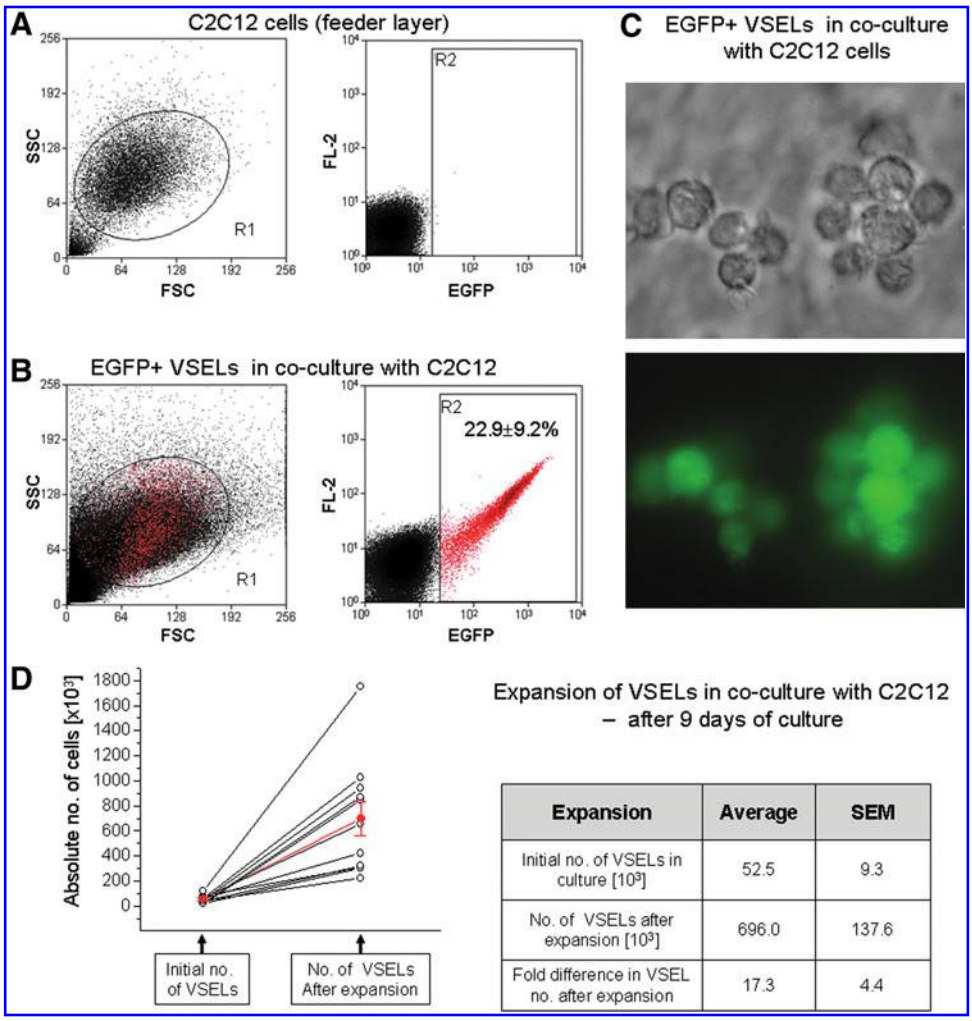
We have shown that both human CB and bone marrow also harbor a very primitive VSEL population that may be identified and purified based on the expression of CXCR4, CD34, or CD133 antigens, and a lack of hematopoietic lineage markers (Lin) and CD45 (37). The CD133$^+$/Lin$^-$/CD45$^-$ cells were noted to be the fraction most enriched in markers of pluripotency, and perhaps represent the most suitable fraction for potential future clinical applications (37, 84). By using imaging cytometry, we established that CB-derived VSELs coexpressing both CD133 and SSEA-4 markers represent the rarest fraction with the smallest size and the highest N/C ratio when compared with other fractions coexpressing CD133 and Oct-4 or CD34 antigens (84).

However, we also observed that approximately 50% of these very small VSELs may be lost during standard clinical procedures of preparation of CB units before cryopreservation and storage for clinical use (84). With the same conditions, the fraction of HSPCs may be recovered with a high yield. We postulate that the loss of VSELs during clinical-preparation procedures as well as centrifugation on Ficoll gradient may be related to the very small size and high density of VSELs, which predispose them to cross the gradient of separation media (84). This potential significant loss of VSELs should be considered during the processing of CB, BM, and mobilized peripheral blood units with erythrocyte and volume-depletion protocols for further storage.

VSELs can be expanded in vitro

VSELs are characterized as cells exhibiting mostly quiescent status. Freshly isolated BM-derived VSELs do not proliferate in the presence of any of the well-known media suitable for expansion of other pluripotent stem cells, including ESCs and induced pluripotent stem cells. At the same time, VSELs are highly resistant to severe environmental conditions that are normally lethal for other stem/progenitor cells, including a high dose of gamma-irradiation (1,500 cGy) (unpublished observation). Both of these features of VSELs confirm the unique primitive status of these cells.

However, we found that when cultured in the presence of a feeder-layer of C2C12 myoblast cell line, VSELs begin to aggregate, proliferate, and form spherical structures resembling ELBs (38, 59, 94) (Fig. 4). Importantly, such cellular clusters stain positive for placental alkaline phosphatase (PALP), a marker of ESCs, indicating the true embryonic characteristics



Expansion	Average	SEM
Initial no. of VSELs in culture [10³]	52.5	9.3
No. of VSELs after expansion [10³]	696.0	137.6
Fold difference in VSEL no. after expansion	17.3	4.4

FIG. 4. Expansion of VSELs in coculture with C2C12 cells before transplantation into infarcted myocardium. (A) C2C12 cells from the feeder layer shown in dot-plots. **(B)** EGFP$^+$ VSELs (red) expanding on C2C12 feeder layer (black) detected with flow cytometry. The expanded VSELs are purified from coculture based on their endogenous green fluorescence with FACS. **(C)** Representative images of EGFP$^+$ VSELs expanding over the C2C12 feeder layer and forming spherical structures (green). Lower and upper panels show corresponding bright-field and fluorescence images, respectively. **(D)** The yield after expansion of VSELs in the coculture system with C2C12 cells. The graph shows cell numbers in individual expansion experiments (black) and the mean data (red), whereas the table shows the average expansion yield ($n=10$ experiments). (To see this illustration in color, the reader is referred to the web version of this article at www.liebertonline.com/ars).

of these cells (38, 59, 94). Moreover, cells derived from ELBs preserve their primitive characteristics and vigorously differentiate into derivatives of all three germ layers, including cardiomyocytes *in vitro* (38, 59, 73).

To exclude the possibility of cell fusion with the C2C12 feeder-layer and subsequent proliferation, VSELs were isolated from EGFP transgenic mice, and it was confirmed that the ELBs were formed exclusively from EGFP$^+$ cells exhibiting normal diploid DNA content (58, 59). Similar spheres were also formed by VSELs isolated from murine fetal liver, spleen, and thymus. Interestingly, the formation of ELBs was restricted to VSELs isolated from younger mice, and no ELBs were observed with VSELs isolated from older mice (older than 2 years) (38, 39, 94). Moreover, we also observed that the number of VSELs in BM decreased with the increasing age of mice (94). This age-dependent decrease in VSEL numbers in BM and their capacity for sphere formation may explain a more-efficient regeneration process in younger individuals. It would be interesting to identify the genes responsible for tissue distribution and expansion of VSELs, as these may be involved in determining the life span of mammals.

This coculture system with C2C12 represents one of the mechanisms for VSEL expansion before further experimental and transplantation application. As mentioned earlier, VSELs may also successfully be expanded in presence of OP-9 cells for further applications in experimental hematology.

Therapeutic Potential of VSELs for Cardiac Repair

The ability of adult stem cells to repair injured and dysfunctional myocardium in humans has been established. However, the ideal cell type for such therapy and several related variables are being evaluated in ongoing clinical trials. In this regard, VSELs offer several major advantages over the currently available cellular substrates. First, given their pluripotent nature and their ability to differentiate into cardiomyocytes and endothelial cells, VSELs appear to be particularly well suited for cellular-replacement therapy. Second, the expression of various angiogenic and protective factors in VSELs renders them suitable for myocardial repair via paracrine actions. Third, unlike the currently available pluripotent cells (embryonic stem cells, induced pluripotent cells), VSELs do not form tumors during extended follow-up. Finally, because VSELs can be isolated from adult tissues, the use of autologous VSELs circumvents rejection and other potential immunologic consequences. Consistent with these attributes, our results from animal models of infarct repair after acute MI

indicate that transplanted VSELs are able to induce cardiac repair with improvement in LV structure and function.

VSELs are mobilized during various pathologic conditions

An important consideration with regard to the use of VSELs for therapeutic purposes is the mobilization of these cells during various pathologic states. This phenomenon signifies that VSELs are naturally intended for the repair of damaged tissues. In a recent study, we reported for the first time that pluripotent VSELs expressing the embryonic marker Oct-4 are mobilized in the early phase after acute MI (87). In mice subjected to ischemia/reperfusion injury, the levels of circulating Sca-1$^+$/Lin$^-$/CD45$^-$ VSELs were elevated at 24 and 48 h after I/R injury followed by a decrease to the levels observed in untreated control mice at 7 days (87). In this study, we confirmed the presence of pluripotent VSELs in peripheral blood (PB) after MI through a comprehensive approach. First, by using flow cytometry, VSELs were identified in the PB by the typical phenotype (Sca-1$^+$/Lin$^-$/CD45$^-$). Second, greater mRNA levels of markers of pluripotency (Oct-4, Nanog, Rex1, Rif-1, and Dppa1) were detected with quantitative RT-PCR. Finally, by using confocal microscopy, we verified the expression of Oct-4, a marker of pluripotency, at the protein level in VSELs, but not in the control population (Sca-1$^+$/Lin$^-$/CD45$^+$ HSCs) (87).

In this regard, the mobilization of VSELs has been confirmed in patients after acute MI (3, 74). For the first time in humans, Wojakowski *et al*. (74) reported the circulation of Oct-4$^+$/SSEA-4$^+$ pluripotent VSELs in the early phase (24 h) after an acute event in patients with ST-segment elevation MI (STEMI) (Fig. 5). Interestingly, the mobilization of VSELs was significantly reduced in older patients (older than 50 years) and in those with diabetes in comparison with younger and nondiabetic patients (74). In another study by Abdel-Latif *et al*. (3), we investigated the kinetics of the mobilization of VSELs and other pluripotent cells in STEMI patients when compared with non-STEMI and in patients with chronic ischemic heart disease (angina). Consistent with the previous data, these results showed that an acute ischemic event provides the strongest stimulus for VSEL mobilization, which occurred in the early postinfarction phase (3, 74).

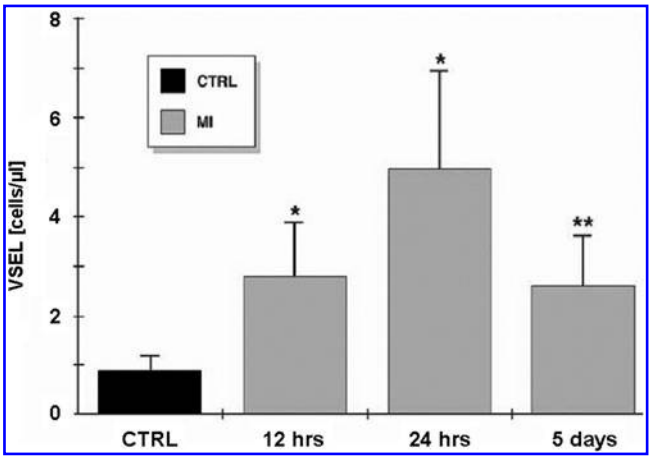


FIG. 5. Mobilization of Lin-/CD133$^+$/CD45$^-$ VSELs in humans. Mobilization of cells shown as change in absolute number of cells per microliter of peripheral blood in patients with acute myocardial infarction (MI) in comparison with healthy control subjects (CTRL). *$p < 0.001$ vs. CTRL; **$p < 0.003$ vs. CTRL. VSELs, very small embryonic-like stem cells. [Reproduced from (74), with permission from Elsevier.]

These results are concordant with those of several studies reporting the mobilization of various types of BM-derived cells after acute myocardial ischemic injury. These include the mobilization of hematopoietic stem cells (43, 51), mesenchymal stem cells (9), endothelial progenitor cells (43, 66), and other distinct subpopulations characterized by surface markers. Circulating CD34$^+$ progenitors (51, 67) and CD34$^+$/CXCR4$^+$, CD34$^+$/c-kit$^+$, and c-met$^+$ subpopulations (75, 76) have been observed in patients after an acute MI. Studies in animals have also shown the presence of BM-derived c-kit$^+$, CD31$^+$ cells in the infarcted myocardium after MI (71). The progenitor cells detected in the PB of patients with acute MI express increased levels of mRNA of early cardiac (GATA-4, Nkx2.5/Csx, and MEF2C) and endothelial (VE-cadherin and von Willebrand factor) markers (75). Similar results have been obtained in mice (36). However, the content of pluripotent cells (determined by the expression of markers of pluripotency) in these mobilized cell types was not investigated in these studies.

We also reported that murine BM-derived VSELs are mobilized after G-CSF stimulation as well as after various forms of tissue injury, including muscle injury, stroke, and acute MI in both animal models and patients (3, 36, 50, 74, 87). Collectively, these data suggest a teleologically important function of VSELs in tissue repair after injury. We believe that additional comprehensive analysis of VSEL mobilization after MI in animal and human models would bring us closer to establishing the time window during which the endogenous mechanisms of heart repair are highly activated and would facilitate the determination of the optimal timing for stem cell transplantation after acute MI.

VSEL transplantation improves cardiac structure and function after acute MI

The presence of circulating VSELs in PB after tissue injury indicated their potential contribution in regeneration of injured tissues. Therefore, we investigated the regenerative potential of these cells in animal models of acute MI. In the first study by Dawn *et al*. (21), we investigated the regenerative efficacy of freshly isolated BM-derived Sca-1$^+$/Lin$^-$/CD45$^-$ VSELs after intramyocardial transplantation in mice that underwent I/R injury. After 35 days of follow-up, VSEL-treated mice exhibited improved global and regional left ventricular (LV) systolic function by echocardiography (Fig. 6) and attenuated myocyte hypertrophy in surviving tissue (histology and echocardiography) when compared with vehicle-treated controls. In contrast, transplantation of Sca-1$^+$/Lin$^-$/CD45$^+$ HSPCs failed to confer any functional or structural benefits (21). Because VSELs isolated from EGFP transgenic mice were used for transplantation, we could track the fate of injected cells in the myocardium, and observed only a small number of scattered EGFP$^+$ myocytes and capillaries in the infarct region and border zone in VSEL-treated mice (21).

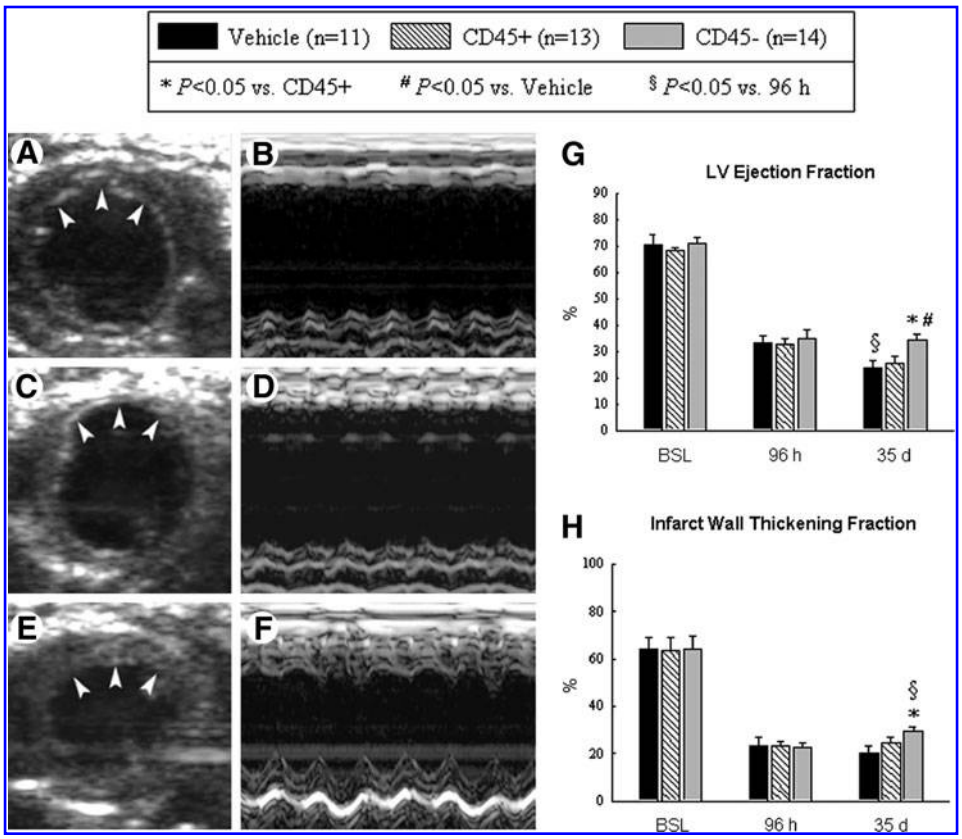


FIG. 6. Echocardiographic assessment of LV function. Representative two-dimensional **(A, C, E)** and M-mode **(B, D, F)** images from vehicle-treated **(A, B)**, $CD45^+$ cell-treated **(C, D)**, and very small embryonic-like stem cell (VSEL)-treated **(E, F)** mice 35 d after coronary occlusion/reperfusion. The infarct wall is delineated by *arrowheads*. **(A, C, E)**. Compared with the vehicle-treated and $CD45^+$ cell–treated hearts, the VSEL-treated heart exhibited a smaller LV cavity, a thicker infarct wall, and improved motion of the infarct wall. **(G, H)** Transplantation of VSEL improved the LV ejection fraction and systolic-thickening fraction of the infarct wall at 35 days after myocardial infarction. Data are expressed as mean ± SEM; $n = 11$–14 mice per group. BSL, baseline; d, days; h, hours; LV, left ventricular. [Reproduced from (21), with permission from John Wiley & Sons, Inc.]

In subsequent short- (35 days) (83) and long-term (6 months) (82) follow-up studies, we investigated the reparative capacity of VSELs that were processed *ex vivo* to increase both their number and their cardiac commitment. Because the frequency of VSELs in the marrow is extremely low, we first examined whether they can be expanded in culture without loss of therapeutic efficacy (82, 83). Accordingly, $EGFP^+$ VSELs were isolated from transgenic mice and further propagated *in vitro* over a C2C12 feeder layer to increase the number of cells before transplantation. These expanded VSELs were isolated by flow cytometry based on EGFP fluorescence (Fig. 4), and predifferentiated in a medium containing TGF-β1, IGF-1, and VEGF-α, a combination known to increase cardiac commitment of stem cells (2, 38, 81). At 35 days after MI, mice treated with expanded and predifferentiated VSELs exhibited improved global and regional LV systolic function by echocardiography and less LV hypertrophy with both histology and echocardiography when compared with vehicle-treated controls (83).

Because improvement in cardiac function after cell therapy has been reported to be transient in a few studies, in the next experiment in VSEL transplantation, we followed up cardiac structure and function over a 6-month period (82). After a 60-min coronary occlusion and reperfusion, mice received intramyocardial injection of vehicle, $CD45^+$ HSPCs, or $CD45^-$ VSELs. During follow-up, VSEL-treated mice exhibited persistently improved LV ejection fraction (EF), smaller LV end-systolic diameter, and greater diastolic infarct wall thickness (82). Results from this study revealed that the observed beneficial effects of VSEL therapy on LV function and anatomy were sustained for at least 6 months after VSEL injection (82). Importantly, no tumor formation was observed during this sufficiently long follow-up (82). Consistent with our observations in the previous studies (21, 83), only a small number of scattered $EGFP^+$ cells expressing α-sarcomeric actin or PECAM-1 or von Willebrand factor were noted in the myocardium of VSEL-treated mice (82).

Potential mechanisms of VSEL-mediated myocardial repair

The three independent studies discussed above established that VSEL transplantation after MI is associated with a consistent and significant beneficial effect on myocardial anatomy and global function (21, 82, 83). Although VSEL transplantation resulted in isolated new myocytes and capillaries in the infarct region, their numbers were too small to account for all of the observed benefits (21, 83). On the basis of these observations, it seems likely that cytokines and growth factors released by differentiating VSELs may directly or indirectly be responsible for the improvements in cardiac structure/function (Fig. 7). It has already been postulated that such "paracrine" effects may be predominantly responsible for the benefits observed with other stem/progenitor cells transplanted for heart repair (26). These released factors often exert antiapoptotic actions and salvage injured yet alive cells from apoptosis, or healthy cells that are negatively affected by products of inflammation known to occur in damaged tissues (14, 63, 70). These secreted factors may also influence the activity of endogenous progenitor cells that are already present

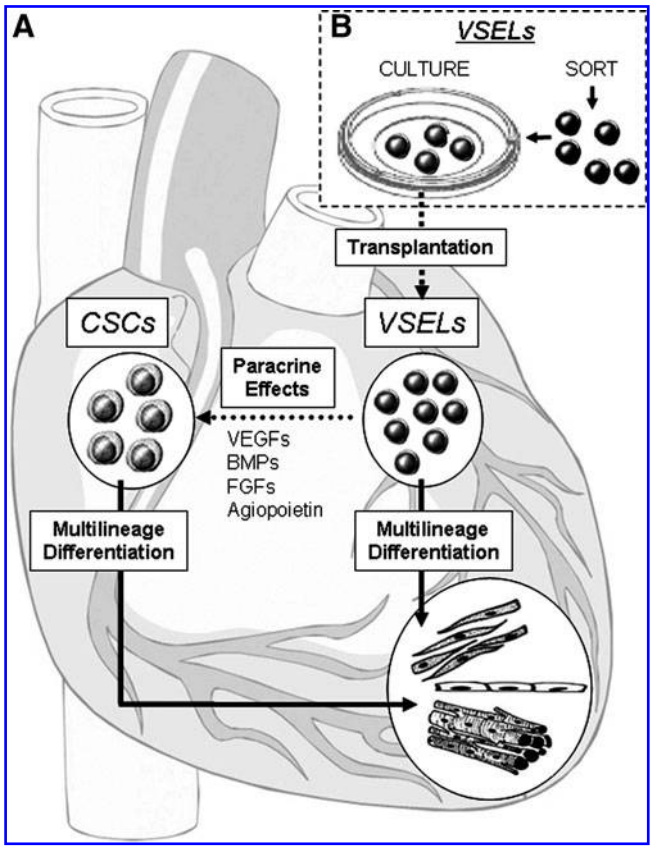


FIG. 7. The potential mechanisms underlying the observed structural and functional benefits after VSEL transplantation after acute myocardial infarction. (A) The *ex vivo* preparation of VSELs before transplantation includes (a) isolation of VSELs by FACS; (b) expansion over the C2C12 feeder layer; and (c) predifferentiation toward cardiac lineages with a growth-factor cocktail. **(B)** Based on our findings, we postulate that after transplantation, expanded and predifferentiated VSELs may lead to a combination of events. VSELs may (a) directly differentiate into cardiomyocytes, smooth muscle cells, and endothelial cells; (b) initiate a protective paracrine response; and (c) activate endogenous cardiac stem/progenitor cells (CSCs) through secreted growth factors. All of these events may be responsible, in part, for the observed benefits.

in myocardial tissue, leading to their proliferation or differentiation or both (30). Whether VSELs influence the endogenous cardiac precursors in an analogous fashion remains to be investigated.

In this regard, the recent microarray data reported by Wojakowski *et al*. (73) provide important insight into the secretome of VSELs at different stages of cardiac differentiation. Among several growth factors released by differentiating VSELs, particular attention should be focused on growth factors, morphogens, and signaling intermediaries that are well known to stimulate both cardiac and endothelial differentiation of primitive cells, including FGFs (FGF1, FGF4, FGF6), BMPs (BMP-4), VEGF-α, angiopoietin, Notch, and sonic hedgehog (73). We postulate that VSELs that have been predifferentiated into a cardiomyogenic pathway before transplantation may also produce similar cytokines/growth factors following intramyocardial injection and stimulate endogenous proliferation and differentiation of cardiac stem/progenitor cells (CSCs) into myocytes and endothelial cells. Because the presence of CSCs in adult hearts has been well documented (7, 20), it is likely that growth factors produced by transplanted VSELs activate endogenous CSCs, leading to their proliferation, differentiation, and incorporation into the myocardium, resulting in functional improvement (Fig. 7).

Conclusions

Although the safety and efficacy of cell therapy for cardiac repair has been established in early clinical trials, the overall progress is hindered by the lack of an ideal cellular substrate for such approaches. The biologic features of VSELs, including the pluripotent nature and the ability to secrete growth factors, make them attractive for cell-therapy strategies in humans. The promising and persistent benefits in LV function and anatomy and the conspicuous lack of tumor formation after VSEL transplantation in animal models of acute MI predict success with similar strategies in humans. It is anticipated that further mechanistic studies in animal models will soon delineate a safe and effective approach for VSEL therapy for cardiac repair in humans.

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Abbreviations Used

BM = bone marrow
BMP = bone morphogenic protein
CB = cord blood
CSC = cardiac stem cell
Dpc = days post coitum
EGFP = enhanced green fluorescent protein
ELB = embryoid-like body
ESC = embryonic stem cell
FACS = fluorescence-activated cell sorting
FGF = fibroblast growth factor
HSPC = hematopoietic stem and progenitor cell
ISS = ImageStream System
Lin = lineage
LT-HSC = long-term repopulating hematopoietic stem cell
LV = left ventricular
MI = myocardial infarction
MSC = mesenchymal stem/stromal cells
N/C = nuclear-to-cytoplasmic ratio
PB = peripheral blood
PGC = primordial germ cell
RT-PCR = reverse transcriptase-polymerase chain reaction
Sca-1 = stem cell antigen-1
STEMI = ST-segment elevation myocardial infarction
TEM = transmission electron microscopy
USSC = unrestricted somatic stem cell
VEGF = vascular endothelial growth factor
VSEL = very small embryonic-like stem cell

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